THE TRANSDERMAL DELIVERY OF ARGININE VASOPRESSIN WITH PHEROID™ TECHNOLOGY

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Dissertation submitted in fulfilment of the requirements for the degree Master of Science in the Department of Pharmaceutics at the Potchefstroom Campus of the North-West University

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Jeremiah 29:11
For I know the plans I have for you,
declares the LORD,
plans for wholeness and not for evil,
to give you a future and a hope.
English Standard Version

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ABSTRACT

The aim of this study was to investigate *in vitro* transdermal diffusion of a small peptide namely arginine vasopressin (AVP) with the aid of the novel Pheroid™ drug delivery system. Generally, peptides seem unfit for transdermal permeation, but it was thought prudent to explore the suitability of this lipid-based system after success was achieved with entrapment of tuberculostatics, bacteria and viruses. Bestatin (a selective aminopeptidase inhibitor) was employed to circumvent any skin-related degradation of the active. Therefore, the effect of bestatin on the preservation of AVP during diffusion was investigated. Vertical Franz cell diffusion studies were conducted with female abdominal skin, with AVP at a concentration of 150 µg/ml in the donor phase and Hepes buffer as the receptor phase over a twelve-hour period. To prove entrapment of AVP within the lipid structures of the Pheroids™, fluorescently-labelled samples were monitored by means of confocal laser scanning microscopy (CLSM), which revealed definite entrapment. *In vitro* permeation profiles for AVP exhibited a biphasic character, with the majority of permeation occurring during the first two hours. The Pheroid™ delivery system proved to be advantageous when applied as delivery medium. The inclusion of bestatin has an enhancing effect on permeation probably due to its protection of AVP.

Keywords: Arginine vasopressin, transdermal diffusion, confocal microscopy, Pheroid™, delivery system, bestatin

UITTREKSEL

Die doelwit van hierdie studie was om *in vitro* transdermale diffusie van 'n klein peptied geneesmiddel, naamlik arginien vasopressin (AVP), met behulp van die Pheroid™ geneesmiddel afleweringsisteem te ondersoek. Oor die algemeen is peptiede nie geskikte kandidate vir transdermale permeasie nie, maar dit is goedgedink om hierdie lipied-gebaseerde sisteem te gebruik in die bevordering van AVP diffusie nadat daar alreeds sukses behaal is met tuberkulostatika, bakterieë en virusse. Bestatien ('n selektiewe aminopeptidase inhibeerder) is gebruik om enige velverwante afbraak van AVP teen te werk en dus is bestatien se effek op die behoud van AVP gedurende diffusie ook ondersoek. Diffusies is uitgevoer oor 'n periode van twaalf ure met behulp van vertikale Franz selle, vroulike abdominale vel, AVP met 'n konsentrasie van 150 µg/ml in die donorfase en Hepes buffer as die reseptorfase. Om bewys te lewer dat die AVP molekules geïnkorporeer word binne die lipied vesikels van die Pheroid™ sisteem is konfokale laser skandeer mikroskopie (CLSM) aangewend. Hierdie mikroskopie het duidelik die teenwoordigheid van die AVP molekules in die Pheroids™ aangedui. *In vitro* permeasieprofiele vir AVP het 'n bifasiese karakter aangedui, met die meerderheid van diffusie wat plaasgevind het gedurende die eerste twee ure. Die Pheroid™ afleweringsisteem blyk voordelig te wees as 'n afleweringsmedium en bestatien het 'n positiewe effek in die bevordering van diffusie getoon, moontlik vanweë beskerming van AVP.

Sleutelwoorde: Arginien vasopressien, transdermale diffusie, konfokale mikroskopie, Pheroid™, afleweringsisteem, bestatien

FOREWORD

During this study we aimed at investigating the transdermal delivery of a protein drug, preferably a peptide hormone, with the aid of the novel Pheroid™ therapeutic drug delivery system. The latter is being researched in combination with several peptides such as insulin, calcitonin and human growth hormone via different administration routes in the subprogram: Drug Delivery of the Unit for Drug Research and Development of the North-West University. Due to successes with the delivery of antibacterials such as rifampicin and other tuberculostatics, and the entrapment of large molecules such as bacteria and viruses in the lipid structures of the Pheroids™, we thought it prudent to ascertain whether or not the Pheroids™ could entrap peptide molecules as well. Initial selection of a model compound to be used in the studies involved porcine somatotropin (pig growth hormone) and desmopressin (DDAVP, an analogue of arginine vasopressin), but due to procurement difficulties of these substances the choice fell on arginine vasopressin (AVP), which was easily obtained from Sigma-Aldrich (Sigma, St. Louis, USA).

It was decided that this dissertation should be written in the so-called article format, which includes an introductory chapter with sub-chapters and a full-length article for publication in a pharmaceutical journal. The article in this dissertation is to be submitted for publication in The European Journal of Pharmaceutical Sciences, and therefore the complete guide for authors is included.

In spite of the many difficulties encountered during the course of this Master's degree study (change of topic two times in a row due to unavailability of drugs, instrumentation problems and a whole lot of unforeseen little inconveniences throwing a spanner in the works!), the end result is finally here. Now I can look forward to the future with a qualification which undoubtedly will open doors for me in the world of pharmacy.

Hanneri Coetzee
6 August 2007

THE TRANSDERMAL DELIVERY OF ARGININE VASOPRESSIN WITH PHEROID™ TECHNOLOGY

CHAPTER 1: INTRODUCTION AND STATEMENT OF THE PROBLEM

In recent times, advances in recombinant DNA biotechnology and the production of increasing numbers of synthetic macromolecules have opened up new possibilities in the pharmaceutical industry. These advances have led to large scale production and increased cost-effective commercialisation of, among others, peptides and proteins. They are commonly classified as macromolecules due to their large sizes, the majority of compounds weighing in at more than 1 kDa. These entities previously had to be extracted from the pituitaries of humans or animals at high cost.

It is commonly known that macromolecules such as peptides and proteins are poorly bioavailable when administered orally due to extensive enzymatic degradation and poor penetration of the gut wall. Thus, these molecules are delivered invasively through methods such as parenteral (intravenous (i.v.), intramuscular (i.m.) and subcutaneous (s.c.)) injections. In spite of advantages presented by parenteral delivery, such as 100 % bioavailability, it has obvious limitations. It is an uncomfortable method of administration with chronic therapy, and patient acceptability and compliance always remain issues to be dealt with (Hamman *et al.*, 2005:165). Therefore, the search for alternative, viable routes of administration and cost-effective dosage forms is an ongoing labour.

Transdermal protein and peptide delivery is an example of an alternative delivery method due to its non-invasiveness, ease of administration and improved patient compliance. Additionally, this administration route avoids gastrointestinal degradation and the first-pass effect of the liver (Medi & Singh, 2003:25). Previously, the formidable barrier properties of the skin had prevented transport of macromolecules across human skin and therefore transdermal delivery was not a sensible option (Prausnitz, 1997b:124). However, recent developments involving the modification of the skin's barrier properties indicate that the transdermal delivery of macromolecules (with molecular masses of >1 kDa) may now be possible.

In order to test the feasibility of transdermal delivery of macromolecules, we made use of the peptide hormone arginine vasopressin (AVP) (MW = 1084.23 Da), which is regarded as a relative 'small' macromolecule, as our model compound in transdermal diffusion studies.

AVP is an endogenous neurohypophyseal, nonapeptide hormone and is commonly utilised in the diagnosis and treatment of, among others, diabetes insipidus and nocturnal enuresis in the synthetic form of ℓ-deamino-8-D-arginine-vasopressin (DDAVP or desmopressin) (Jackson, 2001:789-804).

Due to its large molecular size and aqueous solubility (clear odourless to faint yellow solution at 20 mg/ml in water) (Sigma-Aldrich Corporation, 2006a), arginine vasopressin is not a favourable candidate for transdermal delivery, and additional measures such as penetration enhancement must be taken to ensure effective absorption of the active. In this regard, iontophoresis, electroporation and phonophoresis/sonophoresis (electrically assisted transdermal delivery techniques) provide the only known mechanisms to enhance the penetration of large, hydrophilic or charged molecules across the skin (Kalia *et al.*, 2004:619 and Prausnitz, 1997b:125). Several studies (for example Nair & Panchagnula, 2003b, 2004a) investigated transdermal delivery of AVP as the model compound by making use of iontophoresis in combination with chemical penetration enhancers. Iontophoretic systems are currently incorporated into disposable patch systems (Chiarello, 2004:48), but to the common man the cost of these devices makes this option inaccessible.

In view of the difficulties, another cost-effective dosage form with easier application would be the ideal, such as a gel or cream. Due to the mentioned drawbacks in the delivery of protein pharmaceuticals, penetration enhancement is necessary. During our study we conducted vertical Franz cell diffusion studies with female abdominal skin, and applied the active in combination with the selective aminopeptidase inhibitor bestatin in aqueous solutions of a novel therapeutic drug delivery system, Pheroid™, as well as in HEPES buffer as a control. One of the components of this delivery system is the fatty acid, oleic acid, which have been shown to be an effective chemical penetration enhancer of AVP (Nair & Panchagnula, 2003b) and other peptides (Bhatia & Singh, 1997). Bestatin was employed in the hope that it would inhibit any dermal enzymatic degradation during the diffusion studies, as demonstrated in a previous study by Bi and Singh (2000).

The aim of this study was therefore to investigate the possibility of *in vitro* transdermal diffusion of a small peptide such as arginine vasopressin in combination with the enzymatic inhibitor bestatin, with the aid of the novel therapeutic drug delivery system Pheroid™. The effect of bestatin on the retention of AVP during permeation through the skin was also investigated.

CHAPTER 2: TRANSDERMAL DELIVERY OF PEPTIDE DRUGS

1 TRANSDERMAL DRUG DELIVERY

1.1 INTRODUCTION

The human skin, along with the mucosal linings of the urogenital, digestive and respiratory tracts, comprises the epithelial system of the body. This system has the function of encasing, isolating and protecting the internal organs and structures of the human body from the hostile external environment (Barry, 2002:500 and Franz & Lehman, 2000:15). To survive this adverse environment, preserve its own integrity and fulfil its functional obligation to the rest of the body, the skin has developed the stratum corneum - a specialised structure of "unique physical-chemical composition" (Franz & Lehman, 2000:15). In spite of this highly effective, continually self-repairing obstacle (Williams, 2003:1), the skin seems to be a large and accessible organ ideal for the transdermal administration of a small number of therapeutic agents due to its multiple sites (Franz & Lehman, 2000:16). In the following sections the structure, functions, advantages and limitations, routes of permeation, influencing factors on the transdermal delivery of actives and penetration enhancement, as applicable to arginine vasopressin, will be discussed in short.

1.2 STRUCTURE OF THE SKIN

Covering an area of 15 000 to 20 000 cm^2, varying in thickness from approximately 1.5 to 4 mm and weighing approximately 2 kg, the skin can truly be seen as one of the largest organs of the adult body (Franz & Lehman, 2000:16). The skin is categorised into four main layers (figure 1):

- *The innermost subcutaneous fat layer – the hypodermis or subcutis (1):* The subcutis contains adipocytes (fat cells), which main functions are to provide energy for insulation of the body and to provide mechanical protection against physical shock (Lund, 1994:137).

- *The inner, relatively acellular, connective tissue layer - the dermis or corium (2):* The dermis is situated directly below the epidermis. This largely integrated fibroelastic structure is the largest of the three skin layers (Lund, 1994:137) and predominantly provides support and flexibility to the skin. Nerves, blood vessels and lymphatics are found throughout this layer and skin appendages such as pilosebaceous units and sweat glands pierce it (Barry, 2002:502).

For the purpose of transdermal drug delivery appendages offer a potential route for molecules to enter the lower layers of the skin via the so-called 'shunt routes'. These routes may have a role to play in the permeation process of large polar molecules, as well as the electrical enhancement of transdermal drug delivery (Williams, 2003:5). According to Franz and Lehman (2000:24) there is clear evidence that permeation via hair follicles is of importance during iontophoresis (Franz & Lehman, 2000:25). The transappendageal pathway (described later) represents a significant penetration route for those compounds whose permeation through unbroken stratum corneum is limited (Franz & Lehman, 2000:24).

In terms of percutaneous absorption, the vasculature of the dermis is its most important feature (Franz & Lehman, 2000:24). It serves as a 'sink' for the absorption of diffusing molecules reaching the capillaries. This sink-effect keeps penetrant concentrations in the dermis at a minimum, therefore maximising epidermal concentration gradients and promoting percutaneous absorption (Barry, 2002:502).

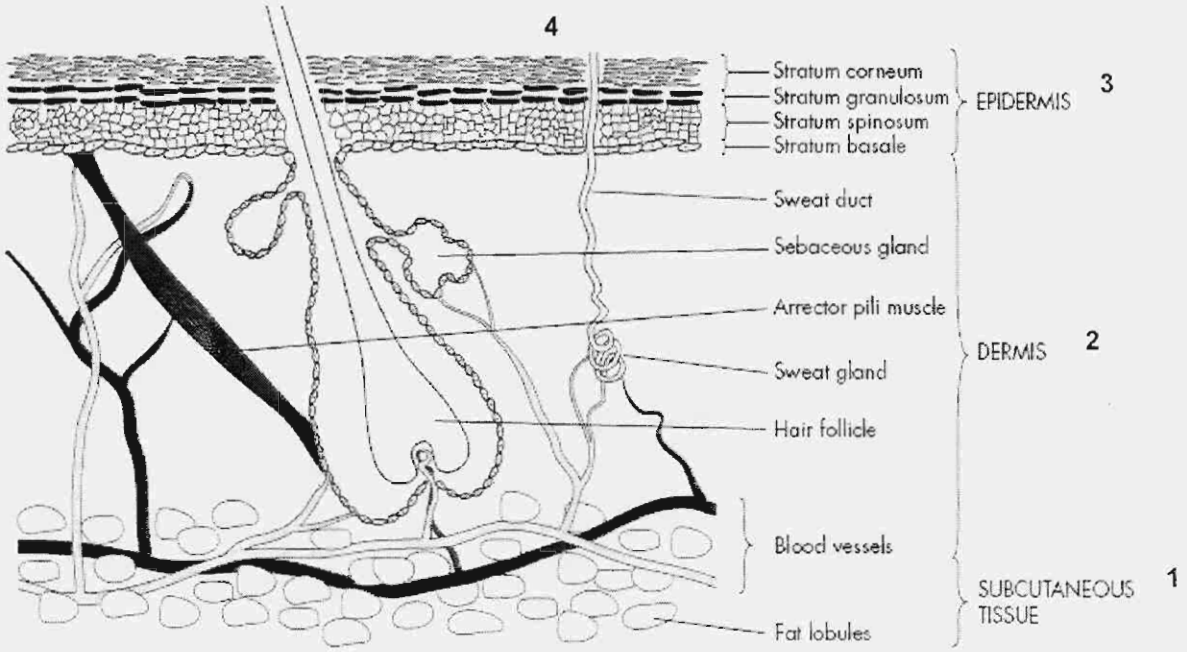


Figure 1: A diagrammatical cross-section through human skin (reproduced from Williams, 2003:3)

- *The viable, stratified, cellular, avascular epidermis (3):* The epidermis is a complex, multi-layered, stratified squamous epithelium membrane which is continually renewing itself and covers the entire outer surface of the body. The chief cell (80%) of the epidermis is the keratinocyte. It derives its name from the keratin (fibrous protein) contained within these cells (Franz & Lehman, 2000:17).

The primary function of these viable cells of the epidermis is to move progressively through a differentiation process, eventually to die (terminal differentiation). Through this mechanism the barrier layer is generated (Franz & Lehman, 2000:16).

Four histologically distinct layers, from inside to outside, can be distinguished: the stratum germinativum (also known as the stratum basale), the stratum spinosum, the stratum granulosum, and finally the stratum corneum (described hereafter as a separate layer of the skin). A fifth layer, the stratum lucidum, is sometimes mentioned but it is mostly considered to be a part of the lower layers of the stratum corneum (Williams, 2003:5).

- *The outermost non-viable epidermal layer - the stratum corneum/horny layer (4):* At the final stage of differentiation, epidermal cells construct this most superficial layer of the epidermis. The stratum corneum is recognised as the rate-limiting barrier to the ingress of materials (Lund, 1994:136), and is often viewed as a separate membrane in topical and transdermal drug delivery studies (Williams, 2003:5). Resistance to the diffusion of molecules is greater in the stratum corneum than in any of the other skin tissues and is therefore chiefly responsible for the remarkable impenetrability of the skin (Lund, 1994:136).

1.3 FUNCTIONS OF THE SKIN

The skin serves a number of invaluable functions. Table 1 presents a brief digest of its biological role. The skin's involvement in the regulation of body temperature and blood pressure, together with its barrier capabilities against damage, are considered its main functions (Lund, 1994:136).

Table 1: The main functions of the skin (reproduced from Barry, 1983:14)

1. To contain body fluids and tissues – the mechanical function
2. To protect from potentially harmful external stimuli – the protective or barrier function
3. To receive external stimuli, that is, to mediate sensation: tactile (pressure), pain or heat
4. To regulate body temperature
5. To synthesise and to metabolise compounds
6. To dispose of chemical wastes (glandular secretions)
7. To provide identifications by skin variations
8. To attract the opposite sex (apocrine secretions are defunct in this role)
9. To regulate blood pressure

1.4 ADVANTAGES AND LIMITATIONS OF TRANSDERMAL DRUG DELIVERY

The ideal transdermal administration provides a constant blood concentration, which is effective but not toxic, that can be maintained for the desired time (Kydonieus *et al.*, 2002:2).

Advantages of this system are (1) reproducibility and prolonged constant delivery rates, (2) convenient and less frequent administrations and (3) reduced side effects because the dose does not exceed the toxic level (Kydonieus *et al.*, 2000:2). The following paragraphs give accounts of the major advantages and limitations of transdermal drug delivery (Roberts *et al.*, 2002:90-92):

1.4.1 ADVANTAGES

- First-pass metabolism via the liver is minimised through transdermal delivery.
- Due to the highly acidic environment of the stomach some drugs degrade to a large extent, which leads to variability in plasma concentration. Some drugs such as NSAIDs also cause gastrointestinal bleeding or irritation. The transdermal route therefore provides a more controlled, non-invasive method of delivery.
- Transdermal delivery systems such as patches can be removed and absorption ceased in the event of an overdose or other problematic situations.
- Because of the reduced frequency of administration and avoidance of the trauma associated with parenteral therapy, patient compliance is improved.

1.4.2 LIMITATIONS

- The major drawback associated with transdermal delivery is the unsuitability of several compounds due to their large molecular weight, aqueous solubility, charge and other factors.
- Several physicochemical parameters influence the diffusion process and variations in permeation rates occur between individuals, different races and persons of different ages.
- Diseased skin as well as the extent of the disease affects permeation rates.
- Some drugs such as peptides and proteins (for example arginine vasopressin) are metabolised before reaching the cutaneous vasculature due to the action of several metabolic enzymes (such as trypsin and aminopeptidases) in the skin.
- Bacteria living on the skin surface can break down some drugs even before penetration through the stratum corneum.

1.5 PERCUTANEOUS ABSORPTION AND ROUTES OF PERMEATION

According to Lund (1994:135), percutaneous absorption is "the term used to describe the penetration of a substance through the skin and subsequent movement into the systemic circulation." Chemicals or drugs permeate the skin via two possible routes (Figure 2):

- The transepidermal route, subdivided in the transcellular and intercellular pathways.
- The transappendageal or 'shunt' route, which involves the hair follicles and sweat glands.

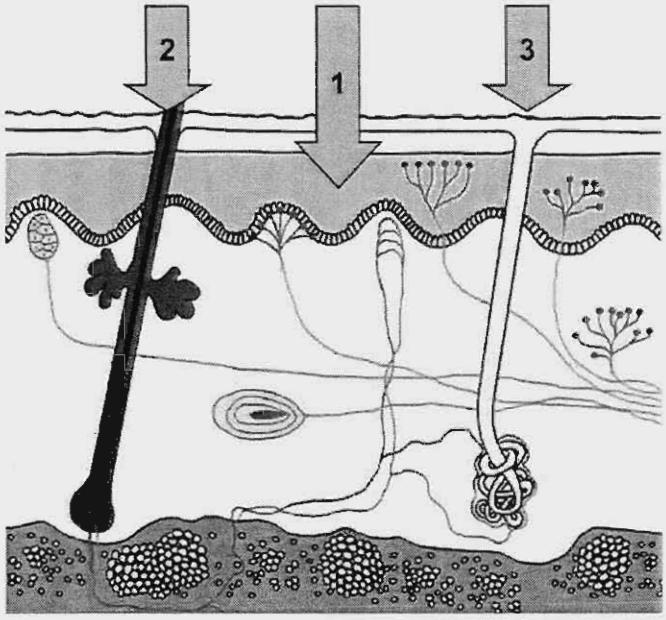


Figure 2: Routes of permeation
Transepidermal route (1) and transappendageal route via hair follicles (2) and sweat ducts (3) (Daniels, 2004)

The transcellular pathway is often regarded as a polar route due to the highly hydrated keratin (the main cellular component of the keratinocytes) which does indeed provide an essentially aqueous environment (Williams, 2003:32). This route could therefore be ideal in the delivery of hydrophilic molecules such as proteins and peptides, but due to the hydrophilic and hydrophobic domains within the stratum corneum, including the almost impenetrable keratinocyte intracellular matrix of keratin and keratohyalin (Roberts *et al.*, 2002:94), this route seems generally unfavourable.

The transappendageal pathway is important for large polar molecules and ions that poorly traverse the stratum corneum. Iontophoretic drug delivery (commonly employed in the delivery of protein pharmaceuticals) largely depends on the presence of shunt routes (as mentioned earlier). Charge is carried through the stratum corneum via the path of least resistance and this route provides less resistance than the bulk of the stratum corneum (Williams, 2003:32).

1.6 PENETRATION ENHANCEMENT

Substances which temporarily diminish the impermeability of the skin are known as penetration enhancers, accelerants or sorption promotors (Barry, 2002:522). Any chemical which is pharmacologically inactive, safe, non-toxic and promotes stratum corneum hydration can be considered a penetration enhancer (Barry, 2002:523).

Penetration enhancement is however not limited to chemicals; vesicles (such as liposomes), modifications of drug molecules and electrically assisted enhancement methods are a few expedients which are utilised to enhance penetration of drugs. Figure 3 gives a summary of commonly used penetration enhancement techniques.

For the purposes of this dissertation, emphasis will be placed on the chemical penetration enhancer oleic acid, and the electrically assisted penetration technique, iontophoresis. Both have been used extensively in transdermal diffusion studies involving peptide pharmaceuticals, and oleic acid is a key component of the novel drug delivery system Pheroid™, described at a later stage in this dissertation.

In the following paragraphs however, mention will be made of the best known chemical enhancers and physical/technological penetration enhancement techniques. A complete description of these enhancers and techniques falls outside the scope of this dissertation, but several reliable sources regarding transdermal research can be consulted for further comprehensive information.

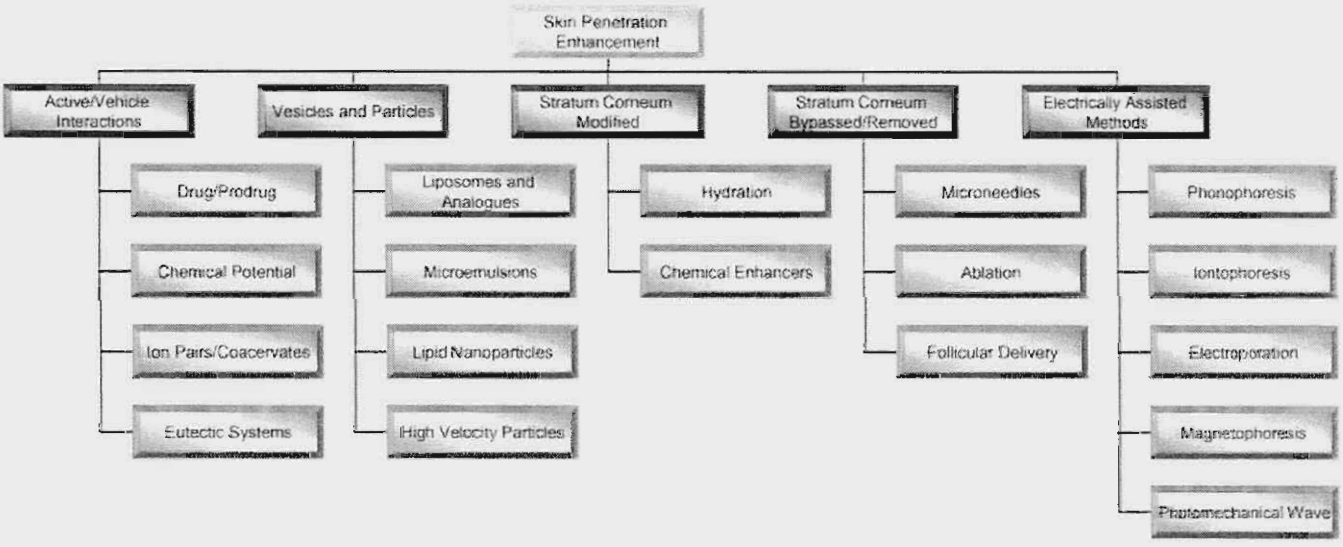


Figure 3: A schematic representation of the most commonly used penetration enhancement techniques (Daniels, 2004)

1.6.1 CHEMICAL PENETRATION ENHANCERS

Chemical penetration enhancers are classified according to the polarity of the drug to be delivered. It is believed that enhancers of the non-polar route act by fluidising skin lipids and those enhancers of the polar route act by inducing conformational changes in hydrated proteins of the skin.

There are a few inert vehicles or enhancers which do not react with the skin. The safest, simplest and most commonly utilised penetration enhancer is water, as evidenced by the widespread use of occlusive dressings and vehicles (Lund, 1994:144).

Other chemical penetration enhancers include the following (Williams, 2003:87-102, Lund, 1994:144-145 and Büyüktimkin *et al.*, 1997:417-441):

- Sulfoxides, such as dimethylsulfoxide (DMSO).
- Azone®, also known as 1-Dodecyl-Hexahydro-2H-azepin-2-one or laurocapram.
- Pyrrolidones, such as *N*-Methyl-2-pyrrolidone (NMP) and 2-pyrrolidone (2-P).
- Alcohols, fatty alcohols (alkanols) and glycols, such as ethanol and propylene glycol (PG).
- Terpenes, such as d-Limonene, X-terpineol, carveol, menthol, carvone, menthone, ascaridole, 1,8-cineole and limonene oxide.
- Phospholipids, such as 1% egg phosphatidylcholine and soybean phospholipids.
- Surfactants.
- Urea.

1.6.2 OLEIC ACID

Several fatty acids have been employed as putative penetration enhancers. Fatty acids have been used to facilitate transdermal delivery of several compounds and they are enhancers of both lipophilic and hydrophilic permeants (Williams, 2003:92). The fatty acids most commonly used are lauric acid (C_{12}, and the most potent straight-chain fatty acid) and the *cis*-unsaturated oleic acid (C_{18}). Figure 4 depicts the chemical structure of oleic acid.

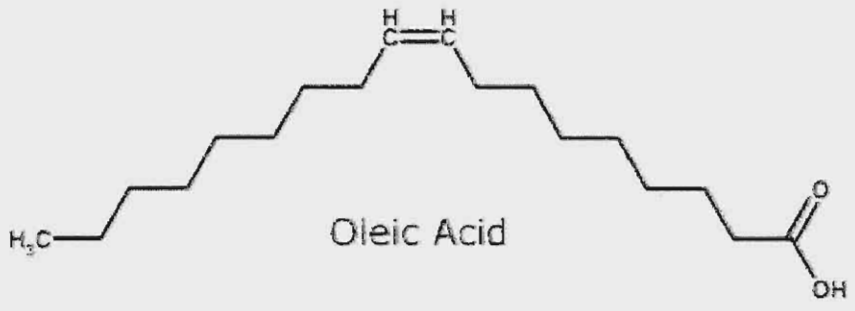


Figure 4: The chemical structure of the fatty acid oleic acid (reproduced from Daniels, 2004)

The mechanism of action of oleic acid has been considerably investigated and it is apparent that the enhancer interacts with the stratum corneum lipid domains. Two modes of action were identified which may clarify the action of this enhancer: 1) lipid fluidization, and 2) lipid phase separation (Naik *et al.*, 1995:299). It is suggested that a novel lipid domain is induced in the barrier lipids on exposure to this enhancer through predominantly the second mechanism. These domains would provide permeability defects within the bilayer lipids, thus facilitating hydrophilic drug permeation via these defects (Williams, 2003:93).

Transdermal successes with oleic acid are described in Bhatia and Singh (1997) and Nair and Panchagnula (2003b) where this enhancer was employed on both occasions in tandem with iontophoresis. The transdermal delivery of the peptide drugs luteinising hormone releasing hormone (LHRH) and arginine vasopressin (AVP) were enhanced in the individual cases.

1.6.3 PHYSICAL AND TECHNOLOGICAL PENETRATION ENHANCERS

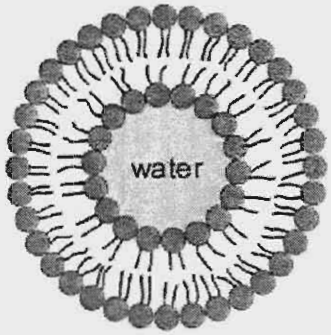
In this group, enhancers such as lipid vesicles, needleless injections, microneedles and electrically assisted penetration techniques can be found.

The best known lipid vesicles are liposomes, spherical structures that fully enclose aqueous volumes (Figure 5). They are colloidal particles (Barry, 2002:523) with the lipid molecules usually being phospholipids or non-ionic surfactants (Büyüktimkin *et al.*, 1997:437) with or without cholesterol. These lipid molecules form concentric bimolecular layers to produce vesicles that trap hydrophilic molecules within their aqueous regions or incorporate lipophilic molecules within the bilayered membrane (Williams, 2003:124). Other lipid vesicles closely related to liposomes are:

- *Niosomes*: Liposomes incorporating non-ionic surfactants such as polyoxyethylene alkyl ethers and may be prepared as single or multilamellar vesicles (Davis *et al.*, 2002:306).

- *Transferosomes:* Highly deformable, elastic or ultraflexible liposomes with a phospholipid (for example phosphatidylcholine) and a surfactant (for example sodium cholate or deoxycholate) as their main ingredients (Williams, 2003:131).

- *Ethosomes*: Liposomes that are also comprised of phospholipids, but incorporate high levels of an alcohol, usually ethanol (Williams, 2003:134).



Liposome

Lipid bilayer enclosing an aqueous core

Figure 5: Representation of a liposome (reproduced from Daniels, 2004)

The needleless injection is a transdermal delivery system concept that operates by firing high velocity particles into the skin with a gas-powered 'gun' (Williams, 2003:136). Solid particles are fired through the stratum corneum into the underlying skin layers by means of a supersonic shockwave of helium gas travelling at Mach 2-3 (Barry, 2002:524).

The stratum corneum can also simply be circumvented by making use of an injection. This approach is underlined by an interesting development: a device consisting of microneedles which insert drug just below the stratum corneum (Barry, 2002:522). Needles used for this purpose range from 100-1000 µm in length and are arranged in arrays of 1000 microneedles or more. These arrays make microscopic punctures in the skin that are large enough for the ingress of macromolecules, but small enough that the patient does not feel the penetration or experience any pain (Chiarello, 2004:50). However, several formulation issues still needs to be addressed such as flow, pressure, absorption, interaction between the microneedle and the molecule at hand. Skin thickness and application site may also affect transdermal delivery via microneedles (Chiarello, 2004:54).

Electrically assisted enhancement techniques are being researched by several workers in the field, and results are acquired with a wide variety of drugs. However, instrumentation for home use of these methods remains problematic and expensive, and concern related to possible irreversible skin damage necessitates further investigation. These enhancement techniques include the following:

- *Ultrasound (phonophoresis or sonophoresis)*: This technique is usually used in physiotherapy and sports medicine to apply a preparation topically to the skin. The application site is then massaged with an ultrasonic source (Barry, 2001:108). Ultrasound is a pressure wave with a frequency too high to be heard by the human ear (> 16 kHz).

Ultrasound echoes off internal structures enabling diagnostic imaging (Prausnitz, 1997a:467), but in terms of transdermal delivery, ultrasonic heating increases transport by fluidising stratum corneum lipids and/or increasing convective flow (Prausnitz, 1997a:468). Therapeutic ultrasound usually makes use of very high frequencies (>1 MHz) and low intensities (<1 W/cm^2), but transdermal studies make use of lower frequencies (for example 20-100 kHz). These lowered frequencies enables delivery of macromolecules at therapeutically relevant rates (Prausnitz, 1997a:469).

- *Electroporation/electropermeabilization:* Electroporation creates transient aqueous pathways in the lipid bilayers by application of a short (μs to ms) electrical pulse of approximately 100-1000 V/cm (Barry, 2001:109). Fluxes for neutral and highly charged molecules of up to 40 kDa in size are increased up to 10-10^4-fold. The process is thought to be able to transport vaccines, liposomes, nonapeptides and microspheres into the skin. Electroporation combined with iontophoresis may even enhance the penetration of peptides such as vasopressin, neurotensin, calcitonin and LHRH (Barry, 2001:109).

Iontophoresis, which forms part of the latter group, will be discussed in the following section.

1.6.4 IONTOPHORESIS

Through iontophoresis (figure 6) a charged molecule is electrically driven into skin tissue. A small direct current (approximately 0.5 mA/cm^2) passes through a drug-containing electrode in contact with the skin and a grounding electrode completes the circuit elsewhere on the skin (Barry, 2001:108). The skin carries above pH~4 a net fixed negative charge, therefore, transdermal transport of positively charged ions are favoured. Iontophoresis therefore induces a convective driving force for transport across the skin by means of a net flux of ions from the anode to the cathode (Prausnitz, 1997a:462).

Iontophoresis has been employed in the electrical enhancement of macromolecules, but it has proven to be more difficult than electrically assisted delivery of small compounds. Success has been achieved with transport studies across animal skin for a wide variety of macromolecules and detectable, sometimes therapeutically useful fluxes have been observed during human skin transport studies.

Arginine vasopressin and some of its analogues have successfully been transported during *in vitro* studies (described at a later stage in this dissertation). Transporting macromolecules across human skin via iontophoresis seems to be limited to smaller compounds in the size range of approximately 1kDa. Arginine vasopressin adheres to this requirement (Prausnitz, 1997a:463).

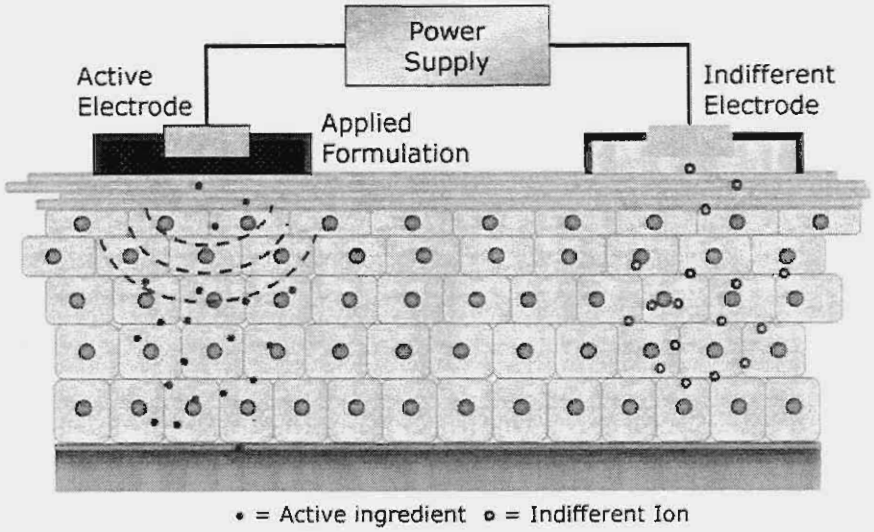


Figure 6: Graphical representation of iontophoresis (Daniels, 2004)

Unfortunately, no delivery system is without problems. Although the apparent current density per unit area is low during iontophoresis, most of the current penetrates via the low resistance route namely the hair follicles. The actual current density in the follicle may be high enough to damage growing hair. Home use of an iontophoretic device is problematic, but work is being done on the miniaturising of iontophoretic systems (Barry, 2001:109 and Chiarello, 2004:48).

Now that we have briefly examined potential means of enhancing penetration of drug molecules, let us look at other factors influencing the percutaneous absorption of drugs, especially those factors which have an impact on large molecules such as proteins and peptides.

1.7 FACTORS INFLUENCING PERCUTANEOUS ABSORPTION

1.7.1 PHYSICOCHEMICAL FACTORS

1.7.1.1 PARTITION COEFFICIENT (P)

According to Williams (2003:27) partition coefficient is "a measure of the distribution of molecules between two phases". An octanol/water partition coefficient is often used as a guide in transdermal studies to predict a molecule's distribution between stratum corneum lipids and water. In some texts, the symbol K is used for this parameter. Most molecules with an intermediate partition coefficient of log P (octanol/water) of one to three shows some solubility in both water and oil phases. Highly lipophilic molecules possess a log P of more than three and hydrophilic molecules a log P of less than one (Williams, 2003:36).

The higher the partition coefficient of the drug for the membrane, the greater the concentration of drug introduced to the skin. Partitioning between the vehicle and stratum corneum often constitutes the rate-limiting step in transdermal drug delivery (Smith & Surber, 2000:28).

1.7.1.2 DIFFUSION COEFFICIENT (D)

Diffusion coefficient is a term sometimes used interchangeably with diffusivity: a property of the diffusing molecule in the membrane and a measure of how easily it will traverse through the tissue. It is expressed in units of area/time (usually cm^2/h or cm^2/s). The diffusion coefficient of a drug in a topical vehicle or in skin depends, at a constant temperature, on the properties of the drug and the diffusion medium and the interaction between them (Smith & Surber, 2000:29). The value of D measures the rate at which a molecule penetrates under specified conditions (Barry, 2002:512).

1.7.1.3 CONCENTRATION DIFFERENCE/DIFFUSANT SOLUBILITY (C)

Diffusion takes place with the aid of a concentration gradient from a region of high diffusant concentration to one of lower concentration. The concentration difference across the stratum corneum provides the driving force for the net movement of the drug molecules between the donor and receptor environments. In *in vivo* (biological) and *in vitro* (flow-through diffusion cell) situations, the drug concentration on the distal side of the barrier always tends to zero as the molecules are immediately swept away by the receptor environment. This phenomenon is termed 'sink'-diffusion and under these conditions the parameters of Fick's Law (described later) are simplified to a certain extent (Smith & Surber, 2000:25).

1.7.1.4 DRUG CONCENTRATION

Increasing the concentration of an active substance in a vehicle usually has an increase in thermodynamic activity as a result. Subsequently, there is also an increase in the quantity of material absorbed (Lund, 1994:141). It has also been observed that the drug flux is proportional to the concentration gradient across the skin. The donor solution should be saturated with permeant to ensure maximal flux in a thermodynamically stable situation (Barry, 2002:512). However, it has been ascertained during transdermal studies with arginine vasopressin, that an increase in drug concentration did not necessarily result in a significantly improved drug flux (Nair & Panchagnula, 2003c:178).

1.7.1.5 DRUG SOLUBILITY AND MELTING POINT

Most organic materials exhibit high melting points; such materials have relatively low aqueous solubilities at normal temperatures and pressures such as those encountered under typical transdermal circumstances. There is thus a close relationship between melting point and solubility (Williams, 2003:37).

As stated elsewhere, the arginine vasopressin product used in this study possesses a satisfactory aqueous solubility of 20 mg/ml, but the melting point of this drug is unknown or not available (according to the Material Safety Data Sheet of the drug) (Sigma-Aldrich Corporation, 2004).

Transdermal candidates must possess lipophilicity, but also need to demonstrate a degree of aqueous solubility since topical preparations are usually applied as an aqueous formulation. Protein pharmaceuticals such as arginine vasopressin are generally hydrophilic compounds, thus additional measures must be employed to transport these drugs across the skin.

1.7.1.6 MOLECULAR WEIGHT, SIZE, VOLUME OR SHAPE

In order to study the influence of molecular size on permeation, molecular volume should be regarded as the most appropriate measure of permeant bulk. Molecular weight is generally taken as an approximation of molecular volume and it is generally assumed that most molecules are essentially spherical (Williams, 2003:36). Absorption is inversely related to molecular weight: small molecules penetrate faster and more efficiently than large ones (Barry, 2002:513).

There is a tendency among conventional therapeutic agents chosen as transdermal candidates to lie within a narrow range (100-500 Da) with regard to their molecular weights. When larger molecules such as peptides and proteins are to be transported by transdermal means the influence of their molecular weights must be taken into account (Williams, 2003:37). In our case, we made use of arginine vasopressin which is approximately 1 kDa in size, thus molecular size of the active is consequently a factor to be reckoned with.

1.7.1.7 IONIZATION, pH AND pK

It is widely believed that ionisable drugs are poor transdermal candidates due to higher aqueous solubilities and the charge they carry. It is however likely that these charged drugs can cross the stratum corneum via the previously mentioned shunt routes. The amounts of drugs that permeate via these routes are just somewhat less than if the species were unionised and were to pass via the larger intercellular route. It should also be mentioned that the stratum corneum is remarkably resistant to pH alterations, tolerating a range of 3 to 9 (Barry, 2002:512). Arginine vasopressin is a charged molecule with a pK value of 10.9 and is +1 charged at a pH of 5.4. All of the facts mentioned above explain why large protein molecules prefer the shunt routes of permeation.

1.7.1.8 VEHICLE FORMULATION

The nature of the vehicle in which the permeant is dissolved or suspended, definitely has an effect on the release of the active substance. The thermodynamic activity of the active substance and its potential for absorption by the skin are more important factors than its ability to penetrate the skin.

The amount of active substance, as well as the diffusion coefficient of the substance and its partition coefficient, are influenced by the nature of the vehicle. If a substance has a high affinity for its vehicle, the substance will have a low thermodynamic activity and will be released at a slower pace. If the solubility of the substance in the vehicle is reduced, more favourable releasing conditions can be obtained (Lund, 1994:141).

In our scenario, arginine vasopressin is dissolved in Hepes buffer and the Pheroid™ delivery system. The nature of both of these vehicles must have an influence on the drug, therefore its further investigation seems necessary.

1.7.2 BIOLOGICAL/BIOMEDICAL FACTORS

There are several physiological factors that can influence the rate of drug delivery to and through healthy skin. Some of these factors include skin age, anatomical site, sex and race, skin condition and hydration, temperature of the skin, blood circulation, skin metabolism, dermatological/pathological disorders or diseases of the skin and species differences (Barry, 2002:509-511, Williams, 2003:14-17 and Lund, 1994:139-140).

Some of the above factors could have played a role in the permeation of arginine vasopressin during our *in vitro* transdermal studies in view of the fact that we made use of Caucasian, female, abdominal skin obtained after cosmetic surgery. However, sex and race do not seem to have such a significant effect on drug permeation (Lund, 1994:140 and Williams, 2003:17).

In preparation of the skin we aimed at keeping the samples as intact as possible, thus ensuring acceptable skin condition. We also conducted experiments in a water bath with a temperature of 37 °C to provide an epidermal surface temperature of 32 °C, thus imitating the *in vivo* situation. To circumvent skin metabolism by the main enzymes of the skin, aminopeptidase and trypsin (Bi & Singh, 2000:92), the enzymatic inhibitor bestatin was introduced.

The following section (2) deals with challenges in the transdermal delivery of peptide drugs. Most of the previously mentioned factors have direct influences on the permeation of protein and peptide pharmaceuticals.

1.7.3 MATHEMATICAL APPROACH TO DRUG PERMEATION

1.7.3.1 FICK'S LAW OF DIFFUSION

Drug permeation through the skin is essentially a passive diffusion process from a region of high drug concentration (the formulation applied on the surface of the stratum corneum) to a region of lower (negligible) drug concentration (within the skin strata).

In order to describe the major parameters that govern the solute diffusion process, some of them being those factors discussed in the previous sections, Fick's Law of diffusion is especially useful. It can be written in the following generalised form (equation 1) (Smith & Surber, 2000:24):

Equation 1: Fick's Law of Diffusion

$$J = \frac{K \cdot D}{h} \Delta C$$

Where J = flux ($\mu g/cm^2.h$)
K = partition coefficient
D = diffusion coefficient (cm^2/h)
ΔC = concentration difference ($\mu g/cm^3$)
h = membrane thickness (cm)

The total amount of permeant that diffuses through the skin in a predetermined amount of time is described by flux. Flux is dependent on the surface area of the membrane to which the drug-containing formulation is applied and the total time of contact between the delivery vehicle and the skin. This parameter becomes important when *in vitro* diffusion studies are undertaken in research. The flux is usually measured by analytical means such as high-performance liquid chromatography (HPLC) or scintillation-counting procedures in permeation experiments (Smith & Surber, 2000:25).

2 *CHALLENGES IN THE TRANSDERMAL DELIVERY OF PEPTIDE DRUGS*

2.1 DELIVERY OF PROTEIN AND PEPTIDE PHARMACEUTICALS

2.1.1 SOURCES OF PHARMACEUTICAL PROTEINS

The era of biotechnology and biotech products is here with most pharmaceutical peptides and proteins being manufactured by means of recombinant DNA- or hybridoma technology. Examples of such 'biotech' products are human insulin, erythropoietin, monoclonal antibodies, cytokines and interferons. The assumption can be made that most of the pharmaceutical proteins are basically endogenous, but unfortunately some of the currently used biotech products are not exactly identical to the endogenous protein or peptide. However, these biotech-derived products continue to make up a majority of the products on the market. In some instances proteins of major therapeutic importance still need to be isolated from humans or animals. Examples are albumin, blood clotting factors and antisera (Crommelin *et al.*, 2002:545).

2.1.2 PEPTIDES AS DRUGS

It is the opinion of Edwards *et al.* (1999:1-4), who wrote an editorial for QJM: An International Journal of Medicine, that we are "on the brink of a therapeutic revolution due to the rapid expansion in the use of peptides as drugs". It is a well known fact that most physiological processes are regulated by peptides through action at some sites as endocrine or paracrine signals and at others as neurotransmitters or growth factors. Protein pharmaceuticals have been and still are utilised in such diverse areas as neurology, endocrinology and haematology (Edwards *et al.*, 1994:1).

The ideal therapeutic agent would be a small-molecular-mass chemical mimic of a large receptor ligand, such as a protein. This agent must be inexpensive to manufacture and able to reach the site of action with ease after oral administration. The problem with this idyllic picture, however, is the large size and many binding sites of the specific cell surface receptors that peptides bind to in order to initiate their action. In addition, peptides have complex tertiary structures which are difficult to mimic. As a result, production of peptide mimics has not achieved success and endogenous peptides are still relied upon in therapeutic situations (Edwards *et al.*, 1994:1).

In their editorial, Edwards and his colleagues referred to luteinising hormone releasing hormone (LHRH), growth hormone (GH), arginine vasopressin (AVP) and cyclosporin as older therapies and proceeded to discuss the newer therapeutic peptides such as insulin, leptin, octreotide, interferons, nerve growth factors (NGFs), recombinant human erythropoietin (EPO), recombinant human growth factors (G-CSF and GM-CSF) and peptide antibiotics (Edwards *et al.*, 1999:1-4). These peptides are but a few of the growing number of compounds being investigated. Other peptides of interest will be mentioned in the sections hereafter.

2.1.3 SPECIFIC CHALLENGES FACING PEPTIDE DELIVERY

Here follows a summary of some of the most important challenges posed by proteins and peptides (Crommelin *et al.*, 2002:545 and Pettit & Gombotz, 1998:343):

- Proteins are delicate entities of large molecular size with numerous functional groups.
- Their structures are stabilised by relatively weak physical bonds which can be readily and irreversibly changed.
- They possess relative instability in environments of extreme pH and temperature; denaturisation of the secondary and tertiary structures takes place.
- Proteolytic enzyme activity, whether in the gastrointestinal tract or on the surface of the skin, pose a serious threat to the viability of peptide delivery.
- Proteins' epithelial penetration capacity is very low unless proper transfer molecules are available.
- The electrical charge and relatively hydrophilic nature of proteins generally diminish membrane transport.

The above facts clearly indicate that delivery of therapeutic peptides is not an uncomplicated achievement. We will therefore examine in the following paragraph the possible routes of administration that have been and still are profusely investigated.

2.1.4 ROUTES OF ADMINISTRATION FOR THERAPEUTIC PEPTIDES

The oral route offers very low bioavailability due to enzymatic degradation and poor gastrointestinal tract penetration. The intravenous (i.v.) route has been rendered useful due to fast clearance (half-lives of minutes) and slow clearance (half-lives of days) from the blood compartment, as well as 100% bioavailability. Subcutaneous or intramuscular administration is more patient-friendly and the injection process easier than with i.v. administration, but proteins are not instantaneously drained to the blood compartment and the bioavailability is not as excellent as with i.v. administration (Crommelin *et al.*, 2002:550).

Alternative routes of delivery, including inhalation, buccal, intranasal and transdermal routes, as well as novel delivery systems such as protective liposomes, are investigated in ongoing research. Neuropeptide systems in the brain are also being explored as likely targets for therapeutics (Edwards *et al.*, 1999:1).

Table 2 lists the different possible routes of delivery for proteins and their relative advantages and disadvantages (Crommelin *et al.*, 2002:551).

Table 2: Alternative routes of administration to the oral route

Route	Relative advantage	Relative disadvantage
Nasal	Easily accessible, fast uptake, proven track record with a number of 'conventional' drugs, probably lower proteolytic activity than in the GI tract, avoidance of first-pass effect, spatial containment of absorption enhancers is possible	Reproducibility (in particular under pathological conditions), safety (e.g. ciliary movement), low bioavailability for proteins
Pulmonary	Relatively easy to access, fast uptake, proven track record with 'conventional' drugs, substantial fractions of insulin are absorbed, lower proteolytic activity that in the GI tract, avoidance of hepatic first-pass effect, spatial containment of absorption enhancers	Reproducibility (in particular under pathological conditions, smokers/ non-smokers), safety (e.g. immunogenicity), presence of macrophages in the lung with high affinity for particulates
Rectal	Easily accessible, partial avoidance of hepatic first-pass effect, probably lower proteolytic activity than in the upper parts of the GI tract, spatial containment of absorption enhancers is possible, proven track record with a number of 'conventional' drugs	Low bioavailability of proteins User-unfriendly method of administration
Buccal	Easily accessible, avoidance of hepatic first-pass effect, probably lower proteolytic activity than in the lower parts of the GI tract, spatial containment of absorption enhancers is possible, option to remove formulation if necessary	Low bioavailability of proteins, no proven track record yet
Transdermal	Easily accessible, avoidance of hepatic first-pass effect, removal of formulation is possible if necessary, spatial containment of absorption enhancers is possible, proven track record with 'conventional' drugs, sustained/controlled release possible	Low bioavailability of proteins

As can be observed in the table above, the pulmonary route is the only exception in contrast to the other delivery options in terms of bioavailability. All the delivery routes however present significant advantages in comparison with the oral route.

Researchers have made use of the following approaches to improve bioavailability of pharmaceutical proteins when considering alternative routes (Crommelin *et al.*, 2000:551):

- Concomitant administration of protease inhibitors to slow down metabolic degradation.
- Excipients (often those with amphipatic character) added to enhance passage through epithelial barriers.
- The use of mucoadhesives to prolong the presence of proteins at absorption surfaces.

While exploring the transdermal route for the delivery of arginine vasopressin, we made use of the first approach by including the enzyme inhibitor bestatin (discussed in a later section) to the formulation.

With cyclosporin (a cyclic decapeptide) as the only successful *oral* delivery peptide candidate, it is evident that much research still needs to be done regarding peptides and this mode of delivery (Pettit & Gombotz, 1998:344). Several traditional peptide hormones have been delivered by the *nasal* route, including desmopressin (DDAVP), LHRH and its analogues, oxytocin and salmon calcitonin (Pontiroli, 1998:85). Despite challenges in *pulmonary* delivery, several proteins and peptides are under investigation, such as insulin, LHRH analogues, G-CSF and growth hormone (Pettit & Gombotz, 1998:344). A variety of peptide pharmaceuticals have been evaluated for *buccal* absorption. Some of these include (Veuillez *et al.*, 2001:93):

- Gastro-intestinal peptides (secretin, substance P).
- Pancreatic hormones (insulin, glucagon).
- Anterior pituitary hormones (adrenocorticotropins, growth hormone).
- Posterior pituitary oligopeptides (oxytocin, vasopressin and their analogues).
- Hypothalamic-releasing-hormones (protirelin, gonadorelin, growth hormone-releasing factor hormone, somatostatin) and derivatives (gonadorelin agonists, buserelin, histrelin and nafarelin).
- Enkephalins, calcitonin and interferons.

Great advances have been made in the *transdermal* delivery of peptides and proteins, using ultradeformable liposomes, electroporation and low-frequency ultrasound (Prausnitz, 1997b:125). For a delivery method to be successful, it must contribute to two important functions: (1) modify the skin barrier and (2) provide a driving force for transport. The mentioned delivery methods in this paragraph adhere to these requirements. Methods which do not alter the skin's properties, such as passive diffusion and iontophoresis, electroporesis and/or electroosmosis, have weaker ability to transport large compounds. Chemical enhancement sometimes increases macromolecular delivery, but safety concerns inhibit its use (Prausnitz, 1997b:139).

Various studies employing peptides as model compounds have been described in earlier papers. Examples are those studies described in this dissertation using arginine vasopressin as model compound, as well as a study into the transdermal delivery of interferon (Foldvari *et al.*, 1999:129-137).

Further reading concerning the different delivery routes and peptides concerned may include:

- Oral delivery: Hamman *et al.* (2005) and Sood & Panchagnula (2001)
- Parenteral delivery: Rothen-Weinhold & Gurny (1997)
- Nasal delivery: Pontiroli (1998)
- Pulmonary delivery: Pettit & Gombotz (1998) and Smith (1997)
- Buccal delivery: Veuillez *et al.* (2001)
- Transdermal delivery: Nair & Panchagnula (2003, 2004) and Foldvari *et al.* (1999)

In the following section emphasis will be placed on arginine vasopressin and its characteristics. We used this drug hoping to illustrate that transdermal delivery of a peptide can be a realistic enterprise.

2.2 ARGININE VASOPRESSIN AS MODEL PEPTIDE FOR TRANSDERMAL DELIVERY

2.2.1 PHYSIOLOGY OF ENDOGENOUS VASOPRESSIN

Vasopressin (Beta-hypophamine; Nichols, 2000:1363) or anti-diuretic hormone (ADH) is a cyclical nonapeptide hormone, with a disulphide bridge joining the two cysteine molecules at position 1 and 6 in the structure (figure 7) (Russell & Glover, 2002:181). The structure can also be seen as a six amino acid ring with a three amino acid side chain (Fitzgerald, 2001:640). Human, and most mammalian vasopressin, is called arginine vasopressin (AVP) due to the arginine residue at position 8 (Nichols, 2000:1363).

This hormone is synthesised in the magnocellular secretory neurons located in the supraoptic nuclei (SON) and the paraventricular nuclei (PVN) of the hypothalamus (Russell & Glover, 2002:181). The biologically inactive precursor macromolecule, pre-pro-vasopressin, paves the way for the biologically active peptide, vasopressin. This 168 amino acid precursor protein (Jackson, 2001:793) is sequentially cleaved to pro-vasopressin and then to vasopressin.

Vasopressin becomes associated with its binding protein, neurophysin II, as well as the glycoprotein, co-peptin. It is then transported in neurosecretory granules or vesicles via non-myelinated axons to the internal infundibular zone of the posterior pituitary.

Here it accumulates in the axon terminals until required for release. The granules are released directly into the circulation or onto cells adjoining the vessel wall (Russell & Glover, 2002:181).

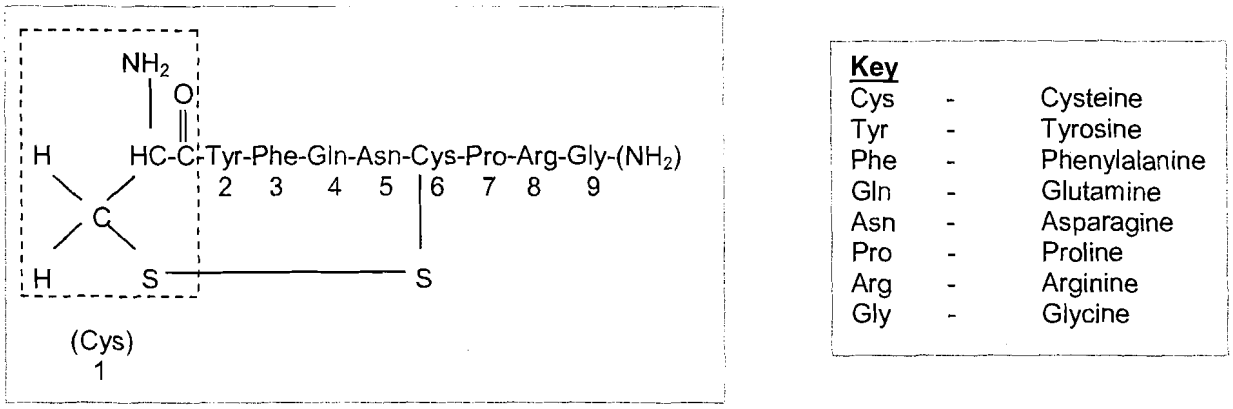


Figure 7: Arginine vasopressin (reproduced from Jackson, 2001:790)

Neurophysin dissociates from the vasopressin molecule prior to secretion, and does not seem to have any physiological role (Russell & Glover, 2002:181). The hormone enters the capillaries and is carried away by blood returning to the heart.

Normal plasma concentrations of vasopressin are as follows (Murray *et al.*, 2000:870):

- With a serum osmolality of 285 mOsmol/kg → 0-2 pg/ml
- With a serum osmolality of >290 mOsmol/kg → 2-12 pg/ml

However, according to Messina *et al.* (2003:468), normal physiological serum levels are 20-30 pg/ml.

The release from the axon terminals appears to be triggered mainly by nervous reflex responses or stimuli (Sweetman, 2002:1271) and occurs via exocytosis. The primary physiological stimulus is increased osmolality of plasma due to water deprivation, as well as hypovolemia and/or hypotension (Jackson, 2001:789). The stimuli are mediated by osmoreceptors in the hypothalamus and baroreceptors in the heart and vascular system.

Vasopressin release can also occur in the absence of changes in plasma osmolality. The non-osmotic regulation of vasopressin is modulated by afferent vagal and glossopharyngeal pathways from the left atrium and carotid sinus baroreceptors (Russell & Glover, 2002:182). Alcohol inhibits the release of vasopressin, resulting in enhanced diuresis (Fleming *et al.*, 2001:433).

Vasopressin is produced in other areas of the brain, where it serves as a neurotransmitter and –modulator (Vander *et al.*, 2001:275). It is also synthesised by the heart, where elevated wall stress increase vasopressin synthesis severalfold, and the adrenal medulla, where vasopressin stimulate catecholamine secretion. This action promotes adrenal cortical growth and stimulates aldosterone synthesis (Jackson, 2001:794).

The structure of human vasopressin and that of the vasopressin in most mammal species is similar to that of another hypothalamic hormone, oxytocin, differing only in two amino acid residues. The successful synthesis of these endogenous posterior pituitary hormones has given rise to the synthesis of a number of analogs of both oxytocin and vasopressin (Nichols, 2000:1362). An example of such a substance is ℓ-deamino-8-D-arginine vasopressin, more commonly known as desmopressin or DDAVP. This synthetic analogue differs from vasopressin in that the amino group has been removed from the *N*-terminal cysteine and ℓ-arginine at position 8 has been replaced by the D-enantiomer (Nichols, 2000:1363). DDAVP is preferred to vasopressin due to its greater anti-diuretic activity and more prolonged action. It also evokes minimal vasoconstrictor effects (Sweetman, 2002:1282).

2.2.2 PHARMACOLOGY

The physiological target cells of vasopressin can be found in the distal convoluted collecting tubules of the kidney. Vasopressin increases the permeability of the cells to water and therefore permits the osmotic equilibration of the collecting tubule contents with the hypertonic interstitial fluid (Granner, 2000:558-559). Molecular cloning has revealed that all of the known vasopressin and oxytocin receptors are members of the G-protein coupled receptor superfamily and they show significant structural homology to one another (Russell & Glover, 2002:182). With regard to vasopressin, two specific subtypes of membrane receptors exist:

- *V_1 receptors*: They are found extrarenally and mediate the vasoconstrictor action of the peptide (Reid, 2001:301). These receptors are subclassified into V_{1a} and V_{1b}, the latter also known as V_3. V_{1a} receptors are the most widespread in vascular smooth muscle, the adrenal gland, hepatocytes, platelets, renal medullary interstitial cells, some CNS structures, to name but a few. The adenohypophysis and adrenal medulla are known to contain V_{1b} receptors (Jackson, 2001:797).

  V_1 effects are mediated by the activation of phospholipase C through the binding of vasopressin to the receptor. Generation of inositol triphosphate and diacylglycerol takes place, which leads to an increased intracellular calcium concentration and activation of proteinkinase C with subsequent vasoconstricting effects (Reid, 2001:301).

- *V_2 receptors:* They are found mainly in the basolateral membrane of the principal cells of the distal tubule and mediate the antidiuretic action of the peptide (Russell & Glover, 2002:182 and Reid, 2001:301). With binding of vasopressin to the receptor, adenylcyclase (coupled to the G-protein G_s) is stimulated and the intracellular levels of cyclic AMP (cAMP) are increased. The latter triggers the insertion of a water channel, aquaporin$_2$ (AQP_2), into the luminal surface of the collecting tubule, thus increasing water reabsorption.

  Aquaporins are preformed, functional water channels located in the apical membranes of collecting duct cells and within the cytoplasm inside vesicles known as aggraphores. Only one aquaporin of a family of five is vasopressin-sensitive (i.e. AQP_2). In the presence of vasopressin, there is an immediate increase in the presence of AQP_2 in the apical membranes and increased movement of aggraphores towards these membranes, followed by a longer-term increase in AQP_2 production within the cells (Russell & Glover, 2002:182).

2.2.3 FUNCTION IN THE HUMAN BODY

Vasopressin has two major physiological roles to play in the human body (Russell & Glover, 2002:182 and Vander *et al.*, 2001:266):

- Serum osmoregulation, via control of water excretion by the kidneys.
- Mediation of baroreceptor reflexes, thus controlling blood pressure.

Vasopressin has a direct antidiuretic action on the kidney. Urine flow is decreased by increased resorption of water from the distal convoluted tubules and collecting ducts of the kidney (Nichols, 2000:1362). Urinary concentration usually takes place when the tubular fluid flows through the medullary collecting ducts. In the presence of a higher plasma concentration of vasopressin, water diffuses passively down an osmotic gradient across the collecting ducts into the hyperosmotic interstitial fluid surrounding the ducts. The water then enters the medullary capillaries and is carried out of the kidneys by venous blood. Reabsorption takes place until the fluid in this segment becomes isoosmotic to plasma in the peritubular cortex, thus 300 mOsmol per litre (Vander *et al.*, 2001:523,524). The total effect is thus a decrease in extracellular fluid osmolality. Vasopressin affords humans and mammals longer thirst free periods through this retention of water and allows the organism to survive longer periods of water deprivation (Jackson, 2001:794).

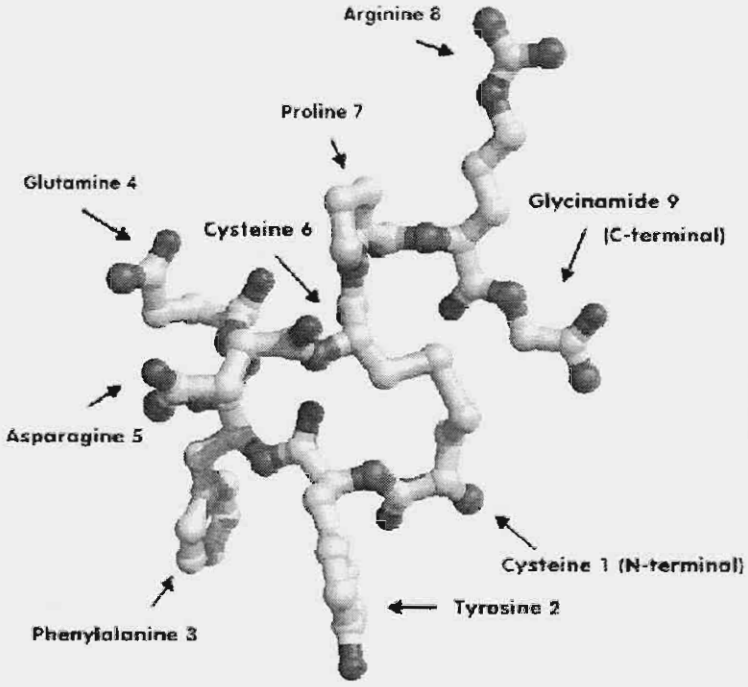
Regarding the control of blood pressure, vasopressin plays a lesser role under normal physiological circumstances. It only becomes important in pathophysiological states such as haemorrhage, hypovolemia and severe hypotension where it acts as a vasoconstrictor.

The action is mediated by V_1 receptors in vascular smooth muscle of the skin, skeletal muscle, fat, pancreas, thyroid gland, gastrointestinal tract, coronary vessels and the brain (Jackson, 2001:800). When cardiovascular pressures decrease (when blood volume decreases), baroreceptors decrease their rate of firing. This decrease in discharge rate to the cardiovascular centre in the medulla oblongata has increased vasopressin secretion as a result. When the plasma vasopressin concentrations becomes very high, widespread peripheral arteriolar constriction takes place. Arterial blood pressure is thus restored to its normal state (Vander *et al.*, 2001:531).

Apart from its main functions in the kidney and with regards to blood pressure, vasopressin also has positive effects on other organs of the body such as the central nervous system (Jackson, 2001:800-801 and Vander *et al.*, 2001:532), the gastrointestinal tract (Nichols, 2000:1362), the blood (Jackson, 2001:801), the uterus and the liver (Jackson, 2001:801).

2.2.4 PHYSICOCHEMICAL CHARACTERISTICS

[Arg^8]vasopressin acetate salt (Sigma, St. Louis, USA) was used during this study.



Approximate diameter in aqueous solution: 16 Å (1.6 nm), compared to 20 – 100 µm of those of sweat glands and hair follicles (Lelawongs *et al.*, 1989:14)

Figure 8: 'Ball-and-stick' model of arginine vasopressin (Crumley, 2003)

Name:	Vasopressin
Synonyms:	AVP; arginine vasopressin; [8-Arginine]vasopressin; argipressin
	ADH; antidiuretic hormone
	Beta-hypophamine, β-hypophamine
Amino-acid sequence:	Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-Gly-NH2
	(Disulphide bridge: 1-6)
Molecular formula:	$C_{46}H_{65}N_{15}O_{12}S_2$
CAS number:	113-79-1
Molecular weight:	1084.23 Da
pI:	10.9 (Nair & Panchagnula, 2004b:583)
pKa:	10.9 (Nair & Panchagnula, 2003b:564)
Partition coefficient:	Not available (According to Material Safety Data Sheet (MSDS))
Melting point:	Not available (According to Material Safety Data Sheet (MSDS))
Vasopressor activity:	Not less than 300 USP units per mg
Storage:	In airtight containers at 2-8 °C

Specifications of Sigma synthetic product used (Sigma-Aldrich Corporation, 2006a):

Appearance:	White powder
Solubility:	Clear odourless to faint yellow solution at 20 mg/ml in water
Water by Karl Fischer:	Not more than 10%
Acetate content:	Not more than 15%
Peptide content by amino acid analysis:	Not less than 75%
Purity by HPLC:	Not less than 95%
Shelf life:	4 years
Storage:	-20 °C

2.2.5 DISEASES ASSOCIATED WITH ARGININE VASOPRESSIN AND THERAPEUTIC APPLICATIONS OF THE DRUG

Several diseases transpire due to deficiencies associated with endogenous arginine vasopressin. These include for example a lack of adequate levels of the drug or the secretion of vasopressin when it is not suitable or necessary.

The most commonly known vasopressin-related disease is diabetes insipidus. According to Beers and Berkow (1999:1068) it is "a temporary or chronic disorder of the neurohypophyseal system due to deficiency of vasopressin (anti-diuretic hormone, ADH) and characterised by excretion of excessive quantities of very dilute (but otherwise normal) urine and by excessive thirst". Dilute urine with an osmolality of < 250 mOsmol/kg with hyponatremia is characteristic of diabetes insipidus.

Diabetes insipidus deprives the kidney of its capacity to produce concentrated urine due to the lowering of collecting duct permeability to water regardless of the state of body fluids (Vander *et al.*, 2001:522). Two major types of diabetes insipidus (DI) have been identified:

- *Cranial, pituitary or neurogenic DI*: Most commonly identified by a marked decrease in hypothalamic nuclei and subsequent secretion of vasopressin, due to pathological lesions involving the supraoptic and paraventricular nuclei as well as a major portion of the pituitary (Beers & Berkow, 1999:1068).

- *Nephrogenic or hereditary DI*: Caused by a failure in activity of normally secreted vasopressin on the renal tubules, presumably due to deflective expression of renal V_2 receptors or vasopressin-sensitive water channels (Fitzgerald, 2003:1075). It is a familial X-linked recessive disorder in male infants, but is more often secondary to hypercalcaemia, hypokalaemia and drug therapy (including lithium salts, foscarnet, clozapine and demeclocycline) (Sweetman, 2002:1273).

In general, DI is very rarely caused by abnormally high rates of degradation of vasopressin by circulating proteolytic enzymes (Jackson, 2001:801).

The diagnosis of DI is made by administering a dose of vasopressin in the form of desmopressin to test renal responsiveness and treatment for cranial DI entails replacement therapy with vasopressin in the form of desmopressin acetate intranasally, s.c. or i.v. (Beers & Berkow, 1999:1070).

SIADH is the abbreviation for the syndrome of inappropriate antidiuretic hormone secretion, and is "a disease of impaired water excretion with accompanying hyponatremia and hypoosmolality caused by inappropriate secretion of vasopressin (ADH)" (Jackson, 2001:803). It is called inappropriate because vasopressin is produced at a normal or increased rate without osmolarity- or volume-dependent physiological stimulation (Fukugawa *et al.*, 2003:841), thus causing a persistent and progressive dilutional hyponatremia with excretion of hypertonic urine (Granner, 2000:559).

Initial treatment is aimed at the underlying cause, but if symptoms persist, water restriction is considered. This is however an unpleasant form of treatment for patients who retain an inappropriate thirst (Sweetman, 2002:1277). The vasopressin antagonists demeclocycline and lithium carbonate (Ives, 2001:259) are utilised to manage and treat SIADH.

Another disease related to vasopressin is nocturnal enuresis. According to Eisendrath and Lichtmacher (2003:1049), nocturnal enuresis can be described as "involuntary micturition during sleep in a person who usually has voluntary control". This disorder is known to be more common in children and occurs usually 3-4 hours after bedtime, but it is not limited to a specific stage of sleep.

Two types of enuresis have been identified: Monosymptomatic/primary nocturnal enuresis (Müller *et al.*, 2004:718) and secondary nocturnal enuresis (Humphreys & Reinberg, 2005:152). The first involves nightly bedwetting persisting beyond the ages of 5 or 6 years with a frequency of at least two episodes per week, and the second has to do with the development of night-time enuresis after uninterrupted dryness was achieved for at least 6 months.

Nocturnal enuresis (NE) is thought to be caused by a delay in functional bladder maturation, interruption of the normal circadian rhythm of vasopressin secretion, abnormal sleep/arousal mechanisms or a combination of these (Humphreys & Reinberg, 2005:152). There seems to be a definite correlation between reduced nocturnal vasopressin secretion and the consecutive increase in nocturnal urine production (Müller *et al.*, 2004:719).

Treatment entails the administration of bedtime desmopressin to ameliorate NE by lowering the nocturnal urine production (Fitzgerald, 2001:641).

Apart from the use of arginine vasopressin in the diagnoses and treatments of diseases closely related to the drug, it has also been employed in a wide variety of other therapeutic circumstances. These medical situations include the following (Russell & Glover, 2002:183-186 and Barlow, 2002:307-310):

- Cardiac disease.
- Cardiopulmonary resuscitation (CPR).
- Vasodilatory shock.
- Portal hypertension and variceal haemorrhage.
- Brain death.
- Von Willebrand's disease (Beers & Berkow, 1999:1215 and Linker, 2003:509-510)

2.3 DEVELOPMENTS AND RESEARCH IN THE TRANSDERMAL DELIVERY OF ARGININE VASOPRESSIN

Arginine vasopressin is but one of several peptide hormone pharmaceuticals that are placed under the transdermal delivery spotlight. In this section we aimed at giving a summary of the most recent developments and research conducted using this drug as the peptide compound of choice.

In five different studies by Nair and Panchagnula (2003a, b, c and 2004a, b) arginine vasopressin (AVP) was used as the model peptide drug to demonstrate the transdermal delivery of a small peptide. AVP was chosen as representative of peptides in the molecular weight range of 1000-1500 Da. Three of these studies were conducted via rat epidermal skin and with the aid of iontophoresis coupled with a variety of chemical penetration enhancers. The remainder of the studies by these two researchers involved investigations into the electrical parameters and physicochemical considerations of AVP transdermal permeation, with references to earlier studies by Lelawongs *et al.* (1989, 1990).

Other studies that involved AVP as model peptide focused on the effects of buffer pH and concentration, as well as proteolytic enzyme inhibitors, on the stability of AVP and its degradation in rat and human cadaver skin (Bi & Singh, 2000, Banga *et al.*, 1995 and Morimoto *et al.*, 1992).

An earlier study by Banerjee and Ritschel in 1989 investigated the effects of pH, concentration of the drug, shaving of abdominal rat skin and surfactant on passive permeation of AVP.

2.3.1 TANDEM USE OF IONTOPHORESIS AND CHEMICAL PENETRATION ENHANCERS

The reasoning behind the execution of these experiments was that chemical enhancers in combination with transdermal iontophoresis (TI) might offer additional advantages over using each mode separately (Nair & Panchagnula, 2003a). It was determined that meaningful permeation rates of peptides could not be achieved solely by iontophoresis and it was therefore thought that combining two enhancement strategies acting by different mechanisms might overcome iontophoresis' shortcomings (Nair & Panchagnula, 2003b).

The first study by Nair & Panchagnula (2003a) regarding the tandem use of enhancers involved stable poloxamer 407 gels as opposed to aqueous solutions of AVP. These gels were evaluated for appearance, drug content and permeation across a PVDF membrane.

The gels were also evaluated *in vivo* by determination of blood levels of AVP in Sprague-Dawley (SD) rats, evaluation of pharmacodynamic activity by determination of urine osmolality in SD rats after gel application and quantification of AVP after release from the gel matrix across a PVDF membrane. Iontophoresis alone and in combination with the chemical enhancers (oleic acid and cineole) were used to augment transdermal permeation. Histopathological studies and an investigation into the metabolism of the drug in the presence of intact skin were also conducted. Regarding the results, the following are important: The maximum plasma concentration attained with iontophoresis alone was 0.047 ± 0.024 µg/ml, whereas a combination of oleic acid (2.5% w/v) and TI produced levels of 0.033 ± 0.008 µg/ml. The passive flux was 0.021 ± 0.002 µg/cm^2. During passive delivery, no significant levels of AVP were observed in the plasma. The stability studies of AVP in the presence of intact skin demonstrated cutaneous metabolism by skin enzymes during transport through the skin. The histopathological studies revealed that skin toxicity caused by either iontophoresis or chemical enhancers alone was lessened by using the two techniques in tandem.

The second study (Nair & Panchagnula, 2003b) involved the use of three different fatty acids (lauric acid, oleic acid and linoleic acid) in ethanol water (EtOH:W, 2:1) separately or together with TI. Sprague-Dawley (SD) rat skin was used in vertical Franz cell diffusion studies after being pretreated with the fatty acids and the skin washed with EtOH:W and ultra-pure water. The results indicated that all the fatty acids increased the flux of AVP and their effectiveness in flux enhancement was comparable. However, oleic acid in combination with TI significantly increased the permeation of AVP both in comparison to pretreatment with fatty acids and iontophoresis alone. Flux valves (in µg/cm^2/h) were the following:

Passive:	0.021
EtOH:W:	0.018
Iontophoresis:	0.270
Fatty acids:	0.409-0.413
Fatty acids + TI:	0.304 (linoleic acid) and 0.830 (oleic acid)

The third study (Nair & Panchagnula, 2004a) involved the use of four different terpenes (5% w/v carvone, pulegone, cineole and menthol) from three different classes separately or in combination with TI. During this study only the epidermis and SC from SD rat skin were used. Three sets of diffusion studies were performed. The first with only iontophoretic involvement, the second with terpene pretreated skin and the third with 2.5% w/v terpenes in tandem with TI. The results indicated that the terpenes increased the flux of AVP in comparison to the control (skin not pretreated with enhancer), but was not significantly different ($P > 0.05$) in comparison to iontophoresis.

Of the terpenes studied, maximum enhancement ratio was observed with cineole. Iontophoresis further increased AVP permeation through enhancer pretreated skin, but was according to Nair and Panchagnula not significant ($P > 0.05$) and therefore indicated no synergism in action between terpenes and iontophoresis. Flux values (in $\mu g/cm^2/h$) were the following:

Passive, EtOH:W and iontophoresis:	As for the second study
Terpenes:	0.083-0.237
Terpenes + TI:	0.271 (menthol) and 0.353 (cineole)

2.3.2 ELECTRICAL PARAMETERS AND PHYSICOCHEMICAL CONSIDERATIONS

An investigation into the influence of electrical parameters on iontophoretic transdermal delivery of AVP was undertaken by Nair & Panchagnula (2004b). They conducted studies on epidermis skin from SD rats to assess the effects of different current densities (CD's), durations, duty cycles and alternating polarity on vasopressin. To ensure the electrochemical stability of the peptide, HPLC was used. Fourier-transform infrared spectroscopy (FT-IR) was used to understand the biophysical changes caused in the skin due to the passage of current.

Nair & Panchagnula (2003c) also investigated the effects of various physicochemical parameters on the TI of vasopressin. The permeation of AVP showed a linear dependence on the concentration of drug in the donor medium. The iontophoretic flux was strongly influenced by the pH of the donor solution. Ionic strength was increased by varying the amount of NaCl from 0.05-0.25 M and flux was subsequently found to decrease. The receptor solution (normal saline) was replaced with HEPES buffer which induced a resultant four times increase in drug permeation. It was concluded that the physicochemical parameters of both the donor and receptor mediums played a significant role in the efficiency of drug delivery of AVP by iontophoresis.

2.3.3 EFFECTS OF BUFFER pH AND -CONCENTRATION AND PROTEOLYTIC ENZYME INHIBITORS

In a study by Bi and Singh (2000) the stability of AVP was investigated as a function of buffer pH, buffer concentration, salt concentration, temperature and skin with or without enzyme inhibitors. The effect of various pHs (2.8-8.5) of 0.1 M phosphate buffer containing 0.3 M NaCl was studied at 50 °C. Phosphate buffer concentrations (0.05, 0.1 and 0.15 M) and salt concentration (0.26, 0.4 and 0.6 M) were also studied at 50 °C. The stability of AVP in phosphate buffer (0.1M) and salt concentration (0.3 M) at pH 3.35 was studied at various temperatures (50, 75, 80 and 90 °C).

Fresh and viable pieces of pig ear skin were added to test tubes containing 25 µg/ml AVP and 0.3 mg/ml (300 µg/ml) of the enzyme inhibitors aprotinin, bestatin and leupeptin. Results indicated the buffer's pH affected the degradation rate of AVP, buffer ions and salt concentrations had no effect on degradation and maximum stability was achieved at pH 3.35. Bestatin was found to have the most stabilising effect on the degradation of AVP among the three enzyme inhibitors investigated. The high temperatures employed in this study seem absurd in the light of degradation of peptides and proteins that shows a marked dependence on temperature. However, during this study, these temperatures were employed to obtain an Arrhenius plot of log k against $1/T$. The slope was equalled to $-E_a/2.303R$ and from this equation the activation energy was calculated. Based on the Arrhenius equation, the shelf life of AVP (1.38 years) could be calculated at room temperature (25 °C) and at a pH of 3.35.

An earlier study by Morimoto *et al.* in 1992 was performed using the proteolytic enzyme inhibitors aprotinin, soybean trypsin inhibitor and camostat mesilate. During this study, camostat mesilate proved to be the most effective in inhibiting the activities of the main enzymes of the skin namely aminopeptidase and trypsin. This result was attributed to the high molecular weights of aprotinin (6500 Da) and soybean trypsin inhibitor (8000 Da) and the subsequent inability to permeate through the skin.

Banga *et al* (1995) investigated transdermal iontophoretic transport and the degradation of vasopressin across human cadaver skin. Degradation of vasopressin was studied in intact skin as well as skin homogenates. To also observe the degradation in the skin, vasopressin was spiked with [^{3}H]vasopressin and transported across skin with an anode in the donor. Samples were analysed by HPLC using a radiochromatography detector. For the iontophoretic studies, excised human skin, either full thickness or dermatomed to 250 µm, was thawed and mounted in Valia-Chien half-cells. Sampling was carried out for 12 hours for the study of degradation by intact skin. The donor solution of 0.25 mg/ml (250 µg/ml) AVP was exposed to the stratum corneum side of a piece of skin. An aluminium foil was placed between these two pieces to hinder any transdermal transport. Samples were taken from both half-cells and analysed by HPLC.

Results indicated that the cumulative amounts of intact vasopressin that permeated during iontophoresis (8 hours) was 15.37 (± 5.31; n = 3) µg/cm^2, which corresponds to only about 1% of permeation. During the degradation experiments, greater degradation was observed in the receptor where the peptide was in contact with the dermal side of the skin. With the aid of HPLC and the radiochromatography detector, only 40% of the intact vasopressin was found to have permeated in 12 hours. Several degradation peaks was seen in the chromatogram and no intact vasopressin permeated under passive conditions.

3 *POSSIBLE EXPEDIENTS TO FACILITATE TRANSDERMAL PERMEATION OF PEPTIDE DRUGS*

In this section of Chapter 1 prominence will be given to the two expedients which were utilised during this study. The first is bestatin hydrochloride, an enzyme inhibitor we employed to inhibit any enzymatic degradation of vasopressin in the skin. The second (and certainly the most important) is the Pheroid™ drug delivery system, a novel approach to the delivery of peptide drugs.

3.1 USE OF BESTATIN HYDROCHLORIDE AS AN ENZYME INHIBITOR

3.1.1 INTRODUCTION

Bestatin is a potent, competitive and specific aminopeptidase inhibitor with affinity for leucine aminopeptidase (LAP), aminopeptidase B (APB) and tri-aminopeptidase. It was originally isolated from culture filtrates of *Streptomyces olivoreticuli* by Umezawa and his colleagues in 1976 (Katragadda *et al.*, 2006:112, Scornik & Botbol, 2001:67 and Umezawa *et al.*, 1976:97-99). LAP and APB, as well as specific binding sites allocated solely for bestatin, are present on the surface of mammalian cells (Petrov *et al.*, 2000:538).

Bestatin was used in this study to selectively inhibit aminopeptidases present inside and on the surface of the skin, which could potentially degrade the studied active, arginine vasopressin (AVP). Bestatin was decided on as the aminopeptidase inhibitor of choice due to its higher stabilising effect on the degradation of AVP among the three enzyme inhibitors investigated in the article by Bi and Singh (2000). During all experimental work we made use of a Fluka (Sigma-Aldrich, St. Louis, USA) bestatin product, namely bestatin hydrochloride (MW = 344.83).

3.1.2 BASIC STRUCTURE AND CHARACTERISTICS

The structure of bestatin has been determined as *N*-[(2S-3R)-3-amino-2-hydroxy-4-phenyl-butanoyl]-L-leucine. Bestatin is a member of a class of natural products that contain β-amino-α-hydroxy amide residues which are the key units in their biological activity (Wasserman *et al.*, 1999:6163).

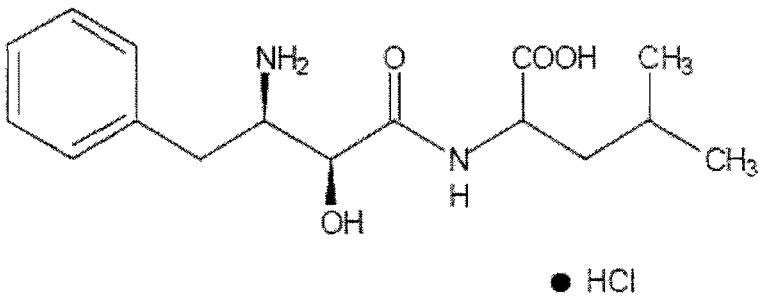


Figure 9: Chemical structure of bestatin hydrochloride (Sigma-Aldrich Corporation, 2005)

The following physicochemical properties of the bestatin hydrochloride used in experiments are of importance (Sigma-Aldrich Corporation, 2006b):

Synonyms:	Bestatin hydrochloride; Ubenimex; [(2S,3R)-3-amino-2-hydroxy-4-phenylbutanoyl]-L-leucine hydrochloride; *N*-[(2S,3R)-3-amino-2-hydroxy-4-phenylbutyryl]-L-leucine hydrochloride
Molecular formula:	$C_{16}H_{24}N_2O_4.HCl$
Molecular weight:	344.83
CAS Number:	65391-42-6
Melting point:	216-218 °C
Solubility:	H_2O: 25 mg/ml, clear, colourless
Storage temperature:	-20 °C
Appearance:	White powder
Purity:	Not less than 98%
pKa values:	8.1 and 3.1

Full IR and UV spectra have been reported (Umezawa *et al.*, 1976:97-99 and Suda *et al.*, 1976:100-101).

3.1.3 MECHANISM OF INHIBITION

Bestatin binds to the catalytic site of the enzyme and therefore competes with the substrate. The inhibitory effect of bestatin is however delayed. With low inhibitor concentration, lag time may be up to one hour before full inhibition is established. This delay is caused by slow binding of the inhibitor to the catalytic site. Inversely, higher concentrations of inhibitor yields shorter lag times. The inhibitor-enzyme complex is tightly bound and bestatin exhibits a competitive kinetics with the substrate. Full inhibition is reached with concentrations of bestatin much lower than that of the substrate (Scornik & Botbol, 2001:71). In the case of metal-dependent enzymes, the 3-amino-2-hydroxy acyl residue takes part in the chelation of the Zn^{2+} in the active site of the enzyme (Stöckel-Maschek *et al.*, 2005:4806).

3.1.4 UTILISATION AS ENZYME INHIBITOR AND THERAPEUTIC AGENT

The main proteolytic enzymes active in the human skin are aminopeptidase and trypsin (Bi & Singh, 2000:92). Being a pseudopeptide, bestatin is recognised by these enzymes as a possible substrate. Bestatin contains a β-amino-α-hydroxyamino acid as the *N*-terminal residue, and this moiety provides a firm interaction with zinc ions at the active sites of aminopeptidases (Tarnus *et al.*, 1996:1287).

According to Botbol and Scornik (1997:1149), bestatin permits the degradation of cellular proteins to di- and tripeptides but interferes with the further degradation of these peptides to amino acids. Some examples of the use of the inhibiting effect of bestatin are the following:

- Bestatin was employed to inhibit the actions of the oligopeptidases contained in the venom of the Taiwan cobra (*Naja naja atra*) (Anderson *et al.*, 1998:719-728).

- More recently bestatin was used in a study, among other inhibitors, to modulate transport and metabolism of amino acids and dipeptide prodrugs of acyclovir (ACV) across rabbit cornea (Katragadda *et al.*, 2006:104-113).

- Bestatin was utilised in nasal (Morimoto *et al.*, 1995:1) and transdermal (Bi & Singh, 2000:87) delivery studies as proteolytic enzyme inhibitor.

Apart from its obvious use in the inhibition of proteolytic enzymes, bestatin has also been made use of in other therapeutic situations. Bestatin has been shown to exhibit antitumor- (Ezawa *et al.*, 1996, Grujić & Renko, 2002, Fujisaki *et al.*, 2003 and Aozuka *et al.*, 2004) as well as antimalarial activity (Gardiner *et al.*, 2006). Bestatin has a multitude of effects on the immune system, both *in vivo* and *in vitro* (Scornik & Botbol, 2001:72) and acts as an analgesic by enkephalinase inhibition (De *et al.*, 1997:8383). Bestatin is also utilised in several miscellaneous situations of which some are somewhat out of the ordinary. They are the following:

- Cardiac fibroblast contraction (Lijnen *et al.*, 2004).
- Aminopeptidase inhibition in alcohol consumption (Szczepanska *et al.*, 1996).
- Nasal and tracheal absorption enhancement (Agu *et al.*, 2003 and Morimoto *et al.*, 2000).
- Ocular permeation and metabolism (Ranta *et al.*, 1998).
- Use in plants (Zheng *et al.*, 2006).

3.2 USE OF PHEROID™ TECHNOLOGY AS A THERAPEUTIC DRUG DELIVERY SYSTEM FOR PEPTIDE DRUGS

3.2.1 INTRODUCTION TO PHEROID™ TECHNOLOGY

Pheroid™ technology, previously known as Emzaloid™ technology, concerns a therapeutic delivery system which comprises of a distinctive submicron oil/water emulsion type formulation. A Pheroid™ is a stable, spherical or vesicular structure within this system that can be manipulated with regard to its morphology, structure, size and function (Schlebusch, 2002:8).

The intellectual property on which Pheroid™ technology is based, was purchased by the North-West University, South Africa in December 2003 from MeyerZall (Pty) Ltd, whose founder, Piet Meyer, had initially developed the technology for the treatment of his own psoriasis.

The novelty of Pheroid™ technology is underlined by patents registered in Europe, the USA, South Africa and China. These patents describe the use of Pheroid™ technology as a delivery system to promote the absorption and increase the efficacy of dermatological, biological and oral medicines in various pharmacological groups. This delivery system is trademarked, but will be referred to as Pheroid(s) throughout this chapter.

Pheroids are thought of to be closely related to other lipid-based delivery systems, such as liposomes, and are often confused with the latter. Some of the similarities and differences between Pheroids and these lipoidal systems will be described in a later section. The components of the Pheroid are manipulated in a very specific manner to ensure its high entrapment capabilities, very fast rate of transport, delivery and stability; making the Pheroid unique among its counterparts (Schlebusch, 2002:9). A formulation of this stature, which entraps its actives, is thought to be a safer option than one involving only an active ingredient. Pheroids are able to enhance the absorption and/or efficacy of several active ingredients and compounds, and have exhibited major improvements in the control of size, charge and hydrophilic-lipophilic characteristics of therapies, when compared to other systems (Grobler *et al.*, 2006:3).

A few of the key advantages of Pheroids are the following (Grobler, 2004:9-14 and Schlebusch: 2002:4):

- Increased delivery of active compounds.
- Decreased time to onset of action.
- Reduction of minimal effective concentration.
- Increased therapeutic efficacy.
- Reduction in cytotoxicity.
- Penetration of most known barriers in the body and in cells.
- Ability to target treatment areas.
- Lack of immunological response.
- Ability to transfer genes to cell nuclei.
- Reduction of drug resistance.

The uptake mechanism of the Pheroids by cells is still unclear, but it may be actively facilitated by a protein-mediated transfer process: the fatty acid membrane binding proteins generally present within lipid rafts in the cell membrane plays a role in this process.
Drugs are entrapped in the Pheroid vesicles with high efficiency and delivered with remarkable speed to target sites in the body. The Pheroid penetrates the keratinised tissue, skin, intestinal lining, vascular system, fungi, bacteria, and parasites (Grobler, 2004:4).

We decided on the use of the Pheroid drug delivery system because of its effectiveness and versatility. It is also one of the most inexpensive delivery systems in commercial use. All components used in the manufacturing of Pheroids are pharmaceutically safe and the system is based on the naturally occurring molecules of the body. The stability of the Pheroid delivery system has been proven for Pheroid-based commercialised products (Grobler *et al.,* 2006:27). In the light of successes achieved in the entrapment of antimalarials (Grobler, 2004:14) and even bacteria and viruses, we thought it wise to employ this system in the transdermal delivery of a peptide drug.

3.2.2 STRUCTURE/COMPOSITION OF PHEROIDS

Pheroids consists mainly of plant and essential fatty acids, amongst which is oleic acid (described in an earlier section). There are various types of Pheroids and each type of Pheroid has a particular composition. The different types of Pheroids are the following (Schlebusch, 2002:8):

- Lipid bilayer vesicles with nano- and micrometer diameters.
- Micro-sponges.
- Depots or reservoirs that contain pro-Pheroids.

The Pheroid delivery system is a colloidal system with the lipid-based submicron- and micron-sized spherical structures (the Pheroids), uniformly dispersed in the formulation. A typical colloidal system usually boasts with ultra-fine particles that are usually between 1-100 nm in diameter, but the Pheroids are typically formulated to have a diameter of between 200 nm and 2 µm (Grobler *et al.,* 2006:4).

Pheroids generally contain a lipid bilayer, as its peer the liposome, but it contains no phospholipids or cholesterol. In contrast to liposomes, Pheroids are formed by a self-assembly process similar to that of low-energy emulsions and microemulsions and no lyophilization or hydrations of the lipid components is necessary. The Pheroids are, like emulsions, dispersed within a dispersion medium, but it contains not only two liquid phases, but also a dispersed gas phase which is associated with the fatty acid dispersed phase (Grobler *et al.,* 2006:7).

The primary components of Pheroids are ethylated and pegylated polyunsaturated fatty acids, including the omega-3 and -6 fatty acids but excluding arachidonic acid. The fatty acids are in the *cis*-formation and therefore compatible with the orientation of the fatty acids in man (Grobler *et al.,* 2006:5). The essential fatty acids component included in the Pheroid can not be manufactured by human cells, but are necessary for various cell functions and have to be ingested.

Some of the advantages of these fatty acids are (Grobler, 2004:4):

- Maintenance of membrane integrity of cells.
- Energy homeostasis.
- Modulation of the immune system through the prostaglandin/leukotrin cascade.

Apart from the fatty acids, the Pheroid also contains nitrous oxide (N_2O), which is found distributed in close association with the dispersed phase throughout the continuous phase. Another dimension is added to the formulation of the basic Pheroid by the addition of this dispersed gas to the respective oil and water phases. The association of N_2O with the dispersed phase has been shown to have at least three functions:

- It contributes to the miscibility of the fatty acids in the dispersal medium.
- It contributes to the self-assembly process of the Pheroids.
- It contributes to the stability of the formed Pheroids.

Some interaction between the fatty acids and the nitrous oxide has been indicated by means of molecular modelling. This interaction has stable vesicular Pheroid structures as a result. The nitrous oxide essential fatty acid (NOEFA) matrix thus provides a functional model for the transport of hydrophobic and hydrophilic drugs. Controlled experiments were performed on various formulations and it was determined that if either the N_2O or the EFA's were absent from the formulations, a dramatic decline in efficacy and stability of the formulations would be observed (Grobler *et al.,* 2006:8-9).

The Pheroid delivery system is ideal in a transdermal situation due to the fact that the composition support the binding of the EFAs (essential fatty acids) to the fatty acid binding proteins of the keratinocyte, the chief cell of the stratum corneum. Furthermore, the intercellular lipid layer that maintains the barrier properties of the skin are supported and the trans-epidermal water loss inhibited by the essential fatty acid component of Pheroids (Grobler *et al.,* 2006:25).

3.2.3 PHEROIDS VERSUS OTHER LIPID-BASED DELIVERY SYSTEMS

The Pheroid delivery system differs significantly from conventional macromolecular carriers, such as liposomes and other lipid vesicular structures. The following table provides some of the similarities and differences between Pheroids and other lipid-based or liposomal drug delivery systems, and highlights advantages of both systems (adapted from Grobler, 2004:6-8):

Table 3: Similarities and differences between Pheroids and other lipid-based delivery systems

Pheroid	Other delivery systems
Consists mainly of essential fatty acids, a natural and essential ingredient of the body	Generally contain substances foreign to the body
Cytokine studies demonstrated that the Pheroid elicits no immune responses in man	Some liposomal formulations have been shown to elicit immune responses
Pheroid can be manipulated in terms of size, charge, lipid composition and membrane packing	Problems with the degree of repeatability of liposomal systems, liposomal types and sizes have been described
Pheroid consists of fatty acids and an affinity exists between the Pheroid and cell membranes	Specific binding and uptake mechanisms have not been described for other delivery systems
The Pheroid is polyphilic and drugs that have different solubilities as well as insoluble drugs can be entrapped	Most delivery systems are either lipophilic or hydrophilic
Sterically stabilised without the disadvantages of increased size or decreased elasticity	Delivery systems generally need to be sterically stabilised
Entrapment in Pheroid changes the pharmokinetics of active compounds, resulting in a decrease in the time needed to achieve maximum concentration levels	Liposomes have similarly been shown to change the pharmacodynamics of active compounds
The type of Pheroid formulated for a specific compound determines the loading capacity of that Pheroid	The loading capacity of most lipid-based delivery systems is dependent on the interior or intra-membrane volume and is therefore limited
The Pheroid showed *in vivo* stability during vaccine animal studies and in initial phase I volunteer trials	Both product and *in vivo* chemical and physical instability are problematic for some lipid-based delivery systems
Pheroids with entrapped small peptides and antibodies have been shown to interact with specific micro-domains on cells in culture. The purpose of the peptides and antibodies was drug targeting	Antibody-containing liposomes for drug targeting have been described

Due to the several advantages associated with the Pheroid delivery system, we thought it wise to employ it in the transdermal delivery of arginine vasopressin. The journal article included in this dissertation gives an account of our findings.

4 SUMMARY

During this study we made use of the peptide hormone pharmaceutical, arginine vasopressin (AVP), which is currently administered in its synthetic form via the parenteral route. Although this route offers advantages such as 100 % bioavailability and the avoidance of oral route limitations (enzymatic, gastric degradation, poor penetration of the gut wall and the first-pass effect of the liver), it still demonstrates shortcomings such as poor patient compliance and the inability of being employed in chronic situations. We aimed at exploring the transdermal application of AVP due to this route's non-invasive nature and ease of application. AVP is utilised in the diagnosis and treatment of, among others, diabetes insipidus and nocturnal enuresis, and transdermal delivery of this drug would surely facilitate more effective and uncomplicated treatment if applied via a gel or cream.

The transdermal delivery of protein and peptide pharmaceuticals is however no easy accomplishment due to proteins generally being of large molecular weight and their inability to traverse the skin via the transepidermal route (pores). Furthermore, the physical-chemical properties of peptides such as their electrical charge and relative hydrophilic nature, as well as their chemical instability due to relatively weak physical bonds, all contribute to the difficulty of delivery of peptides to the systemic circulation.

With the above complexities of transdermal peptide delivery in mind, we considered the Pheroid™ therapeutic drug delivery system as a possible solution. This system incorporates submicron-sized spheres or vesicles which 'entrap' and transport drug molecules across the skin and nasal mucosae. The major role players in this formulation are modified ethylated and pegylated polyunsaturated fatty acids, essential fatty acids and nitrous oxide (N_2O). The essential fatty acid component not produced by the human body should be ingested for normal cell function. One of these essential fatty acids deserves a special mention: Oleic acid has been proven by several studies as an effective penetration enhancer. Thus, we hypothesised that the Pheroid™ system would be able to transport a peptide molecule across the skin due to this dual advantage of a microsphere incorporating a penetration enhancer.

In a further effort to lessen the factors that restrain transdermal delivery of a peptide active, we included a proteolytic enzyme inhibitor together with the active in the donor solution during Franz cell diffusion studies. This inhibitor was bestatin, a selective aminopeptidase inhibitor, and previously proven deterrent of the degradation of AVP on the surface of the skin and within. We also investigated the effect of bestatin on the retention of AVP during diffusion studies.

The principal objectives of this study were the following:

- To investigate the possible *in vitro* transdermal permeation of AVP with the aid of the therapeutic drug delivery system Pheroid™.
- To investigate the effect of the proteolytic enzyme inhibitor bestatin on the retention of AVP during vertical Franz cell diffusion studies.

The journal article included in this dissertation gives an account of our findings in regards to the above objectives.

REFERENCES

AGU, R.U., JORISSEN, M., WILLEMS, T. KINGET, R. & VERBEKE, N. 2003. Mechanistic appraisal of the effects of some protease inhibitors on ciliary beat frequency in a sequential cell culture system of human nasal epithelium. *European journal of pharmaceutics and biopharmaceutics*, 55:283:289.

ANDERSON, L., BUSSLER, B. MARTINS, H. & DUFTON, M. 1997. Enkephalin-processing oligopeptidases in cobra venom: inhibition by thiorphan and bestatin reveals co-operative actions. *Toxicon,* 36(5):719-728.

AOZUKA, Y., KOIZUMI, K., SAITOH, Y., VEDA, Y., SAKURAI, H. & SAIKI, I. 2004. Anti-tumor angiogenesis effect of aminopeptidase inhibitor bestatin against B16-BL6 melanoma cells orthotopically implanted into syngeneic mice. *Cancer letters*, 216:35-42.

BANERJEE, P.S. & RITSCHEL, W.A. 1989. Transdermal permeation of vasopressin. I. Influence of pH, concentration, shaving and surfactant on permeation. *International journal of pharmaceutics*, 49:189-197.

BANGA, A.K., KATAKAM, M. & MITRA, R. 1995. Transdermal iontophoretic delivery and degradation of vasopressin across human cadaver skin. *International journal of pharmaceutics*, 116:211-216.

BARLOW, M. 2002. Vasopressin. *Emergency medicine*, 14:304-314.

BARRY, B.W. 1983. Dermatological formulations: percutaneous absorption. New York, N.Y.: Dekker. 480 p.

BARRY, B.W. 2001. Novel mechanisms and devices to enable successful transdermal drug delivery. *European journal of pharmaceutical sciences,* 14:101-114.

BARRY, B.W. 2002. Transdermal drug delivery. (*In* Aulton, M.E., *ed.* Pharmaceutics: The science of dosage form design. 2nd ed. Edinburgh; New York: Churchill Livingston. p.499-533)

BEERS, M.H. & BERKOW, R. 1999. The Merck manual of diagnosis and therapy. 17th ed. Whitehouse Station, N.J.: Merck Research Laboratories. 2833 p.

BHATIA, K.S & SINGH, J. 1998. Synergistic effect of iontophoresis and a series of fatty acids on LHRH permeability through porcine skin. *Journal of pharmaceutical sciences*, 87(4):462-469.

BI, M. & SINGH, J. 2000. Effect of buffer pH, buffer concentration and skin with or without enzyme inhibitors on the stability of [Arg^8]-vasopressin. *International journal of pharmaceutics*, 197:87-93.

BOTBOL, V. & SCORNIK, O.A. 1997. Measurement of muscle protein degradation in live mice by accumulation of bestatin-induced peptides. *American Journal of physiology: endocrinology and metabolism*, 273(36):E1149-E1157.

BüYüKTIMKIN, N., BüYüKTIMKIN, S. & RYTTING, J.H. 1997. Chemical means of transdermal drug permeation enhancement (*In* Ghosh, T.K., Pfister, W.R. & Yum, S.I., *eds.* Transdermal and topical drug delivery systems. Buffalo Grove, Illinois: Interpharm Press. p. 357-475)

CHIARELLO, K. 2004. Breaking the barrier. *Pharmaceutical technology*, 10:46-56, Oct.

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. Edinburgh; New York: Churchill Livingston. p. 544-553)

CRUMLEY, J. 2003. Nephrogenic and neurogenic diabetes insipidus. http://www.medicine.mcgill.ac/nephros Date of access: 30 Sep. 2006.

DANIELS, R. 2004. Strategies for skin penetration enhancement. *Skin care forum*, 37. http://www.scf-online.com/english/37_e/skinpenetration37_e.htm Date of access: 12 Jul. 2005.

DAVIS, A.F., GYURIK, R.J., HADGRAFT, J., PELLETT, M.A. & WALTERS, K.A. 2002. Formulation strategies for modulating skin permeation. (*In* Walters, K.A., *ed.* Dermatological and transdermal formulations. New York: Dekker. p. 271-317)

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(48):8383-8386.

EDWARDS, C.M.B., COHEN, M.A. & BLOOM, S.R. 1999. Peptides as drugs. *QJM:An international journal of medicine*, 92:1-4.

EISENDRATH, S.J. & LICHTMACHER, J.E. 2003. Psychiatric disorders. (*In* Tierney, L.M (Jr), McPhee, S.J. & Papadakis, M.A., *eds.* Current medical diagnosis & treatment 2003. 42nd ed. New York: McGraw-Hill. p. 1006-1066)

EZAWA, K., MINATO, K. & DOBASHI, K. 1996. Induction of apoptosis by ubenimex (bestatin) in human non-small-cell lung cancer cell lines. *Biomedicine & pharmacotherapeutics*, 50:283-289.

FINNIN, B.C. & MORGAN, T.M. 1999. Transdermal penetration enhancers: applications, limitations and potential. *Journal of pharmaceutical sciences,* 88(10):955-958.

FITZGERALD, P.A. 2001. Hypothalamic & pituitary hormones. (*In* Katzung, B.G., *ed.* Basic & clinical pharmacology. 8th ed. New York: Lange Medical Books/McGraw-Hill. p. 625-643)

FITZGERALD, P.A. 2003. Endocrinology. (*In* Tierney, L.M (Jr), McPhee, S.J. & Papadakis, M.A. *eds.* Current medical diagnosis & treatment 2003. 42nd ed. New York: McGraw-Hill. p. 1067-1149)

FLEMING, M., MIHIC, S.J. & HARRIS, R.A. 2001. Ethanol. (*In* Hardman, J.G. & Limbard, L.E., *eds.* Goodman & Gilman's: the pharmacological basis of therapeutics. 10th edition. New York: McGraw-Hill. p. 429-445)

FOLDVARI, M., BACA-ESTRADA, M.E., HE, Z. HU, J., ATTAH-POKU, S. & KING, M. 1999. Dermal and transdermal delivery of protein pharmaceuticals: lipid-based delivery systems for interferon α. *Biotechnology and applied biochemistry*, 30:129-137.

FRANZ, T.J. & LEHMAN, P.A. 2000. The skin as a barrier: Structure and function. (*In* Kydonieus, A.F. & Wille, J.J., *eds.* Biochemical modulation of skin reactions: transdermals, topicals, cosmetics. Boca Raton: CRC Press. p. 15-33)

FUJISAKI, T., OTSUKA, T., GONDO, H., OKAMURA, T., NIHO, Y., OHHINATA, A. & ABE, F. 2003. Bestatin selectively suppresses the growth of leukemic stem/progenitor cells with BCR/ABL MRNA transcript in patients with chronic myelogeneous leukemia. *International immunopharmacology*, 3:901-907.

FUKAGAWA, M., KUROKAWA, K. & PAPADAKIS, M.A. 2003. Fluid & electrolyte disorders. (*In* Tierney (Jr), L.M., McPhee, S.J. & Papadakis, M.A. *eds.* Current medical diagnosis & treatment 2003. 42nd ed. New York: McGraw-Hill. p. 839-866)

GARDINER, D.L., TRENHOLME, K.R., SKINNER-ADAMS, T.S., STACK, C.M. & DALTON, J.P. 2006. Overexpression of leucyl aminopeptidase in *Plasmodium falciparum* parasites: Target for the antimalarial activity of bestatin. *The journal of biological chemistry*, 281(3):1741-1745.

GHOSH, T.K. & PFISTER, W.R. 1997. Transdermal and topical delivery systems: an overview and future trends. (*In* Ghosh, T.K., Pfister, W.R. & Yum, S.I., *eds.* Transdermal and topical drug delivery systems. Buffalo Grove, Illinois: Interpharm Press. p. 1-32).

GRANNER, D.K. 2000. Pituitary and hypothalamic hormones. (*In* Murray, R.K., Granner, D.K., Mayes, P.A. & Rodwell, V.W., *eds.* Harper's Biochemistry. 25th ed. Stamford, Connecticut: Appleton & Lange. p. 550-559)

GROBLER, A. 2004. Emzaloid™ technology. (Confidential concept document presented to Ferring Pharmaceuticals) 20 p.

GROBLER, A., KOTZE, A. & DU PLESSIS, J. 2006. The design of a skin-friendly carrier for cosmetic compounds using Pheroid™ technology. (*In* Wiechers, J., *ed.* Delivery system technologies. Wheaton, IL.: Allured Publishing Corporation. *In press*)

GRUJIĆ, M. & RENKO, M. 2002. Aminopeptidase inhibitors bestatin and actinonin inhibit cell proliferation of myeloma cells predominantly by intracellular interactions. *Cancer letters*, 182:113-119.

HAMMAN, J.H., ENSLIN, G.M. & KOTZE, A.F. 2005. Oral delivery of peptide drugs: Barriers and development. *Biodrugs*, 19(3):165-177.

HUMPHREYS, M.R. & REINBERG, Y.E. 2005. Contemporary and emerging drug treatments for urinary incontinence in children. *Pediatric drugs*, 7(3):151-162.

IVES, H.E. 2001. Diuretic agents. (*In* Katzung, B.G., *ed.* Basic & clinical pharmacology. 8th ed. New York: Lange Medical Books/McGraw-Hill. p. 245-264)

JACKSON, E.K. 2001. Vasopressin and other agents affecting the renal conservation of water. (*In* Hardman, J.G. & Limbard, L.E., *eds.* Goodman & Gilman's: the pharmacological basis of therapeutics. 10th edition. New York: McGraw-Hill. p. 789-808)

KALIA, Y.N., NAIK, A., GARRISON, J. & GUY, R.H. 2004. Iontophoretic drug delivery. *Advanced drug delivery reviews*, 56:619-658.

KATRAGADDA, S., TALLURI, R.S. & MITRA, A.K. 2006. Simultaneous modulation of transport and metabolism of acyclovir prodrugs across rabbit cornea: An approach involving enzyme inhibitors. *International journal of pharmaceutics*, 320:104-113.

KYDONIEUS, A.F., WILLE, J.J. & MURPHY, G.F. 2000. Fundamental concepts in transdermal delivery of drugs. (*In* Kydonieus, A.F. & Wille, J.J., *eds.* Biochemical modulation of skin reactions: transdermals, topicals, cosmetics. Boca Raton: CRC Press. p. 1-14)

LELAWONGS, P., LIU, J. & CHIEN, Y.W. 1990. Transdermal iontophoretic delivery of arginine vasopressin (II): Evaluation of electrical and operational factors. *International journal of pharmaceutics*, 61:179-188.

LELAWONGS, P., LIU, J., SIDDIQUI, O. & CHIEN, Y.W. 1989. Transdermal iontophoretic delivery of arginine vasopressin (I): Physicochemical considerations. *International journal of pharmaceutics*, 56:13-22.

LIJNEN, P., PETROV, V., DIAZ-ARAYA, G. & FAGARD, R. 2004. Effect of bestatin on angiotensin I-, II-, and III-induced collagen gel contraction in cardiac fibroblasts. *Journal of the renin-angiotensin-aldosterone system,* 5(4):183-188.

LINKER, C.A. 2003. Blood. (*In* Tierney, L.M (Jr), McPhee, S.J. & Papadakis, M.A. *eds.* Current medical diagnosis & treatment 2003. 42nd ed. New York: McGraw-Hill. p. 469-521)

LUND, W. 1994. The Pharmaceutical Codex. 12th ed. London: The Pharmaceutical Press. 1117 p.

MEDI, B.M. & SINGH, J. 2003. Electronically facilitated transdermal delivery of human parathyroid hormone (1 - 34). *International journal of pharmaceutics*, 263:24-33.

MESSINA, L.M., PAK, L.K. & TIERNEY, L.M (JR) 2003. Blood vessels & lymphatics. (*In* Tierney, L.M (Jr), McPhee, S.J. & Papadakis, M.A. *eds.* Current medical diagnosis & treatment 2003. 42nd ed. New York: McGraw-Hill. p. 435-468)

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 of pharmaceutics*, 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 of pharmaceutics*, 113:1-8.

MORIMOTO, K., UEHARA, Y., IWANAGA, K. & KAKEMI, M. 2000. Effects of sodium glycocholate and protease inhibitors on permeability of TRH and insulin across rabbit trachea. *Pharmaceutica acta helvetiae*, 74:411-415.

MüLLER, D., ROEHR, C.C. & EGGERT, P. 2004. Comparative tolerability of drug treatment for nocturnal enuresis in children. *Drug safety*, 27(1):717-727.

MURRAY, R.K., GRANNER, D.K., MAYES, P.A. & RODWELL, V.W. 2000. Appendix. (*In* Murray, R.K., Granner, D.K., Mayes, P.A. & Rodwell, V.W., *eds.* Harper's Biochemistry. 25th ed. Stamford, Connecticut: Appleton & Lange. p. 867-872)

NAIK, A., PECHTOLD, L.A.R.M., POTTS, R.O. & GUY, R.H. 1995. Mechanism of oleic acid-induced skin penetration enhancement *in vivo* in humans. *Journal of controlled release*, 37:299-306.

NAIR, V.B. & PANCHAGNULA, R. 2003a. Poloxamer gel as vehicle for transdermal iontophoretic delivery of arginine vasopressin: evaluation of *in vivo* performance in rats. *Pharmacological research*, 47:555-562.

NAIR, V.B. & PANCHAGNULA, R. 2003b. Effect of iontophoresis and fatty acids on permeation of arginine vasopressin through rat skin. *Pharmacological research*, 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. *Pharmacological research,* 48:175-182.

NAIR, V.B. & PANCHAGNULA, R. 2004a. The effect of pretreatment with terpenes on transdermal iontophoretic delivery of arginine vasopressin. *IL 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. *IL farmaco*, 59:583-593.

NICHOLS, W.K. 2000. Hormones and hormone antagonists. (*In* Gennaro, A.R., *ed.* Remington: The science and practice of pharmacy. 20th ed. Baltimore, Md.; [Great Britain]: Lippincott Williams & Wilkins. p. 1358-1394)

PETROV, V., FAGARD, R. & LIJNEN, P. 2000. Effect of protease inhibitors on angiotensin-converting enzyme activity in human T-lymphocytes. *American Journal of hypertension*, 13(5):535-539.

PETTIT, D.K. & GOMBOTZ, W.R. 1998. The development of site-specific drug-delivery systems for protein and peptide biopharmaceuticals. *Tibtech*, 16:343-349.

PONTIROLI, A.E. 1998. Peptide hormones: Review of current and emerging uses by nasal delivery. *Advanced drug delivery reviews*, 291:81-87.

PRAUSNITZ, M.R. 1997a. Reversible skin permeabilization for transdermal delivery of macromolecules. *Critical reviews™ in therapeutic drug carrier systems,* 14(4):455-483.

PRAUSNITZ, M.R. 1997b. 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. Washington, D.C.: American Chemical Society. p. 124-153)

RANTA, V-P., HÄMÄLÄINEN, K.M., AURIOLA, S. & URTTI, A. 1998. Gradient high-performance liquid chromatographic analysis of enkephalin peptides, their metabolites, and enzyme inhibitors using combined ultraviolet and electrochemical detection. Application to ocular permeability studies *in vitro. Journal of chromatography B,* 709:1-10.

REID, I.A. 2001. Vasoactive peptides. (*In* Katzung, B.G., *ed.* Basic & clinical pharmacology. 8th ed. New York: Lange Medical Books/McGraw-Hill. p. 292-310)

ROBERTS, M.S., CROSS, S.E. & PELLETT, M.A. 2002. Skin transport. (*In* Walters, K.A., *ed.* Dermatological and transdermal formulations. New York: Dekker. p. 89-195)

ROTHEN-WEINHOLD, A. & GURNY, R. 1997. Controlled and/or prolonged parenteral delivery of peptides from the hypothalamic pituitary axis. *European journal of pharmaceutics and biopharmaceutics,* 435:115-131.

RUSSELL, J.C. & GLOVER, P.J. 2002. The physiology and clinical applications of vasopressin in critical illness. *Critical care and resuscitation*, 4:181-191.

SCHLEBUSCH, J. 2002. A briefing document on the use of the MeyerZall therapeutic systems, based on Emzaloid™ technology, to increase the absorption of active ingredients. George: MeyerZall Laboratories. (Unpublished document, confidential and classified) 39 p.

SCORNIK, O.A. & BOTBOL, V. 2001. Bestatin as an experimental tool in mammals. *Current drug metabolism*, 2(1):67-85.

SIGMA-ALDRICH CORPORATION. 2004. Material safety data sheet for arginine vasopressin. http://www.sigma-aldrich.com Date accessed: 3 Jul. 2006.

SIGMA-ALDRICH CORPORATION. 2005. Product information for bestatin hydrochloride. http://www.sigma-aldrich.com Date accessed: 13 Mar. 2006.

SIGMA-ALDRICH CORPORATION. 2006a. Specification sheet for arginine vasopressin.

SIGMA-ALDRICH CORPORATION. 2006b. Specification sheet for bestatin hydrochloride. http://www.sigma-aldrich.com Date accessed: 13 Mar. 2006.

SMITH, E. & SURBER, C. 2000. The absolute fundamentals of transdermal permeation (drug delivery for dummies). (*In* Gabard, B., Elsner, P., Surber, C. & Treffel, P., *eds.* Dermatopharmacology of topical preparations: a product development oriented approach. Berlin; New York: Springer. p. 23-35)

SMITH, P.L. 1997. Peptide delivery via the pulmonary route: a valid approach for local and systemic delivery. *Journal of controlled release*, 46:99-106.

SOOD, A. & PANGHAGNULA, R. 2001. Peroral route: an opportunity for protein and peptide drug delivery. *Chemical reviews*, 101:3275-3303.

STöCKEL-MASCHEK, A., STIEBITZ, B., KOELSCH, R. & NEUBERT, K. 2005. Novel 3-amino-2-hydroxy acids containing protease inhibitors. Part 1: Synthesis and kinetic characterization as aminopeptidase P inhibitors. *Bioorganic & medical chemistry*, 13:4806-4820.

SUDA, H., TAKITA, T, AOYAGI, T. & UMEZAWA, H. 1976. The structure of bestatin. *The journal of antibiotics (Tokyo)*, 29:100-101.

SWEETMAN, S.C. 2002. Martindale: the complete drug reference. 33rd ed. London: Pharmaceutical Press. 2483 p.

SZCZEPANSKA, R., HARDING, S. & GRUPP, L.A. 1996. Characterization of the decline in alcohol consumption by aminopeptidase inhibition. *Drug and alcohol dependence*, 43:133-141.

TARNUS, C., REMY, J.M. & d'ORCHYMONT, H. 1996. 3-Amino-2-hydroxy-propionaldehyde and 3-amino-1-hydroxy-propan-2-one derivates: New classes of aminopeptidase inhibitors. *Bioorganic & medicinal chemistry*, 4(8):1287-1297.

UMEZAWA, H, AOYAGI, T., SUDA, H., HAMADA, M. & TAKEUCHI, T. 1976. Bestatin, an inhibitor of aminopeptidase B, produced by actinomycetes. *The journal of antibiotics (Tokyo)*, 29:97-99.

VANDER, A., SHERMAN, J. & LUCIANO, D. 2001. Human physiology: the mechanisms of body function. 8th ed. New York: McGraw-Hill. 800 p.

VEUILLEZ, F., KALIA, Y.N., JACQUES, Y., DESHUSSES, J. & BURI, P. 2001. Factors and strategies for improving buccal absorption of peptides. *European journal of pharmaceutics and biopharmaceutics,* 51:93-109.

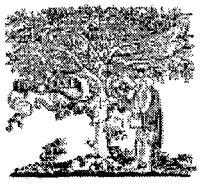
WASSERMAN, H.H., XIA, M., PETERSEN, A.K., JORGENSEN, M.R. & CURTIS, E.A. 1999. Synthesis of the peptidic α-hydroxy amides phebestin, probestin and bestatin from α-keto amide precursors. *Tetrahedron letters*, 40:6163-6166

WILLIAMS, A.C. 2003. Transdermal and topical drug delivery: from theory to clinical practice. London; Chicago: Pharmaceutical Press. 242 p.

ZHENG, W., ZHAI, Q., SUN, J., LI, C-B., ZHANG, L., LI, H., ZHANG, X., LI, S., XU, Y., JIANG, H., WU, X. & LI, C. 2006. Bestatin, an inhibitor of aminopeptidases, provides a chemical genetics approach to dissect jasmonate signalling in Arabidopsis. *Plant physiology*, 141:1400-1413.

CHAPTER 3: ARTICLE FOR PUBLICATION IN THE EUROPEAN JOURNAL OF PHARMACEUTICAL SCIENCES

GUIDE FOR AUTHORS: EUROPEAN JOURNAL OF PHARMACEUTICAL SCIENCES



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Abbreviations are a hindrance for the reader. Use as few abbreviations as possible and write out names of compounds, receptors, etc., in full throughout the text of the manuscript, with the exceptions given below. Unnecessary and nonsense abbreviations are not allowed. Generic names should not be abbreviated. As an example, AMP, HAL, HIST, RAMH, TAM, SST, for amphetamine, haloperidol, histamine, (R)-α -methylhistamine, tamoxifen, somatostatin, are not accepted. Abbreviations which have come to replace the full term (e.g., GABA, DOPA, PDGF, 5-HT, for Y -aminobutyric acid, 3,4-dihydroxyphenylalanine, PDGF, 5-hydroxytryptamine) may be used, provided the term is spelled out in the abstract and in the body of the manuscript the first time the abbreviation is used. Unwieldy chemical names may be abbreviated. As an example, 8-OH-DPAT, DOI, DTG, BAPTA, for 8-hydroxy-2-(di-*n*-propylamino)tetralin, 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane, 1,3-di(2-tolyl)-guanidine, 1,2-bis(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid, are acceptable; however, the full chemical name should be given once in the body of the manuscript and in the abstract, followed in both cases by the abbreviation. Code names may be used, but the full chemical name should be given in the text and in the abstract. *Authors not conforming to these demands may have their manuscripts returned for correction with delayed publication as a result.*

Some abbreviations may be used without definition:

ADP,CDP, GDP, IDP	5'-pyrophosphates of adenosine
UDP	cytidine, guanosine, inosine, uridine
AMP etc.	adenosine 5'-monophosphate etc.
ADP etc.	adenosine 5'-diphosphate etc.
ATP etc.	adenosine 5'-triphosphate etc.
CM-cellulose	carbo methylcellulose
CoA and acetyl-CoA	coenzyme A and its acyl derivatives

DEAE-cellulose	O-(diethylaminoethyl)-cellulose
DNA	deoxyribonucleic acid
EGTA	ethylene glycol-bis(β-aminoethyl ether)*N,N,N',N'*-tetraacetic acid
FAD	flavin-adenine dinucleotide
FMN	flavin mononucleotide
GSH, GSSG	glutathione, reduced and oxidized
Hepes	4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid
NAD	nicotinamide-adenine dinucleotide
NADP	nicotinamide-adenine dinucleotide phosphate
NMN	nicotinamide mononucleotide
Pi, PPi	orthophosphate, pyrophosphate
RNA	ribonucleic acid
Tris	2-amino-2-hydroxymethylpropane-1,3-diol

Two alternative conventions are currently in use in some cases. For example, for the phosphoinositides there are both the abbreviations recommended by the IUPAC-IUB and those of the Chilton Convention (e.g., PtdIns(4,5)P_2 vs. PIP_2 for phosphatidylinositol 4,5-biphosphate). The journal will accept either of these forms but not their combination.

Abbreviations of units of measurements and other terms are as follows:

Units of mass

kilogram	kg
gram	g
milligram	mg
microgram	µg
nanogram	ng
mole (gram-molecule)	mol
millimole	mmol
micromole	µmol
nanomole	nmol
picomole	pmol
femtomole	fmol
equivalent	eq

Units of time

hour	h
minute	min
second	s
millisecond	ms
microsecond	µs

Units of volume

litre	l
millilitre	ml
microlitre	µl

Units of length

metre	m
centimetre	cm
millimetre	mm
micrometre	µm
nanometre	nm

Units of concentration

molar (mol/l)	M
millimolar	mM
micromolar	µM
nanomolar	nM
picomolar	pM

Units of heat, energy, electricity

joule	J
degree Celsius (centigrade)	°C
coulomb	C
ampere	A
volt	V
ohm	Ω
siemens	S

Units of radiation

curie	Ci
counts per minute	cpm
disintegrations per minute	dpm
becquerel	Bq

Miscellaneous

gravity	*g*
dissociation constant	K_d
median doses	LD_{50}, ED_{50}
probability	*P*
routes of drug administration	i.v., i.p., s.c., i.m.
square centimetre	cm^2
standard deviation	S.D.
standard error of the mean	S.E.M.
Svedberg unit of sedimentation coefficient	S
Hill coefficient	n_H

The isotope mass number should appear before the atomic symbol, e.g., [^{3}H]noradrenaline, [^{14}C]choline. Ions should be written: Fe^{3+}, Ca^{2+}, Mg^{2+}. The term absorbance (A) is preferred to extinction or optical density. For abbreviations not included in this list consult: *Units, Symbols and Abbreviations, A Guide for Biological and Medical Authors and Editors*, 1994 (The Royal Society of Medicine, London), ISBN 0-905958-78-0, or *Scientific Style and Format. The CBE Manual for Authors, Editors, and Publishers*, 6th edn (Cambridge University Press, Cambridge), ISBN 0-521-47154-0.

1.5. Nomenclature

Only generic and chemical names of drugs should be used, although a proprietary equivalent may be indicated once, in parentheses. *Pharmacological and Chemical Synonyms*, E.E.J. Marler, 9th edn (Elsevier, Amsterdam, 1990) may be consulted.

The nomenclature of chemical substances should be consistent, clear and unambiguous, and should conform to the usage of the American Chemical Society and the convention recommended by the International Union of Pure and Applied Chemistry (IUPAC).

When in doubt, writers should consult the indexes of *Chemical Abstracts*; the various reports and pamphlets of the American Chemical Society Committee on Nomenclature, Spelling and Pronunciation; and from the International Union of Biochemistry and Molecular Biology (IUBMB): *Biochemical Nomenclature and Related Documents* (Portland Press, London).

When drugs, which are mixtures of stereoisomers, are used, the fact that they have a composite nature and the implication of this for interpretation of the data and drawing of conclusions should be made clear. The use of the appropriate prefix is essential. Use of the generic name alone without prefix would be taken to refer to agents with no stereoisomers. The nomenclature of the various isomers and isomeric mixtures can be found in: (i) *IUPAC, Nomenclature of Organic Chemistry*, eds. J. Rigaudy and S.P. Klesney (Pergamon Press, London), 1979, p. 481; (ii) *Signs of the times: the need for a stereochemically informative generic name system*, Simonyi, M., J. Gal and B. Testa, 1989, Trends Pharmacol. Sci. 10, 349. For nomenclature of peptides, see *Neuropeptides*, Vol. 1, 1981, p. 231.

The nomenclature of receptors and their subtypes should conform to the *TIPS 1995 Receptor & Ion Channel Nomenclature Supplement (Trends Pharmacol. Sci.* Receptor Nomenclature Supplement 1995). Copies of this supplement are available from the publisher (Elsevier Trends Journals, Oxford Fulfilment Centre, P.O. Box 800, Kidlington, Oxford OX5 1DX, UK. Tel.: (44-1865) 843-699; Fax: (44-1865) 843-911).

The trivial name of the enzyme may be used in the text, but the systematic name and classification number according to *Enzyme Nomenclature*, rev. edn (Academic Press, New York, NY, 1984) should be quoted the first time the enzyme is mentioned.

1.6. Editorial review

All manuscripts are generally submitted to 2-3 referees who are chosen for their ability to evaluate the work. Supplementary material may be included to facilitate the review process. Authors may request that certain referees should not be chosen. Members of the editorial board will usually be called upon for advice when there is disagreement among the referees or between referees and authors, or when the editors believe that the manuscript has not received adequate consideration by the referees.

All referees' comments must be responded to, and suggested changes be made. The author should detail the changes made in response to the referees' comments and suggestions in an accompanying letter. If the author disagrees with some changes, the reason, supported by data, should be given. The editors may refuse to publish manuscripts from authors who persistently ignore referees' comments. Handwritten additions or corrections will not be accepted. Only complete retyping of the pages affected by revision is acceptable. A revised manuscript should be received by the editorial office no later than 4 months after the editorial decision was sent to the author(s); otherwise it will be processed as a new manuscript.

2. Organization and style of manuscripts

Authors should consult a current issue of the journal for the general manner of presentation. Manuscripts should be written in clear, concise English (see section 1.1), bearing in mind that English is not the native language of many of the readers. Terms that are not generally understood should be avoided; however if it is absolutely necessary to use such terms, they must be defined.

2.1. Research articles

2.1.1. General

The manuscript of a research article should be arranged as follows.

First page: title, surname(s) and full first name(s) of each author; name and address of the establishment where the work was done; name, full postal address, telephone and telefax numbers and e-mail of author to whom proofs and other correspondence should be sent. Next page: abstract and keywords (indexing terms, normally 3-6 items). Pages 3 to end: 1. Introduction; 2. Materials and methods; 3. Results; 4. Discussion; Acknowledgements; References; Tables; Figure legends and Figures. Parts 3 & 4 may be combined into one item: Results & Discussion. Subdivisions of a section should also be numbered within that section: 2.1., 2.2., 2.3., etc. All pages should be numbered consecutively, the title page being p. 1. See section 2.7 for further information.

Supplementary material for electronic publication can be published on the journal website alongside the article. In the print version, a URL reference will be made to point readers to the location of the article and supplementary material.

2.1.2. Abstract and keywords

The abstract with keywords should be typed on a separate sheet. The abstract should include: the reason why the experiments were done, a very brief description of the experiments (including species, tissue, etc.), followed by the main results, and finally, a conclusion giving the relevance of the results to the question asked.
The abstract must be completely self-explanatory. The abstract should not exceed approximately 200 words. No footnotes may be used and a reference, if cited, must be given in full. Standard terms and scientific nomenclature should be used. Abbreviations and contractions, except those for weights and measures and those explained, should not be used. Below the abstract, type 3-6 keywords or short phrases suitable for indexing. These terms will be printed at the end of the abstract. If possible, keywords should be selected from *Index Medicus* or *Excerpta Medica Index*.

2.1.3. Introduction, Materials and Methods, Results, Discussion

The introduction should not be an extensive review of the literature but should refer only to previous work which has a direct bearing on the topic to be discussed.

Materials and methods should be written clearly and in such detail that the work can be repeated by others. Procedural detail that has been published previously should be referred to by citation. When a modified procedure is used, only the author's modifications of the previously published method need to be given in detail.

Results should be described concisely. Text, tables and figures must be internally consistent.

The discussion should involve the significant findings presented. Wide digressions are unacceptable because of the limitations of space.

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Authors are responsible for the accuracy and completeness of their references as these will not be checked by the editorial office.

References should be listed alphabetically (see sample references) according to the "Harvard" system. Articles written by the same first author with different second authors should be listed according to the second author's surname.

Articles written by the same first author with more than one co-author should be listed alphabetically according to the first author's surname and then according to the year of publication. Two or more references to the same first author with the same publication year should have a, b, c, etc., suffixed to the year indicating the alphabetical order of the second or third author, etc.

References to journals should contain the names and initials of the author(s), the year, the full title, the abbreviation of the name of the periodical according to those in the Bibliographic Guide for Editors and Authors (American Chemical Society, Washington, DC.) followed by the volume and page numbers.

References to books should include the title and name and city of the publisher.

References in the text should be cited by the author's name and the year of publication. For 3 or more authors the name of the first author followed by et al.. should be used, e.g., Davis, Robinson (1990) or (Davis, Illum, 1984; de Ber et al.., 1988, 1989; Borchardt et al.., 1990, 1991a,b,c).

Journals:

Fagerholm, U., Lennernas, H., 1995. Experimental estimation of the effective unstirred water layer thickness in the human jejunum and its importance in oral drug absorption. Eur. J. Pharm. Sci. 3, 247-253.

Lipinski, C.A., Lombardo, F., Dominy, B.W., Feeney, P.J., 1997. Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. Adv. Drug. Deliv. Rev. 23, 3-25.

Books:

Alderborn, G., Nystrom, C., 1998. Pharmaceutical Powder Compaction Technology. Marcel Dekker, New York.

Kissel, T., Koneberg, R., 1996. Injectable biodegradable microspheres for vaccine delivery. In: Cohen, S. and Bernstein, H (Eds.), Microparticulate systems for the delivery of proteins and vaccines. Marcel Dekker, New York, pp. 51-87.

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The number of illustrations should be limited to the essential.

(a) It is important to allow for reduction to fit a single column, 8.4 cm wide or at most a double column, maximally 17.6 cm wide. Of preference, illustrations, especially photomicrographs, should be submitted in their final size (single or double column). When possible, all key symbols should be explained in the figures. All letters and numerals appearing in a particular illustration should be of the same size (approximately 1.4-2.0 mm height when reduced to 8.4 cm width). Comparable illustrations should carry letters, figures and numerals of the same size when reduced to 8.4 cm width.

(b) Graphs should be prepared by a skilled photographer so that the dark, cross-hatched background is eliminated, the faint portions of the graphs are intensified, and a sharp print is obtained. This process may be avoided by using blue-ruled instead of black-ruled recording paper for the originals.

(c) Drawings of chemical structures should as far as possible be produced with the use of a drawing program such as ChemDraw. Authors using the current versions of ChemDraw, ChemIntosh and ChemWindows should use the JOC format.

(d) A calibration bar should be drawn on the micrographs instead of giving a magnification factor in the figure legend.

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Tables should be prepared for use in a single column (8.4 cm wide) or be of page width (17.6 cm).

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(b) Tables should not duplicate material in text or illustrations.

(c) Short or abbreviated column headings should be used and, if necessary, explained in footnotes, and indicated as [a], [b], [c], etc.

(d) Statistical measures of variation, S.D., S.E., etc. should be identified.

(e) Tables should be numbered separately in Arabic numerals (Table 1, 2, etc.).

2.5. Formulas and equations

Structural chemical formulas, process flow diagrams and complicated mathematical expressions should be very clearly presented. All subscripts, superscripts, Greek letters and unusual characters must be identified. Structural chemical formulas and process flow diagrams should be prepared in the same way as graphs.

2.6. GenBank accession numbers

Gene accession numbers refer to genes or DNA sequences about which further information can be found in the databases at the National Center for Biotechnical Information (NCBI) at the National Library of Medicine. Authors wishing to enable other scientists to use the accession numbers cited in their papers via links to these sources, should reference this information in the following manner:

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Example 3: "GenBank accession nos. AI631510, AI631511, AI632198, and BF223228), a B-cell tumor from a chronic lymphatic leukemia (GenBank accession no. BE675048), and a T-cell lymphoma (GenBank accession no. AA361117)".

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One page suggestions for commentaries and mini-reviews should be sent directly to the Editor-in-Chief at mailto:ejps-journal@helsinki.fi for consideration. Please see detailed information on commentaries and mini-reviews below.

2.9.1 Commentaries (Guidance)

The definition of a Commentary for EJPS is three-fold. Firstly, it can be an argued piece of provocative scientific writing purporting to take a balanced position on a controversial pharmaceutical science topic. A second option is for the author to approach the topic from a particular viewpoint on one side of an argument. A third option is to provide a topical update on a hot topic in Pharmaceutical Sciences and this can be more informative than controversial.

Commentaries will be commissioned by the editors in advance or invited from non-commissioned authors if they wish to initially submit a one page summary of the intended Commentary to the editors in advance. All manuscripts will be assessed by 2-3 independent referees.

The journal is looking for stimulating and provoking essays, with referenced material, but without an extensive reference list. Commentaries can contain one summary figure and/or table and should have no more than 30 references to preferably recent peer-reviewed material. The word count should be approximately 2,000 words maximum. The commentary should have a short abstract summary of 150 to 200 words and 4-5 key words should be included, The text should be broken down into 4-5 numbered sections beginning with an Introduction and ending with a Conclusions section. A model of the structures is to be found in Eur. J. Pharm. Sci. 19, 1-11 by R.D. Combes.

2.9.2 Mini-review (Guidance)

Mini-reviews are thought provoking reviews of contemporary pharmaceutical research. Themes are as described in the Scope of the Journal section.

Mini-reviews will usually be commissioned by the editors in advance, but contributions are invited from non-commissioned authors if they wish to initially submit a one page summary of the intended review to the editors in advance. All manuscripts will be assessed by 2-3 independent referees.

The structure of the mini-review is as follows: a title page followed by a 200-300 word abstract with 4-5 key words. The text is then divided into numbered sections finishing with a Summary section. References should be kept to a maximum of 60 and should be mostly to recent peer-reviewed material.

There is a combined maximum of 5 figures / tables. Authors are encouraged to submit their original unpublished work as part of the review if appropriate. The total length of the review should be a maximum of 4,000 words.

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JOURNAL ARTICLE: TRANSDERMAL DELIVERY OF ARGININE VASOPRESSIN WITH PHEROID™ TECHNOLOGY

THE TRANSDERMAL DELIVERY OF ARGININE VASOPRESSIN WITH PHEROID™ TECHNOLOGY

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Abstract

The aim of this study was to investigate *in vitro* transdermal diffusion of a small peptide namely arginine vasopressin (AVP) with the aid of the novel Pheroid™ drug delivery system. Generally, peptides seem unfit for transdermal permeation, but it was thought prudent to explore the suitability of this lipid-based system after success was achieved with entrapment of tuberculostatics, bacteria and viruses. Bestatin (a selective aminopeptidase inhibitor) was employed to circumvent any skin-related degradation of the active. Therefore, the effect of bestatin on the preservation of AVP during diffusion was investigated. Vertical Franz cell diffusion studies were conducted with female abdominal skin, with AVP at a concentration of 150 µg/ml in the donor phase and Hepes buffer as the receptor phase over a twelve-hour period. To prove entrapment of AVP within the lipid structures of the Pheroids™, fluorescently-labelled samples were monitored by means of confocal laser scanning microscopy (CLSM), which revealed definite entrapment. *In vitro* permeation profiles for AVP exhibited a biphasic character, with the majority of permeation occurring during the first two hours. The Pheroid™ delivery system proved to be advantageous when applied as delivery medium. The inclusion of bestatin has an enhancing effect on permeation probably due to its protection of AVP.

arginine vasopressin

transdermal diffusion

confocal microscopy

Pheroid™

delivery system

bestatin

1. Introduction

In recent times, advances in recombinant DNA biotechnology and the production of an increasing number of synthetic macromolecules have led to large-scale production and increased cost-effective commercialisation of, among others, peptide and protein pharmaceuticals. It is commonly known that macromolecules such as peptides and proteins are poorly bio-available when administered orally, due to extensive enzymatic degradation and poor penetration of the gut wall. In this regard, transdermal delivery of peptide drugs can be considered as a viable route due to its non-invasiveness and ease of administration with concomitant avoidance of the first-pass effect of the liver (Medi & Singh, 2003).

However, the stratum corneum is known to be a nearly impenetrable barrier to transport (Prausnitz, 1997), thus a considerable amount of resistance against percutaneous absorption of most substances is experienced. Furthermore, protein and peptide pharmaceuticals generally illustrate poor penetrability due to their large molecular sizes and relatively hydrophilic nature (Crommelin et al., 2002, Pettit and Gombotz, 1998). These large molecules are consequently too 'bulky' to passively traverse intact skin.

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 work done on the transdermal delivery of arginine vasopressin includes five studies by Nair and Panchagnula (2003a,b,c and 2004a,b). Three of these studies were conducted using rat skin and with the aid of iontophoresis coupled with a variety of chemical penetration enhancers. The remainder of the studies by these two researchers involved investigations into the electrical parameters and physicochemical considerations of AVP transdermal permeation, with references to earlier studies by Lelawongs et al. (1989, 1990). Other transdermal studies that involved AVP as the model peptide focused on the effects of buffer pH and concentration, as well as proteolytic enzyme inhibitors, on the stability of AVP and its degradation in rat and human cadaver skin (Bi and Singh, 2000, Banga et al., 1995 and Morimoto et al., 1992). An earlier study by Banerjee and Ritschel in 1989 investigated the effects of pH, concentration of the drug, shaving of abdominal rat skin and surfactant on passive permeation of AVP.

Bestatin is a potent, competitive and specific aminopeptidase inhibitor with an affinity for leucine aminopeptidase (LAP), aminopeptidase B (APB) and tri-aminopeptidase. It was originally isolated from culture filtrates of *Streptomyces olivoreticuli* by Umezawa and his colleagues in 1976. 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 was used in the present study to selectively inhibit aminopeptidases present inside and on the surface of the skin, which could potentially degrade the studied active. Bestatin was decided on as the aminopeptidase inhibitor of choice due to its higher stabilising effect on the degradation of AVP among the three enzyme inhibitors investigated in the article by Bi and Singh (2000).

Pheroid™ technology is a therapeutic drug delivery system involving a unique submicron oil/water emulsion type formulation. The intellectual property on which Pheroid™ technology is based belongs to the North-West University, South Africa and is patented in Europe, the USA, South Africa and China.

Pheroids™ are stable, spherical structures within a delivery system that can be manipulated in terms of morphology, structure, size and function (Grobler et al., 2006. In press). This delivery system is trademarked, but will be referred to as Pheroid(s) throughout this article. Previous transdermal studies involving AVP used iontophoresis at lower currents and chemical enhancers in lower quantities in tandem to circumvent any potential adverse reactions, toxicity and irreversible structural changes (Nair and Panchagnula, 2004a). The Pheroid delivery system, however, is totally safe and skin-friendly (Grobler et al., 2006. In press).

Hepes buffer 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 Pheroid delivery system. Hepes buffer was decided on, as opposed to phosphate buffer saline (PBS) or normal saline, due to previously repeated use of this buffer in transdermal studies involving arginine vasopressin. Also of interest is the four times increase in iontophoretic drug permeation achieved when saline was replaced with Hepes buffer in the receptor compartment of the diffusion cell (Nair and Panchagnula, 2003c). Furthermore, a pilot study comparing PBS and Hepes in terms of their different pHs, AVP stability and -solubility, provided parallel results (data not shown).

In this study the *in vitro* transdermal delivery of arginine vasopressin (AVP) was investigated whilst incorporated in the Pheroid delivery system and in combination with an enzyme inhibitor, bestatin. The effect of bestatin on the preservation of AVP during transdermal diffusion studies, was also explored.

2. Materials and methods

2.1 Materials

[^{8}Arg]vasopressin (AVP) (acetate salt, MW = 1084.23), bestatin hydrochloride (N-[(2S-3R)-3-amino-2-hydroxy-4-phenyl-butyryl]-L-leucine hydrochloride) and 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (Hepes) buffer at a concentration of 1 M were purchased from Sigma-Aldrich (St. Louis, MO, USA). This buffer was used at 25 mM (0.025 M) by dilution in HPLC (deionised) water (Nair and Panchagnula, 2003a,b,c). Pheroids were supplied by the subprogram: Drug Delivery of the Unit for Drug Research and Development, North-West University. The fluorophores Nile Red and Alexa Fluor® 430 (Protein Labelling Kit (A10171) (Invitrogen, Leiden, The Netherlands) were used in the confocal microscopy. Vertical Franz diffusion cells (PermeGear Inc., Bethlehem, PA, USA) were utilised during diffusion studies. 'Far UV' grade BDH HiPerSolv® for HPLC acetonitrile (VWR International, Poole, England) and reagent grade trifluoroacetic acid (TFA) (Sigma, USA) were used as solvent systems in HPLC analyses. HPLC analyses were performed with the aid of a Macherey-Nagel LiChrospher® 100 RP18 ec column (4 mm x 250 mm, 5 µm particle size, pore size 100 Å, endcapped). All solutions were prepared in HPLC (deionised) water, purified by a Milli-Q Academic purification system (Millipore, Milford, USA). All other chemicals and solvents were of analytical grade.

2.2 Methods

2.2.1 Skin preparation method

Abdominal skin was obtained from Caucasian female patients after cosmetic surgery (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). The full-thickness skin was frozen at -20 °C not longer than 24 hours after removal. Before preparation, the skin was thawed at room temperature, remaining blood wiped off with tissue paper and all excess adipose tissue carefully removed with a scalpel.

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 with forceps. Special care was taken to ensure that the integrity of the stratum corneum remained intact. The skin sections were floated on top of Whatman® filter paper after ensuring that the stratum corneum side of the epidermal skin faced upwards. The skin sections were then left to air dry. The prepared skin samples were wrapped in aluminium foil and sealed in plastic bags. The samples were kept frozen at -20 °C until used. Before a diffusion study was conducted the frozen pieces of skin were thawed at room temperature and examined for defects. The skin was cut into circles with a diameter of ± 10 mm before mounting them in the diffusion apparatus.

2.2.2 Franz cell diffusion method

Vertical Franz diffusion cells with a receptor capacity of approximately 2 ml and a 1,075 cm^2 diffusion area were used. The epidermal layer was mounted on the lower half of the diffusion cell with the stratum corneum facing the donor half-cell. For each individual diffusion study a single source of skin was employed for all the cells to minimise variation between skin samples. A small magnetic stirring bar was placed in each receptor compartment to accomplish stirring. Stirring was continued throughout the entire experiment at 950 rpm with the aid of a Variomag® magnetic stirrer plate. The donor compartment was placed on the lower half, with the skin acting as a seal between the two halves, sealed with high vacuum grease and fastened together 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.

After equilibration 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 (R), in the parallel equivalent circuit mode (PAR) and with an alternating current (AC) frequency of 1000 hertz (Hz) (Fasano et al., 2004). Impedance measurements were taken in the donor and receptor compartments simultaneously as an indication of the relative integrity of the skin sample. These impedance measurements were repeated after completion of the diffusion study.

Physiological saline was used in the integrity assessments instead of Hepes buffer for the following reasons: Firstly, Hepes buffer ions have much lower mobility through rat skin during iontophoresis as compared to the major counter ion chloride (Nair and Panchagnula, 2003c). It was therefore hypothesised that if Hepes was unable to transfer a charge during iontophoresis it would probably not be able to do so during impedance measurements. This hypothesis was confirmed during pilot studies (data not shown) where impedance measurements, with Hepes buffer as the donor and receptor phases, were attempted. Secondly, physiological saline was used in the validation of the method by Fasano et al., (2004).

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. Care was taken to ensure 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 Pheroid delivery system, 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. This was done to ensure that sink conditions existed throughout the experiment. 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.

2.2.3 Entrapment of arginine vasopressin in Pheroids

When the drug is dissolved in the prepared Pheroid solution, it must be shaken for approximately 30 minutes at room temperature and kept in a fridge at 2-8 °C for 24 hours before commencement of an experiment. This period of time ensures adequate entrapment of the active in the spherical structures within this system. Entrapment of the peptide inside the lipid structures of the Pheroids was monitored with the aid of confocal laser scanning microscopy (CLSM). For this purpose a Nikon PCM 2000 confocal laser scanning microscope with a Hamamadzu CCD camera, realtime imaging, a medium (10µm) pinhole and a 60x, 1.4D ApoPlanar oil immersion objective was used. 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).

Pheroids are usually, according to protocol, labelled with the fluorophore Nile Red. The latter has an emission wavelength of between 640 and 650 nm. It is the fluorophore of choice as it predominantly accumulates in lipid rich domains, and therefore readily stains phospholipid components of the Pheroid formulation (Saunders et al., 1999).

The AVP was labelled with the reactive dye Alexa Fluor® 430, which exhibits appreciable fluorescence emission to a maximum of 540 nm in the green spectrum. Labelling was carried out according to the comprehensive instructions accompanying the kit. The required 2 mg/ml concentration of AVP was obtained by accurately weighing 2 mg of AVP and dissolving the drug in 1 ml of Pheroid solution.

Ten microlitres (10 µl) of the fluorescently-labelled drug sample was layered on a glass slide and covered with a glass cover-slip. Digital images were obtained through capturing by photomultipliers.

2.2.4 Permeation of AVP with the aid of the Pheroid drug delivery system

Two different sets of diffusion studies were conducted with the donor solutions prepared in the Pheroid delivery system. The first set involved a diffusion study with 15 diffusion cells. Eight cells contained AVP as the donor phase at a concentration of 150 µg/ml, and the remaining seven cells contained bestatin as the donor phase at a concentration of 300 µg/ml.

An AVP concentration of 150 µg/ml was decided upon after preliminary diffusion studies with 75 µg/ml (as used by Nair and Panchagnula, 2003a,b and 2004a) yielded no noticeable results. It should be borne in mind that these researchers made use of Sprague-Dawley rat skin. Rat and hairless mouse skin are inherently more penetrable than human skin (Koch et al., 1987). For the purposes of this study the initial concentration was doubled, but the concentration can be raised to as much as 250 µg/ml (Banga et al., 1995). The mentioned bestatin concentration was chosen in accordance with the success in inhibition of enzymatic activity achieved with 300 µg/ml in the articles by Bi and Singh (2000) and Morimoto et al. (1995).

Solutions were prepared to reach a concentration of exactly 150 µg/ml AVP and 300 µg/ml bestatin. The drugs were dissolved in the Pheroid delivery system solvent and to ensure adequate dissolution, the solutions were vortexed for a few seconds.

The second set of experiments involved three diffusion studies with a total of 29 diffusion cells containing AVP and bestatin dissolved in combination at the abovementioned concentrations.

The results of these two sets of diffusion studies were compared with the passive flux (AVP in Hepes buffer) and control (AVP in combination with bestatin in Hepes buffer). The passive and control diffusion studies are described in the following section.

2.2.5 The effect of bestatin on the preservation of AVP during passive permeation

Two different sets of diffusion studies were conducted with the donor solutions prepared in Hepes buffer (0.025 M, pH = 5.5). The first set involved a diffusion study with 14 diffusion cells. Seven cells contained AVP as the donor phase at a concentration of 150 µg/ml, and the remaining seven cells contained bestatin as the donor phase at a concentration of 300 µg/ml. The diffusion study involving solely AVP in Hepes buffer served as the representation of the passive flux (in section 2.2.4).

Solutions were prepared to reach a concentration of exactly 150 µg/ml AVP and 300 µg/ml bestatin. The drugs were dissolved in the Hepes buffer solvent and to ensure adequate dissolution, the solutions were vortexed for a few seconds.

The second set involved three diffusion studies with a total of 30 cells containing AVP and bestatin dissolved in combination at the abovementioned concentrations. This diffusion study also served as the control for the diffusion experiments in section 2.2.4.

2.2.6 High-performance liquid chromatography (HPLC) analyses

HPLC analyses of AVP were performed by employing a method developed and validated in conjunction with Prof Jan du Preez from the Analytical Technology Laboratory (ATL) at the North-West University, Potchefstroom Campus, South Africa. This method involved the use of an Agilent 1100 series HPLC equipped with a gradient pump, autosampler and diode array UV detector. This apparatus was interfaced with Chemstation Rev. A.08.03 data acquisition and analysis software.

A reversed phase chromatography column was used in all analyses. The mobile phase consisted of 100% acetonitrile (ACN) and the aqueous phase of 0.1% trifluoroacetic acid (TFA) in HPLC grade water. Injection volume was set at a default value of 100 µl.

Gradient elution was used with the gradient being the following: 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 kept 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.

2.2.7 Data analysis

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.

3. Results and discussion

3.1 Entrapment of arginine vasopressin in Pheroids

Fig. 1 illustrates the vesicular structures of the Pheroid delivery system and the entrapment of arginine vasopressin inside the lipid structures of this system. Micrograph A represents the control: the Pheroid drug delivery system analysed without the inclusion of AVP. The Pheroids are clearly visible as red spheres. Micrographs B to D depict the same image of the fluorescently-labelled sample with an AVP concentration of 2 mg/ml, but with co-localisation. Micrograph B is the actual image which illustrates the red spherical Pheroid structures with the green AVP molecules within, micrograph C reveals the Pheroids with exclusion of the AVP molecules, and in micrograph D only the green AVP molecules are visible. The latter three micrographs prove that the arginine vasopressin is in fact entrapped within the spherical lipid structures of the Pheroid delivery system.

3.2 Permeation of AVP with the aid of the Pheroid delivery system

The *in vitro* permeation of AVP with the aid of the Pheroid delivery system was investigated in the absence of the aminopeptidase inhibitor bestatin (the first set of diffusion studies) and compared to the permeation of AVP whilst incorporated into the delivery system together with bestatin (the second set of diffusion studies). 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 *in vitro* permeation profiles of AVP under the different circumstances are shown in Fig. 2. Only the data of cells with an AVP yield of 2% and less were included. Thus, graph 1 in Fig. 2 represents average data from 3 cells, graph 2 from 18 cells, graph 3 from 6 cells and graph 4 from 21 cells.

Table 1 gives a rendition of the most recent available data in the literature regarding the transdermal permeation of AVP as compared to the data of the present study. In the present study the graphs seemed to exhibit a biphasic character, with the first phase from time zero to two hours, and the second from time two to twelve hours. The fluxes for the different phases can be seen in Table 2. The S.D. values are in some cases higher than the mean values of the fluxes due to inclusion of several zero flux values in the data set. The majority of AVP flux seemed to take place during the first two hours of diffusion. This biphasic character can be ascribed to gradual depletion of the AVP after two hours or, in the case of 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 inclusion of bestatin in the receptor fluid might circumvent further degradation, therefore even higher AVP fluxes are possible. The latter scenario thus requires further investigation.

The Pheroid delivery system significantly increased the flux of AVP when compared to the passive flux. With the inclusion of bestatin, an even more distinct increase in the flux of AVP was observed. In the case of the exclusion of bestatin (graphs 1 and 3 of Fig. 2), the AVP flux approaches steady-state, indicating a decline in AVP permeation. The passive flux obtained in the present study (0.0175 and 0.0374 $\mu g/cm^2/h$) correlates well with the passive fluxes (0.021 $\mu g/cm^2/h$) observed during experiments done by Nair and Panchagnula, where passive diffusion was also carried out with the AVP dissolved in Hepes buffer, but through enhancer pre-treated skin.

The results thus clearly indicate that the Pheroid delivery system is capable of enhancing delivery of a peptide to the skin *in vitro*. It is, however, still far removed from the flux obtained with iontophoretic penetration enhancement of AVP at a concentration of 150 µg/ml through Sprague-Dawley rat skin (Nair and Panchagnula, 2003c).

Utilising human cadaver skin yielded rather high fluxes, which may be attributed to the AVP concentration of 250 µg/ml. The diffusion of AVP under the conditions of the present study, but with the AVP at the latter concentration, might render further investigation.

3.3 Effect of bestatin on the preservation of AVP during passive permeation

These diffusion studies were carried out under passive conditions in order to eliminate the possible effect of the Pheroid delivery system on the permeation process. We investigated the passive flux of AVP (the first set of diffusion studies), and compared it to the flux obtained when AVP was dissolved together with bestatin in the Hepes buffer (the second set of diffusion studies). By way of these studies we also investigated the flux of bestatin under passive conditions while dissolved on its own in Hepes buffer and in combination with the AVP. The *in vitro* permeation profiles of AVP can be seen in Fig. 2 (graphs 1 and 2). The *in vitro* profiles for bestatin can be seen in Fig. 3. The data of cells with a bestatin yield of 20% and less were included. Graphs 1 and 2 in Fig. 3. represent average data from 6 and 28 cells, respectively.

The effect of bestatin on the permeation of AVP can clearly be seen in graphs 1 and 2 of Fig. 2. Graph 2 illustrates a steady climb in flux while the flux of graph 1 approaches a steady state. The latter may be ascribed to the absence of bestatin in the donor phase, therefore AVP reaches the maximum permeation rate. The presence of bestatin in the donor phase clearly enhances the permeation of AVP by inhibiting its degradation, as can be observed from the steep climb in flux.

Bestatin, when dissolved individually in Hepes buffer, displays substantial fluxes in comparison to the fluxes obtained when incorporated together with AVP (Table 3). The high permeation of bestatin dissolved in Hepes buffer might be a result of poor skin integrity (as illustrated by electrical impedance measurements taken before and after diffusion studies, data not shown), or it can be ascribed to the small molecular weight of bestatin (344.83 Da), placing it in the ideal molecular weight range (100 – 500 Da) for passive permeation.
From the data presented it can be concluded that the inclusion of bestatin in the formulation has the ability to improve AVP passive permeation, and therefore bestatin has a positive effect on the preservation of AVP. With regard to the fluxes obtained for bestatin, the flux of bestatin seems to lower in the presence of AVP. This observation is predictable, as bestatin inhibits proteolytic enzymatic efforts in the presence of AVP.

Conclusions

The following observations were made during the course of this study:

- Confocal microscopy revealed definite entrapment of the AVP in the spherical structures of the Pheroid™ delivery system.
- *In vitro* permeation profiles for AVP exhibited a biphasic character, with the absence of a lag-time.
- The Pheroid™ system proved to be advantageous when applied as delivery medium during transdermal diffusion studies.
- Bestatin had an enhancing effect on the permeation of AVP, probably due to inhibition of degradation by skin enzymes and subsequent protection of the AVP.

The following aspects might render further investigation:

- It is postulated that the skin enzymes might be able to traverse the skin in conjunction with the AVP and therefore continue their proteolytic activity in the receptor phase of the diffusion cell. The inclusion of bestatin, not only in the donor phase but also in the receptor phase, might thwart any further degradation of the AVP when it has already diffused through the stratum corneum barrier.
- In order to raise AVP flux, higher concentrations of the drug in the donor phase might be sensible.
- The possibility of protection of the AVP molecules by the Pheroid™ structure against degradation by skin enzymes should be looked into.
- Confocal microscopy can be used to trace the process of entrapment of the active inside the Pheroid™ system, and the passage of the vesicles through the skin can be traced.

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References

Banerjee, P.S., Ritschel, W.A., 1989. Transdermal permeation of vasopressin. I. Influence of pH, concentration, shaving and surfactant on permeation. Int. J. Pharm. 49, 189-197.

Banga, A.K., Katakam, M., Mitra, R., 1995. Transdermal iontophoretic delivery and degradation of vasopressin across human cadaver skin. Int. J. Pharm. 116, 211-216.

Bi, M., Singh, J., 2000. Effect of buffer pH, buffer concentration and skin with or without enzyme inhibitors on the stability of [Arg^8]-vasopressin. Int. J. Pharm. 197, 87-93.

Blank, I.H., McAuliffe, D.J., 1985. Penetration of benzene through human skin. J. Invest. 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.

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.

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 Livingston, 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 Lett. 38, 8383-8386.

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. Eur. J. Pharm. Sci. 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.

Koch, R.L., Palicharla, P., Groves, M.J., 1987. Diffusion of [2-^{14}C] Diazepam across hairless mouse skin and human skin. J. Invest. Dermatol. 88, 582-585.

Lelawongs, P., Liu, J., Chien, Y.W., 1990. Transdermal iontophoretic delivery of arginine vasopressin (II): Evaluation of electrical and operational factors. Int. J. Pharm. 61, 179-188.

Lelawongs, P., Liu, J., Siddiqui, O., Chien, Y.W., 1989. Transdermal iontophoretic delivery of arginine vasopressin (I): Physicochemical considerations. Int. J. 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. Int. J. Pharm. 269, 323-328.

Medi, B.M., Singh, J., 2003. Electronically facilitated transdermal delivery of human parathyroid hormone (1 - 34). Int. J. Pharm. 263, 24-33.

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. Int. J. Pharm. 81, 119-125.

Morimoto, K., Miyazaki, M., Kakemi, M., 1995. Effects of proteolytic enzyme inhibitors on nasal absorption of salmon calcitonin in rats. Int. J. Pharm. 113, 1-8.

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. Il 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. Il Farmaco 59, 583-593.

Pettit, D.K., Gombotz, W.R., 1998. The development of site-specific drug-delivery systems for protein and peptide biopharmaceuticals. Tibtech 16, 343-349.

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.

Saunders, J.C.J., Davis, H.J., Coetzee, L., Botha, S., Kruger, A.E, Grobler, A., 1999. A novel skin penetration enhancer: evaluation by membrane diffusion and confocal microscopy. J. Pharm. Pharmaceut. Sci. 2, 99-107.

Umezawa, H, Aoyagi, T., Suda, H., Hamada, M., Takeuchi, T., 1976. Bestatin, an inhibitor of aminopeptidase B, produced by actinomycetes. J. Antibiot. 29, 97-99.

Tables

Table 1: Comparison between data from transdermal studies with the model compound AVP. Data presented in the literature are compared to data of the present study in terms of enhancement methods, AVP concentration, the diffusion membrane, receptor solutions and fluxes obtained with various methods of penetration enhancement.

Study	Method of enhancement	AVP concen-tration	Membrane	Receptor solution (buffer)	Flux (µg/cm²/h)						Reference
					Iontophoresis	Iontophoresis and chemical enhancers	Chemical enhancers	AVP in Pheroids	AVP (+bestatin) in Pheroids	Passive	
1	Iontophoresis and terpenes (carvone, pulegone, cineole and menthol	75 µg/ml	Sprague-Dawley rat skin	0.9% NaCl	0.27	0.271 (menthol) 0.353 (cineole)	0.083 to 0.237 [1]	-	-	0.021[5]	Nair & Panchagnula (2004a)
2	Iontophoresis	75 µg/ml	Sprague-Dawley rat skin	0.9% NaCl	0.041 to 1.371 [2]	-	-	-	-	0.0194 ± 0.0031	Nair & Panchagnula (2004b)
3	Iontophoresis and chemical enhancers (oleic acid and cineole) in poloxamer gels	75 µg/ml	PVDF membrane	0.9% NaCl	0.273 ± 0.079	-	-	-	-	0.021 ± 0.002	Nair & Panchagnula (2003a)
4	Iontophoresis and fatty acids (lauric, oleic and linoleic acid)	75 µg/ml	Sprague-Dawley rat skin	0.9% NaCl	0.27	0.304 (linoleic) 0.830 (oleic)	0.409 to 0.413 [3]	-	-	0.021[5]	Nair & Panchagnula (2003b)
5	Iontophoresis	25, 50, 75, 100 and 150 µg/ml	Sprague-Dawley rat skin	0.9% NaCl	0.079 to 0.787 (150 µg/ml = 0.787)	-	-	-	-	0.021	Nair & Panchagnula (2003c)
6	Iontophoresis	250 µg/ml	Human cadaver skin	HEPES buffer	Ranging from approximately 1.5 to 2.8[4]	-	-	-	-	None	Banga et al. (2000)
7	Pheroid™ drug delivery system	150 µg/ml	Human abdominal epidermis	HEPES buffer	-	-	-	0.0376, 0.1495[6]	0.0575, 0.2182[6]	0.0175, 0.0374[6,7]	Present study

[1] Flux in the order carvone < pulegone < menthol < cineole
[2] Current density (CD) values ranging from 0.35 to 1.25 mA/cm2. All other iontophoretic studies conducted with 0.5 mA/cm2
[3] Flux in the order linoleic acid < oleic acid < lauric acid
[4] Only fluxes at specific time intervals were reported
[5] Passive flux = AVP dissolved in Hepes buffer, permeated through enhancer pre-treated skin
[6] Higher value at time 0 – 2 hours, lower value at time 2 – 12 hours
[7] Passive flux = Passive permeation of AVP dissolved in HEPES buffer

Table 2: Fluxes for AVP in Hepes buffer and Pheroids with or without the inclusion of bestatin. Flux was calculated for two different periods of time: t = 0 – 2 hours and t = 2 – 12 hours. Numbers in brackets refer to graph numbers in Fig. 2. (mean ± S.D.)

Donor phase	Flux (µg/ml/h): 0 – 2 hours	Flux (µg/ml/h): 2 – 12 hours
AVP in Hepes buffer (1)	0.0374 ± 0.065	0.0175 ± 0.028
AVP in Pheroids (3)	0.1495 ± 0.188	0.0376 ± 0.050
AVP (+bestatin) in Hepes buffer (2)	0.1576 ± 0.169	0.0621 ± 0.063
AVP (+bestatin) in Pheroids (4)	0.2182 ± 0.186	0.0575 ± 0.051

Table 3: Fluxes for bestatin in Hepes buffer with or without the inclusion of AVP. Flux was calculated for two different periods of time: t = 0 – 2 hours and t = 2 – 12 hours. Numbers in brackets refer to graph numbers in Fig. 3. (mean ± S.D.)

Donor phase	Flux (µg/ml/h): 0 – 2 hours	Flux (µg/ml/h): 2 – 12 hours
Bestatin in Hepes buffer (1)	9.137 ± 4.297	1.8778 ± 0.939
Bestatin (+AVP) in Hepes buffer (2)	2.6034 ± 3.543	0.4515 ± 0.682

Figure legends and figures

Fig. 1: Confocal laser scanning micrographs of arginine vasopressin and the Pheroid™ delivery system. AVP was labelled with the fluorophore Alexa Fluor® 430, which emits in the green spectrum. The Pheroids were labelled with Nile Red, which emits in the red spectrum. A: control, B: AVP within Pheroids, C: Pheroids, D: AVP

Fig. 2: *In vitro* permeation profiles of arginine vasopressin

◆ (1): AVP dissolved in Hepes buffer with exclusion of bestatin (passive flux of AVP).

■ (2): AVP and bestatin dissolved simultaneously in Hepes buffer (control flux of AVP). The average flux of AVP is shown.

▲ (3): AVP dissolved in the Pheroid™ delivery system with exclusion of bestatin.

× (4): AVP and bestatin dissolved simultaneously in the Pheroid™ delivery system. The average flux of AVP is shown.

Fig. 3: *In vitro* permeation profiles of bestatin

◆ (1): Bestatin dissolved in Hepes buffer with exclusion of AVP.

■ (2): AVP and bestatin dissolved simultaneously in Hepes buffer. The average flux of bestatin is shown.

Figure 1

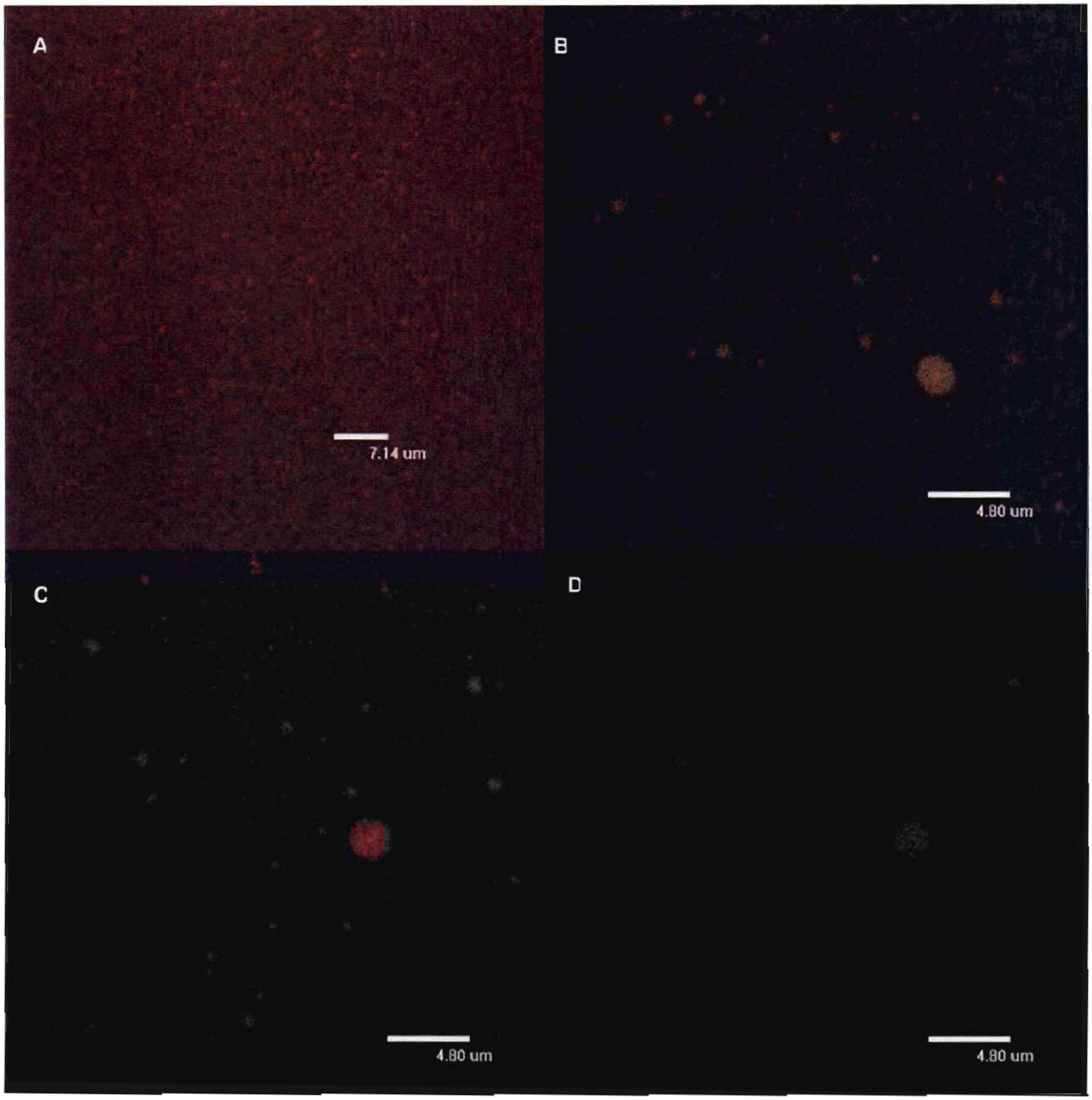


Figure 2

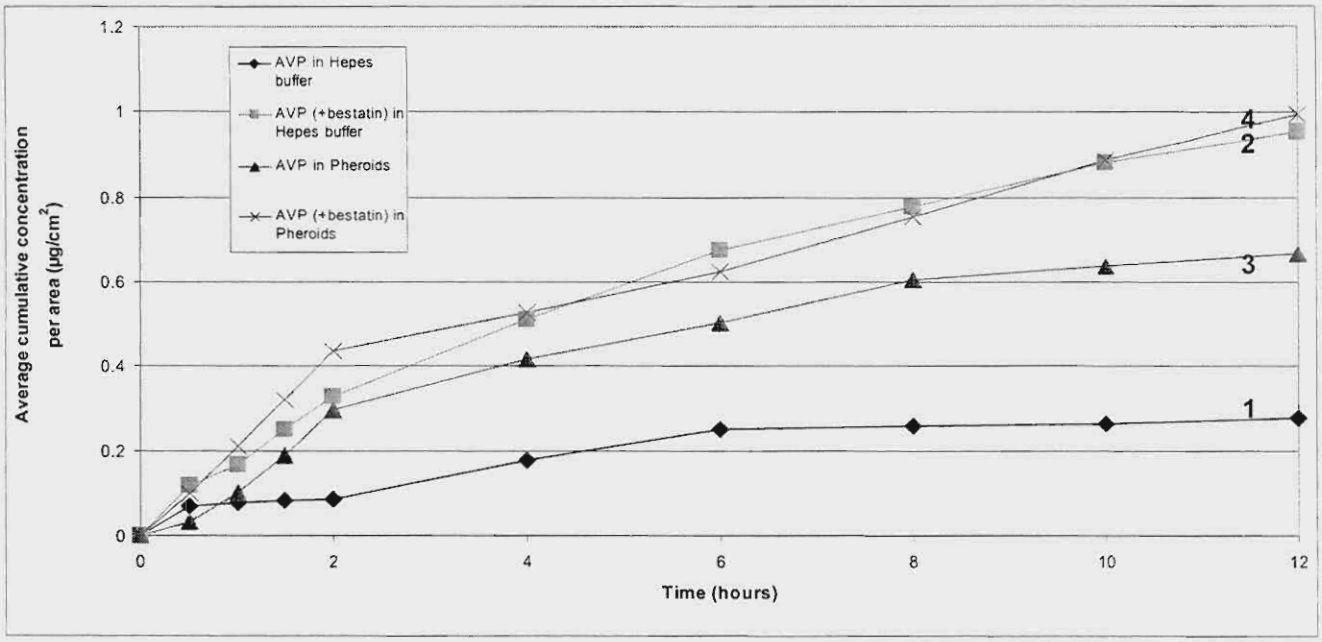
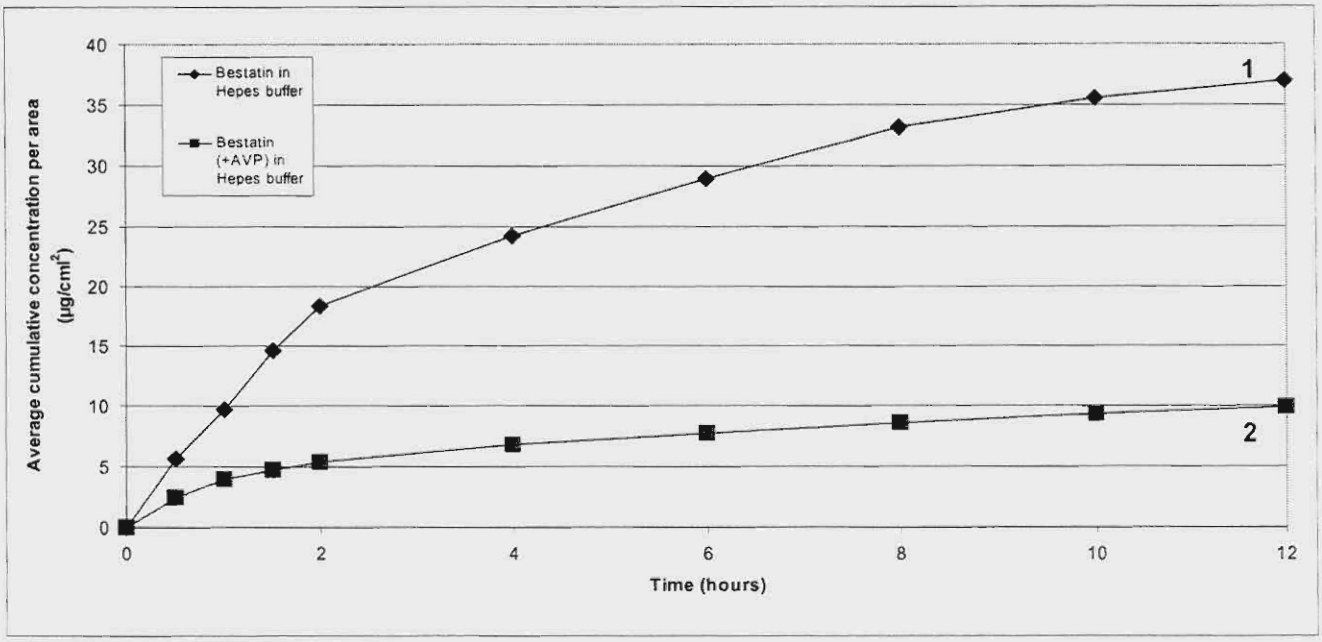

AVP in Hepes buffer
AVP (+bestatin) in Hepes buffer
AVP in Pheroids
AVP (+bestatin) in Pheroids
Average cumulative concentration per area (µg/cm²)
Time (hours)
1.2
1
0.8
0.6
0.4
0.2
0
0
2
4
6
8
10
12
1
2
3
4

Figure 3


Bestatin in Hepes buffer
Bestatin (+AVP) in Hepes buffer
Average cumulative concentration per area (µg/cm²)
Time (hours)
1
2
0
5
10
15
20
25
30
35
40
0
2
4
6
8
10
12

CHAPTER 4: FINAL CONCLUSIONS

The aim of this study was to investigate the possible *in vitro* transdermal diffusion of the peptide hormone arginine vasopressin (AVP) with the aid of the novel Pheroid™ therapeutic drug delivery system. Bestatin, a selective aminopeptidase inhibitor, was introduced to hinder AVP degradation, therefore its effect on the preservation of AVP during diffusion studies was examined as well.

AVP (MW = 1084.23) is a representative of protein and peptide pharmaceuticals in the molecular weight range of 1000 – 1500 Da and was therefore the model compound of choice. In general, transdermal administration of peptide drugs presents an array of difficulties due to their large molecular size and hydrophilic character. The search for efficient dosage forms is an ongoing labour, and several means of penetration enhancement are employed to deliver these compounds.

The Pheroid™ system is a therapeutic drug delivery system which has been applied in the administration of various entities, including peptide hormones, tuberculostatics, bacteria and viruses. In the light of the complications encountered with the delivery of peptides, this unique lipid-based system, somewhat related to liposomes and other lipid vesicles, presented a solution to the problem.

Bestatin was employed to circumvent any degradation of AVP on the surface of the skin and within. Success had been achieved with this inhibitor during previous transdermal and oral delivery studies.

All the above elements were combined with the aid of several diffusion experiments. These experiments were conducted over periods of twelve hours while vertical Franz diffusion cells, female abdominal epidermal skin and Hepes buffer as the receptor phase were utilized. To monitor the entrapment of AVP within the lipid-rich domains of the vesicular Pheroid structures, confocal laser scanning microscopy (CLSM) was employed.

The following observations were made during the course of this study:

- Confocal microscopy revealed definite entrapment of the AVP in the spherical structures of the Pheroid™ delivery system.
- *In vitro* permeation profiles for AVP exhibited a biphasic character, with the absence of a lag-time.

- The Pheroid™ system proved to be advantageous when applied as delivery medium during transdermal diffusion studies.
- Bestatin had a pseudo enhancing effect on the permeation of AVP through inhibition of degradation by skin enzymes.

The following aspects might render further investigation:

- It is postulated that the skin enzymes might be able to traverse the skin in conjunction with the AVP and therefore continue their proteolytic activity in the receptor phase of the diffusion cell. The inclusion of bestatin, not only in the donor phase but also in the receptor phase, might thwart any further degradation of the AVP when it has already diffused through the stratum corneum barrier.
- In order to raise AVP flux, higher concentrations of the drug in the donor phase might be sensible.
- The possibility of protection of the AVP molecules by the Pheroid™ structure against degradation by skin enzymes should be looked into.
- Confocal microscopy can be made use of to trace the process of entrapment of the active inside the Pheroid system, and the passage of the vesicles through the skin can be traced.

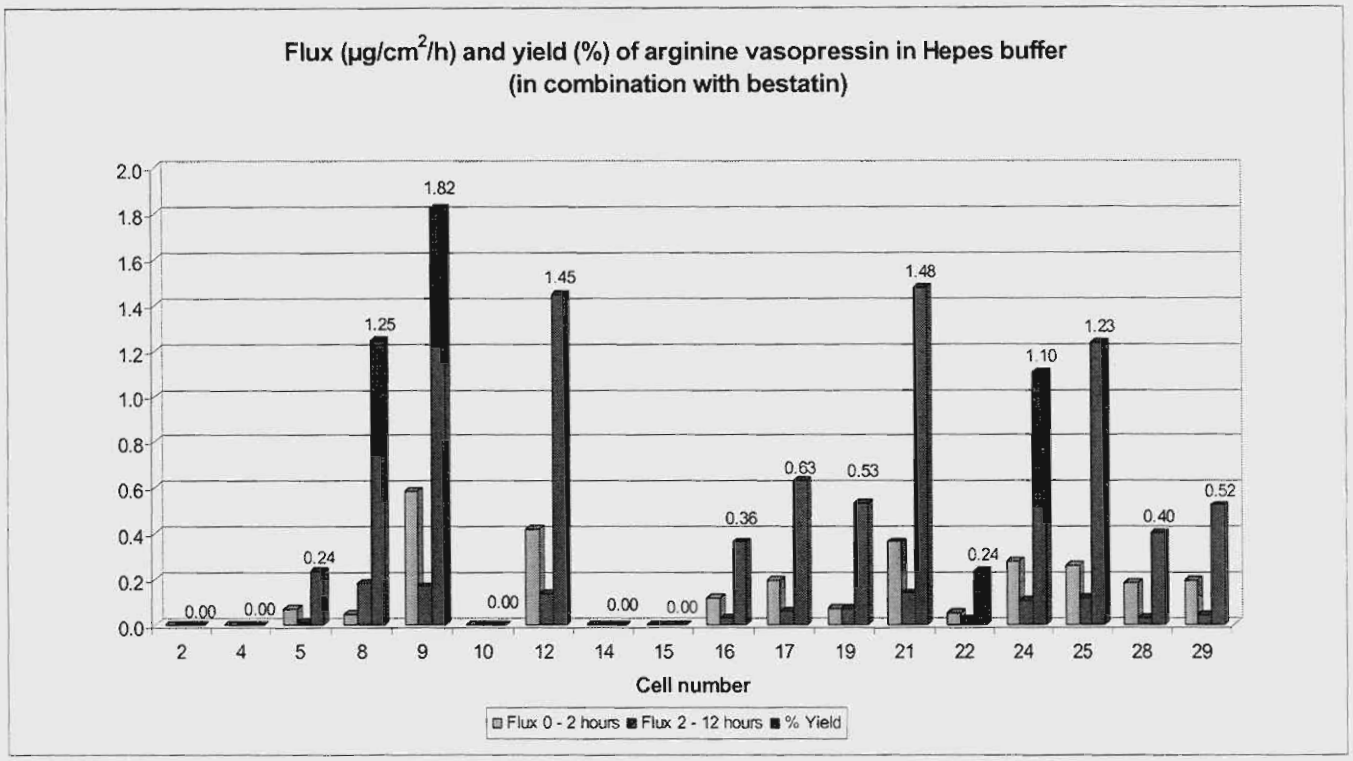
APPENDICES

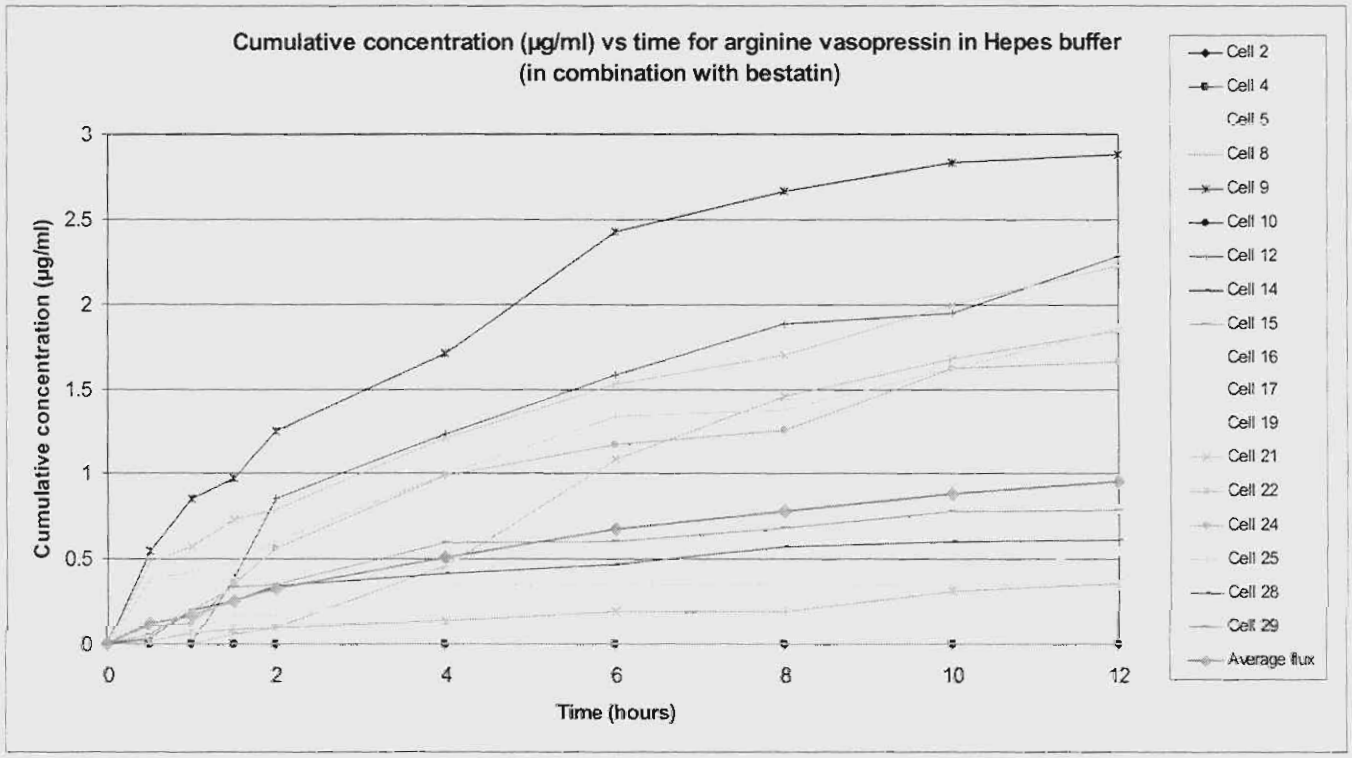
APPENDIX 1: DATA OF FRANZ CELL DIFFUSION STUDIES

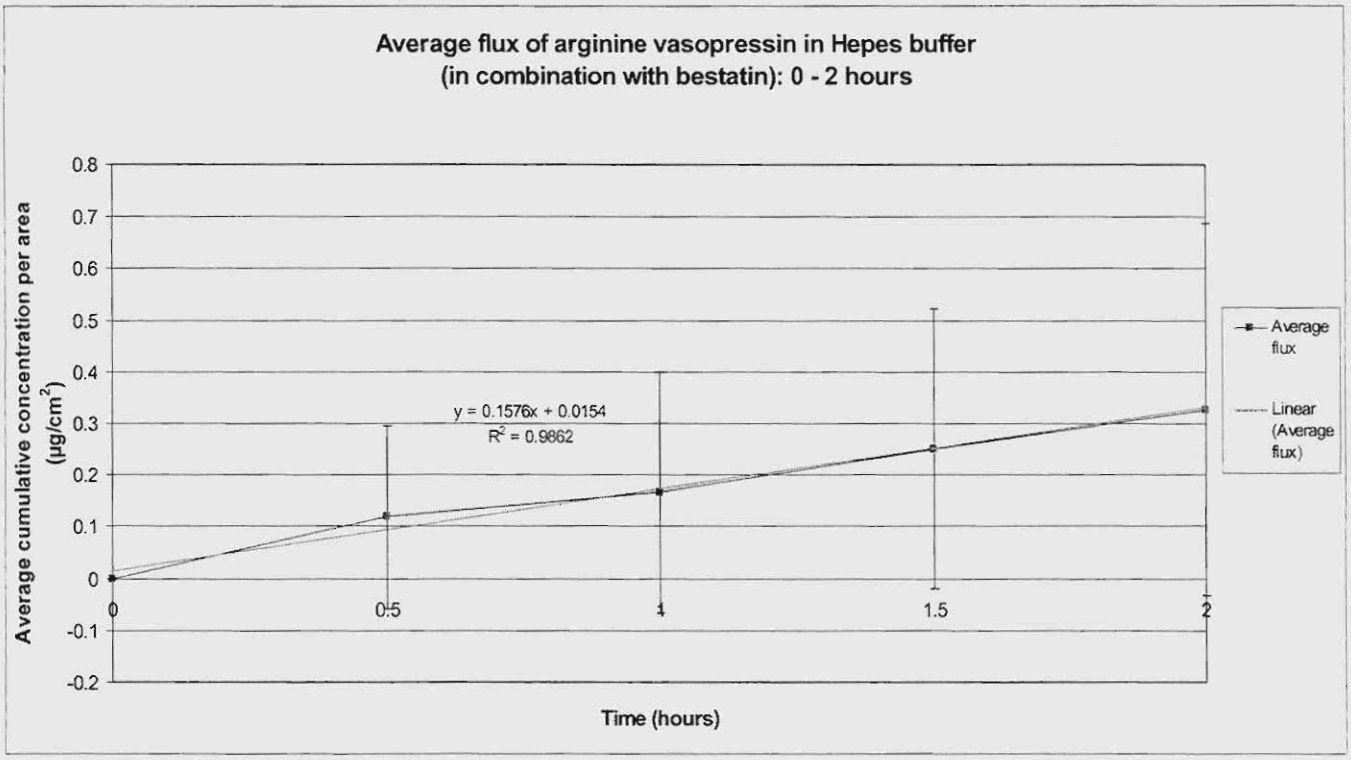
ARGININE VASOPRESSIN (IN COMBINATION WITH BESTATIN) IN HEPES BUFFER (CONTROL)

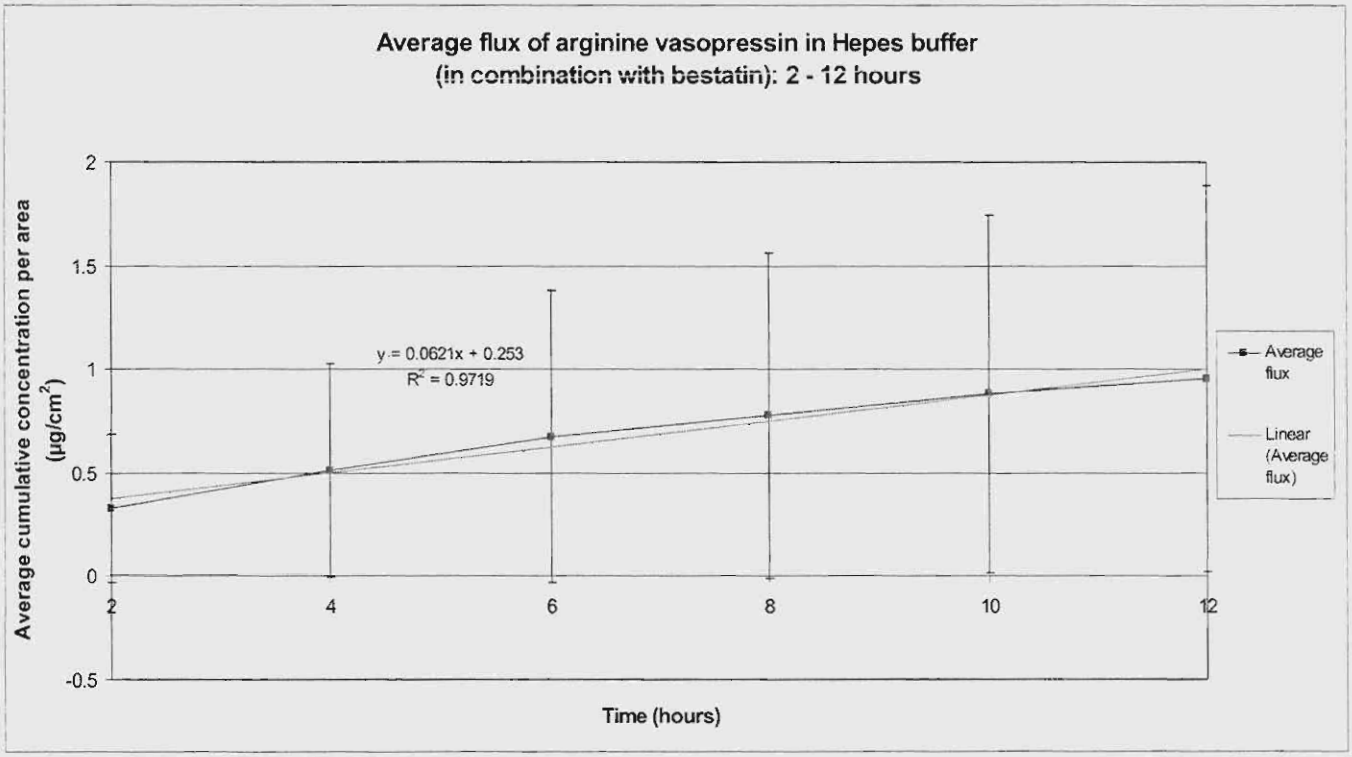
Arginine vasopressin and the enzymatic inhibitor bestatin were dissolved simultaneously in Hepes buffer. The data for arginine vasopressin reported here serves as the control:

Cell #	Integrity (KΩ)		Cum. conc (µg/ml)	% yield	Flux 0-2h (µg/cm^2/h)	Flux 2-12h (µg/cm^2/h)
	Before	After				
1	7.205	8.742	3.198126822	2.16	0.457	0.224
2	20.92	22.18	0	0	0	0
3	4.002	4.661	5.081865703	3.43	1.280	0.216
4	7.638	10.26	0	0	0	0
5	6.089	7.929	0.348910869	0.24	0.069	0.013
6	9.236	11.28	6.103546635	4.12	0.682	0.475
7	3.824	3.707	7.542853562	5.10	2.274	0.255
8	5.177	5.559	1.843009579	1.25	0.048	0.183
9	10.66	13.22	2.881148745	1.82	0.582	0.168
10	24.14	46.62	0	0	0	0
11	3.57	3.27	53.843412874	34.08	9.994	0.142
12	8.55	11.42	2.286910966	1.45	0.421	0.137
13	13.48	19.62	15.934264285	10.08	1.885	0.413
14	13.07	13.74	0	0	0	0
15	24.61	30.80	0	0	0	0
16	9.69	11.00	0.547076248	0.36	0.117	0.030
17	13.13	15.46	0.955702075	0.63	0.196	0.059
18	8.29	8.46	3.850971327	2.55	0.628	0.156
19	9.59	8.84	0.799570675	0.53	0.071	0.070
20	13.86	5.41	9.975389819	6.61	1.331	0.733
21	5.75	8.25	2.228449451	1.48	0.364	0.139
22	15.53	14.46	0.357579980	0.24	0.051	0.026
23	9.25	11.85	5.377810473	3.56	0.726	0.361
24	12.15	12.67	1.664541103	1.10	0.276	0.107
25	8.11	10.04	1.861444333	1.23	0.262	0.117
26	5.09	6.95	88.971419432	58.92	14.480	5.896
27	5.94	7.19	8.106268989	5.37	1.873	0.356
28	24.30	24.87	0.609287519	0.40	0.184	0.028
29	16.10	14.96	0.788402883	0.52	0.197	0.040
30	4.91	3.86	12.519953922	8.29	2.163	0.733


Flux (µg/cm²/h) and yield (%) of arginine vasopressin in Hepes buffer
(in combination with bestatin)
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0.00
0.00
0.24
1.25
1.82
0.00
1.45
0.00
0.00
0.36
0.63
0.53
1.48
0.24
1.10
1.23
0.40
0.52
2
4
5
8
9
10
12
14
15
16
17
19
21
22
24
25
28
29
Cell number
Flux 0 - 2 hours
Flux 2 - 12 hours
% Yield


Cumulative concentration (µg/ml) vs time for arginine vasopressin in Hepes buffer
(in combination with bestatin)
Cumulative concentration (µg/ml)
3
2.5
2
1.5
1
0.5
0
0
2
4
6
8
10
12
Time (hours)
Cell 2
Cell 4
Cell 5
Cell 8
Cell 9
Cell 10
Cell 12
Cell 14
Cell 15
Cell 16
Cell 17
Cell 19
Cell 21
Cell 22
Cell 24
Cell 25
Cell 28
Cell 29
Average flux

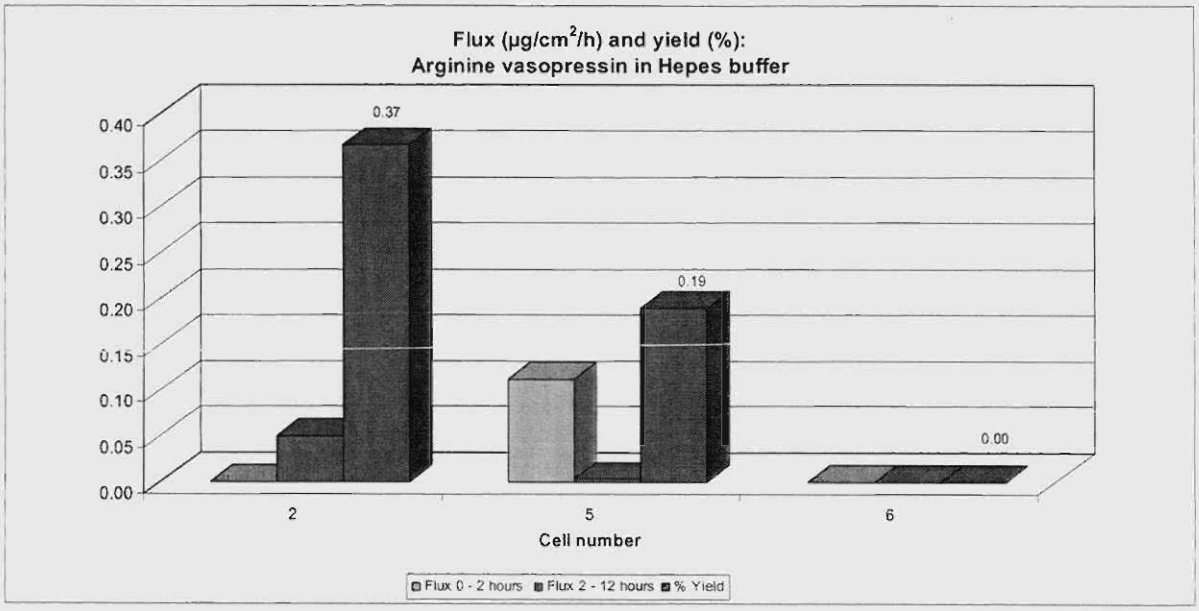

Average flux of arginine vasopressin in Hepes buffer
(in combination with bestatin): 0 - 2 hours
Average cumulative concentration per area (µg/cm²)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
-0.1
-0.2
y = 0.1576x + 0.0154
R² = 0.9862
0
0.5
1
1.5
2
Time (hours)
Average flux
Linear (Average flux)

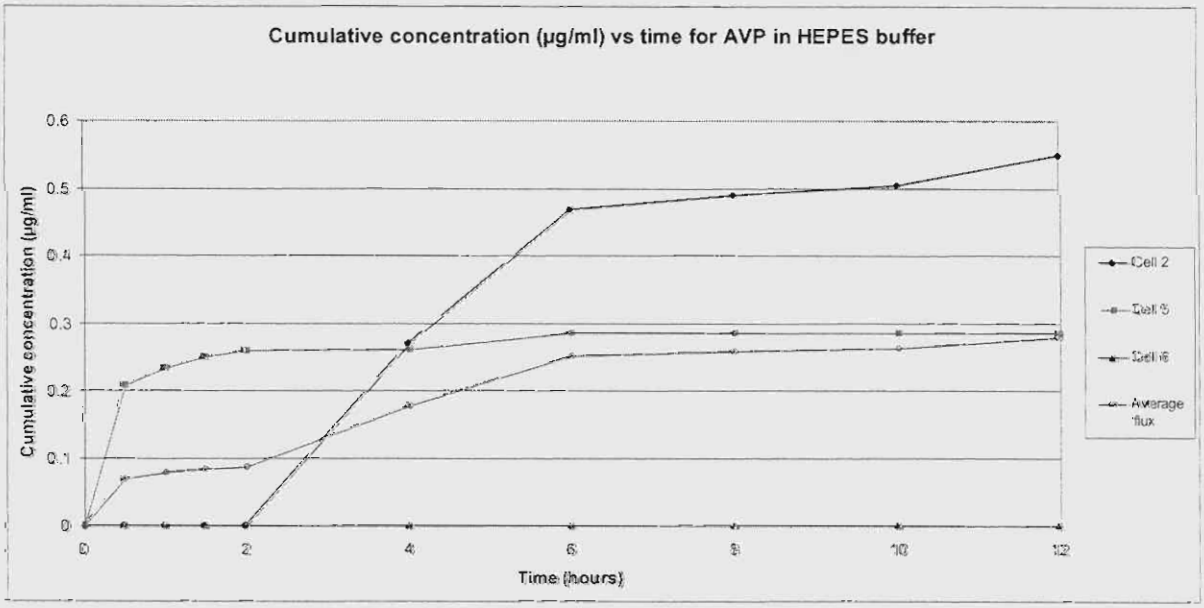

Average flux of arginine vasopressin in Hepes buffer
(in combination with bestatin): 2 - 12 hours
Average cumulative concentration per area (µg/cm²)
2
1.5
1
0.5
0
-0.5
y = 0.0621x + 0.253
R² = 0.9719
2
4
6
8
10
12
Time (hours)
Average flux
Linear (Average flux)

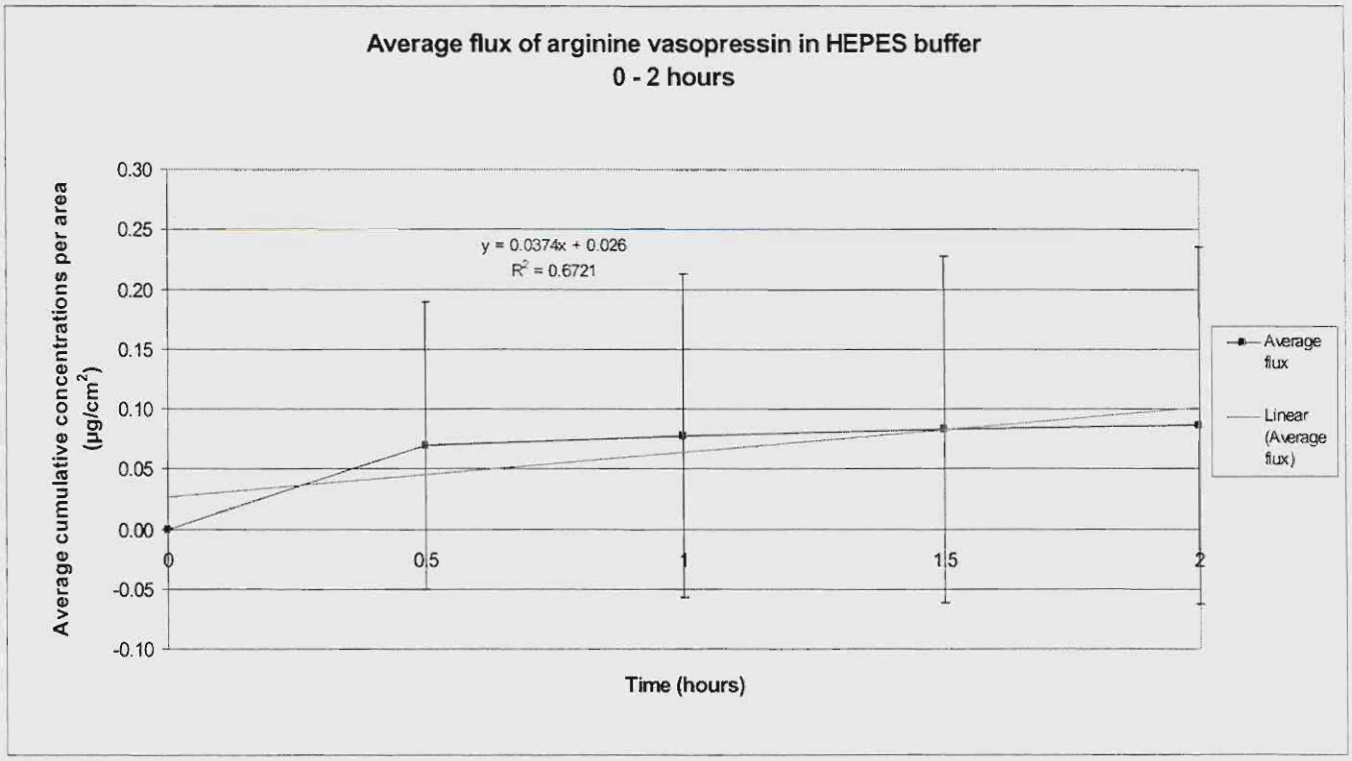
ARGININE VASOPRESSIN IN HEPES BUFFER (PASSIVE FLUX)

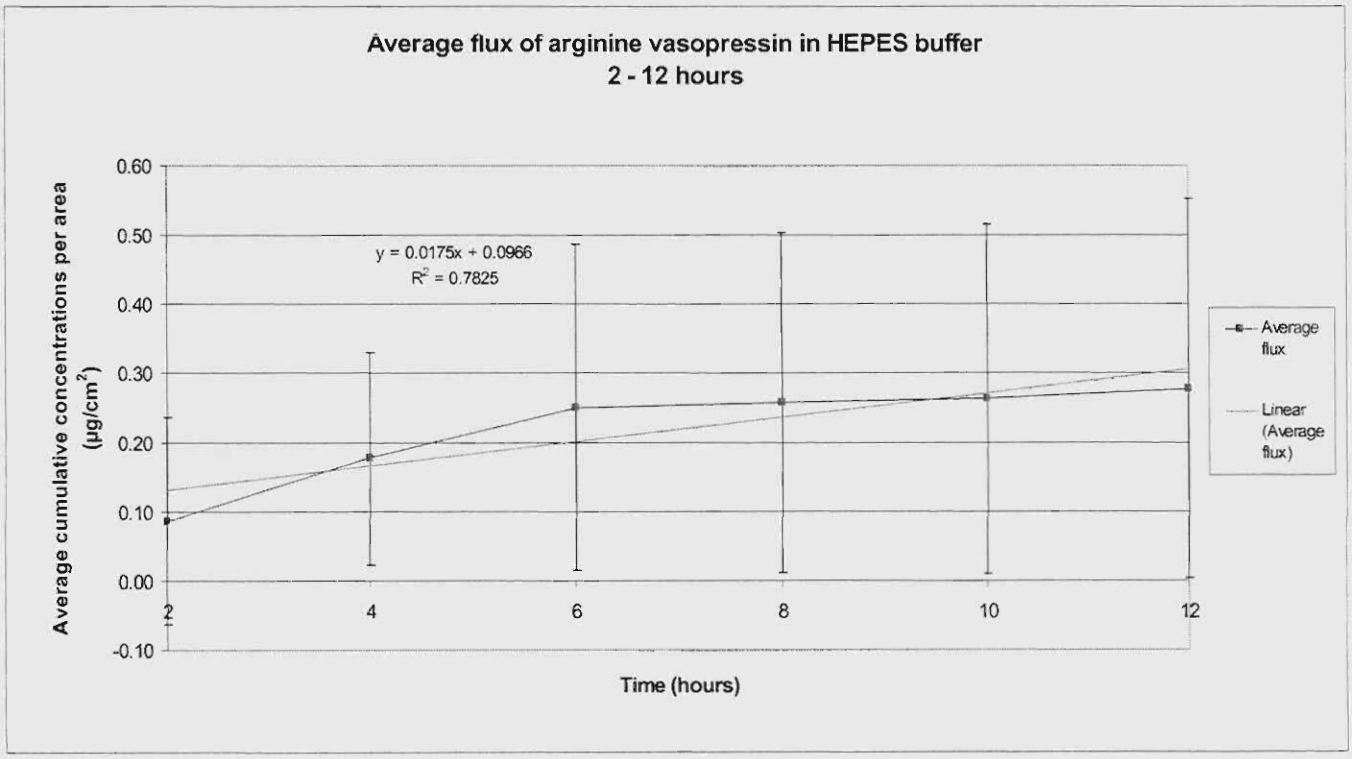
Arginine vasopressin was dissolved in Hepes buffer (omitting bestatin) and the data hereafter gives an account of the passive flux of AVP:

Cell #	Integrity (KΩ)		Cum. conc (µg/ml)	% yield	Flux 0-2h (µg/cm^2/h)	Flux 2-12h (µg/cm^2/h)
	Before	After				
1	6.85	7.78	7.046324474	4.70	1.332	0.445
2	4.57	7.31	0.549570095	0.37	0	0.050
3	10.01	12.57	11.677096129	7.78	2.274	0.679
4	15.04	20.88	9.804066023	6.54	1.262	0.697
5	15.25	15.45	0.284393814	0.19	0.112	0.003
6	9.66	11.84	0	0	0	0
7	10.07	17.07	3.826432420	2.55	0.439	0.285





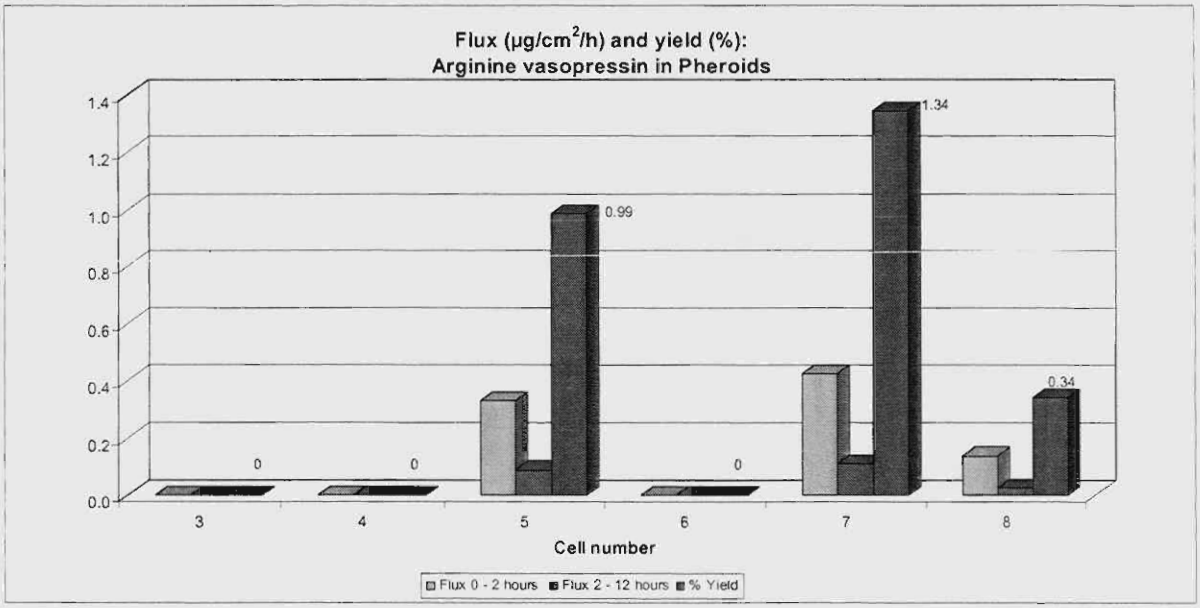

Average flux of arginine vasopressin in HEPES buffer
0 - 2 hours
y = 0.0374x + 0.026
R² = 0.6721
Average cumulative concentrations per area (µg/cm²)
0.30
0.25
0.20
0.15
0.10
0.05
0.00
-0.05
-0.10
0
0.5
1
1.5
2
Time (hours)
Average flux
Linear (Average flux)

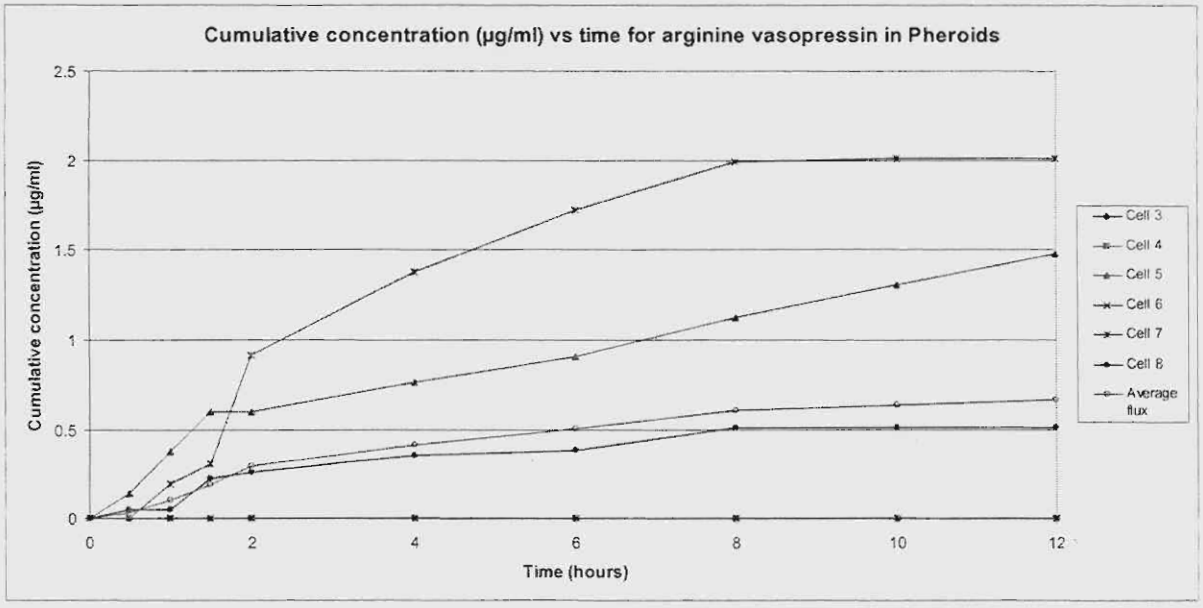

Average flux of arginine vasopressin in HEPES buffer
2 - 12 hours
y = 0.0175x + 0.0966
R² = 0.7825
Average cumulative concentrations per area (µg/cm²)
0.60
0.50
0.40
0.30
0.20
0.10
0.00
-0.10
2
4
6
8
10
12
Time (hours)
Average flux
Linear (Average flux)

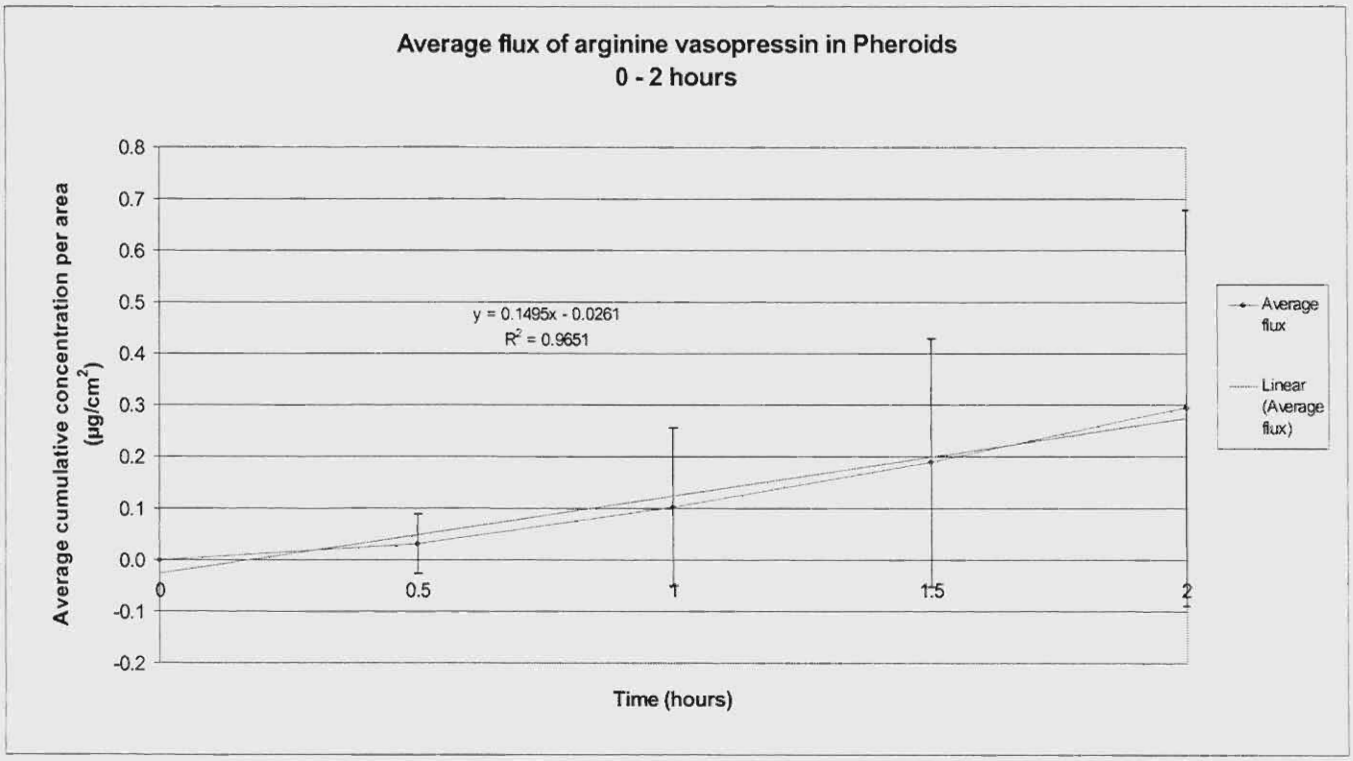
ARGININE VASOPRESSIN IN THE PHEROID™ DELIVERY SYSTEM

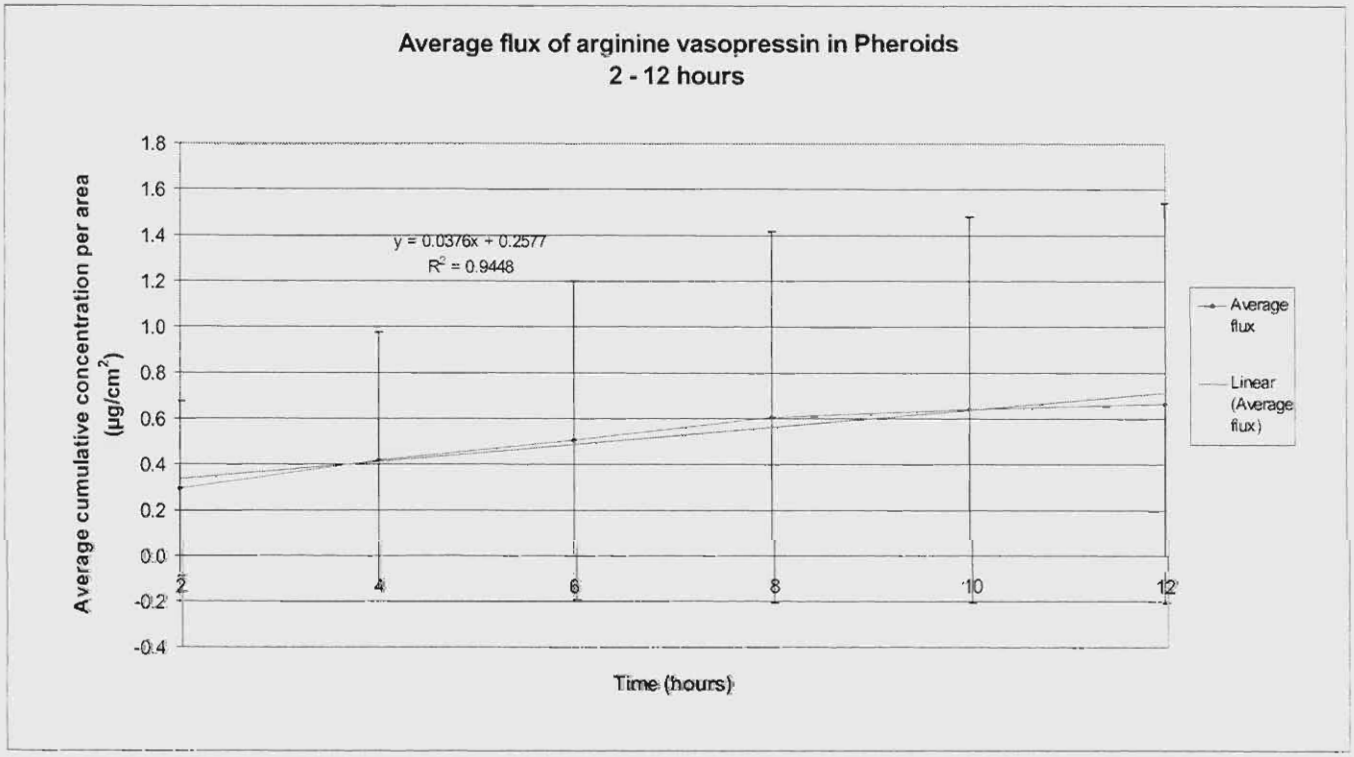
Arginine vasopressin was dissolved in the Pheroid™ drug delivery system (omitting bestatin). The data for arginine vasopressin is reported here:

Cell #	Integrity (KΩ)		Cum. conc (µg/ml)	% yield	Flux 0-2h (µg/cm^2/h)	Flux 2-12h (µg/cm^2/h)
	Before	After				
1	8.4	8.92	15.213611525	10.14	0.342	0.845
2	5.74	6.25	17.582783880	11.72	0.677	1.639
3	14.04	14.78	0	0	0	0
4	11.67	15.25	0	0	0	0
5	5.79	5.53	1.478364208	0.99	0.331	0.089
6	8.82	11.28	0	0	0	0
7	7.15	7.28	2.014356474	1.34	0.426	0.110
8	4.51	7.62	0.512369929	0.34	0.139	0.027





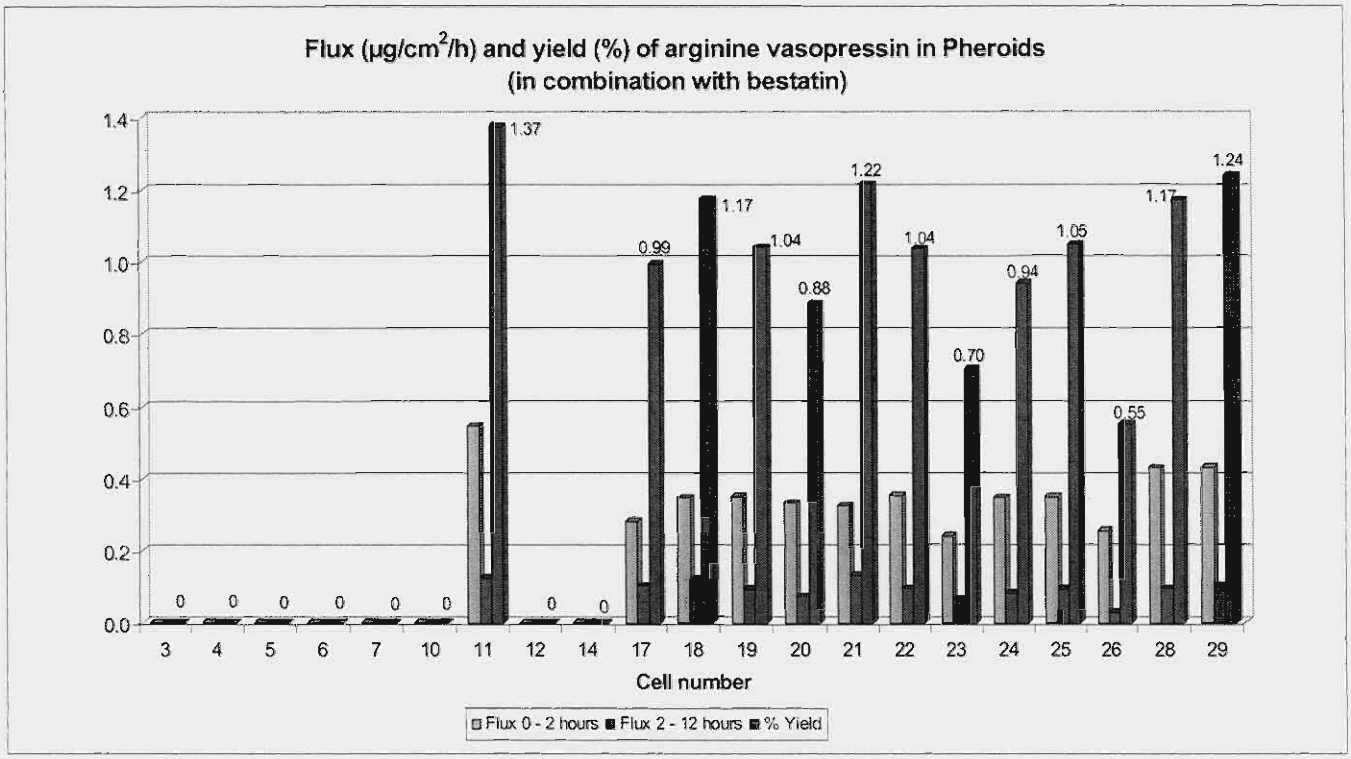

Average flux of arginine vasopressin in Pheroids
0 - 2 hours
Average cumulative concentration per area (µg/cm²)
y = 0.1495x - 0.0261
R² = 0.9651
Average flux
Linear (Average flux)
Time (hours)

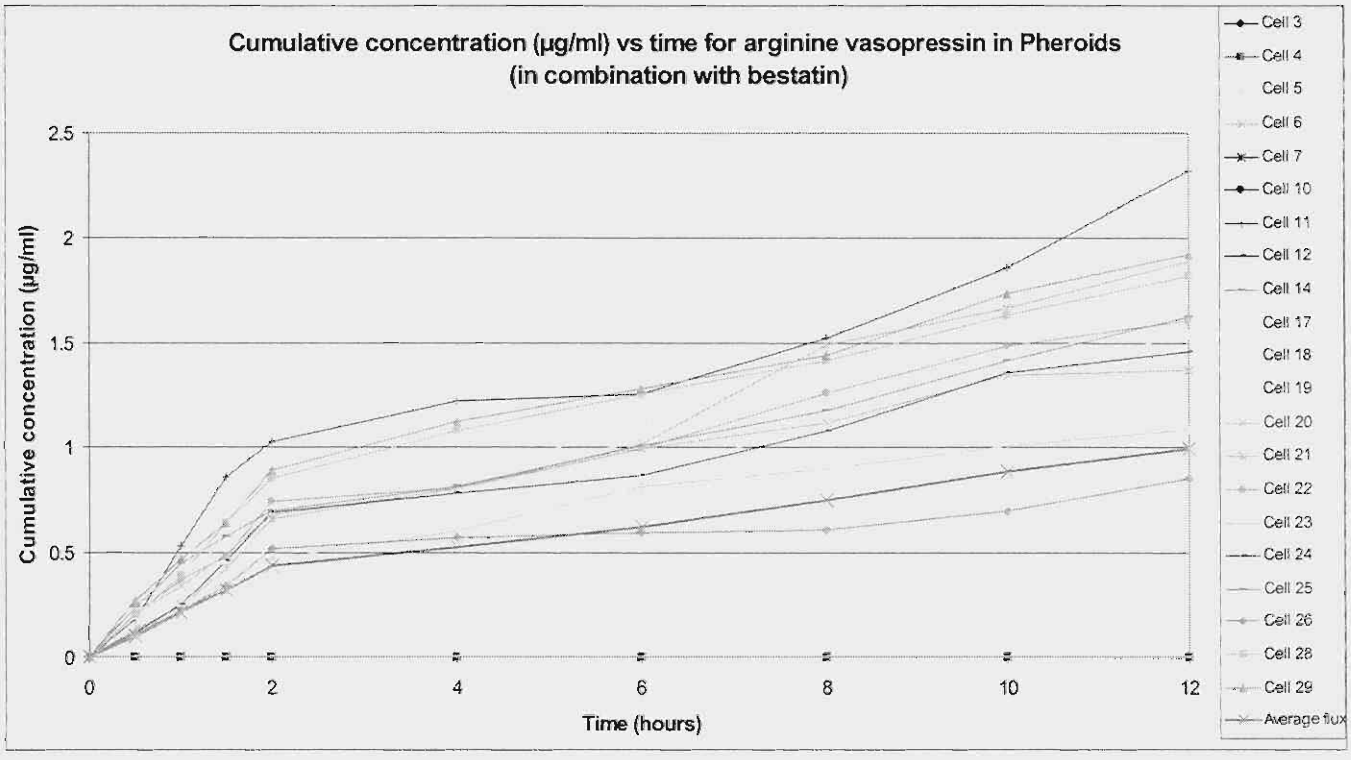

Average flux of arginine vasopressin in Pheroids
2 - 12 hours
Average cumulative concentration per area (µg/cm²)
y = 0.0376x + 0.2577
R² = 0.9448
Average flux
Linear (Average flux)
Time (hours)

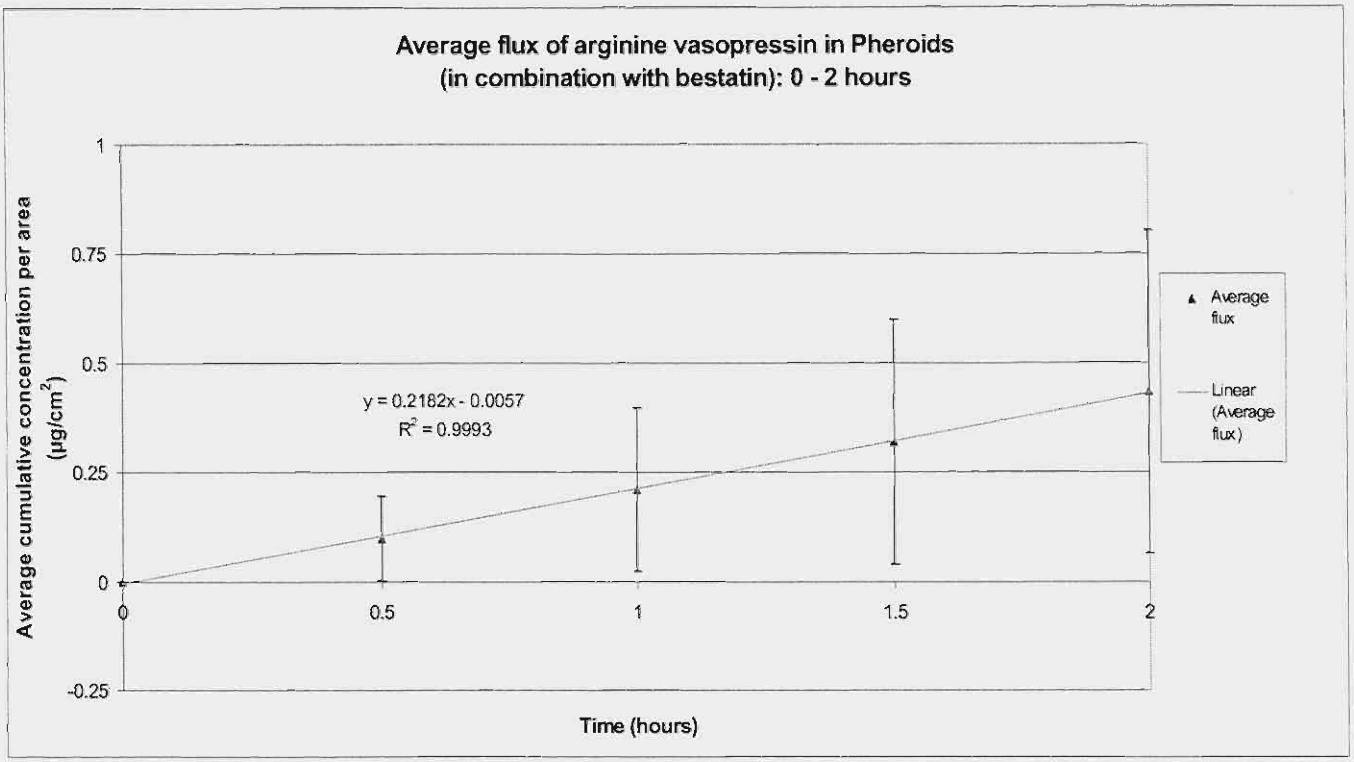
ARGININE VASOPRESSIN (IN COMBINATION WITH BESTATIN) IN THE PHEROID™ DRUG DELIVERY SYSTEM

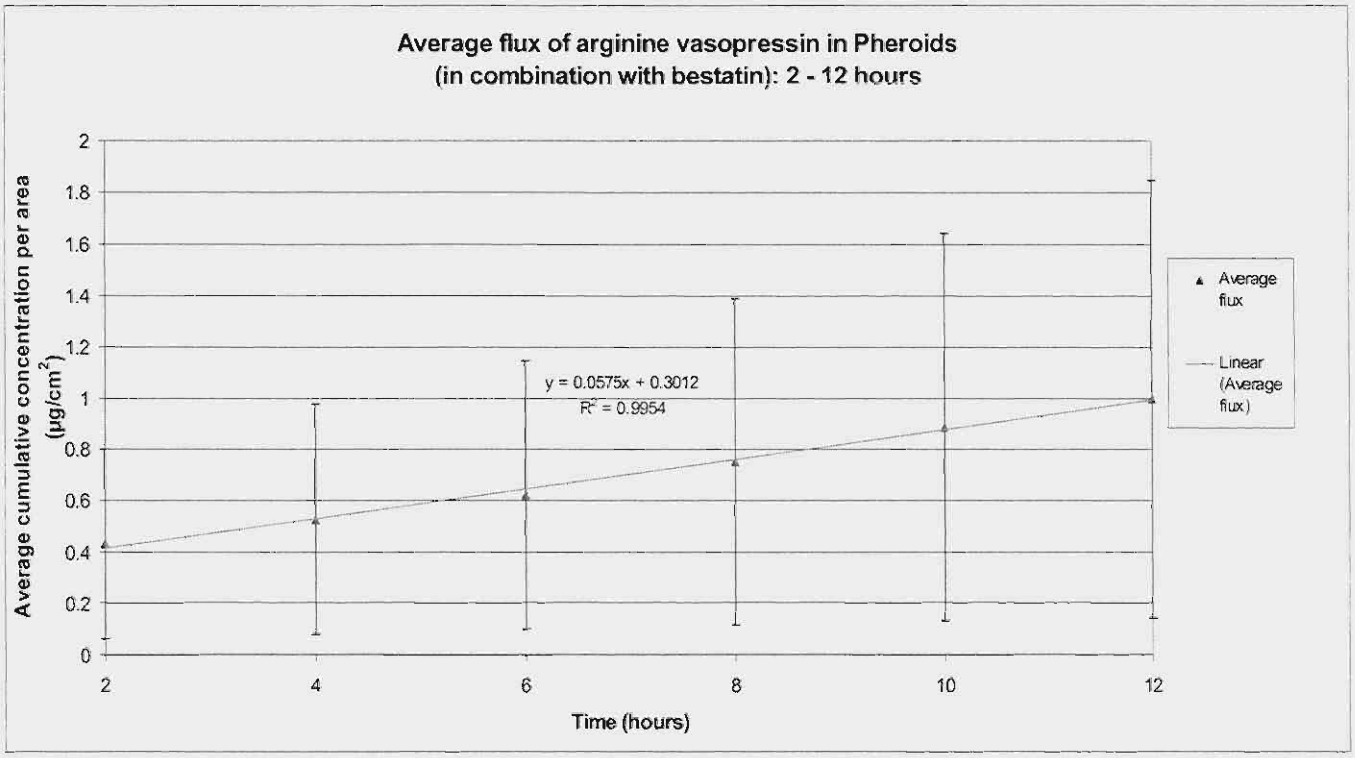
Arginine vasopressin and the enzymatic inhibitor bestatin were dissolved simultaneously in the Pheroid™ delivery system. The data for arginine vasopressin is reported here:

Cell #	Integrity (KΩ)		Cum. conc (µg/ml)	% yield	Flux 0-2h (µg/cm^2/h)	Flux 2-12h (µg/cm^2/h)
	Before	After				
1	12.64	3.94	111.917917508	81.10	16.998	7.472
2	5.89	8.92	3.468948015	2.51	0.633	0.197
3	30.16	20.62	0	0	0	0
4	4.56	5.60	0	0	0	0
5	19.54	23.12	0	0	0	0
6	13.94	13.66	0	0	0	0
7	8.02	8.91	0	0	0	0
8	9.23	13.32	11.531052297	8.36	2.540	0.612
9	10.58	13.47	4.135282010	2.45	0.509	0.324
10	19.64	25.40	0	0	0	0
11	6.63	8.51	2.320879855	1.37	0.547	0.124
12	28.30	46.28	0	0	0	0
13	19.02	16.01	6.936435959	4.10	0	0.761
14	20.17	24.62	0	0	0	0
15	6.04	6.14	14.747340247	8.73	5.220	0.410
16	4.55	6.76	24.108580015	14.27	1.972	2.005
17	47.54	36.47	1.541889592	0.99	0.282	0.104
18	45.88	33.31	1.816585734	1.17	0.346	0.120
19	14.04	22.02	1.615892082	1.04	0.349	0.094
20	36.06	27.87	1.371709926	0.88	0.332	0.074
21	32.74	26.30	1.887292620	1.22	0.324	0.131
22	23.75	21.84	1.609546837	1.04	0.353	0.095
23	33.45	24.98	1.087144614	0.70	0.240	0.063
24	31.52	22.36	1.461206587	0.94	0.347	0.082
25	32.87	21.24	1.626963321	1.05	0.350	0.094
26	37.23	26.49	0.854847129	0.55	0.255	0.029
27	10.05	8.37	7.405849548	4.78	0.572	0.640
28	43.82	29.94	1.819511297	1.17	0.429	0.095
29	21.54	21.68	1.920191343	1.24	0.432	0.102


Flux (µg/cm²/h) and yield (%) of arginine vasopressin in Pheroids
(in combination with bestatin)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0 0 0 0 0 0 1.37 0 0 0.99 1.17 1.04 0.88 1.22 1.04 0.70 0.94 1.05 0.55 1.17 1.24
3 4 5 6 7 10 11 12 14 17 18 19 20 21 22 23 24 25 26 28 29
Cell number
Flux 0 - 2 hours
Flux 2 - 12 hours
% Yield


Cumulative concentration (µg/ml) vs time for arginine vasopressin in Pheroids
(in combination with bestatin)
Cumulative concentration (µg/ml)
2.5
2
1.5
1
0.5
0
0 2 4 6 8 10 12
Time (hours)
Cell 3
Cell 4
Cell 5
Cell 6
Cell 7
Cell 10
Cell 11
Cell 12
Cell 14
Cell 17
Cell 18
Cell 19
Cell 20
Cell 21
Cell 22
Cell 23
Cell 24
Cell 25
Cell 26
Cell 28
Cell 29
Average flux


Average flux of arginine vasopressin in Pheroids
(in combination with bestatin): 0 - 2 hours
Average cumulative concentration per area (µg/cm²)
1
0.75
0.5
0.25
0
-0.25
y = 0.2182x - 0.0057
R² = 0.9993
0
0.5
1
1.5
2
Time (hours)
Average flux
Linear (Average flux)


Average flux of arginine vasopressin in Pheroids
(in combination with bestatin): 2 - 12 hours
Average cumulative concentration per area (µg/cm²)
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
y = 0.0575x + 0.3012
R² = 0.9954
2
4
6
8
10
12
Time (hours)
Average flux
Linear (Average flux)

COMPARISON BETWEEN FLUXES OF ARGININE VASOPRESSIN UNDER DIFFERENT CIRCUMSTANCES

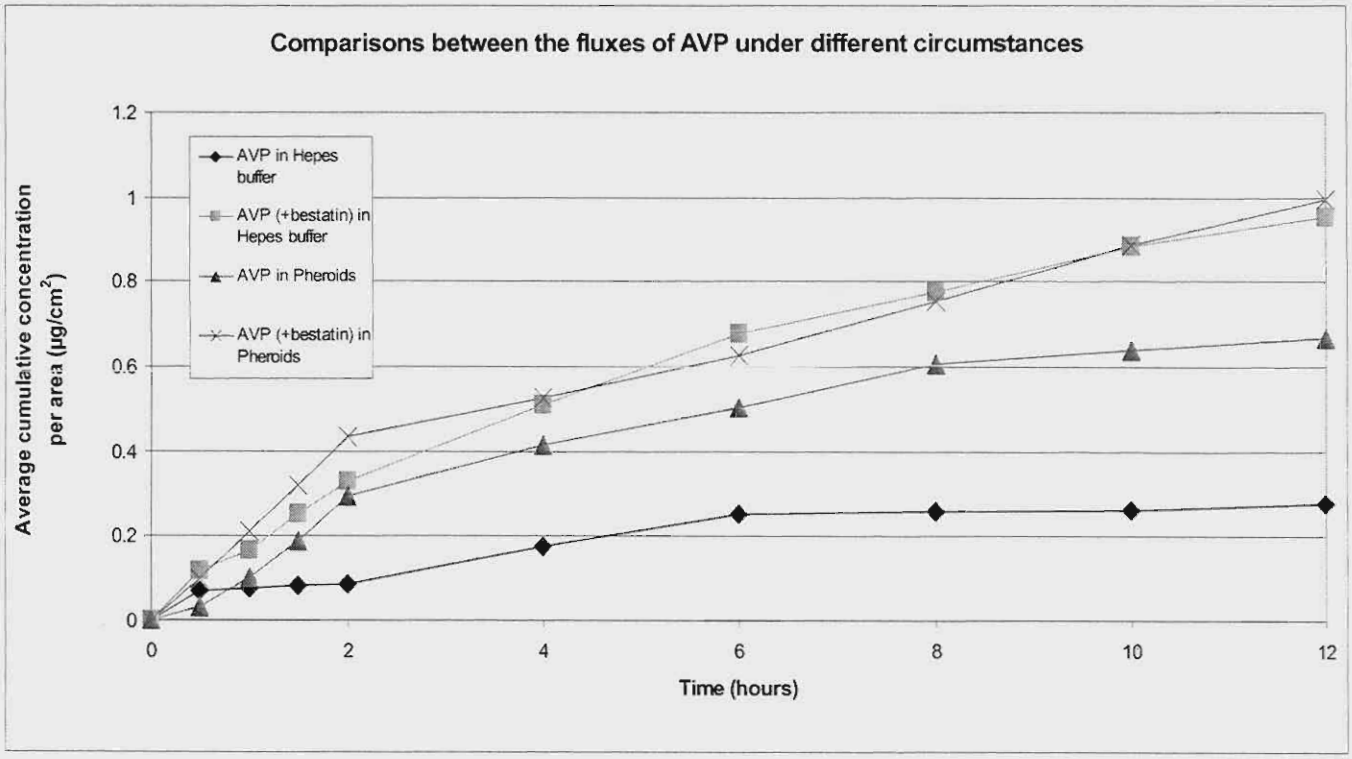
By means of this graph, the fluxes of arginine vasopressin are portrayed under different circumstances:

Blue diamond: AVP dissolved in Hepes buffer with exclusion of bestatin (passive flux of AVP).

Orange square: AVP and bestatin dissolved simultaneously in Hepes buffer (control flux of AVP). The average flux of AVP is shown.

Purple triangle: AVP dissolved in the Pheroid™ delivery system with exclusion of bestatin.

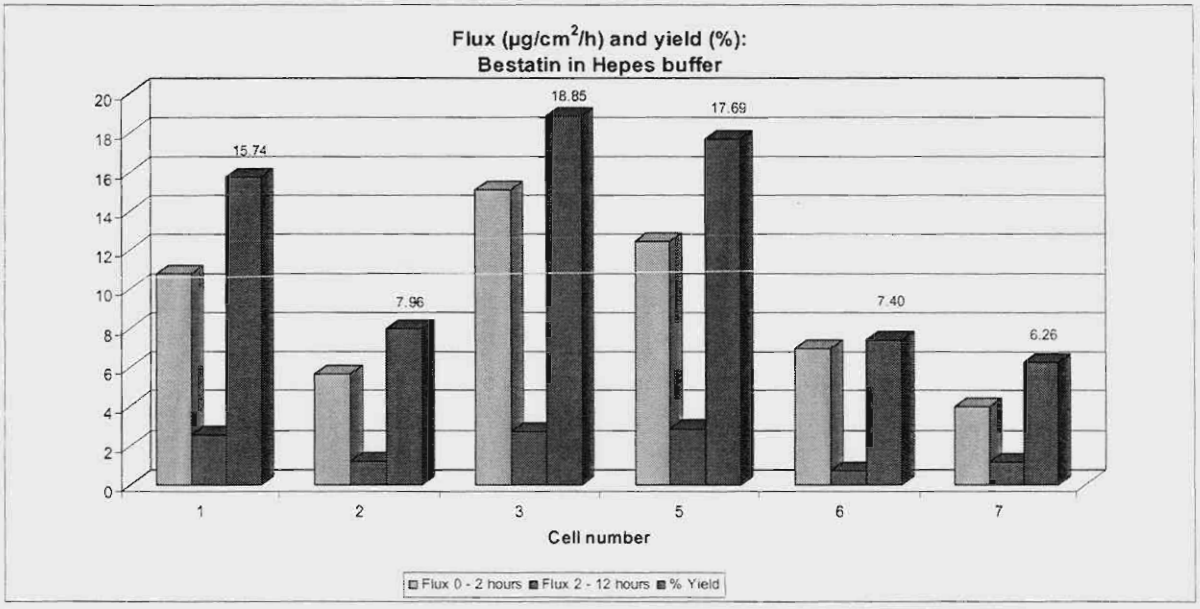
Green cross: AVP and bestatin dissolved simultaneously in the Pheroid™ delivery system. The average flux of AVP is shown.

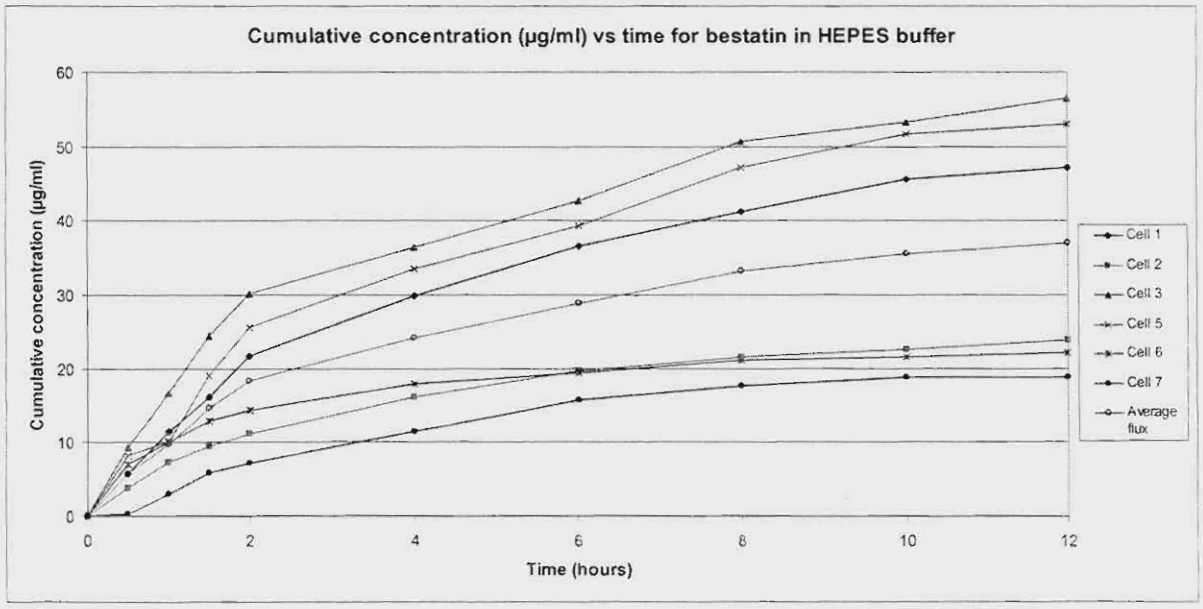


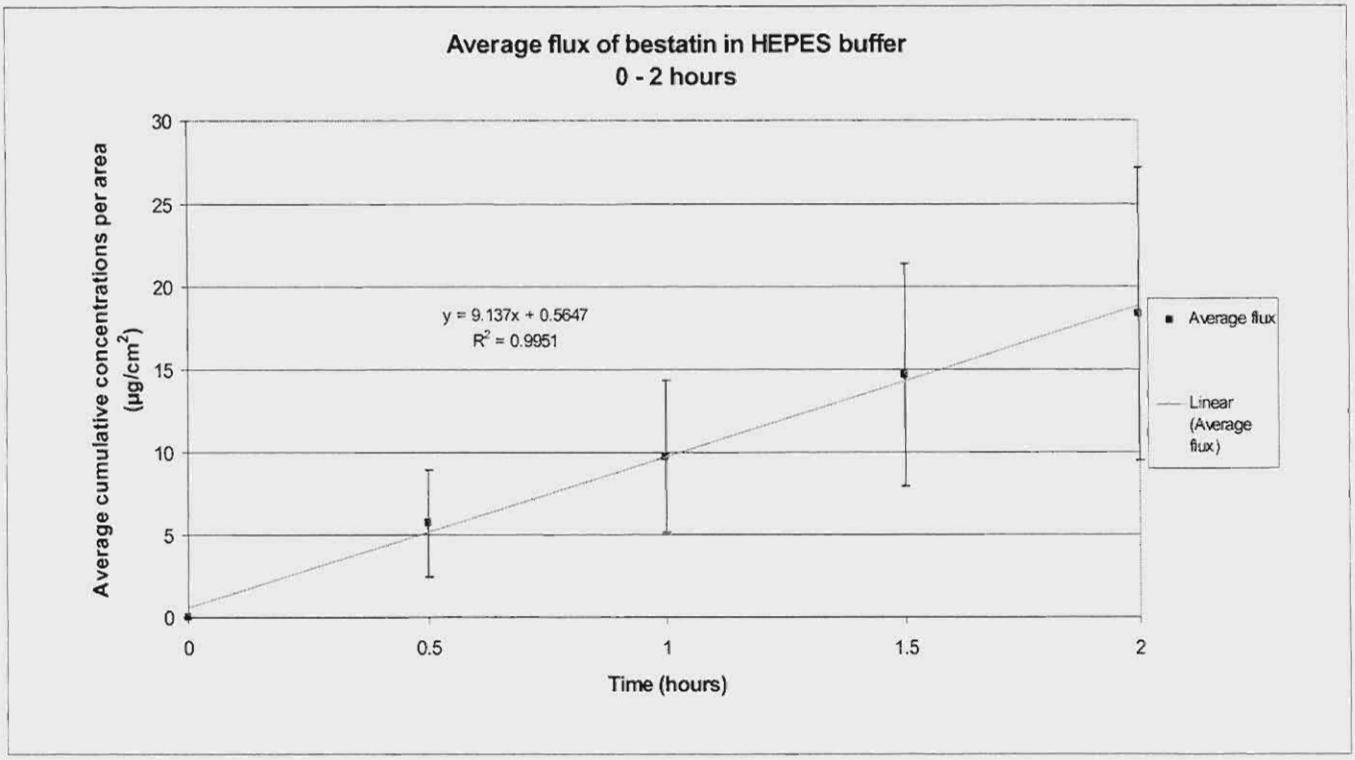
BESTATIN IN HEPES BUFFER

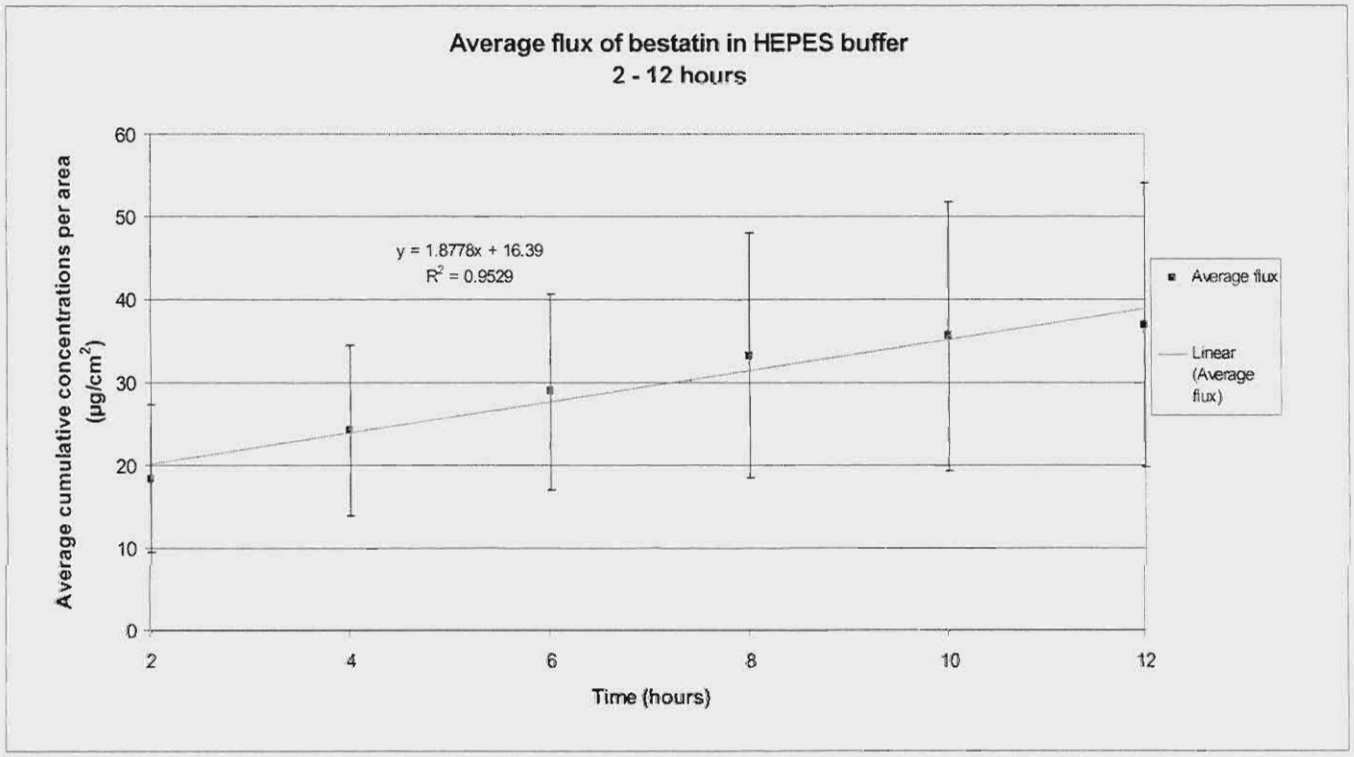
Bestatin was dissolved in Hepes buffer (omitting arginine vasopressin). The data for bestatin is reported here:

Cell #	Integrity (KΩ)		Cum. conc (µg/ml)	% yield	Flux 0-2h (µg/cm^2/h)	Flux 2-12h (µg/cm^2/h)
	Before	After				
1	4.53	6.64	47.221319018	15.74	10.775	2.559
2	3.47	3.91	23.878386556	7.96	5.637	1.203
3	3.87	4.55	56.549346865	18.85	15.076	2.734
4	4.10	3.53	125.301583340	41.77	34.538	5.312
5	4.14	6.77	53.077859781	17.69	12.409	2.859
6	6.10	8.07	22.196048975	7.40	6.948	0.741
7	10.45	7.65	18.779244134	6.26	3.978	1.170





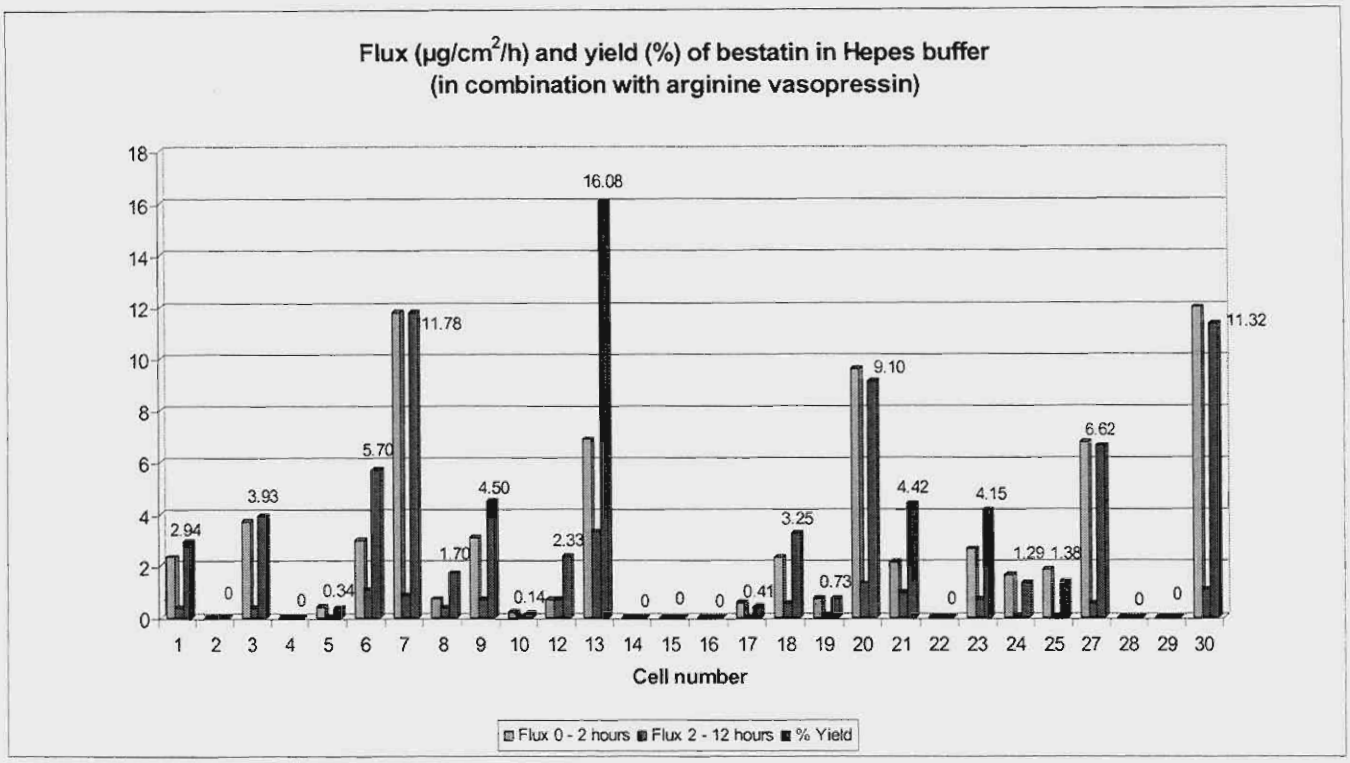

Average flux of bestatin in HEPES buffer
0 - 2 hours
Average cumulative concentrations per area (µg/cm²)
y = 9.137x + 0.5647
R² = 0.9951
Average flux
Linear (Average flux)
Time (hours)
0
5
10
15
20
25
30
0
0.5
1
1.5
2

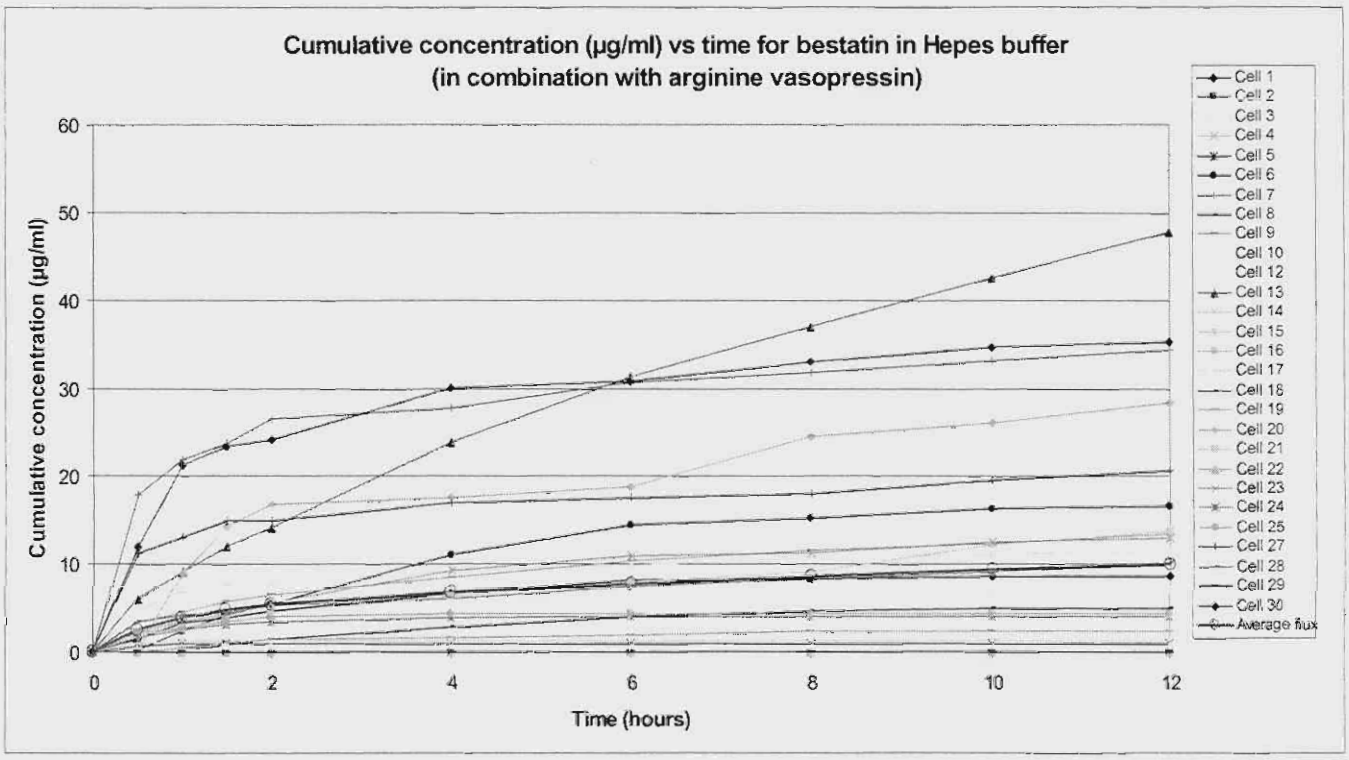

Average flux of bestatin in HEPES buffer
2 - 12 hours
Average cumulative concentrations per area (µg/cm²)
y = 1.8778x + 16.39
R² = 0.9529
Average flux
Linear (Average flux)
Time (hours)
0
10
20
30
40
50
60
2
4
6
8
10
12

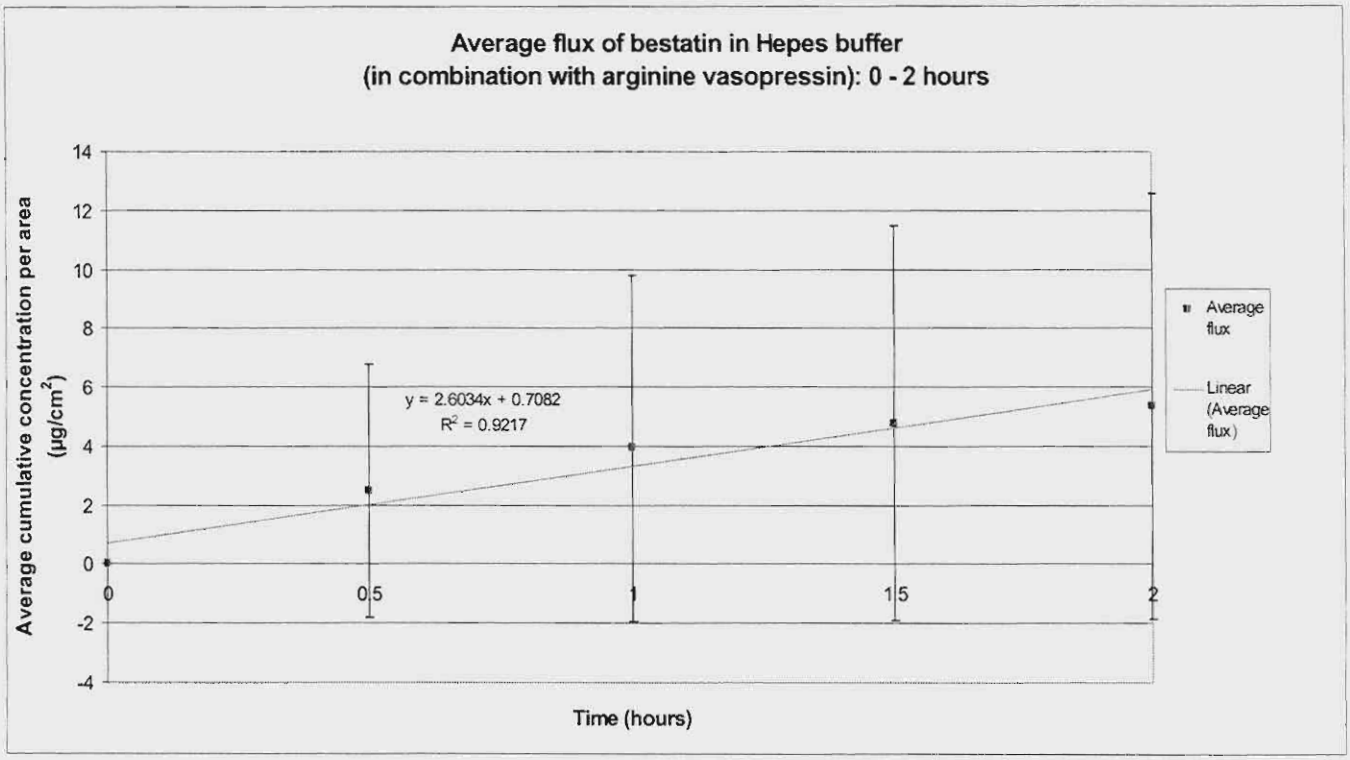
BESTATIN (IN COMBINATION WITH ARGININE VASOPRESSIN) IN HEPES BUFFER

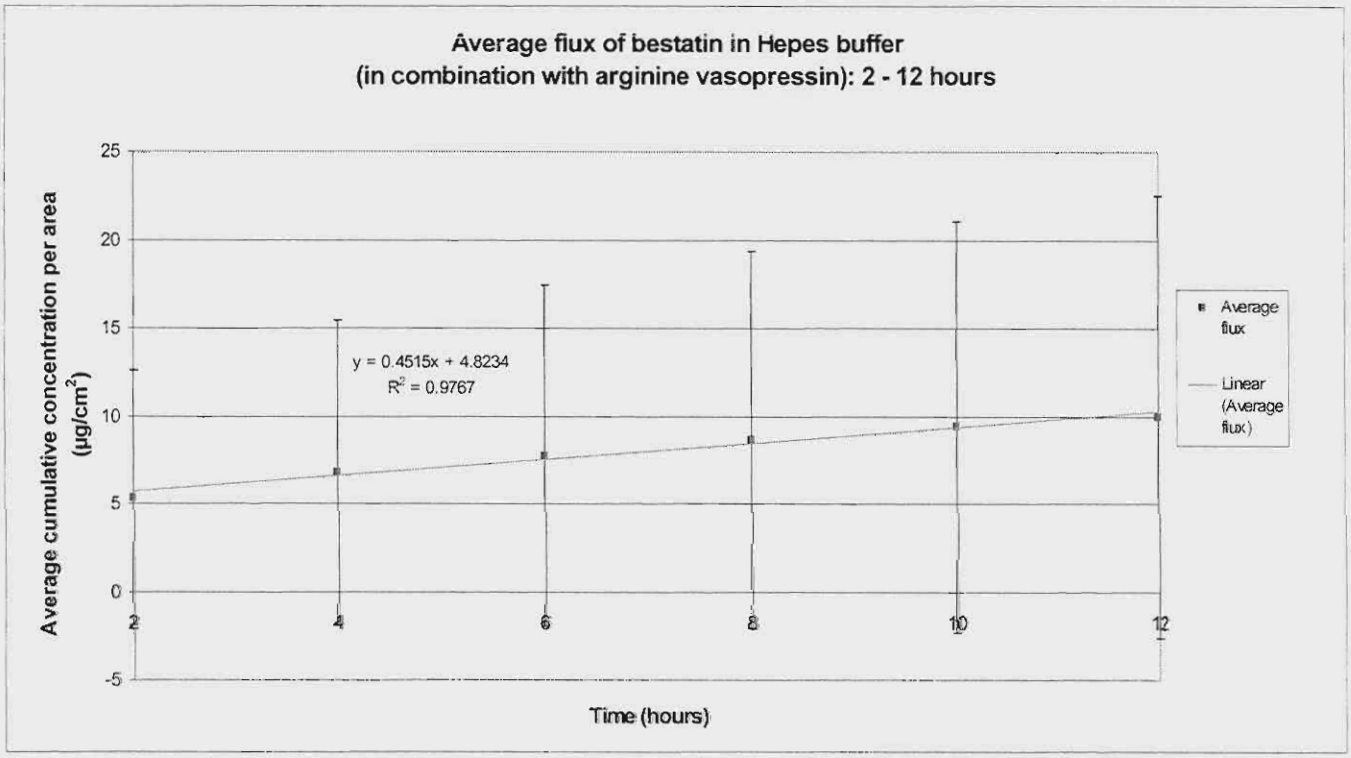
Arginine vasopressin and the enzymatic inhibitor bestatin were dissolved simultaneously in Hepes buffer. The data for bestatin is reported here:

Cell #	Integrity (KΩ)		Cum. conc	% yield	Flux 0-2h	Flux 2-12h
	Before	After	(µg/ml)		(µg/cm^2/h)	(µg/cm^2/h)
1	7.205	8.742	8.592187244	2.94	2.315	0.375
2	20.92	22.18	0	0	0	0
3	4.002	4.661	11.476498775	3.93	3.727	0.347
4	7.638	10.26	0	0	0	0
5	6.089	7.929	0.987149257	0.34	0.418	0.005
6	9.236	11.28	16.641993378	5.70	2.970	1.030
7	3.824	3.707	34.397085627	11.78	11.768	0.808
8	5.177	5.559	4.957466883	1.70	0.694	0.353
9	10.66	13.22	13.355127689	4.50	3.076	0.684
10	24.14	46.62	0.409637248	0.14	0.215	0
11	3.57	3.27	156.542930614	52.71	36.394	8.312
12	8.55	11.42	6.923527653	2.33	0.688	0.651
13	13.48	19.62	47.754351420	16.08	6.882	3.278
14	13.07	13.74	0	0	0	0
15	24.61	30.80	0	0	0	0
16	9.69	11.00	0	0	0	0
17	13.13	15.46	1.268389613	0.41	0.581	0
18	8.29	8.46	10.129506812	3.25	2.288	0.494
19	9.59	8.84	2.268475106	0.73	0.730	0.102
20	13.86	5.41	28.385567821	9.10	9.594	1.272
21	5.75	8.25	13.784196773	4.42	2.138	0.939
22	15.53	14.46	0	0	0.000	0.000
23	9.25	11.85	12.945069348	4.15	2.640	0.671
24	12.15	12.67	4.033548829	1.29	1.628	0.058
25	8.11	10.04	4.298652524	1.38	1.837	0.025
26	5.09	6.95	197.835858512	63.41	35.737	11.814
27	5.94	7.19	20.648714639	6.62	6.750	0.525
28	24.30	24.87	0	0	0	0
29	16.10	14.96	0	0	0	0
30	4.91	3.86	35.320732852	11.32	11.956	1.024


Flux (µg/cm²/h) and yield (%) of bestatin in Hepes buffer
(in combination with arginine vasopressin)
18
16
14
12
10
8
6
4
2
0
2.94
0
3.93
0
0.34
5.70
11.78
1.70
4.50
0.14
2.33
16.08
0
0
0
0.41
3.25
0.73
9.10
4.42
0
4.15
1.29
1.38
6.62
0
0
11.32
1 2 3 4 5 6 7 8 9 10 12 13 14 15 16 17 18 19 20 21 22 23 24 25 27 28 29 30
Cell number
Flux 0 - 2 hours
Flux 2 - 12 hours
% Yield


Cumulative concentration (µg/ml) vs time for bestatin in Hepes buffer
(in combination with arginine vasopressin)
Cumulative concentration (µg/ml)
60
50
40
30
20
10
0
0
2
4
6
8
10
12
Time (hours)
Cell 1
Cell 2
Cell 3
Cell 4
Cell 5
Cell 6
Cell 7
Cell 8
Cell 9
Cell 10
Cell 12
Cell 13
Cell 14
Cell 15
Cell 16
Cell 17
Cell 18
Cell 19
Cell 20
Cell 21
Cell 22
Cell 23
Cell 24
Cell 25
Cell 27
Cell 28
Cell 29
Cell 30
Average flux


Average flux of bestatin in Hepes buffer
(in combination with arginine vasopressin): 0 - 2 hours
Average cumulative concentration per area (µg/cm²)
14
12
10
8
6
4
2
0
-2
-4
y = 2.6034x + 0.7082
R² = 0.9217
0
0.5
1
1.5
2
Time (hours)
Average flux
Linear (Average flux)

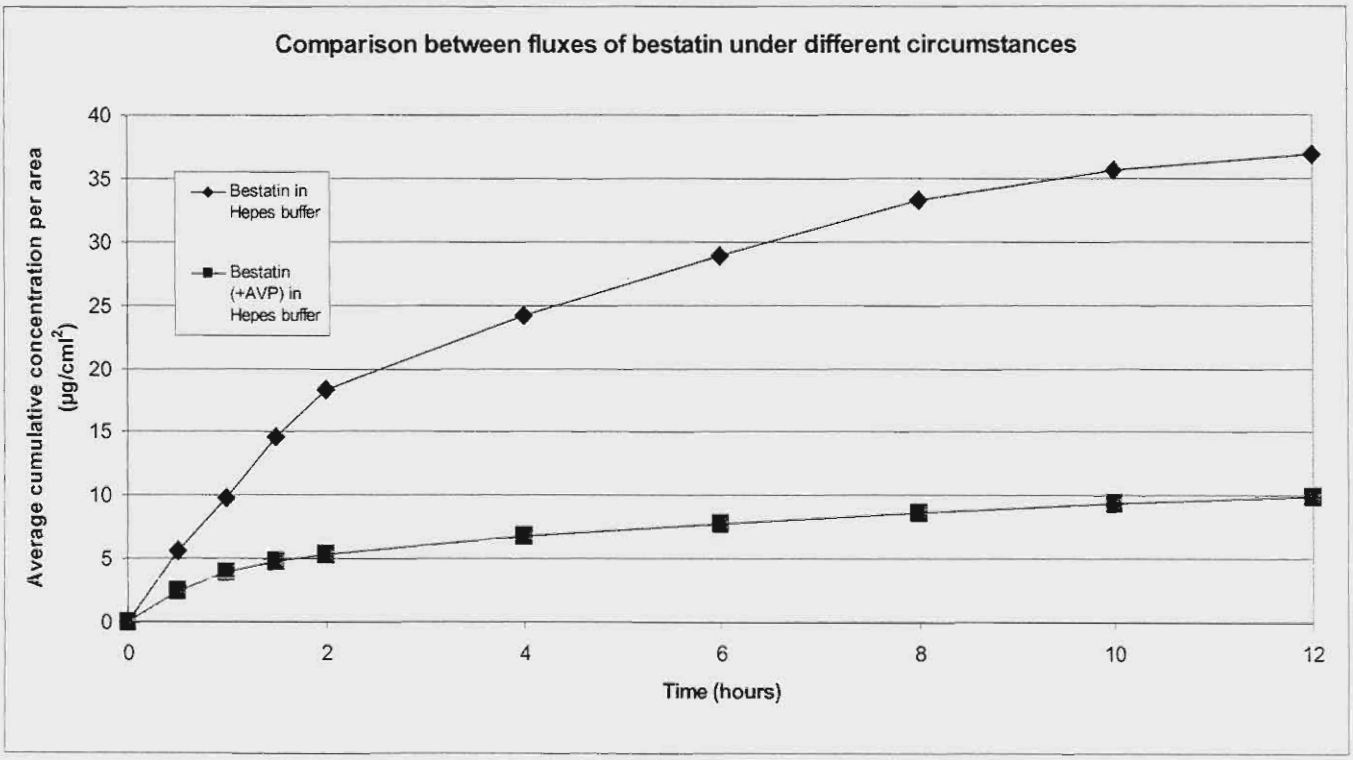

Average flux of bestatin in Hepes buffer
(in combination with arginine vasopressin): 2 - 12 hours
Average cumulative concentration per area (µg/cm²)
25
20
15
10
5
0
-5
y = 0.4515x + 4.8234
R² = 0.9767
2
4
6
8
10
12
Time (hours)
Average flux
Linear (Average flux)

COMPARISON BETWEEN FLUXES OF BESTATIN UNDER DIFFERENT CIRCUMSTANCES

By means of this graph, the fluxes of bestatin are portrayed under different circumstances:

Blue diamond: Bestatin dissolved in Hepes buffer with exclusion of AVP.

Purple square: AVP and bestatin dissolved simultaneously in Hepes buffer. The average flux of bestatin is shown.



APPENDIX 2: VALIDATION OF EXPERIMENTAL METHODS

VALIDATION OF THE HPLC ANALYTICAL METHOD

PURPOSE OF THE VALIDATION

To ensure that the analytical method was sensitive and reliable in the determination of the amount of drug that permeated the skin.

CALIBRATION CURVE

A calibration curve for arginine vasopressin was established by using the standard solutions with the following concentrations: 0.1, 0.2, 1, 2, 10, 20, and 100 µg/ml. The solutions were prepared as follows:

1. Weighed approximately 1 mg (1000 µg) arginine vasopressin accurately and dissolved in a 10 ml volumetric flask. Made up to volume with Hepes buffer and dissolved by means of vortex for a few seconds until dissolved (*100 µg/ml* – mother solution)
2. Diluted 1 ml of the mother solution to 10 ml with Hepes buffer (*10 µg/ml)*
3. Diluted 2 ml of the mother solution to 10 ml with Hepes buffer (*20 µg/ml)*
4. Diluted 1 ml of the 10 µg/ml solution to 10 ml with Hepes buffer (*1 µg/ml)*
5. Diluted 1 ml of the 20 µg/ml solution to 10 ml with Hepes buffer (*2 µg/ml)*
6. Diluted 1 ml of the 1 µg/ml solution to 10 ml with Hepes buffer (*0.1 µg/ml)*
7. Diluted 1 ml of the 2 µg/ml solution to 10 ml with Hepes buffer (*0.2 µg/ml)*

VALIDATION PARAMETERS

LINEARITY

The linearity of an analytical method is described as its ability (within a given range) to obtain results that are directly proportional to the concentration (amount) of analyte in the sample. The linearity of arginine vasopressin was determined by performing linear regression analysis on the plot of the peak area ratios versus concentration (µg/ml). The standard concentrations were prepared as described. The data is best described by a linear equation y = mx + c where:

y	=	peak area ratios of arginine vasopressin
m	=	slope
x	=	concentration of arginine vasopressin in µg/ml
c	=	y-intercept

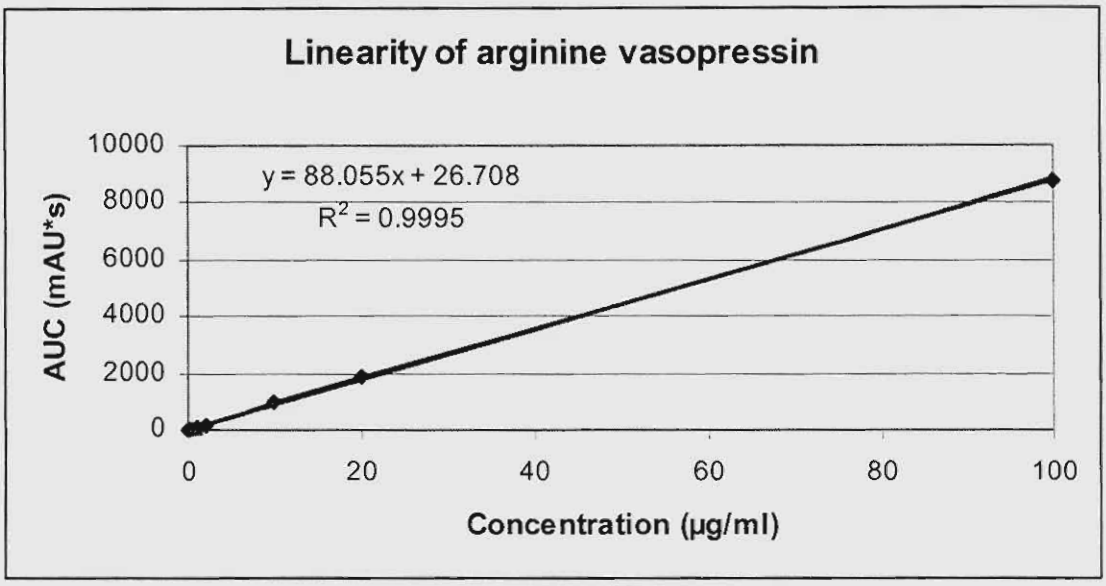


Figure 10: Linear regression curve of arginine vasopressin standards

The regression value (r^2) obtained indicates a high degree of linearity and therefore demonstrates the good stability of the analysis system.

Table 4: Peak area ratio values of AVP standards

Standard (µg/ml)	Peak area ratio
0.1	9.07
0.2	13.58
1	68.72
2	147.61
10	936.89
20	1920.06
100	8801.71
Slope **Y-intercept** **r^2**	**88.055** **26.708** **0.9995**

PRECISION

The precision of an analytical procedure expresses the proximity of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Precision was investigated in terms of intraday (repeatability) variation and interday (reproducibility).

Intraday precision (repeatability)

The intraday precision was determined by performing HPLC analyses (n = 3) on three different samples of known standards (1, 10, 100 µg/ml) of arginine vasopressin three times during the same day. The results can be seen in Table 5, with all the results complying with acceptable pharmaceutical standards/meeting the requirements of the USP. The recovery was found to be ranging between 99.95 % and 100 %.

Table 5: Intraday precision parameters of arginine vasopressin standards

Standards (µg/ml)	Mean peak area ratios			Mean	SD	%RSD
	t = 1	t = 2	t = 3			
1	78.41	67.02	60.72	68.72	8.97	13.05
10	936.46	947.86	926.35	936.89	10.76	1.15
100	8752.14	8825.58	8813.00	8796.91	39.28	0.45

Interday precision (reproducibility)

The interday precision was determined by performing HPLC analyses (n = 3) on 3 different samples of known standards (1, 10, 100 µg/ml) of arginine vasopressin on three consecutive days. The results can be seen in Table 6, with all the results complying with acceptable pharmaceutical standards/meeting the requirements of the USP. The recovery was found to be ranging between 97.61 % and 101.85 %.

Table 6: Interday precision parameters of arginine vasopressin standards

Standards (µg/ml)	Mean peak area ratios			Mean	SD	%RSD
	Day 1	Day 2	Day 3			
1	76.52	72.72	60.72	69.99	8.25	11.78
10	938.46	942.16	926.35	935.66	8.27	0.88
100	8297.72	8684.68	8788.86	8590.42	258.78	3.01

SENSITIVITY

The sensitivity of the analytical method can be assessed by determining the limit of detection, as well as the lowest limit of quantification. The %RSD for both measurements should be <15%. The sensitivity of a method can be improved by increasing the sample volume.

LIMIT OF DETECTION (LOD)

Limit of detection (LOD) is defined as the lowest concentration of an analyte in a sample that can be detected, but not necessarily quantified as an exact value. On a signal-to-noise ratio of 3:1 (the analyte peak is approximately three times the height of the baseline noise), the LOD for arginine vasopressin was 0.04 µg/ml (40 ng/ml).

LIMIT OF QUANTIFICATION (LOQ)

The limit of quantification (LOQ) is defined as the lowest concentration of an analyte in a sample that can be quantitatively determined with acceptable precision and accuracy (%RSD <15%). The LOQ of arginine vasopressin was expressed as a concentration at a signal-to-noise ratio of 10:1 and was determined at 0.1µg/ml (100 ng/ml).

SELECTIVITY

Selectivity is the ability of the method to selectively detect the analyte in the presence of other compounds that may interfere with analyte detection. The method is selective when no interfering peaks with the same retention time as the drug are detected. Bestatin and Hepes buffer were analysed together with arginine vasopressin and illustrated no interference. There was also no interference from the components of the Pheroid™ formulation that permeated the skin. Figures 11 to 13 depict HPLC chromatograms emphasising the above facts.

Figures 11 to 13: HPLC chromatograms of arginine vasopressin and bestatin in the Pheroid™ delivery system manufactured with Hepes buffer as the aqueous phase. (AVP: t=±7.3-7.5 min, Bestatin: t=±8.2-8.5 min. Peaks at approximately 2 and 3 minutes: Hepes buffer components)

Figure 11

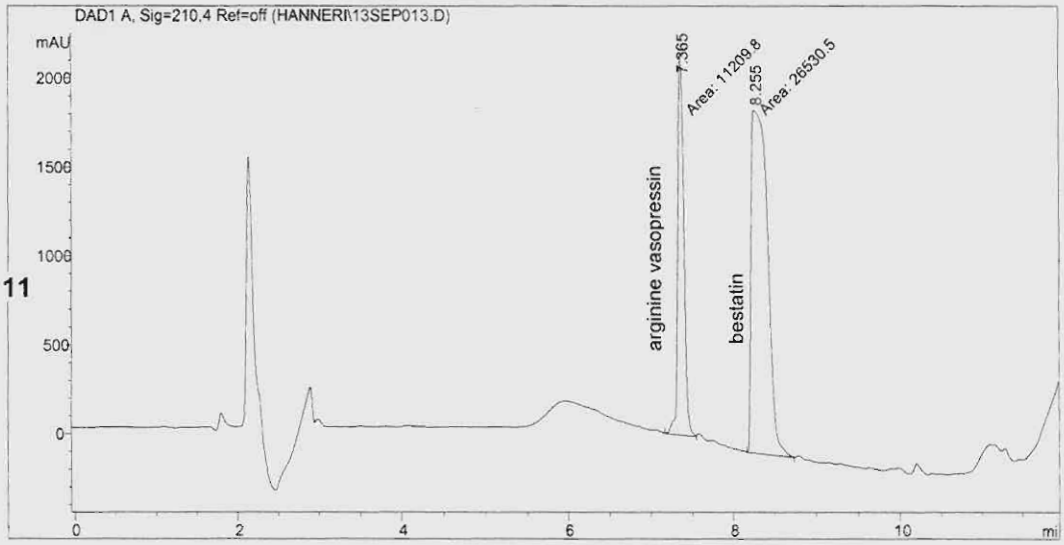


Figure 12

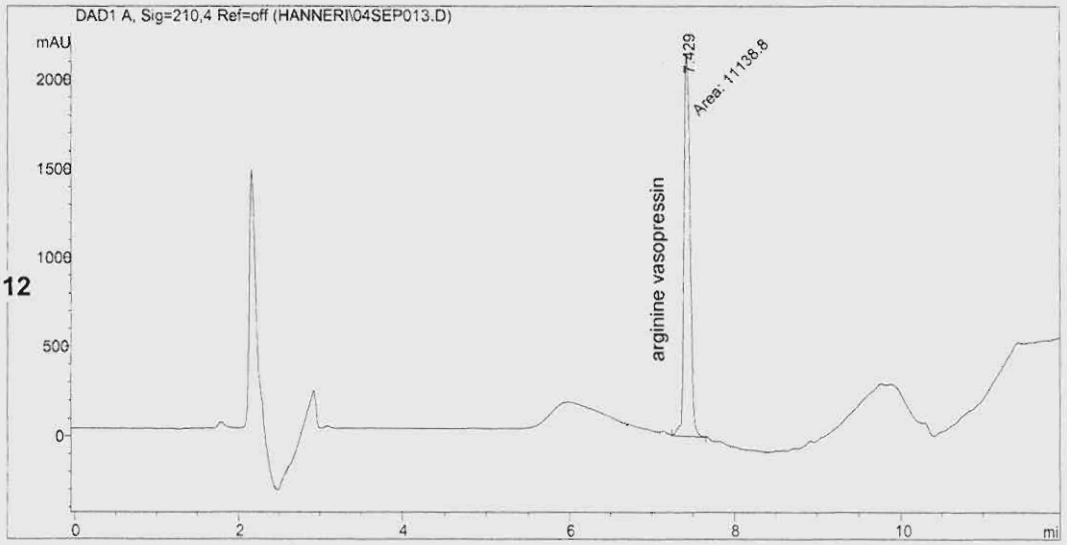
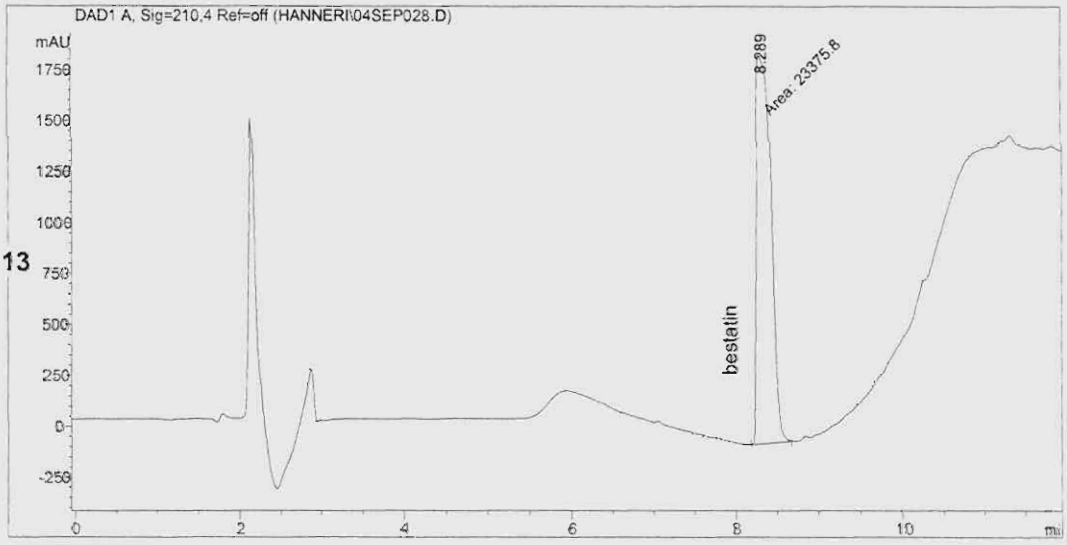


Figure 13



SYSTEM REPEATABILITY

In an attempt to evaluate the repeatability of the peak area and retention time, samples of arginine vasopressin with a known concentration (100 µg/ml) were injected six times. The variations in response (%RSD) of the detection system when six determinations of arginine vasopressin were made on the same day, and under the same conditions, were the following:

Table 7: Variations in response (% RSD) of the detection system regarding peak area and retention time

Injection	Concentration (µg/ml)	Peak area (mAU*s)	Retention time (min)
1	100	8752.14	7.475
2	100	8825.58	7.473
3	100	8813.00	7.474
4	100	8802.87	7.475
5	100	8816.20	7.472
6	100	8800.48	7.472
	Mean	8801.71	7.474
	SD	25.95089	0.0014
	%RSD	0.2948	0.0184

CONCLUSION

The HPLC method has been found to be reliable and sensitive enough for the determination of the concentration of arginine vasopressin.

APPENDIX 3: PHOTOS OF INSTRUMENTATION USED IN DIFFUSION STUDIES AND ANALYSES

Photo 1: Vertical Franz diffusion cells in stand

Photo 2: Metal clamps, magnetic stirrers, Parafilm® and Dow Corning vacuum grease

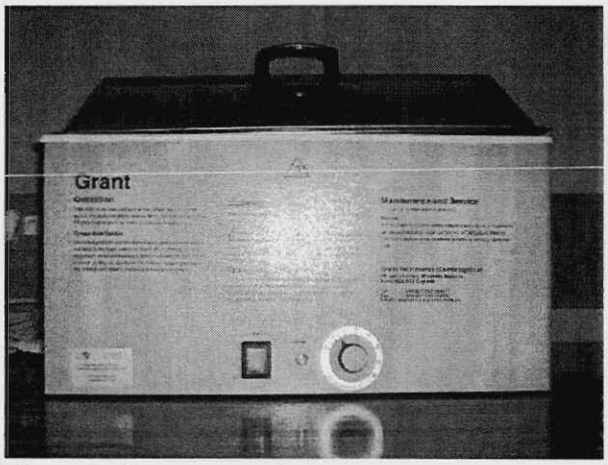


Photo 3: Grant water bath

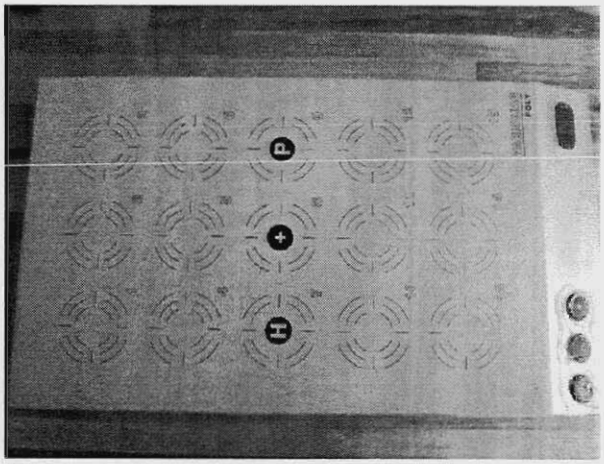


Photo 4: Variomag® magnetic stirrer plate

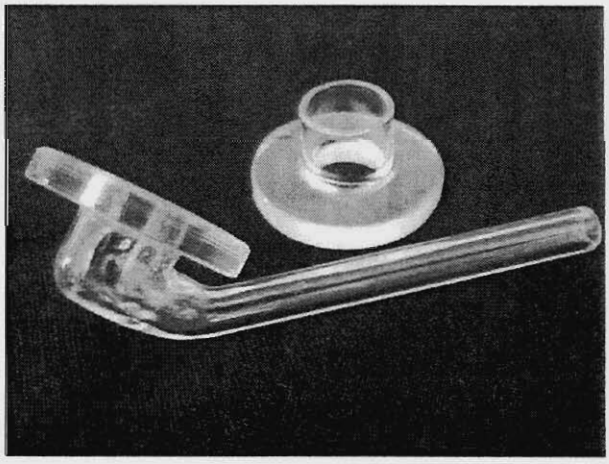


Photo 5: Franz diffusion cell: Donor and receptor compartments

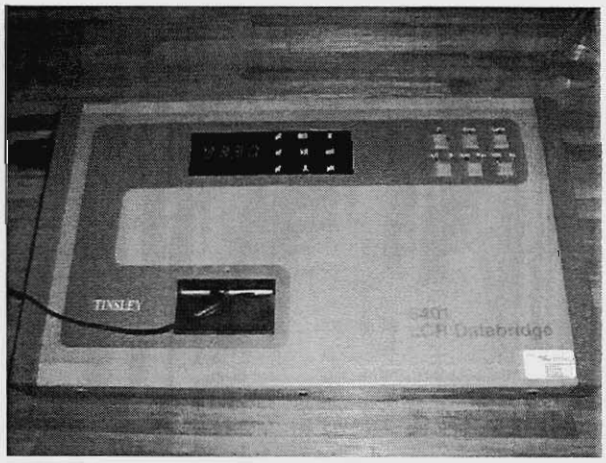


Photo 6: Tinsley Model 6401 LCR Databridge

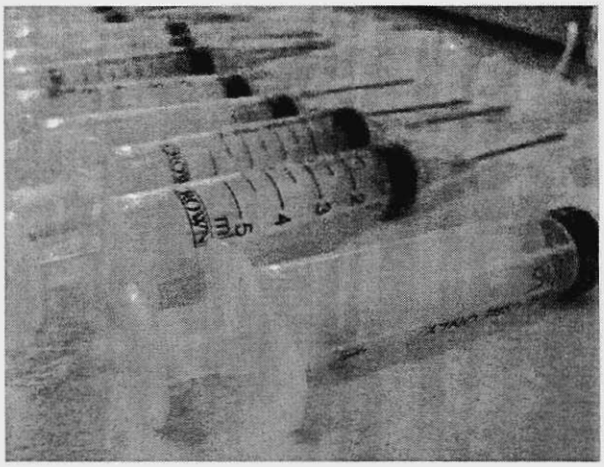


Photo 7: Syringes for removal of samples from receptor compartments of diffusion cells

Photo 8: Separations® HPLC Vials with samples

Photo 9: Agilent Series 1100 HPLC

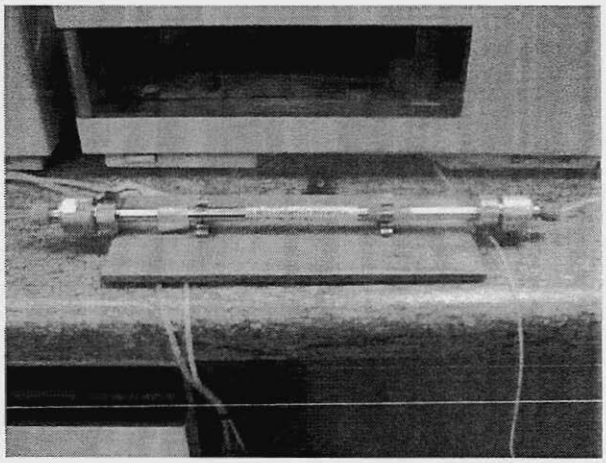


Photo 10: Macherey-Nagel LiChrospher® 100 RP ec column