

# Evaluation of the oral delivery of goserelin with Pheroid<sup>®</sup> technology

**L Erasmus**



**[orcid.org/ 0000-0001-8625-1131](https://orcid.org/0000-0001-8625-1131)**

Dissertation submitted in fulfilment of the requirements for the degree *Magister Scientiae* in Pharmaceutical Sciences at the North-West University

Supervisor: Prof R Hayeshi

Co-supervisor: Ms L Scholtz

Graduation Ceremony

Student number: 23389907

# ACKNOWLEDGEMENTS

I would like to express my sincerest appreciation to the following people, all of who played an integral role during this study.

My supervisor **Prof. Rose Hayeshi**. First of all, thank you for the guidance, support and motivation during these 2 years, you made the research and writing up a lot easier. Thank you very much for the opportunity to have completed a study such as this under your supervision. Thank you for everything you have done to support me throughout my study.

**Prof. Anne Grobler**, thank you for the opportunity to be one of the PCDDP students, your passion for this field is a great inspiration.

My co-supervisor **Liezl-Marié Scholtz**, thank you for your suggestions and input you had in my study.

**The NWU-vivarium staff (Cor Bester, Antoinette Fick, Kobus Venter, Jacob Mabuta)**, thank you for assisting me with the *in vivo* experiments of my study.

**Dr. John Takyi-Williams**, thank you for the hard work and contribution you made towards my study.

My co-workers (**Lerato Thindisa, Stephanie Erasmus, Bianca van Lingen, Riaan van Wyk, Drieke van der Merwe and Jennifer Williams**), thank you for the positivity and motivation, and just overall the laughs and friendship in the office. It made the end a lot easier. I hope the best for your future.

**Dr. Adrienne Leussa**, thank you for the confocal analysis of my samples.

**My parents**. Thank you for the love, support and motivation. Thank you for always believing in me. You are the best parents anyone could ask for.

**Wihan Landsberg**, thank you for all the moral and emotional support. Thank you for the constant motivation throughout this period.

**Mr Martin Magwaza** from BGM Pharmaceuticals for providing the goserelin implants, goserelin acetate and for financial support

The **North-West University** and the **DST/NWU PCDDP** for financial support to make this study possible.

With God all things are possible' (Matthew 19:26). It is the power of God that makes what seems impossible possible. Nothing is impossible with God (Luke 1:37)

# PREFACE

This master's dissertation is submitted in fulfilment of the requirements of Masters Science in Pharmaceutical Sciences by using the article format in accordance with the North-West University (NWU). References and citations were completed in accordance with the Harvard style format.

I **Linnè Erasmus**, the student, did the following in the work presented in this dissertation:

- Planned the study in consultation with study promoters
- Carried out all the experimental work and participated in all the experiments done at the Vivarium of NWU and DST/NWU Preclinical Drug Development Platform (PCDDP)
- Interpreted the results and discussed them with statistician and promoter
- Wrote the complete dissertation with the assistance of promoter
- Drafted the manuscripts

**Student digital signature:**



A handwritten signature in black ink, appearing to read 'Linnè Erasmus', written in a cursive style. The signature is positioned above a solid horizontal line.

---

## ABSTRACT

The therapeutic amphiphilic peptide, goserelin, being investigated in these studies, is a type of hormone therapy generally used in addition to standard adjuvant therapy. Goserelin serves as treatment for numerous hormone-dependent disorders, for instance benign conditions (e.g. uterine fibroids, menorrhagia, endometriosis etc.) or malignant tumours (breast, ovarian, endometrial and prostate carcinoma). Goserelin is a synthetic analogue of the naturally occurring gonadotropin-releasing hormone (GnRH), which is also commonly known as a GnRH agonist. Goserelin acts directly on the hypothalamic-pituitary axis, consequently leading to receptor down-regulation, which in turn leads to the inhibition of the secretion of the pituitary gonadotropins. This causes a decrease of endogenous testosterone in males and oestrogen in females. Through this, a hypogonadal status is achieved. Goserelin in combination with menotropins or recombinant follicle-stimulating hormone (FSH) is commonly used for women undergoing *in vitro* fertilisation (IVF), as it aids in inducing folliculogenesis for controlled ovarian hyperstimulation. Currently, goserelin is administered as a slow releasing subcutaneous depot (slow releases goserelin over a period of 28 days); this is typically a more invasive administration regimen and commonly not usually tolerated well by patients, thus changing the administration route from subcutaneous to oral may yield great advantages.

Goserelin, being a peptide, will face several challenges after oral administration, such as the acidic and enzymatic degradation in the gastrointestinal tract (GI) tract; however, these can be overcome by different strategies, such as formulation and chemical technologies. Using a particulate drug delivery system (DDS) to protect peptide drugs is an example of a formulation technology. Pheroid<sup>®</sup> is a novel colloidal DDS, with the capability of protecting a therapeutic drug and consequently increasing the bioavailability. In this study, goserelin was used in combination with Pheroid<sup>®</sup> technology to determine the pharmacokinetics and pharmacodynamics of goserelin after administration of an oral dose of a novel pro-Pheroid<sup>®</sup>-goserelin formulation, in comparison to the existing goserelin subcutaneous implant (Zoladex<sup>®</sup>) in male and female BALB/c mice. The physicochemical characteristics, i.e. particle size, colloidal stability and morphology, of this novel Pheroid<sup>®</sup> formulation of goserelin were also investigated. The effect of entrapment of goserelin in Pheroid<sup>®</sup> on its gastrointestinal stability was determined by comparing the stability of goserelin and pro-Pheroid<sup>®</sup>-goserelin in simulated gastric fluid (SGF) and simulated intestinal fluid (SIF).

The characteristics of Pheroid<sup>®</sup> revealed no change after the addition of goserelin to pro-Pheroid<sup>®</sup>. The stability study was non-conclusive due to loss of sample during centrifugation of the SIF and SGF of the pro-Pheroid<sup>®</sup>-goserelin samples. An optimised and revised stability study will be conducted in the near future. The male BALB/c mice were divided into three groups: group 1 received the peptide subcutaneously, group 2 received pro-Pheroid<sup>®</sup>-goserelin as an oral dose of 2 mg/kg and group 3 received pro-Pheroid<sup>®</sup>-goserelin as an oral dose of 4 mg/kg. Blood samples were collected at different time points and the plasma was analysed for testosterone and goserelin

concentrations using LC-MS/MS. From the goserelin concentration-time profile, the observed peak plasma concentration ( $C_{max}$ ) and time to maximum concentration ( $T_{max}$ ) were reported and statistically compared. An expected significantly higher  $C_{max}$  and  $T_{max}$  were observed in the subcutaneous group. The 2 mg/kg pro-Pheroid<sup>®</sup> goserelin resulted in a significantly higher  $C_{max}$  compared to the 4 mg/kg dose. An initial (0 - 30 minutes) increase in the testosterone concentration for each group was observed, thus adhering to the flare-up effect. Both the 2 mg/kg and 4 mg/kg pro-Pheroid<sup>®</sup> groups proved to have an effect on the testosterone concentration. The 4 mg/kg pro-Pheroid<sup>®</sup>-goserelin was chosen for further investigation in female BALB/c mice. Hence, it can be concluded that goserelin did succeed in reaching the blood and consequently caused a decrease in the testosterone concentration after the oral administration with pro-Pheroid<sup>®</sup>.

The female BALB/c mice were also divided into three groups; group 1 received goserelin subcutaneously, group 2 received 4 mg/kg pro-Pheroid<sup>®</sup>-goserelin through oral gavage and group 3, the negative control group, received only pro-Pheroid<sup>®</sup>. Evaluation for the daily vaginal smears was done by vaginal cytology for 21 days, to track the disruption or lack thereof of the 4-stage oestrous cycle. The vaginal cytology revealed disruption in the oestrous cycle in groups 1 and 2. Thus it can be concluded that the oral administration of goserelin in pro-Pheroid resulted in sufficient uptake to cause the hormonal effects.

The pro-Pheroid<sup>®</sup>-goserelin formulation in both the male and female studies resulted in bioavailable goserelin as indicated by the related pharmacodynamic (PD) results. In summary, therefore, Pheroid<sup>®</sup> technology proved to protect goserelin from the degradation in the GI tract and could thus be a helpful DDS for oral delivery of therapeutic drugs.

**Keywords:** Goserelin, Pheroid<sup>®</sup> technology, drug delivery, peptide therapeutics, pharmacokinetics, pharmacodynamics, oral drug delivery.

## UITTREKSEL

Die terapeutiese amfifiele peptied, goserelin, wat in hierdie studies ondersoek word, is 'n tipe hormoonterapie wat algemeen benewens standaard adjuvante terapie gebruik word. Goserelin dien as behandeling van talle hormoonafhanklike afwykings, soos byvoorbeeld; goedaardige gewasse (bv. baarmoeder fibroïede, menorrhagie, endometriose ens.) of kwaadaardige gewasse (bors, eierstok, endometriale en prostaatkarsinoom). Goserelin is 'n sintetiese analoog van die natuurlik voorkomende gonadotropien-vrystelling hormoon (GnRH), wat ook algemeen bekend staan as 'n GnRH-agonis. Goserelin tree direk op die hipotalamus-pituïtêre klier, wat gevolglik lei tot reseptor-afregulering wat dan weer lei tot die inhibisie van die afskeiding van die pituïtêre gonadotropiene. Dit veroorsaak 'n afname in endogene testosteroon by mans en estrogeen by vroue. Hierdeur word 'n hipogonadale status behaal. Goserelin in kombinasie met menotropiene of rekombinante follikelstimulerende hormoon (FSH) word algemeen gebruik vir vroue wat in vitro-bevrugting (IVB) ondergaan, hierdie hulpmiddels word in die algemeen gebruik om follikulogenese te bewerkstellig vir beheerde ovariumhiperstimulasie. Tans word goserelin geadministreer as 'n stadige vrystelling van die subkutane depot (die peptied word stadig oor 'n tydperk van 28 dae vrygestel). Hierdie manier van administrasie toediening is 'n meer indringende toedienings metode en word gewoonlik nie goed deur pasiënte geduld nie, dus deur die toedieningsroete van subkutane tot 'n orale toediening te verander sal tot groot voordeel lei.

As gevolg van goserelin wat 'n peptied is, beteken dit dat daar verskeie uitdagings ervaar sal word ná orale toediening, soos bv. die suur teenwoordig en ensimitiese afbraak in die spysverteringskanaal. Dit kan egter oorkom word deur verskillende strategieë soos formulering en chemiese tegnologie. Die gebruik van 'n medisinale afleweringstelsel om die terapeutiese peptiedmiddel te beskerm, is 'n bekende voorbeeld van 'n formuleringstegnologie. Pheroid<sup>®</sup> is 'n nuwe kolloïdale afleweringstelsel, met die vermoë om 'n terapeutiese middel te beskerm en gevolglik die biobeskikbaarheid te verhoog. In hierdie studie is goserelin gebruik in kombinasie met Pheroid<sup>®</sup>-tegnologie om die biobeskikbaarheid van goserelin te vergelyk met die toediening van die goserelin subkutane implantaat (Zoladex<sup>®</sup>) teen 'n orale dosis van pro-Pheroid<sup>®</sup>-goserelin-formulering in beide manlike en vroulike BALB/c muis. Die karakterisering, soos deeltjiegrootte, stabiliteit (in terme van Zeta potensiaal meting) en morfologie van Pheroid<sup>®</sup> en Pheroid<sup>®</sup>-goserelin is ondersoek asook 'n gesimuleerde stabiliteitsstudie van goserelin in kombinasie met Pheroid<sup>®</sup>, in vergelyking met goserelin alleenlik. Gesimuleerde maagvloeistof en gesimuleerde dermvloeistof is gebruik vir die stabiliteitsstudie.

Die eienskappe van Pheroid<sup>®</sup> het geen groot verandering na die byvoeging van goserelin in pro-Pheroid<sup>®</sup> geopenbaar nie. Die stabiliteitsstudie was nie-voldoende as gevolg van die verlies van monster na inkubasie in die pro-Pheroid<sup>®</sup>-groepe. 'n Geoptimaliseerde en hersiene stabiliteitsstudie sal in die nabye toekoms gedoen word. Die manlike BALB/c-muis is in 3 groepe verdeel. Groep 1

het goserelin subkutane ontvang, groep 2 het pro-Pheroid®-goserelin ontvang as 'n orale dosis van 2 mg/kg en groep 3 het pro-Pheroid®-goserelin as 'n orale dosis van 4 mg/kg. Bloedmonsters is op verskillende tydpunkte versamel en die plasma is geanaliseer vir testosteroon- en goserelin-konsentrasies met behulp van LC-MS/MS. Vanaf die goserelin konsentrasie-tydprofiel is die waargenome maksimum plasmakonsentrasie en tyd tot maksimum konsentrasie aangemeld en statisties vergelyk. 'N Verwagte aansienlik hoër maksimum konsentrasie en tyd tot maksimum konsentrasie was waargeneem in die subkutane groep. Die 2 mg/kg pro-Pheroid®-goserelin het 'n beduidende hoër konsentrasie punt in vergeleke met die 4 mg/kg dosis tot gevolg gehad. Die aanvanklike (0 – 30 minute) testosteroonkonsentrasie vir elke groep is waargeneem, en sodoende die “flare-effek” gehandhaaf. Beide die 2 mg/kg en 4 mg/kg pro-Pheroid®-goserelin groepe het 'n verandering in die testosteroon konsentrasie voorgebring. Die 4 mg/kg pro-Pheroid®-goserelin is gekies vir verdere ondersoek in vroulike BALB/c-muise. kan dus afgelei word dat goserelin daarin geslaag het om die bloed te bereik en gevolglik 'n afname in testosteroonkonsentrasie na die orale toediening met pro-Pheroid® veroorsaak het.

Die vroulike BALB/c-muise is ook in drie groepe verdeel; waarvan groep 1 goserelin subkutaan ontvang het, groep 2 het 'n orale dosis van 4 mg/kg pro-Pheroid®-goserelin ontvang het en laastens het groep 3, die negatiewe kontrole groep, slegs pro-Pheroid® oraal ontvang. Evaluering vir hierdie was gedoen deur daaglikse vaginale smere wat vir 21 dae geneem was, dit was sodoende deur vaginale sitologie geanaliseer. Dit is gebruik om die ontwrigting of gebrek daarvan tydens die 4-stadium-oestrous siklus op te spoor. 'n Ontwrigting in die oestrous siklus was waar geneem in beide groep 1 en 2. Dit kan dus afgelei word dat die orale toediening van goserelin in pro-Pheroid® voldoende opname tot gevolg gehad het om 'n effektiewe hormonale effek te veroorsaak.

Die pro-Pheroid®-goserelin-formulering in beide die manlike en vroulike studies het gelei tot 'n verhooging in die bio-beskikbaar van goserelin, soos aangedui deur die verwante farmakodinamiese (PD) resultate. Kortom, Pheroid®-tegnologie het gevolglik goserelin beskerm teen die agteruitgang in die gastrointestinale-kanaal en kan dus 'n nuttige afleweringstelsel wees vir toediening van terapeutiese middels

**Sleutelwoorde:** Goserelin, Pheroid® tegnologie, peptied terapie, farmakokinetika, farmakodinamika, orale geneesmiddelaflewering.

# Table of Contents

ACKNOWLEDGEMENTS .....	i
PREFACE.....	ii
ABSTRACT.....	iii
UITTREKSEL.....	v
LIST OF FIGURES .....	xi
LIST OF TABLES.....	xi
ABBREVIATIONS.....	xv
<b>CHAPTER 1: INTRODUCTION AND PROBLEM STATEMENT .....</b>	<b>1</b>
1. BACKGROUND.....	1
2. PROBLEM STATEMENT .....	1
3. AIM AND OBJECTIVES .....	1
REFERENCES .....	3
<b>CHAPTER 2: LITERATURE REVIEW.....</b>	<b>4</b>
2.1 ORAL DELIVERY OF PEPTIDE DRUGS .....	4
2.1.1 INTRODUCTION.....	4
2.1.2 PROTEIN AND PEPTIDE CHEMICAL CHARACTERISTICS.....	5
2.1.3 HISTORY AND DEVELOPMENT OF THERAPEUTIC PEPTIDES AND PROTEINS..	6
2.1.4 ORAL DELIVERY AND STABILITY OF PEPTIDE DRUGS IN THE GASTROINTESTINAL (GI) TRACT .....	7
2.1.5 STRATEGIES TO IMPROVE ORAL BIOAVAILABILITY OF PEPTIDE AND PROTEIN DRUGS	10
2.1.6 BIOEQUIVALENCE .....	14
2.2 GONADOTROPINS AND THEIR ANALOGUES.....	14
2.2.1 PHARMACOLOGY OF ENDOGENOUS GONADOTROPIN.....	14
2.2.2 GONADOTROPIN (GnRH) ANALOGUES.....	15
2.2.3 THE GnRH AGONIST GOSERELIN .....	17
2.2.4 PROSPECTS OF CHANGING THE ADMINISTRATION OF GOSERELIN .....	21

2.3	PHEROID® TECHNOLOGY .....	21
2.3.1	INTRODUCTION .....	21
2.3.2	THE HISTORY OF PHEROID® TECHNOLOGY .....	22
2.3.3	PHEROID® FUNCTION, CHARACTERISTICS AND TYPES .....	23
2.3.4	COMPONENTS OF PHEROID® .....	25
2.3.5	CHARACTERISTICS THAT MAKE PHEROID® TECHNOLOGY AN INTERESTING CARRIER .....	26
2.3.6	PHARMACEUTIC APPLICATIONS OF PHEROID® TECHNOLOGY .....	29
2.3.7	CHARACTERISATION OF PHEROID® .....	30
2.3.8	CONCLUSION.....	31
	REFERENCES .....	32
	<b>CHAPTER 3: CHARACTERISATION AND STABILITY OF GOSERELIN ENTRAPPED IN PHEROID®</b> .....	<b>42</b>
	ABSTRACT.....	42
1.	INTRODUCTION.....	42
2.	MATERIALS AND METHODS.....	43
2.1	Chemicals.....	43
2.2	Preparation of pro-Pheroid®-goserelin.....	44
2.3	Particle size, zeta potential and morphological measurement.....	44
2.4	Preparation of simulated gastric and intestinal fluid .....	45
2.5	Preparation of goserelin saline stock and pro-Pheroid®-goserelin stock solution.....	45
2.6	Stability test .....	46
2.7	Measurement of drug content .....	46
2.8	Extraction and LC-MS/MS analysis of the supernatant .....	47
3.	RESULTS AND DISCUSSION .....	48
3.1	Characterisation of Pheroid® and Pheroid®-goserelin .....	48
3.2	Stability test .....	49
4.	CONCLUSION .....	50

REFERENCES .....	51
<b>CHAPTER 4: COMPARISON OF THE PHARMACOKINETICS AND PHARMACODYNAMICS OF AN ORAL FORMULATION OF GOSERELIN IN PHEROID® RELATIVE TO A SUBCUTANEOUS IMPLANT OF GOSERELIN, IN MALE MICE .....</b>	<b>54</b>
ABSTRACT.....	54
1. INTRODUCTION.....	54
2. MATERIALS AND METHODS.....	55
2.1 Chemicals.....	55
2.3 Animal experiments .....	56
2.4 Animal groups.....	56
2.5 Measuring of goserelin and testosterone concentration .....	57
2.6 Data analysis .....	58
3. RESULTS AND DISCUSSION .....	58
3.1 Characterisation of Pheroid® and Pheroid®-goserelin formulations.....	58
3.2. Data analysis .....	59
4. CONCLUSION .....	62
REFERENCES .....	63
<b>CHAPTER 5: THE EFFECT OF A GOSERELIN ORAL FORMULATION WITH PHEROID® TECHNOLOGY COMPARED TO A GOSERELIN SUBCUTANEOUS IMPLANT ON THE OESTRUS CYCLE OF FEMALE MICE .....</b>	<b>66</b>
ABSTRACT.....	66
1. INTRODUCTION.....	66
2. MATERIALS AND METHODS.....	67
2.1 Chemicals.....	67
2.2 Preparation of pro-Pheroid®-goserelin.....	68
2.3. Animal experiments .....	68
2.4 Oestrous cycle analysis .....	69
3. RESULTS AND DISCUSSION .....	69
3.1 Characterisation of Pheroid® and Pheroid® goserelin.....	69

3.2	Pharmacodynamic analysis .....	70
	CONCLUSION.....	72
	REFERENCES.....	74
	<b>CHAPTER 6: RESEARCH OUTCOMES, STUDY LIMITATIONS AND FUTURE RECOMMENDATION</b> .....	<b>77</b>
	RESEARCH OUTCOMES .....	77
	STUDY LIMITATIONS .....	77
	FUTURE RECOMMENDATIONS .....	77
	REFERENCES .....	78
	ANNEXURES.....	77
	ANNEXURE A – Experimental Design Diagram.....	79
	ANNEXURE B – Characterisation Results.....	81
	ANNEXURE C – Statistical Analysis .....	86
	ANNEXURE D – Oestrous cycles of female mice .....	89
	ANNEXURE E – Certificate of analysis of Vitamin F Ethyl Ester.....	92
	ANNEXURE F - Poster for Drug Safety Africa 2018 Conference (20 – 22 November 2018).....	94
	ANNEXURE G – Certificate of Proof Reading.....	96

# List of Figures

<b>CHAPTER 2: LITERATURE REVIEW</b> .....	4
<b>Figure 1:</b> Basic structure of an amino acid (adapted from: Langel <i>et al.</i> , 2009).....	6
<b>Figure 2:</b> The intestinal barriers of oral peptide delivery, which includes the paracellular and transcellular routes (Adapted from Fasano, 1998).....	9
<b>Figure 3:</b> Pathway of endogenous GnRH (adapted from: Yeo & de Croft, 2016). ....	15
<b>Figure 4:</b> Historical timeline of the development of GnRH analogues (adapted from: Hayden, 2008).....	16
<b>Figure 5:</b> Molecular structure of goserelin acetate. ....	18
<b>Figure 6:</b> Examples of confocal laser scans of the Pheroid® types: (A) Lipid Bilayer vesicle with entrapped API (Rifampicin); (B) Reservoir containing multiple particles of coal tar - the reservoirs are good entrappers of insoluble compounds; (C) The formation of small pro-Pheroid®s used for some oral formulations (Source: Grobler, 2004; with permission). ....	24
<b>Figure 7:</b> Schematic representation of the differences of basic Pheroid® and pro-Pheroid®: (A) Pro-Pheroid®, (B) Pheroid® (adapted from Grobler, 2009). Green: N <sub>2</sub> O, Blue: α-tocopherol, Dark Purple: long chain fatty acids and Light Purple: aqueous phase. A typical Pheroid® formulation consists of 96% aqueous and 4% oil. ....	25
<b>Figure 8:</b> Key advantages of Pheroid® technology as a drug delivery system (DDS) (adapted from: Grobler, 2009; van Huyssteen, 2010). ....	27
<b>Figure 9:</b> Confocal images: (A) Pheroid® sample, (B) Pheroid® goserelin sample. Frames: 1) Image obtained with a tungsten source light, 2) Red fluorescence signal detected in wavelengths between 500 and 530 merged with that detected between 568 and 642 nm, 3) Red fluorescence signal detected in the 568 and 642 nm waveband only and 4) Green fluorescence signal detected of the 500 to 530 nm waveband only (scale = 20 μm). ....	49
<b>Figure 10:</b> A. Goserelin AUC for the preliminary indication of goserelin degradation over a period of 120 minutes. B. Chromatogram of goserelin from SGF. Red peak: goserelin, Blue peak: alarenin (internal standard).....	49
<b>CHAPTER 3: CHARACTERISATION AND STABILITY OF GOSERELIN ENTRAPPED IN PHEROID®</b> .....	42
<b>Figure 1:</b> Confocal images: (A) Pheroid® sample, (B) Pheroid® goserelin sample. Frames: 1) Image obtained with a tungsten source light, 2) Red fluorescence signal detected in wavelengths between 500 and 530 merged with that detected between 568 and 642 nm, 3) Red fluorescence	

signal detected in the 568 and 642 nm waveband only and 4) Green fluorescence signal detected of the 500 to 530 nm waveband only (scale = 20  $\mu$ m).....49

**Figure 2:** A. Goserelin AUC for the preliminary indication of goserelin degradation over a period of 120 minutes. B. Chromatogram of goserelin from SGF. Read peak: goserelin, Blue peak: alarenin (internal standard).....49

**CHAPTER 4: COMPARISON OF THE PHARMACOKINETICS AND PHARMACODYNAMICS OF AN ORAL FORMULATION OF GOSERELIN IN PHEROID® AND A SUBCUTANEOUS IMPLANT OF GOSERELIN IN MALE MICE** .....54

**Figure 1:** Demonstration of A) oral gavage of pro-Pheroid® and pro-Pheroid®-goserelin, B) goserelin subcutaneous implant (Zoladex®).....58

**Figure 2:** Confocal images of different formulations visualised by labelling with Nile red: (A) Pheroid® sample (B) 2 mg/kg Pheroid® goserelin sample (C) 4 mg/kg Pheroid® goserelin sample. 1) Image obtained with a tungsten source light, 2) Red fluorescence signal detected in wavelengths between 500 and 530 merged with that detected between 568 and 642 nm, 3) Red fluorescence signal detected in the 568 and 642 nm waveband only and 4) Green fluorescence signal detected in the 500 to 530 nm waveband only (scale bar =20  $\mu$ m).....59

**Figure 3:** Representation of goserelin and related testosterone plasma-concentration curves following the administration of goserelin subcutaneous implant (Zoladex®) (n = 6), pro-Pheroid®-goserelin (n = 6) and 4 mg/kg pro-Pheroid®-goserelin in male BALB/c mice (n = 6).....61

**CHAPTER 5: THE EFFECT OF A GOSERELIN ORAL FORMULATION WITH PHEROID® TECHNOLOGY COMPARED TO A GOSERELIN SUBCUTANEOUS IMPLANT ON THE OESTRUS CYCLE OF FEMALE MICE** .....66

**Figure 1:** Morphological visualisation of Pheroid® vesicles, through confocal laser scanning of A) Pheroid® formulation and B) Pheroid®-goserelin formulation. The scale represents 20  $\mu$ m. The red dots represent the Nile red-stained Pheroid® vesicles (scale = 20  $\mu$ m).....70

**Figure 2:** Vaginal cytology representing each stage of oestrous of female BALB/c mice. Three cell types are identified: the nucleated cells are indicated by the blue arrow; the red arrow represents the cornified cells and the purple circle indicates the leukocytes. Stages: A) Pro-oestrous; predominantly consists of nucleated and cornified epithelial cells, B) Oestrous; predominantly cornified epithelial cells C) Metroestrous; consists of all three cell types (cornified epithelial, nucleated epithelial and leukocytes) and D) Dioestrous; predominantly leukocytes but nucleated epithelial also present.....71

**Figure 3:** Representation of oestrous cycle after disruption by goserelin (pro-oestrous).....72

**Figure 4:** Representative disruptive effects on the oestrous cycle in one mouse from each group after A) goserelin subcutaneous implant B) pro-Pheroid® goserelin oral administration and C) pro-Pheroid® oral administration. The arrow indicates the treatment start day. The oestrous stage is indicated by 1. Pro-oestrous, 2. Oestrous, 3. Metoestrous and 4. Dioestrous. (Rest of oestrous cycles of all groups are provided in Annexure D).....72

# LIST OF TABLES

## CHAPTER 2: LITERATURE REVIEW

**Table 1:** Advantages and disadvantages of oral delivery (Hillery *et al.*, 2002; Wang *et al.*, 2016).....5

**Table 2:** GnRH-a and GnRH-ant respective commercial name and route of administration (Anon, 2018; Padula, 2005; Dun & Taylor, 2017; Lambalk *et al.*, 2005; Tur-Kaspa & Ezcurra, 2009; Van Poppel *et al.*, 2008; Weisman & Goldberg, 2001; WHO, 2016). ..... 17

**Table 3:** Pharmacokinetics (ADME) of goserelin (Zoladex<sup>®</sup>) (Butler & Govindan, 2010; Chabner & Longo, 2011). ..... 20

**Table 4:** Average particle size diameter, zeta potential measurement and CLSM average particle size results of Pheroid<sup>®</sup> and Pheroid<sup>®</sup>-goserelin. .... 48

## CHAPTER 3: CHARACTERISATION AND STABILITY OF GOSERELIN ENTRAPPED IN PHEROID<sup>®</sup>

**Table 1:** Average particle size diameter, zeta potential measurement and CLSM average particle size results of Pheroid<sup>®</sup> and Pheroid<sup>®</sup>-goserelin. .... 48

## CHAPTER 4: COMPARISON OF THE PHARMACOKINETICS AND PHARMACODYNAMICS OF AN ORAL FORMULATION OF GOSERELIN IN PHEROID<sup>®</sup> AND A SUBCUTANEOUS IMPLANT OF GOSERELIN IN MALE MICE

**Table 1:** MS parametres.....57

**Table 2:** Particle size, Zeta potential and confocal imaging average size results of Pheroid<sup>®</sup> and Pheroid<sup>®</sup>-goserelin. .... 59

## CHAPTER 5: THE EFFECT OF A GOSERELIN ORAL FORMULATION WITH PHEROID<sup>®</sup> TECHNOLOGY COMPARED TO A GOSERELIN SUBCUTANEOUS IMPLANT ON THE OESTRUS CYCLE OF FEMALE MICE

**Table 1:** Mean particle size diameter, zeta potential measurement and confocal laser scanning imaging average size results of Pheroid<sup>®</sup> and Pheroid<sup>®</sup>-goserelin. .... 70

## ABBREVIATIONS

®	registered trademark
%	percentage
°C	degrees Celsius
x g	gravitational constant
µL	microlitres
µm	micrometres
API	active pharmaceutical ingredient
AUC	area under the curve
C <sub>max</sub>	maximum plasma concentration
EDTA	ethylenediaminetetraacetic acid
<i>et al.</i>	<i>et alii</i>
g	gram
GnRH	gonadotropin releasing hormone
GnRH-a	gonadotropin releasing hormone agonist
GnRH-ant	gonadotropin releasing hormone antagonist
GI	gastrointestinal
h	hours
HEPA	high-efficiency particle arrestance
HCl	hydrochloric acid
IS	internal standard
IVC	individually ventilated cages
IVF	<i>In vitro</i> fertilisation
kg	kilogram
KH <sub>2</sub> PO <sub>4</sub>	monobasic potassium phosphate
L	litres
LC-MS/MS	liquid chromatography– tandem mass spectrometry
mg	milligram
min	minute
mL	millilitre
mM	millimolar
mV	millivolt
m/z	mass to charge ratio

N <sub>2</sub> O	nitrous oxide
NaCl	sodium chloride
NaOH	sodium hydroxide
nm	nanometre
NWU	North-West University
Prof.	Professor
PK	pharmacokinetics
PD	pharmacodynamics
rpm	revolutions per minute
SGF	simulated gastric fluid
SIF	simulated intestinal fluid
T <sub>max</sub>	time to maximum concentration
T <sub>1/2</sub>	half-life
WHO	World Health Organisation

# CHAPTER 1

# **CHAPTER 1: INTRODUCTION AND PROBLEM STATEMENT**

## **1. BACKGROUND**

The peptide and protein therapeutic market has developed significantly in the past few decades, but their use has been limited due to the difficulties associated with the delivery routes alternative to parenteral routes (intravenous, subcutaneous and intramuscular), such as the oral route. (Bruno *et al.*, 2013). The most preferred administration route for therapeutic drugs is the oral route, as this is the easiest and most convenient way for drug delivery, especially in the case of chronic administration. However, due to the limited absorption and degradation (by the presence of enzymes and pH-dependent factors) of peptides and proteins in the gastrointestinal (GI) tract, the bioavailability of the oral peptides and proteins are too low (Bruno *et al.*, 2013; Renukuntla *et al.*, 2013; Muheem *et al.*, 2016). The limitation of the oral delivery in the oral delivery of peptides and proteins can be overcome by chemical transformation, absorption enhancers, enzyme inhibitors, carrier systems, stability enhancers, etc. This will facilitate and protect the therapeutic peptide and proteins for oral delivery (Bruno *et al.*, 2013; Muheem *et al.*, 2016). Although to develop available oral drug delivery system for proteins and peptides careful consideration is required (Renukuntla *et al.*, 2013).

This investigation focused on the evaluation of the novel drug delivery system, Pheroid® technology, for the oral delivery of the amphiphilic peptide goserelin, a gonadotropin-releasing (GnRH) antagonist. Pheroid® technology is a colloidal delivery system, which has the ability to capture, protect, transport and deliver pharmaceutical active compounds and may play a key role in ensuring effective delivery and enhanced bioavailability of peptide drugs (Strauss, 2005).

## **2. PROBLEM STATEMENT**

Goserelin is currently only available as a subcutaneous implant hence the need for a less invasive method exists that is bioequivalent to the subcutaneous implant. It is clear from the literature that Pheroid® technology can be advantageous in improving oral bioavailability and can influence the pharmacokinetics of the active pharmaceutical ingredient (API), in this case, goserelin. Therefore, it could be used to overcome the barriers of oral delivery of goserelin and could consequently lead to an oral delivery of goserelin less invasive than the current subcutaneous depot.

## **3. AIM AND OBJECTIVES**

The aim of the study was to evaluate the oral delivery of goserelin with Pheroid® technology and what effect this would have on the pharmacokinetics and pharmacodynamics of goserelin in mice by:

1. Preparing a pro-Pheroid<sup>®</sup> formulation of goserelin (pro-Pheroid<sup>®</sup>-goserelin) and characterising it by measuring the size, stability and morphology.
2. Determining the effect of entrapment of goserelin in Pheroid<sup>®</sup> on its gastrointestinal stability by comparing the stability of goserelin and pro-Pheroid<sup>®</sup>-goserelin in simulated gastric and simulated intestinal fluids.
3. Performing a study in male mice to determine the dose of pro-Pheroid<sup>®</sup>-goserelin formulation that could maintain sufficient levels of goserelin to ensure castrate levels of testosterone in the mice.
4. Performing a pharmacodynamic study comparing the effect of the oral pro-Pheroid<sup>®</sup>-goserelin formulation and that of a subcutaneous implant of goserelin, on the oestrous cycle of female mice.

## REFERENCES

- Bruno, B.J., Miller, G.D. & Lim, C.S. 2013. Basics and Recent Advances in Peptide and Protein Drug Delivery. *Therapeutic Delivery*, 4(11), pp.1443-1467
- Fosgerau, K. & Hoffmann, T. 2015. Peptide Therapeutics: Current Status and Future Directions. *Drug Discovery Today*, 20(1):122-128.
- Muheem, A., Shakeel, F., Jahangir, M.A., Anwar, M., Mallick, N., Jain, G.K., Warsi, M.H. & Ahmad, F.J. 2016. A Review of the Strategies for Oral Delivery of Proteins and Peptides and Their Clinical Perspectives. *Saudi Pharmaceutical Journal*, 24(4):413-428.
- Renukuntla, J., Vadlapudi, A.D., Patel, A., Boddu, S.H. & Mitra, A.K. 2013. Approaches for Enhancing Oral Bioavailability of Peptides and Proteins. *International Journal of Pharmaceutics*, 447(1):75-93.
- Strauss, T. 2005. Oral Delivery of Calcitonin with Pheroid® Technology. Potchefstroom: NWU (Dissertation – M.Sc.)

# CHAPTER 2

## CHAPTER 2: LITERATURE REVIEW

### 2.1 ORAL DELIVERY OF PEPTIDE DRUGS

#### 2.1.1 INTRODUCTION

The appreciation of the role that peptides play in therapeutics by the pharmaceutical industry has gained a renewal of interest, this has led to a scientific momentum experienced in peptide drug discovery (Henninot *et al.*, 2017). Peptide compounds can be an excellent endorsement and are even preferred to other small molecules and biological therapeutics (Henninot *et al.*, 2017). The therapeutic market for peptide drugs has been increasing since 2000 (Pytlik, 2014). The predominant peptide therapeutic drugs on the market are hormones or derivatives of peptides, which stimulate hormone action (Pytlik, 2014). A primary reason for the growing interest in the peptide therapeutic market may be due to the exquisite binding specificity to their *in vivo* targets, which leads to high potencies of action and proportionately low off-target side effects (Craik *et al.*, 2013). Peptides can be manipulated and designed to reach or target a wide range of molecules, making the peptide therapeutic market almost limitless in fields such as, infectious disease, endocrinology and immunology (Bruno *et al.*, 2013). However, the use of peptides and proteins for therapeutics also raises some disadvantages which include: short *in vivo* half-life and low oral bioavailability due to degradation by enzymes present in the GI tract. These disadvantages could either occur at the site of administration or on the way to the target site. This makes the oral route an unsuitable alternative to parenteral routes (intravenous, subcutaneous and intramuscular) (Almeida & Souto, 2007; Bruno *et al.*, 2013; Strauss, 2005). The route of administration of drugs has a significant impact on the therapeutic outcome (Jitendra *et al.*, 2011). Different routes of administration for peptide drugs have previously been explored (Strauss, 2005). Such routes include nasal, buccal, rectal, vaginal, percutaneous and ocular (Lee, 1990).

The rectal route offers several advantages for peptide and protein delivery, such as avoidance of the first pass effect (Baviskar *et al.*, 2013; Strauss, 2005). The rectum offers a relatively constant environment for drug delivery, although the drug should be in a well absorbable form (Baviskar *et al.*, 2013), however, patient compliance could be a problem due to the reluctance to use rectal formulations (Strauss, 2005). The nasal route is a needle-free, non-invasive, painless route and can be self-administered, but similar to mucosal routes, the physical barrier of the nasal epithelium presents this route with its main limitation, especially for larger molecules such as proteins and peptides (Van Der Walle, 2011). A direct entry into the systemic circulation is provided by the buccal route, however, the amount of drugs that can be administered through buccal routes is limited, as the membrane permeation is a limiting factor, resulting in a much less efficient route (Şenel & Hıncal, 2001; Jensen & Peppers, 2013).

The peptide and protein drug market for vaginal application has gained some attention in the last few years (Strauss, 2005). Advantages of the vaginal route include; a large permeation area, rich vascularisation, avoidance of first-pass metabolism and low enzymatic activity (Choudhury *et al.*, 2011). Due to these advantages, peptide and protein drugs are less degraded in the vaginal tract compared to the the GI tract (Choudhury *et al.*, 2011). However, this route offers limitations as well, such as it being specific and limited to the female gender, the route is less desirable in terms of convenience and the oestrogen concentration influences the permeability of the vagina (Vermani & Garg, 2000).

The oral route is therefore worth exploring as an alternative route for peptide and protein drug delivery, as it offers advantages, however, there are also a few challenges associated with the delivery of peptides for this route (Table 2.1). For many years the bioavailability enhancement of orally administered therapeutic peptides and protein drugs has been an on-going and popular topic in pharmaceutical research (Hamman *et al.*, 2005). Various strategies to improve the low bioavailability of therapeutic peptides and proteins have been investigated and success has been reached, however, this has only been accomplished with particular peptide drugs. Overall, no single strategy has been established thus far (Hamman *et al.*, 2005; Strauss 2005).

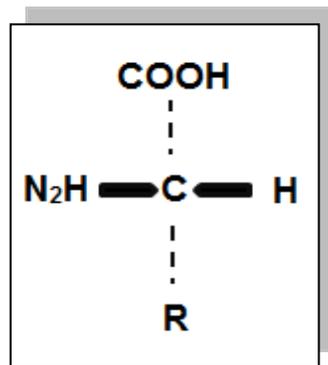
**Table 1:** Advantages and disadvantages of oral delivery (Hillery *et al.*, 2002; Wang *et al.*, 2016).

Advantages	Disadvantages
Less invasive method of administration	Low pH of gastric juices
Higher patient compliance	First pass-effect of the liver
More cost-effective and easy to manufacture	The mucus barrier of the intestinal tract
Accessibility (the drug can be self-administered)	Metabolic activity in the intestinal lumen (the activity of proteases and peptidases)

## 2.1.2 PROTEIN AND PEPTIDE CHEMICAL CHARACTERISTICS

Proteins are an abundant and diverse class of biomolecules, which play a big role in growth and development of the structural regulation and integrity of the body and are also expressions of genetic information (Hettiarachchy *et al.*, 2012; Garrett & Grisham, 2013). Proteins consist of amino acids, a group of organic molecules (chiral molecule) consisting of a basic amino group (NH<sub>2</sub>), a carboxyl group (COOH), a hydrogen molecule and a side chain (also referred to as R); central to this structure is a tetrahedral alpha (α) carbon (C<sub>α</sub>) (Figure 2.1) (Garrett & Grisham, 2013; Reddy, 2018). There are 20 standard amino acids (Langel *et al.*, 2009). Amino acids are structural elements of

polypeptides, but they also play an important role as precursors of proteins and peptides (Langel *et al.*, 2009).



**Figure 1:** Basic structure of an amino acid (adapted from: Langel *et al.*, 2009).

According to Garret and Grisham, (2013) the amino and carboxyl groups of the amino acids can bind and react in a head to tail fashion (through the condensation of the water molecule), this then forms a covalent amide linkage, called a peptide bond, to form a chain of polypeptides and in turn, proteins. Peptide is the name accredited to short polymers of amino acids and is classified according to the number of amino acid units in the chain (when amino acid amount is lower than 50).

### **2.1.3 HISTORY AND DEVELOPMENT OF THERAPEUTIC PEPTIDES AND PROTEINS**

The utilisation of peptides and proteins as therapeutics has evolved greatly over time and still continues to evolve (Lau & Dunn, 2018). Proteins and peptides offer a promising brand of therapeutics as they are the building blocks of life (Muheem *et al.*, 2016). Over 7000 natural peptides which play critical roles in the human physiology have been established, some of these roles include; neurotransmitters, hormones, ion channel ligands, growth factors and anti-infectives (Fosgerau & Hoffmann, 2015). Peptides as therapeutics offer excellent safety, tolerability and efficacy (Fosgerau & Hoffmann, 2015). Metabolic diseases and oncology are the current main disease drivers for the therapeutic market (Fosgerau & Hoffmann, 2015). In 1923, the first recombinant protein therapeutic, viz., insulin, was introduced. Subsequent to its first isolation in 1921, the therapeutic market has increased dramatically (Muheem *et al.*, 2016; Henninot *et al.*, 2017). Insulin was considered a miracle drug and a key aspect of the societal value of scientific research (Henninot *et al.*, 2017). The approval of recombinant insulin (laid down in 1982 by the US Food and Drug Administration (FDA)) was another pivotal moment for this field. The clinical success of insulin has encouraged the field of therapeutic proteins into far-reaching horizons (Muheem *et al.*, 2016). In the first half of the 20<sup>th</sup>-century scientists aimed to understand the basic structure and the physiology of peptide hormones such as insulin, oxytocin, gonadotropin-releasing hormones and vasopressin (Henninot *et al.*, 2017).

Consequently, this catalysed many major advances in biology, pharmacology and chemistry (Henninot *et al.*, 2017).

After venom of cephalopods and arthropods became recognised as a treatment regimen, the isolation of exotic natural products became an attractive strategy for the identifying of original potential therapeutics (Lau & Dunn, 2018). However, certain limitations of native peptides such as negligible oral bioavailability, short half-life and poor physical and chemical stability consequently hampered the enthusiasm for peptide therapeutics (Fosgerau & Hoffmann, 2015; Lau & Dunn, 2018). This led to investigators utilising medicinal chemistry techniques to make peptides and proteins more drug-like by aiming to improve and optimise these characteristics (Lau & Dunn, 2018). In the latter part of the 20<sup>th</sup>-century “biologics”, a new class of therapeutics, became known (Craik *et al.*, 2013). At the turn of the 21<sup>st</sup> century, genome-sequencing efforts became possible and were originally thought to have the potential to lead to an increase in drug development (Craik *et al.*, 2013). The future drug development of ‘omics’ may also be a contributor to personalised medicines based on an individual’s genetic make-up (Craik *et al.*, 2013).

According to Lau and Dunn, (2018) currently more than 60 peptide drugs have been approved in Europe, Japan and the United States to date, and more than double that amount has already been tested in human clinical trials with 150 in active clinical development (Lau & Dunn, 2018). Since 1990, the number of peptides entering clinical trials has been fluctuating, with the lowest amount being 0 peptides and the highest 20, throughout the following years up until 2015. During this period, the cumulative peptide approval has been steadily increasing (Lau & Dunn, 2018). According to Henninot *et al.*, (2017), 28 new, non-insulin peptide drugs have been approved worldwide since 2000, and several of these achieved significant market success. Henninot *et al.*, (2017) also stated that by 2019 the peptide sales are anticipated to exceed \$70 billion purely based on a projected annual growth rate of 9 to 10%. The status of approved and peptide drugs in active development as of 2017 includes 55, 94 and 29 in phase 1,2 and 3 clinical trials respectively and a total of 56 approved peptide drugs (Henninot *et al.*, 2017) Research continues to expand and increase the range of potential peptide-based drugs (Lau & Dunn, 2018), and screening of peptides and computational biology improvement will continue the support of peptide drugs (Lau & Dunn, 2018), Nevertheless, drug development is a lengthy and intricate process (Henninot *et al.*, 2017)

#### **2.1.4 ORAL DELIVERY AND STABILITY OF PEPTIDE DRUGS IN THE GASTROINTESTINAL (GI) TRACT**

One of the major barriers to the successful oral delivery of peptide and protein molecules is their fundamental instability in the GI tract (Wang *et al.*, 2015). There are also barriers in the GI tract that limit absorption, which will be discussed in section 2.1.4.1. The current broad majority of marketed peptide drugs are parenterally administered due to the associated stability issues (Wang *et al.*,

2015). These routes are often unacceptable due to the pain and discomfort associated with the administration, such as injections and implants, which leads to low patient compliance. As mentioned in section 2.1.1, oral administration of drugs can offer some advantages in terms of lower manufacturing costs, increase of patient compliance and ease of administration (Wang *et al.*, 2015). However, several challenges are faced after oral administration, for instance, enzymatic and acidic degradation of peptides in the GI tract (Fosgerau & Hoffmann, 2015). Wang *et al.*, (2015) indicated that goserelin rapidly degraded within 2 to 10 minutes in human gastric fluid (HGF), pig gastric fluid (PGF) and simulated gastric fluid (SGF). Proteins also present with several unfavourable physicochemical properties contributing to their poor oral bioavailability (less than 10%), for instance, their size (sizes above 500 Da commonly experience difficulty with membrane permeability by means of passive diffusion) (Hamman, 2012). Therefore, it is important to improve on the physicochemical properties of peptides for peptide drug design for successful oral delivery.

#### **2.1.4.1 BARRIERS LIMITING PEPTIDE AND PROTEIN ORAL BIOAVAILABILITY**

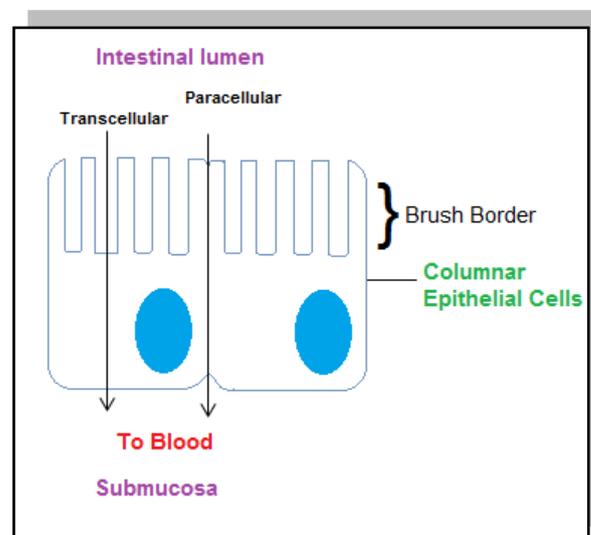
The intestinal absorption of drugs is seriously hampered by the biochemical and physical barriers of the GI tract, this contributes to the low oral bioavailability of peptide and protein drugs (Hamman *et al.*, 2005) The impermeable GI epithelium is one of the main representatives of the physical barrier and includes cell membranes and tight junctions between the adjacent epithelial cells. The absorption and regulation of substances such as drugs and molecules are also greatly influenced by efflux systems and the mucus layer. The biochemical barrier, on the other hand, comprises enzymatic degradation by peptidases (Hamman *et al.*, 2005; Renukuntla *et al.*, 2013). The understanding of these barriers is necessary to improve the oral delivery of drugs (Renukuntla *et al.*, 2013).

##### **2.1.4.1.1 THE PHYSICAL BARRIER**

Before absorption, peptides and proteins have to pass through the mucus layer, apical and basal cell membranes plus cell contents, the basement membrane, tight junctions and the lymph and blood capillary walls (Sarciaux *et al.*, 1995; Strauss, 2005). The small intestine epithelial barrier poses a greater challenge for oral delivery of protein therapeutics and forms the physical barrier (Bruno *et al.*, 2013). One of the major functions of the intestinal epithelium is to act as a selective permeable barrier which limits the interactions between the luminal contents and supporting vectorial transport of nutrients, water and waste products (Groschwitz & Hogan, 2009; Odenwald & Turner, 2017). The intestinal epithelium consists of a single layer of columnar epithelial cells (Carino & Mathiowitz, 1999). Molecules that cross the epithelium can cross by either the transcellular or the paracellular routes (Bruno *et al.*, 2013) (Figure 2.2). When peptides cross the transcellular pathway, they have to pass through the epithelial cell layer by either passive diffusion, vesicular transport or carrier-mediated transport (Zhu *et al.*, 2017). Lipophilic molecules are mostly transported by means of

passive diffusion, on the other hand, hydrophobic molecules and highly charged molecules require a specific transport mechanism, thus molecules need specific physicochemical properties in terms of their charge, size, lipophilicity, hydrogen bonding potential and solution conformation to cross these barriers (Hamman *et al.*, 2005; Zhu *et al.*, 2017).

When peptides and proteins are absorbed in the GI tract, efflux transporters such as P-glycoprotein (P-gp) could expel peptide drugs back into the intestinal lumen (Cayen, 2011; Carino & Mathiowitz, 1999). One of the important physiological functions of P-gp is the export of hydrophobic peptides and transport of peptides in both prokaryotes and eukaryotes (Sharom, *et al.*, 1995). Poor oral bioavailability may also be significantly contributed to by the combination with the intracellular metabolism, and thus this combination act as a barrier that limits the intestinal transport of peptide drugs (Hunter & Hirst, 1997; Pauletti *et al.*, 1996; Witt *et al.*, 2001).



**Figure 2:** The intestinal barriers of oral peptide delivery, which includes the paracellular and transcellular routes (Adapted from Fasano, 1998).

#### 2.1.4.1.2 THE BIOCHEMICAL BARRIER

The presence of drug metabolising enzymes, mucosa and drug transporters together form the biochemical barrier (Mitra *et al.*, 2015). Dietary proteins are broken down in the stomach and intestine by enzymes (peptidase) into a smaller mixture of subunits (peptides) that are sufficiently small for absorption; however, in order for therapeutic proteins to reach and enter the target area to exert their pharmacological action, they should be transported intact (Hamman *et al.*, 2005). One of the formidable obstacles for oral delivery of peptides and proteins is the enzymatic barrier. Hydrolysis of peptides occurs luminally, at the brush border, and intracellularly (Sarciaux *et al.*, 1995; Strauss, 2005). The digestive process is catalysed by enzymes through hydrolytic cleavage of the

peptide bonds such as phosphorylation and oxidation (Sarciaux *et al.*, 1995; Strauss, 2005). The enzymes responsible for the terminal stage of digestion of proteins and peptides are not found freely in the intestinal lumen but are bound to the plasma membrane of the enterocyte (Hooton *et al.*, 2015). These enzymes consist of numerous microvilli, which extend from the cell and constitute the brush border; hence they are referred to as brush border enzymes (Hooton *et al.*, 2015). The majority of the peptides are degraded in the brush border membrane by enzymes such as peptidase (Carino & Mathiowitz, 1999). However, much work has gone into improving these aspects in order to overcome these barriers and such strategies will be discussed in section 2.1.5.

## **2.1.5 STRATEGIES TO IMPROVE ORAL BIOAVAILABILITY OF PEPTIDE AND PROTEIN DRUGS**

As mentioned, peptide and protein drugs have low oral bioavailability due to the harsh environment of the GI tract. However, there are numerous strategies that can and have been implemented to prolong the plasma half-life of long-acting peptide and protein drugs. Chemical modification of a peptide or protein drug may lead to improved pharmacokinetic (PK) profiles of established peptide and protein drugs (Werle & Bernkop-Schnürch, 2006). A study was carried out by Novikov *et al.*, (2010) where the pharmacokinetic (PK) profile and immunogenic profile of organophosphorus hydrolase (OPH) was improved by chemical modification. This study examined the effects of polyethylene glycol (PEG) on the biochemical and pharmacological characteristics on OPH in male Dunkin Hartley guinea pigs and found that the PEGylated OPH revealed a significant improvement of pharmacokinetic properties (Novikov *et al.*, 2010). Hence, to develop a successful and viable peptide or protein drug, careful consideration of the physicochemical properties is required (Renkuntla *et al.*, 2013). Absorption enhancers, enzyme inhibitors, drug delivery system (DDS) and stability enhancers, and other strategies can be used to facilitate oral peptide delivery to reach or enter target cells (Almeida & Souto, 2007; Bruno *et al.*, 2013).

### **2.1.5.1 FORMULATION TECHNOLOGIES**

#### **2.1.5.1.1 ABSORPTION ENHANCERS**

According to Hamman *et al.*, (2005) absorption enhancers are compounds that only reversibly remove or temporarily disrupts the intestinal barrier with minimal tissue damage, this then allows the drug to cross the epithelial cells and subsequently reach the blood circulation. The selection and efficacy of absorption enhancers depend on the peptide's and protein's physicochemical properties, regional differences in the intestinal membrane, nature of the vehicle and other excipients (Renkuntla *et al.*, 2013). The various absorption enhancers that have been investigated for enhanced absorption of peptide and protein drugs can be grouped as surfactants, chelating agents, cationic and anionic polymers, acylcarnitines, fatty acids and their derivatives (Renkuntla *et al.*, 2013).

### 2.1.5.1.2 ENZYME INHIBITORS

Enzyme inhibitors bring about a decline in enzyme-related processes, enzyme production or enzyme activity. Widely employed inhibitors include amastatin, aprotinin (trypsin/chymotrypsin inhibitor), boroleucine, bestatin and puromycin (aminopeptidase inhibitors) (Renukuntla *et al.*, 2013). A study by Yamamoto *et al.*, (1994) indicated the effect of protease enzyme inhibitors, such as sodium glycocholate, camostat mesilate, bacitracin, soybean trypsin inhibitor, and aprotinin, on the intestinal metabolism of insulin in rats. It was reported that sodium glycocholate, camostat mesilat, and bacitracin are more efficient in improving the physiological availability of insulin in the large intestine (Yamamoto *et al.*, 1994). However, the use of these enzyme inhibitors still remains a questionable method as they can cause possible adverse effects, stimulation of protease secretion and disturbances of digestion of the nutritive proteins, all due to the result of feedback regulation (Hamman *et al.*, 2005).

### 2.1.5.1.3 PARTICULATE CARRIER SYSTEMS

Particulate drug delivery systems (DDS) consist mainly of lipids/ and or polymers (Jana & Jana, 2017). Many therapeutic and diagnostic applications may be offered by particulate DDS of various shapes and sizes (liposomes, oil-in-water emulsions, polymeric nano-and microspheres and metallic nanoparticles (NPs)) (Moghimi & Farhangrazi, 2014). The size range of particulate DDS generally covers less than 10 nm to a few millimetres (Jana & Jana, 2017). When the drug is attached to/entrapped in the carrier, the result may be a decrease of drug clearance; this consequently leads to the lowering of the volume of distribution and an increase in the area under the curve (AUC). This means that the PK parameters of the entrapped drug will be controlled by the physiochemical properties of the carrier, which regulates the drug release and biological factors that modulate carrier performance (Moghimi & Farhangrazi, 2014).

Several different types of particulate drug carriers include (Srikanth *et al.*, 2012):

- Microspheres/particles: These characteristically free flowing powders consist of proteins or synthetic polymers which are naturally biodegradable and ideally have a particle size lower than 200  $\mu\text{m}$ . They are developed for an array of applications such as, but not limited to, controlled drug delivery, vaccine delivery etc (Chouhan *et al.*, 2017)

A study by Zhang *et al.*, (2018) illustrated the use of microspheres for the oral delivery of insulin; these insulin-loaded microspheres were evaluated in rats with diabetes induced by streptozotocin. This study observed an obvious dose-dependent hypoglycaemic effect (Zhang *et al.*, 2018). One limitation of using microspheres for a DDS is that they tend to migrate away from the injection site, this might lead to the potential risk of an embolism and further organ damage (Trivedi *et al.*, 2008; Rajput *et al.*, 2012).

- Nanospheres/particles: These are particulate dispersions with a typical size range of 10 to 1000 nm. The drug is entrapped, dissolved, encapsulated or attached to a nanoparticle matrix. Either nanoparticles, nanocapsules or nanospheres can be obtained depending on the preparation method. It has been previously shown that nanospheres are absorbed intact across the intestinal epithelium and subsequently taken up in the liver where degradation occurs and the entrapped drugs are released (Owens *et al.*, 2003). Although nanospheres have a few advantages for drug delivery, the disadvantages cannot be ignored, such as the change of morbidity and mortality due to cardiovascular effects of nanospheres (Gwinn & Vallyathan, 2006).
- Liposomes: Microscopic vesicles which consist of one or more concentric lipid bilayers. These layers are separated by water or an aqueous buffer, and have a typical diameter range between 25 to 100 µm and are classified accordingly. Liposomes are acknowledged as small unilamellar vesicles (SUV, 10 -100 nm) or large unilamellar vesicles (LUV, 100 – 3000 nm). Lee *et al.*, (2015) conducted a study with vasopressin entrapped in a liposome DDS to increase the half-life of vasopressin in rats. It was concluded that the concentration of vasopressin for the liposomal vasopressin treated group was higher at 60 minutes, although they observed that animals treated with liposomal vasopressin performed indifferently to vasopressin treated rats in serum lactate level, oedema profile and inflammatory profile. A few disadvantages of using liposomes as a drug delivery system includes their short half-life, low solubility, high production cost, tendency to leak and fuse with the encapsulated drug/molecules (Akbarzadeh *et al.*, 2013).
- Microemulsions: Thermodynamically stable liquid dispersions of oil and water which are transparent and homogenous. Microemulsions have a typical droplet diameter range between 100 to 1000 nm. Microemulsions have also been utilised as a DDS for peptide and protein drugs, one such example includes a study by Sharma *et al.*, (2010), where the use of microemulsions for oral delivery of insulin was investigated. The conclusion of this study was that the microemulsions formulation displayed a 10-fold enhancement in bioavailability compared with plain insulin solution administered orally. However, using microemulsions as a DDS may present with drawbacks such as the surfactant must be non-toxic for pharmaceutical use when the challenge is that large concentrations of surfactants and co-surfactants are necessary for stabilisation of the microemulsion.
- Pheroid® technology: Pheroid® is a complex polydisperse technology based on a colloidal emulsion system, composed of an organic carbon backbone of unsaturated fatty acids and containing unique and stable lipid-based submicron and micron-sized structures (Grobler, 2009; Grobler, 2013). Calcitonin, vasopressin and insulin are just a few examples of peptide and protein drugs previously used in Pheroid® technology studies. This will be further discussed in full in section 2.3.6.

#### **2.1.5.1.4 SITE-SPECIFIC DELIVERY**

Recently a significant increase of strategies of site-specific delivery in the GI tract has been seen (Wilding, 2000). As mentioned, the colon has several attractive features for drug delivery, which include reduced enzymatic activity, natural absorptive characteristics, prolonged residence time and increased tissue responsiveness to absorption enhancers (Hamman *et al.*, 2005). Different approaches employed for this delivery include magnetic systems, expandable systems and mucoadhesive systems. Chitosan, pectin, dextran and chondroitin sulphates are a few examples of polysaccharides exploited for site-specific delivery to the colon (Renukuntla *et al.*, 2013). As another example of site-specific delivery, Dorkoosh *et al.*, (2001) explored the concept of superporous hydrogels (SPH) and SPH composite (SPHC) for the delivery of peptide and protein drug delivery. The study focused on the use of SPH composite with croscarmellose sodium Ac-Di-Sol® as a composite agent. The *in vitro* results proved that both the formulations partially inhibited trypsin. Dorkoosh (2002) conducted a study with non-diabetic pigs, which demonstrated that SPH and SPHC in both external and internal platforms enhanced insulin plasma levels compared with an oral insulin formulation; the glucose-lowering however was not substantial (Dorkoosh *et al.*, 2002).

Site-specific delivery, e.g. colon drug delivery, is associated with specific limitations such as the fact that the colon is situated in the distal part of the GI tract, which makes patient compliance low due to the administration method. The maintenance of the stability of a drug in the colon is also a matter of concern (Amidon *et al.*, 2015).

#### **2.1.5.2 CHEMICAL AND STRUCTURAL MODIFICATIONS**

Chemical modification may alter the properties of the drug, such as the pharmacokinetics, solubility or antigenicity (Jain, 2008). Structural manipulation of a protein or peptide may provide several opportunities not only to improve the pharmacokinetic but also the pharmacodynamic profiles of the drug (Hamman *et al.*, 2005). Structural modification of the peptide primary structure also leads to the improvement of the enzymatic stability and mucosal penetration (Mahajan *et al.*, 2014). A diametrically opposite approach can be implemented to modify proteins with lipids to induce favourable interactions between the peptide and the intestinal cell membranes (Buckley *et al.*, 2016). To demonstrate this, a study was conducted by Wang *et al.*, (2002), where they modified calcitonin by reversible lipidisation through reversible fatty acid-desmopressin (DDAVP) conjugates. This study concluded that through conjugation of DDAVP with fatty acids, the lipophilicity was increased as well as the anti-diuretic activity of this calcitonin, thus, in turn, the increase in permeation (Wang *et al.*, 2002). By chemically modifying a peptide or protein drug, it is likely that the circulation half-life will also be altered, this can be seen as an advantage or disadvantage depending on the therapeutic aim (Buckley *et al.*, 2016).

Another example of a structural modification of drugs is prodrugs. Prodrugs are a pharmacologically inactive chemical derivative and need to undergo a transformation in the body to become active (Pauletti *et al.*, 1996). A prodrug compound is assembled by chemical modifications of a biologically active compound which will liberate the active compound *in vivo* by chemical or enzymatic processes (Lohar *et al.*, 2012). The carrier released *in vivo* and the metabolic fragment apart from the drug must be non-toxic. Prodrugs increase the chemical stability, alter aqueous solubility, improve bioavailability and provide hydrolytic stability to the drug (Mahajan *et al.*, 2014). An example of a peptide prodrug found to protect the C-terminal amide bond against cleavage is  $\alpha$ -chymotrypsin (Hamman *et al.*, 2005). A few limitations of the pro-drug approach include (Bharati, 2015):

- There may be formation of unexpected metabolites that can be toxic.
- The inert carrier that is generated following cleavage may form a toxic metabolite.
- Vital cell constituents might be consumed during the activation stage, which leads to the depletion of the cell.

## 2.1.6 BIOEQUIVALENCE

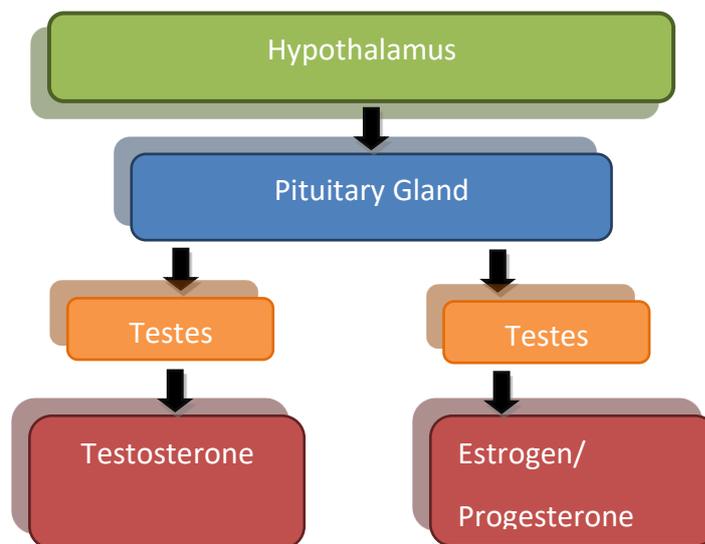
When changing the administration method for drugs, the desired target plasma drug concentration of the changing dose must be bioequivalent to that of the given dose, and the multiple-dose regimen must be designed within the therapeutic window (Shargel *et al.*, 2012). Bioequivalence and bioavailability studies are essential for an oral dose formulation and are required to ensure the therapeutic equivalence of a pharmaceutically equivalent test product and a reference product (Krishnaiah *et al.*, 2011; Zhu *et al.*, 2017). Bioequivalence can be used to support new formulations of previously approved products (Shargel *et al.*, 2012). A bioequivalence study can be done by comparing the measured drug concentration in the blood or plasma for both the new and reference formulation. Pharmacokinetic parameters can be derived from the blood levels, such as the maximum concentration ( $C_{max}$ ) and the area under the curve (AUC) (Shargel *et al.*, 2012). The test product is claimed to be bioequivalent to the reference if the calculated 90% confidence interval around the AUC and  $C_{max}$  is totally within the bioequivalence limits of 80% to 125% (Krishnaiah *et al.*, 2011). The fundamental bioequivalence assumption states that if two drugs are shown to be bioequivalent they are assumed to have the same therapeutic and adverse effect, meaning that they are therapeutically equivalent (Krishnaiah *et al.*, 2011).

## 2.2 GONADOTROPINS AND THEIR ANALOGUES

### 2.2.1 PHARMACOLOGY OF ENDOGENOUS GONADOTROPIN

The gonadotropin-releasing hormone (GnRH) (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH<sub>2</sub>) plays a role in the regulation of the neurohormonal control of reproduction in mammals and also plays a crucial role in the control of the ovarian cycle in women (Coccia, 2004; Counis *et al.*, 2005).

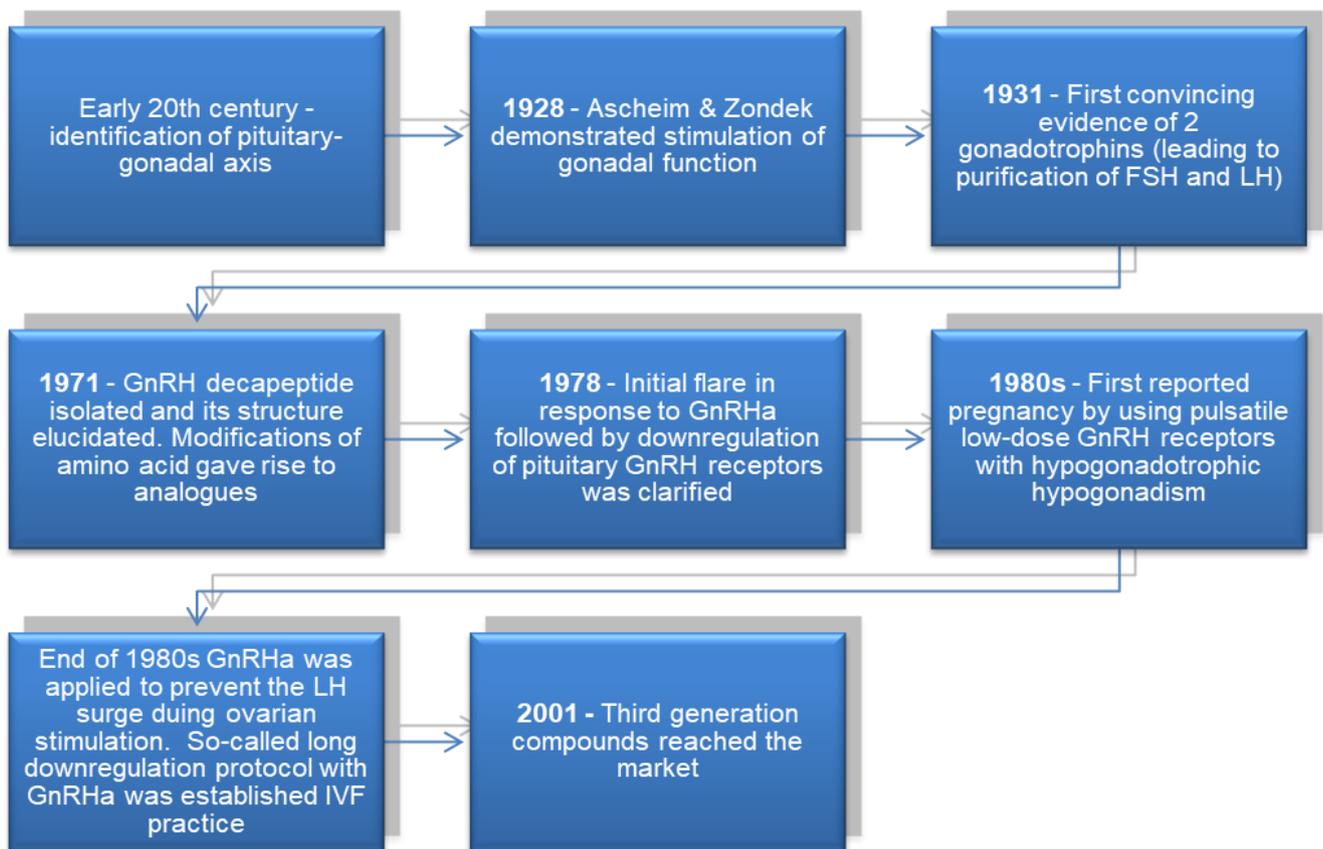
The primary physiological site of action of GnRH is on pituitary gonadotropins (Figure 2.3) (Clayton, 1988). Synthesis of GnRH occur in hypothalamic neuron cell bodies and are secreted by their terminals into the hypophysiportal circulation (Conn & Crowley Jr, 1991). These cell bodies are localised in the preoptic area, anterior hypothalamic area, and medial basal hypothalamus (Smith, 2010). GnRH stimulates the gonadotropin cells to release heterodimeric gonadotropins luteinising hormone (LH) and follicle-stimulating hormone (FSH), upon arrival at the anterior pituitary gland (Conn & Crowley Jr, 1991). LH and FSH then stimulate the gonadal production of sex steroids and gametogenesis respectively (Conn & Crowley Jr, 1991). However, the release of GnRH is not continuously just from the hypothalamus, but is also secreted in a pulsating fashion approximately every hour from the hypophyseal portal system (Jones & Lopez, 2013). Thus, FSH and LH are released in a pulsatile fashion (Jones & Lopez, 2013). This secretion is controlled by the activity of cells in the area known as the GnRH pulse generator (Jones & Lopez, 2013). The receptor concentration of GnRH is negatively regulated by testosterone and progesterone and positively regulated by oestradiol *in vivo* as well as by the ligand itself (Clayton, 1988).



**Figure 3:** Pathway of endogenous GnRH (adapted from: Yeo & de Croft, 2016).

### 2.2.2 GONADOTROPIN (GNRH) ANALOGUES

GnRH analogues are a group of drugs which influence the hypothalamus and pituitary, this includes GnRH agonists (GnRH-a) and antagonists (GnRH-ant) (Naheed *et al.*, 2014). Widespread clinical use of the control of reproduction has been achieved and over 2000 analogues have been synthesised and tested over the last 30 years (Padula, 2005). Figure 2.4 demonstrates the history of the development of GnRH analogues. GnRH analogues have an oral bioavailability of 0.1%, thus GnRH analogues are administered through other routes such as intranasally, subcutaneous injections and intramuscular depot preparations (Naheed *et al.*, 2014). However, with a highly variable 4 to 21% availability, the intranasal route is inefficient relative to the other two administration routes and frequent large doses are also necessary (Chrisp & Goa, 1990).



**Figure 4:** Historical timeline of the development of GnRH analogues (adapted from: Hayden, 2008).

Many preparations of GnRH-a exist, such as buserelin, histrelin, goserelin, deslorelin, nafarelin, leuprorelin (leuprorelin) and triptorelin (Table 2.2) (Naheed *et al.*, 2014). Of these, goserelin, nafarelin and leuprorelin are most commonly used (Naheed *et al.*, 2014). Deslorelin is a superagonist, being 100 times more efficacious than the natural GnRH (Padula, 2005). Padula (2005) stated that the market for treatment of human prostate cancer with GnRH-a, in conjunction with the control of stimulation programmes for human assisted reproduction, has led to the commercialisation of many agonists (Padula, 2005). This agonist causes a stimulatory action on the pituitary gland, leading to a decrease in LH and FSH and consequently causes the down-regulation of testosterone and oestrogen to castrate and post-menopausal levels respectively (Padula, 2005).

Conversely, GnRH-ant is similar in structure to the natural GnRH but have an antagonistic effect (Naheed *et al.*, 2014). These antagonists compete with the natural GnRH for receptor binding and thus block the action of natural GnRH in the body (Naheed *et al.*, 2014). GnRH-ant produce an immediate effect by a competitive blockade of the GnRH receptors (Coccia *et al.*, 2004). Due to the competitive blocking, LH (and to a lesser effect FSH) concentration drops rapidly (Coccia *et al.*, 2004). The pituitary function normalises immediately following the cessation of the medication (Coccia *et al.*, 2004). Examples of antagonists include Abarelix, Cetrorelix, Elagolix, Ganirelix, and Degarelix (Table 2.2) (Coccia *et al.*, 2004).

**Table 2:** GnRH-a and GnRH-ant respective commercial name and route of administration (Anon, 2018; Padula, 2005; Dun & Taylor, 2017; Lambalk *et al.*, 2005; Tur-Kaspa & Ezcurra, 2009; Van Poppel *et al.*, 2008; Weisman & Goldberg, 2001; WHO, 2016).

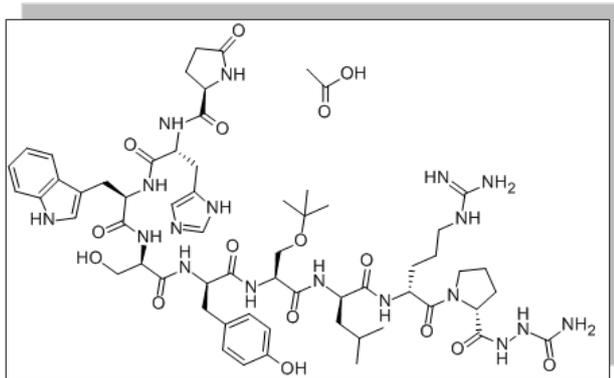
Peptide Common Name	Commercial Product	Route of Administration
<b>GnRH-a</b>		
Buserelin	Receptal®	Implant, nasal spray
Goserelin	Zoladex®, Zoladex®-3-month	Subcutaneous implant
Histrelin	Supprelin® LA, Vantas®	Subcutaneous implant
Leuporide	Lupron® Depot-PED, Lupron®, Lupron® depot, Viadur®	Subcutaneous implant, injection
Nafarelin	Synarel®	Nasal spray
Triptorelin	Trelstar®, Telstar® depot, Triptodur®	Injection
<b>GnRH-ant</b>		
Abarelix	Plenaxis®, Plenaxis® depot	Intramuscular, injection
Cetrorelix acetate	Cetrotide®	Injection
Degarelix	Firmagon®	Subcutaneous implant
Elagolix sodium	Orilissa®	Oral
Ganirelix acetate	Antagon®	Subcutaneous injection

## 2.2.3 THE GnRH AGONIST GOSERELIN

### 2.2.3.1 PHARMACOLOGY

The amphiphilic peptide goserelin (C<sub>59</sub>H<sub>84</sub>N<sub>18</sub>O<sub>14</sub>, molecular weight 1269.433 g/mol) is a synthetic, decapeptide GnRH analogue, also known as a luteinising releasing hormone (LHRH) agonist. The pharmaceutically acceptable form is goserelin acetate (C<sub>61</sub>H<sub>88</sub>N<sub>18</sub>O<sub>16</sub>, molecular weight 1329.485 g/mol) and can be chemically described as an acetate salt of [d-ser(But)<sub>6</sub>, Azgly 10] LHRH with a chemical structure of pro-Glu-Trp-Ser-Tyr-D-Ser(But)-Leu-Arg-Pro-Azgly-NH<sub>2</sub> acetate (Figure 2.5) (Roach III & Izaguirre, 2007; Zhang *et al.*, 2014). It is beneficial to use peptides in the acetate salt form as it facilitates the generation of pH levels between the ranges of 3 to 6, which is known to improve the stability of peptides generally (Laidler & Farkas, 2013). Henceforth, goserelin acetate will be referred to as goserelin. Goserelin, therapeutic trade name Zoladex®, is the most intensively investigated GnRH agonist. It is a type of hormone therapy that can be used in addition to standard

adjuvant therapy (the standard therapy is surgery followed by radiotherapy, chemotherapy and/or tamoxifen) (Cockshott, 2000; Baum *et al.*, 2006). Various hormone-dependent disorders, such as benign conditions (e.g. uterine fibroids, menorrhagia, endometriosis etc.) or malignant tumours (breast, ovarian, endometrial and prostate carcinoma), are treated effectively with GnRH analogues (Kiesel *et al.*, 2002). Bolla *et al.*, (1997) demonstrated that goserelin in conjunction with radiotherapy can be used successfully to improve the survival rate by as much as five years in patients with locally advanced prostate cancer. Zhang *et al.*, (2011) also indicated that GnRH analogues could inhibit the growth of transplanted tumours in nude mice. A study conducted by Moore *et al.*, (2015) established that the administration of goserelin combined with chemotherapy can protect against ovarian failure, reduce the risk of early menopause and improve the prospects of fertility. The GnRH analogues are also used in combination with menotropins or recombinant follicle-stimulating hormone (FSH) to induce folliculogenesis (this is the process where a diplotene oocyte becomes surrounded by a single layer of granulosa cells and enclosed by an intact basal lamina, this leads to the formation of a specific germ cell compartment) for controlled ovarian hyperstimulation in women undergoing IVF (Geber *et al.*, 2002; Polin *et al.*, 2011).



**Figure 5:** Molecular structure of goserelin acetate.

The different doses for different treatments of goserelin are as follows (Gray *et al.*, 2010):

- Prostate cancer: 3.6 mg subcutaneous injection every 28 days, or 10.8 mg subcutaneous injection every 12 weeks. An antiandrogen may be given 3 days before until 3 weeks after.
- Breast cancer: 3.6 mg subcutaneous implant which slowly releases for 28 days.
- Endometriosis: 3.6 mg daily subcutaneous injections every 28 days for a maximum of 6 months (not to be repeated).
- Endometrial thinning before intrauterine surgery: 3.6 subcutaneous injection once.

- Assisted reproduction: 3.6 mg subcutaneous injection is administered once to down-regulate the pituitary gland, this should lead to the serum oestradiol levels being declined. Gonadotropin is then administered following the protocol of the individual clinic.

### **2.2.3.2 MEANS OF ADMINISTRATION AND LIMITATIONS**

One goserelin (Zoladex®) implant, which is a biodegradable sustained release depot to desensitise the pituitary gland and stop the natural production of endogenous FSH and LH, is injected subcutaneously through a 16-gauge needle (Marshall, 2017). A local anaesthetic can also be used to aid in the comfort of the injection. To achieve its therapeutic effect goserelin needs to be administered for several weeks, as the mechanism of action is best suited for a long-term acting formulation (Aulton & Taylor, 2018). Goserelin is hence formulated with the agonist microencapsulated within biodegradable poly (DL-lactide-co-glycolide) microspheres (Aulton & Taylor, 2018; Matson & Lieberman, 1990).

### **2.2.3.3 SIDE EFFECTS**

Side effects with the use of goserelin include hypoestrogenic side effects, such as vaginal dryness, vasomotor symptoms, emotional instability, loss of bone mineral density (BMD) and insomnia (Moghissi *et al.*, 1998). In using goserelin, the patient may experience short-term toxic effects and

long-term effects associated with premature menopause (Jonat *et al.*, 2002). Examples of less or rare common side effects include; fainting, fast/ irregular heartbeat, skin rash, hives or itching, anxiety, depression, numbness in hands etc. (Anon, 2018).

### **2.2.3.4 PHARMACOKINETICS AND PHARMACODYNAMICS**

#### **2.2.3.4.1 PHARMACOKINETICS**

Goserelin treatment is currently only available as a slow releasing depot (Zoladex®), which releases goserelin over a period of either 1 (3.6 mg) or 3 (10.8 mg) months (Cockshott, 2000). The 1 month formulation contains 3.6 mg goserelin in a poly (DL-lactide-co-glycolide) matrix, which consists of 50% lactide and 50% glycolide, while the 3 month depot contains 10.8 mg goserelin in a poly(DL-lactide-co-glycolide) matrix, which consists of 95% lactide and 5% glycolide (Aulton & Taylor, 2018). The subcutaneous depot drug is injected into the extracellular space of the subcutaneous tissue where the drug material resides where it is then transported to the blood or lymph capillaries for absorption prior to reaching the systemic circulation (Richter & Jacobsen, 2014). Initially, goserelin is released at a slower rate for the first eight days, followed by an accelerated rate for the remainder of the dosing period (Ma & Hadzija, 2012). Table 2.3 summarises the absorption, distribution, metabolism and elimination (ADME) of goserelin.

**Table 3:** Pharmacokinetics (ADME) of goserelin (Zoladex®) (Butler & Govindan, 2010; Chabner & Longo, 2011).

Pharmacokinetics	Description
<b>Absorption</b>	<ul style="list-style-type: none"> <li>Goserelin is absorbed subcutaneously from the implant; time to peak concentration varies in male and females: females 8-22 days and males 12-15 days</li> <li>The subcutaneous absorption is rapid and can be detected in serum within 10 minutes</li> </ul>
<b>Distribution</b>	<ul style="list-style-type: none"> <li>The volume of distribution: females; 20.3 ± 4.1 L and males 44.1 ± 13.6 L</li> <li>Protein binding is minimal; 27.3%</li> </ul>
<b>Metabolism</b>	<ul style="list-style-type: none"> <li>The hepatic route is the main pathway of metabolism via the hydrolysis of the C-terminal of amino acids</li> <li>Half-life: 4.2 hr.</li> <li>The major metabolites are in serum (1-7 fragment) and urine (5-10 fragment)</li> </ul>
<b>Elimination</b>	<ul style="list-style-type: none"> <li>Enzymatically degraded by pyroglutamate aminopeptidase, endopeptidase and postproline-cleaving enzymes</li> <li>Renal excretion is more than 90% in urine, with more than 20% unchanged, only 2% found in faeces</li> </ul>

#### 2.2.4.2 PHARMACODYNAMICS

Drugs can interact with receptors at the site of action and initiate a cascade of events resulting in the pharmacodynamic response (Shargel *et al.*, 2012). Goserelin is one of the synthetic GnRH analogues that enhance receptor affinity (mimics endogenous GnRH); it acts on the hypothalamic-pituitary axis where receptor downregulation occurs, leading to the inhibition of the secretion of pituitary gonadotropins (tachyphylaxis) (Baum *et al.*, 2006; Jonat, 1998; Zhang *et al.*, 2014). Initially, goserelin induces a transient increase in LH and FSH levels, which leads to the desensitisation of the GnRH receptors by interrupting its physiological intermittent stimulation (Tan *et al.*, 2015, Perry & Brogden, 1996). Both LH and FSH levels will initially increase, reaching a peak after two or three days (Chrisp & Goa, 1991). Within 14 days, the levels will decrease to pre-treatment values or below (Chrisp & Goa, 1991). This leads to the achievement of castrate levels of testosterone in males and the achievement of post-menopausal levels of oestradiol in females resulting in progesterone synthesis being abolished and ovulation and luteinisation being prevented (Chrisp & Goa, 1991; Cockshott, 2000). Goserelin thus causes suppression of gonadotropins with the continued

administration (Chrisp & Goa, 1991). For IVF, goserelin (subcutaneous implant) is used to desensitise the pituitary gland leading to a reduction of endogenous FSH and LH, lower concentrations of circulating oestradiol and inhibited follicular development. Synthetic FSH, LH and human chorionic gonadotropin (hCG) are then administered to stimulate ovulation artificially (Marshall, 2017).

Zhang *et al.*, (2014) demonstrated the plasma concentration-time curves of goserelin and testosterone after treatment of rats with 0.45 mg of the sustained-release implant of acetate goserelin. From this, it was clear there is an initial increase of goserelin in the first 6 h followed by a decrease. After 12 h, goserelin concentration increases again reaching a peak concentration followed by a decrease in concentration, due to the slow releasing of the subcutaneous depot. Testosterone concentration initially increases followed by a decrease indicating the achievement of castration levels of testosterone as a result of an initial increase of LH and FSH followed by a decrease of LH and FSH; this correlates with the pharmacokinetics and pharmacodynamics of goserelin as stated above

## **2.2.4 PROSPECTS OF CHANGING THE ADMINISTRATION OF GOSERELIN**

Subcutaneous administrations/injections are a more invasive administration method than other preferred administration routes, for example, oral administration, because injections are usually not well tolerated by patients. Hence, patient compliance with drug administration by injection or subcutaneous implants is generally poor, leading to a severely restricted therapeutic value of a drug, thus the need for less invasive administration, such as oral administration, exists (Almeida & Souto, 2007; Shaji & Patole, 2008). Oral administration of drugs can offer some advantages in terms of lower manufacturing costs, an increase of patient compliance and ease of administration as it is generally more comfortable and acceptable, as mentioned in section 2.1.1 (Wang *et al.*, 2015). The pharmaceutical industry is under pressure to maximise the full potential of viable drug candidates, this can be accomplished by incorporating various drug delivery systems (Sastray *et al.*, 2000).

## **2.3 PHEROID® TECHNOLOGY**

### **2.3.1 INTRODUCTION**

For a drug to have a therapeutic effect within the body, it should reach or enter target areas in reasonable quantities, and if it is delivered orally it should be stable within the GI tract, as previously mentioned in section 2.1. The Pheroid® delivery system has been investigated as a carrier for therapeutic drugs to enhance the absorption of drugs, which encounter low bioavailability. Oberholzer (2009) conducted a study aiming to increase the therapeutic response to insulin, through nasal administration, in male Sprague Dawley rats by the use of Pheroid® technology as a drug delivery system. This study concluded that the therapeutic effect of nasal administration of insulin was significantly higher in the insulin Pheroid® formulation. Pheroid® technology is a complex

polydisperse technology based on a colloidal emulsion system, composed of an organic carbon backbone of unsaturated fatty acids and containing unique and stable lipid-based submicron and micron-sized structures emulsified in water saturated in nitrous oxide (Grobler, 2009; Grobler, 2013).

Advantages of using Pheroid® technology as a DDS include (Strauss, 2005):

- Increased bioavailability and therapeutic efficacy.
- Decreased time to onset of action.
- Reduction of the minimal effective concentration, cytotoxicity, immunological response and drug resistance.

Pheroid® can be manipulated in terms of morphology, structure, size and function and can entrap, transport and deliver active pharmaceutical ingredients (APIs) and other useful molecules (Strauss, 2005; Uys, 2006).

### **2.3.2 THE HISTORY OF PHEROID® TECHNOLOGY**

The origin of Pheroid® technology can be traced back to Emzaloid™ technology (Gibhard, 2012). Emzaloid™ is a patented drug delivery system, which consists of a unique submicron-emulsion type formulation, capable of encapsulation of various drugs and delivering therapeutic drugs to the target sites with high efficacy within the body (Anon., 2012). Emzaloid™ technology was discovered and developed by MeyerZall Laboratories (Grobler, 2009) and first used in an effort to treat/cure psoriasis (Grobler, 2009). The psoriasis product proved to be more effective with fewer side effects than the comparable product on the market (Grobler, 2009). Following this discovery, a research programme was initiated in collaboration with the South African Medical Research Council (SAMRC) to assist in the determination of the effectiveness of Emzaloid™ technology and the reason behind the success of this product (Gibhard, 2012; Grobler, 2009). The conclusion made by the SAMRC was that the psoriasis product contained micro-vesicles, which led to the hypothesis (that was proven to be correct) that the vesicles constituted a delivery system capable of possibly enhancing the absorption of the active ingredient/therapeutic drug (Gibhard, 2012; Grobler, 2009). In 2003, North-West University (NWU) obtained intellectual property with regard to Emzaloid™ technology (Gibhard, 2012; Grobler, 2009). This drug delivery system was optimised in terms of morphology and particle size, which sparked the initiation of a solid founding delivery system called Pheroid® technology (Gibhard, 2012; Grobler, 2009).

Nonetheless, it is important to note that Emzaloid™ technology was not renamed to Pheroid® technology. There are distinctive differences found between these two technologies, such as (Gibhard, 2012; Grobler, 2009):

- Emzaloid™-based products are manufactured under low pressure (80 kPa) for four hours.

- Pheroid<sup>®</sup>-based products are manufactured under higher pressure (200 kPa) for the duration of four days.
- Pheroid<sup>®</sup> technology contains  $\alpha$ -tocopherol where Emzaloid<sup>™</sup> does not.

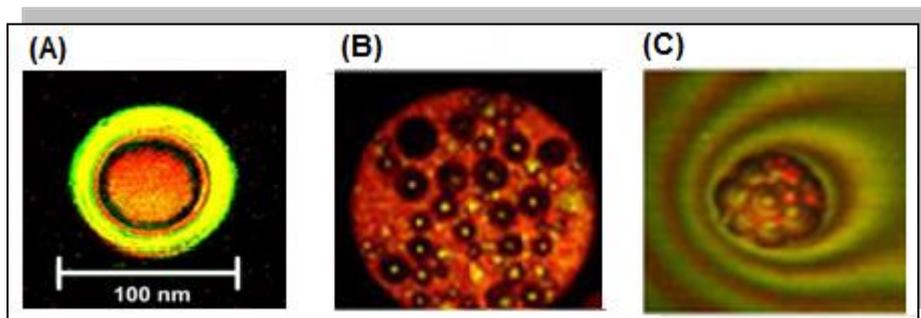
### 2.3.3 PHEROID<sup>®</sup> FUNCTION, CHARACTERISTICS AND TYPES

Pheroid<sup>®</sup> mainly consists of three phases:

1. Aqueous phase: Primarily water.
2. Oil phase: Combination of essential fatty acids.
3. Nitrous oxide (N<sub>2</sub>O): The role of the N<sub>2</sub>O is to ensure the stability of the formulation (Steyn *et al.*, 2011).

Each component of Pheroid<sup>®</sup> will be discussed in full in section 2.4.3.2. Pheroid<sup>®</sup> can be manipulated in terms of size, morphology, lipid composition, charge and membrane packing (Grobler, 2009; van Huyssteen, 2010). Various types of Pheroid<sup>®</sup> structures form as a result of self-emulsifying characteristics as a consequence of side-chain interactions; these main types are (Strauss, 2005; Globler, 2013) (Figure 2.6):

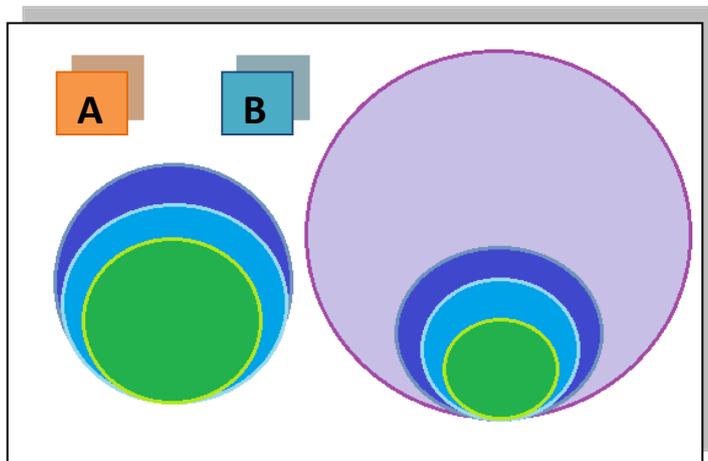
- Lipid bilayer vesicles in both the nano and micro size range: the size and shape of the vesicles can be controlled (typically between 0.5 – 1.5  $\mu$ m). Vesicles can entrap the API thus making it possible to create a safer and more effective formulation.
- Microsponges: the size of microsponges is usually 1.5 to 5 $\mu$ m. The microsponges support prolonged release.
- Depots/reservoirs that contain pro-Pheroid<sup>®</sup>s: the sizes are determined by the amount of pro-Pheroid<sup>®</sup>s contained in the reservoirs.



**Figure 6:** Examples of confocal laser scans of the Pheroid® types: **(A)** Lipid Bilayer vesicle with entrapped API (Rifampicin); **(B)** Reservoir containing multiple particles of coal tar - the reservoirs are good entrappers of insoluble compounds; **(C)** The formation of small pro-Pheroid®s used for some oral formulations (Source: Grobler, 2004; with permission from author).

### 2.3.3.1 PRO-PHEROID®

Pro-Pheroid® lacks the water phase meaning it only consists of the oil phase (Figure 2.7) (Grobler, 2009). Payne *et al.*, (1986) originally described the concept of a pro-delivery system when pro-liposomes were described as a precursor for liposomes (Grobler, 2013). Pro-liposomes can be defined as a free-flowing granular product that on hydration or on contact with biological fluids in the body, forms an isotonic liposomal dispersion (Muneer *et al.*, 2017). Similarly, in the addition of a water phase, vesicles and/or other lipid aggregates of Pheroid® micro- and nanoparticles form spontaneously (Grobler, 2009; Grobler, 2013). When pro-Pheroid® is administered orally, the water phase is added in situ by the fluid present in the stomach content leading to the formation of Pheroid® (Grobler, 2009). Pro-Pheroid® can be a very helpful DDS in the case of drugs or active pharmaceutical ingredients (APIs) that are unstable, such as peptides and proteins (Grobler, 2013). The manufacturing of pro-Pheroid® is relatively simple and side-steps many of the difficulties generally encountered with lipid-based vesicles.



**Figure 7:** Schematic representation of the differences of basic Pheroid<sup>®</sup> and pro-Pheroid<sup>®</sup>: (A) Pro-Pheroid<sup>®</sup>, (B) Pheroid<sup>®</sup> (adapted from Grobler, 2009). **Green:** N<sub>2</sub>O, **Blue:** α-tocopherol, **Dark Purple:** long chain fatty acids and **Light Purple:** aqueous phase. A typical Pheroid<sup>®</sup> formulation consists of 96% aqueous and 4% oil.

## 2.3.4 COMPONENTS OF PHEROID<sup>®</sup>

### 2.3.4.1 FATTY ACID COMPONENT

The fatty acids typically used in Pheroid<sup>®</sup> consist primarily of ethylated and PEGylated polyunsaturated fatty acids (Gibhard, 2012; Globler, 200).

#### 2.3.4.1.1 VITAMIN F ETHYL ESTER

Vitamin F ethyl ester is a combination of unsaturated fatty acids, with typical distribution as follows (Adapted from certificate of analysis: supplied in Annexure E):

- C<sub>16</sub> (stearic acid) 7.0 to 11.0%
- C<sub>18</sub>: (Palmitic acid) 2.0 to 5.0%
- C<sub>18.1</sub>: (oleic acid): 48.0 to 24.0%
- C<sub>18.2</sub> (linoleic acid): 31.0 to 37.0%
- C<sub>18.3</sub>: (α-linoleic acid): 26.0 to 34.0%
- > C<sub>18</sub>: 0.0 to 2.0%

#### 2.3.4.1.2 KOLLIPHOR<sup>®</sup>-EL

Kolliphor<sup>®</sup>-EL is a non-ionic stabiliser and emulsifier, the main component being glycerol polyethylene glycol ricinoleate. The hydrophobic part is made up of fatty acid esters of

polyethylene glycol; the smaller hydrophilic part consists of free polyethylene glycols and ethoxylated glycerol (BASF, s.a.).

#### **2.3.4.2 NITROUS OXIDE COMPONENT**

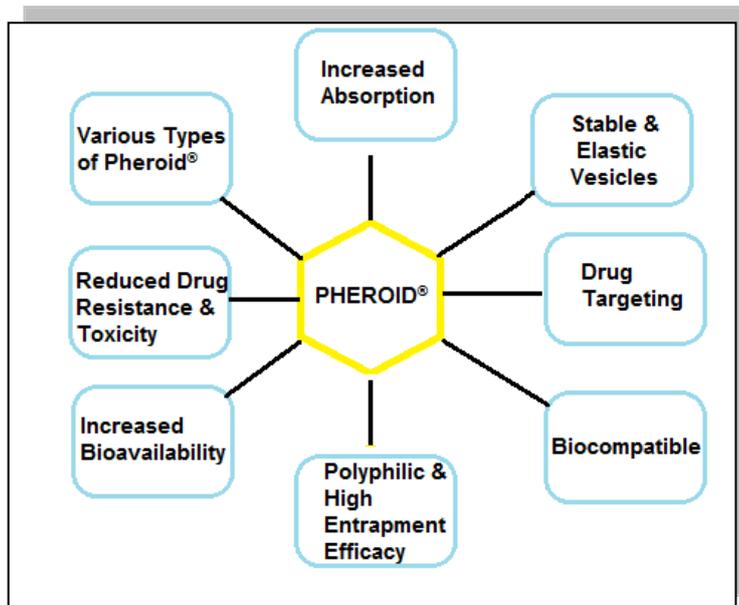
Nitrous oxide (N<sub>2</sub>O) is colourless, non-toxic and very stable, and one of several oxides of nitrogen also known as laughing gas (Lassey & Harvey, 2007). Studies investigating the interactions between the fatty acids and nitrous oxide through molecular modelling revealed a nitrous oxide essential fatty acid matrix that provided a function model for the transportation of hydrophilic and hydrophobic drugs (Gibhard, 2012; Globler, 2008). Nitrous oxide provides stability, miscibility and imparts an ordered structure to the self-assembled vesicle (Gibhard, 2012; Globler, 2009).

#### **2.3.4.3 α-TOCOPHEROL COMPONENT**

Vitamin E is a lipid-soluble component in the cell's antioxidant defence system and is a natural, tolerable and highly cost-effective molecule (Engin, 2009; Rizvi *et al.*, 2014). It can only be obtained from diet and cannot be made in the body (Engin, 2009; Rizvi *et al.*, 2014). According to Traber (2012), α-tocopherol is the most common of the homologs and a biologically active form due to its faster metabolism (Engin, 2009; Rizvi *et al.*, 2014). Vitamin E has numerous important roles within the body due to its antioxidant activity (it interrupts free radical reactions by capturing the free radical) (Engin, 2009; Rizvi *et al.*, 2014). Vitamin E is also known as an emulsion stabiliser (Rizvi *et al.*, 2014).

#### **2.3.5 CHARACTERISTICS THAT MAKE PHEROID® TECHNOLOGY AN INTERESTING CARRIER**

Figure 2.8 demonstrates the key advantages of using Pheroid® technology as a DDS. More detail will be discussed in full in section 2.4.5.1 to 2.4.5.5



**Figure 8:** Key advantages of Pheroid® technology as a drug delivery system (DDS) (adapted from: Globler, 2009; van Huyssteen, 2010).

### 2.3.5.1 TOXICITY OF PHEROID®

As mentioned before, Pheroid® mainly consists of essential fatty acids, which are necessary for normal bodily functions but cannot be produced by the body and thus have to be ingested externally through diet. Hence, these fatty acids form part of the natural biochemistry pathways of the body and because of this, causes no cytotoxicity (Globler, 2009; van Huyssteen, 2010). An extensive *in vivo* study was done by Elgar (2008) on Sprague Dawley rats; this study observed no signs of toxicity at a dose of 50 mg/kg oral administration of pro-Pheroid®. No immune response by man was observed in cytokine studies after the administration of Pheroid® (Globler, 2009). Grobler, (2009) suggested that some drugs (such as proteins) may induce an immunologic response, but masking the protein by using Pheroid® may reduce recognition by the immune system.

### 2.3.5.2 STABILITY

Grobler (2009) stated that in biological environments particles in colloidal systems generally must be stabilised to retain their stability. The interior volume of Pheroid® is stabilised due to its composition through hydrogen bonding and water bridge interactions and not by cholesterol (as in the case of most lipid-based delivery systems) (Grobler *et al.*, 2006). Extremely elastic structures are formed due to the presence of the N<sub>2</sub>O gas as well as the pliable pegylated tails, which are added to the fatty acids of the Pheroid® (Grobler, 2009; van Huyssteen, 2010). Additional PEG can be added to the Pheroid® formulation by PEGylation. This allows the Pheroid®s to be sterically stabilised and maintain their inner spaces (van Huyssteen, 2010). According to Verhoef and Anchordoquy (2013), the presence of PEG used in formulations such as Pheroid® is thought to create a hydrophilic

barrier. In addition to this Pheroid<sup>®</sup> is not known to be shattered when under moderate pressure (Grobler *et al.*, 2006).

### **2.3.5.3 PHEROID<sup>®</sup> CELL MEMBRANE INTERACTION**

An affinity exists between cell membranes and Pheroid<sup>®</sup> vesicles. Pheroid<sup>®</sup> is presumed to interact with the cell membrane and subsequently penetrate the cell through an endosome sorting mechanism, which leads to effective and fast delivery of the active ingredient (Grobler, 2009; van Huyssteen, 2010). As stated by Grobler *et al.*, (2006), the fluidity of the Pheroid<sup>®</sup> membrane should increase the movement of hydrophobic and hydrophilic molecules or compounds laterally in the membrane to connecting cells. Pheroid<sup>®</sup> can infiltrate keratinised tissue, skin, intestinal lining, vascular system, fungi, bacteria and parasites (Grobler, 2009).

### **2.3.5.4 DRUG TARGETING AND PHARMACOKINETICS**

The biodistribution of Pheroid<sup>®</sup> can be influenced depending on the type and extent of the fatty acid modifications, according to Grobler *et al.*, (2008). Various native interactions between fatty acids and cells can influence the cellular uptake of Pheroid<sup>®</sup>, these include the binding between the fatty acids and the fatty acid binding proteins in the cell membrane and the interaction between Pheroid<sup>®</sup> and the lipid rafts present in the cell membrane. The APIs are subsequently released due to the metabolism of Pheroid<sup>®</sup> in either the mitochondria or the peroxisomes of the cells, and this depends on the composition of the Pheroid<sup>®</sup>. This was confirmed by co-localisation studies of Pheroid<sup>®</sup> and various sub-cellular organelles, conducted by Britz, (2009).

Pheroid<sup>®</sup> containing small peptides and antibodies has previously shown to interact with specific micro-domains on cells in culture (Grobler, 2004). Through entrapment of the API in Pheroid<sup>®</sup> vesicles or microsponges, the pharmacokinetics of the API can be altered; this may lead to a decrease of the time needed for the API to reach the maximum plasma concentration ( $C_{max}$ ). The entrapment may also lead to the reduction of the volume of distribution, therefore leading to the increased concentration of the API at the specific target site. Hence, the narrow therapeutic index is enhanced with less toxicity (Grobler, 2009). The bioavailability and absorption of the API can be enhanced for oral, topical, parenteral and nasal administration routes by using Pheroid<sup>®</sup> technology. Examples of this are discussed in section 2.4.6. The increase in the bioavailability of the API can lead to an increase of the minimal inhibitory concentration (MIC) and therapeutic effect (Grobler, 2009; van Huyssteen, 2010).

### **2.3.5.5 DRUG PROTECTION**

Before drugs can reach or enter the target site, many drugs may present with a reduced therapeutic effect due to partial degradation of the drug (Vogelson, 2001). Pheroid<sup>®</sup> may protect entrapped APIs from metabolism, degradation and inactivation in the GI tract.

## 2.3.6 PHARMACEUTIC APPLICATIONS OF PHEROID® TECHNOLOGY

Pheroid® technology has been investigated as a DDS for a number of peptides and proteins. Some examples of the successful delivery of a therapeutic protein include an *in vivo* study done by Steyn *et al.*, (2010) to evaluate the use of Pheroid® technology for nasal delivery of recombinant human growth hormone (rhGH) in male Sprague Dawley rats. All formulations were administered directly into the nasal cavity of the left nostril of the rats. The conclusion made was that the Pheroid® vesicles improved the nasal absorption and the bioavailability of rhGH. Another study, conducted by Oberholzer (2009) using Sprague Dawley rats, demonstrated the advantages of using Pheroid® technology and insulin. All the formulations were directly administered in the stomach, ileum and duodenum. Results obtained indicated that the ileum showed undoubtedly to be the best area of absorption for Pheroid® and insulin.

An *in vitro* transtandard deviationormal study of a peptide prohormone was conducted by Coetzee (2007) as another example of the delivery of a peptide therapeutic ingredient with the use of Pheroid® technology. This study focused on the *in vitro* transtandard deviationormal delivery of arginine vasopressin (AVP) and used abdominal skin obtained from Caucasian female patients after cosmetic surgery. Confocal imaging revealed entrapment of AVP in the Pheroid® structures furthermore the *in vitro* permeation profiles of the AVP indicated that the majority of permeation occurred during the first two hours. This study proved that Pheroid® as a delivery system was advantageous when applied as a delivery medium. Another study was carried out by Strauss (2005) to evaluate the absorption enhancing capabilities of N-trimethyl chitosan chloride (TMC) and Pheroid® technology for intestinal salmon calcitonin absorption. The peak plasma concentration ( $C_{max}$ ) for Pheroid® vesicles and calcitonin and Pheroid® microsponges and calcitonin was 386.25 and 432.14 pg/mL respectively. TMC increased the  $C_{max}$  2-fold with a concentration of 738.96 pg/mL. The calcitonin in the saline solution presented with the lowest  $C_{max}$  of 249.09 pg/mL. The results of this study indicated that both Pheroid® technology and TMC showed greater concentrations of salmon calcitonin in comparison with salmon calcitonin in saline. This is an indication of the potential that Pheroid® technology holds as a drug delivery system for drugs unstable in conventional routes.

### 2.3.6.2 TREATMENT OF INFECTIOUS DISEASES

The applicability of Pheroid® technology has been previously shown for infectious diseases. For proof of concept, examples of studies done with treatments for infectious agents include a transtandard deviationormal study done by van der Walt (2007). This study used Pheroid® technology to deliver acyclovir, an antiviral agent. It compared a Pheroid® micro-formulation *in vitro* with a reference sample and found that the Pheroid®-based acyclovir formulation had a better drug delivery across the skin compared to the reference sample. A study conducted by Steyn *et al.*, (2011) with Pheroid® technology and artemisinins (antimalarial lactone) with C57BL/6 mice via oral gavage, demonstrated the artemisin concentrations entrapped in Pheroid® in the blood concentration

were increased by 4.57 compared to the drug only formulation. Pheroid® technology has also been used in conjunction with anti-tuberculosis drugs (TB); such studies include research by Matthee, (2007), which aimed to determine if a pro-Pheroid® formulation would increase the absorption of the anti-TB drug rifampicin in mice. An increase (300%) of the absorption of pro-Pheroid® rifampicin formulation was found when compared to the plasma concentrations of the commercial product (Rifafour e-275®).

### **2.3.6.3 VACCINES**

For more than 90 years adjuvants have been used in human vaccines for the enhancement of immunogenicity of highly purified antigens (Di Pasquale *et al.*, 2015). An urgent need for effective vaccine adjuvants exists because non-living vaccine antigens are generally poorly immunogenic and thus require supplementary components to help stimulate protective immunity (Coffmann *et al.*, 2010). According to Grobler (2004), the Pheroid® carrier is, *per se*, an adjuvant. A study conducted by Krause *et al.*, (2015) demonstrated the use of Pheroid® technology as an alternative to Freund's adjuvant to raise antibodies in chickens against *Plasmodium falciparum*. Pheroid® indicated a 2-fold increase for the antibodies relative to the controls. The conclusion the study made was that Pheroid® was well tolerated in the chickens and hence proves to have great potential for the development of a safe adjuvant for the testing of alternative stimulatory factors (Krause *et al.*, 2015).

### **2.3.7 CHARACTERISATION OF PHEROID®**

#### **2.3.7.1 CONFOCAL LASER SCANNING MICROSCOPY (CLSM)**

CLSM provides a method for visualisation of the emulsion droplets and has been traditionally used as a valuable tool for the obtaining of high-resolution images and three-dimensional reconstructions of an array of biological specimens (de Lange Davies, s.a). Approximate size and morphology of the Pheroid® vesicles and microsponges are also established by CLSM.

#### **2.3.7.2 ZETA POTENTIAL MEASUREMENT**

Zeta potential indicates the stability of an emulsion and, according to Roland *et al.*, (2003), can be defined as the difference in potential between the surface of the tightly bound layer of ions on the particle surface and the electro neutral region of the solution. When the emulsion is more stable the Zeta potential will have a high positive or negative value (+/-25 mV), in contrast, if the Zeta potential has a low positive or negative value (< 25 mV) stability will be low (Roland *et al.*, 2003).

#### **2.3.7.3 PARTICLE SIZE DISTRIBUTION**

Roland *et al.*, (2003) stated that for the safety of the preparation of formulations or the release properties of the API, the median size and the distribution of sizes are very important. The typical diameter range for Pheroid® formulations is between 200 nm and 2 µm (Grobler, 2009). It is

important to know the particle size of the formulation as it is generally known that particles that present with a particle size smaller than 7  $\mu\text{m}$  may be retained by the phagocytotic mononuclear cells of the reticuloendothelial system (RESO) in the liver, spleen and bone marrow. Thus, the smaller the particles the higher the chance of the accumulation in the bone marrow (Buszello & Muller, 2000).

### **2.3.8 CONCLUSION**

From the literature it is clear that Pheroid<sup>®</sup> technology offers many advantages for drug delivery. The Pheroid<sup>®</sup> delivery system can be manipulated and optimised in terms of size, morphology, structure and function. Pheroid<sup>®</sup> technology shows great potential to enhance bioavailability, especially cases where the API has poor absorption, such as in the case of peptide and protein therapeutic drugs, which will ultimately lead to higher plasma levels of the drug. In conclusion, the application of Pheroid<sup>®</sup> technology is quite broad with major applications for the delivery of drugs with low bioavailability such as peptide and protein drugs. This study will, therefore, use Pheroid<sup>®</sup> technology to attend to the poor oral bioavailability of goserelin.

## REFERENCES

- Akbarzadeh, A., Rezaei-Sadabady, R., Davaran, S., Joo, S.W., Zarghami, N., Hanifehpour, Y., Samiei, M., Kouhi, M. & Nejati-Koshki, K. 2013. Liposome: Classification, Preparation, and Applications. *Nanoscale Research Letters*, 8(1):102-102.
- Almeida, A.J. & Souto, E. 2007. Solid Lipid Nanoparticles as a Drug Delivery System for Peptides and Proteins. *Advanced Drug Delivery Reviews*, 59(6):478-490.
- Aulton, M. & Taylor, K. 2018. *The Design and Manufacture of Medicines* 5th ed. China: Elsevier. 11,310, 645, 773-774,
- Amidon, S., Brown, J.E. & Dave, V.S. 2015. Colon-Targeted Oral Drug Delivery Systems: Design Trends and Approaches. *AAPS PharmSciTech*, 16(4):731-741.
- Anon. 2012. Emalaziod™. <http://www.mzl.co.za/emzaloid/> Date of access: 5 April 2018.
- Anon. 2018. Gonadotropin Releasing Hormones. <https://www.drugs.com/drug-class/gonadotropin-releasing-hormones.html>. Date of access: 9 July 2018 2018.
- Anon. 2018. Goserelin Side Effects <https://www.drugs.com/sfx/goserelin-side-effects.html>. Date of access: 23 July 2018 2018
- BASF. s.a. Kolliphor® EL. <https://pharmaceutical.basf.com/en/Drug-Formulation/Kolliphor-EL.html> Date of access: 3 October 2018.
- Baviskar, P., Bedse, A., Sadique, S., Kunde, V. & Jaiswal, S. 2013. Drug Delivery on Rectal Absorption: Suppositories. *Journal of Pharmacological Science Review*, 21(1):70-76.
- Bharati, A. 2015. Prodrug strategy. <https://www.slideshare.net/anvitaj1/prodrug-strategy>. Date of access: 10 October 2018 2018.
- Bolla, M., Gonzalez, D., Warde, P., Dubois, J.B., Mirimanoff, R.-O., Storme, G., Bernier, J., Kuten, A., Sternberg, C., Gil, T., Collette, L. & Pierart, M. 1997. Improved Survival in Patients with Locally Advanced Prostate Cancer Treated with Radiotherapy and Goserelin. *New England Journal of Medicine*, 337(5):295-300.
- Britz, C. 2009. Nevirapine in pro-Pheroid®: A Preservative Efficacy and Stability Test. Potchefstroom (NWU). (Thesis - M.Sc)
- Bruno, B.J., Miller, G.D. & Lim, C.S. 2013. Basics and Recent Advances in Peptide and Protein Drug Delivery. *Therapeutic Delivery*, 4(11):1443-1467.

- Buckley, S.T., Hubálek, F. & Rahbek, U.L. 2016. Chemically Modified Peptides and Proteins - Critical Considerations for Oral Delivery. *Tissue Barriers*, 4(2):e1156805.
- Buszello, K. & Müller, B.W. 2000. Emulsions as Drug Delivery Systems. *Pharmaceutical Emulsions and Suspensions*:191-228.
- Butler, S.K. & Govindan, R. 2010. Essential Cancer Pharmacology: The Prescriber's Guide. Vol 1: *Lippincott Williams & Wilkins*: 205
- Carino, G.P & Mathiowitz, E. 1999. Oral Insulin Delivery. *Advance Drug Delivery Review*, 35(2-3):249-257.
- Cayen, M.N. 2011. Early Drug Development: Strategies and Routes to First-in-Human Trials: John Wiley & Sons.p 36
- Chabner, B.A. & Longo, D.L. 2011. Cancer chemotherapy and biotherapy: principles and practice: *Lippincott Williams & Wilkins*: 655
- Chouhan, R., Goswami, S. & Bajpai, A.K. 2017. Recent Advancements in Oral Delivery of Insulin: from Challenges to Solutions. *Nanostructures for Oral Medicine*. Elsevier. p. 435-465).
- Chrisp, P. & Goa, K.L. 1991. Goserelin. A Review of its Pharmacodynamic and Pharmacokinetic Properties, and Clinical use in Sex Hormone-Related Conditions. *Drugs*, 41(2):254-288.
- Choudhury, A., Das, S. & Kar, M. 2011. A Review on Novelty and Potentiality of Vaginal Drug Delivery. *International Journal of PharmaTech Research*, 3:1033-1044.
- Clayton, R. 1988. Mechanism of GnRH Action in Gonadotrophs. *Human Reproduction*, 3(4):479-483.
- Coffman, R.L., Sher, A. & Seder, R.A. 2010. Vaccine Adjuvants: Putting Innate Immunity to Work. *Immunity*, 33(4):492-503.
- Coccia, M.E., Comparetto, C., Bracco, G.L. & Scarselli, G. 2004. GnRH Antagonists. *European Journal of Obstetrics & Gynecology and Reproductive Biology*, 115:S44-S56.
- Conn, P.M. & Crowley Jr, W.F. 1991. Gonadotropin-Releasing Hormone and its Analogues. *New England Journal of Medicine*, 324(2):93-103.Craik, D.J., Fairlie, D.P., Liras, S. & Price, Craik, D.J., Fairlie, D.P., Liras, S. and Price, D., 2013. The Future of Peptide-Based Drugs. *Chemical Biology & Drug Design*, 81(1):136-147.
- Counis, R., Laverrière, J.-N., Garrel, G., Bleux, C., Cohen-Tannoudji, J., Lerrant, Y., Kottler, M.-L. & Magre, S. 2005. Gonadotropin-Releasing Hormone and the Control of Gonadotrope Function. *Reproduction Nutrition Development*, 45(3):243-254.

de Lange Davies, C Confocal Laser Scanning Microscopy (CLSM).

[https://www.ntnu.edu/physics/biophysmedtech/methods/-/asset\\_publisher/UczAeR4bgbvq/content/confocal-laser-scanning-microscopy-clsm-?redirect=http%3A%2F%2Fwww.ntnu.edu%2Fweb%2Fphysics%2Fbiophysmedtech%2Fmethods%3Fp\\_p\\_id%3D101\\_INSTANCE\\_UczAeR4bgbvq%26p\\_p\\_lifecycle%3D0%26p\\_p\\_state%3Dnormal%26p\\_p\\_mode%3Dview%26p\\_p\\_col\\_id%3Dcolumn-3%26p\\_p\\_col\\_pos%3D1%26p\\_p\\_col\\_count%3D2](https://www.ntnu.edu/physics/biophysmedtech/methods/-/asset_publisher/UczAeR4bgbvq/content/confocal-laser-scanning-microscopy-clsm-?redirect=http%3A%2F%2Fwww.ntnu.edu%2Fweb%2Fphysics%2Fbiophysmedtech%2Fmethods%3Fp_p_id%3D101_INSTANCE_UczAeR4bgbvq%26p_p_lifecycle%3D0%26p_p_state%3Dnormal%26p_p_mode%3Dview%26p_p_col_id%3Dcolumn-3%26p_p_col_pos%3D1%26p_p_col_count%3D2). Date of access: 8 June 2018.

Derendorf, H., Lesko, L.J., Chaikin, P., Colburn, W.A., Lee, P., Miller, R., Powell, R., Rhodes, G., Stanski, D. & Venitz, J. 2000. Pharmacokinetic/Pharmacodynamic Modelling in Drug Research and Development. *Journal of Clinical Pharmacology*, 40(12 Pt 2):1399-1418.

Di Pasquale, A., Preiss, S., Tavares Da Silva, F. & Garçon, N. 2015. Vaccine Adjuvants: from 1920 to 2015 and Beyond. *Vaccines*, 3(2):320-343.

Dorkoosh, F.A., Verhoef, J., Borchard, G., Rafiee-Tehrani, M. & Junginger, H.E. 2001. Development and characterization of a novel oral peptide drug delivery system. *Journal of Controlled Release*, 71(3):307-318.

Dorkoosh, F.A., Borchard, G., Rafiee-Tehrani, M., Verhoef, J.C. & Junginger, H.E. 2002. Evaluation of Superporous Hydrogel (SPH) and SPH composite in Porcine Intestine ex-vivo: Assessment of Drug Transport, Morphology Effect, and Mechanical Fixation to Intestinal Wall. *European Journal of Pharmaceutics and Biopharmaceutics*, 53(2):161-166.

Dun, E.C. & Taylor, H.S. 2017. Elagolix: a Promising Oral GnRH antagonist for Endometriosis-Associated Pain. *Oncotarget*, 8(59):99219.

Engin, K.N. 2009. Alpha-Tocopherol: Looking Beyond an Antioxidant. *Molecular Vision*, 15:855.

Fasano, A. 1998. Innovative Strategies for the Oral Delivery of Drugs and Peptides. *Trends in Biotechnology*, 16(4):152-157.

Fosgerau, K. & Hoffmann, T. 2015. Peptide Therapeutics: Current Status and Future Directions. *Drug Discovery Today*, 20(1):122-128.

Gibhard, L. 2012. The Effect of Pheroid® Technology on the Bioavailability of Quinoline- Mased Anti-Malarial Compounds in Primates. Potchefstroom: NWU (Thesis – PhD.)

Gray, A.H., Wright, J., Goodey, V. & Bruce, L. 2010. Injectable Drugs Guide: *Pharmaceutical Press*: 399

Grobler, A.F. 2004. Emzaloid™ technology. Potchefstroom North West Univeristy [Confidential: concept document]

- Grobler, A.F. 2008. Pharmaceutical Applications of Pheroid® Technology. Potchefstroom: North West University
- Grobler, A.F. 2009. Pharmaceutical Applications of Pheroid® Technology. North-West University.
- Grobler, A.F. 2013. Nano & Micro-Delivery Systems : the Pheroid® story: 2013.
- Gwinn, M.R. & Vallyathan, V. 2006/ Nanoparticles: Health Effects—Pros and Cons. *Environmental Health Perspectives*, 114(12):1818-1825.
- Hamman, J.H. 2012. Creating Opportunities from Pharmacokinetic Challenges: Potchefstroom : Noordwes-Universiteit, Potchefstroomkampus.
- Hamman, J.H., Enslin, G.M. & Kotzé, A.F. 2005. Oral Delivery of Peptide drugs. *BioDrugs*, 19(3):165-177.
- Henninot, A., Collins, J.C. & Nuss, J.M. 2017. The Current State of Peptide Drug Discovery: Back to The Future? *Journal of Medicinal Chemistry*, 61(4):1382-1414.
- Hayden, C. 2008. GnRH analogues: Applications in Assisted Reproductive Techniques. *European Journal of Endocrinology*, 159(suppl 1):17-25.
- Hettiarachchy, N.S., Sato, K., Marshall, M.R. & Kannan, A. 2012. Food Proteins and Peptides: Chemistry, Functionality, Interactions, and Commercialization: CRC Press.p 410
- Hillery, A.M., Lloyd, A.W. & Swarbrick, J. 2002. Drug Delivery and Targeting: for Pharmacists and Pharmaceutical Scientists: CRC Press. p. 164 - 166
- Hooton, D., Lentle, R., Monro, J., Wickham, M. & Simpson, R. 2015. The Secretion and Action of Brush Border Enzymes in the Mammalian Small Intestine. *Reviews of Physiology, Biochemistry and Pharmacology. Springer.* p. 59-118.
- Houde, A., Kademi, A. & Leblanc, D. 2004. Lipases and their Industrial Applications. *Applied Biochemistry and Biotechnology*, 118(1-3):155-170.
- Jain, K. 2008. Methods in Molecular Biology-Drug Delivery Systems. *Drug Delivery Systems-an Overview*, ed. KK Jain. Hatfield, Herts, UK: Humana Press: 20.
- Jana, S. & Jana, S. 2017. Particulate Technology for Delivery of Therapeutics: *Springer*.p. 10 - 30
- Jensen, S.C. & Peppers, M.P. 2013. Pharmacology and Drug Administration for Imaging Technologists-E-Book: *Elsevier health sciences*.

Jonat, W., Kaufmann, M., Sauerbrei, W., Blamey, R., Cuzick, J., Namer, M., Fogelman, I., Haes, J.C.d., Matteis, A.d., Stewart, A., Eiermann, W., Szakolczai, I., Palmer, M., Schumacher, M., Geberth, M. & Lisboa, B. 2002. Goserelin versus Cyclophosphamide, Methotrexate, and Fluorouracil as Adjuvant Therapy in Premenopausal Patients with Node-Positive Breast Cancer: The Zoladex Early Breast Cancer Research Association Study. *Journal of Clinical Oncology*, 20(24):4628-4635.

Jones, R.E. & Lopez, K.H. 2013. Human reproductive biology. 4<sup>th</sup> ed. London: Academic Press: 15 - 21

Jitendra, Sharma, P.K., Bansal, S. & Banik, A. 2011. Noninvasive Routes of Proteins and Peptides Drug Delivery. *Indian Journal of Pharmaceutical Sciences*, 73(4):367-375.

Krishnaiah, Y., Perry, G., Hong, H., Sörgel, F., Smith, M.A., Bhoop, B.S., Seto-Young, D., Avery, B.A., Kanwar, J.R. & Geng, B. 2011. Statistical Issues in Bioavailability/Bioequivalence Studies. *Journal of bioequivalence & bioavailability*:

[https://www.researchgate.net/profile/Laszlo\\_Endrenyi/publication/270063965\\_Statistical\\_Issues\\_in\\_BioavailabilityBioequivalence\\_Studies/links/55e51f2108aecb1a7ccb9586.pdf](https://www.researchgate.net/profile/Laszlo_Endrenyi/publication/270063965_Statistical_Issues_in_BioavailabilityBioequivalence_Studies/links/55e51f2108aecb1a7ccb9586.pdf). Date of access: 17

May 2018

Krause, R.G., Grobler, A.F. & Goldring, J.D. 2015. Comparing Antibody Responses in Chickens Against *Plasmodium Falciparum* Lactate Dehydrogenase and Glyceraldehyde-3-phosphate Dehydrogenase with Freund's and Pheroid® Adjuvants. *Immunological investigations*, 44(7):627-642.

Lambalk, C., Leader, A., Olivennes, F., Fluker, M., Andersen, A.N., Ingerslev, J., Khalaf, Y., Avril, C., Belaisch-Allart, J. & Roulier, R. 2005. Treatment with the GnRH Antagonist Ganirelix Prevents Premature LH Rises and Luteinization in Stimulated Intrauterine Insemination: Results of a Double-Blind, Placebo-Controlled, and Multicentre Trial. *Human Reproduction*, 21(3):632-639.

Langel, U., Cravatt, B.F., Graslund, A., Von Heijne, N., Zorko, M., Land, T. & Niessen, S. 2009. Introduction to peptides and proteins: CRC Press: p 5 – 12

Laidler, P. & Farkas, I. 2013. *Hydrochloride salt of peptide and its use in combination with other peptides for immunotherapy*: (Patent: US 9657061B2)

Lasseby, K. & Harvey, M. 2007. Nitrous Oxide: the Serious Side of Laughing Gas. *Water Atmosphere*, 15(2):10-11.

Lau, J.L. & Dunn, M.K. 2018. Therapeutic Peptides: Historical perspectives, current development trends, and future directions. *Bioorganic & medicinal chemistry*.p. 2700-2707

- Lee, M.-T.G., Wang, H.-M., Ho, J.-A.A., Fan, N.-C., Yang, Y.-L., Lee, C.-C. Chen, S.-C. 2015. Resuscitation Using Liposomal Vasopressin in an Animal Model of Uncontrolled Hemorrhagic Shock. *PloS One*, 10(7):p. e0130655.
- Lee, V. 1990. Peptide and Protein Drug Delivery. Vol. 4: CRC Press.p. 480 - 490
- Liu, D.Z., Lecluyse, E.L. & Thakker, D.R. 1999. Dodecylphosphocholine-Mediated Enhancement of Paracellular Permeability and Cytotoxicity in Caco-2 Cell Monolayers. *Journal of Pharmaceutical Sciences*, 88(11):1161-1168.
- Lohar, V., Singhal, S. & Arora, V. 2012. Approach to Better Drug Delivery. *International Journal of Pharmaceutical Research*, 4(1):15-21.
- Ma, J.K. & Hadzija, B. 2012. Basic physical pharmacy: Jones & Bartlett Publishers: 387
- Mahajan, A., Rawat, A.S., Bhatt, N. & Chauhan, M.K. 2014. Structural Modification of Proteins and Peptides. *Indian Journal of Pharmaceutical Education and Research*, 48(3):34-47.
- Matthee, L.I. 2007. Preclinical Evaluation of the Possible Enhancement of the Efficacy of Antituberculosis Drugs by Pheroid™. Potchefstroom: NWU (Thesis- M.Sc)
- Moghimi, S.M. & Farhangrazi, Z.S. 2014. Chapter 6 - Nanoparticles in Medicine: Nanoparticle Engineering for Macrophage Targeting and Nanoparticles that Avoid Macrophage Recognition. Nanoparticles and the Immune System. *San Diego: Academic Press*: p. 77-89.
- Moghissi, K.S., Schlaff, W.D., Olive, D.L., Skinner, M.A. & Yin, H. 1998. Goserelin Acetate (Zoladex) with or without Hormone Replacement Therapy for the Treatment of Endometriosis. *Fertility and Sterility*, 69(6):1056-1062.
- Moore, H.C.F., Unger, J.M., Phillips, K.-A., Boyle, F., Hitre, E., Porter, D., Francis, P.A., Goldstein, L.J., Gomez, H.L., Vallejos, C.S., Partridge, A.H., Dakhil, S.R., Garcia, A.A., Gralow, J., Lombard, J.M., Forbes, J.F., Martino, S., Barlow, W.E., Fabian, C.J. & Minasian, L. 2015. Goserelin for Ovarian Protection During Breast-Cancer Adjuvant Chemotherapy. *New England Journal of Medicine*, 372(10):923-932.
- Muheem, A., Shakeel, F., Jahangir, M.A., Anwar, M., Mallick, N., Jain, G.K., Warsi, M.H. & Ahmad, F.J. 2016. A Review on the Strategies for Oral Delivery of Proteins and Peptides and their Clinical Perspectives. *Saudi Pharmaceutical Journal*, 24(4):413-428.
- Muneer, S., Masood, Z., Butt, S., Anjum, S. & Zainab, H. 2017. Proliposomes as Pharmaceutical Drug Delivery System: A Brief Review. *Journal of Nanomedical and Nanotechnology*, 8(448):2.

- Naheed, B., Uthman, O.A., O'Mahony, F., Kuiper, J.H. & O'Brien, P.M.S. 2014. Gonadotropin-Releasing Hormone (GnRH) Analogues for Premenstrual Syndrome (PMS). *The Cochrane Library*.
- Novikov, B.N., Grimsley, J.K., Kern, R.J., Wild, J.R. & Wales, M.E. 2010. Improved Pharmacokinetics and Immunogenicity Profile of Organophosphorus Hydrolase by Chemical Modification with Polyethylene Glycol. *Journal of Controlled Release*, 146(3):318-325.
- Oberholzer, I.D. 2009. Oral and Nasal Delivery of Insulin with Pheroid® Technology. Potchefstroom: NWU.(Thesis – PhD)
- Odenwald, M.A. & Turner, J.R. 2017. The Intestinal Epithelial Barrier: a Therapeutic Target? *Nature Reviews Gastroenterology and Hepatology*, 14(1):9.
- Owens, D.R., Zinman, B. & Bolli, G., 2003. Alternative Routes of Insulin Delivery. *Diabetic Medicine*, 20(11):886-898.
- Padula, A. 2005. GnRH Analogues—Agonists and Antagonists. *Animal Reproduction Science*, 88(1-2):115-126.
- Pauletti, G.M., Gangwar, S., Knipp, G.T., Nerurkar, M.M., Okumu, F.W., Tamura, K., Siahaan, T.J. & Borchardt, R.T. 1996. Structural Requirements for Intestinal Absorption of Peptide Drugs. *Journal of Controlled Release*, 41(1-2):3-17.
- Perry, C.M. & Brogden, R.N. 1996. Goserelin. *Drugs*, 51(2):319-346.
- Pytlík, W. 2014. The Growing Significance of Peptide Therapeutics. <https://www.gesundheitsindustriew.de/en/article/news/the-growing-significance-of-peptidetherapeutics/>. Date of access: 23 October 2017.
- Rajput, S., Agrawal, P., Pathak, A., Shrivastava, N., Baghel, S.S. & Baghel, R.S. 2012. A Review on Microspheres: Methods of Preparation and Evaluation. *World Journal of Pharmacy and Pharmaceutical Sciences*, 1(1):422-438.
- Reddy, M.K. 2018. Amino Acid Chemical Compound. Encyclopedia Britannica
- Renukuntla, J., Vadlapudi, A.D., Patel, A., Boddu, S.H. & Mitra, A.K. 2013. Approaches for Enhancing Oral Bioavailability of Peptides and Proteins. *International Journal of Pharmaceutics*, 447(1-2):75-93.
- Richter, W.F. & Jacobsen, B. 2014. Subcutaneous Absorption of Biotherapeutics: Knowns and Unknowns. *Drug Metabolism and Disposition*, 42(11):1881-1889.
- Rizvi, S., Raza, S.T., Ahmed, F., Ahmad, A., Abbas, S. & Mahdi, F. 2014. The Role of Vitamin E in Human Health and Some Diseases. *Sultan Qaboos University Medical Journal*, 14(2):e157.

- Roland, I., Piel, G., Delattre, L. & Evrard, B. 2003. Systematic Characterization of Oil-in-Water Emulsions for Formulation Design. *International Journal of Pharmaceutics*, 263(1–2):85-94.
- Sarciaux, J., Acar, L. & Sado, P. 1995. Using Microemulsion Formulations for Oral Drug Delivery of Therapeutic Peptides. *International Journal of Pharmaceutics*, 120(2):127-136.
- Sastry, S.V., Nyshadham, J.R. & Fix, J.A. 2000. Recent Technological Advances in Oral Drug Delivery—a Review. *Pharmaceutical Science & Technology Today*, 3(4):138-145.
- Şenel, S. & Hincal, A.A. 2001. Drug Permeation Enhancement via Buccal Route: Possibilities and Limitations. *Journal of Controlled Release*, 72(1):133-144.
- Shaji, J. & Patole, V. 2008. Protein and Peptide Drug Delivery: Oral Approaches. *Indian Journal of Pharmaceutical Sciences*, 70(3):269-277.
- Shargel, L., Andrew, B. & Wu-Pong, S. 2012. Applied Biopharmaceutics and Pharmacokinetics. 7<sup>th</sup>. Vol. 270: McGraw-Hill New York:.
- Sharma, P. & Asthana, A. 2016 Hydrogel Advancement: A New Approach for Gastroretentive Drug Delivery. *International Journal of Pharmaceutical and Clinical Research*; 8(10): 1418-1422
- Sharom, F.J., DiDiodato, G., Yu, X. & Ashbourne, K.J. 1995. Interaction of the P-Glycoprotein Multidrug Transporter with Peptides and Ionophores. *Journal of Biological Chemistry*, 270(17):10334-10341.
- Smith, M.S. 2010. Estrus and Menstrual Cycles: Neuroendocrine Control. *Encyclopedia of Neuroscience. Elsevier Ltd*
- Srikanth, K., Vankadari, R.M.G. & Devanna, N. 2012. Particulate Carrier Systems: A Review. *International Research. Journal of Pharmacy*, 3, pp.22-26.
- Stadnyk, A.W. 2009. Intestinal Epithelial Cells: Immunological Aspects. *Essential for Life Science*. <https://onlinelibrary.wiley.com/doi/abs/10.1038/npg.els.0003816>. Date of access: 11 May 2018
- Steyn, D., du Plessis, L.H. & Kotzé, A. 2010. Nasal Delivery of Recombinant Human Growth Hormone: in vivo Evaluation with Pheroid® Technology and N-trimethyl Chitosan Chloride. *Journal of pharmacy & pharmaceutical sciences*, 13(2):263-273.
- Steyn, J.D., Wiesner, L., Du Plessis, L.H., Grobler, A.F., Smith, P.J., Chan, W.-C., Haynes, R.K. & Kotzé, A.F. 2011. Absorption of the Novel Artemisinin Derivatives Artemisone and Artemiside: Potential Application of Pheroid™ Technology. *International Journal of Pharmaceutics*, 414(1-2):260-266.
- Strauss, T. 2005. Oral Delivery of Calcitonin with Pheroid® Technology. Potchefstroom (NWU). (Thesis - M.Sc)

- Tan, M.E., Li, J., Xu, H.E., Melcher, K. & Yong, E.-I. 2015. Androgen Receptor: Structure, Role in Prostate Cancer and Drug Discovery. *Acta Pharmacologica Sinica*, 36(1):3-23.
- Thakur, D. 2016. Microspheres. <https://www.slideshare.net/DivyaThakur17/microspheres-67311278>. Date of access: 9 October 2018.
- Trivedi, P., Verma, A. & Garud, N. 2008. Preparation and Characterization of Aceclofenac Microspheres. *Asian Journal of Pharmaceutics*: 2(2).110-115
- Tur-Kaspa, I. & Ezcurra, D. 2009. GnRH Antagonist, Cetrorelix, for Pituitary Suppression in Modern, Patient-Friendly Assisted Reproductive Technology. *Expert Opinion on Drug Metabolism & Toxicology*, 5(10):1323-1336.
- Van Der Walle, C. 2011. Peptide and protein delivery: Academic Press.
- van Huyssteen, E. 2010. Efficacy Enhancement of the Antimalarial Drugs, Mefloquine and Artesunate, with Pheroid™ Technology. Potchefstroom (NWU). (Thesis – M.Sc)
- Van Poppel, H., Tombal, B., Jean, J., Persson, B.-E., Jensen, J.-K. & Olesen, T.K. 2008. Degarelix: a Novel Gonadotropin-Releasing Hormone (GnRH) Receptor Blocker—Results from a 1-yr, Multicentre, Randomised, Phase 2 Dosage-Finding Study in the Treatment of Prostate Cancer. *European Urology*, 54(4):805-815. p. 102 - 105
- Verhoef, J.J.F. & Anchordoquy, T.J. 2013. Questioning the Use of PEGylation for Drug Delivery. *Drug Delivery and Translational Research*, 3(6):499-503.
- Vermani, K. & Garg, S. 2000. The Scope and Potential of Vaginal Drug Delivery. *Pharmaceutical Science & Technology Today*, 3(10):359-364.
- Vogelson, C.T. 2001. Advances in Drug Delivery Systems. *Modern Drug Discovery*, 4(4):49-50.
- Wang, J., Yadav, V., Smart, A.L., Tajiri, S. & Basit, A.W. 2015. Toward Oral Delivery of Biopharmaceuticals: An Assessment of the Gastrointestinal Stability of 17 Peptide Drugs. *Molecular Pharmaceutics*, 12(3):966-973.
- Wang, B., Hu, L. & Siahaan, T.J. 2016. Drug Delivery: Principles and Applications: John Wiley & Sons.
- Wang, J., Wu, D. & Shen, W.C. 2002. Structure-Activity Relationship of Reversibly Lipidized Peptides: Studies of Fatty Acid-Desmopressin Conjugates. *Pharmaceutical Research*, 19(5):609-614.

- Wang, J., Yadav, V., Smart, A.L., Tajiri, S. & Basit, A.W. 2015. Toward Oral Delivery of Biopharmaceuticals: an Assessment of the Gastrointestinal Stability of 17 peptide drugs. *Molecular Pharmaceutics*, 12(3):966-973.
- Weisman, K. & Goldberg, M.E. 2001. Therapeutic Uses of Abarelix. (Patent : US 6197337B1)
- Werle, M. & Bernkop-Schnürch, A. 2006. Strategies to Improve Plasma Half Life Time of Peptide and Protein Drugs. *Amino Acids*, 30(4):351-367.
- WHO. 2016. WHO/EMP/RHT/TSN/2016.1 International Nonproprietary Names (INN) for Biological and Biotechnological Substances. Date of access 18 Sep 2018 2018.
- Wilding, I. 2000. Site-Specific Drug Delivery in the Gastrointestinal Tract. *Critical Review Therapeutic Drug Carrier System*, 17(6):557-620.
- Witt, K.A., Huber, J.D., Egleton, R.D., Roberts, M.J., Bentley, M.D., Guo, L., Wei, H., Yamamura, H.I. & Davis, T.P. 2001. Pharmacodynamic and Pharmacokinetic Characterization of Poly (Ethylene Glycol) Conjugation to Met-Enkephalin Analog [D-Pen2, D-Pen5]-Enkephalin (DPDPE). *Journal of Pharmacology and Experimental Therapeutics*, 298(2):848-856.
- Yamamoto, A., Taniguchi, T., Rikyuu, K., Tsuji, T., Fujita, T., Murakami, M. & Muranishi, S. 1994. Effects of Various Protease Inhibitors on the Intestinal Absorption and Degradation of Insulin in Rats. *Pharmaceutical Research*, 11(10):1496-1500.
- Yin, N., Brimble, M., Harris, P. & Wen, J. 2014. Enhancing the Oral Bioavailability of Peptide Drugs by Using Chemical Modification and Other Approaches. *Medical Chemistry*, 4:763-769.
- Zhang, H., Wang, W., Li, H., Peng, Y. & Zhang, Z. 2018. Microspheres for the Oral Delivery of Insulin: Preparation, Evaluation and Hypoglycaemic Effect in Streptozotocin-Induced Diabetic Rats. *Drug Development and Industrial Pharmacy*, 44(1):109-115.
- Zhu, H., Li, B.V., Uppoor, R.S., Mehta, M. & Yu, L.X. 2017 Chapter 14 - Bioavailability and Bioequivalence. *Developing Solid Oral Dosage Forms (Second Edition) Boston: Academic Press*. p. 381-397).
- Zhu, L., Lu, L., Wang, S., Wu, J., Shi, J., Yan, T., Xie, C., Li, Q., Hu, M. & Liu, Z 2017. Chapter 11 Oral Absorption Basics: Pathways and Physicochemical and Biological Factors Affecting Absorption A2 - Qiu, Yihong. (In Chen, Y., Zhang, G.G.Z., Yu, L. & Mantri, R.V., eds. *Developing Solid Oral Dosage Forms (Second Edition)*. Boston: Academic Press. p. 297-329).

# CHAPTER 3

# CHAPTER 3: CHARACTERISATION AND STABILITY OF GOSERELIN ENTRAPPED IN PHEROID®

## ABSTRACT

Successful oral delivery of therapeutic peptides and proteins is hindered by several factors, such as the extensive hepatic first-pass metabolism, the harsh environment of the gastrointestinal tract, and poor permeation across the mucosa. Different techniques and strategies can be implemented to improve the bioavailability and protect the peptides and proteins from degradation, such as using a drug delivery system, chemical modifications, enzyme inhibitors, etc. This study focused on the use of a colloidal drug delivery system, termed Pheroid® technology, to protect the peptide goserelin in a simulated gastric fluid (SGF) and simulated intestinal fluid (SIF). The aim of this study was to evaluate the effect of entrapment of goserelin in Pheroid®, on the particle size, stability and morphology of Pheroid®. A study was also conducted to determine the effect of entrapment of goserelin in Pheroid® on the stability in gastrointestinal fluids by using SGF and SIF; although results could not be obtained for this due to speculated sample loss. The addition of goserelin to Pheroid® resulted in a slight increase in particle size from 0.187 µm to 0.217 µm and the zeta potential slightly increased from -25.1 mV to -22.3 mV. In contrast, the confocal particle size increased from 844 nm to 699 nm, however, confocal was only used as a tool for visualisation of morphology. In this sense, confocal imaging revealed no change in the morphology of the Pheroid® vesicles and microsponges with the addition of goserelin. In conclusion, the addition of goserelin did not have a considerable effect on the physicochemical characteristics of Pheroid®.

## 1. INTRODUCTION

The amphiphilic decapeptide goserelin is a synthetic analogue of the gonadotropin-releasing hormone (GnRH), also known as a GnRH agonist (Kiesel *et al.*, 2002). Various hormone-dependent disorders, such as benign conditions (e.g. uterine fibroids, menorrhagia, endometriosis etc.) or malignant tumours (breast, ovarian, endometrial and prostate carcinoma), are effectively treated with GnRH analogues (Kiesel *et al.*, 2002). GnRH agonists, such as goserelin, are primarily administered via parenteral routes, such as the intravenous, subcutaneous and intramuscular routes (Padula, 2005), however, these routes are a more invasive administration method due to injections and implants not being well tolerated by patients. This results in patient compliance being generally poor, leading to a restricted therapeutic value of the drug. Therefore, a need for less invasive administration routes exists, such as peroral route (Almeida & Souto, 2007; Shaji & Patole, 2008).

Therapeutic peptide drugs, such as goserelin, face several challenges after peroral administration, such as degradation due to enzymes present in the gastrointestinal (GI) tract and crossing the intestinal mucosa (Fosgerau & Hoffmann, 2015). Wang *et al.*, (2015) demonstrated that goserelin rapidly degraded within 2 to 10 minutes in human gastric fluid (HGF), pig gastric fluid (PGF) and

simulated gastric fluid (SGF). However, strategies can be implemented to improve the bioavailability of peptide drugs, such as formulation and chemical technologies. One example of formulation technology is the use of a particulate drug delivery system (DDS) to protect the drug from degradation. This study focuses on the use of one such system called Pheroid<sup>®</sup> technology. Pheroid<sup>®</sup> technology is novel, colloidal drug delivery system, typically consisting of an oil phase ( $\alpha$ -tocopherol, Kolliphor<sup>®</sup>-EL and vitamin F ethyl ester) and an aqueous phase (Steyn *et al.*, 2011). The Pheroid<sup>®</sup> system is capable of targeting specific treatment areas, transporting genetic material to the cell nucleus and decreasing drug resistance, all this while improving the delivery of dynamic complexes, reducing the time of onset of action, decreasing the minimal effective drug concentration and enhancing the therapeutic effect (Grobler *et al.*, 2008). Pheroid<sup>®</sup> technology has previously been investigated as a DDS for other peptides successfully. For example, Steyn *et al.*, (2010) showed that Pheroid<sup>®</sup> improved the nasal absorption and the bioavailability of recombinant human growth hormone. Oberholzer (2009) conducted a study with Pheroid<sup>®</sup> technology and insulin and found that with Pheroid<sup>®</sup> as a DDS, insulin had sufficient therapeutic effect, proving that the effectiveness of oral administration of proteins can be enhanced by Pheroid<sup>®</sup> technology. Pro-Pheroid<sup>®</sup>, an alternative to Pheroid<sup>®</sup> technology, lacks the water phase, meaning it only consists of the N<sub>2</sub>O-saturated oil phase (Grobler, 2009). On addition of a water phase, vesicles and/or other lipid aggregates of Pheroid<sup>®</sup> micro and nanoparticles form spontaneously (Grobler, 2009; Grobler, 2013). When pro-Pheroid<sup>®</sup> is administered orally, the water phase is added *in situ* by the fluid present in the stomach and intestinal fluid, thus leading to the Pheroid<sup>®</sup> formation (Grobler, 2009). The particle size analysis will give an average diameter of the vesicles; this is important to know as the particle size can influence the absorption and dissolution of a drug. Pheroid<sup>®</sup> is typically formulated to have a diameter of between 200 nm and 2  $\mu$ m (Grobler, 2009).

The aim of the study was to explore the use of Pheroid<sup>®</sup> technology as a DDS for goserelin. Physicochemical characterisations were used to prove entrapment of goserelin in Pheroid<sup>®</sup> and stability tests in simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) were used to investigate whether entrapment of goserelin in Pheroid<sup>®</sup> would protect it from degradation in the gastrointestinal (GI) environment.

## **2. MATERIALS AND METHODS**

### *2.1 Chemicals*

Goserelin acetate powder (C<sub>61</sub>H<sub>88</sub>N<sub>18</sub>O<sub>16</sub>) molecular weight: 1269.41 g/mol was obtained from Bachem AG Budendorf. Pro-Pheroid<sup>®</sup> ingredients: vitamin F ethyl ester, dl- $\alpha$ -tocopherol and Kolliphor<sup>®</sup> EL were obtained from Chemipo (South Africa), Chempure (South Africa), BASF Chemicals (South Africa) respectively. Potassium phosphate monobasic, n-hexane, methyl alcohol reagent grade, pepsin from porcine gastric mucosa, pancreatin from porcine pancreas (activity at

least three times more than USP specification), sodium chloride, sodium hydroxide was obtained from Sigma-Aldrich® (South Africa). Dimethyl sulfoxide (DSMO) was bought from Molecular Probes Inc. (United States of America). Alarelin as an internal standard (ISTD) was obtained from MedChem Express, USA (CAS number 79561-22-1, Purity > 99.61%). HPLC water was obtained from Milli-Q water purification system (Millipore SAS 67120 Molsheim, France). LC-MS grade acetonitrile, water and formic acid and HPLC grade n-butanol were obtained from Merck (Pty) Ltd, South Africa.

## *2.2 Preparation of pro-Pheroid®-goserelin*

The pro-Pheroid® was prepared by weighing and heating vitamin F ethyl ester (70 % w/w) to 70°C and Kolliphor® EL (29% w/w) to 120°C individually; when Kolliphor® EL cooled down to 70°C the two were combined and allowed to cool to 55°C; dl- $\alpha$ -tocopherol (1% w/w) was weighed and added to the cooled mixture. After the preparation of the oil phase, the goserelin was added to appropriate concentrations, this was achieved by adding the goserelin acetate into the oil phase followed by sonication to ensure a homogenous suspension. The mixture was then gassed with nitrous oxide (N<sub>2</sub>O) at 150 kPa for 4 days and afterwards stored in an amber glass bottle at 4°C until used.

## *2.3 Particle size, zeta potential and morphological measurement*

The pro-Pheroid® and pro-Pheroid®-goserelin formulation were analysed and assessed by confocal laser scanning microscopy (CLSM) for morphology, zeta-potential measurement for stability analysis and particle size distribution. The samples were prepared by diluting each individual sample with 0.1 M HCl to simulate the acid in the stomach (4% oil phase; 96% of 0.1M HCl). By the addition of 0.1 M HCl, the pro-Pheroid® is converted to Pheroid® and vesicles and microsponges are spontaneously formed. Lastly goserelin in saline was analysed by CLSM to observe and detect fluorophore characteristics.

### *2.3.1 Particle size analysis*

The mean particle size and particle size distribution of the Pheroid® vesicles and microsponges after goserelin entrapment were measured by light scattering with a particle size analyser using the Malvern Mastersizer Nano ZS (Malvern Instruments, Worcestershire, United Kingdom), and indicated as a polydisperse index. After the instrument laser was aligned with deionised water, approximately 1 mL of the diluted Pheroid® sample was added to the Malvern Mastersizer tank; three measurements were taken by the Mastersizer and the average particle diameter was reported. The samples were stirred continuously to obtain a homogeneous dispersion of the vesicles and microsponges.

### *2.3.2 Zeta potential*

To determine the stability of the emulsion, a Malvern Zetasizer Nano ZS (Malvern Instruments, Worcestershire, United Kingdom) was used to measure the zeta potential. Firstly, the Pheroid<sup>®</sup> sample was diluted 50000× with deionised water; 1 mL of the diluted sample was loaded into the cuvette and then placed in the measurement chamber of the Zetasizer. The Zetasizer analysed three measurements of zeta potential (in mV) and reported the average.

### *2.3.3 Confocal laser scanning microscopy analysis*

The morphological features of the Pheroid<sup>®</sup> vesicles and microsponges were assessed by CLSM. The images were taken with a Nikon D – eclipse microscope with a DXM 1200 digital camera with real-time imaging. 50 µL of the sample (either pro-Pheroid<sup>®</sup> or pro-Pheroid<sup>®</sup> goserelin) was labelled with 1 µL of a 1 mg/mL Nile Red fluorescent marker dissolved in dimethyl sulfoxide (DSMO). The samples were then vortex-mixed before and after incubation (15 min in the dark). For the morphology of the Pheroid<sup>®</sup> vesicles and microsponges, 20 µL of the samples was analysed on a microscope slide. This was done as follows: each of the three lasers emits a light with wavelengths 405, 488 and 543 nm respectively. Fluorescence light emissions were collected within the three wavelength bands (above 650 nm, 540-640nm, and 485-545 nm).

## *2.4 Preparation of simulated gastric and intestinal fluid*

### *2.4.1 Simulated gastric fluid*

The SGF was prepared according to USP specifications: 0.2 g sodium chloride (NaCl) was dissolved in 50 mL deionised water by gently shaking the mixture, 0.67 mL of 10 M hydrochloric acid (HCl) was added to adjust the pH of the solution to 1.21, 0.321 g pepsin was added to this and dissolved by gently shaking and stirring the mixture, subsequently, deionised water was added to make up the volume to 100 mL.

### *2.4.2 Simulated intestinal fluid*

The SIF was prepared according to USP specifications: 0.682 g monobasic potassium phosphate (NaH<sub>2</sub>PO<sub>4</sub>) was dissolved in 25 mL deionised water by gently shaking the mixture, 13.3 mL of 0.2 N sodium hydroxide (NaOH) was added to adjust the pH to 6.81 and to this 1 g pancreatin was added and dissolved by stirring gently. After the pancreatin was dissolved deionised water was added to a final volume of 100 mL.

## *2.5 Preparation of goserelin saline stock and pro-Pheroid<sup>®</sup>-goserelin stock solution*

### *2.5.1 Pro-Pheroid<sup>®</sup>-goserelin stock solution*

A stock solution of pro-Pheroid® goserelin was prepared by adding 0.7 mg of goserelin to 700 µL of pro-Pheroid® (1mg/mL) to a concentration of 0.79 mM. A 10x dilution was prepared by adding 20 µL of stock solution to 180 µL of pro-Pheroid®. 85 µL of the diluted sample was topped up to 10 mL with pro-Pheroid® to obtain a final concentration of  $6.7 \times 10^{-4}$  mM.

### 2.5.2 Goserelin saline stock solution

A stock solution of goserelin saline was prepared by adding 0.8 mg of goserelin to 800 µL of 0.9% sodium chloride (NaCl) (1mg/mL) to a concentration of 0.79 mM. A 10x dilution was prepared from this by adding 20 µL of the stock solution to 180 µL of 0.9% NaCl, 85 µL of the diluted sample was then aliquoted and topped up to 10 mL with 0.9% NaCl to obtain the final concentration of  $6.7 \times 10^{-4}$  mM.

## 2.6 Stability test

0.507 mL of the goserelin saline stock and the pro-Pheroid®-goserelin stock solution were added to 10 mL of SGF and 10mL SIF in triplicate to a concentration of  $3.4 \times 10^{-5}$  mM. This was incubated in a shaking incubator (Scientific shaking incubator) at 37°C at 9.6 g (100 rpm) for 120 minutes. 75 µL of sample was withdrawn at frequent time intervals (0, 2, 5, 10, 30, 60, 90 and 120 minutes). To this, 225 µL of ice-cold acetonitrile was added to inactivate the enzymes and allow for the quantitative determination of intact peptide remaining. The samples were centrifuged for 10 minutes at  $9\ 600 \times g$ , the supernatant was slowly removed and stored at -80°C until liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis.

## 2.7 Measurement of drug content

### 2.7.1 LC-MS/MS analysis

The chromatographic separation and mass spectrometric detection method were developed and validated according to previously described methods with some modifications (Kim *et al.*, 2010; Zhang *et al.*, 2014)

LC analyses were performed on Agilent 1290 Infinity HPLC system consisting of a binary pump with two identical high pressure (1200 bar) pumps, a two-channel solvent degasser and four-channel inlet solvent selection valve and CTC PAL HTx-xt auto sampler with a 20 µL sample loop. The analytes were separated at 25°C on Phenomenex Kinetex™ C18 column (30 mm × 2.1 mm, 1.7 µm) using gradient elution with water (A) and acetonitrile (B), both with 0.1% formic acid. The flow rate was 0.5mL/min and the gradients were as follows: 0.0 to 0.2 min: 10%B; 0.2 to 1.0 min: linear from 10 to 90%B; 1.0 to 1.40 min: 90% B; 1.40 to 1.41 min: linear from 90 to 10%B; 1.41 to 2.0 min: 10%B.

Tandem mass spectrometry was performed using SCIEX API 4000 QTRAP mass analyser equipped with a Turbo Ion Spray source (SCIEX, Toronto, Canada) operating in electrospray ionization (ESI) positive mode. The Analyst software (version 1.6) was used for instrument control, data acquisition and quantitative analyses. Detection and quantitation of analytes were achieved using multiple reaction monitoring (MRM) mode due to high selectivity and sensitivity. Optimised instrument settings were as follows: ionisation mode, positive; curtain gas, 30 psi; collision activated dissociation (CAD) gas, medium; nebuliser gas (GS1), 50psi, heater gas (GS2), 50 psi; ion spray voltage, 5.5 kV; temperature, 550°C. The nitrogen flow was produced by a gas generation system (Peak Scientific Nitrogen Generator, model AB-3G, PA, USA).

A dwell time of 200 msec was used for all transitions. MS/MS parameters were optimised by direct infusion of goserelin and alarelin standard solutions at a flow rate of 10 µL/min, in the positive electrospray ionisation (ESI) mode. Two MRM transitions were selected for goserelin, the most intense being used for quantification and the other for confirmation. One MRM transition was selected for the internal standard (alarelin). MRM of the precursor product transitions were  $m/z$  635.5 → 607.4/249.3 for goserelin and  $m/z$  584.5 → 249.1 for the internal standard. The MS/MS settings were: collision energy at 27/45volts for goserelin and 43volts for alarelin; collision exit cell potential at 30/12volts for goserelin and 18volts for alarelin; declustering potential and entrance potential were 101.0 and 10.0 volts for both goserelin and its internal standard, respectively.

## *2.8 Extraction and LC-MS/MS analysis of the supernatant*

### *2.8.1 SIF and SGF with saline goserelin*

Goserelin was extracted by a liquid-liquid extraction method. The samples were spiked with 5 µL of internal standard (alarelin) and vortex-mixed (Vortex Genie 2) briefly. To this, 300 µL of acetonitrile was added and again vortex mixed for 1 minute. Afterwards, the samples were centrifuged (Lasec HermLe Z326K) at 13 680 × g for 10 minutes and 360 µL of the supernatant were dried in an evaporator (SpeedVac) at 40°C for ± 2 hours. Next the samples were reconstituted in 10% acetonitrile and spun down at 21 380 × g. The upper layer was transferred to a vial and analysed by LC-MS/MS (4000 Q TRAP).

### *2.8.2 SIF and SGF with Pheroid®-goserelin*

The peptide was extracted from the supernatant by double protein extraction. The samples were spiked with 5 µL of the internal standard. To this, 300 µL of acetonitrile was added, vortex-mixed for 1 minute and sonicated on high for 5 minutes; 300 µL of *n*-hexane was added to the samples and vortex-mixed again for 3 minutes. After this, the samples were left for 5 minutes at room temperature. Subsequently, the samples were centrifuged at 13 680 × g for 10 minutes. The *n*-hexane layer (upper layer) was carefully removed and discarded. The supernatant was then transferred to

Eppendorf tubes. Next, the samples were dried down in the evaporator and then reconstituted in 10% acetonitrile and spun down at 21 380 × g. The upper layer was transferred to a vial and analysed by LC-MS/MS.

### 3. RESULTS AND DISCUSSION

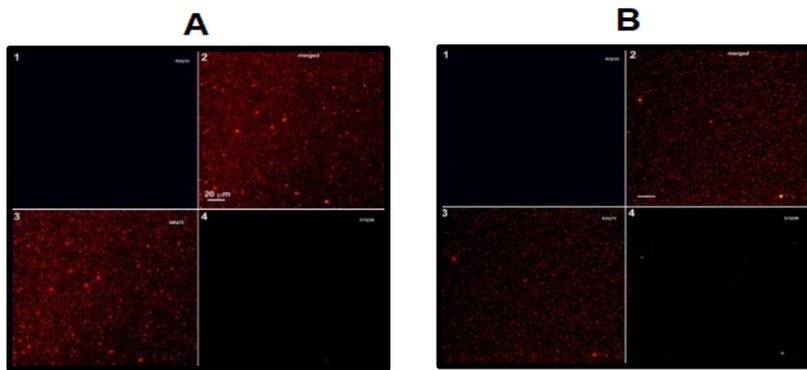
#### 3.1 Characterisation of Pheroid® and Pheroid®-goserelin

The particle size, zeta potential and confocal image average size are summarised in Table 1. D(0.5) represents the particles of which 50% of the samples were smaller and 50% larger than this size in diameter (also known as the mass median diameter(MMD)). By the addition of goserelin, the MMD increased from 0.187 µm to 0.217 µm. The zeta potential analysis demonstrated a higher zeta potential for the Pheroid®-goserelin sample compared to the Pheroid® vesicles. This indicates that by the addition of goserelin, the particle size of the Pheroid® vesicles and microsponges slightly increased, but in contrast, colloidal stability slightly decreased. According to Roland *et al.*, (2003), when the zeta potential has a high positive or negative value (+/- 25 mV), it means the emulsion is more stable, and when zeta potential has a low positive or negative value (< 25 mV) stability will consequently be lower. Hence, according to this information, the two samples are considered both stable. The average size according to the CLSM decreased with the addition of goserelin to Pheroid® from 844 nm to 699 nm. However, microscopy is not often used as a quantitative tool to determine particle size (Uys, 2006). According to Grobler *et al.*, (2009), Pheroid® vesicles and microsponges have a typical diameter range between 200 nm and 2 µm, thus the particle diameter range of these two formulations still corresponded with this criterion. The morphology of the Nile red-stained Pheroid® vesicles can be observed and confirmed as spherical shapes by the CLSM results (Figure 1). From this information, it can be concluded that the addition of goserelin to Pheroid® did not alter the typical spherical morphology of Pheroid® vesicles. When goserelin saline formulation was imaged on CLSM, no fluorescence was emitted.

**Table 4:** Average particle size diameter, zeta potential measurement and CLSM average particle size results of Pheroid® and Pheroid®-goserelin.

Pheroid® Sample	Particle size D(0.5) (mean ± standard deviation, µm) *	Zeta potential (mV) (mean ± standard deviation, mV)*	Confocal image average size (nm)
Pheroid®	0.187 ± 0.62	-25.1 ± 4.2	844
Pheroid®-goserelin	0.217 ± 0.22	- 22.3 ± 5.08	699

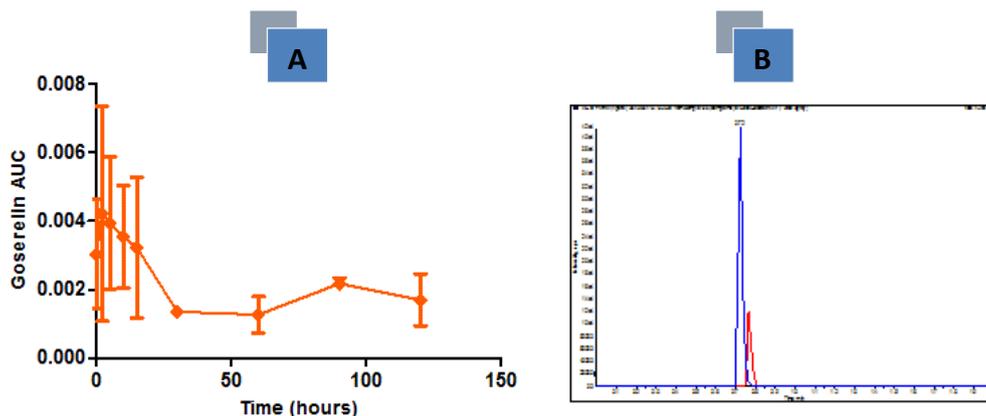
\* n = 3 measurements made by the Malvern Zetasizer and Malvern Mastersizer



**Figure 9:** Confocal images: **(A)** Pheroid<sup>®</sup> sample, **(B)** Pheroid<sup>®</sup> goserelin sample. Frames: **1)** Image obtained with a tungsten source light, **2)** Red fluorescence signal detected in wavelengths between 500 and 530 merged with that detected between 568 and 642 nm, **3)** Red fluorescence signal detected in the 568 and 642 nm waveband only and **4)** Green fluorescence signal detected of the 500 to 530 nm waveband only (scale = 20 µm).

### 3.2 Stability test

Firstly, extracted samples from the goserelin saline in SGF were analysed by LCMS/MS (a demonstration of a chromatogram of goserelin extracted from SGF is indicated in Figure 2B), and as demonstrated in Figure 2A, the area under the curve (AUC) was used for the preliminary indication of the degradation of goserelin over the period of 120 minutes. A similar degradation pattern of goserelin was described in the study done by Wang *et al.*, (2015). Complications were experienced when the SGF pro-Pheroid<sup>®</sup>-goserelin samples were analysed on the LC-MS/MS. No detectable peaks of goserelin were observed.



**Figure 10:** A. Goserelin AUC for the preliminary indication of goserelin degradation over a period of 120 minutes. B. Chromatogram of goserelin from SGF. **Red** peak: goserelin, **Blue** peak: alareinin (internal standard).

This is speculated to be due to loss of sample during centrifugation. When the samples were spun down, the Pheroid<sup>®</sup> was presumed to settle at the bottom, thus trapping the goserelin. Due to this,

the analysis of the study was halted so that the method could be modified and optimised in the near future. One proposed solution for this would be to avoid centrifuging the samples directly after incubation and subsequently adapting the extraction method accordingly to adequately dissolve the Pheroid® releasing the presumed entrapped goserelin so it can be quantified on the LC-MS/MS and effectively compared to the SIF and SGF goserelin concentration without Pheroid®.

#### **4. CONCLUSION**

From the confocal imaging, it is clear that the morphology did not change with the addition of goserelin to Pheroid®. No considerable difference was observed between the pro-Pheroid® formulation and the pro-Pheroid®-goserelin formulation particle sizes and colloidal stability. Hence, it can be concluded that the presumed entrapment of goserelin had no effect on the typical characteristics of Pheroid®. As mentioned, the stability study will have to be repeated to prevent sample loss; the extraction method of the Pheroid® samples should also be adjusted and optimised to assure detectable concentration of goserelin on the LC-MS/MS.

## REFERENCES

- Almeida, A.J. & Souto, E. 2007. Solid Lipid Nanoparticles as a Drug Delivery System for Peptides and Proteins. *Advanced Drug Delivery Reviews*, 59(6):478-490.
- Baum, M., Hackshaw, A., Houghton, J., Fornander, T., Nordenskjold, B., Nicolucci, A., Sainsbury, R. & Collaborators'Group, Z.I. 2006. Adjuvant Goserelin in Pre-Menopausal Patients with Early Breast Cancer: *European Journal of Cancer*, 42(7):895-904.
- Cho, S.B., Nakanishi, K., Kokubo, T., Soga, N., Ohtsuki, C. & Nakamura, T. 1996. Apatite Formation on Silica Gel in Simulated Body Fluid: it's Dependence on Structures of Silica Gels Prepared in Different Media. *Journal of Biomedical Materials Research: An Official Journal of The Society for Biomaterials and The Japanese Society for Biomaterials*, 33(3):145-151.
- Cockshott, I.D. 2000. Clinical Pharmacokinetics of Goserelin. *Clinical Pharmacokinetics* 39(1):27-48.
- Fosgerau, K. & Hoffmann, T. 2015. Peptide Therapeutics: Current Status and Future Directions. *Drug Discovery Today*, 20(1):122-128.
- Grobler, A., Kotzé, A. & Du Plessis, J. 2008. The Design of a Skin-Friendly Carrier for Cosmetic Compounds Using Pheroid™ Technology. *Science and Applications of Skin Delivery System Technologies Allured*. Ed. Johann Wiechers: 283-311.
- Grobler, A.F. 2009. Pharmaceutical Applications of Pheroid® Technology. North-West University (Thesis – PhD)
- Grobler, A.F. 2013. Nano & Micro-Delivery Systems: the Pheroid® story: 2013.
- Kaufmann, M., Jonat, W., Schachner-Wünschmann, E., Bastert, G. & Maass, H. 1991. The Depot GnRH Analogue Goserelin in the Treatment of Premenopausal Patients with Metastatic Breast Cancer-A 5-year Experience and Further Endocrine Therapies. *Oncology Research and Treatment*, 14(1):22-30.
- Kokubo, T., Ito, S., Huang, Z., Hayashi, T., Sakka, S., Kitsugi, T. & Yamamuro, T. 1990. Ca, P-Rich Layer Formed on High-Strength Bioactive Glass-Ceramic A-W. *Journal of Biomedical Materials Research*, 24(3):331-343.
- Kiesel, L.A., Rody, A., Greb, R.R. & Szilágyi, A. 2002. Clinical use of GnRH Analogues. *Clinical Endocrinology*, 56(6):677-687.

- Kim, M.K., Lee, T.H., Suh, J.H., Eom, H.Y., Min, J.W., Yeom, H., Kim, U., Jung, H.J., Cha, K.H., Choi, Y.S. and Youm, J.R., 2010. Development and Validation of a Liquid Chromatography–Tandem Mass Spectrometry Method for the Determination of Goserelin in Rabbit Plasma. *Journal of Chromatography B*, 878(24), pp.2235-2242.
- Laidler, P. & Farkas, I. 2013. Hydrochloride Salt of Peptide and its Use in Combination with Other Peptides for Immunotherapy: Google Patents.
- Marques, M.R., Loebenberg, R. & Almukainzi, M. 2011. Simulated Biological Fluids with Possible Application in Dissolution Testing. *Dissolution Technology*, 18(3):15-28.
- Muheem, A., Shakeel, F., Jahangir, M.A., Anwar, M., Mallick, N., Jain, G.K., Warsi, M.H. & Ahmad, F.J. 2016. A Review on the Strategies for Oral Delivery of Proteins and Peptides and Their Clinical Perspectives. *Saudi Pharmaceutical Journal*, 24(4):413-428.
- Oberholzer, I.D. 2009. Oral and Nasal Delivery of Insulin with Pheroid™ Technology. Potchefstroom: NWU. (Thesis – M.Sc.)
- Padula, A. 2005. GnRH Analogues—Agonists and Antagonists. *Animal Reproduction Science*, 88(1-2):115-126.
- Penwell, A., Sharp, K., Mansour, M. and Sammatur, L., 2012. Development and Validation of an HPLC/UV Assay for Separation and Quantification of Peptide Antigens from a Liposomal Vaccine Delivery Platform. *Journal of Pharmaceutical and Biomedical analysis*, 66, pp.176-182.
- Roach III, M. & Izaguirre, A. 2007. Goserelin Acetate in Combination with Radiotherapy for Prostate Cancer. *Expert Opinion on Pharmacotherapy*, 8(2):257-264.
- Roland, I., Piel, G., Delattre, L. & Evrard, B. 2003. Systematic Characterization of Oil-in-Water Emulsions for Formulation Design. *International Journal of Pharmaceutics*, 263(1–2):85-94.
- Shaji, J. & Patole, V. 2008. Protein and Peptide Drug Delivery: Oral Approaches. *Indian Journal of Pharmaceutical Sciences*, 70(3):269-277.
- Steyn, D., du Plessis, L.H. & Kotzé, A. 2010. Nasal Delivery of Recombinant Human Growth Hormone: *in vivo* Evaluation with Pheroid™ Technology and N-trimethyl Chitosan Chloride. *Journal of Pharmacy & Pharmaceutical Sciences*, 13(2):263-273.
- Steyn, J.D., Wiesner, L., du Plessis, L.H., Grobler, A.F., Smith, P.J., Chan, W.-C., Haynes, R.K. & Kotzé, A.F. 2011. Absorption of the Novel Artemisinin Derivatives Artemisone and Artemiside: Potential Application of Pheroid™ technology. *International Journal of Pharmaceutics*, 414(1):260-266.

Vertzoni, M., Diakidou, A., Chatziliias, M., Söderlind, E., Abrahamsson, B., Dressman, J.B. & Reppas, C. 2010. Biorelevant Media to Simulate Fluids in the Ascending Colon of Humans and Their Usefulness in Predicting Intracolonic Drug Solubility. *Pharmaceutical Research*, 27(10):2187-2196.

Wang, J., Yadav, V., Smart, A.L., Tajiri, S. & Basit, A.W. 2015. Toward Oral Delivery of Biopharmaceuticals: An Assessment of the Gastrointestinal Stability of 17 Peptide Drugs. *Molecular Pharmaceutics*, 12(3):966-973.

Werle, M. & Bernkop-Schnürch, A. 2006. Strategies to Improve Plasma Half Life time of Peptide and Protein Drugs. *Amino acids*, 30(4):351-367.

Zhang, S., Han, J., Leng, G., Di, X., Sha, C., Zhang, X., Li, Y. & Liu, W. 2014. An LC-MS/MS Method for the Simultaneous Determination of Goserelin and Testosterone in Rat Plasma for Pharmacokinetic and Pharmacodynamic Studies. *Journal of Chromatography B Analytical Technology Biomedical Life Science*, 965:183-189

Zhou, Q., Yan, H., Li, R., Li, X. and Wei, J., 2018. Quantitative Determination of Monotropein in Rat Plasma and Tissue by LC-MS/MS and its Application to Pharmacokinetic and Tissue Distribution Studies. *Revista Brasileira de Farmacognosia*, 28(4): 451 - 456

# CHAPTER 4

# CHAPTER 4: COMPARISON OF THE PHARMACOKINETICS AND PHARMACODYNAMICS OF AN ORAL FORMULATION OF GOSERELIN IN PHEROID® RELATIVE TO A SUBCUTANEOUS IMPLANT OF GOSERELIN, IN MALE MICE

## ABSTRACT

The peptide goserelin has low oral bioavailability as a result of the rapid degradation in the gastrointestinal (GI) tract. When peptides are used in combination with a drug delivery system, the oral bioavailability can be enhanced. Pheroid® technology is one such delivery system which has the ability to transport, entrap and deliver pharmaceutical active ingredients. Pharmacokinetic parameters were compared for goserelin subcutaneous implant and pro-Pheroid®-goserelin oral formulations. Pro-Pheroid®-goserelin was administered at a once-off dose of 2 mg/kg and 4 mg/kg respectively to male BALB/c mice via oral gavage. Drug and testosterone concentrations in plasma were determined by LC-MS/MS analysis. The goserelin subcutaneous group had a significantly higher  $C_{max}$  and  $T_{max}$  compared to the pro-Pheroid®-goserelin 4 mg/kg group, although the 2 mg/kg resulted in a significantly higher  $C_{max}$  than the 4 mg/kg group. All formulations displayed an initial (0 – 30 min) increase in testosterone concentration. Thus, Pheroid® technology was successfully implemented for the oral delivery of goserelin. Both 2 mg/kg and 4 mg/kg proved to have an effect on the testosterone concentration. Future studies need to be conducted to extend the time of blood sampling as well as adjust the dose for a clearer concentration-time profile.

## 1. INTRODUCTION

Peptide drugs are rapidly degraded and hydrolysed by proteolytic enzymes such as peptidase, in the gastrointestinal (GI) tract, and therefore ineffective when administered orally (Cockshot, 2000). One such drug is goserelin, which is one of the most intensively investigated gonadotropin-releasing hormone (GnRH) agonist (GnRH-a) drug and is used in addition to standard adjuvant as a type of hormone therapy (Cockshott, 2000; Baum *et al.*, 2006). GnRH agonists, such as goserelin, are safe and effective for the clinical treatment and management of advanced prostate cancer (Eckstein & Haas, 2014). Goserelin acts on the hypothalamic-pituitary axis, and because of this interaction, receptor down-regulation occurs. This then leads to the inhibition of the secretion of the pituitary gonadotropin, which in turn decrease the endogenous testosterone and oestrogen (Baum *et al.*, 2006; Jonat, 1998; Zhang *et al.*, 2014). This considerable decrease in the testosterone concentration, leads to hypogonadal status, also known as “castration level” (Eckstein & Haas, 2014.) Therapeutic goserelin, also named Zoladex®, is currently administered as a subcutaneous implant, which is injected in the extracellular space of the subcutaneous tissue, this slowly releases goserelin over a period of either one (3.6 mg) or three (10.8 mg) months (Cockshott, 2000; Steyn, 2014). Parenteral administration, such as, injections and implants are usually not well tolerated by patients due to the stress, pain and discomfort associated with the invasive administration method.

Due to this, patient compliance is generally poor, which leads to a hampered therapeutic value of the drug, hence there is a clear requirement for a less invasive administration method.

The poor peroral bioavailability of peptide and protein drugs can be overcome by implementing different strategies such as formulation and chemical technologies. Using a particulate drug delivery system (DDS) to protect peptide drugs is an example of a formulation technology. Pheroid® is one such formulation technology. Pheroid® is a colloidal drug delivery system, primarily made up of fatty acids (Kolliphor® EL, Vitamin F ethyl ester),  $\alpha$ -tocopherol and water (Steyn *et al.*, 2011). The Pheroid® system is capable of transporting genetic material to the cell nucleus, targeting specific treatment areas and decreasing drug resistance (Grobler *et al.*, 2008). Pheroid® vesicles and microsponges can entrap both lipophilic and hydrophilic drugs (Grobler *et al.*, 2007). Pheroid® has previously been successfully employed as a DDS for the nasal delivery of recombinant human growth hormone (Steyn *et al.*, 2010), for cosmetic application (Grobler *et al.*, 2007) and the transdermal delivery of insulin growth factor (van Niekerk., 2009) just to name a few. Pro-Pheroid®, an alternative to Pheroid® technology, lacks the water phase meaning it consists only of the oil phase (Grobler, 2009). By the addition of an aqueous phase, vesicles or other lipid aggregate of the Pheroid® micro and nanoparticles, form spontaneously (Grobler, 2009; Grobler, 2013). When pro-Pheroid® is administered perorally, the aqueous phase is added *in situ* by the stomach and intestinal fluid, subsequently leading to the formation of Pheroid® vesicles and microsponges (Grobler, 2009). The purpose of this study was to compare the pharmacokinetic (PK) and pharmacodynamic (PD) of the current goserelin subcutaneous implant (Zoladex®) vs. goserelin entrapped in Pheroid® vesicles and microsponges in male mice.

## 2. MATERIALS AND METHODS

### 2.1 Chemicals

Goserelin acetate powder (C<sub>61</sub>H<sub>88</sub>N<sub>18</sub>O<sub>16</sub>) molecular weight: 1269.41 g/mol was obtained from Bachem AG Budendorf. Goserelin acetate implant (Zoladex®) [D-Ser(But)<sub>6</sub>,Azgly<sub>10</sub>]LHRH molecular weight: 1269.433 g/mol was obtained from BGM Pharma (South Africa). Pro-Pheroid® ingredients, vitamin F ethyl ester, dl- $\alpha$ -tocopherol and Kolliphor®-EL, were obtained from Chemipo (South Africa), Chempure (South Africa) and BASF chemicals (South Africa) respectively. Dimethyl sulfoxide (DSMO) was purchased from Molecular Probes Inc. (United States of America).

### 2.2 Preparation of pro-Pheroid®-goserelin

Pheroid® was prepared as specified in Chapter 3. Pro-Pheroid® consists of vitamin ethyl ester (70% w/w), Kolliphor® EL (29% w/w) and  $\alpha$ -tocopherol (1% w/w). To the finished pro-Pheroid® formulation, goserelin was added for respective doses. The pro-Pheroid®-goserelin was then gassed for four days, at 150 kPa, in a small pressure vessel with nitrous oxide (N<sub>2</sub>O). The pro-Pheroid®-goserelin

formulation was analysed and assessed for the morphology using a confocal laser scanning microscopy CLSM (Nikon D-Eclipse C1), stability testing by analysing the Zeta potential with a Malvern Zetasizer NS (Malvern instruments) and the mean particle size distribution with a Malvern (Malvern instruments). The characterisation was done according to the characterisation methods described in Chapter 3

### 2.3 Animal experiments

All mouse experimental procedures were approved by the ethics committee of the North-West University, NWU-AnimCare REC, (ethics approval no. NWU-00268-16-A5). The animals utilised were male BALB/c mice weighing approximately  $15 \pm 1.0$  g, and between the ages of 6 to 8 weeks. The mice were housed in individually ventilated cages (IVC) (Techniplast) at the PCDDP Vivarium, at the Potchefstroom campus of the North-West University. These cages were equipped with rack isolator systems with input and exhaust fan filter units providing HEPA filtered inlet and outlet air. The room temperature was  $22 \pm 1$  °C with a relative humidity of 55% ( $\pm 10\%$ ) and a light/dark cycle of 12 hours, ventilation of 20 air changes per hour under positive pressure. They received water *ad libitum* and were fed standard rodent maintenance chow (NutritionHUB (Pty) Ltd), and housed on bedding derived from dust free and non-toxic exfoliated corncob chips.

### 2.4 Animal groups

The mice were randomly divided into three groups and allocated to each group as follows: Group 1 (n = 48) was the control group: each mouse in this group received a 3.6 mg subcutaneous implant (Figure 1B) of goserelin, which slowly released goserelin over a period of 28 days, resulting in 3.6 mg/28 days = 0.128 mg/day. All mice in groups 2 (n = 42) and 3 (n = 42) were fasted for 12h before the start of the study, but water was provided *ad libitum*. Group 2 received a once-off oral formulation of pro-Pheroid<sup>®</sup>-goserelin at a dose of 2 mg/kg through oral gavage, and group 3 received a once-off oral formulation of pro-Pheroid<sup>®</sup>-goserelin at a dose of 4 mg/kg through oral gavage, thus a final concentration of 2 mg/kg goserelin was administered to each mouse in group 2 and 4 mg/kg goserelin was administered to each mouse in group 3. After oral administration of the pro-Pheroid<sup>®</sup>-goserelin, the pro-Pheroid<sup>®</sup> was converted to Pheroid<sup>®</sup> by the fluids present in the stomach of the mice, which lead to the formation of vesicles and microsponges thus entrapping goserelin in these vesicles to protect it from degradation in the stomach and to aid absorption in the intestine. Blood collections for group 1 were staggered at time points: t = 0.5; 6; 24 hours and 2; 4; 7; 9; 10; 11; 13; 15; 18; 21; 24 days. Terminal blood was collected at day 28 in tubes containing ethylenediaminetetraacetic acid (EDTA). Groups 2 and 3 received a single oral pro-Pheroid<sup>®</sup>-goserelin formulation by oral gavage (Figure 1A). Terminal blood was collected for groups 1 and 2 at time points: t = 0.5; 1; 6; 24; 2; 4; 7; 9; 10; 11; 13; 15; 18; 21 and 24 hours in tubes containing EDTA. All the samples were centrifuged at  $448 \times g$  for 15 minutes; the plasma was carefully removed

and kept at  $-80^{\circ}\text{C}$  until liquid chromatography-tandem mass spectrometry (LC-MS /MS) analysis could be performed.

## 2.5 Measuring of goserelin and testosterone concentration

### 2.5.1 LC-MS/MS analysis

LC analyses were performed on a Q-Exactive Plus High-resolution Orbitrap Mass spectrometry, coupled with Ultimate 3000 UPLC. Table 1 indicates the MS parameters. The samples were separated by an Agilent Eclipse Plus C18 RRHD  $1.8\ \mu\text{m}$ ,  $2.1 \times 50\ \text{mm}$ , with a column oven temperature of  $40^{\circ}\text{C}$  using gradient elution with Millipore water 0.1 % Acetic acid (A) and Methanol 0.1 % Acetic acid (B). The flow rate was 0.5 mL/min with a total run time of 7.1 mins. Multiple reaction monitoring (MRM) of the precursor product transitions were  $m/z\ 289.21620 \rightarrow 298.21620/97.065$  for testosterone and  $m/z\ 292.23503 \rightarrow 292.23503/97.065$  for the internal standard (testosterone-D3). For goserelin and the internal standard (alarelin), the precursor product transitions were  $m/z\ 635.32800 \rightarrow 635.32800/249.0983$  and  $584.30653 \rightarrow 584.30653/249.0983$  respectively.

**Table 1:** MS parameters (electron Spray Ionization ( ESI ) -Positive Ion Mode)

<b>Sheath gas flow</b>	55 psi
<b>Auxiliary Gas flow</b>	10 psi
<b>Sweep Gas flow</b>	0 psi
<b>Capillary Temperature</b>	$320^{\circ}\text{C}$
<b>Auxiliary Heater Temp</b>	$350^{\circ}\text{C}$
<b>S-Lens RF</b>	55 v
<b>Spray Voltage</b>	4000 v

### 2.5.2 Extraction of goserelin and testosterone from plasma

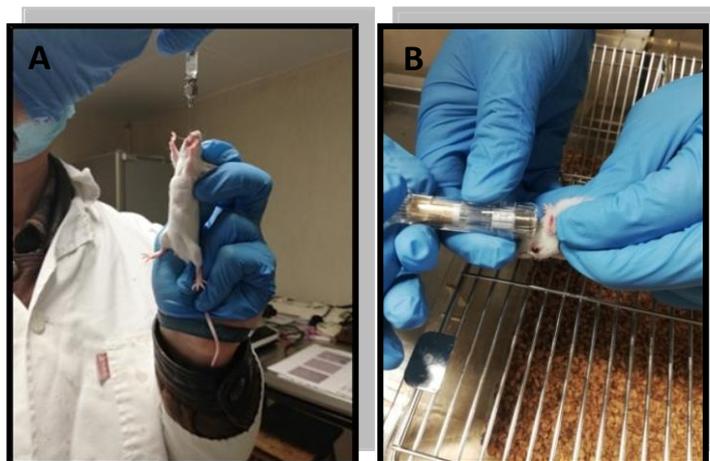
The LC-MS/MS method for the simultaneous determination of goserelin and testosterone was done similar to the previously published method by Zhang *et al.*, (2014). A  $100\ \mu\text{L}$  of rat plasma sample was pipetted into in a 15 mL centrifuge tube, then  $100\ \mu\text{L}$  of internal standard working solution,  $100\ \mu\text{L}$  of methanol-water-formic acid (60:40:0.08 v/v/v) and  $500\ \mu\text{L}$  of methanol was added. After vortexing, the mixture was centrifuged at  $5000 \times g$  for 15 min. The supernatant was mixed with  $500\ \mu\text{L}$  of water and then loaded onto the SPE cartridge, which was previously activated by successive applications of 1 mL of methanol and 1 mL of Milli-Q water. The SPE cartridge was washed with 1 mL of Milli-Q water and 1 mL of methanol-water (60:40 v/v), followed by elution with 1 mL of methanol (containing 0.01% of formic acid). The eluate was collected in a clean glass reactive vial and evaporated to dryness at  $50^{\circ}\text{C}$  under a stream of nitrogen for 10 min. The dry residue was

reconstituted with 100  $\mu$ L of methanol–water–formic acid (60:40:0.08 v/v/v), and then a 10  $\mu$ L was injected into the LC–MS/MS system.

## 2.6 Data analysis

The experimental data were evaluated in terms of the goserelin and testosterone plasma concentration vs. time and were analysed by means of the one-compartment open model analysis method using Phoenix<sup>®</sup> WinNonlin<sup>®</sup> software (Certara L.P., USA). However, WinNonlin could not fit the data, therefore the  $C_{\max}$  and  $T_{\max}$  of each individual mouse was compared and statistically analysed using a non-parametric one-way ANOVA Kruskal-Wallis test (SAS Institute Inc. 2016). Differences were considered statistically significant if  $p < 0.05$ .

The effect of goserelin on testosterone plasma concentration of all the groups was determined by measurement and comparison of the plasma testosterone levels in the initial 0 to 30 minutes. Statistical significance was assessed by a t-test using SAS software (SAS Institute Inc. 2016). Differences were considered statistically significant if  $p < 0.05$ .



**Figure 1:** Demonstration of **A)** oral gavage of pro-Pheroid<sup>®</sup> and pro-Pheroid<sup>®</sup>-goserelin, **B)** goserelin subcutaneous implant (Zoladex<sup>®</sup>).

## 3. RESULTS AND DISCUSSION

### 3.1 Characterisation of Pheroid<sup>®</sup> and Pheroid<sup>®</sup>-goserelin formulations

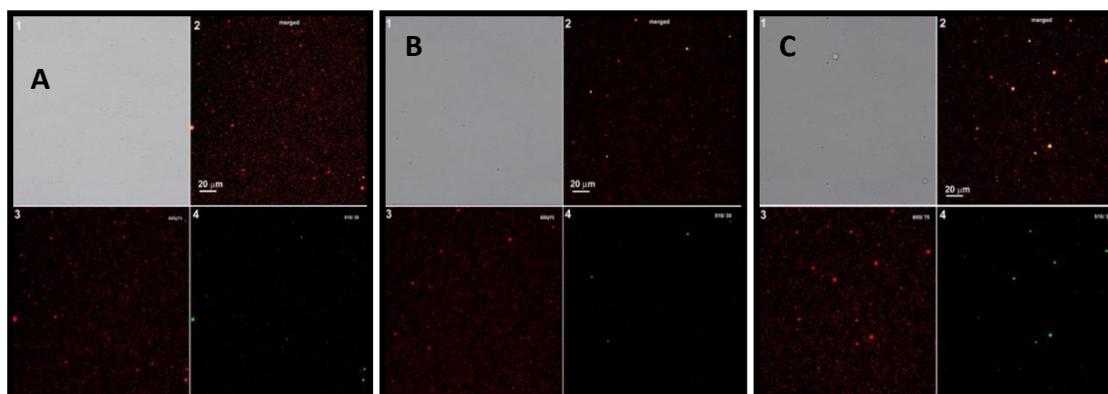
The particle size, zeta potential and confocal image average size are summarised in Table 1. D(0.5) represents the particles at which 50% of the samples are smaller and 50% larger than this size in diameter (also known as the mass median diameter(MMD)). By the addition of goserelin, the MMD decreased for both the 2 mg/kg and 4 mg/kg encapsulated vesicle dose from 0.145  $\mu$ m to 0.126 and 0.133  $\mu$ m respectively. All the formulations agreed with the typical Pheroid<sup>®</sup> diameter presented in the literature, as stated in Grobler *et al.*, (2009), thus a successful formulation

according to particle size was prepared. The slight increase of the particle size diameter may be due to dynamic nature of the Pheroid<sup>®</sup> system of the formation and disbanding of Pheroid<sup>®</sup> vesicles, higher leads to the new formation of these vesicles (Grobler *et al.*, 2007). The zeta potential was lower for the Pheroid<sup>®</sup>-goserelin 2 mg/kg formulation than the Pheroid<sup>®</sup> formulation but lower for the 4 mg/kg dose.

**Table 2:** Particle size, Zeta potential and confocal imaging average size results of Pheroid<sup>®</sup> and Pheroid<sup>®</sup>-goserelin.

Sample	D(0.5) ( $\mu\text{m}$ ) (mean $\pm$ standard deviation, $\mu\text{m}$ )*	Zeta potential (mV) (mean $\pm$ standard deviation, mV)*	Confocal image average size (nm)
Pheroid <sup>®</sup>	0.145 $\pm$ 0.120	-19.8 $\pm$ 4.73	535
2 mg/kg Pheroid <sup>®</sup> goserelin	0.126 $\pm$ 0.110	-18.3 $\pm$ 4.81	491
4 mg/kg Pheroid <sup>®</sup> goserelin	0.133 $\pm$ 122	-22.3 $\pm$ 8.18	428

\*(n = 3) measurements of the Malvern Zetasizer and Malvern Mastersizer



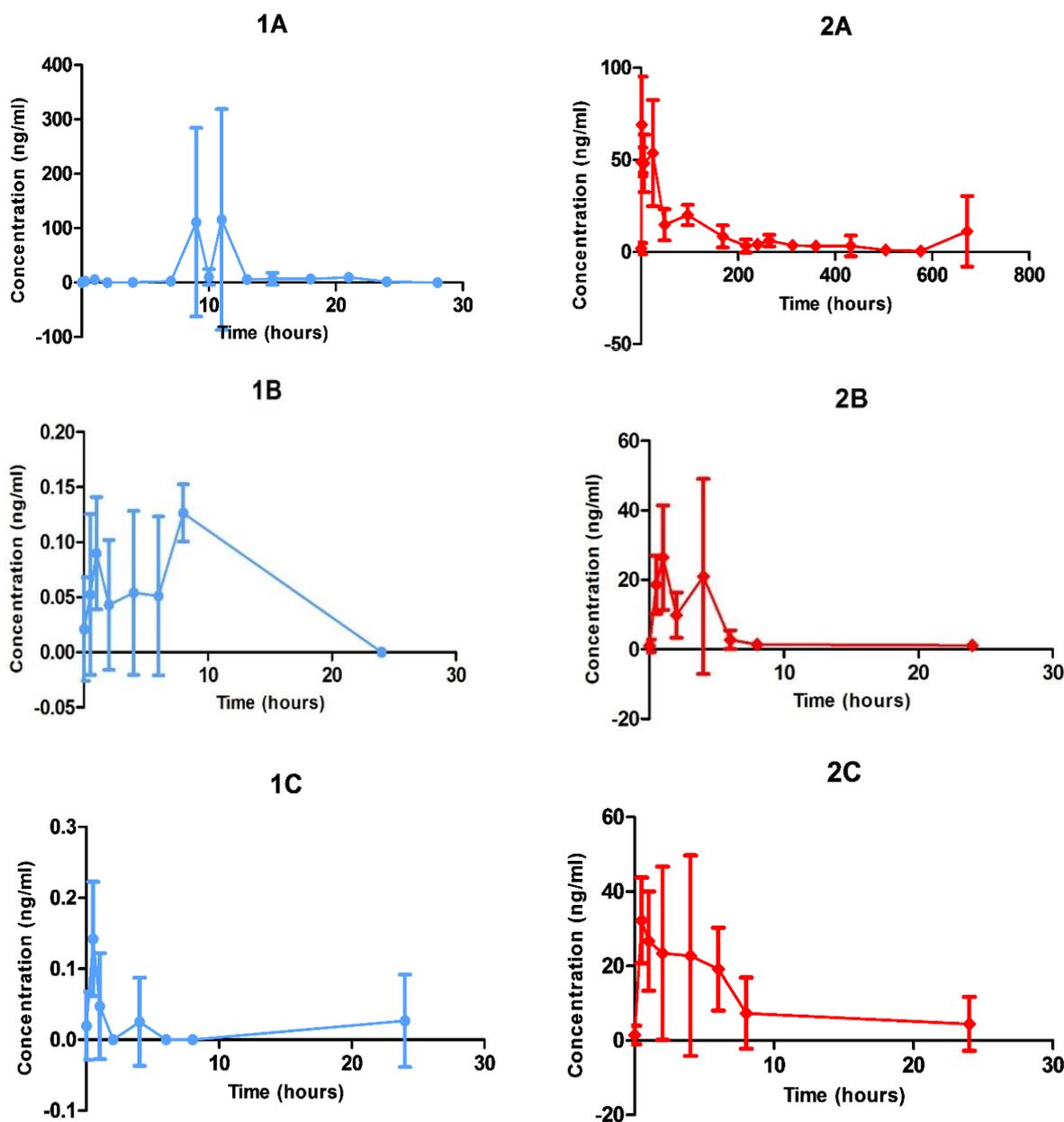
**Figure 2:** Confocal images of different formulations visualised by labelling with Nile red: (A) Pheroid<sup>®</sup> sample (B) 2 mg/kg Pheroid<sup>®</sup> goserelin sample (C) 4 mg/kg Pheroid<sup>®</sup> goserelin sample. 1) Image obtained with a tungsten source light, 2) Red fluorescence signal detected in wavelengths between 500 and 530 merged with that detected between 568 and 642 nm, 3) Red fluorescence signal detected in the 568 and 642 nm waveband only and 4) Green fluorescence signal detected in the 500 to 530 nm waveband only (scale bar =20  $\mu\text{m}$ ).

### 3.2. Data analysis

The pharmacokinetic parameters of single goserelin 3.6 mg subcutaneous implant (Zoladex<sup>®</sup>), 2 mg/kg and 4 mg/kg oral administration of pro-Pheroid<sup>®</sup>-goserelin of male BALB/c mice were

compared. The plasma concentration-time curves for all the groups are presented in Figure 3. As mentioned previously, WinNonlin could not fit the data.

For the subcutaneous group, an initial goserelin concentration spike was observed (Figure 3, 1A). On days 9 and 11 goserelin peak concentrations were followed by a decrease in concentration up until day 28. The testosterone concentration followed a similar pattern with an initial increase at 1h this was followed by a decrease in concentration at 2h (69.03 ng/mL). A spike in concentration was subsequent to this decrease, followed by a slow decrease (Figure 3, 2A). This concentration-time profile correlates with a previous study conducted by Zhang *et al* (2014) in rats; this study indicated the concentration-time profiles of goserelin and testosterone following the goserelin subcutaneous injection implant. The concentration-time curves of pro-Pheroid<sup>®</sup>-goserelin oral administration at a dose of 2 mg/kg are presented in Figure 3. An initial increase of goserelin concentration can be seen at 0.5h, this is followed by a slow decrease at 8h. In comparison, the 4 mg/kg produced fewer promising results (presented in Figures 3, 3A & B), however, this dose did demonstrate a higher initial spike of goserelin concentration, but the following concentrations up until 24h were lower compared to that of the 2 mg/kg group. This could possibly be attributed to the faster release of the higher dose of goserelin in GI tract or first-pass metabolism, thus leading to faster degradation before reaching the blood circulation as a result of more loaded vesicles, this may also be lower due to a slower release of higher doses. According to the particle size analysis (Table 1), 2 mg/kg presented with a smaller mass median, this could also possibly contribute to the higher concentration. A study conducted by Ong *et al.*, (2016) indicated the influence of the size of liposomes on the oral bioavailability. They concluded that a small liposomal size can enhance the oral bioavailability and should thus be a key factor to keep in mind for effective formulations (Ong *et al.*, 2016). All these factors could be possible reasons for the differences in concentrations of the two oral doses, but due to the timings of this study, it is difficult to speculate what happened to the goserelin and testosterone concentrations subsequent to 24 hours; this could be solved by further investigation of a longer study period. The testosterone initial concentration of the 4 mg/kg group had a similar spike in concentration as the other groups, however, an overall higher concentration of testosterone is seen compared to the other groups throughout the 24-hour period. This could be due to the effect of the overall lower concentration of goserelin during the 24 period. In conclusion, in this sense, although the 2 mg/kg is a lower dose it seems to be more effective in terms of the ultimate goal of lowered testosterone concentrations. In addition, no signs of toxicity or adverse effects were observed after the administration of either subcutaneous implant, 2mg/kg or 4 mg/kg pro-Pheroid<sup>®</sup>-goserelin.



**Figure 3:** Representative plasma-concentration curves of male BALB/c mice for 1A) goserelin concentration of subcutaneous implant (Zoladex®) group, 2A) testosterone concentration of subcutaneous implant group (n=6), 1B) goserelin concentration of 2 mg/kg pro-Pheroid®-goserelin group, 2B) testosterone concentration of 2 mg/kg pro-Pheroid®-goserelin group (n=6), 1C) goserelin concentration of 4 mg/kg pro-Pheroid®-group and 2C) testosterone concentration of 4 mg/kg pro-Pheroid-goserelin group in (n=6). Data is mean  $\pm$  SD.

As previously mentioned, the PK parameters could not be derived from the concentration-time profiles thus the observed  $C_{max}$  and  $T_{max}$  of each group were statistically compared. There was no statistically significant difference found between the goserelin  $C_{max}$  of the subcutaneous group and 2 mg/kg group ( $p = 0.006$ ). However, the  $C_{max}$  of the goserelin subcutaneous group was significantly higher than that of the pro-Pheroid®-goserelin 4 mg/kg ( $p = 0.017$ ). The  $C_{max}$  of the pro-Pheroid®-goserelin 2 mg/kg was also significantly higher to that of the 4 mg/kg ( $p = 1$ ). There was no

statistically significant difference between the  $T_{max}$  of the subcutaneous group and 4 mg/kg group, ( $p = 0.0003$ ), yet, the  $T_{max}$  of the subcutaneous group was significantly higher than the pro-Pheroid<sup>®</sup>-goserelin 2 mg/kg ( $p = 0.145$ ). A significantly higher  $T_{max}$  was also observed in the pro-Pheroid<sup>®</sup>-goserelin 4 mg/kg group compared to the 2 mg/kg group ( $p = 0.175$ ). Due to the subcutaneous administration route, the bioavailability of goserelin was higher and thus the goserelin concentration-time profile presented with a higher  $C_{max}$  and  $T_{max}$  compared to the oral pro-Pheroid<sup>®</sup>-goserelin 4 and 2 mg/kg. The smaller particle size of the pro-Pheroid<sup>®</sup>-goserelin 2 mg/kg (as seen from the particle size analysis) could be a possible explanation for the higher  $C_{max}$  compared to the 4 mg/kg because as mentioned, smaller particle size means better absorption. The higher  $T_{max}$  of pro-Pheroid<sup>®</sup>-goserelin 4 mg/kg could mean a slower release of goserelin from the Pheroid<sup>®</sup> vesicles. A t-test was done with the testosterone 2 and 4 mg/kg groups to compare the effect of goserelin on testosterone concentration between the initial times, 0 to 30 minutes. According to the p-value (0.04), a significant difference was found in the initial differences between the two doses. From this, it is clear that 4 mg/kg resulted in a higher initial increase of testosterone than 2 mg/kg. This can be due to the dose of 4 mg/kg being higher than that of the 2 mg/kg, thus having an initial higher effect on testosterone.

According to the overall pharmacokinetic and pharmacodynamic outcome, the 2 mg/kg resulted in a more favourable dose for the sufficient maintenance of goserelin in plasma and in return, provided more preferable testosterone concentration depletion in male BALB/c mice compared to the initial period of goserelin subcutaneous implant. Due to the doses being single administration compared to the subcutaneous implant, the  $C_{max}$  and  $T_{max}$  values were lower. Nonetheless, the concentration values of goserelin and testosterone in the plasma were above detection levels, meaning the use of Pheroid<sup>®</sup> technology as a drug delivery system for the oral administration of goserelin did improve the absorption of goserelin in blood and therefore may be a useful drug delivery system for drugs with low bioavailability, such as peptides and proteins.

#### **4. CONCLUSION**

The aim of this study was met. Meaning that the study successfully demonstrated an increase in goserelin concentration in the plasma of mice, which consequently lead to a decrease in testosterone levels. Thus, the results of the study indicated the potential of Pheroid<sup>®</sup> technology to improve the oral bioavailability of goserelin. Future in-depth studies will have to be done to compare the effectiveness of long-term use of pro-Pheroid<sup>®</sup>-goserelin to maintain sufficient levels of goserelin to ensure castrate levels of testosterone.

## REFERENCES

- Almeida, A.J. & Souto, E. 2007. Solid Lipid Nanoparticles as a Drug Delivery System for Peptides and Proteins. *Advance Drug Delivery Review*, 59(6):478-490.
- Baum, M., Hackshaw, A., Houghton, J., Fornander, T., Nordenskjold, B., Nicolucci, A., Sainsbury, R. & Collaborators'Group, Z.I. 2006. Adjuvant Goserelin in Pre-Menopausal Patients with Early Breast Cancer. *European Journal of Cancer*, 42(7):895-904.
- Chrisp, P. & Goa, K.L. 1991. Goserelin. *Drugs*, 41(2):254-288.
- Clayton, R. 1988. Mechanism of GnRH Action in Gonadotrophs. *Human Reproduction*, 3(4):479-483.
- Coccia, M.E., Comparetto, C., Bracco, G.L. & Scarselli, G. 2004. GnRH Antagonists. *European Journal of Obstetrics & Gynecology and Reproductive Biology*, 115: S44-S56.
- Cockshott, I.D. 2000. Clinical Pharmacokinetics of Goserelin. *Clinical Pharmacokinetics*, 39(1):27-48.
- Conn, P.M. & Crowley Jr, W.F. 1991. Gonadotropin-Releasing Hormone and its Analogues. *New England Journal of Medicine*, 324(2):93-103.
- Fosgerau, K. & Hoffmann, T. 2015. Peptide Therapeutics: Current Status and Future Directions. *Drug Discovery Today*, 20(1):122-128.
- Eckstein, N. & Haas, B. 2014. Clinical Pharmacology and Regulatory Consequences of GnRH Analogues in Prostate Cancer. *European Journal of Clinical Pharmacology*, 70(7):791-798.
- Grobler, A., Kotzé, A. & Du Plessis, J. 2007. The Design of a Skin-Friendly Carrier for Cosmetic Compounds using Pheroid™ Technology. *Science and Applications of Skin Delivery System Technologies Allured*. Ed. Johann Wiechers: 283-311.
- Grobler, A., Kotzé, A. & Du Plessis, J. 2008. The Design of a Skin-Friendly Carrier for Cosmetic Compounds Using Pheroid™ Technology. *Science and Applications of Skin Delivery System Technologies Allured*. Ed. Johann Wiechers:283-311.
- Grobler, A.F. 2009. Pharmaceutical Applications of Pheroid® Technology. North-West University.(Thesis – PhD).
- Grobler, A.F. 2013. Nano & Micro-Delivery Systems: the Pheroid® story: 2013.

- Jennes, L., Ulloa-Aguirre, A., Janovick, J., Adjan, V. & Conn, P. 2009. *The Gonadotropin-Releasing Hormone and its Receptor* p.51.
- Jonat, W. 1998. Luteinizing Hormone-Releasing Hormone Analogues--the Rationale for Adjuvant Use in Premenopausal Women with Early Breast Cancer. *British Journal of Cancer*, 78(Suppl 4): p.5.
- Kiesel, L.A., Rody, A., Greb, R.R. & Szilágyi, A. 2002. Clinical Use of GnRH Analogues. *Clinical Endocrinology*, 56(6):677-687.
- Laidler, P. & Farkas, I. 2013. Hydrochloride Salt of Peptide and its Use in Combination with Other Peptides for Immunotherapy: Google Patents.
- Ma, J.K. & Hadzija, B. 2012. *Basic Physical Pharmacy*: Jones & Bartlett Publishers.p.387
- Naheed, B., Uthman, O.A., O'Mahony, F., Kuiper, J.H. & O'Brien, P.M.S. 2014. Gonadotropin-Releasing Hormone (GnRH) Analogues for Premenstrual Syndrome (PMS). *The Cochrane Library Database of Systematic Reviews*. (10).
- Ong, S.G.M., Ming, L.C., Lee, K.S. & Yuen, K.H. 2016. Influence of the Encapsulation Efficiency and Size of Liposome on the Oral Bioavailability of Griseofulvin-Loaded Liposomes. *Pharmaceutics*, 8(3):25.
- Padula, A. 2005. GnRH Analogues—Agonists and Antagonists. *Animal Reproduction Science*, 88(1-2):115-126.
- Perry, C.M. & Brogden, R.N. 1996. Goserelin. *Drugs*, 51(2):319-346.
- Rani, S. and Pargal, A., 2004. Bioequivalence: An Overview of Statistical Concepts. *Indian Journal of Pharmacology*, 36, pp.209-216.
- Richter, W.F. & Jacobsen, B. 2014. Subcutaneous Absorption of Biotherapeutics: Knowns and Unknowns. *Drug Metabolism and Disposition*, 42(11):1881-1889.
- Roach III, M. & Izaguirre, A. 2007. Goserelin Acetate in Combination with Radiotherapy for Prostate Cancer. *Expert Opinion on Pharmacotherapy*, 8(2):257-264.
- Shaji, J. & Patole, V. 2008. Protein and Peptide Drug Delivery: Oral Approaches. *Indian Journal of Pharmaceutical Sciences*, 70(3):269-277.
- Smith, M.S. 2010. Estrus and Menstrual Cycles: Neuroendocrine Control. *Encyclopaedia of neuroscience*. Elsevier Ltd.

Steyn, D., du Plessis, L.H. & Kotzé, A. 2010. Nasal Delivery of Recombinant Human Growth Hormone: *in vivo* Evaluation with Pheroid™ Technology and N-trimethyl Chitosan Chloride. *Journal of Pharmacy & Pharmaceutical Sciences*, 13(2):263-273.

Tan, M.E., Li, J., Xu, H.E., Melcher, K. & Yong, E.-I. 2015. Androgen Receptor: Structure, Role in Prostate Cancer and Drug Discovery. *Acta Pharmacologica Sinica*, 36(1):3-23.

Van Niekerk, L. 2009. Pheroid™ technology for the Topical Delivery of Insulin Growth fFactor 1 (IGF – 1), Keratinocyte Growth Factor (KGF) and Vascular Endothelial Growth Factor (VEGF). Potchefstroom: NWU. (Thesis – M.Sc.)

Zhang, S., Han, J., Leng, G., Di, X., Sha, C., Zhang, X., Li, Y. & Liu, W. 2014. An LC-MS/MS Method for the Simultaneous Determination of Goserelin and Testosterone in Rat Plasma for Pharmacokinetic and Pharmacodynamic Studies. *Journal of Chromatography B Analytical Technology Biomedical Life Science*, 965:183-189.

# CHAPTER 5

# CHAPTER 5: THE EFFECT OF A GOSERELIN ORAL FORMULATION WITH PHEROID® TECHNOLOGY COMPARED TO A GOSERELIN SUBCUTANEOUS IMPLANT ON THE OESTRUS CYCLE OF FEMALE MICE

## ABSTRACT

Peptide therapeutics have restricted therapeutic value due to the current administered method via parenteral routes; overall this is a more invasive and stressful for patients, and thus the need for a less invasive administration route exists. This study focused on the use of Pheroid® technology to protect the peptide, goserelin, from degradation in the gastrointestinal tract and subsequently improve the peroral bioavailability. An oral formulation of pro-Pheroid®-goserelin was compared to the current goserelin subcutaneous implant (Zoladex®) in terms of the effect and disruption of the oestrus cycle in female BALB/c mice. Both the subcutaneous group and pro-Pheroid-goserelin groups displayed a disruption in the oestrous cycle. The pro-Pheroid®-goserelin group took longer to disrupt the cycle, but it still caused a disruption after  $\pm 15$  days. This demonstrates that Pheroid® technology did protect goserelin and consequently increased the bioavailability. The control pro-Pheroid® group did not present any disruption. In conclusion, goserelin was successfully entrapped in the Pheroid® vesicles and microsponges and the disruption of the oestrus cycle suggested that Pheroid® increased the oral bioavailability of goserelin by protecting it from degradation in the gastrointestinal tract of the mice.

## 1. INTRODUCTION

Goserelin ( $C_{59}H_{84}N_{18}O_{14}$ , molecular weight 1269.433 g/mol) is an amphiphilic peptide, known as an agonist analogue of gonadotropin-releasing hormone (Bolla *et al.*, 1997). Goserelin is one of the most intensively investigated gonadotropin-releasing hormone (GnRH) agonist (GnRH-a) drugs and is approved for the treatment of prostate cancer, metastatic breast cancer and endometriosis, however, it is also used for controlled polycystic, erratic menstrual bleeding and to assist in the harvesting of eggs for *in vitro* fertilisation (Cockshott, 2000; Baum *et al.*, 2006; Upfal, 2006). The development of GnRH analogues was one of the most important advantages in the field of assisted reproductive technologies (Fleming *et al.*, 1982). For controlled ovarian hyperstimulation in women undergoing *in vitro* fertilisation (IVF), GnRH-a is used in combination with recombinant follicle stimulating hormone (FSH) or menotropins to induce folliculogenesis (Geber *et al.*, 2002). GnRH analogues are also widely used in combination with human menopausal gonadotropins for IVF (Tapanainen *et al.*, 1993).

Goserelin acts on the hypothalamic-pituitary axis and, because of this receptor, downregulation occurs. This leads to the inhibition of the secretion of the pituitary gonadotropins (Baum *et al.*, 2006; Jonat, 1998; Zhang *et al.*, 2014). Luteinising hormone (LH) and FSH initially increase reaching a

peak after two to three days, however, within 14 days the levels decrease to the pre-treatment values or even below and consequently, production of oestradiol from the ovaries decreases to post-menopausal levels (Baum *et al.*, 2006). The current administration regime of goserelin is a subcutaneous depot injected into the extracellular space of the subcutaneous tissue and slowly releases goserelin over a period of either one (3.6 mg) or three (10.8 mg) months (Cockshott, 2000; Richer & Jacobsen, 2014). The susceptibility of the GnRH-a to gastrointestinal peptidase degradation makes other routes such as peroral unsuitable (Conn and Crowley, 1991; Chrisp and Goa 1990). Subcutaneous implants and injections are usually not well tolerated and are considered stressful for the patients due to this administration method being more invasive than other more conventional routes, such as the preferred peroral route. This results in patient compliance being generally poor, leading to a restricted therapeutic value of the drug. Physiological factors such as stress may influence the chance of obtaining a pregnancy from IVF (Eugster and Vingerhoets, 1999; Klonoff-Cohen, 2005; Boivin and Schmidt, 2005). For IVF to be successful it is important to minimise stress and the inconvenience related to the administration as much as possible (Tapanainen *et al.*, 1993). Hence, the need for a less stressful administration method is clear.

To overcome this, different strategies can be implemented, such as formulation and chemical technologies. Particulate drug delivery systems (DDS), such as Pheroid<sup>®</sup> technology, are an example of a formulation technology for the protection of drugs from degradation. This delivery system is a patented, colloidal drug delivery system, which consists of fatty acids (vitamin F ethyl ester, kolliphor<sup>®</sup> EL) and  $\alpha$ -tocopherol, this makes up 4% of the formulation, and a water, which makes up 96% of the total formulation (Steyn *et al.*, 2011). Pheroid<sup>®</sup> technology is capable of targeting specific areas for treatment, transportation of genetic material to the cellular nucleus and the decrease of drug resistance, all this while improving the delivery of dynamic complexes (Grobler *et al.*, 2008). Pro-Pheroid<sup>®</sup> is an alternative to Pheroid<sup>®</sup> technology, which lacks the water phase thus only consisting of an N<sub>2</sub>O-saturated oil phase (Grobler, 2009). On addition of an aqueous phase, e.g in the peroral administration of pro-Pheroid<sup>®</sup>, the pro-Pheroid<sup>®</sup> is converted to Pheroid<sup>®</sup> by the gastric fluid present in the stomach (aqueous phase) spontaneously forming Pheroid<sup>®</sup> vesicles and microsponges (Grobler, 2009). The aim of this study was to evaluate a peroral delivery of goserelin with the use of Pheroid<sup>®</sup> technology versus the current subcutaneous implant to observe the disruption of the oestrous cycles of female BALB/c mice, which signifies the down-regulation of oestrous.

## 2. MATERIALS AND METHODS

### 2.1 Chemicals

Goserelin acetate powder (C<sub>61</sub>H<sub>88</sub>N<sub>18</sub>O<sub>16</sub>) molecular weight: 1269.41 g/mol was obtained from Bachem AG Budendorf. Goserelin acetate implants (Zoladex<sup>®</sup>) [D-Ser(But)<sub>6</sub>,Azgly<sub>10</sub>]LHRH molecular weight: 1269.433 g/mol were obtained from BGM Pharma (South Africa). Pro-Pheroid<sup>®</sup>

ingredients: vitamin F ethyl ester, dl- $\alpha$ -tocopherol, Kolliphor<sup>®</sup> EL were obtained from Chemipo (South Africa), Chempure (South Africa), BASF chemicals (South Africa) respectively. Acetonitrile (LCMS analytical grade) was purchased from Merck (Pty) Ltd, South Africa. Dimethyl sulfoxide (DSMO) was purchased from Molecular Probes Inc. (United States of America). The internal standard (Alarelin) was sourced from MedChem Express, USA (Purity > 98%).

## 2.2 Preparation of pro-Pheroid<sup>®</sup>-goserelin

Pheroid<sup>®</sup> was produced as previously described in Chapter 3 and Pro-Pheroid<sup>®</sup> was prepared by heating vitamin ethyl ester (70% w/w) to 70°C and Kolliphor<sup>®</sup> EL (29% w/w) to 120°C. The two were combined when Kolliphor<sup>®</sup> EL cooled down to 70°C, they were further left to cool to 55°C, then  $\alpha$ -tocopherol (1% w/w) was added and mixed. After the preparation of pro-Pheroid<sup>®</sup>, the appropriate dose of goserelin was added and sonicated for  $\pm$  15 seconds. The formulations were then gassed for four days, at 150 kPa, in a small pressure vessel with nitrous oxide (N<sub>2</sub>O). For quality control purpose the formulations were analysed and assessed for the visualisation of the morphology by using confocal laser scanning microscopy CLSM (Nikon D-Eclipse C1), stability by analysing the Zeta potential using a Malvern Zetasizer Nano ZS (Malvern Instruments, Worcestershire, United Kingdom) and the mean particle size distribution using Malvern Mastersizer nano ZS (Malvern Instruments, Worcestershire, United Kingdom). The characterisation was done as mentioned in Chapter 3. In order for the analysis of these characteristics, the pro-Pheroid<sup>®</sup> was first converted to Pheroid<sup>®</sup> vesicles and microsponges by the addition of 0.1M HCl to simulate the stomach environment.

## 2.3. Animal experiments

All mouse experimental procedures were approved by the Ethics Committee (NWU-AnimCareREC) of the North-West University (ethics number: NWU-00268-16-A5). Eighteen BALB/c mice (female) between the ages of 5 and 8 weeks were used in this study. The mice were housed in individually ventilated (IVC) rat cages (Techniplast equipped with rack isolator systems (equipped with input and exhaust fan filter units that provided HEPA filtered inlet and outlet air) within the PCDDP Vivarium at the Potchefstroom campus of the North-West University. The room temperature was  $22 \pm 1$  °C with a relative humidity of  $55 \pm 10\%$ , a light/dark cycle of 12 hours and ventilation of 20 air changes per hour under positive pressure. They were provided water *ad libitum* and fed standard rodent maintenance chow (NutritionHUB (Pty) Ltd), and housed on bedding derived from dust free and non-toxic exfoliated corncob chips in order to absorb urine, excessive moisture, and potentially hazardous ammonia vapours.

### 2.3.1 Administration of formulations

The mice were randomly assigned to three groups with six mice per group. Group 1 received a 3.6 mg subcutaneous implant of goserelin, which slowly released over a period of 28 days; this results in 3.6 mg/28 days = 0.128 mg/day. Groups 2 and 3 received a peroral formulation of pro-Pheroid<sup>®</sup> and pro-Pheroid<sup>®</sup>-goserelin (4 mg/kg) respectively through oral gavage. For five consecutive days prior to the administration of goserelin and pro-Pheroid<sup>®</sup> goserelin, vaginal smears were taken from each mouse to determine the pattern of the oestrous cycle (the smears were taken at approximately the same time every day at mid-morning). The smears were collected using a swab wetted with 0.9 %saline. The swab was inserted into the vaginal tract of the mice (to a depth of approximately 1 cm), gently turned and rolled against the vaginal wall (at an angle of about 45°) then removed. This was transferred to a clean dry glass slide by rolling the swab across the slide. After administration of the test substances, smears were collected daily for five consecutive days at approximately the same time.

#### 2.4 Oestrous cycle analysis

The changes in the oestrous cycle in mice can be identified by changes occurring in the animal's physiology and anatomy. These changes can be investigated using a variety of methods to determine the oestrous cycle stages, such as evaluating vaginal cytology (Allen, 1922; Nelson *et al.*, 1982; Caligioni, 2009). Vaginal cytology is a type of endocrine assay, which was the method of identification in this study's four oestrous stages (pro-oestrous, oestrous, metoestrous and dioestrous). After the application of the vaginal smear on a dry glass slide, it was air-dried and analysed using a light microscope with a magnification range between 80X and 100X (direct cytology). The identification and classification of the four stages of the oestrous cycle were based on the proportion and type of cells observed in the vaginal smear (leukocytes, cornified epithelial and nucleated cells), and assessed and compared as demonstrated by Caligioni (2009) and Byers *et al.*, (2012). Cornified epithelial cells can be identified by large, angular and irregularly shaped, mostly non-nucleated cells, leukocytes are very small, round cells and epithelial cells are not quite as large as the cornified cells and much more rounded in shape.

### 3. RESULTS AND DISCUSSION

#### 3.1 Characterisation of Pheroid<sup>®</sup> and Pheroid<sup>®</sup> goserelin

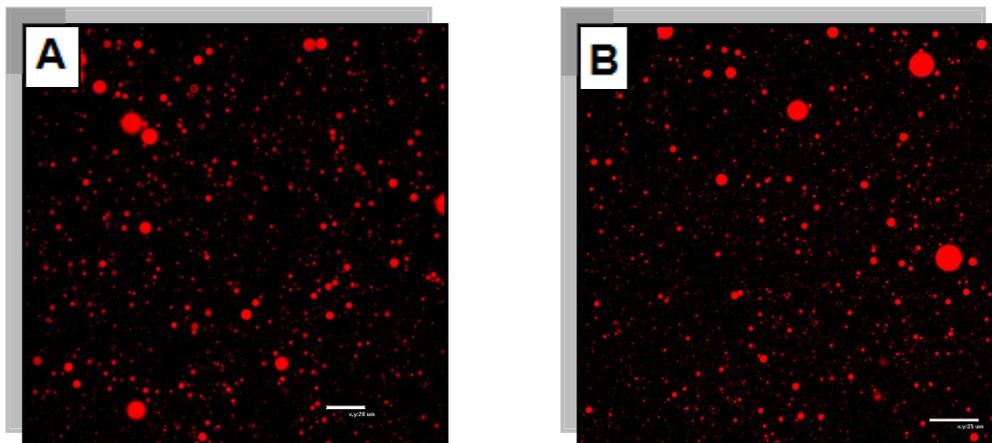
The particle size, Zeta potential and confocal image average size are summarised in Table 1. D(0.5) represents the particles at which 50% of the samples are smaller and 50% larger than this size in diameter (also known as the mass median diameter(MMD)). The MMD increased by the entrapment of goserelin in Pheroid<sup>®</sup> vesicles and microsponges increased from 0.694 µm to 0.947µm. The active pharmaceutical ingredient can alter the morphology, size and stability of the Pheroid<sup>®</sup> vesicles. The Zeta potential was lower by 5.5 mV for the Pheroid<sup>®</sup>-goserelin sample than for the Pheroid<sup>®</sup> sample, meaning that the addition of goserelin to Pheroid<sup>®</sup> slightly decreased the stability. As indicated in

Roland *et al.*, (2003), if the Zeta potential has a high positive or negative value ( $\pm 25$  mV), it means the emulsion is more stable, if it has a low positive or negative value ( $< 25$  mV) stability will be lower. Correspondingly, the average size according to the confocal laser scanning microscopy decreased with the addition of goserelin to Pheroid<sup>®</sup> from 339 nm to 282 nm, although. Grobler *et al.*, (2009) stated the Pheroid<sup>®</sup> particle diameter ranges from 200 nm to 2  $\mu$ m. Thus, the mean particle size and confocal average particle size did adhere with this. The spherical shape and separation of the Pheroid<sup>®</sup> vesicles are confirmed by the CLSM for both formulations (Figure 1).

**Table 1:** Mean particle size diameter, zeta potential measurement and confocal laser scanning imaging average size results of Pheroid<sup>®</sup> and Pheroid<sup>®</sup>-goserelin.

Pheroid <sup>®</sup> Sample	Particle size D(0.5) ( $\mu$ m) (mean $\pm$ standard deviation, $\mu$ m)*	Zeta potential (mV) (mean $\pm$ standard deviation, mV)*	Confocal image average size (nm)
Pheroid <sup>®</sup>	0.694 $\pm$ 0.52	-21.3 $\pm$ 5.15	339
Pheroid <sup>®</sup> -goserelin	0.947 $\pm$ 0.78	-15.8 $\pm$ 9.05	282

\* (n = 3) measurements by the Malvern Zetasizer and Malver Mastersizer

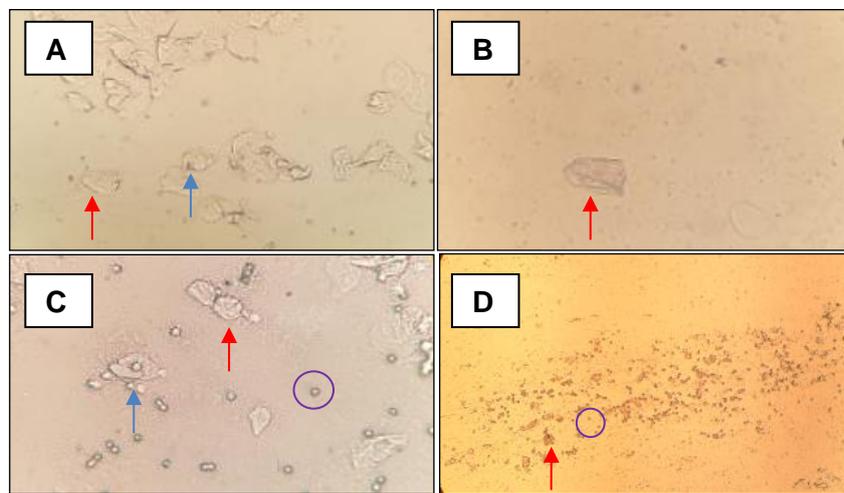


**Figure 1:** Morphological visualisation of Pheroid<sup>®</sup> vesicles, through confocal laser scanning of A) Pheroid<sup>®</sup> formulation and B) Pheroid<sup>®</sup>-goserelin formulation. The scale represents 20  $\mu$ m. The red dots represent the Nile red-stained Pheroid<sup>®</sup> vesicles (scale = 20  $\mu$ m).

### 3.2 Pharmacodynamic analysis

The four different oestrous stages of the female BALB/c mice are demonstrated in Figure 2. From this figure, the different cell types are also visible. Figure 3 is an example of the change that occurred after depletion of oestrogen and down-regulation of the pituitary gland with the continuous and subcutaneous administration of goserelin. This figure exclusively consists of nucleated cells and cornified cells from the repetition of the proestrous stage, as represented in Figures 4 A & B. The

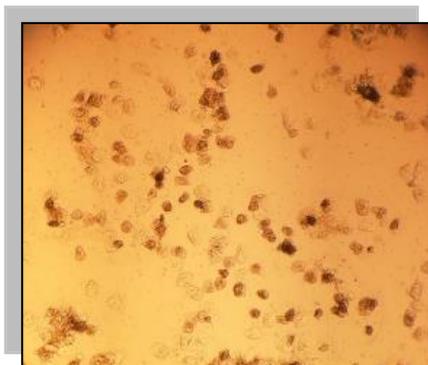
oestrous cycle of the goserelin subcutaneous implant group (Figure 4 A) changed three days after the administration of the implant; this was followed by a repetitive prooestrous stage up until study termination at day 21. A similar finding was also indicated by a study conducted by Horicks *et al.*, (2015). The oestrous cycle of the 4 mg/ kg pro-Pheroid<sup>®</sup>-goserelin group was normal up until day 15  $\pm$  1, 10 days after the oral administration of pro-Pheroid<sup>®</sup>-goserelin as demonstrated in Figure 4 B. This is seven days later compared to the goserelin subcutaneous group; this was due to the higher bioavailability of subcutaneous implants and the lower peroral bioavailability of pro-Pheroid<sup>®</sup>-goserelin. There was no effect on the oestrous cycle in the control group mice that received oral pro-Pheroid<sup>®</sup> (Figure 4 C), however, one mouse from this group showed an irregular pattern which was thought to be due to stress caused by the animal handling, or environmental factors (Goldman *et al.*, 2007).



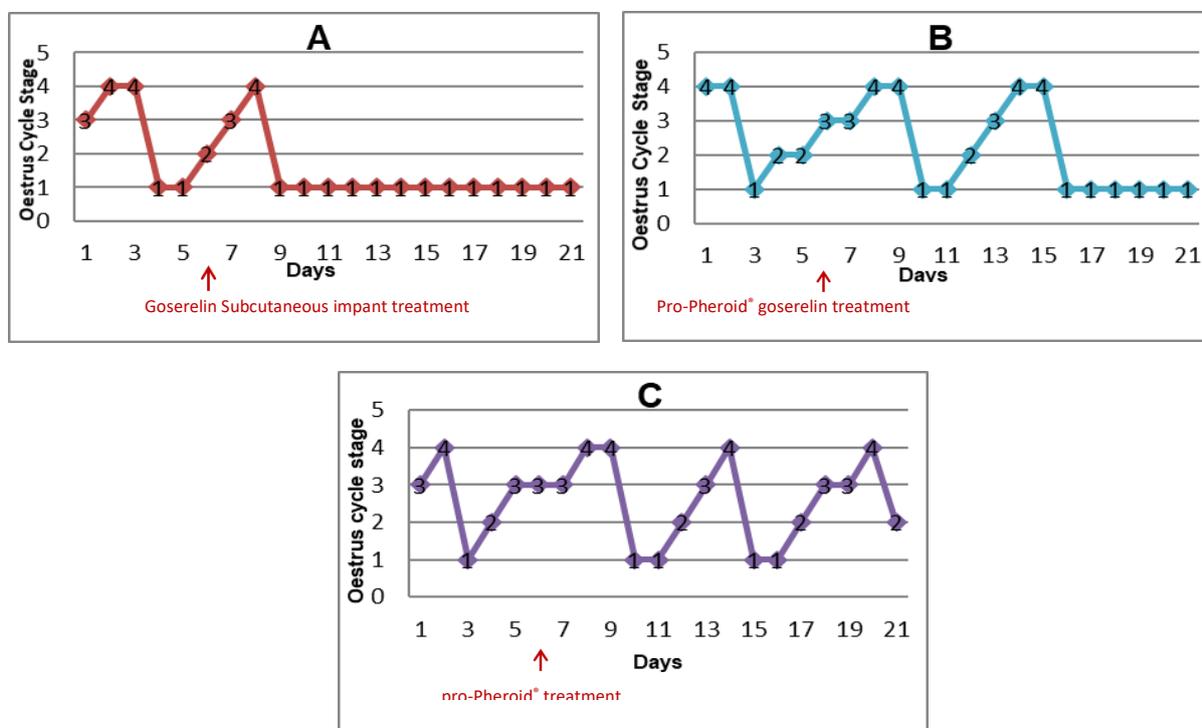
**Figure 2:** Vaginal cytology representing each stage of oestrous of female BALB/c mice. Three cell types are identified: the nucleated cells are indicated by the blue arrow; the red arrow represents the cornified cells and the purple circle indicates the leukocytes. Stages: **A)** Pro-oestrous; predominantly consists of nucleated and cornified epithelial cells, **B)** Oestrous; predominantly cornified epithelial cells **C)** Metroestrous; consists of all three cell types (cornified epithelial, nucleated epithelial and leucocytes) and **D)** Dioestrous; predominantly leucocytes but nucleated epithelial also present.

From the results, it is clear that the peroral pro-Pheroid<sup>®</sup> goserelin formulation was successfully taken up in the blood of the mice and thus proved to be successful in disrupting the oestrous cycle when compared to the pro-Pheroid<sup>®</sup> alone group. A similar pattern of disruption can also be seen in the goserelin subcutaneous group. One limitation of this study was that there was no goserelin alone group to evaluate if goserelin alone would have an effect on the oestrous cycle. This will be an indication of the peroral absorption and stability of goserelin. A study conducted by Wang *et al.*, (2015) indicated that goserelin was stable in simulated gastric fluid, but not simulated intestinal fluid. If this is the case, no expected disruption will occur. In addition, no signs of toxicity or adverse effects

were observed after the administration of either subcutaneous implant or 4 mg/kg pro-Pheroid<sup>®</sup>-goserelin.



**Figure 3:** Representation of oestrous cycle after disruption by goserelin (repetitive pro-oestrous).



**Figure 4:** Representative disruptive effects on the oestrous cycle in one mouse from each group after **A)** goserelin subcutaneous implant **B)** pro-Pheroid<sup>®</sup> goserelin oral administration and **C)** pro-Pheroid<sup>®</sup> oral administration. The arrow indicates the treatment start day. The oestrous stage is indicated by 1. Pro-oestrous, 2. Oestrous, 3. Metoestrous and 4. Dioestrous. (Rest of oestrous cycles of all groups are provided in Annexure D).

## CONCLUSION

Although the oral pro-Pheroid<sup>®</sup> goserelin formulation took longer to disrupt the oestrous cycle compared to the goserelin subcutaneous implant, the goserelin entrapped in Pheroid<sup>®</sup> vesicles, formed after oral gavage *in situ*, still proved to be successful in disrupting the cycle. This means that

the Pheroid® vesicles presumably successfully entrapped goserelin and subsequently increased the bioavailability, and the aim of this study was met. The use of Pheroid® technology for a DDS of goserelin is promising for the improvement and protection of the bioavailability of drugs with low oral bioavailability due to the harsh gastric environment. Future studies will have to be conducted to optimise the dose frequency and dosage of goserelin to be used in pro-Pheroid® and to include an additional goserelin, alone group. It is clear that Pheroid® technology has potential in future peptide studies for the improvement of bioavailability to exploit this system's full potential.

## REFERENCES

- Allen, E. 1922. The Oestrous Cycle in the Mouse. *American Journal of Anatomy*, 30(3):297-371A
- Bolla, M., Gonzalez, D., Warde, P., Dubois, J.B., Mirimanoff, R.-O., Storme, G., Bernier, J., Kuten, A., Sternberg, C., Gil, T., Collette, L. & Pierart, M. 1997. Improved Survival in Patients with Locally Advanced Prostate Cancer Treated with Radiotherapy and Goserelin. *New England Journal of Medicine*, 337(5):295-300.
- Boivin, J. & Takefman, J.E. 1995. Stress Level Across Stages of *In Vitro* Fertilization in Subsequently Pregnant and Nonpregnant Women. *Fertility and Sterility*, 64(4):802-810.
- Caligioni, C.S. 2009. Assessing Reproductive Status/Stages in mice. *Current Protocols in Neuroscience*, 48(1): A. 4l. 1-A. 4l. 8.
- Chrisp, P. & Goa, K.L. 1990. Nafarelin. *Drugs*, 39(4):523-551.
- Conn, P.M. & Crowley Jr, W.F. 1991. Gonadotropin-Releasing Hormone and its Analogues. *New England Journal of Medicine*, 324(2):93-103.
- Cooper, R. 1993. Monitoring of Estrous Cycle in the Laboratory Rodent by Vaginal Lavage. *Female Reproductive Toxicology*:45-56.
- Fleming, R., Adam, A., Barlow, D., Black, W., MacNaughton, M. & Coutts, J. 1982. A New Systematic Treatment for Infertile Women with Abnormal Hormone Profiles. *BJOG: An International Journal of Obstetrics & Gynaecology*, 89(1):80-83.
- Eugster, A. & Vingerhoets, A. 1999. Psychological Aspects of *In Vitro* Fertilization Social. *Science & Medicine*, 48(5):575-589.
- Geber, S., Sales, L. & Sampaio, M.A. 2002. Comparison Between a Single Dose of Goserelin (Depot) and Multiple Daily Doses of Leuprolide Acetate for Pituitary Suppression in IVF Treatment: a Clinical Endocrinological Study of the Ovarian Response. *Journal of Assisted Reproduction and Genetics*, 19(7), pp.313-318
- Goldman, J.M., Murr, A.S. & Cooper, R.L. 2007. The Rodent Estrous Cycle: Characterization of Vaginal Cytology and its Utility in Toxicological Studies. *Birth Defects Research Part B: Developmental and Reproductive Toxicology*, 80(2):84-97.
- Horicks, F., Van Den Steen, G., Houben, S., Englert, Y. & Demeestere, I. 2015. Folliculogenesis is Not Fully Inhibited During GnRH Analogues Treatment in Mice Challenging their Efficiency to Preserve the Ovarian Reserve During Chemotherapy in this Model. *PloS One*, 10(9):e0137164.

- Kuten, A., Sternberg, C. & Gil, T. 1997. Improved Survival in Patients with Locally Advanced Prostate Cancer Treated with Radiotherapy and Goserelin. *New England Journal of Medicine*, 337(5):295-300.
- Klonoff-Cohen, H. 2005. Female and Male Lifestyle Habits and IVF: What is Known and Unknown. *Human Reproduction Update*, 11(2):180-204.
- Nelson, J.F., Felicio, L.S., Randall, P.K., Sims, C. & Finch, C.E. 1982. A Longitudinal Study of Estrous Cyclicity in Aging C57BL/6J Mice: I. Cycle Frequency, Length and Vaginal Cytology. *Biology of Reproduction*, 27(2):327-339.
- Tapanainen, J., Hovatta, O., Juntunen, K., Martikainen, H., Ratsula, K., Tulppala, M. & Tuomivaara, L. 1993. Endocrinology: Subcutaneous Goserelin Versus Intranasal Buserelin for Pituitary Down-Regulation in Patients Undergoing IVF: a Randomized Comparative Study. *Human Reproduction*, 8(12):2052-2055.
- Upfal, J. 2006. The Australian Drug Guide: Every Person's Guide to Prescription and Over-the-counter Medicines, Street Drugs, Vaccines, Vitamins and Minerals: *Black Inc.* p 133
- Baum, M., Hackshaw, A., Houghton, J., Fornander, T., Nordenskjold, B., Nicolucci, A., Sainsbury, R. & Collaborators' Group, Z.I. 2006. Adjuvant goserelin in pre-menopausal patients with early breast cancer: results from the ZIPP study. *European Journal of Cancer*, 42(7):895-904.
- Boivin, J. & Takefman, J.E. 1995. Stress level across stages of in vitro fertilization in subsequently pregnant and nonpregnant women. *Fertility and Sterility*, 64(4):802-810.
- Chrisp, P. & Goa, K.L. 1990. Nafarelin. *Drugs*, 39(4):523-551.
- Cockshott, I.D. 2000. Clinical pharmacokinetics of goserelin. *Clinical Pharmacokinetics*, 39(1):27-48.
- Conn, P.M. & Crowley Jr, W.F. 1991. Gonadotropin-releasing hormone and its analogues. *New England Journal of Medicine*, 324(2):93-103.
- Eugster, A. & Vingerhoets, A. 1999. Psychological aspects of in vitro fertilization: a review. *Social science & medicine*, 48(5):575-589.
- Grobler, A.F. 2008. Pharmaceutical applications of Pheroid® technology. Potchefstroom: North West University
- Grobler, A.F. 2009. Pharmaceutical applications of Pheroid™ technology. North-West University.
- Jonat, W. 1998. Luteinizing hormone-releasing hormone analogues--the rationale for adjuvant use in premenopausal women with early breast cancer. *British journal of cancer*, 78(Suppl 4):5.

Richter, W.F. & Jacobsen, B. 2014. Subcutaneous absorption of biotherapeutics: knowns and unknowns. *Drug Metabolism and Disposition*, 42(11):1881-1889.

Steyn, J.D., Wiesner, L., du Plessis, L.H., Grobler, A.F., Smith, P.J., Chan, W.-C., Haynes, R.K. & Kotzé, A.F. 2011. Absorption of the novel artemisinin derivatives artemisone and artemiside: Potential application of Pheroid™ technology. *International Journal of Pharmaceutics*, 414(1):260-266.

Zhang, S., Han, J., Leng, G., Di, X., Sha, C., Zhang, X., Li, Y. & Liu, W. 2014. An LC-MS/MS method for the simultaneous determination of goserelin and testosterone in rat plasma for pharmacokinetic and pharmacodynamic studies. *Journal of Chromatography B Analytical Technology Biomed Life Sciences*, 965:183-189.

# CHAPTER 6

## CHAPTER 6: RESEARCH OUTCOMES, STUDY LIMITATIONS AND FUTURE RECOMMENDATION

### RESEARCH OUTCOMES

1. The characterisation results revealed no considerable changes in either the particle size, stability or the morphology by the addition of goserelin to Pheroid<sup>®</sup>. This is, therefore, an indication that goserelin did not alter or have any effect on the Pheroid<sup>®</sup> characteristics and structure. Stability of goserelin in simulated fluids could not be confirmed due to loss of sample during centrifugation of the Pheroid<sup>®</sup> containing samples subsequent to incubation.
2. The  $C_{max}$  and the  $T_{max}$  of the goserelin concentration-time profile of the goserelin subcutaneous group was significantly higher than that of both 2 mg/kg and 4mg/kg pro-Pheroid<sup>®</sup>-goserelin; this was due to the higher bioavailability of the implant compared to that of the peroral formulations. Although Pheroid<sup>®</sup> did aid in the improvement of the goserelin concentration in the blood, as demonstrated from the LC-MS/MS result, 2 mg/kg pro-Pheroid<sup>®</sup>-goserelin proved to be more effective than the 4mg/kg pro-Pheroid-goserelin dose in terms of a higher  $C_{max}$  of goserelin as well as a higher observed overall goserelin concentration; the testosterone concentration was also maintained lower throughout the 24 hours. The results thus provide proof of Pheroid<sup>®</sup> technology as a DDS for drugs with low bioavailability such as peptides.
3. The pro-Pheroid<sup>®</sup>-goserelin and goserelin subcutaneous implant had an effect on the oestrous cycle, as shown by a disruption in the cycle on day  $\pm 15$  and  $\pm 9$  respectively. The disruption in the oestrous cycle is an indication of the curtailing action of goserelin on the oestrogen production. Thus Pheroid<sup>®</sup> presumably increased the oral bioavailability of goserelin.

### STUDY LIMITATIONS

1. The animal studies did not include a goserelin only formulation group, thus no comparison could be made between goserelin alone and goserelin entrapped in Pheroid<sup>®</sup> to study the effect on the testosterone concentration and the oestrous cycle. The reasoning behind this was the oral bioavailability of GnRH analogues, such as goserelin, was only 0.1% (Naheed *et al.*, 2014), thus it was expected that no detection of any goserelin in the blood or any subsequent pharmacodynamic effect would be detected.
2. The goserelin and testosterone concentration was only observed over a period of 24 hours for the pro-Pheroid<sup>®</sup>-goserelin peroral groups.

### FUTURE RECOMMENDATIONS

1. Perform an extended study with pro-Pheroid<sup>®</sup>-goserelin in male mice to see if Pheroid<sup>®</sup> has slow release capabilities, and to further observe the concentration of goserelin and

testosterone in the blood beyond the 24- hour period to acquire a clearer and extended goserelin and testosterone concentration-time profile.

2. Include a goserelin saline formulation for all the animal studies to prove the low oral bioavailability of the peptide.
3. Perform a dose ranging and frequency study to ensure the correct peroral dosage of pro-Pheroid<sup>®</sup>-goserelin, which would be bioequivalent to the goserelin subcutaneous implant and thus ensure the prolonged effect on testosterone and oestrogen concentrations.
4. Repetition of the stability study in SIF and SGF to ensure the successful release of goserelin from Pheroid<sup>®</sup> vesicles and microsponges and ensure correct sample handling after incubation and prevent sample loss. The extraction method of the Pheroid<sup>®</sup> samples should also be adjusted and optimised to assure detectable concentration of goserelin on the LC-MS/MS.
5. Perform histology on the female mice ovaries after treatment with goserelin subcutaneous implant and oral pro-Pheroid<sup>®</sup>-goserelin to observe and compare the effect on the ovaries after treatment and to correlate this to the disruption in the oestrous cycle.

## REFERENCES

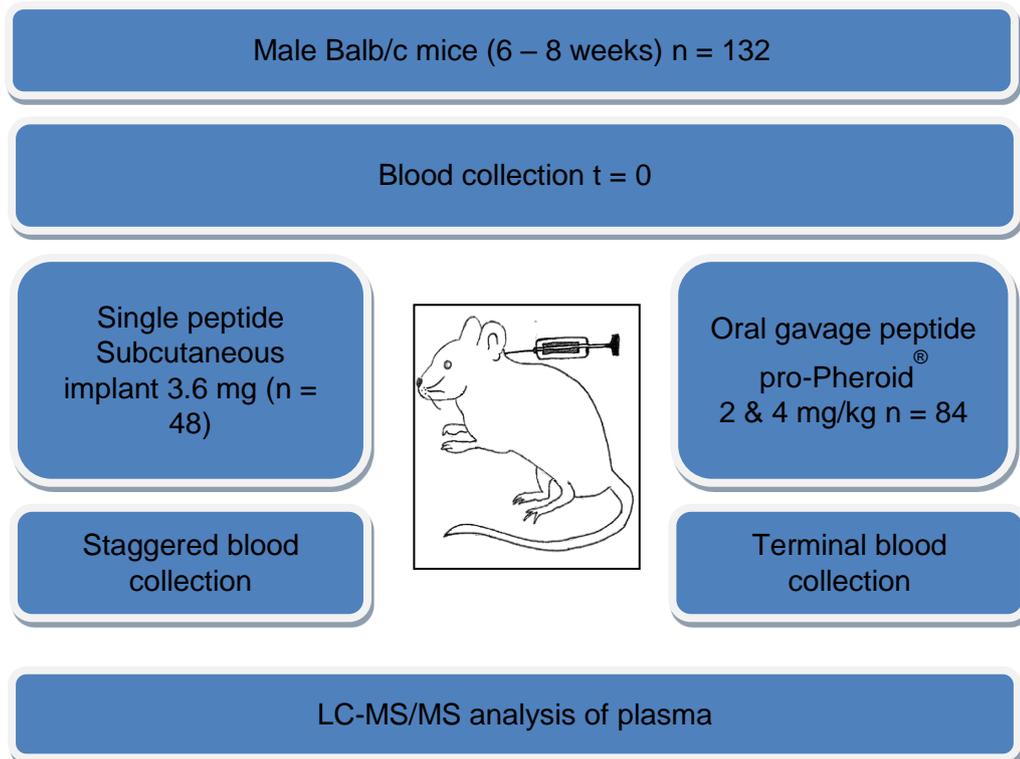
Naheed, B., Uthman, O.A., O'Mahony, F., Kuiper, J.H. & O'Brien, P.M.S. 2014. Gonadotropin-releasing hormone (GnRH) analogues for premenstrual syndrome (PMS). *The Cochrane Library*, (10).

# ANNEXURES

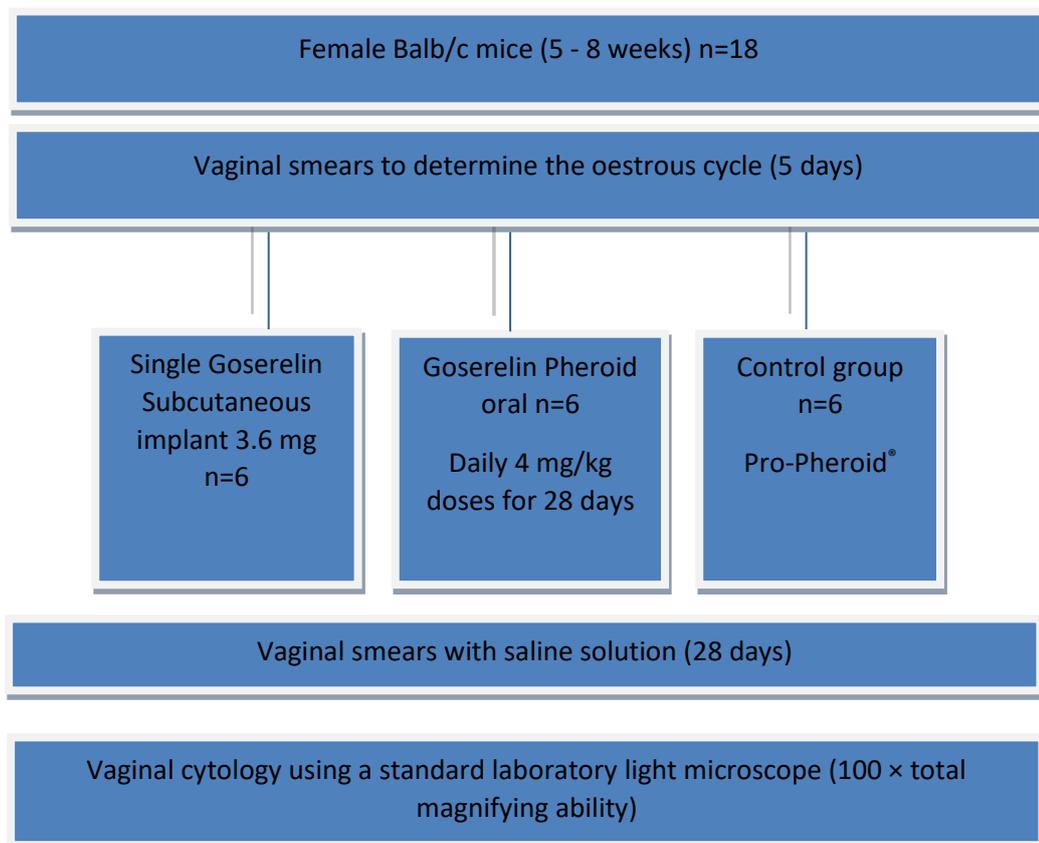
## **ANNEXURE A – Experimental Design Diagram**

The following diagrams represent the experimental flow for:

## 1) Goserelin Male Mice Study



## 2) Goserelin Female Mice Study



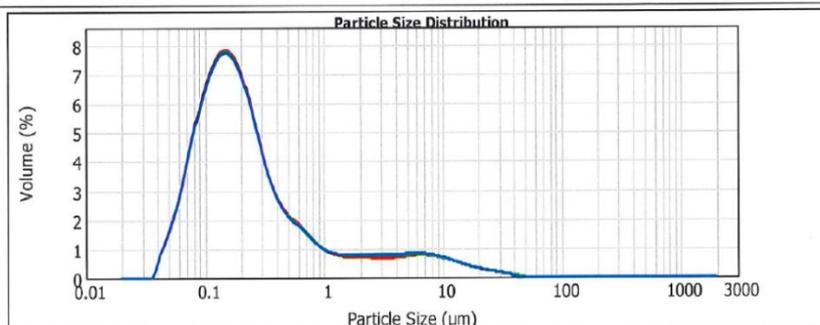
## **ANNEXURE B – Characterisation Results**

The following figures indicate the graphs for particle size analysis and zeta potential as well as their respective values.

## Particle Size Analysis for Stability Study Formulations

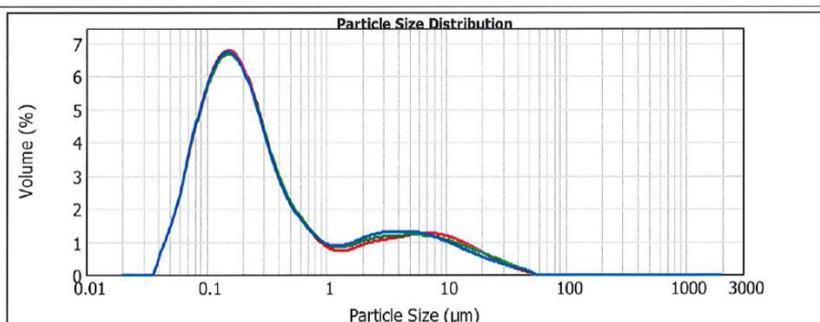
### 1. Pro-Pheroid®

d(0.1): 0.077 um                      d(0.5): 0.187 um                      d(0.9): 2.569 um



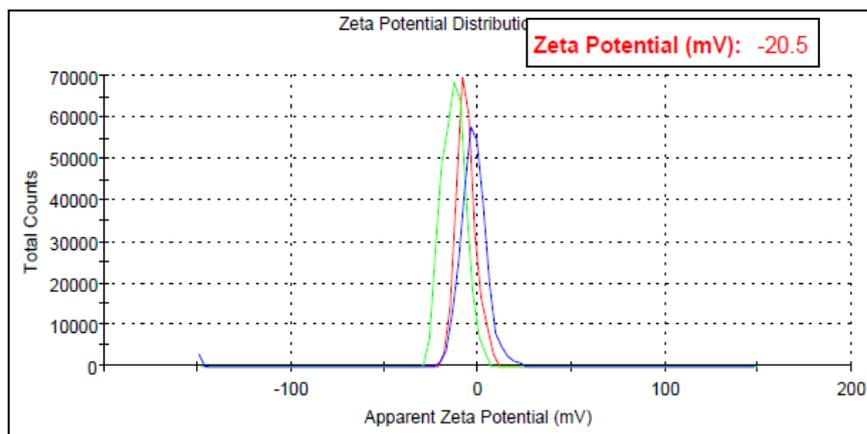
### 2. Pro-Pheroid®-goserelin 4 mg/kg

d(0.1): 0.080 um                      d(0.5): 0.217 um                      d(0.9): 5.399 um

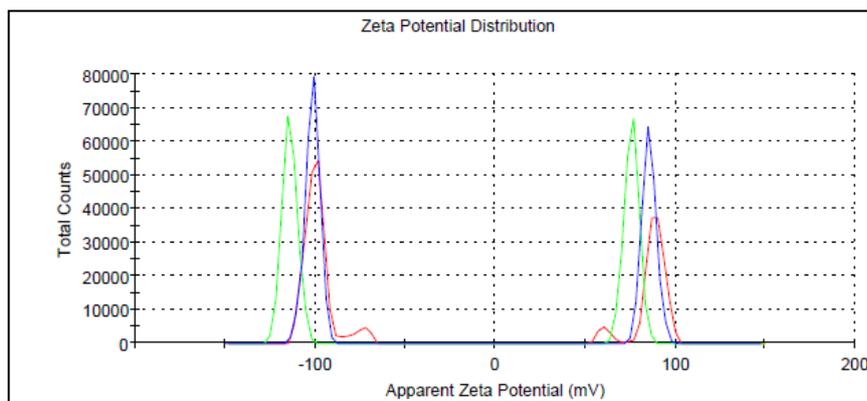


## Zeta Potential Analysis for Stability Study Formulations

### 1. Batch P18015: Pro-Pheroid®



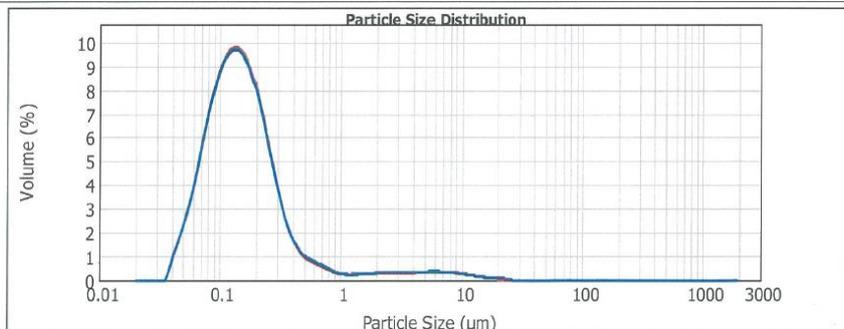
### 2. Pro-Pheroid®-goserelin 4 mg/kg



## Particle Size Analysis for Male Mice Study Formulations

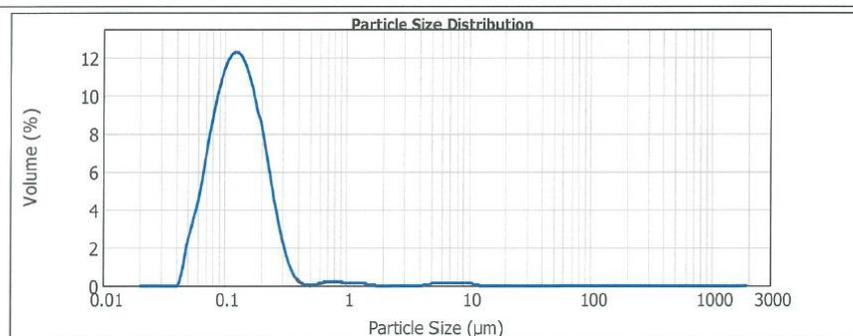
### 1. Pro-Pheroid®:

d(0.1): 0.069 um                      d(0.5): 0.145 um                      d(0.9): 0.406 um



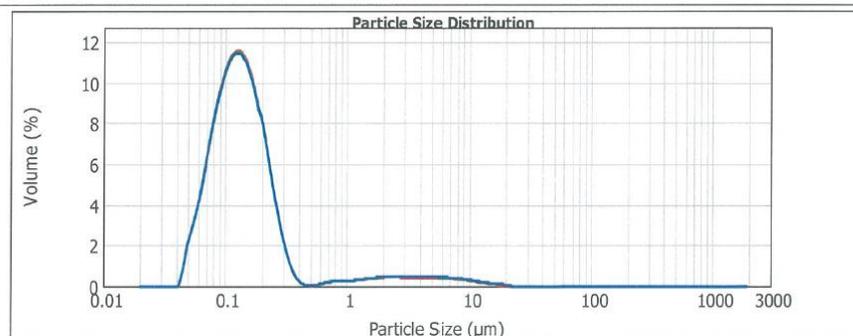
### 2. Pro-Pheroid®-goserelin 2 mg/kg

d(0.1): 0.069 um                      d(0.5): 0.126 um                      d(0.9): 0.234 um



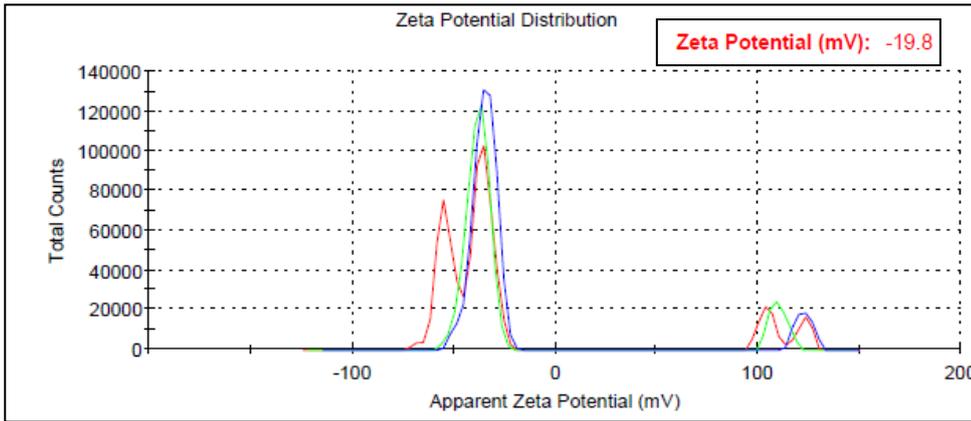
### 3. Pro-Pheroid®-goserelin 4 mg/kg

d(0.1): 0.071 um                      d(0.5): 0.133 um                      d(0.9): 0.293 um

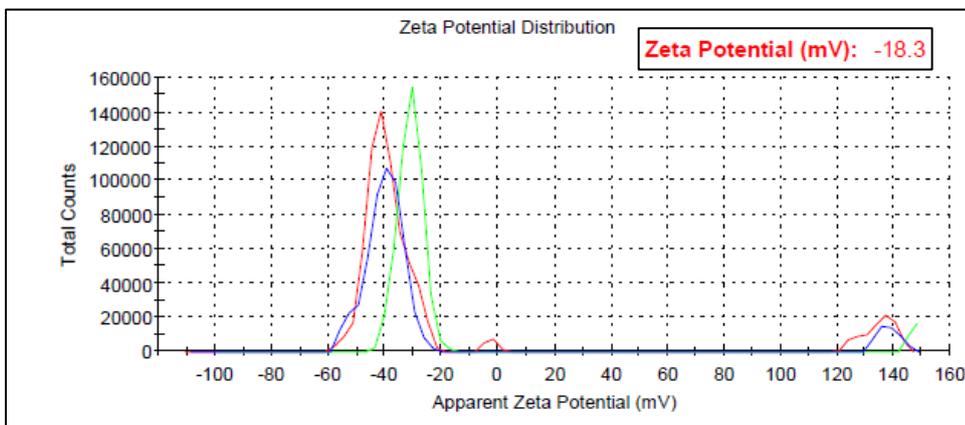


## Zeta Potential Analysis for Male Study Formulations

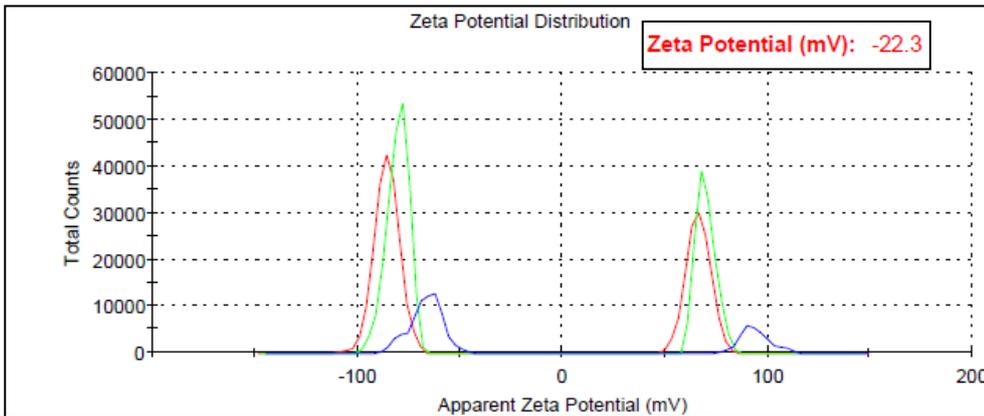
### 1. Pro-Pheroid®



### 2. Pro-Pheroid®-goserelin 2 mg/kg



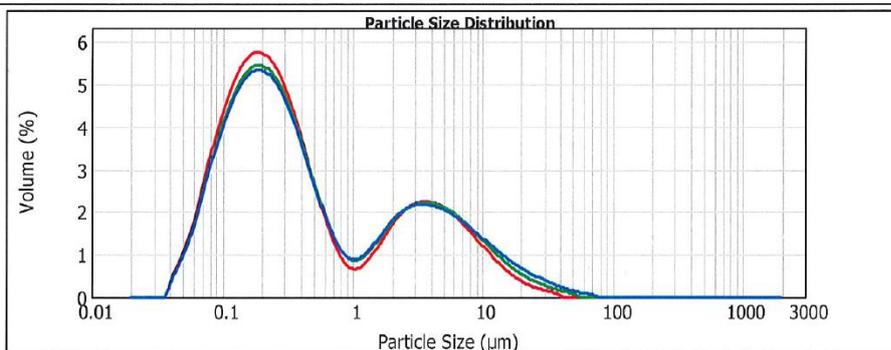
### 3. Pro-Pheroid®-goserelin 4 mg/kg



### Particle Size Analysis for Female Study Formulations

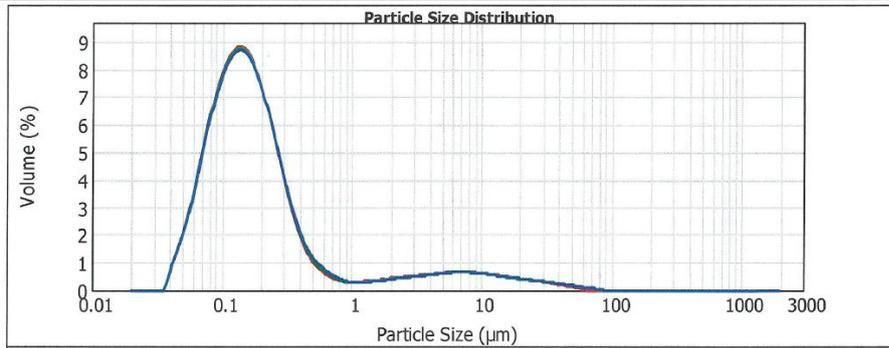
#### 1. Pro-Pheroid®

d(0.1): 0.092 um      d(0.5): 0.325 um      d(0.9): 7.478 um



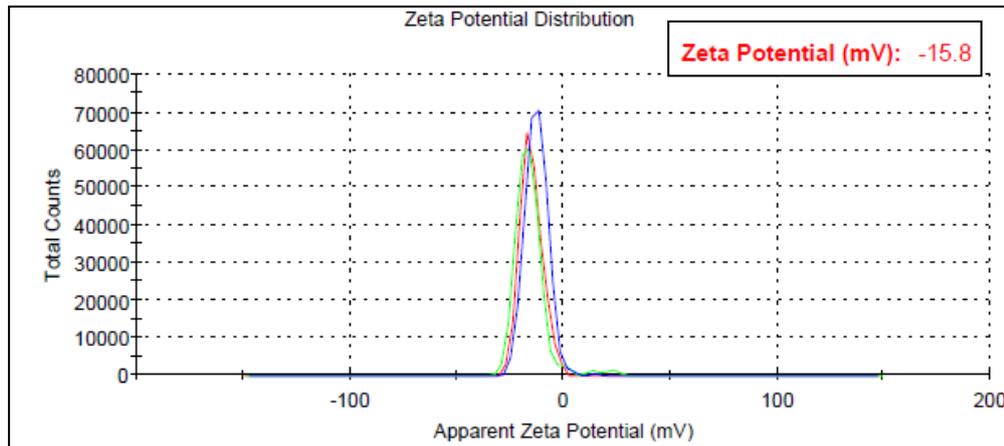
## 2. 4 mg/kg pro-Pheroid®-goserelin

d(0.1): 0.071 um      d(0.5): 0.159 um      d(0.9): 2.244 um

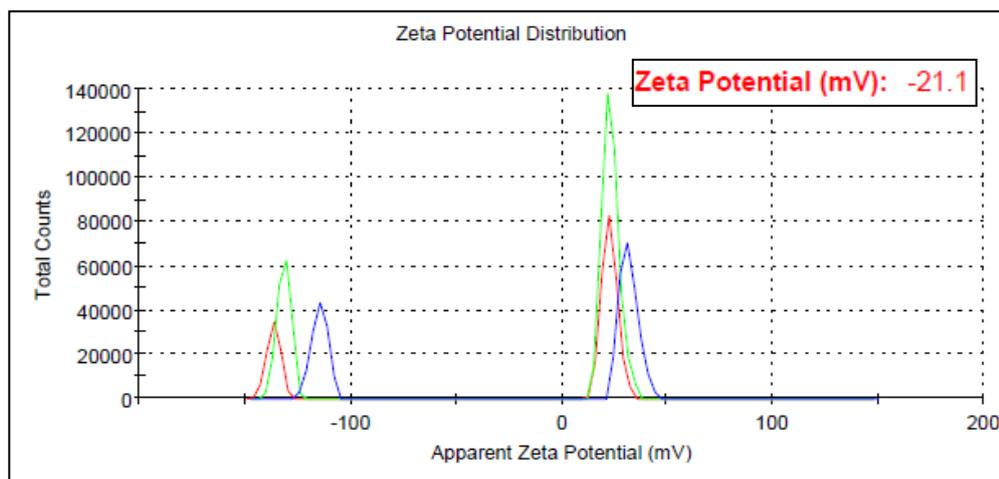


## Zeta potential Analysis for Female Study Formulations

### 1. Pro-Pheroid®



### 2. 4 mg/kg pro-Pheroid®-goserelin



## **ANNEXURE C – Statistical Analysis**

## One-way ANOVA: Assumptions

**Table 1:** Normality test

Variable	Normality (yes/no)
Goserelin C <sub>max</sub>	No
Goserelin T <sub>max</sub>	No

Neither Goserelin C<sub>max</sub> nor T<sub>max</sub> was normally distributed (according to the Normal probability plot)

**Table 2:** Equal variances

Variable	p-value
Goserelin C <sub>max</sub>	0.02
Goserelin T <sub>max</sub>	0.15

The  $p < 0.05$  thus the null

hypothesis ( $H_0$ ) is rejected.

## One-way ANOVA: Kruskal-Wallis

**Table 3:** Kruskal-Wallis

Variable	p-value
Goserelin C <sub>max</sub>	0.003
Goserelin T <sub>max</sub>	0.0005

The p-value  $< 0.05$  thus there is a significant difference between the mean groups of goserelin C<sub>max</sub> and goserelin T<sub>max</sub>, thus meaning that the  $H_0$  is rejected

## Non-parametric test: Multiple variants

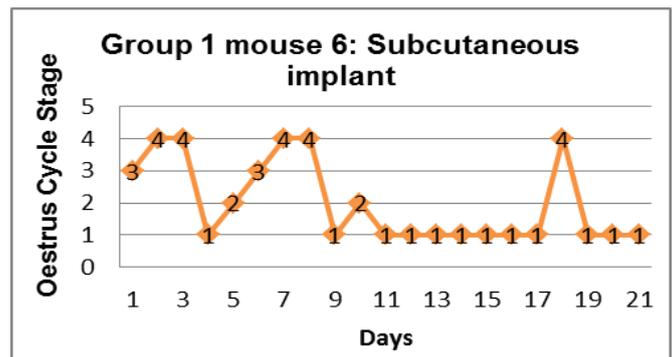
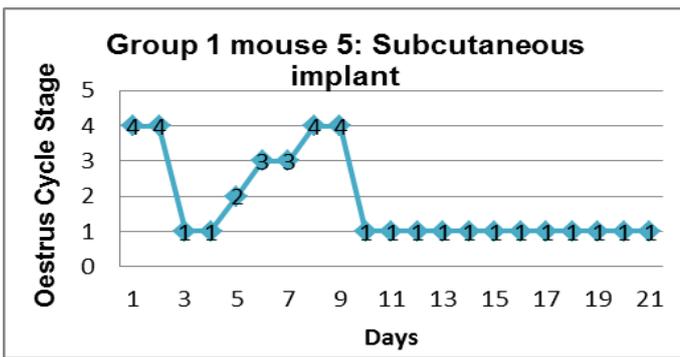
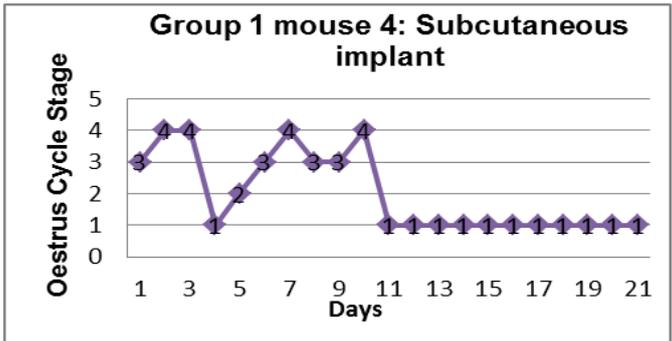
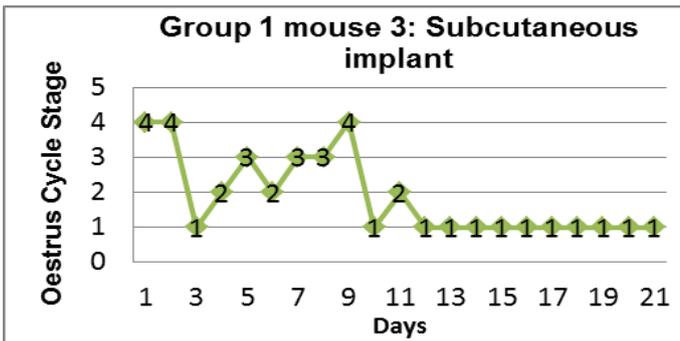
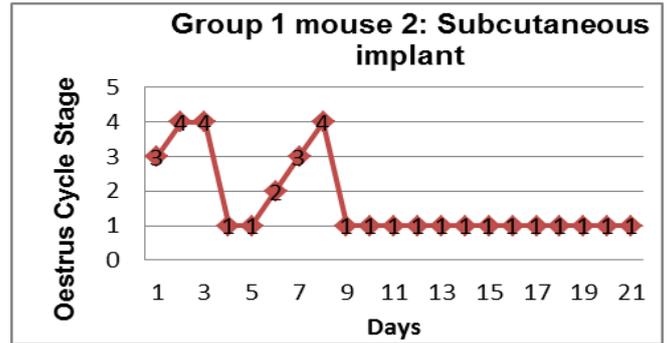
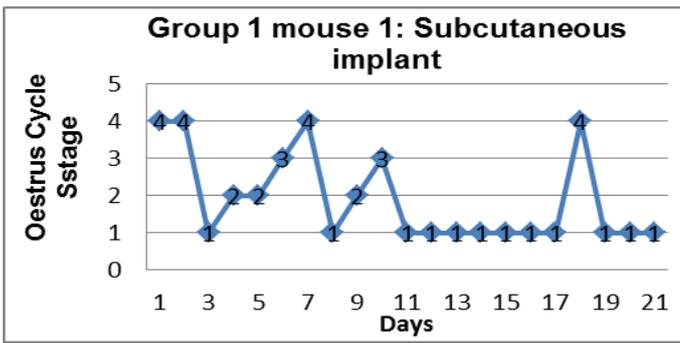
Variable	Group - Group	P value
Goserelin C <sub>max</sub>	1 - 2	0.006
	1 - 3	0.017
	2 - 3	1.00
Goserelin T <sub>max</sub>	1 - 2	0.145

	1 - 3	0.0003
	2 - 3	0.175

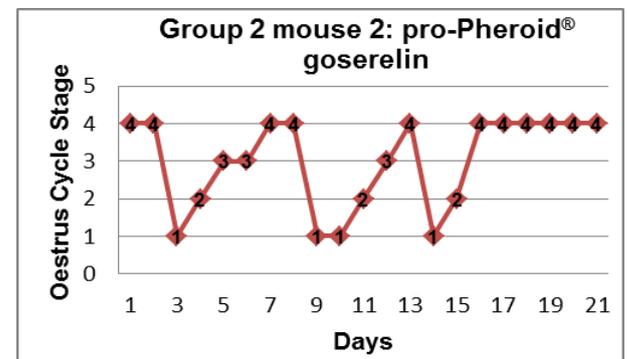
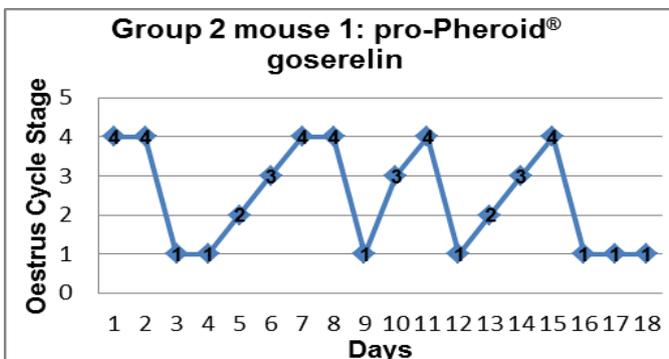
## **ANNEXURE D – Oestrous cycles of female mice**

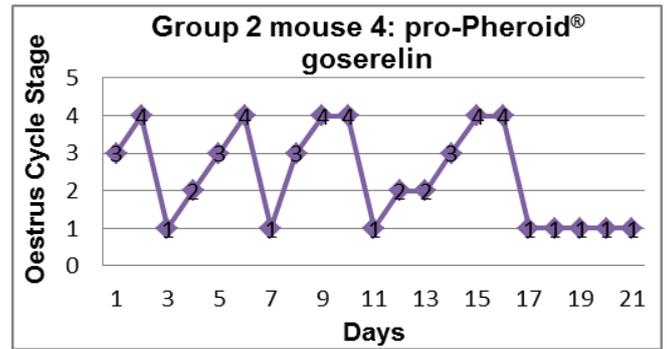
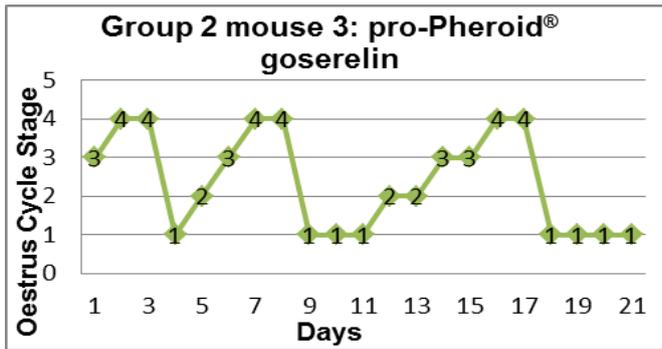
The diagrams below demonstrate the oestrous cycle of all female mice in each formulation group (administration of formulations was given at day 6).

1) Subcutaneous groups

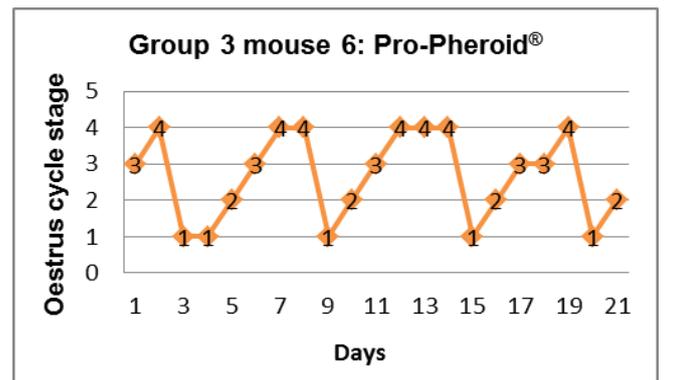
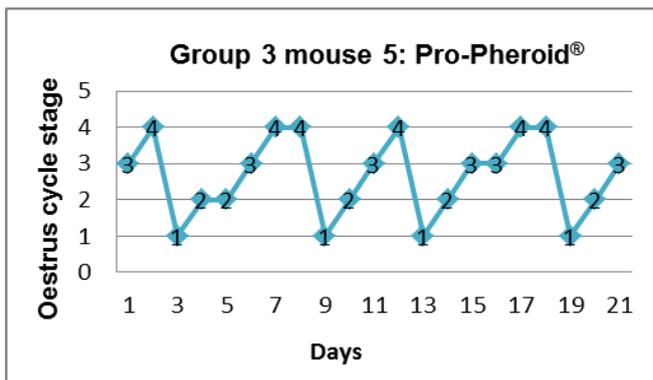
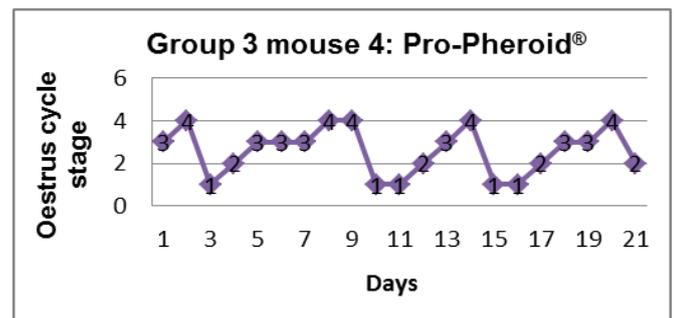
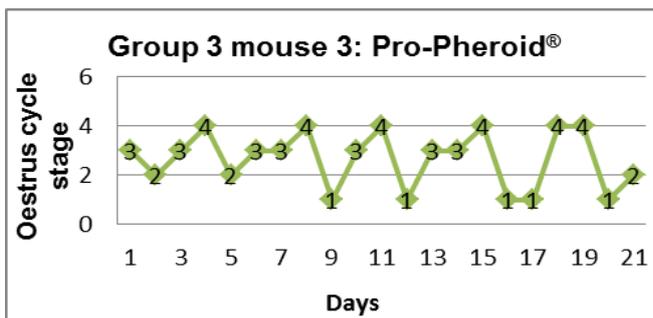
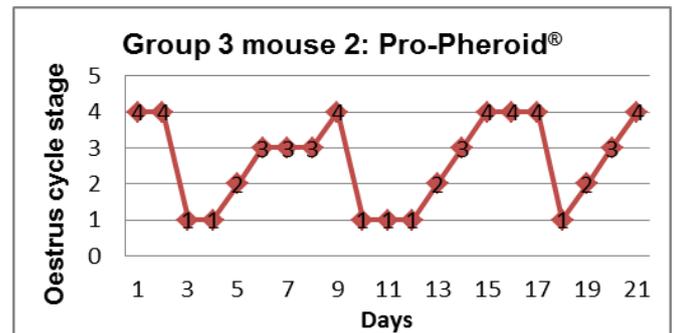
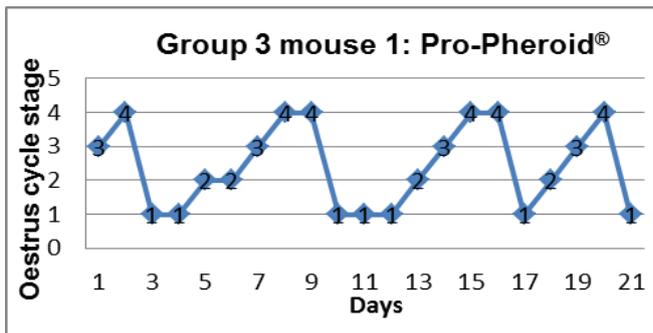
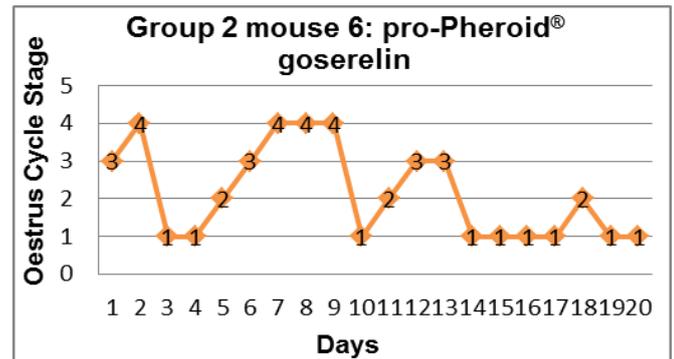
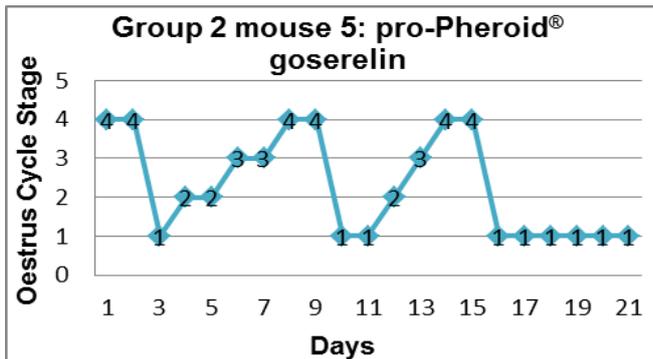


2) pro-Pheroid-goserelin® 4 mg/kg group





3) Pro-Pheroid® group



## **ANNEXURE E – Certificate of analysis of Vitamin F Ethyl Ester**

for fine cosmetics · since 1926



CLR · Postfach 410480 · 12114 Berlin · Germany  
**IMCD SOUTH AFRICA (PTY) LTD.**  
co Turners Shipping, Edenvale  
275 Oak Avenue  
2194 Johannesburg

Chemisches Laboratorium Dr. Kurt Richter GmbH  
Bennigsenstraße 25  
12159 Berlin  
Germany  
Tel +49 (0)30 85 10 26-0  
Fax +49 (0)30 85 10 26-85  
info@clr-berlin.com  
www.clr-berlin.com

### Certificate of Analysis Vitamin F Ethyl Ester CLR

Article	523	Customer	30135
Lot	3153386	Order No.	105889
Production date	24.11.2015	Retest Date	18.11.2016
Remark			

	Method	Specification	Result	Unit
<b>General Parameters</b>				
Refractive index (nD20)	CLR-pa001ch	1,4570 - 1,4590	1,4582	
Density (20°C)	CLR-pa002ch	0,8770 - 0,8800	0,8798	g/ml
Acid value	CLR-pa014ch	0,0 - 1,0	1,0	
Iodine value (Hanus)	CLR-pa018ch	145,0 - 155,0	150,7	
<b>Fatty acid distribution</b>				
< C16	CLR-pa042ch	0,0 - 1,0	0,0	%
C16-0	CLR-pa042ch	7,0 - 11,0	8,1	%
C18-0	CLR-pa042ch	2,0 - 5,0	4,1	%
C18-1	CLR-pa042ch	18,0 - 24,0	21,9	%
C18-2	CLR-pa042ch	31,0 - 37,0	32,6	%
C18-3	CLR-pa042ch	26,0 - 34,0	32,7	%
> C18	CLR-pa042ch	0,0 - 2,0	0,5	%
<b>Microbiological tests</b>				
Colony forming units			< 100	/ ml
Pathogenic germs			Not detectable	

2016/06263

Commerzbank AG · BIC: DRESDE33 · Page 0 Pages 1 of 1  
Deutsche Postbank AG · BIC: PBNKDE33 · IBAN: DE41 1001 0010 0008 1351 07  
HRB 53488 · Amtsgericht Charlottenburg · S.z. Berlin · USt-IdNr. DE 136763182 · Geschäftsführer Dr. Stefan Borchert

**ANNEXURE F - Poster for Drug Safety Africa 2018  
Conference (20 – 22 November 2018)**

# Evaluation of the oral delivery of goserelin with Pheroid® technology

Linné Erasmus<sup>1</sup>, Liezl-Marié Scholtz<sup>1</sup>, Kobus Venter<sup>1</sup>, Cor Bester<sup>1</sup>, Antoinette Fick<sup>1</sup>,  
Jacob Mabena<sup>1</sup>, Martin Magwaza<sup>2</sup>, Anne Grobler<sup>1</sup>, Rose Hayeshi<sup>1</sup>

<sup>1</sup>DST/NWU Preclinical Drug Development Platform (PCDDP), North-West University, Potchefstroom, South Africa  
<sup>2</sup>BGM Pharmaceuticals, Florida Park, South Africa

## Introduction

Various hormone-dependent disorders, such as benign conditions (e.g. uterine fibroids, menorrhagia, endometriosis etc.) or malignant tumours (breast, ovarian, endometrial and prostate carcinoma), are treated effectively with GnRH analogues. The therapeutic amphiphilic peptide, goserelin, is a type of hormone therapy that can be used in addition to standard adjuvant therapy (the standard therapy is surgery followed by radiotherapy, chemotherapy and/or tamoxifen). Currently, goserelin is only available as a slow release subcutaneous implant which is an invasive and generally not well-tolerated method of administration, thus the potential for changing the route to a less invasive route such as oral will yield great advantage. However, other alternative routes than parenteral administration like the oral route are not suitable for peptides and proteins due to degradation by enzymes and other factors present in the gastrointestinal (GI) tract. Although by the use of a drug delivery system (DDS), a pharmaceutical active ingredient can be protected from the harsh environment of the GI tract. Pheroid® technology is one such example of a DDS. Pheroid® is a novel colloidal DDS which has the ability and potential to improve the oral bioavailability of therapeutic drugs especially drugs such as peptides and proteins (Grobler et al, 2008; Steyn et al., 2011). Thus the prospect of changing to a less invasive administration method will yield a great advantage.

## Materials and Methods

Pheroid® technology was investigated as a drug delivery system (DDS) for goserelin in female Balb/c mice (ethics approval no: NWU-00268-16-A5). An oral pro-Pheroid®-goserelin formulation was compared the oral pro-Pheroid® vehicle. The following experimental design was used:

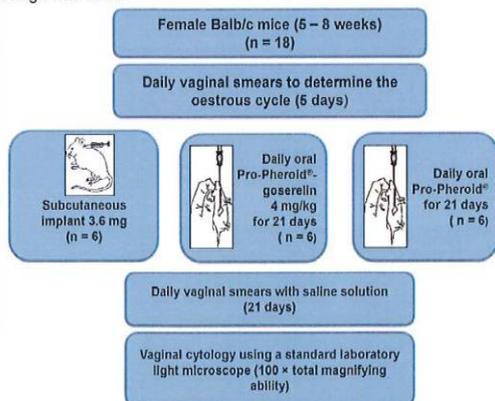


Figure 1: Experimental design

## Acknowledgments

- Study Supervisor: Prof. Rose Hayeshi
- Study Co-Supervisor: Liezl-Marie Scholtz
- BGM Pharmaceuticals, DST/NWU PCDDP & NWU for financial support
- BGM Pharmaceuticals for providing the goserelin

## Results

The goserelin subcutaneous implant and pro-Pheroid®-goserelin resulted in a disruption in the oestrous cycle on day  $\pm 9$  and  $\pm 15$  respectively, changing to a constant pro-oestrous stage (Figure 3). The representation of a vaginal smear after the disruption of the oestrus cycle is demonstrated in Figure 2.

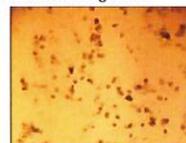


Figure 2: Representation of oestrus stage (pro-oestrous) after disruption by the goserelin from female vaginal smear

Pro-Pheroid® had no effect on the oestrous cycle, thus the disruptive effect seen in the pro-Pheroid®-goserelin group was due to the effective action of goserelin.

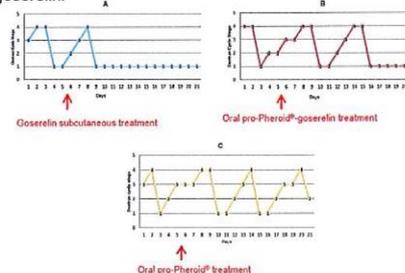


Figure 3: Representative effects on the oestrus cycle in one mouse from each group after A) goserelin subcutaneous implant, B) pro-Pheroid® goserelin oral administration and C) pro-Pheroid® oral administration. The arrow indicates the treatment start day. The oestrus stage is indicated by 1. Pro-oestrus 2. Oestrus 3. Metoestrus and 4. Dioestrus. Disruption can be seen in A and B.

## Conclusions

- Pro-Pheroid®-goserelin brought on a disruption in the cycle, thus Pheroid® presumably increased the oral bioavailability of goserelin.
- The use of Pheroid® technology for a DDS of goserelin is promising for the improvement and protection of the bioavailability of drugs with low oral bioavailability due to the harsh gastric environment.
- It is clear that Pheroid® technology has potential in future goserelin studies for the improvement of bioavailability to exploit this system's full potential.

## References

- Grobler, A., Kotzé, A. & Du Plessis, J. 2008. The Design of a Skin-Friendly Carrier for Cosmetic Compounds Using Pheroid™ Technology. *Science and Applications of Skin Delivery System Technologies* Allured. Ed. Johann Wechers: 283-311.
- Steyn, D., du Plessis, L.H. & Kotzé, A. 2010. Nasal Delivery of Recombinant Human Growth Hormone: in vivo Evaluation with Pheroid™ Technology and N-trimethyl Chitosan Chloride. *Journal of Pharmacy & Pharmaceutical Sciences*, 13(2):263-273.
- Kiesel, L.A., Rody, A., Greb, R.R. & Szilágyi, A. 2002. Clinical use of GnRH analogues. *Clinical Endocrinology*, 56(6):677-687.

## **ANNEXURE G – Certificate of Proof Reading**

# *Gill Smithies*

## *Proofreading & Language Editing Services*

59, Lewis Drive, Amanzimtoti, 4126, KwaZulu Natal

Cell: 071 352 5410 E-mail: [moramist@vodamail.co.za](mailto:moramist@vodamail.co.za)

### *Work Certificate*

<b>To</b>	Linnè Erasmus
<b>Address</b>	DST/NWU Preclinical Drug Development Platform, Potchefstroom Campus, North-West University
<b>Date</b>	16/11/2018
<b>Subject</b>	Evaluation of the oral delivery of goserelin with Pheroid® technology
<b>Ref</b>	GS/LE/01

I, Gill Smithies, certify that I have proofed the following dissertation, Evaluation of the oral delivery of goserelin with Pheroid® technology, to the standard as required by NWU, Potchefstroom Campus.

*Gill Smithies*

16/11/2018