

CHAPTER 7: THE USE OF PHEROID™ IN THE DELIVERY OF PEPTIDE DRUGS

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7.1 Chapter summary

Studies with four proteins of different sizes and administered by three different administration routes support the subject of this invention. These studies were designed, planned, initiated and supervised and the results were interpreted by the inventors, but the studies were performed mainly as part of postgraduate studies (Strauss, 2005; de Bruin, 2005; Kotze, 2005; Steyn, 2006; Coetzee, 2007; Oberholzer, 2009).

The studies reported in this patent application specifically concern the *peroral* administration of a peptide drug or therapeutic protein. Some additional studies that support the intranasal and topical administration of a therapeutic protein by Pheroid™ technology formed part of the original provisional patent submission but could not be included in the final formal application as the studies were reported on in the dissertations of the above-named postgraduate students and the dissertations were housed in the open university library some months before submission of the application to the EPO.

An important advantage of Pheroid™ technology is that it allows administration of therapeutic proteins or peptide drugs by administration routes other than that by injection. Despite the conventional wisdom that any protein/peptide in the gastrointestinal tract would be

destroyed rapidly by the digestive process (either by stomach acid or intestinal enzymes), or that the molecular sizes of these drugs are too large for nasal or topical delivery, entrapment of therapeutic proteins/peptides into a fatty acid matrix, followed by intracellular release of the therapeutic proteins from such matrices have been shown to be successful in the Pheroid™. Using this invention therapeutic proteins /peptide drugs are shown to be

- (a) absorbed better into the circulation as confirmed by its presence in the plasma of the animals, and
- (b) undegraded as confirmed by means of antibody assays and
- (c) effective as indicated by its enhancement of therapeutic effect.

The flexibility of Pheroid™ technology allows for the absorption, distribution and delivery of a wide variety of therapeutic protein pharmaceuticals, systemically as well as locally, making it well suited for a broad spectrum of therapeutic applications. The FA-based particles can be manipulated in terms of their structure, size, morphology and function, depending on the type and size of the drug molecules to be delivered, the therapeutic indication and the required circulating half life of the drug.

Yet another advantage of the Pheroid™ invention is the possibility of increasing the circulating half life of the therapeutic protein drug. This increase may be the result of:

- (a) an inhibition of enzymatic degradation;
- (b) a decrease in therapeutic protein recognition of immune cells, leading to an immunogenic response - no humoral immune responses against the invention itself were found after either oral, nasal, topical or subcutaneous administration;
- (c) a stabilization of the stereochemistry of the therapeutic protein by entrapment into the FA matrix of the invention.

The above then also indicates another advantage of the invention: that potential deleterious side-effects due to immunogenic response are minimized by masking of therapeutic protein/peptide. One of the most prominent advantages of Pheroid™ technology is the use of essential and other therapeutic fatty acids in its composition. It is well known from the literature that these fatty acids (FA) contribute *inter alia* to the maintenance of cell membrane integrity, modulation of the immune system, energy homeostasis, and the antioxidant status of the cell. The FA component contributes to the transport of the particles of the invention and their entrapped drugs across the cell membrane. These characteristics of the FA-based particles afford it significant advantages over other delivery systems.

In terms of the various administration routes, the ability to use Pheroid™ for nasal delivery is yet another advantage. 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 as fatty acid based drops or even sprays. Various factors synergistically enhance the permeation of nasally administered drugs: the nasal cavity offers a highly vascularized epithelium, a porous endothelial membrane and a relatively large surface area due to the presence of a large number of microvilli. Fatty acid-based gels may be a viable option when longer lasting drug release is required.

In a similar fashion, Pheroid™ technology holds promise for topical administration. 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 it is not invasive. Pheroid™ 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. As shown below, the FA-based particles are able to penetrate human skin, which means that it is possible to administer active compounds via the topical route. These and other objects, advantages and features of the Pheroid™ will become apparent to those persons skilled in the art upon reading the formal patent application and additional studies below.

References for Chapter Summary

- COETZEE, H. 2007. The transdermal delivery of arginine vasopressin with pheroid technology. NWU (Dissertation M.Sc.) 158p
- DE BRUYN, T. 2005. Nasal delivery of insulin with Pheroid technology. NWU (Dissertation M.Sc.) 107p
- KOTZÉ, J. C. 2005. Nasal drug delivery of calcitonin with pheroid technology. Potchefstroom: NWU (Dissertation M.Sc.) 105p.
- OBERHOLZER, I. D. 2009. Peroral And nasal delivery of insulin with Pheroid™ technology. Potchefstroom: NWU (Thesis Ph.D.) 174p.
- STEYN, D. 2006. Nasal delivery of recombinant human growth hormone with Pheroid technology. Potchefstroom: NWU (Dissertation M.Sc.) 115p.
- STRAUSS, T. 2005. Peroral delivery of calcitonin with pheroid technology. Potchefstroom: NWU (Dissertation M.Sc.) 105p.

7.2 Bibliographic details on file at WIPO

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Applicants: **NORTH-WEST UNIVERSITY** [ZA/ZA]; 1 Hoffman Street, Joon van Rooy Building, 2531, Potchefstroom (ZA) (*All Except US*).
GROBLER, Anne Frederica [ZA/ZA]; (ZA) (*US Only*).
KOTZE, Abraham Frederik [ZA/ZA]; (ZA) (*US Only*).
DU PLESSIS, Jeanetta [ZA/ZA]; (ZA) (*US Only*).

Inventors: **GROBLER, Anne Frederica**; (ZA).
KOTZE, Abraham Frederik; (ZA).
DU PLESSIS, Jeanetta; (ZA).

Agent: **D M KISCH INC**; P O Box 781218, 2146 Sandton (ZA).

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Abstract: The invention provides a formulation for the administration of at least one therapeutic mammalian protein to a mammal, and for enhancing the absorption, distribution and release of the at least one therapeutic mammalian protein in or on the mammal, the formulation consisting of at least one therapeutic mammalian protein in a micro-emulsion which micro-emulsion is constituted by a dispersion of vesicles or microsponges of a fatty acid based component in an aqueous or other pharmacologically acceptable earner in which nitrous oxide is dissolved, the fatty acid based component comprising at least one long chain fatty acid based substance selected from the group consisting of free fatty acids and derivatives of free fatty acids It further provides a method of the effective delivery of at least one therapeutic mammalian protein to a mammal and for enhancing the therapeutic efficacy of such at least one therapeutic mammalian protein, the method comprising the step of administering the at least one therapeutic mammalian protein to the mammal in such a formulation

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7.3 Description of the patent WO/2009/004595: Enhancement of the efficacy of therapeutic proteins

7.3.1 Field of the invention

This invention relates generally to the field of drug administration, more particularly to the oral, nasal, topical or parenteral delivery of peptide or protein drugs by entrapment into a fatty acid (hereinafter also referred to as FA) based nitrous oxide saturated matrix in the form of a vesicles or microsponges. The invention further relates to the enhancement in the efficacy of protein or peptide drugs by its entrapment into the fatty acid- based vesicles and microsponges of the invention. In addition, the invention relates to an increase in the therapeutic window of the administered protein or peptide drug.

7.3.2 Definitions and background to the invention

Peptides and proteins are both composed of amino acid residues linked together by amide or peptide bonds. The distinction between these two classes of compounds is based on different conventions, none of which is universally satisfactory. The terms protein and peptide will accordingly be used interchangeably in this specification to signify compounds that contain multiple amino acid residues linked by amide bonds.

As used herein, "protein" is not limited to native (i.e. naturally-occurring) or full-length proteins, but is meant to encompass protein fragments having a desired activity or other desirable biological characteristic, as well as mutants or derivatives of such proteins or protein fragments that retain a desired activity or other biological characteristic. Mutant proteins encompass proteins having an amino acid sequence that is altered relative to the native protein from which it is derived, where the alterations can include amino acid substitutions (conservative or non-conservative), deletions, or additions (e.g., as in a fusion protein).

Derivatives of proteins include proteins that have been modified by the binding of other molecules such as carbohydrates to the protein. Reference to "peptide" herein is intended to have a corresponding meaning.

Also in this specification the expressions "therapeutic mammalian protein" and "therapeutic mammalian peptide" when used in the context of the invention to be disclosed herein are intended to signify proteins or peptides (as qualified above) which, in their naturally-occurring form are produced by a mammalian body and which have therapeutic properties when administered to a mammal, and are thus intended to exclude proteins and peptides which are produced by micro-organisms such as proteins and peptides that have antigenic properties and may thus be used in the preparation of vaccines, and also to exclude salmon calcitonin and human growth hormone as well as any protein or peptide agent specifically named in WO9717978 in respect of the invention entitled ADMINISTRATION MEDIA FOR ANALGESIC, ANTIINFLAMMATORY AND ANTI-PYRETIC DRUGS CONTAINING NITROUS OXIDE AND PHARMACEUTICAL COMPOSITIONS CONTAINING SUCH MEDIA AND DRUGS, or in WO0205850 in respect of the invention entitled ENHANCEMENT OF THE ACTION OF ANTI-INFECTIVE AGENTS (including ramoplanin, teicoplanin, vancomycin and interferon alpha), or in WO0205851 in respect of the invention entitled ENHANCEMENT OF THE ACTION OF CENTRAL AND PERIPHERAL NERVOUS SYSTEM AGENTS, and is further also to exclude proteins and peptides that are incorporated in a dosage form for the purpose of targeting a specific receptor to which any therapeutically active agent also present in such dosage form (or precursor of such agent or nucleic acid substance coding for such agent) is intended to be delivered. The aforementioned WO patent publications are accordingly incorporated by reference in this description.

The proteins with which this invention is specifically concerned is the group consisting of insulin, parathyroid hormone, parathyroid-like hormone, glucagon, insulinotrophic hormone, vasopressin and hormones involved in the reproductive system, chemotactins, cytokines including interleukins 1, 2 and RA but excluding the interferons, chemokines, enzymes including proteases and protease inhibitors, growth factors including acidic and basic fibroblast growth factors, epidermal growth factor, tumor necrosis factors, platelet derived growth factor, granulocyte macrophage colony stimulating factor, neurite growth factor and insulin-like growth factor-1, hormones including the gonadotrophins and somatomedians, immunoglobulins, lipid-binding proteins and soluble CD4, urokinase, streptokinase, superoxide dismutase (SOD), catalase, phenylalanine ammonia lyase, L-asparaginase, pepsin, uncage, trypsin, chymotrypsin, elastase, carboxypeptidase, lactase, sucrase, ciliary neurite transforming factor (CNTF), clotting factor VIII, erythropoietin, thrombopoietin, insulintropin, cholecystokinin, glucagon-like peptide I,

intrinsic factor, Ob gene product, tissue plasminogen activator (tPA), brain-derived neurite factor, phenylalanine transporter (for phenylketonuria), brush border enzymes and transporters.

Proteins are essential to virtually all biological functions, including metabolism, growth, reproduction, and immunity. As such, they have a potential role as pharmaceutical agents for the treatment of a wide range of human diseases. Indeed, they have already been used to treat diseases such as cancer, hemophilia, anemia and diabetes successfully, and for a number of diseases is the only effective treatment. Because many congenital and acquired medical disorders result from inadequate production of various gene products, protein or peptide therapy, such as hormone replacement therapy, provides a means to treat these diseases through their supplementation to the patient. As with almost all therapies, the therapy that is most easily administered, least expensive, and most likely to realize patient compliance is the therapy of choice.

Although protein drugs have enormous therapeutic potential, their more widespread use has been limited by several restrictive technical factors. These include the following considerations:

- Proteins remain difficult and expensive to manufacture compared to other pharmaceuticals. Large- scale purification of proteins in bioactive form can be a limiting step in the commercialization of these drugs. The production of these drugs may be cost prohibitive in developing countries.
- Many proteins are metabolized in the body, resulting in a short circulating half life and a need for frequent dosing. Due to the hydrophilic nature and molecular size of protein drugs they are poorly absorbed across mucosal epithelia, both transcellularly and paracellularly, leading to poor bioavailability.
- Proteins are often degraded in the harsh gastric environment after oral administration. Protection against degradation may make the per oral administration of these drugs viable.
- Clearance of proteins is generally fast, and they are eliminated quickly in the patient. This results in the need for frequent re-administration, contributing to cost. An increase in the circulating half life of the peptide at therapeutic concentrations would therefore be advantageous.
- Generally, protein drugs must be given by injection. This increases the complexity and expense of the treatment. The disagreeable nature of administration also limits potential clinical applications and decrease patient compliance.

An enhancement in either the absorption, or the efficacy of these drugs should accordingly contribute to more cost effective and hence affordable peptide or protein drugs.

Administration of therapeutic protein products (such as hormones, growth factors, signaling molecules, neurotransmitters, cytokines or polypeptides for protein replacement therapy) by administration routes other than the parenteral route has attracted wide attention as a method to treat various mammalian diseases. Due to the described problems with other administration routes, it is necessary to employ a drug delivery system or penetration enhancer for administration of these drugs via alternative administration routes. Poor bioavailability may be partly overcome by the inclusion of absorption enhancers in protein drug formulations although that is not necessarily the best solution.

With the advent of Human Genome Organisation (HUGO) new proteins are discovered at an increasing pace, and known proteins become available as therapeutic agents. New ways and methods to expand the application of these molecules as drugs by improving the feasibility and convenience of their use are now required. The present invention addresses precisely such an effort.

The oral route is the most common, simple, convenient and physiological way of administering traditional active compounds. The oral route generally does not lend itself to the administration of protein drugs due to the problems described above. A variety of delivery systems have been developed to try to accomplish therapeutic peroral delivery of proteins (for reviews, see Chang *et al.* 1994 *Gastroenterol.* 106: 1076-84, Morsey *et al.* 1993 *JAMA* 270: 2338-45, and Ledley 1992 *J. Pediatr. Gastroenterol. Nutr.* 14: 328-37).

The ease of administration via an oral or other lumenal route, or the nasal or topical route allows administration of peptides through non-invasive procedures. These routes share a number of significant physiological characteristics:

- (a) The administration sites can be regarded as existing "outside" of the body as the anatomical receptacle of the drug is separated from the circulation by a continuous biological barrier.
- (b) The sites are separated from the circulation by (a) continuous layer(s) of cells, in the case of oral administration the intestinal epithelium, in the case of nasal administration the nasal epithelium and underlying layers and in the case of topical administration the epidermis and to some extent the dermis. The layer(s) thus form a continuous biological barrier with various levels of penetrability.
- (c) Any drug administered thus remains in the exterior space, and cannot enter the body proper and its bloodstream, unless it first crosses the described cell layer(s). However,

once the drug is absorbed into the epithelial cells of the nasal tract, or GI lumen, or has penetrated through the epidermis, it can be transported into the bloodstream where the therapeutic protein will presumably act in the same manner as current, injectable forms of the drug.

- (d) The cells lining the biological barriers described all secrete a fluid (i.e. mucus or sweat) that may interfere with the stability or complicate absorption of the drug. Proteases present in such fluid may in each case cause degradation of the protein.

Thus past efforts to administer proteins through the oral, nasal or topical route have met with severe obstacles. Many of the existing delivery systems either have their own inherent drawbacks or are not entirely suitable for protein delivery. Moreover, delivery of the drugs via the bloodstream of the individual results in exposure of the proteins and any carrier associated with it to the immune system, which can result in immunological adverse reactions.

7.3.2.1 The therapeutic mammalian protein: insulin

Insulin therapy is still the mainstay of the treatment of Type 1 and 2 diabetes and is the most widely used protein drug. Despite advances, it is still administered by subcutaneous injection or microneedles which cause disruption of the skin. Subcutaneous or microneedle administration suffers from disadvantages such as time lag between peak insulin levels and postprandial glucose levels, hypoglycemia, weight gain, peripheral hyperinsulineamia and poor patient compliance. An overdose of insulin may cause secondary effects such as the release of glucagon, growth hormone, catecholamines and corticosteroids as a result of pronounced hypoglycemia. Efforts to develop dosage forms that may circumvent or at the very least decrease these problems are ongoing.

Insulin is normally synthesized as pro-insulin by the β -cells of the islets of Langerhans found in the endocrine pancreas. In its processed form, it consists of an A and B chain with a combined a molecular weight of 5807.7dalton and an amino acid number of 51. Insulin is released from secretory granules in the β -cells of the islets directly into the blood stream at a low basal rate. A variety of stimuli, such as glucose, sugars, certain amino acids and vagal activity stimulates release of insulin. Under normal fasting conditions, the pancreas secretes about 40 μ g (1 IU) of insulin per hour into the hepatic portal vein The insulin concentration of portal blood averages between 2 - 4 ng/ml, and the peripheral blood 0.5 ng/ml (12 μ IU/ml). The plasma half-life of insulin is around 5 to 6 minutes in healthy people, with the degradation of insulin occurring mainly in the liver, kidneys and muscle. It is estimated that 50% of the insulin

that reaches the liver by the hepatic portal vein is degraded and does not reach the general circulation.

Commercially available insulin preparations that are currently available can mostly be classed as:

- Ultra-short-acting insulin, with fast onset and short duration of action;
- Short-acting insulin, with fast onset of action;
- Intermediate acting insulin, and
- Long-acting insulin, with slow onset and a longer duration of action.

All of the above preparations are stabilized by the addition of zinc and/or protamines. Conventional subcutaneous insulin therapy mainly consists of split-dose injections of mixtures of short-acting and intermediate-acting preparations with the addition of long-acting insulin for prolonged duration of action to sustain overnight basal levels.

The oral route is attractive for insulin therapy because of both pragmatic and physiological reasons. In practice, it is associated with simplicity and comfort. Besides the discomfort of injections, the reuse of needles carries a risk of infection. Oral preparations are generally cheaper to manufacture, as they do not have to be sterile. A physiological advantage lies in the fact that it mimics the endogenous secretion of insulin more closely: insulin is absorbed from the intestine and reaches the liver via the hepatic portal vein, with a direct effect on the hepatic glucose production and the maintenance of energy levels by the liver, avoiding in this fashion hyperinsulinemic effects. Insulin administered parenterally on the other hand, does not simulate the normal dynamics of endogenous insulin secretion. Despite these advantages of peroral insulin, this route has not been used successfully, as less than 0.5% of the orally administered dose is absorbed from the GI tract and less than 0.1% reaches the central bloodstream intact. The oral delivery of complex drug molecules such as hormones is currently receiving attention, with an interest in increasing the intestinal permeability of such large molecules and molecules with known poor bioavailability.

7.3.3 Object of the invention

A primary object of the present invention is to provide a method of administration of a therapeutic mammalian protein as herein defined, and certain named proteins, through non-invasive means. The primary object is extended to provide a method whereby the efficacy of the

administered therapeutic mammalian proteins, and certain named proteins, is enhanced and the amount of expensive active drug needed is reduced.

A secondary object is the stabilization of a therapeutic mammalian protein, and certain named proteins, against degradation by a) masking of the protein against protease action and b) by the concomitant incorporation of a protease inhibitor as hereinafter described. The present invention is advantageous in that it may be used to protect therapeutic mammalian proteins drugs, and certain named protein drugs from enzyme action.

7.3.4 Description of the invention

According to the present invention there is further provided a therapeutic mammalian protein formulation for the administration of one or more therapeutic mammalian proteins to a mammal, and for enhancing the absorption, distribution and release of such delivered substance(s) in or on the mammal, the formulation consisting of at least one therapeutic mammalian protein in a micro-emulsion which micro-emulsion is constituted by a dispersion of vesicles or microsponges of a fatty acid based component in an aqueous or other pharmacologically acceptable carrier in which nitrous oxide is dissolved, the fatty acid based component comprising at least one long chain fatty acid based substance selected from the group consisting of free fatty acids and derivatives of free fatty acids.

The invention also provides for a formulation for the administration to a mammal of at least one protein selected from the group consisting of insulin, parathyroid hormone, parathyroid-like hormone, glucagon, insulinotrophic hormone, vasopressin and hormones involved in the reproductive system, chemotactins, cytokines including interleukins 1, 2 and RA but excluding the interferons, chemokines, enzymes including proteases and protease inhibitors, growth factors including acidic and basic fibroblast growth factors, epidermal growth factor, tumor necrosis factors, platelet derived growth factor, granulocyte macrophage colony stimulating factor, neurite growth factor and insulin-like growth factor-1, hormones including the gonadotrophins and somatomedians, immunoglobulins, lipid-binding proteins and soluble CD4, urokinase, streptokinase, superoxide dismutase (SOD), catalase, phenylalanine ammonia lyase, L-asparaginase, pepsin, uricase, trypsin, chymotrypsin, elastase, carboxypeptidase, lactase, sucrase, ciliary neurite transforming factor (CNTF), clotting factor VIII, erythropoietin, thrombopoietin, insulintropin, cholecystokinin, glucagon-like peptide I, intrinsic factor, Ob gene product, tissue plasminogen activator (tPA), brain-derived neurite factor, phenylalanine transporter, brush border enzymes and transporters, and for enhancing the absorption, distribution and release of the at least one protein in or on the mammal, the formulation

consisting of the at least one protein in a micro-emulsion which micro-emulsion is constituted by a dispersion of vesicles or microsponges of a fatty acid based component in an aqueous or other pharmacologically acceptable carrier in which nitrous oxide is dissolved, the fatty acid based component comprising at least one long chain fatty acid based substance selected from the group consisting of free fatty acids and derivatives of free fatty acids.

According to the present invention there is also provided methods for the effective delivery of at least one therapeutic mammalian protein to a mammal by various administration routes and for enhancing the therapeutic efficacy of such therapeutic mammalian protein(s), the method comprising the step of administering the at least one therapeutic mammalian protein to the mammal in a formulation consisting of the at least one therapeutic mammalian protein in a micro-emulsion constituted by a dispersion of vesicles or microsponges of a fatty acid based component in an aqueous or other pharmacologically acceptable carrier in which nitrous oxide is dissolved, the fatty acid based component comprising at least one long chain fatty acid based substance selected from the group consisting of free fatty acids and derivatives of free fatty acids.

The invention further also provides for a method for the effective delivery to a mammal of at least one protein selected from the group consisting of insulin, parathyroid hormone, parathyroid-like hormone, glucagon, insulinotrophic hormone, vasopressin and hormones involved in the reproductive system, chemotactins, cytokines including interleukins 1, 2 and RA but excluding the interferons, chemokines, enzymes including proteases and protease inhibitors, growth factors including acidic and basic fibroblast growth factors, epidermal growth factor, tumor necrosis factors, platelet derived growth factor, granulocyte macrophage colony stimulating factor, neurite growth factor and insulin-like growth factor-1, hormones including the gonadotrophins and somatomedians, immunoglobulins, lipid-binding proteins and soluble CD4, urokinase, streptokinase, superoxide dismutase (SOD), catalase, phenylalanine ammonia lyase, L-asparaginase, pepsin, uricase, trypsin, chymotrypsin, elastase, carboxypeptidase, lactase, sucrase, ciliary neurite transforming factor (CNTF), clotting factor VIII, erythropoietin, thrombopoietin, insulintropin, cholecystokinin, glucagon-like peptide I, intrinsic factor, Ob gene product, tissue plasminogen activator (tPA), brain-derived neurite factor, phenylalanine transporter, brush border enzymes and transporters, and for enhancing the absorption, distribution and release of the at least one protein in or on the mammal, the method comprising the step of administering the at least one protein to the mammal in a formulation consisting of the at least one protein in a micro-emulsion which micro-emulsion is constituted by a dispersion of vesicles or microsponges of a fatty acid based component in an aqueous or other pharmacologically acceptable carrier in which nitrous oxide is dissolved, the fatty acid based

component comprising at least one long chain fatty acid based substance selected from the group consisting of free fatty acids and derivatives of free fatty acids.

In a preferred embodiment, the vesicles or microsponges used in the present invention are designed so as to enhance therapeutic mammalian protein, or above named protein, absorption and therapeutic mammalian protein, or above named protein, systemic circulation time while at the same time decreasing therapeutic mammalian protein, or above named protein, degradation. This combination of necessity results in increased efficacy of the therapy.

Non-invasive routes of administration such as per oral, topical or nasal routes require that any therapeutically active compound must first cross a continuous biological barrier consisting of a layer or layers of cells and sometimes some additional fibrous tissue before it can enter the body proper and its bloodstream. As described above, in this invention therapeutic mammalian protein, or above named protein, drugs are packaged into or entrapped within FA-based nitrous oxide saturated particles. Changes in the composition of the FA result in different types of particles, of which at least two types will specifically be addressed in the examples stated below.

Preferably, the composition also contains the antioxidant dl- α -tocopherol or a stable derivative of this antioxidant. Thus, the vehicle may contain α -tocopherol or one of its derivatives at a concentration of no less than 0.1% and no more than 5% in addition to commercially available antioxidants. For example, the formulation can include one or more antioxidants, such as TBHQ (tert-butyl hydro quinone), BHA (butylated hydroxyanisole) or BHT (butylated hydroxytoluene), which can increase the degree of enhancement of the therapeutic mammalian protein, or above named protein, of interest, particularly where the stability of the drug molecule is at risk.

For purposes of increased shelf life, the composition may also contain protease inhibitors which are commercially available, such as bestatin.

The dispersion is preferably characterized in that at least 50% of the vesicles are of a diametrical size of between 80 nanometer and 3 μ m and that of the microsponges between 1.5 and 6.0 μ m. Both the size and shape of the vesicles are reproducible. It will be understood that the vesicles or microsponges in the dispersion are elastic and not necessarily of perfectly spherical shape and accordingly the term "diametrical size" is not to be understood as a term of geometric precision. It is further to be understood that it is not practicable to determine such diametrical size in three dimensions without the use of highly sophisticated instrumentation. It is accordingly to be determined in two dimensions by means of microscopic observation and thus

refers to the maximum measurement across observed vesicles or microsponges as seen in two dimensions.

Various fatty acids and modified fatty acids (e.g. , ethylated fatty acids) can be used in accordance with the present invention. Techniques for the modification of the fatty acids are known in the art (Villeneuve *et al.* 2000. Journal of Molecular Catalysis Enzymatic, 9: 113-148, Demirbas A, 2007, Energy Conversion and management, in press, available online at www.sciencedirect.com), each of which is hereby incorporated by reference with respect to methods and compositions for the formation of fatty acid based vehicles). For example, polyethylene glycol (PEG) molecules, small peptides or carbohydrate molecules may be linked to the carboxyl group of the fatty acid. The modifications are preferred to be biologically functional and to support a desired characteristic. Some modified fatty acids are commercially available.

Preferably both fatty acids containing ethyl groups and polyethylene groups attached to their carbonyl groups are used. Numerous such modified fatty acids are known in the art and are commercially available. In general, each such commercial preparation consists of a variety of modified fatty acids. The fatty acid based component may be selected from the group consisting of oleic acid, linoleic acid, alpha-linolenic acid, gamma-linolenic acid, arachidonic acid, eicosapentaenoic acid [C20 5 ω 3], decosahexaenoic acid [C22 6 ω 3], and ricinoleic acid, and derivatives thereof selected from the group consisting of the C₁ to C₆ alkyl esters thereof, the glycerol-polyethylene glycol esters thereof, and the reaction product of hydrogenated and unhydrogenated natural oils composed largely of ricinoleic acid based oils, such as castor oil, with ethylene oxide. In one form of the invention the fatty acid component of the micro-emulsion may consist or include a mixture of esterified fatty acids. In this regard use may be made of the commercially available product known as Vitamin F Ethyl Ester. Despite the availability of commercial products containing combinations of esterified fatty acids, the fatty acid based component may be constituted from selected single fatty acids or modified fatty acids according to the cellular and subcellular target of the invention. For microsponges, preferably very long chain polyunsaturated fatty acids are used. The long chain fatty acids may be selected from any of a variety of such fatty acids known in the art. The fatty acid component may thus alternatively include or consist of the long chain fatty acids known as eicosapentaenoic acid [C20:5 ω 3] and decosahexaenoic acid [C22:6 ω 3]. Such a product combination is available from Roche under the trade name "Ropufa '30' n-3 oil". An alternative product that may be used for this purpose is one of the group of Incrome products available from Croda.

The fatty acid component may in addition to the aforementioned substances or mixtures of substances also include the reaction product of hydrogenated natural oils composed largely of

ricinoleic acid based oils with ethylene oxide. It is preferable for this substance to be produced from castor oil of which the fatty acid content is known to be predominantly composed of ricinoleic acid. This product may be modified as to the extent of hydrogenation, ethylation and the addition of groups such as polyethylene glycol. A range of such products is being marketed by BASF under the trade description of Cremophor of various grades. According to a preferred form of the invention for certain applications, the ricinoleic acid molecules are modified by the addition thereto of polyethylene glycol groups which comprise between 35 and 45 ethylene oxide units.

The typical fatty acid profile of the FA-based vesicles is as follows:

- 0.2324% of ethylated C_{16:0} fatty acids
- 0.098% of ethylated C_{18:0} fatty acids
- 0. 6076% of ethylated C_{18:1} fatty acids
- 0. 9744% of ethylated C_{18:2} fatty acids
- 0.784% of ethylated C_{18:4} fatty acids
- 1.00% of glycerol-polyethylene glycol esters of ricinoleic acid.

The typical fatty acid profile of the FA-based microsponges is as follows:

- 0.2324% of ethylated C_{16:0} fatty acids
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- 00. 6076% of ethylated C_{18:1} fatty acids
- 0. 9744% of ethylated C_{18:2} fatty acids
- 0.784% of ethylated C_{18:4} fatty acids
- 1.00% of glycerol-polyethylene glycol esters of ricinoleic acid
- 0. 25% of ethylated C_{20:3} fatty acids
- 0. 25% of ethylated C_{22:3} fatty acids.

The vehicle further contains nitrous oxide gas dissolved in the fatty acid mixture to impart the requisite size distribution of vesicles and the requisite stability to the micro-emulsion. The nitrous oxide gas is sparged through the fatty acid phase or the water phase or the final formulation containing the therapeutic mammalian protein, or above named protein, of the present invention.

In its preferred form, the FA-based particles consist of an oil phase and a water phase, both of which are present in association with nitrous oxide. A precursor form of these particles, consisting of only the oil phase in association with nitrous oxide is generally used for oral

applications. The aqueous phase may consist of sterile water or sterile buffers, depending on the properties of the drug to be entrapped, while the oil phase consists of a combination of modified fatty acids. The fatty acids are manipulated to ensure remarkably high entrapment capabilities, extremely fast rate of transport and cellular delivery.

According to another aspect of the invention there is provided a method for producing a delivery vehicle according to the present invention as defined above, comprising the steps of mixing the fatty acid based component with water to obtain a micro-emulsion, and introducing nitrous oxide gas into the mixture to impart the requisite size distribution of vesicles and the requisite stability to the micro-emulsion. Techniques for production of self-emulsifying micro-emulsions are known in the art (see, for example, Gursoy and Benita, *Biomedicine & Pharmacotherapy*, Volume 58, Issue 3, April 2004, Pages 173-182). In this regard, the mixing of the fatty acid component is preferably effected in the presence of heating, with stirring, preferably by means of a high speed shearer.

According to another aspect of the invention, the therapeutic mammalian protein, or above named protein, drug may be pre-mixed with either the oil phase or the water phase, depending on the hydrophobicity and polarity of the specific therapeutic mammalian protein, or above named protein, to be entrapped. In this case, the mixing of the formulation is preferably effected after cooling the fatty acid component to below 50°C and with some stirring, preferably by means of a low speed shearer in the presence of the nitrous oxide gas. The mixing may also occur after the formation of the particles by gentle mixing.

The nitrous oxide gas may be introduced into the water either before or after the fatty acid based component of the micro-emulsion is mixed with the water. Thus in one form of the invention the nitrous oxide gas may be dissolved in the water to obtain a saturated solution of the nitrous oxide gas in water, and the saturated solution of the nitrous oxide gas is thereafter mixed with the fatty acid component of the micro-emulsion being prepared. The saturated solution of the nitrous oxide gas in water may be prepared by sparging the water with the nitrous oxide gas, or by exposing the water to the nitrous oxide gas at a pressure in excess of atmospheric pressure for a period of time in excess of the time required for the water to become saturated with the nitrous oxide gas. In an alternative form of this aspect of the invention an emulsion of the fatty acid component in water may first be prepared and may thereafter be gassed by exposing the emulsion to the nitrous oxide gas. This is preferably done by sparging.

Formulations are typically available in forms that can be used in dosage devices or formulations used in oral, nasal or topical administrations. Such forms include any additives that further enhance effectiveness, stability, or ease of application such as penetration enhancers,

thickeners and other adjuvants, and any other ingredients including solvents, carriers, or dyes. The application method and species to be treated determine which formulation is preferable.

This invention focuses on an effective method of transport of therapeutic mammalian proteins, or above named proteins across the biological barriers. The formulation comprising the therapeutic mammalian protein, or above named protein, of interest entrapped in a transport vehicle is absorbed into cells lining the anatomical receptacles (i.e. nasal cavity, GI lumen or skin) after being administered externally. Preferably, the therapeutic mammalian protein, or above named protein, of interest is stably entrapped in the transport vehicle. The therapeutic mammalian protein, or above named protein, is then transported to the systemic circulation, preferably in therapeutically effective amounts. Once in the circulation, therapeutic mammalian proteins, or above named proteins that serve as replacement or supplementation of therapeutic mammalian protein, or above named protein, therapy acts in the same manner as if they were naturally expressed by the subject. Furthermore, where the therapeutic mammalian protein, or above named protein, is an exogenous therapeutic mammalian protein, or above named protein, that provides a desired therapeutic effect the drug exhibits the same activity as if it were delivered by conventional injection methods.

In a preferred embodiment, sufficient levels of the therapeutic mammalian protein, or above named protein, of interest are absorbed into the blood for therapeutic mammalian protein, or above named protein, therapy to be effective. The therapeutic effect of the therapeutic mammalian protein, or above named protein, may be enhanced by targeting of the fatty acid matrix through covalent binding of targeting amino acid sequences, motifs or peptides to the carbonyl groups of the fatty acids, or by attaching other elements which mediate specific organ selection.

7.3.4.1 Therapeutic mammalian proteins and conditions amenable to treatment by protein or peptide therapy

Preferably, the therapeutic mammalian protein, or above named protein, entrapped in the particles of the invention can be any therapeutic mammalian protein, or above named protein, that can be used for therapeutic mammalian protein, or above named protein, replacement or supplementation, be it caused by either an inherited or acquired disease associated with a specific therapeutic mammalian protein, or above named protein, deficiency. The aim of the intervention would be to restore the levels of the deficient therapeutic mammalian protein, or above named protein, to normal levels in at least the systemic circulation but preferably also in the applicable organs, tissue or cells. Conditions caused by therapeutic mammalian protein, or

above named protein, deficiencies that can be treated by replacement or supplementation include diabetes, hemophilia, anemia, immunodeficiencies, nutrient absorption deficiencies, and steroid hormone replacements. The therapeutic mammalian protein, or above named protein, may also be any therapeutic mammalian protein, or above named protein, that may regulate or switch on or switch off a specific pathway in the body. Numerous therapeutic mammalian proteins, or above named proteins that are desirable for protein therapy are well known in the art. Proteins commonly used in treatments can be delivered by various administration routes using the present invention. Such therapeutic mammalian proteins, or above named proteins are disclosed in, for example, the Physicians' Desk Reference (1994 Physicians' Desk Reference, 48th Ed, Medical Economics Data Production Co, Montvale, N J, incorporated by reference) and can be dosed using methods in Harrison's Principles of Internal Medicine and/or the AMA "Drug Evaluations Annual" 1993, all incorporated by reference.

Proteins can be either completely lacking or defective in which case complete replacement needs to be undertaken. Alternatively, the protein may be under-expressed in which case the invention would be used for supplementation therapy. A protein may also be over-expressed and therapy may need to supply a therapeutic mammalian protein, or above named protein, to either regulate or degrade the over-expressed protein.

Exemplary preferred therapeutic mammalian proteins, or above named proteins include the hormones and peptide hormones such as insulin, parathyroid hormone, parathyroid-like hormone, glucagon, insulinotrophic hormone, vasopressin and hormones involved in the reproductive system.

The following specific classes of therapeutic mammalian proteins, or above named proteins are specifically included for use with the invention chemotactins, cytokines including interleukins 1, 2 and RA (excluding interferon alpha), chemokines, enzymes including proteases and protease inhibitors, growth factors including acidic and basic fibroblast growth factors, epidermal growth factor, tumor necrosis factors, platelet derived growth factor, granulocyte macrophage colony stimulating factor, neurite growth factor and insulin-like growth factor-1, hormones including the gonadotrophins and somatomedians, immunoglobulins, lipid-binding proteins and soluble CD4.

Exemplary enzymes, as one of the therapeutic mammalian protein, or above named protein drug classes may also be packaged into the vesicles or micro-sponges of the invention for enhanced therapeutic efficacy. Individuals skilled in the art will recognize that the invention may benefit delivery of the following enzymes: urokinase, streptokinase, superoxide dismutase

(SOD), catalase, phenylalanine ammonia lyase, L- asparagmase, pepsin, uricase, trypsin, chymotrypsin, elastase, carboxypeptidase, lactase, sucrase.

Specific therapeutic mammalian proteins, or above named proteins that are included are ciliary neurite transforming factor (CNTF), clotting factor VIII, erythropoietin, thrombopoietin, insulintropin, cholecystokinin, glucagon-like peptide I, intrinsic factor, Ob gene product, tissue plasminogen activator (tPA), brain-derived neurite factor. Administration of a therapeutic mammalian protein, or above named protein by the oral route is indicated where the subject suffers from a condition due to malabsorption of nutrients, e.g. deficiency in digestive enzymes, including lactase, intrinsic factor, sucrase, or transporters.

Where the target for protein therapy is the gastrointestinal tract, the entrapped therapeutic mammalian protein, or above named protein may be phenylalanine transporter (for phenylketonuria), lactase for lactase deficiency, intrinsic factor, or other brush border enzymes and transporters.

The therapeutic mammalian protein, or above named protein may be modified by posttranslational modification or applicable mutations of the gene coding for such protein or by synthetic attachment of carbohydrate groups to such protein. The protein therapy concerned in this invention is aimed at therapy of mammalian subjects, be it bovine, canine, feline, equine, or human, or rodent subjects. Preferably the therapeutic mammalian protein, or above named protein used in the therapy is specific to man, i.e. obtained from recombinant production or chemical synthesis, but this requirement is not absolute, particularly if the amino acid sequence of the therapeutic mammalian protein, or above named proteins is highly conserved and non-immunogenic. Alternatively, the mammalian subject may have a condition which is amenable to treatment by a protein which is foreign to the mammalian subject, but may for instance enhance a normal metabolic process.

Exemplary diseases that are amenable to treatment using the subject invention, and exemplary, appropriate therapeutic mammalian protein, or above named proteins which can be used in treating these diseases, are discussed below. The intestinal epithelium is the major absorptive surface in animals, and as such transports substances preferentially from the intestinal lumen into blood. It has been described in the literature that larger molecules may also be absorbed: in newborn animals immune responses are the result of the absorption of antibody proteins, various digestive enzymes from the pancreas, and other therapeutic mammalian protein, or above named proteins such as insulin, has been shown to cross the intestinal epithelium. Permeability to proteins has been seen primarily in the duodenum and

terminal ileum, but proteins are also known to be absorbed from the lower portions of the large bowel, and suppositories have been used for this purpose therapeutically.

Proteins that are manufactured in the gut and targeted for secretion into the blood and are included in the ambit of the invention include hormones such as CCK (cholecystokinin), gastric inhibitory peptide (GIP), glucagon-like peptide I (GLPI), gut glucagon, islet amyloid polypeptide (IAPP), neuropeptide Y (NPY), polypeptide Y (PPY), secretin, vasoactive intestinal peptide (VIP), and a variety of lipoproteins important in lipid metabolism.

Preferably, the use of active therapeutic mammalian proteins, or above named proteins, which may consist of multiple peptides are included in the invention. Similarly, therapeutic mammalian proteins, or above named proteins or peptides containing posttranslational modifications and processing that would normally occur in specific cells, but which may be absent in the cells targeted for treatment, are included. The use of modified forms of the therapeutic mammalian proteins, or above named proteins, where the modification carries a therapeutic advantage, are included in the invention. Such modifications may be aimed at characteristics such as protease resistance or enhanced activity relative to the wild-type protein.

7.3.4.2 Assessment of protein therapy

The fatty acid based vehicles of the present invention can be used in connection with any therapeutic mammalian protein that is desired for administration. While the FA matrix can be used with therapeutic mammalian proteins, or above named proteins whose efficacy in intravenous therapies has not yet been tested, it can also be used with therapeutic mammalian proteins, or above named proteins of well- established efficacy (e.g., insulin, etc.). Furthermore, given the examples below, the ordinarily skilled artisan can readily determine that, since the FA matrix efficiently enhance absorption and efficacy of insulin in the bloodstream in an animal model after administration, then enhancement in the therapeutic effect of other therapeutic mammalian proteins, or above named proteins can also be readily achieved using the claimed fatty acid matrix of the invention. Since the enhancement in efficacy of the claimed invention can be used in connection with a wide variety of therapeutic products, the therapeutic enhancement can be monitored in a variety of ways. Generally, such evaluation would be based on a comparative specific assay of a sample of blood from a subject treated with the same therapeutic molecule in similar concentrations in the presence and absence of the invention. Appropriate assays for detecting a therapeutic mammalian protein, or above named protein of interest in such samples are well known in the art. For example, a sample of blood can be tested for the presence of the therapeutic mammalian protein, or above named protein using an antibody which specifically binds the therapeutic mammalian protein, or above named

protein in a RIA (radio immune assay). Such assays are performed quantitatively to determine the degree of enhancement. The assay may be 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 mIU for IRMA. ELISA systems can increase the sensitivity 100 fold. The ELISA assay, as well as other immunological assays for detecting the therapeutic mammalian protein, or above named protein in a sample, are described in *Antibodies: A Laboratory Manual* (1988, Harlow and Lane, eds. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.)

The amount of a specific therapeutic mammalian protein, or above named protein drug that traverse a biological barrier may be quantified by analytical methods such as high performance liquid chromatography, as exemplified below.

Alternatively, or in addition, the efficacy of the protein therapy can be assessed by measuring an activity associated with the therapeutic mammalian protein, or above named protein. Where the therapeutic mammalian protein, or above named protein is insulin, the efficacy of the therapy can be assessed by examining blood glucose levels of the mammalian subject or by measuring insulin (e.g., by using the human insulin radioimmunoassay kit, Linco Research Inc . St. Louis, Mo.).

7.3.5 Description of preferred embodiments

The invention will now be illustrated, purely by way of examples with reference to the following non-limiting description of Preparations and Examples. The invention concerns itself with the advantages it offers in the enhancement of efficacy of therapeutic mammalian proteins, or above named proteins by routes other than the parenteral route typically used in the administration of these drugs. In the following section some background on problems associated with and factors inherent in the intranasal, peroral and topical routes of administration of therapeutic mammalian proteins, or above named proteins are described.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood to one of ordinary skill in the art to which this invention belongs. Although any methods, devices and materials similar or equivalent to those described herein can be used in the practice or testing of the invention, the preferred methods, formulations and materials are now described.

7.3.6 Examples of the invention

Without thereby limiting the scope of the invention some examples will now be described to illustrate the invention.

7.3.6.1 Preparation 1

Preparation of formulations suitable for use as a delivery vehicle for use in delivering a therapeutic mammalian protein, or above named protein to mammals

A formulation according to the invention may be made up as follows:

- Step 1: A desired volume of water is saturated with nitrous oxide gas at ambient pressure using a pressure vessel and sparger. The vessel is connected to a supply of nitrous oxide via a flow control valve and pressure regulator. The closed vessel is supplied with nitrous oxide at a pressure of 2 bar for a period of 96 hours, it having been determined that at the aforementioned temperature the water is saturated with nitrous oxide over such period of time under the above-mentioned pressure. In the case of the preparation of the basic or stock formulation to be used as a carrier medium comprising a dispersion of vesicles unchlorinated water is used. The water is phosphate buffered to a pH of 5.8.
- Step 2: The following fatty acid based compositions were made up: First, Vitamin F Ethyl Ester CLR 110 000 Sh.L. U./g obtained from CLR (Chemicals Laboratorium Dr. Kurt Richter GmbH of Berlin, Germany) which is composed mainly of 21% oleic acid, 34% linolenic acid, and 28% linoleic acid that are modified by esterification with an ethylene group of the carboxy terminal, was heated to 75°C. Secondly, pegylated, hydrogenated fatty acid, ricinoleic acid (also known by the INCI name as PEG-n-Hydrogenated Castor Oil), was heated to 80°C and mixed with the first group of fatty acid based Vitamin F Ethyl Ester at 70°C. The ratio of the first group of fatty acids to the latter fatty acid was generally 2.8:1.
- Step 3: dl- α -Tocopherol of varying percentages (final concentration of between 0.1% when used as general anti-oxidant was added to the heated fatty acids mixture above).
- Step 4: The buffered water was heated to 73°C and mixed with the fatty acid mix with the aid of a high speed shearer to a final concentration of between 3.2 and 4%, depending on the specific use of the preparation. This fatty acid mixture constituted the basic preparation that contains vesicles of sizes in the nanometer range as determined by particle size analysis on a Malvern sizer.

Step 5: To the basic preparation may be added additional ethylated fatty acids DHA (decahexonoic acid) and EPA (eicosapentanoic acid). The preferable amount of the two fatty acids for this invention was 0.5%. The addition of these fatty acids results in the formation of microsponges rather than vesicles, with particles between 2-5 μm in size, as determined by particle size analysis on a Malvern sizer.

7.3.6.2. Example 1: The enhancement in insulin plasma levels and insulin efficacy by its entrapment in the FA-based particles if the invention

7.3.6.2.1 *Animal studies*

Male Sprague Dawley rats with a body mass of between 240 and 336g were used as experimental *in vivo* model to investigate the absorption and efficacy enhancing capabilities of the current invention. Besides other advantages of this animal as model, the anatomical sequence and morphology of the animal's gastro-intestinal and nasal physiology and biochemistry show several similarities to that of the human.

In this study, insulin was directly administered into the stomach, ileum or duodenum of the animals. The experimental procedures of the *in vivo* method are well documented in the literature. Six animals were used for each group in the study. Rats were fasted 18 hours prior to drug administration but water was supplied *ad libitum*. The rats were kept under artificial conditions to create the ideal environment for the optimum growth and health of the animals. Infection with pathogen organisms was also minimised and variables were kept constant. The conditions under which the rats were bred and kept were 21°C with a relative humidity of 55%, a light intensity of between 350-400 lux one meter above floor level with light cycles of 12 hours light and 12 dark and 18 air changes per minute.

The study has a parallel design: the experimental animals were arranged in various test groups according to the different treatments and each animal received a single treatment. The control group received a single dose of insulin in saline solution to determine the absorption without the presence of any enhancing agent. Subcutaneous injection of insulin in one group was used to validate assaying procedures. The normal rat glucose and insulin profile was determined in a group that received only saline; this group acted as biological reference.

Recombinant human insulin was obtained from Sigma-Aldrich (South Africa). Insulin was entrapped in the FA-based vesicles or microsphere preparations of the invention, prepared as described above, according to the concentration and volume of formulation required. Before entrapment the FA-based preparations were heated to 31°C in a waterbath. After addition of the

insulin these formulations were shaken gently for 30 minutes to allow the insulin to be entrapped in the FA preparation. After entrapment the formulations were kept at 4°C until administration. A dose of 50IU/kg was administered in each of the test and control groups, except for the insulin that was administered intravenously, the dose of which was 0.5IU/kg per animal, while that for subcutaneous administration was 4IU/kg.

Cannulation of the *artery carotis communis*, ensured that sufficient blood volumes from the same rat at different time intervals could be obtained for analysis. Anaesthesia was induced by halothane and lasted for ± 3 hours. All surgical procedures necessary for the cannulation of the *carotis communis* were carried out while the rats were under anaesthesia. 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. Anaesthesia was induced in each rat by their inhalation of a concentration of 4.0% v/v liquid halothane (Fluothane®, Zebeca SA (Pty) Ltd, Woodmead, RSA) in a closed glass container. The rats were removed from the container upon loss of consciousness. Anaesthesia was maintained by alternate use of 2.0 and 4.0% halothane and medical oxygen. 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 consciousness, by deepening the anaesthesia with the 4.0% halothane until breathing ceased.

Abdominal surgical procedures were performed under the same conditions. The skin of the ventral abdomen was shaved and disinfected. A midline abdominal incision (laparotomy) of approximately 2 cm was made through the *linea alba caudal* to the sterno without cutting the intestines. The stomach, ileum or colon was identified and lifted out of the incision for administration of the insulin formulations where after it was replaced in the abdominal cavity in its correct anatomical position. The incision was covered with sterile gauze and kept moist with a saline-heparin solution to prevent dehydration.

The relevant formulation was injected directly into the specific area. Intra-gastric injections were made into the lumen of the stomach, after which the stomach was ligated at the start of the duodenum to ensure that the formulations were not transported to the small intestine by peristaltic movement. Intra-ileal administrations were made directly into the lumen of the small intestine, 7 cm from the stomach exit into the intestines. The small intestine was not ligated to ensure normal absorption of insulin with normal transit times. Intra-colonic administrations were made directly into the colon. The colon was ligated at the proximal end to ensure that

formulations did not pass back into the ileum. All administrations were done gently and slowly to prevent any spillage.

In the case of the groups receiving intravenous injections, a single administration with a volume of 200 μ l/body weight containing 0.5IU/kg was made into the tail of each rat. The efficacy of recombinant human insulin in rats was hereby confirmed and the relative bioavailability of the test formulations could be calculated with this group as reference. Subcutaneous injections were made just beneath the abdominal skin in volumes of 300 μ l/250g of body weight, resulting in a dose of 4IU/kg, which seemed comparable with that of commercial preparations.

Blood samples consisting of one ml of blood were collected at 0, 5, 10, 15, 30, 60, 120 and 180 minutes after drug administration for the determination of blood glucose values and insulin plasma levels. Blood glucose levels are a reflection of the therapeutic efficacy of each formulation. Blood glucose levels were measured with a Glucometer® II reflectance meter. A single drop of blood was applied to a Glucostix® reagent strip (Bayer, South Africa), blotted after 30 seconds and the glucose in mmol/l was obtained after 20 seconds.

Plasma insulin levels of the plasma samples were determined by the quantitative measurement of human insulin in the collected plasma using a human specific radioimmunoassay (RIA) kit obtained from LINCO Research, USA. The specificity of the human insulin specific RIA was stated to be 100% for human insulin and 0.1% for rat insulin, with no cross-reactivity with human pro-insulin. The limit of detection of the kit was 2 μ IU/ml.

7.3.6.2.2 Results

Intra-gastric administrations

The enhancement in the absorption of insulin by entrapment in the particles of the invention is illustrated in Table 7.1. Table 7.1 shows the average plasma levels found for two experimental groups of animals consisting of 6 rats each, and each of which have received a single administration of 50IU/kg insulin in the indicated form. The times at which the blood samples were collected are indicated.

Table 7.1: Intra-gastric plasma levels

Time	Insulin/saline (μ IU/ml)	FA vesicles (μ IU/ml)
0	12.95	8.28
5	19.09	49.37
10	15.29	18.85
15	11.1	19.13
30	13.09	19.57
60	11.8	16.06
120	10.48	26.48
180	13.51	32.56
Parameter	Insulin/saline	FA vesicles
C_{\max} (μ IU/ml)	19.09	49.37
T_{\max} (minutes)	5 minutes	5 minutes
AUC	2175	4282
Enhancement in AUC		96.873563%
Enhancement in C_{\max}		158.62%

The vesicles of the invention thus aided the absorption of insulin and maintained a higher concentration of insulin in the blood through the course of the 3-hour experiment. The initial absorption is reflected by the T_{\max} at 5 minutes for both groups, but the results seem to indicate that insulin is protected in the blood against degradation by entrapment into the vesicles and gradually released, as levels appear to still be rising after 3 hours, whereas in the absence of vesicles, insulin levels seem to be at base level. As a result of the still rising trend, the absolute bioavailability as found after intravenous administration of insulin, cannot be accurately calculated but when corrected for dosage the absolute bioavailability is at least doubled by entrapment into the vesicles of the invention. The increase in relative bioavailability and therapeutic levels by the vesicles of the invention are indicated by the enhancement in AUC and C_{\max} respectively.

The enhancement in therapeutic efficacy was measured by determining the effect of the various administrations on the blood glucose levels.

Table 7.2: Decrease in the % of blood glucose levels		
Parameter	Insulin/saline	FA vesicles
T_{\max} (minutes)	30	15
C_{\max} ($\mu\text{IU/ml}$)	4.1	15.3
AUC	275.5	613.5
Enhancement AUC		122.686%
Enhancement C_{\max}		273.1707%

The results portrayed in Table 7.2 confirm that the enhancement in the therapeutic efficacy by the invention is larger than that in the relative absorption/bioavailability (compare enhancement of AUC= 96.8762% in Table 1 and 122.68% in Table 2). This supports the hypothesis that entrapment of insulin in the vesicles is protecting the insulin from proteolytic degradation in the plasma.

Intra-ileal administrations

Results obtained from blood plasma insulin levels after intra-ileal administration of *insulin* in 0.9% saline and entrapped in the vesicles of the invention are reflected in table 7.3. These results indicate that the ileum presents with ideal characteristics for optimum insulin absorption. Compared to the ileum, the stomach did not provide as much insulin absorption. A vast increase of blood plasma insulin levels of up to 243.8 $\mu\text{IU/ml}$ is reached after 5 minutes with vesicle-entrapped insulin, compared to the 39.3 $\mu\text{IU/ml}$ of the same dose in saline.

Table 7.3: Intra-ileal plasma levels		
Time	Insulin/saline ($\mu\text{IU/ml}$)	Insulin/FA vesicle ($\mu\text{IU/ml}$)
0	20.83	7.22
5	37.73	241.96
10	33.16	172.58
15	15.49	47.605
30	19.86	35.0775
60	19.49	26.084
120	14.9	26.236
180	13.88	25.7675

Parameter	Insulin/saline	FA vesicles
AUC	569.1	4178
T _{max}	5	5
C _{max}	37.73	242.8
% enhancement AUC		634.1416%
% enhancement C _{max}		541.3994%

Table 7.4 shows that the enhanced therapeutic efficacy observed for vesicle-entrapped insulin after intra-gastric administration is also present after intra-ileal administration. Again the results confirm that the enhancement in the therapeutic efficacy by the invention is larger than that in the relative absorption/bioavailability (compare enhancement of AUC= 634.1416% in Table 3 and 42774.73% in Table 7.4). The protection of insulin from proteolytic degradation by entrapment of insulin in the vesicles in the plasma is clear.

Table 7.4: Decrease in the % of blood glucose levels		
Parameter	Insulin/saline	FA vesicles
T _{max} (minutes)	10	10
C _{max} (μIU/ml)	4.0	42.3
AUC	13.81	5921
Enhancement AUC		42774.73%
Enhancement C _{max}		957.50%

The enhancement in therapeutic efficacy by entrapment in the vesicles of the invention is 437.7 times. A comparison between the AUC's observed after intravenous insulin administration and intra-ileal administration can be used to determine absolute therapeutic efficacy. The absolute therapeutic efficacy of the insulin entrapped in vesicles was found to be 0.69 times of that observed after IV administration and 0.72 times that of subcutaneously administered insulin. Whilst the therapeutic efficacy of the vesicle-entrapped insulin is still somewhat lower than that of parenteral administrations, no glucagon response was observed for this therapy over the period of the study, with the % blood glucose levels staying under 100%, whilst a glucagon or hyperglycaemic response was observed with both the parenteral administration routes.

Intracolonic administration

Entrapment of insulin in vesicles led to enhanced insulin plasma levels (1.63 times) and therapeutic response (7.8 times) when compared with the insulin in saline administration.

7.3.6.2.3 Conclusion

The entrapment of insulin into the vesicles of invention gave a near ideal insulin response, resulting in sufficient therapeutic efficacy but no hyperglycaemia.

7.4 Claims of the invention

The following is claimed for invention WO2009004595 20090108:

1. A formulation for the administration of at least one therapeutic mammalian protein to a mammal, and for enhancing the absorption, distribution and release of the at least one therapeutic mammalian protein in or on the mammal, the formulation consisting of at least one therapeutic mammalian protein in a micro-emulsion which micro-emulsion is constituted by a dispersion of vesicles or microsponges of a fatty acid based component in an aqueous or other pharmacologically acceptable carrier in which nitrous oxide is dissolved, the fatty acid based component comprising at least one long chain fatty acid based substance selected from the group consisting of free fatty acids and derivatives of free fatty acids.
2. A formulation for the administration to a mammal of at least one protein selected from the group consisting of insulin, parathyroid hormone, parathyroid-like hormone, glucagon, insulinotropic hormone, vasopressin and hormones involved in the reproductive system, chemotactins, cytokines including interleukins 1, 2 and RA but excluding the interferons, chemokines, enzymes including proteases and protease inhibitors, growth factors including acidic and basic fibroblast growth factors, epidermal growth factor, tumor necrosis factors, platelet derived growth factor, granulocyte macrophage colony stimulating factor, neurite growth factor and insulin-like growth factor-1, hormones including the gonadotrophins and somatomedians, immunoglobulins, lipid-binding proteins and soluble CD4, urokinase, streptokinase, superoxide dismutase (SOD), catalase, phenylalanine ammonia lyase, L-asparaginase, pepsin, uncase, trypsin, chymotrypsin, elastase, carboxypeptidase, lactase, sucrase, ciliary neurite transforming factor (CNTF), clotting factor VIII, erythropoietin, thrombopoietin, insulintropin, cholecystikinin, glucagon-like peptide I, intrinsic factor, Ob gene product, tissue plasminogen activator (tPA), brain-derived neurite factor,

- phenylalanine transporter, brush border enzymes and transporters, and for enhancing the absorption, distribution and release of the at least one protein in or on the mammal, the formulation consisting of the at least one protein in a micro-emulsion which micro-emulsion is constituted by a dispersion of vesicles or microsponges of a fatty acid based component in an aqueous or other pharmacologically acceptable carrier in which nitrous oxide is dissolved, the fatty acid based component comprising at least one long chain fatty acid based substance selected from the group consisting of free fatty acids and derivatives of free fatty acids.
3. A method for the effective delivery of at least one therapeutic mammalian protein to a mammal and for enhancing the therapeutic efficacy of such at least one therapeutic mammalian protein, the method comprising the step of administering the at least one therapeutic mammalian protein to the mammal in a formulation consisting of the at least one therapeutic mammalian protein in a micro- emulsion constituted by a dispersion of vesicles or microsponges of a fatty acid based component in an aqueous or other pharmacologically acceptable carrier in which nitrous oxide is dissolved, the fatty acid based component comprising at least one long chain fatty acid based substance selected from the group consisting of free fatty acids and derivatives of free fatty acids.
 4. A method for the effective delivery to a mammal of at least one protein selected from the group consisting of insulin, parathyroid hormone, parathyroid-like hormone, glucagon, insulinotrophic hormone, vasopressin and hormones involved in the reproductive system, chemotactins, cytokines including interleukins 1 ,2 and RA but excluding the interferons, chemokines, enzymes including proteases and protease inhibitors, growth factors including acidic and basic fibroblast growth factors, epidermal growth factor, tumor necrosis factors, platelet derived growth factor, granulocyte macrophage colony stimulating factor, neurite growth factor and insulin-like growth factor-1 , hormones including the gonadotrophins and somatomedians, immunoglobulins, lipid-binding proteins and soluble CD4, urokinase, streptokinase, superoxide dismutase (SOD), catalase, phenylalanine ammonia lyase, L-asparaginase, pepsin, uncase, trypsin, chymotrypsin, elastase, carboxypeptidase, lactase, sucrase, ciliary neurite transforming factor (CNTF), clotting factor VIII, erythropoietin, thrombopoietin, insulintropin, cholecystokinin, glucagon-like peptide I, intrinsic factor, Ob gene product, tissue plasminogen activator (tPA), brain-derived neurite factor, phenylalanine transporter, brush border enzymes and transporters, and for enhancing the absorption, distribution and release of the at least one protein in or on the mammal, the method comprising the step of administering the at least one protein to the mammal in a formulation consisting of the at least one protein in a micro- emulsion which micro-emulsion is constituted by a

dispersion of vesicles or microsponges of a fatty acid based component in an aqueous or other pharmacologically acceptable carrier in which nitrous oxide is dissolved, the fatty acid based component comprising at least one long chain fatty acid based substance selected from the group consisting of free fatty acids and derivatives of free fatty acids .

5. The formulation of claim 1 or 2 or method of claim 3 or 4 wherein the composition also contains the antioxidant dl- α -tocopherol or a stable derivative thereof.
6. The formulation of claim 1 or 2 or method of claim 3 or 4 wherein the composition includes a protease inhibitor.
7. The formulation of claim 1 or 2 or method of claim 3 or 4 wherein the dispersion is characterized in that at least 50% of the vesicles are of a diametrical size of between 80 nanometer and 3 μ m and that of the microsponges between 1.5 and 6.0 μ m.
8. The formulation of claim 1 or 2 or method of claim 3 or 4 wherein the fatty acid based component is selected from the group consisting of oleic acid, linoleic acid, alpha-linolenic acid, gamma-linolenic acid, arachidonic acid, eicosapentaenoic acid [C20 5 ω 3], decosahexaenoic acid [C22 6 ω 3], and ricinoleic acid, and derivatives thereof selected from the group consisting of the C₁ to C₆ alkyl esters thereof, the glycerol-polyethylene glycol esters thereof, and the reaction product of hydrogenated and unhydrogenated natural oils composed largely of ricinoleic acid based oils with ethylene oxide.
9. The formulation of claim 1 or 2 or the method of claim 3 or 4 wherein the fatty acid component of the micro-emulsion is constituted by the mixture of esterified fatty acids known as Vitamin F Ethyl Ester.
10. The formulation of claim 1 or 2 or the method of claim 3 or 4 wherein the dispersion is in the form of microsponges and is constituted by very long chain polyunsaturated fatty acids selected from eicosapentaenoic acid [C20:5 ω 3] and decosahexaenoic acid [C22:6 ω 3] or a mixture of both.
11. The formulation or method of claim 10 wherein the fatty acid component further includes the reaction product of hydrogenated natural oils composed largely of ricinoleic acid based oils with ethylene oxide or modified derivatives thereof.
12. The formulation of claim 1 or 2 or method of claim 3 or 4 wherein the protein is insulin.
13. The formulation of claim 12 which is adapted for nasal administration.
14. The formulation of claim 12, which is adapted for oral administration

7.5 National phases

The patent application has not yet progressed to the national phases in any country.

7.6 Notices and documents under the PCT

No documents besides the original provisional application (available as .pdf file on the WIPO web site), and the subsequent formal submission have as yet been taken up into the notices and documents of the application.

7.7 Additional studies supporting the patent application

For the sake of completeness, and to avoid confusion regarding the differences between the provisional and final applications, the studies included in the provisional patent application and their results are summarized below. The study on the topical administration of the hormone arginine vasopressin is an example of the co-entrapment of a protease inhibitor (bestatin) into the PheroidTM with the aim of preventing degradation.

7.7.1 Transdermal delivery of arginine vasopressin with FA-based vesicles of the invention

The stratum corneum is known to be a nearly impenetrable barrier (Prausnitz, 1997), resulting in a considerable amount of resistance against percutaneous absorption of most substances. Protein or pharmaceuticals generally illustrate poor penetrability due to their large molecular sizes and relatively hydrophilic nature (Crommelin *et al.*, 2002, Pettit and Gombotz, 1998).

In order to test the feasibility of transdermal delivery of macromolecules, the peptide hormone arginine vasopressin (AVP) (MW = 1084.23 Da) was used as a model compound. AVP is regarded as a relative 'small' macromolecule and represents peptides in the molecular weight range of 1000-1500 Da. It is an endogenous neurohypophyseal, nonapeptide hormone and is commonly utilised in the diagnosis and therapy of *diabetes insipidus* and nocturnal enuresis in the synthetic form of *l*-deamino-8-D-arginine-vasopressin (DDAVP or desmopressin).

Previous studies on the transdermal absorption and/or delivery of arginine vasopressin involving AVP used iontophoresis at low currents and chemical enhancers in low quantities in tandem to circumvent any potential adverse reactions, toxicity and irreversible structural changes (Nair and Panchagnula, 2004 a, Lelawongs *et al.*, 1989, 1990). Other transdermal studies that involved AVP as the model peptide focused on the effects of pH and concentration, proteolytic enzyme inhibitors, the stability of AVP and its degradation (Bi and Singh, 2000, Banga *et al.*, 1995 and Morimoto *et al.*, 1992).

Bestatin is a potent, competitive and specific aminopeptidase inhibitor with an affinity for leucine aminopeptidase (LAP), aminopeptidase B (APB) and tri-aminopeptidase. Bestatin has been shown to exhibit antitumor as well as antimicrobial activity, but is also known to act as an immune response modifier and analgesic by enkephalinase inhibition (De *et al.*, 1997). Bestatin hydrochloride was used in the present study to selectively inhibit aminopeptidases present inside and on the surface of the skin, which could potentially degrade the hormone.

In this study the comparative *in vitro* transdermal transport of arginine vasopressin (AVP) across human skin was investigated. Hepes (4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid) at pH 5.5 was used as the receptor phase in the transdermal diffusion studies and as the solvent for all solutions prepared. It was also utilised as the aqueous phase in the manufacturing of the FA-vesicles of the present invention.

7.7.1.1 Study procedures

Abdominal skin was obtained from Caucasian female patients after cosmetic surgery and frozen at -20 °C not longer than 24 hours after removal (Leveque *et al.*, 2004). Ethical approval for the procurement and utilisation of the skin was provided by the Research Ethics Committee of the North-West University (reference number 04D08). To prepare the skin for transdermal diffusion studies, the skin was thawed at room temperature, remaining blood wiped off and excess adipose tissue removed. Epidermal layers were separated by immersing the skin for 1 min in water at 60 °C (Blank and McAuliffe, 1985; Bronaugh and Collier, 1991). The epidermal layer was gently flayed from the underlying tissue, taking care to maintain the integrity of the stratum corneum. Skin sections with the stratum corneum side of the skin facing upwards were floated on top of Whatman® filter paper and then left to air dry. These samples were wrapped in aluminium foil, sealed in plastic bags and kept frozen at -20 °C until utilisation. Before initiating a diffusion study, the frozen pieces of skin were thawed at room temperature and examined for defects. The epidermal skin layer was cut into circles (± 10 mm in diameter) and mounted with the stratum corneum facing the donor half-cell of the lower half of vertical Franz diffusion cells (PermeGear Inc., Bethlehem, PA, USA) with a receptor capacity of approximately 2 ml and a 1,075 cm² diffusion area. A single source of skin was employed for each diffusion study to minimise variation between skin samples. The donor compartment was placed on top of the lower half, with the epidermal layer acting as seal between the two halves, sealed with high vacuum grease and fastened with a metal clamp. After filling both compartments of the diffusion cells with physiological saline, cells were equilibrated for 1 hour in a water bath held at a constant temperature of 37 ± 0.5 °C (Cleary, 1993) giving a membrane temperature of 32 ± 1.0 °C. The integrity of the skin was ascertained with the aid of a Model 6401 LCR Databridge (H. Tinsley, Inc., Croydon, Surrey, UK) set in the resistance mode, in the parallel equivalent circuit

mode and with an alternating current frequency of 1000 hertz (Hz) (Fasano *et al.*, 2004). Impedance measurements taken in donor and receptor compartments simultaneously are an indication of the relative integrity of the skin sample and were taken before and after completion of the diffusion study. Physiological saline and not Hepes buffer was used in the integrity assessments as Hepes buffer ions have a lower mobility through skin during iontophoresis compared to the major counter ion chloride (Nair and Panchagnula, 2003c). The contents of the receptor phases were stirred with a magnetic stirrer bar for the duration of the study. The compartments were emptied after conclusion of the impedance measurements.

The receptor phase, Hepes buffer (0.025 mM, pH = 5.5±0.5), was sonicated for 15 min to remove air bubbles and avoid the build-up of air pockets (Du Plessis *et al.*, 2002), and heated to 37 °C. The receptor compartment was filled with the buffer before adding the drug-containing solution to the donor compartments, ensuring that no air bubbles were trapped in the receptor compartment or underneath the skin. To initiate an experiment, the donor compartment of each cell was charged with 1000 µl (1 ml) of either an aqueous solution of the active in Hepes buffer or the drug dissolved in the FA vesicles, depending on the experiment, and immediately covered with Parafilm® to prevent any liquid from evaporating. At predetermined intervals (0.5; 1; 1.5; 2; 4; 6; 8; 10; and 12 hours), the entire content of the receptor compartment was withdrawn, and replaced with fresh 37°C Hepes buffer to mimic sink conditions. One hundred microlitres (100 µl) of each sample was directly assayed by high-performance liquid chromatography (HPLC) to determine the drug concentration in the receptor fluid.

[⁸Arg]vasopressin (AVP) (acetate salt, MW = 1084.23) was entrapped in the vesicles of the present invention at a concentration of 150 µg/ml AVP for approximately 30 minutes at room temperature. This formulation was stored at 2-8 °C for 24 hours before commencement of an experiment. Entrapment of the peptide in the vesicles was confirmed with the aid of confocal laser scanning microscopy (CLSM) on a Nikon PCM 2000 CLSM, using a medium (10µm) pinhole and a 60x, 1.4D ApoPlanar oil immersion objective. The microscope was equipped with a green krypton laser (wavelengths: excitation 488 nm, emission 515 nm) and a red helium/neon laser (wavelengths: excitation 505 nm, emission 564 nm). AVP was labeled with the reactive dye Alexa Fluor® 430 and the particles of the invention with Nile red according to the instructions of the manufacturer (Invitrogen, Leiden, Netherlands). Alexa Fluor® 430 exhibits maximum fluorescence emission at 540 nm while Nile red labeled particles had an emission wavelength of between 640 and 650 nm. The entrapment efficiency could thus be monitored. The reference solution contained 150 µg/ml AVP dissolved in Hepes buffer at a concentration of 25mM. When included, the concentration of bestatin hydrochloride in both the reference and test formulations was 300 µg/ml.

To determine the amount of AVP transported across the skin epidermis, high-performance liquid chromatography (HPLC) analyses of AVP found in the receptor phases were performed. The method was developed and validated in conjunction with Coetzee (2007) and Prof J du Preez from the North-West University, Potchefstroom Campus, South Africa. An Agilent 1100 series HPLC equipped with a gradient pump, autosampler and diode array UV detector was interfaced with Chemstation Rev. A.08.03 data acquisition and analysis software. A reversed phase chromatography column (Macherey-Nagel LiChrospher® 100 RP18 ec column; 4 mm x 250 mm, 5 µm particle size, pore size 100 Å, endcapped), a mobile phase of 100% acetonitrile (ACN) and the aqueous phase of 0.1% trifluoroacetic acid (TFA) in HPLC grade water was used. Injection volume was set at a default value of 100 µl. The following gradient elution was used: 5 % ACN up until 2 minutes, then a linear increase in ACN to reach 80 % after a further 8 minutes. Stop time was at 10 minutes and a 4-minute post time allowed the instrument to return to the initial ACN concentration. The preservation time of AVP was approximately 7.3-7.5 minutes and that of bestatin approximately 8.2-8.5 minutes. The flow rate was constant at 1 ml/min and analyses were performed at ambient room temperature (25±1 °C). The DA detector was used to detect the absorbance of the effluents at a wavelength of 210 nm.

The cumulative amount of AVP permeated per unit time skin area was plotted against time. With the possible exception of the passive flux, the plots exhibited biphasic character, thus the slopes of the linear portions of the plots between zero and two hours, as well as two and twelve hours, were estimated as the steady-state fluxes for the two time periods. The yield of each cell was depicted as a percentage of the applied concentration and based on these values, data of cells with yield values of 2% and less for arginine vasopressin and values of 20% and less for bestatin were selected for inclusion in the dataset. All the results were expressed as mean ± S.D.

7.7.1.2 Results and discussion

CLSM analysis confirmed entrapment of arginine vasopressin within the vesicles of the invention. The *in vitro* permeation of AVP with the aid of the FA-based vesicles was investigated in the absence and presence of the aminopeptidase inhibitor bestatin. It was also compared to the control (permeation of AVP in combination with bestatin in Hepes buffer) and the passive flux (AVP in Hepes buffer). The average *in vitro* permeation profiles of AVP under the different circumstances are shown Table 5. Only the data of cells with an AVP yield of 2% and less were included. The fluxes of each of the groups assayed were obtained from at least 6 diffusion cells and only means are portrayed. For example, AVP in FA vesicles in table 7.5 represents the mean flux determine from 18 cells, AVP (+bestatin) in Hepes buffer group from 6 cells and AVP (+bestatin) in FA vesicles group from 21 cells.

The transport of AVP across the prepared skin exhibited a biphasic character, with the first phase from time zero to two hours, and the second from time two to twelve hours. Flux was calculated for two different periods of time: $t = 0 - 2$ hours and $t = 2 - 12$ hours. The majority of AVP flux seemed to take place during the first two hours of diffusion.

Table 7.5: Fluxes for AVP in buffer and entrapped in FA vesicles plus/minus bestatin

Donor phase	Flux ($\mu\text{g/ml/h}$): 0 – 2 hours	Flux ($\mu\text{g/ml/h}$): 2 – 12 hours	Total flux
AVP in Hepes buffer	0.0374	0.0175	0.0549
AVP in FA vesicles	0.1495	0.0376	0.1871
AVP (+bestatin) in FA vesicles	0.2182	0.0575	0.2692

The vesicles of the present invention significantly increased the flux of AVP when compared to the observed passive flux. With the inclusion of bestatin, the flux increased even more. Without bestatin, the AVP flux approaches steady-state, indicating a decline in AVP permeation. The biphasic character of the flux may be ascribed to gradual depletion of the AVP after two hours or, in the presence of bestatin, the depletion of bestatin and the consequential decline in AVP flux. It is also possible that the proteolytic enzymes, aminopeptidase and trypsin, might diffuse through the skin concomitantly with the AVP and degrade the active while in the receptor phase.

The results thus indicate that entrapment of AVP in the vesicles of the invention is capable of enhancing delivery of a peptide to the skin *in vitro* at least 2.4 times.

7.7.2 Intranasal administration of protein/peptide drugs

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. Using nasal dosage forms, the risk of overdosing is reduced and self-medication is possible through this route. Furthermore, there is a reduced risk of infection and of infectious disease transmission due to the fact that this is a non-invasive route of administration. It is 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.

Nasal administration and intravenous administration may exhibit very similar concentration-time profiles, suggesting that a rapid onset of pharmacological activity is possible after nasal administration (Hussain, 1998). The bioavailability of various active compounds has been further improved when incorporated in mucoadhesive dosage forms, probably because the residence time of drug carriers at the absorption site is prolonged. This it is possible to formulate sustained release dosage forms that incorporate Pheroid™. The two main barriers for drug permeation in the nasal cavity are the enzymes present and the nasal mucosal lining (Ugwoke *et al.*, 2005). 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.

7.7.2.1 *Physicochemical properties of administered drug and formulations and biological factors that can affect nasal absorption and permeability*

Any invention that has as its aim the enhancement of the efficacy of nasally administered drugs therefore need to take cognizance of at least some of the factors that may influence the nasal permeation and/or absorption of the drug (Merkus *et al.*, 1996). The following physicochemical and biological factors bear a relevance to successful intranasal administration of the therapeutic proteins:

- (i) The bioavailability of compounds with a molecular weight (MW) higher than 1 kDa is low (0.5% - 5%). Without any enhancing factor, the bioavailability can be directly predicted from the MW of the molecule (Donovan & Huang, 1998). Drugs with an MW of less than 300 Da will mostly permeate through the aqueous channels of an epithelial membrane. The rate of permeation of compounds with an MW higher than 300 Da is highly sensitive to both molecular weight and size (Henderson *et al.*, 1988).
- (ii) Drug permeation is also determined by the lipophilicity or hydrophilicity of the specific drug: Lipophilic compounds show a direct relationship between the MW and drug permeation while an inverse relationship exists in the case of hydrophilic compounds. Other transport pathways might be of importance for hydrophilic drugs. The lipid domain plays an important role in the barrier function of the nasal mucosa. The mucosa appears to be primarily lipophilic by nature and although some hydrophilic characteristics are present, excess hydrophilicity leads to a decrease in the systemic bioavailability of some drugs (Corbo *et al.*, 1989).
- (iii) Appropriate aqueous solubility of a drug contributes to increased dissolution and enhances compatibility with the aqueous nature of the nasal secretions (Henderson *et al.*, 1988).

- (iv) The pH partition theory states that unionized species are absorbed much better than their ionized counterparts. A quantitative relationship exists between nasal absorption and the partition coefficient (Jiang *et al.*, 2008). An increase in either the partition coefficient or lipophilicity of a specific drug will generally increase the concentration of that specific drug in the cerebrospinal fluid. The nasal absorption of weak electrolytes is dependent on their degree of ionization and the pH of both the nasal surface and the formulation can affect a drug's permeation - the pH of nasal formulations should ideally be in the range of 4.5 - 6.5 avoid nasal irritation. The nasal pH should preferably be lower than that of the drug's pKa. The slightly acidic pH is also bactericidal to some extent (Rathbone *et al.*, 1994).
- (v) The contact time between the drug and the nasal mucosa increase with an increase in viscosity of the formulation. The ciliary beating and mucociliary clearance are affected by the administration of viscous formulations which in turn may also alter the permeability of the administered drug (Romeijn *et al.*, 1996).
- (vi) 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. The volume within which the compound is administered may influence the permeation of the drug, with over-saturation and/or rapid drainage from the nasal cavity identified as problematic. Only 25 - 200 μ l of drug solution can be administered into the nasal cavity. In the case of nasal sprays, solutions and suspensions are preferred to powder sprays that often cause mucosal irritation. Specialized systems such as lipid emulsions, microspheres, liposomes and pro-liposomes provide prolonged contact between the drug and the mucosal membrane, resulting in a better chance of permeation for the administered drug (Mitra *et al.*, 2000). A drug needs to be solubilized before it can permeate the nasal mucosa and its solubility in nasal secretions is thus an important factor. Nasal secretions contain 90% water, 2% mucin, 1% salts and approximately 1% proteins such as albumin, immunoglobulins, lysozyme, lacto ferrin, and lipids (Kalinin *et al.*, 1984).
- (vii) Pathological conditions such as mucociliary dysfunction, hypo- and hyper- secretion of mucus and irritation of the nasal mucosa, caused by conditions such as rhinitis and polyposis, may decrease permeation of drugs.
- (i) Physiological factors that may influence absorption of drugs after nasal administration include the pH of the nasal cavity, the nasal cycle or diurnal variation, blood supply

and neuronal regulation, mucociliary clearance and ciliary beating frequency and the area of the nasal mucus membrane exposed to the administered drug (Donovan & Huang, 1998; Corbo *et al.*, 1989). *The nasal cycle or diurnal variation* implies that circadian rhythms affect nasal secretions. Nasal clearance and secretion rates were found to decrease during the night thus altering drug permeation (Arora *et al.*, 2002). The viscosity 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 be impaired because contact with the cilia is lost. These variations alter the time of contact between the drug and the mucosa (Mortazavi & Smart, 1994). A reduction in mucociliary clearance enhances drug permeation while an increase in this clearance will generally lead to a decrease in drug permeation (Marttin *et al.*, 1998). Blood supply and neuronal regulation influence intra-nasal drug absorption: 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 (Chiba *et al.* 2007). Parasympathetic stimulation can thus lead to the increased permeability of a compound (Arora *et al.*, 2002).

7.7.2.2 Three therapeutic proteins as model drugs for intra-nasal administration: calcitonin, human growth hormone and insulin

Salmon calcitonin (sCT), used in the treatment of osteoporosis (Colman *et al.*, 2002), is poorly bioavailable after oral administration and requires the use of applicable delivery systems with absorption enhancing properties (Pontioli, 1998). Calcitonin, a natural polypeptide a hormone produced by parathyroids, regulates calcium concentrations in body fluids, lowers the serum calcium and inhibits osteoclast activity. In 1981 the FDA's Endocrinologic and Metabolic Drug Advisory Committee approved Calcimar® for treatment of postmenopausal osteoporosis contingent on a phase-4 study (Colman *et al.*, 2002). This phase-4 fracture study failed due to unreliable results but a calcitonin was subsequently registered for treatment of postmenopausal osteoporosis (Colman *et al.*, 2002) and Paget's disease.

Calcitonin is found in three therapeutic forms (Lee, 2000):

- porcine calcitonin is the most immunogenic and not suitable for long-term use,
- human calcitonin which is less potent although less immunogenic and
- synthetic salmon calcitonin suitable for use in long term treatment.

The molecular formula for human calcitonin is $C_{151}H_{226}N_{40}O_{45}S_3$ with a molecular weight of 2777.9 while salmon calcitonin has a molecular formula of $C_{145}H_{240}N_{44}O_{48}S_2$ (Zaidi *et al.*, 2002). The therapeutic protein consists of a single chain peptide of 32 amino acid residues with one disulfide bond at position 1 and 7. The complete sequence and the C-terminal proline amide are essential for full biological activity (Buttery *et al.*, 2002). The highly purified synthetic salmon calcitonin preparations have high potency and superior analgesic effects and are available in several dosage forms including injections, a nasal spray or suppositories. The potency is assigned in international units (IU), defined by the International Standard for sCT established by the World Health Organization (WHO) (Rafferty *et al.*, 2001).

Calcitonin is used in the treatment of Paget's disease associated with bone mineral loss such as hyperparathyroidism, malignancies and immobilisation and more specifically for its analgesic effect (Lee, 2000). Salmon calcitonin is indicated for treatment of some forms of hypercalcemia and osteoporosis in women who are more than five years past menopause and for whom oestrogen replacement therapy is not an option (Rafferty *et al.*, 2001:). The duration of action of calcitonin varies between 30 minutes and 12 hours after intravenous (IV) administration, while its effects last for 8-24 hours after subcutaneous or intramuscular (IM) injection (Lee, 2000). The adult dose for hypercalcaemia is 5-10 IU/kg/day in 2-4 divided doses for slow IV injection or as a single dose IV infusion diluted in 500 mL normal saline over 6 hours. The dose for Paget's disease is 100 IU subcutaneous or IM daily or every second day with a maintenance dose of 50-100 IU three times a day. For osteoporotic vertebral fracture, a daily dose of 100 IU for 2 weeks is administered subcutaneously (Lee, 2000). Calcitonin is rapidly metabolized in the kidneys, blood and peripheral tissues (Lee, 2000). Intramuscular preparations show poor relative bioavailability of salmon calcitonin (only 1.6 %).

The Ca^{2+} plasma levels regulate the biosynthesis and secretion of calcitonin: when Ca^{2+} levels are high, calcitonin secretion increases and when the Ca^{2+} levels are low, the calcitonin secretion is low. The normal circulating calcitonin levels are lower in women than in men with a circulating half-life of 10 min (Marcus *et al.*, 1996). The calcitonin gene encodes two precursors, one for calcitonin and the other for a related peptide, calcitonin gene-related peptide (CGRP). Through differential expression, calcitonin is expressed mainly in the thyroid gland, whereas CGRP is produced predominantly in the neural tissues (Buttery *et al.*, 2002). Various molecules, including catecholamines, glucagons, gastrin and cholecystokinin, stimulate calcitonin production (Marcus *et al.*, 1996).

The physiological role of calcitonin is not yet completely elucidated but calcitonin is thought to play a major role in the protection of the skeleton during calcium stress like growth, pregnancy and lactation (Buttery *et al.*, 2002). Calcitonin secretion results in a fall in plasma

calcium due to the acute inhibition of osteoclastic activity. Calcitonin modulates osteoclast activity by (a) a direct inhibition of the resorptive activity of mature osteoclasts and (b) by a progressive reduction in osteoclast numbers because of inhibition of osteoclast recruitment (Buttery *et al.*, 2002). A decrease of 60 % in bone resorption after treatment with 200 IU of salmon calcitonin has been reported (Stepan & Zikan, 2003). Calcitonin acts by binding to a G-protein-coupled cell surface receptor of the PTH-secretin receptor family, which results in the activation of either adenylate cyclase or phospholipase C. Several forms of the calcitonin receptors (CTR) are expressed in osteoclasts, the kidneys, and the brain (Buttery *et al.*, 2002). A decrease in bone resorption by salmon calcitonin depends on binding of the hormone receptors on the basolateral membrane of osteoclasts (Stepan & Zikan, 2003).

Lipophilic drugs are generally well absorbed from the nasal cavity and plasma levels are often typical to those obtained after an intravenous injection with bioavailabilities approaching 100%. However, despite the large surface area and extensive blood supply of the nasal cavity, the permeability of the nasal mucosa is normally low for polar molecules and large molecular weight molecules (Costantino, 2007). To enhance nasal absorption efficacy of calcitonin, various enhancers have been tried but without much success (Sinswat & Tengamnuy, 2003).

The therapeutic protein: human growth hormone (hGH) and Somatropin (rhGH)

Human growth hormone (hGH) is a large fragile protein that acts on many different tissues in order to promote a healthy metabolism (Cenegenics Medical Institute, 2005). It is produced by the anterior pituitary gland and consisting of 191 amino-acids, it has a molecular weight of $\pm 22\,000$ Da. The effects of growth hormone are mediated predominantly by insulin-like growth factors 1 and 2 (IGF-1 and IGF-2) or the so called somatomedins, produced by most body tissues in response to an increase in growth hormone levels. IGF-1 is released in response to the presence of hGH (Cenegenics Medical Institute, 2005). The only sources of safe and accurately assembled hGH are those that use recombinant DNA technology for production (Cenegenics Medical Institute, 2005).

Somatropin ($C_{990}H_{1528}N_{262}O_{300}S_7$) is a synthetic hGH, also known as recombinant human growth hormone (rhGH). It is of the same size with the same amino acid sequence, and is similar in conserved sequences to that of human growth hormone (Hansen, 2002). Somatropin stimulate soft tissue and skeletal growth by promoting cell division, protein synthesis and the uptake of amino-acids (Thorner, 1990). 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 (Ballard *et al.*, 1987:398-404, Ihle,

1996: 23-65). The metabolisms of hGH and rhGH are similar (Rosenfeld *et al.*, 1982:202) and hGh and rhGH are henceforth used interchangeably.

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. It is currently administered by daily injections which are both difficult to administer and painful for the patient (Laursen *et al.*, 1996). It is therefore important to explore alternative routes of administration. The nasal delivery of hGH would thus offer many advantages in patient compliance due to easier administration, the elimination of injection pain, and the ease of dosage adjustment or manipulation in the case of children. Administration of hHG is followed by an immediate transient period of hypoglycemia in growth hormone deficient patients but not in normal subjects (Wilton & Sietniks, 1987); 2-4 hours after administration anti-insulin-like actions, such as an increase in serum free-fatty acid levels due to the inhibition of glucose utilization and lipolysis, are observed. In growth hormone deficient children this lipolytic effect is reflected by a loss of subcutaneous fat during the early months of growth hormone treatment (Hansen, 2002).

The physiological secretion of growth hormone is nocturnal: it occurs in a pulsatile manner approximately every 2 - 3 hours at a rate of 0.6 - 1.5 mg in a 24 hour cycle in young men. This equivalent to $1.3 - 3.0 \text{ IU} \cdot 24\text{h}^{-1}$ hGH (Phillip *et al.*, 1996). Optimal growth is probably achieved by simulation of the natural physiological growth hormone pulse frequency and dosing for adult growth hormone deficient patients is best determined by dose titration against clinical characteristics and serum IGF-1 levels (Hansen, 2002). A total weekly dose of $>0.5 - 0.7 \text{ IU} \cdot \text{kg}^{-1}$ or alternatively a dose of $12 \text{ IU} \cdot \text{m}^{-2}$, divided into daily or three-times-weekly doses, administered via subcutaneous or intramuscular injection is recommended. 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. With nasal administration it may be possible to mimic the normal endogenous pulsatile hGH secretory pattern more closely (Ugwoke *et al.*, 2005).

Less obvious pathological conditions, such as AIDS-related wasting / cachexia, may be treated with hGH. Some of the biochemical changes associated with aging are also associated with a progressive decline in the natural levels of hGH and IGF-1 and correcting hGH and IGF-1 levels can delay age-related dysfunction in some organs. Modulation of hGH and IGF-1 can contribute to enhanced skin elasticity and thickness, decreased total body fat and increased lean muscle mass, improved blood flow to the kidneys, increased bone mineral density, decreased LDL cholesterol levels and increased HDL cholesterol levels, enhanced healing, improved energy levels and improved exercise capacity and over-all well-being (Cenegenics Medical Institute, 2005). Most of the compositional changes, such as fat loss or muscle or bone

gain, will probably be observed only after 3 - 11 months of therapy (Cenegenics Medical Institute, 2005).

Whilst pathologies caused by mutations of the hGH gene may be alleviated by the administration of exogenous hormone, similar pathologies caused by mutations of the gene for the growth hormone receptor, such as Laron type dwarfism, will not respond to hGH or rhGH treatment. The expression of specific genes such as somatostatin, growth hormone-releasing hormone, IGF-1, albumin and myosin heavy chains can be directly influenced by hGH (Möller *et al.*, 1994). hGH exerts its action by binding to specific GH receptors, which bears some relation to the cytokine receptor family (Ihle, 1996). Receptor expression is enhanced by insulin and sex steroids but reduced by fasting and renal insufficiency. The receptors with a half-life of around 45 minutes, are found in fibroblasts, the gastrointestinal tract and the brain, osteoblasts and chondrocytes, adipocytes and hepatocytes (Möller *et al.*, 1994).

Somatropin is mainly administered by means of subcutaneous and intramuscular injection. Considerable variation exists between individuals with respect to both magnitude and timing of the rise in serum somatropin levels but peak serum levels are generally achieved 2 - 8 hours after injection and return to baseline after 8 - 16 hours in man (Wilton & Sietniks, 1987; Albertsson-Wikland, 1976; Johansson, 1998). Although higher levels of the hormone reach the systemic circulation after intramuscular injection compared to subcutaneous injection, 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, 1976; Johansson, 1998). There is no significant difference in the observed metabolic effects of subcutaneous and intramuscular injection (Jorgensen *et al.*, 1987). Generally, rhGH is not absorbed in an active form from the gastrointestinal tract due to the enzymatic degradation of the active compound.

Somatropin is unstable when exposed to water, especially at physiological conditions of pH 7.4 and 37 °C. Aggregation and decomposition of concentrated solutions at physiological temperatures result in irreversible aggregation, rapid destruction of intact protein, and loss of its biological activity (Foster, 1999). A prerequisite for the successful development of a somatropin administration system is to find a way to stabilize the hormone as hydration of the administration system will also result in hydration of the protein, causing subsequent aggregation through formation of disulfide cross-links, isopeptide bonds and hydrophobic interactions (Schulga *et al.*, 2002). Somatropin may be stabilized by precipitation with bivalent ions such as zinc or copper (Franklin & Geffner, 2009). Such bivalent ion-precipitated somatropin could be combined with a biocompatible oil or a reservoir could be formed with one to several layers of polymers, such as paraffin or cellulose acetate (Franklin & Geffner, 2009).

Information on the therapeutic protein insulin is incorporated in the formal patent application.

7.7.3 Nasal delivery of calcitonin

In this study the absorption enhancing properties of the FA-based vesicles and microsponges of the invention, when administered with calcitonin was studied using the nasal route. Calcitonin was entrapped into the fatty acid-based formulations of the invention and administered nasally in rats. Plasma levels of calcitonin and plasma calcium levels were obtained to evaluate the effect of the entrapment of calcitonin in FA based formulations on its nasal permeability. These permeation properties were compared to permeation profiles obtained from commercially available preparations. The following section describes the experimental methods used in this study.

7.7.3.1. Animal studies

Calcitonin was obtained from Merck (South Africa). 750 µg of calcitonin was entrapped in 45 ml of either a FA vesicle formulation prepared as described above or a similar volume of FA microsphere formulations prepared as described above. Before entrapment, the FA-based preparations were heated to 31 °C in a water-bath. After addition of the calcitonin, these formulations were shaken gently for 30 minutes and kept on ice until administration. The final preparations contained the equivalent amount of calcitonin of 10 IU/kg body weight when administered in a volume of 100 µl/kg body weight. The control solution was prepared in the same way by dissolving 750 µg of calcitonin in 45 ml phosphate buffer solution (PBS; pH 7.3). All solutions were freshly prepared before nasal administration.

The morphology and approximate size and of the vesicles and microsponges in the presence and absence of entrapped calcitonin were determined using a Confocal Laser Scanning Microscopy (CLSM) (PCM 2000 CLSM with Nikon digital camera DXM 1200, realtime imaging, and He/Ne laser with a 60 x ApoPlanar oil immersion objective, excitation at 505 nm and oil emission at 568 nm). The particles were fluorescently labelled with Nile red. The vesicles were shown to be spherical in nature with a homogenous intensity. The droplet sizes of the particles were measured with a Malvern Mastersizer (Malvern Instruments, United Kingdom), using Milli Q water (800 ml) to wash and align the laser. The background was measured and then 2 ml of the sample was added for measurement of volume sizes. No increase in the size or change in the morphology of the particles containing calcitonin when compared to particles without entrapped calcitonin, indicating that the molecular size of this therapeutic protein does not influence the superficial properties of the particles.

To obtain statistical meaningful results between different treatments, six male Sprague

Dawley rats with a body weight of approximately 250 g - 350 g rats were used per group per experiment type (Statistical Consultation Service (North-West University, South Africa). Rats were bred and kept at the Animal Research Centre (North-West University) as described above in the patent application.

An Eppendorf® micropipet (10-100 µl) was used for the nasal administration of calcitonin reference standard and test formulations after the rats were fasted 18 hours prior but water was supplied *ad libitum*. The formulations were administered at a dose of 10 IU/kg body-weight in the right nasal cavities of the rats in a volume of 100 µl/kg body-weight. Care was taken not to induce epithelial injury in administration of the reference and test formulation. Both the FA-based vesicle and microsphere formulations were used as test formulations in this study. Sufficient blood volumes from the same rat at different time intervals could be obtained for analysis of drug content after cannulation of the *artery carotis communis*, as described. To determine the plasma drug profile, 8 blood samples with a volume of 1000 µl were collected at 0, 5, 10, 15, 30, 60, 120 and 180 minutes after nasal administration, with time 0 taken 1 minute prior to administration. The collected samples were kept at 0 °C. To obtain plasma, 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.

Immunoreactive calcitonin was quantified in a volume of 100 µl of each plasma sample with the use of the standard preparations and procedures supplied in a radioimmunoassay (RIA) commercial kit obtained from Diagnostic Systems Laboratories (Laboratory Specialties, Johannesburg, South Africa). The radioactivity was detected with a Gammatec™ II gamma counter (A Canberra Company, South Africa), with results presented as plasma calcitonin concentration (pg/ml) against time. Plasma calcium levels were determined spectrophotometrically using a calcium assay kit (Roche Diagnostics, Germany). A volume of 50 µl of plasma of each sample was used for analysis, with results presented as plasma calcium concentration (mmol/l) against time.

7.7.3.2 Results

The plasma calcitonin and plasma calcium concentrations obtained after a single nasal administration of 10 IU/kg bodyweight calcitonin with FA based vesicles and microspheres to rats were determined and are shown in figure 7.1 and tables 7.6 and 7.7.

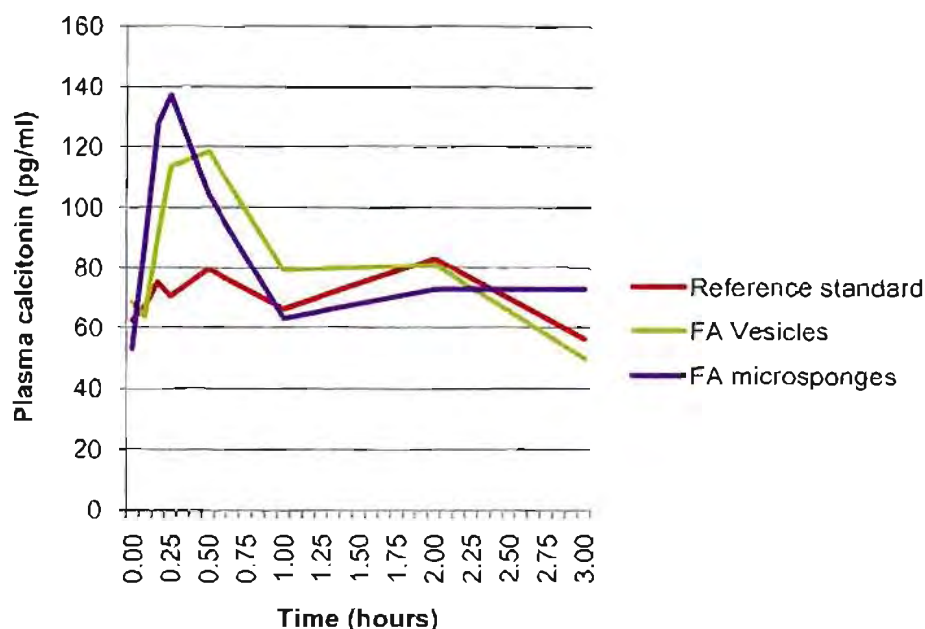


Figure 7.1: The comparative plasma profiles after administration of calcitonin.

The enhancement in the calcitonin plasma levels over time after a single dosage by entrapment of the calcitonin entrapped in the FA based particles, with an enhancement of in the highest plasma levels (C_{max}) by 42% and 65% for by for the FA vesicles and microsponges of the invention respectively.

Table 7.6: Observed bioavailability parameters			
	Reference std.	Vesicles	Microsponges
Total Area	12985	14640	14295
Peak area	4341	5923	5867
T_{max}	120	30	15
C_{max}	82.94	118.36	136.9
% enhanced above endogenous levels		36.44	35.15

Although the C_{max} of the vesicles and microsponges differed significantly, the peak area (AUC) obtained for those two groups of rats was nearly similar, but was significantly higher than that of the reference standard ($p < 0.001$). An interesting observation is that entrapment of calcitonin led to a shorter T_{max} , which would translate in faster onset of action in practice.

No major differences were observed against time in group that received the reference standard and it can be concluded that nasal administration of calcitonin alone, in the dose

given, did not lead to any therapeutic effect. The plasma levels of both calcitonin and calcium obtained are probably the endogenous levels maintained in the experimental animals under normal conditions.

Similar results were obtained for the plasma calcium concentrations (table 7.7). The plasma levels in percentage (%) were also calculated from the plasma calcium concentrations (mmol/l). A change in the calcium levels, as a result of calcitonin administration, indicates the efficacy of the treatment.

Table 7.7: Comparative average calcium plasma levels after administration						
	Reference std.		FA vesicles		FA microsponges	
Time	mmol/l	%	mmol/l	%	mmol/l	%
0	3.38 ± 0.13	100.00 ± 0.00	3.56 ± 0.64	100.00 ± 0.00	3.63 ± 0.34	100.00 ± 0.00
5	3.45 ± 0.11	102.07 ± 5.44	3.29 ± 0.17	92.42 ± 11.70	3.48 ± 0.21	95.87 ± 8.36
10	3.43 ± 0.16	101.48 ± 6.87	3.22 ± 0.19	90.45 ± 12.83	3.48 ± 0.37	95.87 ± 14.77
15	3.42 ± 0.13	101.18 ± 6.61	3.18 ± 0.24	89.33 ± 14.31	3.16 ± 0.09	87.05 ± 8.71
30	3.34 ± 0.17	98.82 ± 4.26	3.12 ± 0.18	87.64 ± 14.78	3.00 ± 0.23	82.64 ± 7.99
60	3.20 ± 0.15	94.67 ± 5.50	2.99 ± 0.16	83.99 ± 14.93	2.93 ± 0.16	80.72 ± 6.80
120	3.17 ± 0.28	93.79 ± 10.04	2.80 ± 0.23	78.65 ± 15.01	2.94 ± 0.21	80.99 ± 4.13
180	3.25 ± 0.31	96.15 ± 9.34	2.79 ± 0.22	78.37 ± 14.83	2.77 ± 0.24	76.31 ± 11.53

The rats that received calcitonin entrapped in FA vesicles or microsponges did show a decrease in the calcium plasma levels and thus a therapeutic effect. Despite the difference observed in C_{max} between the vesicles and microsponges, the therapeutic effect is very similar for those two groups and mimics the results on the AUC. The effect was still present and in fact increasingly pronounced towards the end of the monitoring period. It must be noted that the therapeutic effect observed was found after a single administration and should be enhanced by repeated administrations.

In conclusion, both FA vesicles and microsponges are able to increase the nasal absorption of calcitonin. The maximum effect obtained with the particles of the invention was around 15-30 minutes post-administration and was maintained up to 60 min after administration. This could be an important factor in nasal drug delivery as a fast onset of action may be desirable for the

drug to escape the mucociliary clearance mechanism present in the nasal route of drug administration. The exact mechanism of action of particles of the invention is not yet clear but it is believed that a group of fatty acid transporters present on cell membranes actively binds and transports the particles with its entrapped compound across the cell membrane, allowing the entrapped calcitonin to be actively absorbed.

7.7.4. Nasal delivery of rhGH

This investigation focussed specifically on the evaluation of the ability of the fatty acid based matrix to transport recombinant human growth hormone across mucosal epithelia when administered via the nasal cavity.

7.7.4.1 Study design and *in vivo* model

As in the studies described above, this study used 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 received a single dose of rhGH/saline solution each to determine the amount of absorption without any absorption enhancing additive. The rat *in vivo* model described above was again used in this investigation with procedures identical to that described above. The study was approved by the Ethical Committee of the North-West University (05D18). Six rats each were again used to determine the extent of absorption of rhGH in each treatment group, thus ensuring detection of significant statistical differences between the control and experimental groups. A dose of 0.6 IU/kg rhGH was administered subcutaneously in the lower abdominal region. The control group and test groups received a nasally administered dose of 3.6 IU/kg of rhGH. The recombinant human growth hormone (rhGH, 1 mg = 3 IU) was supplied by Warren Chem Chemical Specialities (Midrand, RSA) after authorization from the Department of Health. The different experimental formulations used were as follows:

Formulation A acted as reference standard as described above where 6 mg of the freeze dried rhGH powder was 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. Formulations B to D was prepared similarly to A with the following differenced. Formulation B acted as positive control sample, and consists of 3.6 IU/kg weight made up in physiological saline, which was administered in the absence of any penetration enhancers or release devices. Formulation C consists of 3.6 IU/kg rhGH entrapped in the FA vesicles of the invention and Formulation D consists of 3.6 IU/kg rhGH entrapped in the FA microsponges of the invention.

Between 95 and 105 μl (0.6 IU/kg rhGH) of the subcutaneous formulation (Formulation A) was injected directly under the skin in the abdominal region of each rat in the subcutaneous group. Formulations B – D was administered via the nasal cavity. Between 25 - 35 μl was administered via the left nostril of each test subject at a dose of 100 $\mu\text{l/kg}$ bodyweight. The final concentration rhGH administered nasally to each rat was 3.6 IU/kg as determined by a calculation based on the bodyweight of the rat. Each rat received a single dose.

Formulations Ci and Cii differs in formulation Cii was left to stand in a fridge at 5 °C for a further 24 hours before administration with the aim of allowing further entrapment of rhGH into the vesicular matrix. Similarly formulations Di and Dii differ in time of entrapment. Both formulations C and D show the presence of differently sized particles when measured with a Malvern Mastersizer (Malvern Instruments, UK). Entrapment of rhGH into the fatty acid matrices led to an average increase of 0.88 μm per particle for Formulations Ci and Cii and 2.01 μm in Formulation D. The large increase in particle sizes after entrapment in the sponge matrix may indicate some dimerization of the rhGH.

Nasal administration occurred by direct administration into the nasal cavity, through the left nostril. Rats were fasted for a period of 12 hours prior to nasal drug administration but water was supplied *ad libitum*. Nasal administration was performed at a dose of 100 $\mu\text{l/kg}$ bodyweight. Blood samples with a volume of 800 μl were collected through cannulation at the time intervals indicated in the results, with time 0 taken 1 minute prior to administration as described. The collected samples were kept at 0 °C. Plasma was obtained through centrifugation as described. The recovered plasma samples were stored at -70 °C for not more than 3 weeks before analysis.

7.7.4.2 Analyses of plasma rhGH

The quantitative analyses of the amount of rhGH present in the collected samples were performed using a hGH-IRMA kit obtained from BIOSOURCE™ (South Africa), manufactured by BioSource Europe S.A., Rue de l'Industrie, 8, B-1400 Nivelles, Belgium. The principles underlying this specific immunoradiometric assay is that of coated-tube separation: The capture antibody (Mab 1) 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. The signal antibody (Mab 2) is labelled with ^{125}I . Addition of Mab 2 will complete the system and trigger the immunological reaction. According to the instructions of the manufacturers, the coated tubes were washed with the supplied wash solution and the remaining fraction of radioactivity, bound to the coated tube, reflected the antigen (rhGH) concentration. The radioactivity was determined by counting the radioactivity in the tubes for 60 seconds in an Auto-Gamma® 5 000

series, Packard Gamma counting system. This technique does not distinguish exogenous somatropin from endogenous growth hormone (Veldhuis *et al.*, 2001:S26).

7.7.4.3 Results

Table 7.8 shows the average blood plasma concentrations ($\mu\text{IU/ml}$) of rhGH obtained after administration of the various formulations measured over a period of 180 minutes. The symbol SE represents the standard error values. The blood plasma concentration of rhGH is given in micro-international units per millilitres ($\mu\text{IU/ml}$).

TABLE 7.8: Plasma levels of rhGH obtained (n=6 for each group)								
	Reference std.		Positive control		FA-vesicles Ci		FA-vesicles Cii	
Time (min)	Average ($\mu\text{IU/ml}$)	SE	Average ($\mu\text{IU/ml}$)	SE	Average ($\mu\text{IU/ml}$)	SE	Average ($\mu\text{IU/ml}$)	SE
0	0	0	0.06	0.03	0	0	0.08	0.05
5	2.61	0.52	0.02	0.02	0	0	0.14	0.08
10	3.93	0.59	0.02	0.02	0.02	0.02	0.08	0.05
15	4.32	0.82	0.12	0.04	0	0	0.09	0.05
30	4.78	1.11	0.08	0.05	0.36	0.11	0.26	0.1
60	4.09	0.93	0.11	0.04	2.36	0.86	0.52	0.1
120	2.05	0.47	0.15	0.05	3.42	1.24	3.05	0.89
180	0.72	0.1	0.18	0.04	2.15	0.5	4.14	1.22

After subcutaneous injection, the blood plasma concentration of rhGH reached its maximum concentration, namely $4.78 \mu\text{IU/ml}$, at time $t = 30$ minutes, after which is decreased. These results are in agreement with the results obtained in previous studies for the subcutaneous administration of rhGH. It is evident from the results obtained that there was virtually no absorption of rhGH after the nasal administration of the control formulation (rhGH/saline solution), suggesting that larger peptide molecules, such as rhGH with the high molecular weight of 22 000 Da, need to be administered in conjunction with a penetration enhancer or similar system in order to obtain acceptable therapeutic plasma levels of nasally administered rhGH. In contrast to the control formulation, the nasal administration of rhGH with microsponges did show a slight increase in plasma concentration. From time $t = 0$ tot $t = 30$ minutes the rhGH concentration in the plasma was very low with values in the range $0.00 - 0.11 \mu\text{IU/ml}$. At time $t = 60$ minutes a small, yet very definite, increase in concentration is observed ($0.19 \mu\text{IU/ml}$). The concentration continues to increase and at time $t = 120$ minutes a value of $0.41 \mu\text{IU/ml}$ is

recorded. The concentration is $0.68 \mu\text{IU/ml}$ 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. Nasal administration of rhGH entrapped in FA vesicular matrices led to an increase in the systemic absorption and plasma concentration of rhGH: at time $t = 120$ minutes a significant rise is recorded with a concentration of $3.05 \mu\text{IU/ml}$ and a further rise to $4.14 \mu\text{IU/ml}$ at time $t = 180$ minutes.

A comparison of the plasma levels after the administration of rhGH entrapped for 30 minutes (Formulation Ci) and that of the rhGH administered 24 hours after entrapment (Formulation Cii) shows that Formulation Cii reaches its peak value ($3.42 \mu\text{IU/ml}$) at time $t = 120$ minutes while the levels after administration of Formulation Cii are still on the increase at time $t = 180$ minutes. Whereas maximum plasma levels could be determined for both the reference standard and the positive control, the same is not true for the entrapped rhGH. Entrapment of rhGH shifted the plasma level profile in time, with the time of maximum absorption (T_{max}) being later than with either the reference standard or the nasally administered rhGH that are not entrapped in the FA matrices. The results showed that this study should be conducted over a longer time period and it was repeated with blood samples collected over a 5 hour period instead of the usual 3 hour period. Blood samples were taken at 1 hour intervals, namely 1, 2, 3, 4 and 5 hours respectively after administration. The results obtained are shown in Figure 7.2.

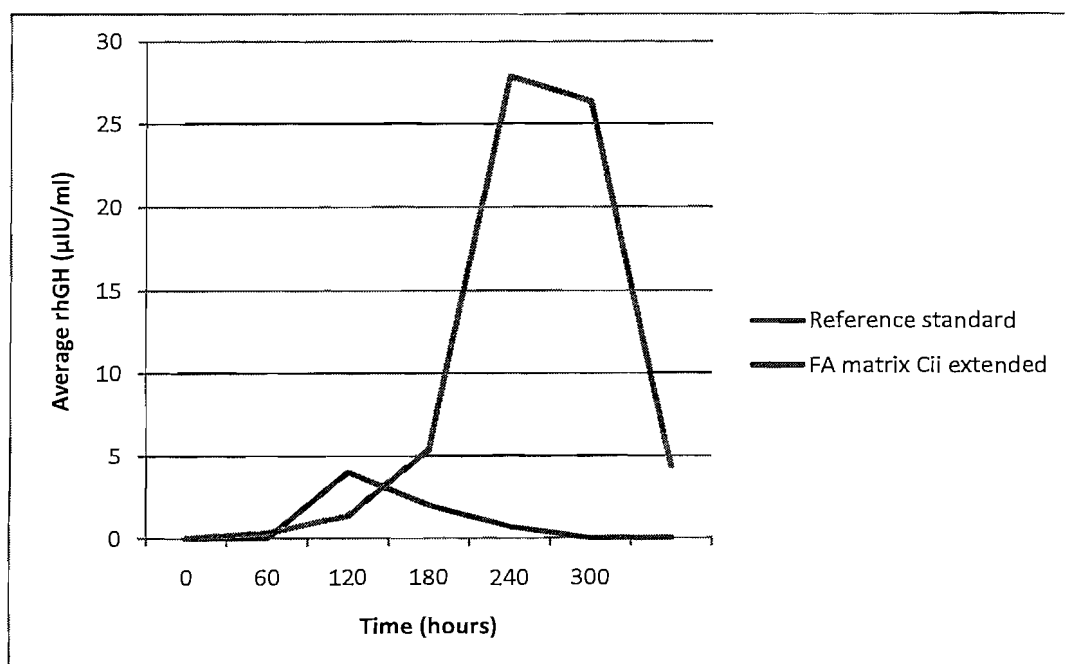


Figure 7.2 shows the average plasma levels rhGH after administration.

This study rendered higher plasma concentrations of rhGH in comparison to the previous experiment with the same formulation, the only difference being that this study 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 $0.31 \mu\text{IU/ml}$ was recorded and at time $t = 1$ hour a plasma concentration of $1.36 \mu\text{IU/ml}$ was measured. The concentration of rhGH continues to rise with a truly amazing increase at time $t = 3$ hours ($27.90 \mu\text{IU/ml}$), after which the concentration stays fairly constant for the next hour. After 5 hours the concentration decreased substantially. A comparison of the average plasma levels obtained at $t = 3$ hours between the first and second studies is very different and it is unclear what the reason for this major difference in plasma levels could be. It is possible that the short time span between blood sampling in the 3 hour study may have an impact on the plasma levels.

7.7.4.4 Comparison of results obtained

The goal of this study was to determine the impact of entrapment of rhGH in the FA-based vesicles and microsponges of the invention with regard to absorption and bioavailability. Since the administered dose was the same in all of the above formulations (rhGH 3.6 IU/Kg) with the exception of the subcutaneous administration, it would be fair to link the effectiveness of each formulation directly to the maximum average rhGH plasma concentration obtained with each formulation. The control formulation, rhGH in saline, clearly shows that virtually no absorption of rhGH took place without the aid of an absorption enhancer. In table 7.9, the average bioavailability parameters obtained for each rhGH formulation are compared.

Table 7.9: Comparative bioavailability parameters		
Parameters	Vesicles (24hr)	Subcutaneous administration
Total Peak Area (AUC)	63.34	6.86
Peak X (T_{\max})	3.2	1
Peak Y (C_{\max})	27.9	4.09

The fatty acid-based formulations may act by transporting the entrapped drug molecules via the transcellular pathways. The difference in the T_{\max} values may be attributed to a difference in the pathways employed for permeation and may indicate a rate-limiting absorption phase in the presence of the FA-based formulation. In man, rhGH exhibits a biphasic clearance curve with a half disappearance time in normal subjects of $9.0 \pm 3.5 \text{ min.}$ ($n = 8$) for the first phase over 60 min., and $30.7 \pm 10.8 \text{ min.}$ ($n = 8$) for the second phase between 60 and 120 min after intravenous injection (Wilton *et al.*, 1988:117). Only one peak has been observed in the rat

model. The plasma half-life of the vesicle-entrapped half-life is increased, resulting in a much longer therapeutic window.

The dosage given intra-nasally was 6 times that administered subcutaneously as literature indicates that lower systemic plasma levels are obtained when not using a parenteral administration route. The AUC obtained for subcutaneous administration and for FA microsponges is about equal. Taking the dosages into account, it would indicate that the systemic absorption of subcutaneously absorbed rhGH is in reality about 6 times that of the intranasal administration. Surprisingly, the AUC obtained using the FA-based vesicles showed a >9.23 times enhancement over that observed after subcutaneous administration, despite the fact that clearance was not complete after 5 hours in the case of the rhGH entrapped in the FA vesicles. Taking the different dosage into account, the FA vesicles showed the capability of enhancing systemic rhGH plasma levels 1.5 times above that observed for subcutaneous administration and 34.7 times that of the comparative intranasal administered of rhGH in the absence of FA vesicles or microsponges.

FA-based vesicles have thus been found to be a most effective absorption enhancer. The FA vesicles performed much better than the FA microsponges. As the vesicles and sponges differs only in a) the composition of the particles with the presence of two additional fatty acids in the microsponges, b) the size of the particle with the microsphere being twice the size of the vesicle, and c) the steric structure of the particle, the results showed the importance of any or all or a combination of these factors in intranasal administration.

The high systemic absorption of rhGH, and the increase in the therapeutic window opens up the possibility of intra-nasal administration of rhGH with the added advantage of increased intervals between dosages without any increase in the dose of the rhGH.

7.7.5. Nasal delivery of insulin

The advantages of nasal administration of therapeutic proteins are described above. In this study, insulin, as described in patent application, was administered nasally, using the same procedures described for calcitonin and rhGH above. The determination of plasma levels and blood glucose levels were performed according to procedures described in the patent application.

7.7.5.1 Results

In figure 7.3 and table 7.10 the comparative observed plasma levels and bioavailability parameters after nasal administration of insulin at a dosage of 8 and 12IU/kg body weight are presented.

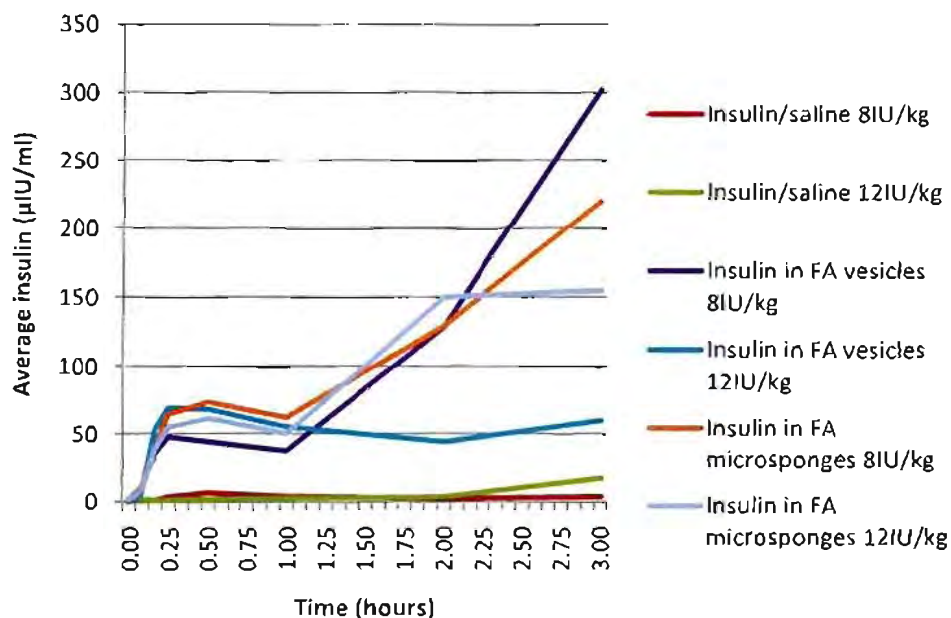


Figure 7.3: The comparative average plasma levels of insulin after nasal administration.

The observed enhancement of the plasma insulin levels by the vesicles and microsponges of the invention after intranasal administration of 8IU/kg body weight, as reflected by the AUCs, is dramatic, with a 29.27 times enhancement in the case of the vesicles and 28.54 times in the case of the microsponges.

Table 7.10: Bioavailability parameters after intra-nasal administration						
Dosage	Insulin/saline		Insulin in FA vesicles		Insulin in microsponges	
	8IU/kg	12IU/kg	8IU/kg	12IU/kg	8IU/kg	12IU/kg
AUC	666.5	948.4	20175	9417	19687	18055
T _{max}	30	180	180	15	180	180
C _{max}	6.734	17.45	302.5	68.79	220.2	154.8
AUC enhancement			29.27007	8.929355	28.53788	18.03733
C _{max} enhancement			43.92129	2.94212	31.69973	7.87106

At this dosage, the particles of the invention seem to enhance the absorption of and transport of insulin into the plasma equally. The enhancements found for the higher dosage (12IU/kg body weight) differ substantially for the two types of particles (8.9 times for the vesicles

and 18.04 times for the microsponges). The difference may be explained by the different release profiles of the entrapped insulin: in the case of the lower dosage administered by vesicles, the insulin plasma level is still increasing, indicating that the transport to or release into the plasma is slower. In fact, in all the groups that received insulin by way of the particles of the invention, the real enhancement is higher than that portrayed, as in none of these cases has the blood drug profiles returned to base level.

The results in table 7.11 reflect the therapeutic efficacy of the administered insulin. These results show that the enhancement in therapeutic efficacy by the particles of the invention is even larger than the enhancement in the drug plasma levels.

The decreases in blood glucose levels are 92.98 times and 141.7 times more for the vesicle and microsponges at a dosage of 8IU/kg body weight respectively when compared to the insulin/saline control. This enhancement in therapeutic efficacy was 147.23 and 189.47 after administration of 12IU/kg body weight times for vesicles and microsponges respectively. The therapeutic efficacy of the microsponges increased more than that of the vesicles, despite the lower plasma levels observed. As the samples used in the determination of the plasma levels and the blood glucose levels are identical, the result is not due to sample- or inter-animal variation. The explanation for this discrepancy is probably that entrapped insulin may not be recognized by the antibodies of the RIA before the insulin is released, but it may still exert its therapeutic effect.

Table 7.11: The extent of the % blood glucose decrease after intranasal administration						
Time	Insulin/saline		Insulin in FA vesicles		Insulin in FA microsponges	
	8IU/kg	12IU/kg	8IU/kg	12IU/kg	8IU/kg	12IU/kg
0	0	0	0	0	0	0
5	-1.18	6.48	12.35	8.16	7.27	3.4
10	5.58	-0.08	11.72	5.65	19.17	13.18
15	10.033	-4.4	18.22	6.3	19.22	8.67
30	5.27	-8.32	12.68	10.32	36.47	13.87
60	3.47	-13.98	16.52	19.8	34.53	30.32
120	2.05	-30.7	27.67	39.525	36.95	35.26
180	-26.58	-40.55	44.38	38.2	54.94	72.375
AUC	46	32.2	4323	4773	6564	6133
T _{max}	15	5	180	120	180	180
C _{max}	10.03	6.48	44.38	39.53	54.94	72.38
AUC enhancement			92.978	147.23	141.7	189.47

7.8 References

- ABS, T., MATSSON, A.F., BENGTSSON, B.-A., FELDT-RASMUSSEN, U., GÓTH, M.I., KOLTOWSKA-HÄGGSTRÖM, M., MONSON, J.P., VERHELST, J., WILTON, P. and on behalf of the KIMS Study Group. 2005. Isolated growth hormone (GH) deficiency in adult patients: Baseline clinical characteristics and responses to GH replacement in comparison with hypopituitary patients. A sub-analysis of the KIMS database. *Growth hormone & IGF research*. 15(5):349-359.
- ALBERTSSON-WIKLAND, A., ISAKSSON, O., 1976. Development of responsiveness of young normal rats to growth hormone. *Metabolism*. 25(7):747-759
- AMA DRUG EVALUATIONS ANNUAL; the *American Medical Association*. 1994. Ed. United States Pharmacopeia: 2364pp
- ANTIBODIES: A LABORATORY MANUAL. 1988. Harlow and Lane, eds. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- ARORA, P., SHARMA, S., GARG, S., 2002. Permeability issues in nasal drug delivery. *Drug Discovery Today*. 7(18): 967-975.
- BANERJEE, P.S., RITSCHER, W.A., 1989. Transdermal permeation of vasopressin. I. Influence of pH, concentration, shaving and surfactant on permeation. *International journal. pharm.* 49: 189-197.
- BANGA, A.K., KATAKAM, M., MITRA, R., 1995. Transdermal iontophoretic delivery and degradation of vasopressin across human cadaver skin. *International journal. pharm.* 116:211-216.
- BALLARD, F.J., FRANCIS, G.L., ROSS, M., BAGLEY, C.J., MAY, B., WALLACE, J.C., 1987. Natural and synthetic forms of insulin-like growth factor-1 (IGF-1) and the potent derivative, destriptide IGF-1: Biological activities and receptor binding. *Biochemical and Biophysical Research Communications*. 149(2): 398-404.
- BI, M., SINGH, J., 2000. Effect of buffer pH, buffer concentration and skin with or without enzyme inhibitors on the stability of [Arg⁸]-vasopressin. *International journal. pharm.* 197:87-93.
- BLANK, I.H., MCAULIFFE, D.J., 1985. Penetration of benzene through human skin. *J. investigative dermatol.* 85:522-526.
- BRONAUGH, R.L., COLLIER, S.W., 1991. Preparation of human and animal skin. In: Bronaugh, R.L. and Maibach, H.I. (Eds.), *In vitro* percutaneous absorption: principles, fundamentals and applications. CRC Press, Boca Raton, FL., p. 1-6.
- BUTTERY, L. D.K., MANCINI, L., MORADI-BIDHENDI, N., O'SHAUGHNESSY, M.C., POLAK, J.M., MACINTYRE, I., 2002. Nitric Oxide and Other Vasoactive Agents. Principles of Bone Biology (Second Edition), 995-1013.
- Cenegenics Medical Institute at <http://www.cenegenics.com/>. Accessed 10/09/2005:1.
- CHANG, A.G., WU, G. Y. 1994 Gene therapy: applications to the treatment of gastrointestinal and liver diseases. *Gastroenterology*. 106(4):1076-84.
- CHIBA, Y., MATSUO, K., SAKAI, H., ABE, K., MISAWA, M. 2007. Regional differences in vascular responsiveness of nasal mucosae isolated from naive guinea pigs. *Auris nasus larynx*. 34(2):197-201.

- CLEARY, G.W., 1993. Transdermal drug delivery. In: Zatz, J.L., (Ed.), Skin permeation: fundamentals and application. Allured Publishing Corporation, Wheaton, IL., p. 207-237.
- COETZEE, H. 2007. The transdermal delivery of arginine vasopressin with pheroid technology. NWU (Dissertation M.Sc.) 158p
- COLMAN, E. HEDIN, R., SWANN, J., ORLOFF, D., 2002. A brief history of calcitonin. *The Lancet*. 359(9309):885-886.
- CORBO, D.C., HUANG, Y.C., CHIEN, Y.W. 1989. Nasal delivery of progestational steroids in ovariectomized rabbits. II. Effect of penetrant hydrophilicity. *International Journal of Pharmaceutics*. 50(3): 253-260.
- COSTANTINO, H.R. ILLUM, L., BRANDT, G., JOHNSON, P.H., QUAY, S.C., 2007. Intranasal delivery: Physicochemical and therapeutic aspects. *International Journal of Pharmaceutics*. 337(1-2):1-24.
- CROMMELIN, D., VAN WINDEN, E., MEKKING, A., 2002. Delivery of pharmaceutical proteins. In: Aulton, M.E., (Ed.), *Pharmaceutics: The science of dosage form design*. 2nd ed. Churchill Livingstone, Edinburgh; New York, p. 544-553.
- DE, A., BASAK, P., IQBAL, J., 1997. Polyaniline supported cobalt catalysed one pot stereoselective synthesis of the structural analogues of aminopeptidase inhibitor bestatin. *Tetrahedron letters*. 38:8383-8386.
- DE BRUYN, T. 2005. Nasal delivery of insulin with Pheroid technology. NWU (Dissertation M.Sc.) 107p
- DEMIRBAS, A. 2007. Importance of rural bioenergy for developing countries. *Energy Conversion & Management*. 48: 2386-2398.
- DONOVAN, M.D., HUANG, Y. 1998. Large molecule and particulate uptake in the nasal cavity: the effect of size on nasal absorption. *Advanced drug delivery reviews*. 29(1-2):147-155.
- DU PLESSIS, J., PUGH, W.J., JUDEFEIND, A., HADGRAFT, J., 2002. Physico-chemical determinants of dermal drug delivery: effects of the number and substitution pattern of polar groups. *European journal pharmaceutical science*. 16:107-112.
- FASANO, W.J., HINDERLITER, P.M., 2004. The Tinsley LCR Databridge Model 6401 and electrical impedance measurements to evaluate skin integrity *in vitro*. *Toxicol in vitro*. 18:725-729.
- FOSTER, T.P., 1999. Somatotropin delivery to farmed animals. *Advanced drug delivery reviews*. 38(2):151-165.
- FRANKLIN, S.L., GEFFNER, M.E., 2009. Growth Hormone: The Expansion of Available Products and Indications. *Endocrinology & metabolism clinics of North America*. 38(3):587-611.
- GURSOY, R.N. & BENITA, S. 2004. Self-emulsifying delivery systems (SEDDS) for improved oral delivery of lipophilic drugs. *Biomedicine & Pharmacotherapy*. 58(3): 173-182.
- HANSEN, T.K., 2002. Pharmacokinetics and acute lipolytic actions of growth hormone: Impact of age, body composition, binding proteins, and other hormones. *Growth hormone & IGF research*. 12(5):342-358.
- HENDERSON, R.F., BECHTOLD, W.E., MEDINSKY, M.A., FISCHER, J.P., LEE, T.T. 1988. The effect of molecular weight/lipophilicity on clearance of organic compounds from lungs. *Toxicology and applied pharmacology*. 95(3):515-521.

- HUSSAIN, A.A., 1998. Intranasal drug delivery. *Advanced drug delivery reviews*. 29(1-2):39-49.
- IHLE, J.N., 1996. Signaling by the Cytokine Receptor Superfamily in Normal and Transformed Hematopoietic Cells. *Advances in cancer research*. 68:23-65.
- JIANG, H-L., KANG, M.L., QUAN, J-S., KANG, S.G., AKAIKE, T., YOO, H.S., CHONG-SU CHO, C-S., 2008. The potential of mannosylated chitosan microspheres to target macrophage mannose receptors in an adjuvant-delivery system for intranasal immunization. *Biomaterials*. 29(12):1931-1939.
- JOHANSSON, G., ALBERTSSON-WIKLAND, K., BENGTSSON, B-A., HANSSON, I., 1998. Discontinuation of GH treatment in adolescent patients: Metabolic effects. *Growth hormone & IGF research*. 8(4):324.
- JORGENSEN, J.H., MAHER, L.A., REDDING, J.S. 1987. In vitro activity of LY146032 (daptomycin) against selected aerobic bacteria. *Eur journal of clin microbiol*. 6(1):91-96.
- KALINER, M., MAROM, Z., PATOW, C., SHELHAMER, J., 1984. Human respiratory mucus. *Journal of allergy and clinical immunology*. 73(3):318-323.
- KOCH, R.L., PALICHARLA, P., GROVES, M.J., 1987. Diffusion of [2-¹⁴C] Diazepam across hairless mouse skin and human skin. *Journal investigative dermatol*. 88:582-585.
- KOTZÉ, J. C. 2005. Nasal drug delivery of calcitonin with pheroid technology. Potchefstroom: NWU (Dissertation M.Sc.) 105p.
- LAURSEN, T., MØLLER, J., JØRGENSEN, J.O., ORSKOV, H., CHRISTIANSEN, J.S. 1996. Bioavailability and bioactivity of intravenous vs subcutaneous infusion of growth hormone in GH-deficient patients. *Clin endocrinol*. 45:333-339.
- LEDLEY, F.D. 1992. Somatic gene therapy in gastroenterology: approaches and applications. *J pediatric gastroenterol nutrition*. 14(3):328-37.
- LEE, Y-H., SINKO, P.J. 2000. Oral delivery of salmon calcitonin. *Advanced drug delivery reviews*. 42(3):225-238.
- LELAWONGS, P., LIU, J., CHIEN, Y.W., 1990. Transdermal iontophoretic delivery of arginine vasopressin (II): Evaluation of electrical and operational factors. *International journal pharm*. 61:179-188.
- LELAWONGS, P., LIU, J., SIDDIQUI, O., CHIEN, Y.W., 1989. Transdermal iontophoretic delivery of arginine vasopressin (I): Physicochemical considerations. *International journal pharm*. 56:13-22.
- LEVEQUE, N., MAKKI, S., HADGRAFT, J., HUMBERT, PH., 2004. Comparison of Franz cells and microdialysis for assessing salicylic acid penetration through human skin. *International journal pharm*. 269:323-328.
- MARCUS, R., FELDMAN, D., KELSEY, J., 2001. Osteoporosis (Second Edition), 819-843.
- MARTTIN, E. SCHIPPER, N.G. M., VERHOEF, J. C., MERKUS, F. W. H. M., 1998. Nasal mucociliary clearance as a factor in nasal drug delivery. *Advanced drug delivery reviews*. 29(1-2):13-38.
- MEDI, B.M., SINGH, J., 2003. Electronically facilitated transdermal delivery of human parathyroid hormone (1 - 34). *International journal pharm*. 263:24-33.

- MERKUS, F. W. H. M., MARTTIN, E., ROMEIJN, S. G., SCHIPPER, N. G. M., VERHOEF, J. 1996. Excipients to enhance the nasal delivery of peptides. *European Journal of Pharmaceutical Sciences*. 4(Supplement 1):S35.
- MITRA, R., PEZRON, I., CHU, W.A., MITRA, A.K., 2000. Lipid emulsions as vehicles for enhanced nasal delivery of insulin. *International journal pharm.* 205(1-2):127-134.
- MÖLLER, C., EMTNER, M., ARNER, P., NORSTEDT, G. 1994. Growth hormone regulation of lipid metabolism in cells transfected with growth hormone receptor cDNA. *Molecular and cellular endocrinology*. 99(1):111-117.
- MONTVALE, N.J. 1994. Physicians' Desk Reference, 48th Ed, Medical Economics Data Production Co.
- MORIMOTO, K., IWAKURA, Y., MIYAZAKI, M., NAKATANI, E., 1992. Effects of proteolytic enzyme inhibitors of enhancement of transdermal iontophoretic delivery of vasopressin and an analogue in rats. *International journal pharm.* 81:119-125.
- MORIMOTO, K., MIYAZAKI, M., KAKEMI, M., 1995. Effects of proteolytic enzyme inhibitors on nasal absorption of salmon calcitonin in rats. *International journal pharm.* 113:1-8.
- MORSEY, M.A., MITANI, K., CLEMENS, P., CASKEY, C.T. 1993. Progress Toward Human Gene Therapy. *JAMA*. 270: 2338-45.
- MORTAZAVI, S.A. AND SMART, J.D., 1994. An in-vitro method for assessing the duration of mucoadhesion. *Journal of controlled release*. 31(2):207-212.
- NAIR, V.B., PANCHAGNULA, R., 2003a. Poloxamer gel as vehicle for transdermal iontophoretic delivery of arginine vasopressin: evaluation of *in vivo* performance in rats. *Pharmacol. res.* 47:555-562.
- NAIR, V.B., PANCHAGNULA, R., 2003b. Effect of iontophoresis and fatty acids on permeation of arginine vasopressin through rat skin. *Pharmacol. res.* 47:563-569.
- NAIR, V.B., PANCHAGNULA, R., 2003c. Physicochemical considerations in the iontophoretic delivery of a small peptide: studies using arginine vasopressin as a model peptide. *Pharmacol. res.* 48:175-182.
- NAIR, V.B., PANCHAGNULA, R., 2004a. The effect of pretreatment with terpenes on transdermal iontophoretic delivery of arginine vasopressin. *II Farmaco*. 59:575-581.
- NAIR, V.B., PANCHAGNULA, R., 2004b. Influence of electrical parameters in the iontophoretic delivery of small peptide: *in vitro* studies using arginine-vasopressin as model peptide. *II Farmaco*. 59:583-593.
- OBERHOLZER, I. D. 2009. Peroral And nasal delivery of insulin with Pheroid™ technology. Potchefstroom: NWU (Thesis Ph.D.) 174p.
- PETTIT, D.K., GOMBOTZ, W.R., 1998. The development of site-specific drug-delivery systems for protein and peptide biopharmaceuticals. *Tibtech*. 16:343-349.
- PHILLIP, M., CHALEW, S.A., MCCARTER R.J.JR, AMIT, T., KOWARSKI, A.A., HOCHBERG, Z., 1996. The relationship of growth rate, plasma growth hormone (GH) concentration, and GH-binding protein. *Metabolism*., 45(4):424-429.
- Physicians' Desk Reference, 48th Ed, MONTVALE, N.J. 1994. Medical Economics Data Production Co.

PONTIROLI, A.E., 1998. Peptide hormones: Review of current and emerging uses by nasal delivery. *Advanced drug delivery reviews*. 29(1-2): 81-87.

PRAUSNITZ, M.R., 1997. Transdermal delivery of macromolecules: Recent advances by modification of skin's barrier properties. In: Shahrokh, Z., Sluzky, V., Cleland, J.L., Shire, S.J. & Randolph, T.W., (Eds.), *Therapeutic protein and peptide formulation and delivery*. American Chemical Society, Washington, D.C., p. 124-153.

RAFFERTY, B., CORRAN, P., BRISTOW A., 2001. Multicenter collaborative study to calibrate salmon calcitonin by bioassay and high-performance liquid chromatography: establishment of the third international standard. *Bone*. 29(1):84-89

RATHBONE, M.J., DRUMMOND, B.K., TUCKER, I.G., 1994. The oral cavity as a site for systemic drug delivery. *Advanced drug delivery reviews*. 13(1-2):1-22.

ROMEIJN, S.G., VERHOEF, J.C., MARTIN, E., MERKUS, F.W. H. M. 1996. The effect of nasal drug formulations on ciliary beating *in vitro*. *International journal pharm*. 135(1-2):137-145.

ROSENFELD, R.G., AGGARWAL, B.B., HINTZ, T.L., DOLLAR, L.A., 1982. Recombinant DNA-derived methionyl human growth hormone is similar in membrane binding properties to human pituitary growth hormone. *Biochemical and biophysical research communications*. 106(1):202-209.

SCHULGA, A.A., MAKAROV, A.A., LEVICHKIN, I.V., BELOUSOVA, Y.V., LOBACHOV, V.M., PROTASEVICH, I.I., PACE, C.N., KIRPICHNIKOV SINSWAT, M.P., & TENGAMNUAY, 2002. Increased stability of human growth hormone with reduced lactogenic potency. *FEBS letters*. 528(1-3):257-260

SINSWAT, P. & TENGAMNUAY, P. 2003. Enhancing effect of chitosan on nasal absorption of salmon calcitonin in rats: comparison with hydroxypropyl- and dimethyl-beta-cyclodextrins. *Int journal of pharmaceutics*. 257(1-2):15-22.

STEPAN, J.J. AND ZIKÁN, V., 2003. Calcitonin load test to assess the efficacy of salmon calcitonin. *Clinica chimica acta*. 336(1-2):49-55.

STEYN, D. 2006. Nasal delivery of recombinant human growth hormone with Pheroid technology. Potchefstroom: NWU (Dissertation M.Sc.) 115p.

STRAUSS, T. 2005. Peroral delivery of calcitonin with pheroid technology. Potchefstroom: NWU (Dissertation M.Sc.) 105p.

THORNER, M. O., VANCE, M.L., HARTMAN, M.L., HOLL, R.W., EVANS, W.S., VELDHUIS, J.D., VAN CAUTER, E., COPINSCHI, G., BOWERS, C.Y., 1985. Physiological role of somatostatin on growth hormone regulation in humans. *Metabolism*. 39(9) Supplement 2:40-42.

UGWOKE, M.I., AGU, R.U., VERBEKE, N., KINGET, R. 2005. Nasal mucoadhesive drug delivery: Background, applications, trends and future perspectives. *Advanced drug delivery reviews*. 57(11):1640-1665.

UMEZAWA, H, AOYAGI, T., SUDA, H., HAMADA, M., TAKEUCHI, T. 1976. Bestatin, an inhibitor of aminopeptidase B, produced by actinomycetes. *Journal antibiot*. 29:97-99.

VELDHUIS, J.D., ANDERSON, S.M., SHAH, N., BRAY, M., VICK, T., GENTILI, A., MULLIGAN, T., JOHNSON, M.L., WELTMAN, A., EVANS, W.S., IRANMANESH, A. 2001. Neurophysiological regulation and target-tissue impact of the pulsatile mode of growth hormone secretion in the human. *Growth hormone & IGF research*. 11(Supplement 1):S25-S37.

VILLENEUVE, P., MUDERHWA, J.M., GRAILLE, J., HAAS M.J. 2000. Customizing lipases for biocatalysis: a survey of chemical, physical and molecular biological approaches. *Journal of Molecular Catalysis Enzymatic.*, 9: 113-148.

ZAIDI, M., INZERILLO, A. M., MOONGA, B. S., BEVIS, P. J. R., HUANG, C. L. -H. 2002. Forty years of calcitonin—where are we now? A tribute to the work of Iain Macintyre, FRS. *Bone*. 30(5):655-663.