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Transdermal Delivery of Progesterone

Sara Zargar-Shoshtari

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy, The University of Auckland, New Zealand

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Abstract

Background and Aims: Vasomotor hot flushes are one of the most common and troublesome symptoms of menopause. The current available therapeutic options for management of hot flushes are associated with either severe adverse effects or lack of therapeutic efficacy. Bioidentical progesterone is considered a safer alternative to its synthetic counterparts. However, transdermal penetration of progesterone is poor and variable due to the physical and enzymatic barriers of the skin. The objective of this thesis is to evaluate the efficacy of Self Microemulsifying Drug Delivery Systems (SMEDDS) together with inhibition of progesterone metabolism across the skin, for enhanced transdermal permeation.

Method: SMEDDS were characterised in terms of their water solubilisation capacity, the ability to form microemulsion, and thermal stability. In vitro diffusion studies across porcine skin was then carried out to measure the penetration rate of progesterone from SMEDDS and selected commercial formulations. Microscopic evaluation of the skin surface together with infrared spectroscopy was used to investigate the mechanism of action of SMEDDS. In the pilot clinical study, the effect of enzyme inhibitor dutasteride on the bioavailability of progesterone was investigated in postmenopausal women.

Results and Discussion: Systems based on Tween 85 and Cremophor EL as the surfactant, Imwitor 308 as the cosurfactant, and Myritol and Isopropyl Myristate as the oil phase were able to solubilise over 70% water. Water in oil microemulsion could solubilise 20-30% water and remained stable at 32°C. Subsequent in vitro studies showed that saturated SMEDDS significantly enhanced the penetration rate of progesterone across the skin when compared to control and commercial creams. The mode of action of SMEDDS was through their penetration enhancing ability rather than supersaturation. Inhibition of 5α-reductase enzymes did not increase the bioavailability of transdermal progesterone.

Conclusion: This thesis explored novel approaches to enhance the transdermal permeation of bioidentical progesterone. In addition to the generation of physiochemical data, the permeation rate of progesterone was significantly improved through development of a novel SMEDDS. The formulations developed could be applied to other steroidal hormones and the
Abstract

data generated provides the background information for further research on SMEDDS as topical or transdermal delivery systems. This thesis also demonstrated that 5α-reductase enzymes do not affect the transdermal bioavailability of progesterone.
Dedication

To my family, who has offered me love and support throughout the course of this thesis
Acknowledgments

I am heartily grateful to my supervisor, Dr Raid Alany, who encouraged me to do a PhD. I attribute what I have accomplished to the support, patience and knowledge he provided throughout, whilst allowing me room to work in my own way. I would also like to thank my co-supervisor, Dr Jing Wen, whose guidance and support from the first day of my PhD enabled me to develop an understanding of the project. Without the help of my supervisors, this thesis would have not been completed or written.

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The work on the clinical pilot study would not have been possible without the team at Sina Hospital Urology Research Center and Mirzakoochak Khan Woman’s Hospital. I would like to thank Dr Abdolrasoul Mehrsai and Dr Hananneh Wahhabaghei for assisting me in the development of the study protocol and application for ethics approval. I owe my deepest gratitude to Dr Firuzeh Akbari (CEO of Mirzakoochak Khan Hospital) for offering me the
hospital’s facilities. I am especially thankful to M Pashazadeh for helping me with the enzyme immunoassay studies.

I cannot find words to express my gratitude to my parents, who have sacrificed everything for me to get this far in life. They have supported me both financially and emotionally in every step of my PhD. I am indebted for their support with the clinical study, and particularly their assistance in recruiting menopausal women.

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Publications Arising From this Thesis

Refereed Journal Articles


Conference Abstracts

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### Lists of Abbreviations

- **Concentration in the Donor Solution**
- **Free Energy**
- **Change in Entropy**
- **Change in the Surface Area**
- **AAE** Average Absolute Error
- **ATR** Attenuated Total Reflectance
- **AUC** Area Under the Curve
- **C_{average}** Average Progesterone Concentration Between 0 and 12 h
- **C_{baseline}** Baseline Progesterone Concentration on Day 0
- **C_{baseline 30}** Baseline Progesterone Concentration on Day 30
- **CBG** Corticosteroid Binding Globulin
- **CD** Cyclodextrins
- **CEE** Conjugated Equine Estrogen
- **LC-MS/MS** Chromatography–Tandem Mass Spectrometry
- **CH** Carbon-Hydrogen
- **CHI_g** Gradient Chromatography Hydrophobic Index
- **CHI_i** Isocratic Chromatography Hydrophobic Index
- **C_{max}** Maximum Progesterone Concentration Achieved in 12 h
- **CMC** Critical Micelle Concentration
- **C_n** Concentration of the Withdrawn Sample
- **C_{octanol}** Concentration in the Octanol Phase
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>$C_t$</td>
<td>Concentration in the Receptor Solution</td>
</tr>
<tr>
<td>$C_{\text{water}}$</td>
<td>Concentration in the Aqueous Phase</td>
</tr>
<tr>
<td>D</td>
<td>Diffusion Coefficient</td>
</tr>
<tr>
<td>DE</td>
<td>Dermal Edema</td>
</tr>
<tr>
<td>DL</td>
<td>Dilution Line</td>
</tr>
<tr>
<td>DLS</td>
<td>Dynamic Light Scattering</td>
</tr>
<tr>
<td>Dr</td>
<td>Dermis</td>
</tr>
<tr>
<td>DS</td>
<td>Degree of Saturation</td>
</tr>
<tr>
<td>DSC</td>
<td>Differential Scanning Calorimetry</td>
</tr>
<tr>
<td>$d\sigma/d\phi$</td>
<td>Maximum Change in the Electrical Conductivity Relative to the Change in the Volume Fraction of Water</td>
</tr>
<tr>
<td>D$\phi$</td>
<td>Change in the Volume Fraction of Water</td>
</tr>
<tr>
<td>EF</td>
<td>Enhancement Factor</td>
</tr>
<tr>
<td>Ep</td>
<td>Epidermis</td>
</tr>
<tr>
<td>EPO</td>
<td>Evening primrose oil</td>
</tr>
<tr>
<td>ERT</td>
<td>Estrogen Replacement Therapy</td>
</tr>
<tr>
<td>E-state index</td>
<td>Electropotopological Index</td>
</tr>
<tr>
<td>F</td>
<td>Fishers Test Value</td>
</tr>
<tr>
<td>Fr</td>
<td>Epidermal Fragmentation</td>
</tr>
<tr>
<td>FSH</td>
<td>Follicle Stimulating Hormone</td>
</tr>
<tr>
<td>FTIR</td>
<td>Fourier Transform Infrared Spectroscopy</td>
</tr>
<tr>
<td>GI</td>
<td>Gastrointestinal</td>
</tr>
<tr>
<td>GSE</td>
<td>Generalised Solubility Equation</td>
</tr>
<tr>
<td>h</td>
<td>Hour(s)</td>
</tr>
<tr>
<td>$H$</td>
<td>Thickness of the Membrane</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td>Hydrogen Peroxide</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric Acid</td>
</tr>
<tr>
<td>HDL</td>
<td>High Density Lipoprotein</td>
</tr>
<tr>
<td>HERS</td>
<td>Heart and Estrogen/Progestin Replacement Therapy</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Hematoxylin–Eosin</td>
</tr>
<tr>
<td>HLB</td>
<td>Hydrophilic-Lipophilic Balance</td>
</tr>
<tr>
<td>HRT</td>
<td>Hormone Replacement Therapy</td>
</tr>
<tr>
<td>IPM</td>
<td>Isopropyl Myristate</td>
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<td>Partition Coefficient</td>
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<tr>
<td>K’</td>
<td>Retention Factor</td>
</tr>
<tr>
<td>KCl</td>
<td>Potassium Chloride</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>Potassium Dihydrogen Phosphate</td>
</tr>
<tr>
<td>Km</td>
<td>Mass Ratio of Surfactant to Cosurfactants</td>
</tr>
<tr>
<td>LDL</td>
<td>Low Density Lipoprotein</td>
</tr>
<tr>
<td>LO</td>
<td>Lecithin Based Organogels</td>
</tr>
<tr>
<td>LOD</td>
<td>Limit of Detection</td>
</tr>
<tr>
<td>logK'$_w$</td>
<td>Measure of Lipophilicity at 100% Water</td>
</tr>
<tr>
<td>logP</td>
<td>Logarithm of Octanol Water Partition Coefficient</td>
</tr>
<tr>
<td>logS$_w$</td>
<td>Logarithm of Aqueous Solubility</td>
</tr>
<tr>
<td>LOO</td>
<td>Leave One Out</td>
</tr>
<tr>
<td>LOQ</td>
<td>Limit of Quantification</td>
</tr>
<tr>
<td>LSER</td>
<td>Linear Solvation Energy Relationship</td>
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<tr>
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<tr>
<td>LTR</td>
<td>Localised Transport Regions</td>
</tr>
<tr>
<td>ME</td>
<td>Microemulsion</td>
</tr>
<tr>
<td>MHPG</td>
<td>3-methoxy-4-hydroxyphenylglycol</td>
</tr>
<tr>
<td>min</td>
<td>Minute(s)</td>
</tr>
<tr>
<td>MP</td>
<td>Melting Point</td>
</tr>
<tr>
<td>MPA</td>
<td>Medroxyprogesterone Acetate</td>
</tr>
<tr>
<td>N</td>
<td>Sample Size</td>
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<tr>
<td>NADPH</td>
<td>Nicotinamide Adenine Dinucleotide Phosphate</td>
</tr>
<tr>
<td>Na₂HPO₄</td>
<td>Disodium Hydrogen Phosphate</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium Chloride</td>
</tr>
<tr>
<td>NaOH</td>
<td>Sodium Hydroxide</td>
</tr>
<tr>
<td>OECD</td>
<td>Organisation for Economic and Cooperation and Development</td>
</tr>
<tr>
<td>o/w</td>
<td>Oil in water</td>
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<tr>
<td>P</td>
<td>Octanol Water Partition Coefficient</td>
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<tr>
<td>PCS</td>
<td>Photon Correlation Spectroscopy</td>
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<tr>
<td>PDI</td>
<td>Polydispersity Index</td>
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<tr>
<td>PEG 400</td>
<td>Propylene Glycol 400</td>
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<tr>
<td>PIT</td>
<td>Phase Inversion Temperature</td>
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<tr>
<td>PSA</td>
<td>Pressure Sensitive Adhesives</td>
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<tr>
<td>QSAR</td>
<td>Quantitative Structural Activity Relationship</td>
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<tr>
<td>QSPR</td>
<td>Quantitative Structure Permeability Relationship</td>
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<tr>
<td>q₁</td>
<td>Cumulative Amount</td>
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<tr>
<td>R₂</td>
<td>Excess Molar Refraction</td>
</tr>
<tr>
<td>R²</td>
<td>Correlation Coefficient</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>-------------</td>
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<tr>
<td>RH</td>
<td>Hydrodynamic Radius</td>
</tr>
<tr>
<td>RP-HPLC</td>
<td>Reversed Phase HPLC</td>
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<tr>
<td>RSD</td>
<td>Relative Standard Deviation</td>
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<tr>
<td>SC</td>
<td>Stratum Corneum</td>
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<tr>
<td>SD</td>
<td>Standard Deviation</td>
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<tr>
<td>SE</td>
<td>Standard Error</td>
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<tr>
<td>SEM</td>
<td>Scanning Electron Microscopy</td>
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<td>SLN</td>
<td>Solid Lipid Nanoparticles</td>
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<tr>
<td>SMEDDS</td>
<td>Self Microemulsifying Drug Delivery systems</td>
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<tr>
<td>SSME</td>
<td>Supersaturated Microemulsion</td>
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<tr>
<td>SSRI</td>
<td>Selective Serotonin Reuptake Inhibitors</td>
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<td>T</td>
<td>Absolute Temperature</td>
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<tr>
<td>t₀</td>
<td>Dead Volume</td>
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<td>T₃</td>
<td>Total Triiodothyronine</td>
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<td>T₄</td>
<td>Thyroxin</td>
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<tr>
<td>Tₜₐₜₚₑₜₜ</td>
<td>Cloud Point</td>
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<tr>
<td>TDDS</td>
<td>Transdermal Drug Delivery Systems</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission Electron Microscopy</td>
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<tr>
<td>Tₘₐₓ</td>
<td>Time to Maximum Progesterone Concentration</td>
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<tr>
<td>tₗ</td>
<td>Retention Time</td>
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<tr>
<td>TR</td>
<td>Gradient Retention Time</td>
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<tr>
<td>TSH</td>
<td>Thyroid Stimulating Hormone</td>
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<tr>
<td>UV</td>
<td>Ultraviolet</td>
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<tr>
<td>UV-VIS</td>
<td>Ultraviolet Visible Spectroscopy</td>
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<tr>
<td>v/v</td>
<td>Volume by Volume</td>
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**Lists of Abbreviations**

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<td>V&lt;sub&gt;n&lt;/sub&gt;</td>
<td>Volume of the Withdrawn Sample</td>
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<tr>
<td>V&lt;sub&gt;r&lt;/sub&gt;</td>
<td>Volume of the Receptor Solution</td>
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<tr>
<td>V&lt;sub&gt;x&lt;/sub&gt;</td>
<td>McGowan Characteristic Volume</td>
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<tr>
<td>w/o</td>
<td>Water in Oil</td>
</tr>
<tr>
<td>w/w</td>
<td>Weight by Weight</td>
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<tr>
<td>WHI</td>
<td>Women’s Health Initiative</td>
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<tr>
<td>W&lt;sub&gt;max&lt;/sub&gt;</td>
<td>Water Solubilisation Capacity</td>
</tr>
<tr>
<td>Z-Average</td>
<td>Average Droplet Size</td>
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<tr>
<td>H</td>
<td>Viscosity</td>
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<tr>
<td>η&lt;sub&gt;o&lt;/sub&gt;</td>
<td>SMEDDS Viscosity</td>
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<tr>
<td>Σ</td>
<td>Electrical Conductivity</td>
</tr>
<tr>
<td>Σα&lt;sub&gt;2&lt;/sub&gt;^H</td>
<td>Hydrogen Bond Acidity</td>
</tr>
<tr>
<td>Σβ&lt;sub&gt;2&lt;/sub&gt;^H</td>
<td>Hydrogen Bond Basicity</td>
</tr>
<tr>
<td>Φ</td>
<td>Volume Fraction of Water</td>
</tr>
<tr>
<td>φ&lt;sub&gt;C&lt;/sub&gt;</td>
<td>Percolation Threshold</td>
</tr>
<tr>
<td>φ&lt;sub&gt;o&lt;/sub&gt;</td>
<td>Volume Fraction of Organic Modifier</td>
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<td>logP Values Based on the HPLC Model</td>
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<td>Experimental logP Value</td>
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<td>Interfacial Tension</td>
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<tr>
<td>η&lt;sub&gt;2&lt;/sub&gt;^H</td>
<td>Polarisability</td>
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<tr>
<td>δ&lt;sub&gt;i&lt;/sub&gt;</td>
<td>Solubility Parameter of the Permeant</td>
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<tr>
<td>δ&lt;sub&gt;s&lt;/sub&gt;</td>
<td>Solubility Parameter of the Skin Lipids</td>
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Introduction

1.1 Menopause

Menopause is the complete cessation of the menstrual cycle for at least 12 months [1]. The average menopausal age is 51.5 years, although natural biological changes begin several years preceding the last menstrual period [2]. Menopause occurs either naturally or as the result of surgery, radiation, or chemotherapy. As a woman approaches menopause, the menstrual cycle becomes irregular and eventually stops. At the same time, the number of oocytes reduce, reaching about 10% in the last few months proceeding menopause [3]. Increased levels of Follicle Stimulating Hormone (FSH) and declining levels of estrogens and progesterone are the most predominant hormonal changes observed during the menopausal transition. Symptoms experienced during this phase are directly related to the deficiency of estrogens and progesterone.

The menopausal transition, shown in Figure 1.1, is defined by three distinctive periods: premenopause, early menopause and late menopause. Premenopause is the time from when the menopausal symptoms start until 12 months after the last menstrual cycle. The most common symptoms experienced during this stage include hot flushes, vaginal dryness, dyspareunia, urinary incontinence, mood instability, and depression [4]. In most women, these symptoms last about 1-2 years, although some may experience them for over 30 years. The rate of bone turnover increases during the transitional phase, predisposing women to an increased risk of vertebral and hip fractures within 5-10 years after menopause (late menopause). Hormone deficiency is correlated with an increased risk of breast cancer. The proceeding sections will further discuss three of the most common symptoms experienced during the menopause, namely urogenital changes, osteoporosis, and vasomotor symptoms.
Figure 1.1: Schematic representation of various stages and symptoms of the menopausal transition period.
The image is redrawn and modified from reference [1].

1.1.1 Urogenital Changes

The healthy vagina is thick with lubricating abilities, containing a large number of estrogen receptors and high levels of glycogens, which are important for maintenance of vaginal acidity [5]. In menopause, reduced estrogen levels will abolish glycogen production leading to an increase in the vaginal pH and the overgrowth of contaminating organisms such as streptococci, staphylococci and diphtheria [6]. As the blood supply to the vagina diminishes, it becomes thin and inelastic. The vaginal lubrication also decreases. These physiological changes contribute to vaginal atrophy, which presents as vaginal dryness, burning, itching, dyspareunia, discharge, and bleeding. Vaginal bleeding and dyspareunia are reported by 27%-55% and 32%-41% of women respectively [7].
1.1.2 Osteoporosis

Osteoporosis is one of the leading causes of disability and death in postmenopausal women [8]. About 20% of postmenopausal women have osteoporosis and 52% have a low bone mineral density in the hip bone [9]. The rate of bone loss during and 4 years after menopause increases to about 1-2% annually [10, 11]. Recker et.al. reported a total bone loss of 10.5% for spine, 7.7% for the total body mass, and 5.3% for femoral neck within 7 years surrounding the menopause [11]. Low bone mineral density increases the risk of fractures within 5-10 years after menopause. Menopausal women ( >50 years old) have a 40% risk of bone fractures [9].

1.1.3 Vasomotor Symptoms

Vasomotor symptoms, including hot flushes and night sweats are one of the most common symptoms associated with menopause. Hot flushes are also problematic in men and women undergoing cancer treatment. Hot flushes are described as the abrupt, transient sensation of warmth, which usually starts in the face and spreads to the upper body. The term night sweats refers to flushes that occur with perspiration during the sleep. About 80% of menopausal women may experience vasomotor symptoms [2] that normally subside within a few years, although for some it may last for more than 30 years [12]. In some women hot flushes come on quickly and reach maximum intensity within a few minutes before gradually fading away. Others may experience a less intense hot flush lasting for up to 30 minutes. Increase in core body temperature (by 0.1°C), skin temperature, heart rate, and cutaneous blood flow are common physiological changes observed prior to a hot flush [13].

Hot flushes have been closely linked to estrogen withdrawal, although no direct correlation has been noted between plasma estrogen levels and occurrence of these symptoms [14]. Furthermore, antidepressants have shown to improve hot flushes significantly without any direct effect on plasma estrogen. Therefore, the involvement of additional mechanisms such as an increase in the core body temperature and changes in the levels of noradrenaline and serotonin have been investigated.
Figure 1.2 represents the physiological changes taking place before the sensation of a hot flush. Increase in the core body temperature is a direct result of changes in the thermoregulatory set point, followed by perspiration and vasodilatation, the classical mechanisms of heat loss. The thermoregulatory set point is a range of temperatures within the sweating and shivering thresholds, at which the major thermoregulatory responses (sweating and shivering) are not activated (Figure 1.2). The narrowing of the set point is associated with the activation of the central α₂ noradrenergic receptors as the result of an imbalance in the levels of the neurotransmitters noradrenaline and serotonin [15]. Following the trigger of a hot flush, high levels of plasma 3-methoxy-4-hydroxyphenylglycol (MHPG), the predominant metabolite of noradrenalin, have been noted [16]. This shows possible changes in the level of noradrenalin prior to and during the onset of a hot flush. Furthermore, yohimbine, an α₂ adrenergic antagonist, elevated the levels of MHPG and noradrenalin, triggering a hot flush whilst clonidine, an α₂ adrenergic agonist, reduced the central noradrenergic activity and hot flushes [17, 18]. All these observations support the possible involvement of noradrenalin in thermoregulation and physiology of hot flushes.

![Figure 1.2: Physiological events taking place before the onset of a hot flush. Modified from [16].](image-url)
1.2 Treatment of Vasomotor Associated Symptoms

1.2.1 Hormone Replacement Therapy (HRT)

Estrogens are C18 endogenous hormones with a wide range of physiological activities. There are three forms of estrogens found in the body, namely estrone, estradiol, and estriol (Figure 1.3). These hormones are synthesised from testosterone or androstenedione through aromatisation of ring A [8]. During the reproductive period, estradiol is the predominant hormone in the body. As the menopausal transition approaches, estradiol concentration drops significantly so that estrone becomes the dominant hormone. Prior to the publication of the Women’s Health Initiative (WHI) and the Heart and Estrogen/Progestin Replacement Therapy (HERS), Estrogen Replacement Therapy (ERT) was considered as the standard treatment for management of vasomotor symptoms.

ERT can reduce menopausal hot flushes by up to 90%. Oral 17β Estradiol (0.5-1 mg/day) and Conjugated Equine Estrogen\(^1\) (CEE) (0.625 mg/day) are the most commonly used ERTs for management of vasomotor symptoms, although the new formulation of oral Estradiol acetate (EA) is also effective [19, 20]. A randomised double-blinded study compared the therapeutic efficacy of EA 0.45mg/day, EA 0.9 mg/day and EA 1.8 mg/day to placebo [19]. After 12 weeks, the frequency of hot flushes reduced significantly by 61% (0.45 mg/day), 78% (0.9 mg/day) and 91% (1.8 mg/day) in the treatment groups, compared to 45% in the placebo group.

Transdermal ERT is used at a lower dose due to the avoidance of first hepatic pass metabolism. In one study, the frequency and severity of hot flushes was reduced significantly by up to 95% after treatment with ultralow dose (14 µg/day) transdermal estradiol for 12 weeks [21]. Vaginal formulations of estrogen are also available, although these are mainly used for treatment of vaginal atrophy and will not be further discussed.

\(^1\) CEE is a complex mixture of more than 10 different estrogens. Estrone sulphate and equilin sulphate are the main constituents.
Unopposed estrogen is associated with an increased risk of endometrial cancer. The incidence of endometrial cancer is 2.8 times higher in ERT users compared to never users [22] and further increases by 6.7 fold when ERT is used for > 5 years [22]. The incidence of endometrial hyperplasia, the marker of endometrial cancer, depends on the dose and formulation of estrogen as well as the duration of treatment [23]. In clinical trials, annual prevalence of up to 53% has been reported [24]. Generally, the risk of endometrial hyperplasia after 1 year of treatment with low dose oral CEE (0.3 or 0.45 mg/day), oral ethinyl estradiol (5 µg/ml) or oral esterified estrogen (0.3 mg/day) is similar to placebo [25]. However, one study showed that the incidence of endometrial hyperplasia with low dose CEE (0.3 mg/day) was comparable to CEE 0.625 mg/day [26] and further increased when ERT was taken for > 8 years. In general, ERT is primarily preserved for women who have had a hysterectomy.

Endometrial cancer or hyperplasia can be prevented when ERT is combined with progestins in a continuous or a sequential regimen [27]. Progestins are differentiated into three main groups: pregnane derivatives, 19-norpregnane derivatives, and 19-nortestosterone derivatives. A critical requirement for their progestronic activity is the presence of a double bond between the C4 and C5 in the A ring and a 3-keto group (Figure 1.4) [28]. Tibolone and norgestimate are examples of prodrugs, which lack this property but are converted to active progestins when administered orally.

Monotherapy with oral (10 mg/day) and depot (150 mg/month) Medroxyprogesterone Acetate (MPA) is as effective as CEE (0.625 mg/ day) in reducing menopausal hot flushes [29-31]. Recently, oral MPA was established as the standard therapy for reducing hot flushes in men.
undergoing androgen suppression treatment [32]. Monotherapy with megestrol acetate is also effective for alleviating hot flushes in men and women [33].

**Figure 1.4:** The chemical structure of progesterone and selected progestins.

The highlighted areas represent A: 3-keto group; and B: double bond between C4 and C5 in the A ring, which are required for progestronic activities.

### 1.2.1.1 Risks associated with Hormone Replacement Therapy (HRT)

Following the publication of several studies in the late 90s and early 2000s, many patients stopped using Hormone Replacement Therapy (HRT). It was found that the incidence of breast cancer, stroke, venous thromboembolism and ovarian cancer significantly increases with HRT. The Million Women Study and the WHI reported a significant increase in the risk of breast cancer and mortality in current HRT users, although treatment with CEE alone did not impose such a major risk [34-36]. The WHI trial also reported a 29% increase in the rate of cardiovascular events in women taking combined HRT [35]. HRT is also associated with
uterine bleeding, breast tenderness, nausea, dizziness, bloating, hirsutism, fluid retention in the extremities, weight gain, depression, and altered lipid and glucose metabolism. HRT is contraindicated in women with a history of breast cancer, hormone induced neoplasia, liver dysfunction, and venous and arterial thromboembolism.

Since the publication of the WHI and the Million Women Study, much attention has been paid to finding novel alternatives for the management of vasomotor hot flushes without imposing a major risk to women’s health. Natural products can be used for management of mild to moderate symptoms or as an add-on therapy in more severe cases. Non-hormonal therapy is reserved for management of more severe symptoms in women where HRT is ineffective or contradicted.

1.2.2 Life Style Modification and Natural Products

Regular exercise is shown to be effective for the management of mild to moderate vasomotor symptoms, with only 5% of active women reporting severe symptoms compared to 16% of women with little or no exercise [37]. Vitamin E supplementation [38], smoking cessation and weight reduction are also effective for managing symptoms. Smoking can predispose the women to severe and frequent hot flushes. The menopausal age is reduced by nearly 1.74 years in smokers [39].

Herbal remedies such as isoflavonoid, black cohosh extract, ginseng and evening primrose oil are commonly used by symptomatic menopausal women. Populations with isoflavonoid rich diets have a lower incidence of hot flushes and coronary heart disease [40]. In fact, 80% of Western women experience hot flushes compared to 9.8-37% of Asian women [6, 41]. Soybean and red clover are a major source of dietary isoflavonoid, a diphenolic phytoestrogen that is converted to estrogen like compounds in the gastrointestinal tract. Improvement in menopausal symptoms has only been demonstrated by soy products [42], with the maximum relief observed in those which are able to convert daidzein, the main component of soy, to a substance known as equol [43].

Of the primary active constituents of black cohosh including flavonoid, aromatics and triterpenes [44], the standardised isopropanolic extract, containing 5% of 27-deoxyacetin
(Remifemin), has been most widely evaluated. Recent trials comparing black cohosh against estrogenic compounds have generated mixed results, with one study showing no improvement in hot flushes with 40 mg of black cohosh extract [45]. In a larger study, back cohosh 40 mg/day (Remifemin) was shown to be as effective as oral tibolone 2.5 mg/day [46].

Ginseng, ginkgo, and evening primrose oil (EPO) are also used for decreasing vasomotor symptoms. Their mechanism of action might be related to the rich supply of γ-linolenic acid, flavonoid and bioflavonoids. However, long-term clinical data are not available to support their efficacy in reducing vasomotor symptoms.

1.2.3 Non-hormonal Therapy

Certain classes of antidepressants and anticonvulsants can significantly improve hot flushes in symptomatic women. These are further discussed below.

Oral gabapentin, a novel γ-aminobutyric acid analogue, significantly reduces the severity and frequency of hot flushes in postmenopausal women, including those with a history of breast cancer [47, 48]. This effect might be due to the modulation of the hypothalamic calcium channels, which are involved in mediating the thermoregulatory set point, although this is not fully understood [47, 49]. The most common side effects of gabapentin reported by postmenopausal women include somnolence (20%), dizziness (13%-50%), headache (50%), rash (7%), heart palpitation, and peripheral edema [47, 48]. Fatigue, nausea, ataxia, nystagmus, and tremor are other common adverse effects associated with this drug.

Venlafaxine, a novel antidepressant, reduces hot flush severity by up to 61% with the maximum improvement observed at an oral dose of 75 or 150 mg daily [50]. In women who attained a significant reduction in physiological hot flushes (>50%), venlafaxine can also improve sleep quality and mental health, although it is not as effective as MPA [51]. The selective Serotonin Reuptake Inhibitors (SSRI), paroxetine and fluoxetine, have been used for managing vasomotor symptoms in menopausal women including those with a history of breast cancer [52-54]. The most frequently reported side effects of SSRI, nausea and vomiting, have led to withdrawal of up to 21% of subjects in clinical trials [55]. Other adverse effects reported include sweating, weight gain, somnolence, and sexual dysfunction. A dose
related increase in blood pressure has been observed in menopausal women treated with venlafaxine, with a mean increase of 7.2 mm Hg at a daily dose of 375 mg [56].

Alpha-adrenergic agonists clonidine hydrochloride (0.1-0.4 mg/day) and methyldopa (250 mg/day) can reduce hot flushes in symptomatic women by up to 65% when taken orally [57-59]. Transdermally, 0.1 mg/day of clonidine reduces the frequency of hot flushes in 80% of subjects [60]. Methyldopa and clonidine are associated with serious side effects including bradycardia (clonidine), hemolytic anemia, and liver disorders (methyldopa).

Veralipride is a synthetic benzamide derivative with antidopaminergic activity. Monotherapy with veralipride (100mg/day) or treatment on alternate days in combination with raloxifene is effective for reducing the frequency and severity of menopausal hot flushes [61, 62]. Veralipride is associated with increased prolactin and galactorrhea.

The efficacy of Bellergal, a combination of ergotamine tartrate, levorotatory alkaloids, and phenobarbital, for management of vasomotor hot flushes is controversial. In one study, both Bellergal and placebo significantly reduced hot flushes, although significant differences were not observed between the groups [63]. About 38% of subjects withdrew from this study due to adverse effects such as skin rash, dizziness, dry mouth, and sleepiness. Thus, due to these toxicities and lack of therapeutic efficacy, Bellergal is no longer recommended for treatment of menopausal related hot flushes [64].

Other agents investigated for alleviating hot flushes include, centrally acting α2 antagonist mirtazapine [65, 66], reversible monoamine oxidase-A inhibitor moclobemide [67], and propanolol [68]. Dry mouth and somnolence are common adverse effects reported for mirtazapine and moclobemide, leading to 18 to 25% withdrawal from clinical studies.

### 1.2.4 Bioidentical Progesterone

Progesterone is C-21 steroid hormone involved in mammary gland development, pregnancy and menstrual cycle. Endogenous progesterone is derived from pregnenolone. Exogenous or
bioidentical progesterone, which is chemically identical to the endogenous form (Figure 1.4), is synthesised from diosgenin, a compound extracted from yam. Yams are part of the Dioscoreaceae family. To date, more than 400 different types of this species have been identified [69]. The major source of diosgenin is from Dioscorea mexicana (Mexican wild yam). However, in recent years diosgenin has been identified in many other species of Dioscoreaceae [69, 70]. In one study, the concentration of diosgenin in Dioscorea polygonoides was reported as 2.45% [69].

Compared to synthetic progestins and the aforementioned non-hormonal therapies, the adverse effects associated with progesterone are mild and limited. Women treated with progesterone (oral or vaginal) frequently complain of cramps, headache, nausea, depression, breast tenderness, and abdominal pain (≥10%) [71, 72]. Dizziness, diarrhoea, and vaginal discharge have also been reported (≤10%) [72]. Progesterone metabolites such as 5α and 5β pregnenolone bind to the GABA receptor, resulting in sedative effects [28, 73, 74]. A double-blind randomised trial in 40 menopausal women showed that application of 25 mg of progesterone cream daily for 15 days inhibited the proliferation of breast epithelia cells [75]. These preliminary data suggested that short-term application of progesterone cream is not associated with breast epithelial cell hyperplasia, as observed with their synthetic counterparts.

The antiproliferative effects of progesterone are well documented. Furthermore, progesterone might be beneficial for management of other related menopausal symptoms such as hot flushes. These benefits together with the lack of severe adverse effects render progesterone ideal for managing of menopausal symptoms.

### 1.2.4.1 Pharmacodynamics

Oral micronised progesterone was the first dosage form marketed, under the trade name Utrogestan [76]. Low dose oral micronised progesterone (100 mg for 25 days per month), in combination with estradiol (1.5 mg) has shown to prevent endometrial bleeding in 67% of postmenopausal women for 12 months [77]. The secretary changes induced by progesterone are dose dependent with oral doses as high as 300 mg/day achieving optimum secretary transformation, comparable to that of premenopausal women [78]. Oral progesterone is also
effective for preserving the effect of estrogen on lipoprotein metabolism. One study examined the effect of percutaneous estradiol in combination with oral progesterone (200 mg for 12 days during each cycle) on serum lipid and lipoprotein levels in 45 postmenopausal women [79]. ERT significantly reduced the low density lipoprotein (LDL) levels, while high density lipoprotein (HDL) and triglyceride levels were maintained constant. Recent data presented at the 92nd annual meeting of the Endocrine Society (2010) showed that oral progesterone is also effective for management of vasomotor hot flushes [80]. In women taking 300 mg of oral progesterone for 12 weeks, hot flushes were reduced by 48% as compared to 22% in the placebo group.

Oral progesterone is subjected to extensive metabolism in the gut wall and the liver. In fact, only under 10% of the oral dose reaches the systematic circulation intact [76]. As such, high doses of up to 300-400 mg daily are required to achieve optimum therapeutic response. This can predispose women to an increased number of side effects. Therefore, much attention has been paid to identify alternative routes of delivery for bioidentical hormones. Current alternative progesterone products available in New Zealand include intramuscular (Gestone Solution for injection), vaginal (Crinone 8% vaginal gel), and transdermal formulations. Nasal sprays have also been developed and investigated [81, 82].

Fanchin et al. reported the secretary transformation of endometrium when progesterone gel was administered vaginally at different doses (45, 90 and 180 mg) [83]. Optimal therapeutic response was observed even in the low dose group, despite low plasma progesterone levels. Generally, vaginal formulations of progesterone are used to achieve local effects, as progesterone will mainly accumulate in the uterine wall when administered vaginally. Pain and discomfort, which may be encountered during the application of vaginal products, renders these formulations less favourable amongst menopausal women.

Nasal progesterone has been investigated as an alternative dosage form for endometrial protection in postmenopausal women [82]. Ten postmenopausal women were randomly allocated to either oral estrogen combined with nasal progesterone (34 mg) or progesterone alone. Endometrial examination was performed following the administration of the last progesterone dose. Clear secretary changes were only observed in the group treated with the combination therapy.
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Transdermal delivery of progesterone has the potential to increase its bioavailability due to avoidance of first pass and gut wall metabolism. Transdermal formulations allow for reduced drug dose, controlled drug delivery, and maintenance of the steady state plasma levels. These formulations are also favourable amongst patients as they are easy to apply. Transdermal delivery of progesterone has been the subject of several investigations, with studies demonstrating mixed results.

In one study, transdermal progesterone cream applied even at relatively high doses (64 mg) did not prevent the proliferative effects of estrogen on the endometrium [84]. Progesterone was only administered for 14 continuous days in three cycles. Such short duration of treatment may not be sufficient to achieve optimum therapeutic response [85]. Conversely, Leonetti et al. demonstrated that transdermal application of 15-30 mg of progesterone daily (for 6 months) can significantly reduce endometrial proliferation in postmenopausal women treated with 0.625 mg of conjugated equine estrogen (CEE) [86]. Additionally, Landes et al. have shown that the endometrial antiproliferative effect of progesterone cream (40 mg/day for 6 months) is comparable to that of oral medroxyprogesterone acetate (2.5 mg/day) [87].

Additionally, transdermal progesterone has been investigated for management of vasomotor symptoms and osteoporosis. In a study by Lee et al., bone mineral density significantly increased in 63% of postmenopausal women treated with progesterone cream (20 mg daily) [88]. Leonetti et al. reported a significant improvement in vasomotor symptoms in 83% of the subjects who applied 20 mg/day of progesterone cream for 1 year [89]. Conversely, a recent study by Benster and colleagues showed that progesterone cream, even at high doses of 60 mg/day (used for 6 months), is no more effective than placebo for the management of vasomotor symptoms [90]. The duration of this study (used for 6 months) was much shorter than the study reported by Leonetti et al., indicating that long-term treatment with transdermal progesterone might be necessary to achieve a therapeutic response.

Overall, the data regarding the therapeutic efficacy of transdermal progesterone are controversial. This might be associated with the complex pharmacokinetic of progesterone, which is further discussed below.
1.2.4.2 Pharmacokinetics

Table 1.1 shows the plasma progesterone concentration during various stages of the menstrual cycle and at menopause. During the follicular phase, plasma progesterone concentration does not surpass 1.4 ng/ml. Conversely, in the luteal phase, the levels sharply increase, ranging from 4-25 ng/ml. It is widely accepted that luteal progesterone levels must be attained to achieve maximum therapeutic benefits [85]. The pharmacokinetics data of progesterone, summarised in Table 1.2, show that luteal progesterone levels are achieved with most formulations other than nasal sprays and transdermal creams.

Table 1.1: Plasma concentration of progesterone at various stages of the menstrual cycle and at menopause

<table>
<thead>
<tr>
<th>Menstrual Phase</th>
<th>Concentration (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Follicular phase</td>
<td>0.20-1.40</td>
</tr>
<tr>
<td>Luteal Phase</td>
<td>4.00-25.00</td>
</tr>
<tr>
<td>Menopause</td>
<td>0.10-1.00</td>
</tr>
</tbody>
</table>

A single oral dose of progesterone (100mg) can result in a maximum plasma progesterone concentration of up to 10 ng/ml over the period of 1-2 h [91-93]. Increasing the dose to 200 mg/day leads to a 2 fold increase in the plasma progesterone level [91].

Following the administration of a single dose vaginal gel (90mg) the plasma progesterone concentration can increase to a maximum of 10 ng/ml within 7 h [92]. The delayed release of progesterone into the systematic circulation is associated with its direct transport to the uterus wall, across the highly vascularised vaginal tissue (first pass uterine effect) [28]. In effect 4 h after vaginal application of progesterone (100 mg), its levels in the endometrial and myocardial tissue increased to 185±155 ng and 254±305 ng/100 mg of the tissue respectively [94]. Many different vaginal formulations have been developed and investigated, including, gels, creams, gelatin capsules, and suppositories. The gels have a distinctive adhesive characteristic that allows progesterone (90 mg) to be released continuously [83]. After 24 h levels of about 3 ng/ml can still be detected in the blood [92].
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A rapid rise in the plasma progesterone concentration is observed when progesterone is administered intramuscularly in an oily solution (100 mg) [95]. Within 6 h the plasma level reaches a maximum of 16.06±1.63 ng/ml with steady state levels obtained by day 21 of the cycle after 6 days of exogenous progesterone administration. The levels obtained with intramuscular progesterone formulations are significantly higher than with vaginal or oral formulations [95, 96]. However, due to local pain and irritation at the administration site, this route of drug delivery is not recommended for regular use.

Pharmacokinetic studies have shown that the luteal progesterone levels cannot be achieved when progesterone is administered transdermally. This in turn has raised concerns about the therapeutic efficacy of transdermal progesterone, including its ability to inhibit the proliferative effect of estrogen or give relief from hot flushes. In a study by Carey et al., 24 postmenopausal women applied progesterone cream for 6 weeks at either 20 mg twice daily or 40 mg once daily [97]. The plasma progesterone levels were measured on days 1 and 42. No considerable differences were observed between the two treatment groups. The average maximum plasma concentration was 0.22 ng/ml on day 1 and increased to 1.6 ng/ml on day 42. In a similar study conducted by Burry et al., 6 postmenopausal women applied 30-60 mg of progesterone cream for 4 weeks in combination with 0.05 mg of transdermal estradiol [98]. At the end of the study the average plasma progesterone level increased significantly (p < 0.05), from 0.17 ng/ml to 1.6-3.3 ng/ml.

In the studies reported by Carey et al. and Burry et al., a significant increase in the plasma progesterone level was noted. However, the average plasma levels were highly variable and did not surpass 3.3 ng/ml. The low plasma progesterone levels observed in these studies may not sufficiently protect the endometrium against estrogenic proliferation or give relief from hot flushes [84]. However, it has to be considered that plasma progesterone may not reflect the progesterone concentration in other tissues [85]. For example, the mean salivary progesterone can rapidly increase to 18 ng/ml within 1 h of treatment with transdermal progesterone while the plasma levels remain constant [99, 100]. Lee et al. suggest that monitoring the salivary level of progesterone is important as this value represents the transdermally bioavailable progesterone level [101].
Table 1.2: Pharmacokinetics profile of various dosage forms of progesterone

<table>
<thead>
<tr>
<th>Route/Form</th>
<th>Dose (mg)</th>
<th>C_{max} (ng/ml)</th>
<th>T_{max} (h)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oral Capsule</td>
<td>100</td>
<td>10.20±8.40</td>
<td>2.70±1.00</td>
<td>[91]</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>19.90±20.50</td>
<td>2.20±1.00</td>
<td></td>
</tr>
<tr>
<td>Vaginal Gel</td>
<td>90</td>
<td>10.51±0.54</td>
<td>7.00±2.70</td>
<td>[92]</td>
</tr>
<tr>
<td>Vaginal Suppository</td>
<td>400</td>
<td>16.30±17.15</td>
<td>4.89±2.28</td>
<td>[102]</td>
</tr>
<tr>
<td>Rectal Suppository</td>
<td>100</td>
<td>15-51.9</td>
<td>4</td>
<td>[103]</td>
</tr>
<tr>
<td>Intramuscular Injection</td>
<td>100</td>
<td>16.06±1.63</td>
<td>6</td>
<td>[95]</td>
</tr>
<tr>
<td>Nasal Spray</td>
<td>11</td>
<td>3.75</td>
<td>1</td>
<td>[104]</td>
</tr>
<tr>
<td>Transdermal Cream</td>
<td>20-60</td>
<td>1-3.3</td>
<td>2-6</td>
<td>[97, 98, 105]</td>
</tr>
</tbody>
</table>

1.2.4.3 Factors Influencing the Bioavailability of Transdermal Progesterone

Less attention has been paid to identifying factors that can affect the bioavailability and therapeutic efficacy of progesterone. Figure 1.5 represents the processes involved in the transport of progesterone across the skin. When progesterone is applied to the skin, a concentration gradient forms between the formulation and the skin, whereby the drug diffuses passively to the epidermis and the dermis into the lymphatic channels or blood capillaries, although its mode of transport is debatable (see Chapter 5). To be successfully absorbed across the skin, progesterone must overcome two main barriers: the Stratum Corneum (SC) and the enzymatic barriers. Progesterone is a substrate for the 5α-reductase enzymes present in the skin. It is hypothesised in this thesis that metabolism by these enzymes may contribute to the low plasma progesterone levels. Moreover, the lipophilicity of progesterone (log P 3.87) may reduce its penetration through the impermeable SC.

The permeation of progesterone through the skin can be optimised by developing a novel transdermal delivery system that can enhance permeation across the SC while preventing its metabolism. Chapters 3 and 4 of this thesis focus on development of a novel transdermal delivery system for progesterone. In Chapter 5 the effect of 5α-reductase inhibition on the bioavailability of transdermal progesterone will be investigated.
Chapter 1-Introduction

The successful development of a transdermal delivery system requires the knowledge of the skin structure, factors that can affect drug permeation, and potential strategies that enhance the permeation rate. These factors will be further discussed in the proceeding sections.

**Figure 1.5:** Schematic of the processes involved in the transport of progesterone across the skin.

### 1.3 Transdermal Drug Delivery

#### 1.3.1 Anatomy of the Skin

The primary function of the skin, the largest organ in the body, is to protect the body against external agents such as chemicals, radiation, free radicals, and microorganisms. The skin is also involved in temperature control and is a sensory organ. The thickness of the skin diverges based on its function and location, generally ranging from 2 mm in the sole of the feet to 0.1 mm in the eyelid [106]. The skin is composed of three main layers including epidermis, dermis, and hypodermis. These layers are shown in Figure 1.6. The dermis is about 3-5 mm thick, containing fibrous proteins, glycosaminoglycans, and interfibrillar gels [107]. Blood and lymphatic vessels, sebaceous glands, sweat glands, and hair follicles are other structures...
located deep within the dermis. Cushioning, stretch, strength, and nutritional support to the epidermis are the predominant functions of the dermis.

The epidermis consists of stratified squamous epithelium and is about 100-150 μm thick [108]. The viable epidermis is made up of four different layers: stratum basale, stratum spinosum, stratum granulosum, and stratum lucidum. These layers are formed, as the columnar epithelial cells in the basal layer differentiate into flat keratinocytes that are pushed to the upper layers of the epidermis. These cells occupy more than 95% of the epidermis and the rest is occupied by melanocytes, Langerhans cells and Merkel cells [108]. SC is the uppermost layer of the skin consisting of terminally differentiated keratinocytes also known as corneocytes. The pH of non-occlusive skin is in the range of 4-6 and can affect the SC lipid structure [109]. In the pH range of 5-6 the SC exists as a mixture of gel phases while at a higher pH (pH 7) it exists as a single gel phase [110]. The structure and function of this layer is further discussed below.
Figure 1.6: Simplified diagram of the skin and the SC structure.

The dashed blue line represents permeation across the SC (intercellular route), and the dashed brown line represents permeation though the corneocytes (transcellular route).

1.3.2 Stratum Corneum (SC)

The SC consist of 10-15 layers of corneocytes and is about 10-15 µm thick [111]. Several models including the brick and the mortar model [112], single gel phase model [113], and domain mosaic model [114] have been used to describe the structure and functions of the SC. Based on the former model, the corneocytes (bricks) are embedded into the lipid rich bilayer (mortar) (Figure 1.6). The numbers of these corneocytes are highly variable ranging from 8 in the cheek to 86 in the heels of the feet [115]. The main function of these cells is to protect the skin from physical and chemical damage. The highly cross-linked keratins, surrounded by the insoluble cornified cell envelope, are critical for this function. Corneodesmosome links the
corneocytes together providing added support and stability for the SC. Together these structures render the corneocytes impermeable to solutes.

The lipid bilayer consists of ceramide, fatty acids, cholesterol, cholesterol esters, and cholesterol sulfate. The former is derived from enzymatic cleavage of linoleic acid and plasma membrane constituents as they enter the SC. The concentration of these lipids is shown in Table 1.3. Of these, ceramide is the most predominant form having nine different subclasses with hydrocarbon chains ranging from 14-36 carbons [116]. The main function of the SC lipids is to control and prevent transepithelial water loss. The natural moisturising factor produced in the corneocytes provides added support for maintenance of this function [116].

Hydration of the SC is essential for desquamation and functioning of the SC enzymes [116]. In general, the concentration gradient of water decreases when moving from the basal layer towards the SC [113]. The water content of the SC is variable and is influenced by the external environment. In its dried state, the SC contains 15-20% water [117]. Most of this water is localised in the keratin layers [118]. Water may also be present within the lipid bilayer, although this has not been fully established [113].

Table 1.3: The concentration of human SC lipids

<table>
<thead>
<tr>
<th>Type of Lipid</th>
<th>Concentration* (µg/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type 1 Ceramide</td>
<td>8.50</td>
</tr>
<tr>
<td>Type 2 Ceramide</td>
<td>25.10</td>
</tr>
<tr>
<td>Type 3 Ceramide</td>
<td>8.10</td>
</tr>
<tr>
<td>Type 4 Ceramide</td>
<td>6.60</td>
</tr>
<tr>
<td>Type 5 Ceramide</td>
<td>5.80</td>
</tr>
<tr>
<td>Type 1 Ceramide</td>
<td>14.60</td>
</tr>
<tr>
<td>Fatty acids</td>
<td>13.20</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>34.70</td>
</tr>
<tr>
<td>Cholesterol Sulphate</td>
<td>3.30</td>
</tr>
<tr>
<td>Cholesterol Ester</td>
<td>10.10</td>
</tr>
</tbody>
</table>

*The values are represented as µg of lipid per mg of the dried weight of SC. Values are obtained from reference [119].
1.3.3 Routes of Penetration

The SC is the main barrier for transdermal permeation of drugs. Transport across the SC is governed by two main pathways, the transappendageal and transepidermal pathways. The transappendageal pathway includes permeation through hair follicles, sweat glands and sebaceous glands. The surface area of this route is considered to be about 0.1% of the total surface area of the skin [120]. As such, the transappendageal pathway does not contribute significantly to the steady state transdermal flux. However, this route can be considered critical for large molecules, ions, vaccines and targeted drug delivery. Fan et al. showed that the hair follicles play a major role in the retention and transport of vector DNA [121]. The follicular route is also involved in the local delivery of liposomes. Liposomes are thought to enter the follicular cells through the lipophilic channels associated with the follicular duct [122]. Liposomal delivery systems have shown to promote targeted drug delivery to the transappendageal pathway and increase local drug concentration [123].

The transepidermal route is further divided into two main pathways: the transcellular route and intercellular route (Figure 1.6). The former pathway involves partition and diffusion through the corneocytes and the lipid bilayer. Although the transcellular route contributes to 99% of the total SC surface area [124], transport via this route is limited. Conversely, the intercellular pathway involves the permeation of the drug through lipid continuous domains. This pathway is considered the main route for transdermal drug delivery, even though it only contributes to < 1% of the total SC surface area [124].

Following the permeation through the epidermis, the drug is distributed to the systemic circulation or peripheral tissues by the dermal lymphatic or blood vessels. Alternatively, the drug may be transported into the deeper tissue. The penetration pathway for drugs is determined by their physiochemical properties including partition and diffusion coefficient into the protein and lipid rich domains [124]. The ideal drug candidate for transdermal drug delivery should have a balanced partition coefficient (1-3). This allows for rapid drug penetration into the lipid rich SC domains. Permeants with low melting points (MP) are also preferable. This translates into a higher solubility, as illustrated by the generalised solubility equation [120]. Oil and water solubility within the formulation and across the SC are also highly critical. This is to ensure a high concentration gradient, which is the main driving force for drug penetration, across the skin. Optimisation of these properties can enhance the
percutaneous permeation of drugs across the SC in accordance with the Fick’s Law of diffusion:

\[
\text{Equation 1.1}
\]

where \( J_{\text{ss}} \) is the steady state flux or permeation rate, \( D \) is the diffusion coefficient, \( k_{d} \) is the drug partition coefficient between the drug and the SC, \( c_{d} \) is the constant drug concentration in the donor solution, and \( l \) is the thickness of the membrane or the diffusion path length. The transport across the skin is via passive diffusion and the factors listed in the above equation will predominantly govern the permeation rate across the skin. However, the transdermal flux can also be affected by several biological and physiochemical factors. These are further discussed in section 1.4.

1.4 Factors Affecting Transdermal Permeation

1.4.1 Biological Factors

The barrier function of the skin can be compromised as the result of exposure to chemicals, radiation, and due to various skin diseases. Depending on the nature of the disease, the permeation rate across the skin can be reduced or enhanced. In ectopic dermatitis, irritants can disrupt the tightly packed lipid bilayers within the SC. Subsequently, these irritants can enter the skin, further damaging the corneocytes and the epidermal bilayers [125]. Ogawa et al. demonstrated that the permeation rate of theophylline in lesional atopic dermatitis was significantly higher compared to the normal skin [126]. Conversely, in psoriasis where the skin thickness is increased, the permeation rate of the drug is expected to decrease.

Most chemicals and solvents can act as penetration enhancers, increasing the rate of drug transport through the disruption of the tightly packed lipid bilayer. Ultraviolet radiation has shown to enhance the permeation rate of various hydrophilic and lipophilic drugs by a similar mechanism. Duracher et al. found that permeation provoked by irradiation was higher for lipophilic drugs [127]. Removal of the SC by tape stripping can also increase the permeation
rate of various compounds across the skin. In one study, the permeation rate of water nearly doubled after removal of the SC by tape stripping [128].

The skin barrier is further compromised by age, with newborn infants of 32 weeks gestation or less, showing a greater rate of transepidermal water loss (TEWL) compared to infants of 37 weeks gestation or more [129]. The thickness of the skin decreases with age. This can result in reduced barrier function and higher drug permeation. However, one study showed that TEWL does not vary significantly between young (19-42 years) and old (69-85 years) subjects with the exception of skin occluded for 24 h [130].

In addition, the regional skin site can also affect transdermal drug permeability. Variation in the skin permeability across different areas in the body can be attributed to skin thickness and the distribution of skin appendages. Feldmann et al. studied the effect of regional skin sites on the permeation rate of model drug hydrocortisone [131]. Drug permeation was evaluated at various skin regions. In general, the absorption was greater in regions with an abundant number of hair follicles and reduced in regions where the SC was thicker. An exception to this was the palm, which is thicker but has no hair follicles. In this study, the permeation rate across the scrotum and the foot arch were the highest and the lowest, respectively. In a similar study, the permeation rate of benzoic acid across various tissues was found to be in the following order: forehead > abdomen > thigh > chest > arm > back [132]. Transdermal scopolamine patches are available in the market for treatment of nausea and motion sickness. These patches are normally applied behind the ear. This region is selected as the facial skin, including the ear is known to be more permeable compared to skin in other regions of the body [133].

Changes in the dermal blood supply can also have a profound effect on the absorption and permeation rate of topically applied drugs. Increase in the dermal blood flow can reduce the residence time of the permeant in the dermis, whilst a decrease in the blood flow will have the opposite effect. The later mechanism is important for drugs in which a local effect is desired. Vasoconstrictors can be used to minimise the systematic absorption of local anaesthetics. Several studies have investigated the effect of the vasoconstrictor phenylephrine on the dermal concentration of lidocaine [134, 135]. A significant increase in the local

---

2 A parameter used for assessing the barrier function of the skin
concentration of lidocaine has been reported with increase in the concentration of phenylephrine [134].

The thermotropic behaviour of the SC is well documented [136, 137]. The average temperature of the SC is around 32°C. Increase in the temperature initially allows for a small rise in the permeation rate [136], which is associated with loosening of the tightly packed gel phases of the SC lipids. At a certain temperature, these gel phases are converted to liquid crystals, which have a greater lipid fluidity. Thereafter a rapid rise in the permeation rate is observed with any further increase in the temperature.

1.4.2 Environmental and Physiochemical Factors

1.4.2.1 Skin Hydration

Hydration of the SC has a profound effect on the rate and extent of drug permeation across the skin. Lambert et al. studied the effect of long term skin hydration on permeability of various lipophilic and hydrophilic drugs [138]. They suggest that long-term skin hydration results in the formation of “time dependent polar channels”, which are aqueous in nature. Subsequently, they showed that the permeation rate of polar permeant (vidarabine and hydrocortisone) were substantially enhanced following the hydration of the skin for over 40 h, while the permeation rate of lipophilic butanol was not affected. SC can be hydrated through application of various formulations, occlusion and back diffusion of water from the underlying tissue. Oily formulations, waxes and emulsions are all known to enhance the hydration of SC. Oil in water (o/w) emulsions infuse the skin with water while water in oil (w/o) emulsions prevent transepithelial water evaporation through occlusion. Occlusion with an impermeable membrane is often used to improve the permeability of drugs across the skin. The concept of occlusion is employed in formulation of transdermal patches that contain an impermeable backing membrane. EMLA cream is an example of a commercial product in which the permeation rate of lignocaine and prilocaine are enhanced by the use of occlusive dressings [117].
1.4.2.2 Drug Solubility and Concentration

Drug solubility can affect its ability to penetrate biological membranes in accordance with Equation 1.1. Solubility can be optimised though addition of cosolvents, surfactants, or cyclodextrins, formation of a prodrug, or changing the polymorphic form of the drug. The solubility of the permeant will in turn affect its thermodynamic activity. This parameter represents the driving force that allows the drug to diffuse across the skin. According to Equation 1.1, the flux across the skin is directly proportional to the initial drug concentration in the formulation \((C_0)\). The thermodynamic activity could be increased by increasing the drug concentration. At saturation, the drug is expected to have a thermodynamic activity close to unity. The level of saturation can be optimised by changing the solutes concentration or decreasing its solubility [139]. Both techniques are effective methods to enhance the thermodynamic activity and the transdermal flux. The flux of betamethasone-17-benzoate from various ointment formulations has been measured [140]. For each vehicle, increasing the concentration of the steroid close to the saturation level increased the flux in accordance with Equation 1.1. When saturated, the flux from all vehicles was the same, thus highlighting the importance of the thermodynamic activity for transdermal permeation.

1.4.2.3 Partition Coefficient (P)

Octanol water partition coefficient (P) is another critical parameter, which can affect drug permeability through the SC. Optimal permeation across this layer is achieved for drugs that have a logP value ranging from 1-3. Highly water-soluble drugs, and those with high lipophilicity, cannot easily pass the SC into the viable epidermis. LogP values above 1.6 have also shown to extensively affect drug permeation through the follicular pathways [141]. Above this value, the follicular permeation decreased with increase in logP.

The SC-vehicle partitioning is another important parameter that can affect the permeation rate across the skin. This parameter can be increased though the addition of agents that can enhance drug solubility in the SC. The SC-vehicle partition coefficient of isoproterenol HCl increased with the addition of propylene glycol [142]. Optimal permeation rate and hence partition coefficient were observed in aqueous systems containing 20% propylene glycol.
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Penetration enhancers can increase partitioning into the SC through disrupting the intercellular lipid conformation.

1.5 Transdermal Formulations

In a broad sense, passive transdermal penetration enhancement can be optimised through two main mechanisms [143]. The first mechanism (push effect) involves increasing the drug concentration gradient across the skin (Section 1.5.1). This could be achieved by either increasing drug solubility (cosolvent effect), increasing drug loading dose (thermodynamic effect), or modifying drug partitioning. The second mechanism is based on diminishing the diffusional resistance of the skin (pull effect). This is a direct consequence of formulation additives partitioning into the skin, thus leading to conformational changes in the epidermis (Section 1.5.2). A combination of these mechanisms is often adopted to produce maximum effect. Figure 1.7 summarises some of the novel strategies used for optimisation of transdermal drug delivery. Several of these strategies have been tested for transdermal drug delivery of progesterone (Table 1.4). In most formulations, progesterone was used as model lipophilic drug alongside other steroidal compounds to investigate transdermal permeation rate. Most studies demonstrate a significant increase in transdermal flux of progesterone. However, in general, optimal transdermal permeation has not been achieved.

1.5.1 Drug Modification and Drug Vehicle Interactions

One of the first steps in the development of a transdermal delivery system is the selection of a compound that has the ideal properties for transdermal delivery. Not all compounds will possess optimal properties such as balanced logP or adequate solubility. In such cases, modifying the physicochemical properties of the active entity through formulation of a supersaturated solution or formation of prodrugs and inclusion complexes can be beneficial. The following are examples of novel formulation approaches that are based on drug modification or drug vehicle interactions.
1.5.1.1 Prodrug/Codrug

The concept of prodrug was first introduced by Adrien Albert who described inert compounds that can be chemically or enzymatically transformed into an active moiety [144]. A prodrug is a pharmacologically active substance, which has been covalently linked to an inactive moiety in order to enhance its physiochemical properties, and hence its permeation rate into and across biological membranes. Once the prodrug passes the SC, it is then converted into the parent drug by the metabolically active dermis and epidermis. Topically applied prednicarbate is an example of a commonly used prodrug that is metabolised into prednisolone upon absorption into the skin [145]. Improved permeation rate and bioavailability, and increased stability are amongst the main benefits of using a prodrug [146]. The prodrug approach can be used either alone or alongside novel and traditional delivery approaches to optimise transport of the drug across the skin.

Ideally, the inactive moiety should be non-irritating, non-toxic and must not accumulate in the body. This has made the selection of an appropriate prodrug quite challenging. As a result, novel alternatives such as codrugs have been introduced. Codrug refers to the co-administration of pharmacologically active compounds that have been linked together with a reversible covalent bond [145]. This approach allows for co-administration of drugs in a single dosage form, whilst at the same time improves the physiochemical properties of drugs without introducing any unwanted moiety into the body.
Figure 1.7: Summary of novel strategies for optimising transdermal drug delivery.
Modified from [120].

1.5.1.2 Eutectic Systems

The MP of a compound is inversely proportional to its SC solubility and lipophilicity [147]. Thus, the solubility, and hence transdermal flux, could increase when the MP is reduced. One method of reducing the MP is through the formation of a eutectic mixture. A binary eutectic mixture is a mixture of two ingredients that have a lower melting point compared to the individual constituents. This is achieved through the inhibition of the crystallisation process of each ingredient by the other constituent present in the mixture. The successful formation of eutectic mixture depends on the comiscibility of constituents with each other [148]. Ideally, the constituents should be able to solubilise each other in the liquid state, and remain immiscible in the solid state. Eutectic mixtures of ibuprofen containing various penetration enhancers have shown to improve its transdermal flux across the skin [148]. Ibuprofen and menthol have a MP of about 80°C and 40°C respectively that reduces to a single MP at 20°C in their eutectic mixture [149].
1.5.1.3 Ion Pairs

Transdermal delivery of charged drug molecules is highly challenging as these agents cannot readily partition or penetrate into the SC. To overcome this problem, formulations based on lipophilic ion pairs have been developed and investigated. In such formulations, an oppositely changed species is added to the charged drug molecule. This creates a neutral species, which can now penetrate across the SC into the viable epidermis where the parent drug is released. Recently, Ma et al. demonstrated that this technique can improve the permeability of the nonsteroidal anti-inflammatory agent flurbiprofen by up to 20 times [150].

1.5.1.4 Supersaturation

As discussed earlier, the flux through the skin is a function of thermodynamic activity as described by the following equation:

\[
\text{Equation 1.2}
\]

where \(A\) is the thermodynamic activity of the drug in the formulation, \(\gamma\) is the effective activity coefficient in the membrane and \(t\) is the thickness of the membrane [117]. The thermodynamic activity can be increased to beyond unity when the solubility of the drug in the formulation exceeds its equilibrium solubility. This can be achieved through several techniques [151]. The first technique involves dissolving the drug in a cosolvent such as propylene glycol and then mixing it with a non-solvent (usually water) to create a supersaturated solution. The second method is based on the evaporation of volatile solvents either during the manufacturing process or upon the application of the formulation to the surface of the skin. Alternatively, supersaturation can be achieved by the uptake of water from the skin into the formulation. Instability associated with crystal growth and precipitation is one of the inherent disadvantages of a supersaturated system. Supersaturation stability can be improved through addition of nucleating polymers such as polyvinyl alcohol, although it is still a limiting factor [152]. Although the polymers can inhibit nucleation, crystal growth may be observed upon long-term storage.
1.5.1.5 **Cyclodextrins (Inclusion Complexes)**

Cyclodextrins (CD) are cyclic chains of glucopyranoside units with a molecular weight of about 1000-1500 [153]. Depending on the number of the glucopyranoside units, they can be classified as α-CD (6 units), β-CD (7 units) and γ-CD (8 units). Other chemical derivatives have also been formulated through alkylation and hydroxy alkylation. Due to their cyclic structure, CD are capable of forming a distinctive three-dimensional structure with a lipophilic cavity and a hydrophilic exterior. This characteristic allows CD to form reversible noncovalent inclusion complexes with the drug molecule (Figure 1.8), modifying its physiochemical properties such as solubility and stability. CD can improve the stability of drug molecules upon storage [154] and at the same time protect the drug from enzymatic degradation within the skin [155]. Depending on the nature of the inclusion complex formed, they may also accelerate certain enzymatic reactions [156]. This mechanism is beneficial when a prodrug is used. CD have been used to enhance both dermal and transdermal drug delivery. This is achieved through modification of SC lipids and by improving the drugs solubility [153]. Reduction of drug irritancy and side effects is an added advantage of CD, although CD themselves may cause cutaneous irritation.

![Figure 1.8: Schematic representation of a drug-CD inclusion complex.](image)

### 1.5.2 Penetration Enhancers

Various chemical and physical enhancers have been developed and investigated for transdermal delivery of lipophilic and hydrophilic drugs. Physical enhancing techniques often involve the application of external stimuli such as a pulse or current to alter the lipid organisation of the SC and generate pathways for drug transport. These enhancers are of a
particular interest for water soluble and high molecular weight proteins and peptides for which transdermal drug delivery is particularly challenging. One of the main advantages of using chemical and physical enhancers is that synergic effects are often seen when one or two of these techniques are used together or along with other types of formulations. Techniques such as iontophoresis can also allow for programmed drug delivery, such that the dose and the rate of the drug administered can be controlled. Despite these benefits, there is concern about the safety of using chemical and physical enhancers. Certain classes of chemical penetration enhancers are toxic (for example ionic surfactants) and can cause skin irritation. Application of a very high voltage or pulse to the skin may also result in permanent tissue damage. Edema and dermal necrosis are reported with application of high ultrasound frequencies [157, 158]. Furthermore, the possibility of pain and discomfort and the need to wear particular devices for physical penetration may reduce patient compliance.

1.5.2.1 Chemical Enhancers

Chemical penetration enhancers have long been used to reduce the barrier functions of the skin and facilitate the penetration of a wide range of drugs molecules. The penetration enhancing effects of different materials have been highly investigated. However, to date, no product with optimal properties for transdermal drug delivery has been indentified [159]. The ideal enhancer should reversibly reduce the barrier properties of the skin without having any possible pharmacological activity in the body. It is also important to select an enhancer that is non-toxic and has minimal adverse effects. Physical characteristics of the product is also important in the view of the cosmetic industry. The mechanism of action of penetration enhancers are variable and generally depend on the physiochemical properties of the individual enhancer and the nature of its interaction with the skin. However, in general, penetration enhancers work by improving the partitioning of the drug, disrupting the packing of the intercellular lipid bilayers and keratin domains, and enhancing skin hydration.
1.5.2.2 Physical enhancers

Iontophoresis

Iontophoresis has been used for transdermal delivery of ionic drugs, proteins and peptides [160, 161]. It involves the application of an electric current to the surface of the skin, in order to aid the transport of charged molecules. The principle mechanism of iontophoresis is based on the concept of “like charges repel each other” [162]. A charged drug solution is placed on the application area with an electrode carrying the same charge placed on top of the solution. At the same time, an electrode carrying the opposite charge is placed on a different area of the body. Following the application of a small current, the charged drug will be pushed away from the electrode into the skin. Likewise, any oppositely charged ions in the body can move into the donor chamber. The electromigration is initiated by electrorepulsion (as described) or electroosmosis where the movement of ions across the skin will initiate solvent flow. Through analysis of published literature, Guy et al. demonstrated that electromigration is dependent on the molecular weight of ions, with small molecular weight ions (Na\(^+\)) mainly transported via electrorepulsion [163]. On the other hand, large molecular weight substances (>1000Da) are primarily transported via electroosmosis. The transappendageal and paracellular routes are the main pathways involved for the transport of drugs by iontophoresis [160]. However, application of a current may result in conformational changes in the skin peptide. It promotes the “flip-flop” motion, in which the helix of the polypeptide chain will form a parallel alignment [164-166]. This in turn will result in repulsion of the adjacent dipoles and form cavities through which the drug is transported.

Electroporation

Transdermal drug delivery with electroporation is similar to iontophoresis. However, it involves the application of a higher voltage (>100V) over a short duration (ms-µs) [167]. Although the technique is normally applied to cell membrane phospholipids, it has been proven to induce positive changes on the multilamellar bilayers of the SC [168]. By applying an electric current to the skin, the SC can become polarised. Above the breakdown threshold of the SC, the lipid bilayers can rearrange themselves, forming reversible aqueous cavities.
through which the drug is transported [169]. Drug transport into the skin can be achieved by several different mechanisms including electrorepulsion, electroosmosis, pressure and diffusion [169, 170]. Transdermal drug delivery with electroporation has been used for a wide range of hydrophilic, charged and large molecular weight compounds.

Sonophoresis

Transdermal drug delivery using sonophoresis has gained a growing interest in the past two decades. In this technique, the ultrasound frequency is used to disrupt the tightly packed lipid bilayers, forming highly permeable cavities or “Localised Transport Regions” (LTR), through which the drug can be transported [158]. Using infrared spectroscopy Alvarez-Roman et al. showed that approximately 30% of the SC lipids are extracted following the application of low frequency ultrasound [171]. The LTR were found to be isolated and highly distinguishable from the neighbouring unaffected regions. It has been further demonstrated that the permeability of the hydrophilic drugs, such as calcein, is approximately 80 times greater in the LTR than in the surrounding areas [172]. In general, ultrasonic frequencies in the range of 20 kHz to 16 MHz can be used. Studies have demonstrated that low frequency ultrasound (48 kHz) is highly effective for transdermal drug delivery and, as such, is now commonly used [173, 174]. Low frequency sonophoresis is divided into two types: simultaneous and pretreatment sonophoresis [175]. Within the clinical setting, the ultrasound and the formulation are simultaneously applied to the surface of the skin. When the ultrasound is switched off, the permeability of the drug will decline as the permeation rate is directly related to the ultrasound intensity and frequency. Alternatively, the ultrasound can be applied to the skin for a short time, prior to the application of the formulation. This method allows for continuous enhanced permeability of the drug for several hours without the need to wear an ultrasound device. Sonophoresis has been investigated for a variety of drug molecules including large proteins and peptides.

Microneedles

Microneedles are transdermal patch like devices made up of materials such as titanium sheet or stainless steel with arrays of vertical projections on their surface. These ~50-1000 μm tall vertical projections are designed to create microscopic pores in the skin through which
macromolecules as well as compounds in the nanometre range can be transported [176]. In this technique, the transport of molecules is generally via passive diffusion but can be further enhanced through application of electric current. There are two types of microneedles: solid design and hollow design. Several variations of the former have been investigated. In the “poke and patch design”, microneedles are used to create holes within the skin and then a transdermal patch is used to deliver the drug [176, 177]. Alternatively, the drug can be coated on the vertical projections, which are directly applied to the skin (“coat and poke”) [176, 178]. Mikszta et al. delivered a naked plasmid DNA into mice skin. The microprojections were dipped into the sample and were then scraped against the skin in order to damage its integrity (“coat and scrape”) [176, 179]. Compared to the solid microneedles, the hollow design allows for the transport of the drug through its microprojection either by diffusion or by applying pressure. Transdermal delivery of insulin has been investigated in hairless rats using these microneedles. It was demonstrated that the blood glucose concentration can be reduced by up to 47% over a short period of time [180].
**Table 1.4:** Examples of novel formulation strategies investigated for transdermal or topical delivery of progesterone

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Additives</th>
<th>Loading Dose (% w/w or w/v*)</th>
<th>Flux (µg.cm⁻².h⁻¹)</th>
<th>Skin Source</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oily Solution</td>
<td>Glycerol Monooleate</td>
<td>0.35*</td>
<td>4.7 ± 0.01</td>
<td>Hairless Mouse (SC)</td>
<td>[181]</td>
</tr>
<tr>
<td>Oily Solution</td>
<td>Medium-Chain Mono and Diglycerides</td>
<td>0.01</td>
<td>NA</td>
<td>Porcine (Ear)</td>
<td>[182]</td>
</tr>
<tr>
<td>Hydration</td>
<td>PEG400 solution</td>
<td>0.016*</td>
<td>0.25 ±0.06</td>
<td>Human</td>
<td>[183]</td>
</tr>
<tr>
<td>Nanoemulsion</td>
<td>α-CD</td>
<td>1.00</td>
<td>0.58 ± 0.14</td>
<td>Porcine (Abdomen)</td>
<td>[184]</td>
</tr>
<tr>
<td>Nanoemulsion</td>
<td>β-CD</td>
<td>1.00</td>
<td>0.63 ± 0.06</td>
<td>Porcine (Abdomen)</td>
<td>[184]</td>
</tr>
<tr>
<td>Nanoemulsion</td>
<td>γ-CD</td>
<td>1.00</td>
<td>1.04 ± 0.10</td>
<td>Porcine (Abdomen)</td>
<td>[184]</td>
</tr>
<tr>
<td>Nanoemulsion</td>
<td>α-CD –SS#</td>
<td>1.00</td>
<td>0.72 ± 0.08</td>
<td>Porcine (Abdomen)</td>
<td>[184]</td>
</tr>
<tr>
<td>Nanoemulsion</td>
<td>β-CD -SS#</td>
<td>1.00</td>
<td>0.86 ± 0.01</td>
<td>Porcine (Abdomen)</td>
<td>[184]</td>
</tr>
<tr>
<td>Nanoemulsion</td>
<td>γ-CD -SS#</td>
<td>1.00</td>
<td>1.21 ± 0.11</td>
<td>Porcine (Abdomen)</td>
<td>[184]</td>
</tr>
<tr>
<td>Hydrogel</td>
<td>Carrageenan</td>
<td>1.00</td>
<td>0.05</td>
<td>Porcine (Abdomen)</td>
<td>[185]</td>
</tr>
<tr>
<td>Hydrogel</td>
<td>Chitosan–EDTA</td>
<td>1.00</td>
<td>0.03</td>
<td>Porcine (Abdomen)</td>
<td>[185]</td>
</tr>
<tr>
<td>Hydrogel</td>
<td>Chitosan-Glycolic Acid</td>
<td>1.00</td>
<td>0.09</td>
<td>Porcine (Abdomen)</td>
<td>[185]</td>
</tr>
</tbody>
</table>

*SS: Sucrose Stearate
Table 1.4 (continued): Examples of novel formulation strategies investigated for transdermal or topical delivery of progesterone

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Additives</th>
<th>Loading Dose (% w/w or w/v*)</th>
<th>Flux (µg.cm$^{-2}$.h$^{-1}$)</th>
<th>Skin Source</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microemulsion</td>
<td>Medium Chain Glyceride</td>
<td>1.00</td>
<td>0.27 ± 0.05</td>
<td>Porcine (Ear)</td>
<td>[186]</td>
</tr>
<tr>
<td>Microemulsion</td>
<td>Pure Microemulsion</td>
<td>1.00</td>
<td>0.68</td>
<td>Porcine (Abdomen)</td>
<td>[187]</td>
</tr>
<tr>
<td>Microemulsion</td>
<td>Silicon Dioxide</td>
<td>1.00</td>
<td>0.50</td>
<td>Porcine (Abdomen)</td>
<td>[187]</td>
</tr>
<tr>
<td>Microemulsion</td>
<td>Polymeric Emulsifier</td>
<td>1.00</td>
<td>0.78</td>
<td>Porcine (Abdomen)</td>
<td>[187]</td>
</tr>
<tr>
<td>Microemulsion</td>
<td>Polycarbophil</td>
<td>1.00</td>
<td>0.76</td>
<td>Porcine (Abdomen)</td>
<td>[187]</td>
</tr>
<tr>
<td>Drug In Adhesive Patch</td>
<td>Urea or Pantothenol</td>
<td>3.12</td>
<td>27-35*</td>
<td>Rat</td>
<td>[188]</td>
</tr>
<tr>
<td>Drug In Adhesive Patch</td>
<td>Silicone X7-2920 Adhesive</td>
<td>5.00</td>
<td>0.90</td>
<td>Hairless Rat</td>
<td>[189]</td>
</tr>
<tr>
<td>Drug In Adhesive Patch</td>
<td>Dow Corning-355 Adhesive</td>
<td>5.00</td>
<td>0.75</td>
<td>Hairless Rat</td>
<td>[189]</td>
</tr>
<tr>
<td>Drug In Adhesive Patch</td>
<td>E8086 Silicone Adhesive</td>
<td>5.00</td>
<td>0.90</td>
<td>Hairless Rat</td>
<td>[189]</td>
</tr>
</tbody>
</table>

* indicates the average amount of progesterone permeated every hour rather than the steady state flux
Table 1.4 (continued): Examples of novel formulation strategies investigated for transdermal or topical delivery of progesterone

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Additives</th>
<th>Loading Dose (% w/w or w/v*)</th>
<th>Flux (µg.cm$^{-2}$.h$^{-1}$)</th>
<th>Skin Source</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liposome</td>
<td>Polycarbophil</td>
<td>1.00</td>
<td>0.40</td>
<td>Porcine (Abdomen)</td>
<td>[190]</td>
</tr>
<tr>
<td>Liposome</td>
<td>Chitosan–EDTA</td>
<td>1.00</td>
<td>0.60</td>
<td>Porcine (Abdomen)</td>
<td>[190]</td>
</tr>
<tr>
<td>Liposome</td>
<td>Polymeric Emulsifier</td>
<td>1.00</td>
<td>0.35</td>
<td>Porcine (Abdomen)</td>
<td>[190]</td>
</tr>
<tr>
<td>Liposome</td>
<td>Carrageenan</td>
<td>1.00</td>
<td>0.74</td>
<td>Porcine (Abdomen)</td>
<td>[190]</td>
</tr>
<tr>
<td>Liposome</td>
<td>Pure Liposome</td>
<td>1.00</td>
<td>0.52</td>
<td>Porcine (Abdomen)</td>
<td>[190]</td>
</tr>
<tr>
<td>DPPC-Liposome</td>
<td>Xylenesulfonic acid</td>
<td>0.50</td>
<td>-</td>
<td>Porcine (Abdomen)</td>
<td>[191]</td>
</tr>
<tr>
<td>DPPC-Liposome</td>
<td>Cholesterol</td>
<td>0.50</td>
<td>-</td>
<td>Porcine (Abdomen)</td>
<td>[191]</td>
</tr>
<tr>
<td>DPPC-Liposome</td>
<td>Stearylamine</td>
<td>0.50</td>
<td>-</td>
<td>Porcine (Abdomen)</td>
<td>[191]</td>
</tr>
<tr>
<td>Deformable Liposome</td>
<td>$\gamma$-CD</td>
<td>1.40</td>
<td>Small</td>
<td>Porcine (Ear)</td>
<td>[192]</td>
</tr>
</tbody>
</table>
1.5.3 Novel Formulations

1.5.3.1 Nanoparticles

Nanoparticles are one of the most commonly investigated colloidal delivery systems. They are classified as solid colloidal suspension with a diameter of < 1 µm. Depending on their chemical composition, nanoparticles can form different structures. Polymeric based nanoparticles are classified as capsules in which the drug is entrapped into a lipophilic or an aqueous core surrounded by a shell. Solid lipid nanoparticles (SLN) consist of a porous lipid matrix in which the drug can be absorbed or entrapped. They are generally considered solid at room and body temperature [193]. SLN dispersions can improve transdermal flux by preventing TEWL through the formation of an occlusive film over the skin surface. The polymer matrix also minimises drug mobility and, as such, allows for controlled drug delivery. Poly lactic acids, poly glycolic acids, triglycerides, polyvinyl alcohol, chitosan, and fatty acids are amongst the most widely used polymers and lipids for fabrication of nanoparticles and microparticles [111]. These agents are generally considered biocompatible and non-toxic.

1.5.3.2 Lipid vesicles

Lipid vesicles consist of a hydrophobic lipid bilayer with an inner aqueous core and a hydrophilic outer shell (Figure 1.9). The bilayer is made from phospholipids or non-ionic surfactants. Depending on the size and number of layers, lipid vesicles can be classified into three different types: multilamellar vesicles (multiple layers), large unilamellar vesicles (> 100 nm), and small unilamellar vesicles (<100 nm) [194]. A range of lipid vesicles such as traditional liposomes, elastic liposomes, niosomes, proliposomes, proniosomes, and ethosomes have been developed and investigated as potential topical or transdermal drug carriers. Due to their unique structure, lipid vesicles are able to entrap both hydrophilic and hydrophobic drugs. These systems have been especially investigated for delivery of small molecules, proteins, peptides [195], and vaccines [196]. Lipid vesicles are non-toxic, biocompatible and biodegradable [197]. The aforementioned properties together with penetration enhancing effects, and the ability to control drug release, makes lipid vesicles
ideal for transdermal or topical drug delivery. Chemical (oxidative and hydrolytic degradation) and physical (aggregation and leakage from the vesicle) instability upon storage are amongst the major drawbacks associated with these systems [198, 199]. Hydrolysis of the phospholipid bilayer and oxidation of the lipids are a possible basis of instability in phospholipid containing systems [197]. The source and the purity of natural phospholipids are also of concern and replacement with synthetic lipids is not considered cost effective. Substitution of the phospholipids with a surfactant may overcome some of these problems.

**Figure 1.9:** Schematic representation of a vesicular system.

Liposomes, Niosomes and Discomes

The aqueous core of a liposome is surrounded by phospholipid bilayers such as phosphatidylcholine, which have structures similar to those lipids found in the biomembrane [124]. Cholesterol is sometimes added to improve the stability and rigidity of the membrane [200]. Applicability of liposomes for transdermal and topical drug delivery was first tested by Mezi et al. in 1980 [201]. Since then, it has been demonstrated that traditional liposomes will only act as drug carriers, deposited on the upper skin layers without penetrating into the deeper layers of the epidermis or the systemic circulation [202, 203]. Traditional liposomes are now considered an effective system for topical drug delivery. As discussed earlier, chemical and physical instability are the major problems associated with traditional liposomes. To overcome this, novel surfactant based lipid vesicles (Niosomes) have been developed and investigated. Niosomes are made from hydration and self assembly of non-ionic surfactants in an aqueous solution [111]. The non-ionic surfactants employed are often single alkyl chains or sorbitan esters [204]. Although the presence of surfactants may enhance the flexibility of these systems, their main application remains similar to that of traditional liposomes [200].
Chapter 1-Introduction

Discomes are a modified version of niosomes. These are large disk-like structures with a diameter of 12-60 µm made from dilution of non-ionic surfactant polyoxyethylene cholesteryl ether (Solulan C24) with niosomes [205]. These lipid vehicles are typically used for ocular drug delivery due to their large size.

Proliposomes and Proniosomes

Proliposomes are solid particles containing a dried mixture of porous powders (such as mannitol) and phospholipids. They can readily form liposomes upon contact with skin sweat or water under occlusion [122]. Proniosomes are designed based on the same concept. They are generally composed of non-ionic surfactants, phospholipids [122]. Some formulations may also contain cholesterol for added stability. They were first reported by Hu and Rodes in 1991 in order to overcome the stability issues observed with standard niosomes [204]. It was shown that these systems were as effective as traditional niosomes, with the added advantage of improved stability.

Transfersomes and Ethosomes

Transfersomes, or highly deformable vesicles, are a novel class of lipid vesicles first introduced in 1992 by Gregor Cevc [206]. The composition of transfersomes is similar to that of traditional liposomes. However, they also contain an edge activator, a substance used to destabilise the lipid bilayers, making these systems more flexible [107, 111, 207]. Surfactants such as Spans, Tweens, dipotassium glycyrrhizinate, and sodium cholate are commonly used as edge activators [200]. Due to their flexibility, transfersomes are able to penetrate the SC intact, moving into the deeper layers of the dermis where they can be absorbed into the systemic circulation [202]. In order to maintain their swollen structure, transfersomes must always remain hydrated [107]. To achieve this, they will follow the skin hydration gradient from the dry surfaces into the water rich regions [206]. Several other mechanisms including transappendageal transport and penetration enhancing effects have been also proposed [202]. According to El Maghraby, shunt routes are not involved in their transdermal delivery [208], although transport into the hair follicles has been reported [107]. Pretreatment of the skin with the empty vesicle was also shown to significantly improve the penetration of model drugs across the skin [209].
Touitou et al. was the first group to combine the penetration enhancing effects of ethanol with the flexibility of deformable liposomes to formulate a novel non-invasive vesicle known as ethosomes [210]. These are essentially traditional liposomes, which contain high levels of ethanol (about 30%). The inclusion of ethanol enhances the flexibility of the lipid bilayer and reduces its melting transition [200]. These, together with penetration enhancing effects of ethanol [211], are thought to be one of the most predominant mechanisms involved in the transport of drugs from ethosomes. Novel Therapeutic Technology Inc. is a chief innovator in the development of ethosomes [212]. Lipoduction™ is an example of their ethosomes formulation, which is used for removal of cellulite and excess body fat.

1.5.3.3 Transdermal drug delivery systems

Transdermal Drug Delivery Systems (TDDS) are “drug loaded adhesive patches” designed to release the drug into the skin at a controlled rate [213]. In general, there are four main types of TDDS (Figure 1.10) [213, 214]. All TDDS consist of an adhesive layer, an impermeable backing membrane, and a release liner. In the most basic type, the drug is dissolved into the adhesive layer, which serves as a rate controlling membrane (drug in adhesive). A modification of this design is the multi-laminate TDDS, which contains an additional layer that controls the rate of drug release (rate controlling membrane). In other similar designs, the drug can be loaded into a reservoir with (liquid reservoir) or without (polymer matrix) a rate controlling membrane present. The polymer matrix system offers the advantage of having the adhesive only on the edge of the patch and as such can minimise skin irritation. In general, the adhesive must firmly hold the TDDS in contact with the skin for the intended duration of application without causing skin damage or irritation. The adhesive must also be inert, non-toxic, and biocompatible with the skin, the drug and other excipients present in the formulation. Pressure Sensitive Adhesives (PSA) are used to adhere the patch on the skin with the application of a light pressure [213]. Polyisobutylene, silicone, acrylics and hydrogels are examples of some commonly used PSA.

Scopolamine (an antiemetic) was one of the first drugs formulated as a TDDS by Alza Corporation in 1980. Since then numerous drugs, including buprenorphine, clonidine, fentanyl, lignocaine, nitroglycerine, nicotine, oxybutynin prilocaine, rivastigmine,
testosterone, norethindrone acetate, and estradiol have been formulated as TDDS. TDDS systems can be used alone or in combination with other formulations to control the drug release rate and enhance penetration. The delivery of drugs from these systems can be achieved via passive or active transport. The former involves the transport of the drug from the patch to the skin down its concentration gradient. In active TDDS, the application of an external energy such as electrical current is used to aid the delivery of the drug across the skin.

**Figure 1.10:** Schematic representation of various types of TDDS.

### 1.5.3.4 Lecithin Based Organogel

Lecithin based organogels (LO) are thermodynamically stable, transparent viscoelastic gel phased systems comprising a surfactant (lecithin), a non-polar solvent, and an aqueous phase. In the presence of certain organic solvents, lecithin forms spherical micelles that transform into cylindrical aggregates and eventually a three-dimensional gel-like network following the
addition of an aqueous medium (Figure 1.11) [215]. The existence of an unsaturated fatty acid residue is prerequisite for the gelation process [215]. Various organic solvents including esters, fatty acids, alkanes, amines, and ethers can be used to formulate LO [216]. LO were first introduced by Scartazzini and Luisi in 1988 [217]. This was followed by the introduction of Pluronic Lecithin based organogel (PLO), which contains lecithin in the oil phase and Pluronic F127 (20-30% w/w) in the aqueous phase [218]. In recent years, PLO and LO have gained a growing interest for transdermal delivery of lipophilic and hydrophilic drugs due to enhanced drug solubility and improved stability [216]. Increased transdermal penetration by these systems is mainly attributed to improved drug solubility and the fluidisation of the SC lipids. However, similar to the liposomal systems, the purity and chemical stability of the phospholipid lecithin is of concern.

**Figure 1.11:** Schematic representation of formation of LO.

### 1.5.3.5 Microemulsions

Microemulsions are thermodynamically stable, transparent, isotropic systems, consisting of oil, water and surfactant, with or without a cosurfactant. Depending on their composition, different types of microemulsions can be formed (Figure 1.12). These include w/o microemulsion, where the water droplets are surrounded by the continuous oil component, and o/w microemulsion, where the water is the continuous component. The transformation from w/o microemulsions into o/w microemulsion can occur through a bicontinuous microstructure, where the oil and water components are running continuously throughout the system with no long-range packing order. These are commonly formed when the ratio of oil and the aqueous phase are similar. The formation of a microemulsion is a thermodynamic
process. In most systems, the surfactant molecules may not adequately reduce the interfacial tension and thus addition of a cosurfactant or a cosolvent is necessary. These agents have the ability to further reduce the interfacial tension, reduce the rigidity of the interfacial film, reduce the long range packing order of the surfactant molecules and optimise the interfacial curvature [219]. The curvature of the interfacial film is temperature dependant, with bicontinuous microemulsions showing a net curvature of zero [220]. As the temperature is increased, the oil component will start to move into the continuous phase and the system will adopt a negative curvature. Conversely, when the temperature is decreased o/w microemulsions with a positive curvature are formed. The term microemulsion should not be confused with emulsions and nanoemulsions, which have a diameter of > 500 nm and > 50 nm, respectively. Table 1.5 summarises the main properties of microemulsions and coarse emulsions.

**Figure 1.12:** Schematic representation of o/w, w/o and bicontinuous microemulsions.
Microemulsions are considered ideal for drug delivery due to their low viscosity, ease of preparation, improved drug stability and solubility, small droplet size ( < 200 nm), surfactant provoked permeability, and protection against enzymatic degradation [221]. The later characteristics are responsible for improved permeability of drugs across biological membranes including the skin. Moreover, microemulsions allow for co-administration of hydrophilic and lipophilic drugs, thus providing synergic effects. Given these properties, microemulsions have been extensively investigated for topical, dermal and transdermal delivery of various compounds. The potential mechanism of action of microemulsions has been related to two main factors: modification of the SC lipid bilayer, and enhanced solubility and thermodynamic activity of the active compound [222]. A number of publications have investigated microemulsions as a potential delivery system for bioidentical progesterone. Nandi et al. have shown that isopropyl myristate (IPM) microemulsions systems can increase the solubility of progesterone by 330 fold [223]. Hosmer et al. further showed that the flux of progesterone across the skin increased by up to 4.8 fold in microemulsions containing medium chain glycerides [186].

Table 1.5: Comparison between coarse emulsions and microemulsions

<table>
<thead>
<tr>
<th>Property</th>
<th>Coarse Emulsion</th>
<th>Microemulsion</th>
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</thead>
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<tr>
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<td>Spontaneous</td>
</tr>
<tr>
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<td>&lt;200 nm</td>
</tr>
<tr>
<td>Microstructure</td>
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<td>Droplets or Bicontinuous</td>
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<td>Ultra Low (Close to 0 mN m⁻¹)</td>
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1.6 Aim and Objective of the Thesis

HRT is the standard treatment for management of vasomotor symptoms including hot flushes. However, HRT is associated with a number of severe adverse effects including endometrial cancer (ERT), heart disease, and breast cancer. Moreover, the therapeutic efficacy of natural products is questionable and non-hormonal therapies are associated with a large number of adverse effects. On the other hand, natural or bioidentical progesterone has been considered a safer alternative to HRT for management of hot flushes and prevention of estrogen associated endometrial cancer. The safety profile and reduced side effects of progesterone are mainly attributed to its chemical structure, which is identical to the form naturally present in the body. Substitution of oral formulations with transdermal creams can further reduce adverse effects, reduce dose and improve patient compliance. However, clinical data concerning the therapeutic efficacy of transdermal progesterone are controversial, with several studies demonstrating low plasma progesterone levels that are insufficient to protect the endometrium or give relief from hot flushes. The low bioavailability of progesterone can be attributed to poor transdermal permeation across the SC and/or its metabolism in the viable epidermis and the dermis by the 5α-reductase enzymes. The aim of this thesis is to investigate two novel strategies for enhancing the transdermal permeation and bioavailability of progesterone. These strategies include:

A. **Formulation approach** based on “water free microemulsions” to enhance the transdermal permeation rate of progesterone across the skin and;

B. **Pharmacokinetic approach** to inhibit the transdermal metabolism of progesterone across the skin.

The specific objectives of this thesis are to:

- develop and validate a stability indicating assay for quantification of progesterone and 5α-reductase enzyme inhibitor dutasteride;
- investigate the physiochemical properties (MP, solubility, and partition coefficient) of progesterone and dutasteride;
- develop and characterise a novel “water free microemulsion” system, capable of solubilising a high percentage of water;
Chapter 1-Introduction

- measure the permeation rate of progesterone from selected formulations across excised porcine skin;
- investigate the mode of action of novel “water free microemulsions”; and
- investigate the effect of 5α-reductase inhibition on the serum and salivary progesterone concentration.
Chapter 2-Preformulation Studies

2 Preformulation Studies

2.1 Introduction

Prior to the development of a dosage form, it is important to establish certain fundamental physiochemical properties of the drug molecule. This information is valuable during the formulation process and can influence the route of drug delivery, choice of excipients, and the type of formulation developed. Many different physiochemical properties should be investigated in the preformulation stage including MP, solubility, stability, powder flow, excipient compatibility. The choice of these fundamental properties depends on the availability of data on the physiochemical properties of the investigated agent and its intended formulation direction.

2.1.1 High Performance Liquid Chromatography (HPLC)

One of the first steps of preformulation is to develop an analytical method for quantifying the analyte of interest. Spectroscopic techniques are often a suitable choice, as most drugs absorb light in the ultraviolet (UV) region. Simple techniques such as ultraviolet visible spectroscopy (UV-VIS) lack specificity and sensitivity. With the development of a High Performance Liquid Chromatography (HPLC) assay, multiple compounds can be rapidly quantified with a high degree of sensitivity and specificity.

Demands on the pharmaceutical industry for increased production of high quality products emphasises the need for development and validation of an accurate and robust analytical method. The aim of the method validation is to ensure that the selected analytical method is accurate, reproducible, rugged, specific, and fit for its intended purpose [224]. Method validation is required prior to the introduction of a new method or when the analytical method and the instrument are changed.
HPLC methods for quantifying and separating progesterone from its active metabolites have been reported [225-229], however, these methods are not stability indicating. The only two spectroscopic assays reported for quantification of dutasteride are based on chromatography–tandem mass spectrometry (LC-MS/MS) and mass spectrum fragmentation [230, 231]. In the current chapter, HPLC methods for quantification of progesterone and dutasteride were developed. ICH guidelines for quantitative determination of analyte were used to validate these methods [232]. The guideline requires the accuracy, precision, specificity, linearity, and range to be tested.

2.1.2 Octanol Water Partition Coefficient (P)

Octanol water partition coefficient (P) is the ratio of the concentration of a substance in the octanol phase (C\text{octanol}) to its concentration in the aqueous phase (C\text{water}) at equilibrium at a given temperature [227, 233]. Logarithm of the partition coefficient (logP) is extensively used as a measure of molecular lipophilicity, a valuable parameter in the field of Quantitative Structural Activity Relationship (QSAR) [233, 234] and Quantitative Structure Permeability Relationship (QSPR). As discussed in Chapter 1, the lipophilicity of a permeant will govern the extent of its partitioning into the lipid rich SC. A parabolic relationship is observed between the lipophilicity and logP [235], where the partitioning and permeation of highly hydrophilic (logP<1) and lipophilic compounds (logP>3) across the SC is limited. Generally, optimum permeation rate is observed for permeants with a logP of 2-3 [235]. Lipophilicity can also influence the choice of excipients and formulation used. The experimental logP of progesterone is 3.87. This value has not been reported for dutasteride.

Various methods have been used for determining logP [236-239]. Of these, the shake flask method is one of the oldest and most widely employed. It involves partitioning of the solute between water and octanol. Once partitioning is achieved, the two phases are separated and the concentration of the solute in each phase is measured. Despite its simplicity, the technique suffers from a number of limitations. Before the partition coefficient can be measured, the two phases must be mutually saturated with each other, which can take a minimum of 24 h to achieve [227]. In addition, the practicality of the technique is limited to compounds that have logP values between -2 to 4 (occasionally up to 5) [240]. The co-miscibility and subsequent formation of an emulsion between the two phases upon shaking is another inherent difficulty.
associated with this technique. The slow stirring technique minimises contamination of the aqueous phase and is particularly useful for compounds with logP values of 5 or higher. Nonetheless, this technique also suffers from long equilibration times of 2-5 days [233].

To overcome the inherent difficulties associated with the above methods, numerous indirect methods for calculation of logP have been investigated [241]. Reversed Phase HPLC (RP-HPLC) has been recognised as a reliable technique for accurate determination of logP. This technique allows for rapid screening of a large number of compounds with high lipophilicity (up to 7). Moreover, a small amount of sample with impurities can be used to obtain reproducible results.

RP-HPLC acts as a surrogate of the shake flask method, where the retention time of the analyte represents its partitioning between octanol and water. The retention factor (K’) is used as an index of lipophilicity. It measures the equilibrium distribution of the analyte between the stationary phase and the mobile phase and is represented as:

\[
K' = \frac{t_r - t_0}{t_0}
\]

Equation 2.1

where \(t_r\) is the retention time of the analyte and \(t_0\) is the dead volume, commonly measured as the retention time of an unretained compound. It has been demonstrated that K’ best correlates with lipophilicity when a pure aqueous eluent is used. However, this can lead to extremely prolonged retention times particularly for lipophilic compounds. Consequently, K’ is calculated at various volume fractions of organic modifier (\(\phi_o\)) and extrapolated to 100% aqueous phase (\(\log K'_w\)), using linear (Equation 2.2) or quadratic relationships [242].

\[
\log K' = Slope \cdot \phi_o + Intercept
\]

Equation 2.2

The intercept in the above equation, also known as \(\log K'_{w0}\), is a measure of lipophilicity when 100% water is employed. \(\log K'_{w0}\) has been shown to correlate well with experimental logP values [1,11]. Based on Equation 2.2 a linear relationship has been observed between logK’ and \(\phi_o\) [243, 244]. However several authors have pointed out that this equation is only valid
when 30-70% organic modifier is employed in the mobile phase [245, 246]. Moreover, a good linear correlation is generally obtained for structurally related compounds [246, 247]. To overcome such limitations, a novel approach known as Isocratic Chromatography Hydrophobic Index (CHI\textsubscript{i}) has been introduced [248]. The index represents the percentage of organic modifier required to obtain an equal distribution of the analyte between the stationary phase and the mobile phase [248, 249]. Rearrangement of Equation 2.2 demonstrates that when the K’ is twice the dead time (K’=1), CHI\textsubscript{i} and logK\textsubscript{w} (intercept) are related through the following equation:

$$CHI_i = \frac{-\text{Intercept}}{\text{Slope}}$$ \hspace{1cm} \text{Equation 2.3}

where the intercept and slope are obtained from Equation 2.2. Applying this equation to more than 500 drug molecules, followed by linear regression analysis, has demonstrated a correlation between CHI\textsubscript{i} values and experimentally determined partition coefficient [248]. For one set of compounds (n=140), moderate correlation (R=0.88), between logP and CHI\textsubscript{i} values were observed. Due to high standard error of estimate, exact measurements of logP could not be carried out.

Valko et al. further extrapolated the CHI\textsubscript{i} to a gradient HPLC method [250]. A strong linear correlation has been obtained between the gradient retention time (T\textsubscript{R}) and CHI\textsubscript{i} [251].

$$CHI_i = \text{Slope}T_R + \text{Intercept}$$ \hspace{1cm} \text{Equation 2.4}

The above equation was subsequently used to convert the T\textsubscript{R} into a gradient CHI (CHI\textsubscript{g}). This approach aligns the CHI\textsubscript{g} index with CHI\textsubscript{i} scale. The CHI\textsubscript{g} is equivalent to volume fraction of organic phase concentration at which the analyte is eluting from the column. In this chapter, these gradient and isocratic HPLC methods were investigated to develop a rapid and reliable method for calculating the logP of dutasteride.

### 2.1.3 Solubility

Solubility is a fundamental parameter in the field of QSPR, a property that relates the permeability of compounds to their physiochemical parameters. A balanced solubility is
prerequisite for optimum transdermal permeation across the skin. Solubility governs the pathway for drug transport, with most small water-soluble molecules transported via the water rich domains of the transcellular route. Solubility screening is also important throughout different stages of drug discovery and formulation development and can aid in the selection of excipients. Inadequate lipid or aqueous solubility (logS\textsubscript{w}) can result in incomplete absorption of drugs across the skin.

Saturation solubility of a compound is defined as the “maximum amount of the most stable form of a compound which can remain in solution at a certain temperature under equilibrium conditions” [252]. This parameter is often measured using traditional equilibration techniques, which require a large amount of sample (2-3 mg), and a long equilibration time (>24 h). For poorly water-soluble compounds, a sensitive analytical technique is required, which may not be available during the early stages of preformulation. To overcome these limitations, numerous \textit{in silico} models for predicting logS\textsubscript{w} have been explored [253, 254]. In this thesis, the following models have been investigated: Generalised Solubility Equation (GSE), Abrahams Linear Solvation Energy Relationship (LSER), and AlogPs.

GSE, developed by Valvani and Yalkowsky, is a well known model for predicting logS\textsubscript{w} [255]. In this model, the solubility of a non-electrolyte solid can be predicted from its MP and logP in accordance with the following equation:

\begin{equation}
\text{Equation 2.5}
\end{equation}

Using a set of 580 non-electrolytes, Jain and Yalkowsky showed that the logS\textsubscript{w} values calculated by Equation 2.5 were in good agreement (R\textsuperscript{2}=0.97) with the experimental logS\textsubscript{w} values [256]. The modified GSE has been used for predicting the solubility of weak acids and bases, although it is less accurate [257].

LSER is another well-established model for predicting logS\textsubscript{w}. In the LSER equation, logS\textsubscript{w} is defined by size, polarity, hydrogen binding parameters involved in forming solvent cavity, and hydrogen bonds between the solvent and the solute molecules [258]. Using a set of 659 training compounds, Abraham and Le developed the following LSER equation for calculating logS\textsubscript{w} [259]:
where $R_2$ is an excess molar refraction; a parameter related to the solute size, $\alpha_2^H$, is the polarisability or the ability of the solvent to stabilise a charge separation; $\Sigma \alpha_2^H$ is the hydrogen bond acidity or the ability to donate a proton when forming a hydrogen bond; $\Sigma \beta_2^H$ is the ability to accept a proton (hydrogen bond basicity); and $V_x$ is the McGowan characteristic volume. The descriptor listed in Equation 2.6 relates to the properties of the solute and the solvent molecules where $V_x$ represents the formation of cavity within the solvent molecules and the remaining descriptors represent the solute-solvent interaction. Using the above equation logS$_w$ of 67 test compounds were calculated with a standard deviation of 0.50 log units [259].

The AlogPs model for predicting logS$_w$ is based on atom type Electrotopological index (E-state index). This parameter is calculated from the electronic and topological environment of an atom in its valance state [260]. Based on this model, each structure is striped into hydrogen-suppressed backbone. The electronic environment represents the number of pi and lone pair electrons, which are associated with long-range molecular interactions. The topological environment is expressed numerically as the closeness of the atom to the surface of the molecule. From the topological and electronic environment of the atom, an “intrinsic index” is calculated. This index is then corrected for the pertubation effects from the neighbouring atoms, and is referred to as the E-state index. The solubility of 1921 sets of compounds have been accurately calculated using their molecular weights and E-state indices [261].

The aqueous (logS$_w$) and SC solubility can be improved by using surfactants and cosolvents. Surfactant molecules are amphiphilic in nature, with a hydrophilic head group and a lipophilic tail. This property allows the surfactants to aggregate with their polar head group in direct contact with the aqueous medium. The lipophilic tail can enhance the logS$_w$ of nonpolar compounds whilst at the same time destroy the lipid arrangement of tightly packed

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3 The influence of one atom on another
SC to improve drug partitioning within this layer. Enhanced formulation solubility is critical for transdermal drug delivery, as the concentration gradient is one of the main driving forces for permeation of drugs across the skin.

In this chapter, the experimental and predicted logS\(_w\) of 25 steroids is compared in order to establish an accurate method for predicting their logS\(_w\). Moreover, the solubility of progesterone and dutasteride in various oils and surfactants is also measured.

### 2.1.4 Melting Point (MP)

When selecting compounds for transdermal drug delivery, it is important to know their MP as this parameter can have a major impact on the transdermal flux across the skin [262]. The ideal candidate for transdermal drug delivery should have a low MP, generally <200°C [263]. This value corresponds to ideal lipid and aqueous solubility, which are critical for optimum permeation. In addition, the MP is important for identifying the polymorphic forms of a drug. Transformation into a different polymorphic form can take place during manufacturing processes, such as milling, homogenisation, compression, and other processes that involve heating and cooling of the sample. To date, five polymorphic forms of progesterone and three polymorphic forms of dutasteride have been identified [264-266].

The MP can be determined using capillary melting, hot stage microscopy and Differential Scanning Calorimetry (DSC). DSC is a versatile technique, which measures the enthalpy of heat transition using a small sample size. MP, crystalline and amorphous transition, sublimations, and evaporation are examples of processes, which can be quantified using DSC. In this chapter, DSC is used to study the melting behaviour of progesterone and dutasteride.
2.2 Chapter Aims

The specific aims of this chapter are to:

- develop and validate a stability indicating HPLC method for the quantification of progesterone and dutasteride;
- develop an accurate HPLC method for measuring the logP of steroids;
- determine the logP of dutasteride;
- identify an accurate method for predicting logS\textsubscript{w} of steroids;
- predict the logS\textsubscript{w} of progesterone and dutasteride;
- measure the saturation solubility of progesterone and dutasteride in common non-ionic surfactants and oils; and
- investigate the MP and thermal behaviour of progesterone and dutasteride.

2.3 Materials and Methods

2.3.1 Materials

Finasteride, dutasteride, hydrocortisone, nandrolone, clostebol, stigmasterol, and MPA were purchased from Hallochem Pharma Co. Ltd. (China); cholesterol was purchased from Acros Organic (Belgium); dexamethasone was from PCCA (Australia); and estriol, estradiol, progesterone, and testosterone were from Pharmaceutical Compounding New Zealand (New Zealand). Isopropyl Myristate (IPM), Tween 85, Tween 80, Tween 40, Brij 30 and propylene glycol 400 (PEG 400) were purchased from Sigma-Aldrich (USA). Soybean oil was purchased from Sigma (UK). Myritol 318 was obtained from Cognis Care Chemicals (Germany). Labrasol and Transcutol P were kindly donated by Gattefossé (Saint Priest Cedex, France). Imwitor 308 was obtained from Sasol (Germany). Cremophor EL was supplied by BAFS (Germany). Ethyl oleate and sesame seed oil were from Fluca (UK). Isopropyl Palmitate (IPP) and safflower oil were kindly donated by Croda Chemicals Ltd. (UK). Evening primrose oil (EPO) was from Soap Kitchen (UK). All samples were used without any further purification. HPLC grade methanol and acetonitrile were obtained from Ajax Finechem (New Zealand). Triple-distilled water was obtained in-house by reverse osmosis (MilliQ, Millipore, USA).
2.3.2 HPLC Method Development

2.3.2.1 Instrument

The UV spectrum of progesterone and dutasteride were recorded on a UV–Vis spectrophotometer (Libra S32, Biochrom Ltd., England) in the range of 200-400 nm. The liquid chromatographic system was an Agilent 1100 series (manufactured by Agilent Technologies, Germany) comprising the following components: a quaternary pump, a vacuum solvent microdegasser, an autosampler with a 100-well tray, and an online Diode array detector. The output signal was monitored using Chemstation software (designed by Agilent Technologies, Germany) on a Pentium computer.

2.3.2.2 Chromatographic Conditions

Dutasteride: Liquid chromatography was carried out at 25°C± 1°C using a Phenomenex Luna (4.6mm×250 mm), 5µm C8 (2) reversed-phase column. The mobile phase consisted of HPLC grade acetonitrile (55%), methanol (25%) and water (20%). Separation was achieved under isocratic conditions at a flow rate of 1 ml/min and the injection volume of 20 µl. Dutasteride and its degradation products were detected at a wavelength of 244 nm with a total run time of 10 min.

Progesterone: Liquid chromatography was carried out at 25°C± 1°C using a Phenomenex Gemini (4.6mm×250 mm), 5µm C18 column. The mobile phase consisted of HPLC grade acetonitrile (75%) and water (25%). Separation was achieved under isocratic conditions at a flow rate of 1 ml/min and the injection volume of 20 µl. Progesterone and its degradation products were detected at a wavelength of 241 nm with a total run time of 10 min.

2.3.2.3 Preparation of Standard Solutions

Stock solutions containing 500 µg/ml of dutasteride or progesterone were prepared by dissolving appropriate amounts of the drug in methanol or dimethyl sulfoxide (for stress studies). Working solutions of 100 µg/ml were prepared from the stock solution.
2.3.2.4 Forced Degradation Studies (Stress Testing)

In order to develop a stability indicating HPLC assay, progesterone and dutasteride were subjected to degradation by acid (1 or 0.2 M HCL), base (1 or 0.2 M NaOH), oxidising agent (3% H_2O_2), and heat (70ºC) for 24 h to 10 days. Photostability studies were performed in accordance with ICH guidelines in which the drug was exposed to UV light (photostability chamber: Binder KBF P series, USA) for a minimum of 24 h. All samples were stored at room temperature, protected from light, with the exception of those samples subjected to heat and light. The analytical conditions were subsequently optimised to allow for the separation of progesterone and dutasteride from their degradation products with a resolution of >1.5.

2.3.2.5 Method Validation

Calibration standards were prepared from working solutions in triplicates. Linearity of the HPLC methods was determined over the concentration range of 1-100 µg/ml and 4-100 µg/ml for progesterone and dutasteride, respectively. Precision of the analytical method was performed at two different levels: repeatability and intermediate precision. The repeatability was determined by analysing three repeated injections of the standard solution at three concentration levels in the lower, middle and upper range. For the intermediate precision, these concentrations were measured on three consecutive days.

The accuracy of the analytical method was tested in triplicate at three-concentration levels within 50-150% of the test concentration, which was set at 50 µg/ml. The percentage recovery was calculated from the slope and the intercept of the calibration curve.

Working concentrations of dutasteride and progesterone were repeatedly diluted and analysed to determine the lowest concentration of the analyte that can be detected (LOD) and quantified (LOQ). The LOD was set as three times the baseline noise level, which was measured by injecting a blank, and LOQ was defined as ten times the signal to noise ratio.
2.3.3 Octanol Water Partition Coefficient (P) Determination

2.3.3.1 Isocratic and Gradient HPLC System Setup and Measurements

The liquid chromatographic system was an Agilent 1200 series comprising components described in section 2.3.2.1. All analytes were dissolved in methanol. The concentrations of steroids used ranged from 10-200 μg/ml. Acetone was used to measure the dead time (t₀). All analytes were detected at 210 nm and 240 nm.

Isocratic and gradient measurements were made at 25°C ±0.5 on a SUPELCOSIL LC-AZB (5 um, 4.6 x 50mm, Sigma-Aldrich, Auckland, New Zealand) column, at a flow rate of 4 ml/min. Duplicate injections (10-50 μl) of each sample were made over a methanol concentration of 95%-97.5%-100% (v/v) (stigmasterol and cholesterol) or 60%-65%-70% (v/v) (all other compounds).

2.3.3.2 Calculation of LogP

Logarithm of the retention factor (logK′) was calculated at 100% water (logK′ₜ). The slope and intercept of Equation 2.2 were then used to calculate CHIᵢ. CHIᵢ was calculated from the gradient Tᵣ. In each gradient run, the methanol fraction was increased linearly from 60%-100% (v/v) over 60 min and held at 100% for a further 10 min. A linear correlation was obtained between Tᵣ and CHIᵢ. The constant of this linear correlation was used to calculate the CHIᵢ values. The experimental logP of model steroids were obtained from ALOGPS database [267]. For those compounds in which the experimental values were not available (stigmasterol and cholesterol), values were calculated using KOWWIN, obtained from the ALOGPS database. Previously, this model has been shown to correlate well with experimental logP values of various compounds [268, 269]. The Leave One Out (LOO) technique, where a reference compound is used as the test compound, was used for calculation of logP of steroids [270, 271]. The logP of dutasteride was calculated from the linear and gradient methods. For each model, the Average Absolute Error (AAE), which measure the closeness of agreement between two sets of parameters, was calculated using the following equation:
where $w_{logP}$ is the logP value based on the HPLC models, $w_{exp}$ is the experimental logP value, and $n$ is the number of compounds evaluated.

### 2.3.4 Solubility Studies

#### 2.3.4.1 Calculation of Aqueous Solubility

The aqueous solubilities of 25 reference steroids were calculated using Equation 2.5 (GSE), Equation 2.6 (LSER) or the AlogPs software. The MP and the logP of steroids were obtained from the literature and the Merk Index [272, 273]. The constant in the Equation 2.6 was calculated using the computer software ABSOLVE (ADME Boxes, version 3.5, USA). For each model, the AAE was calculated using Equation 2.7. The calculated and experimental logSw, MP, logP, and the constants in the LSER are summarised in Table 2.1.

#### 2.3.4.2 Determination of Saturation Solubility

The saturation solubility of dutasteride and progesterone was measured in oils and surfactants. An excess amount of each drug was added to 2 ml of the oil or the surfactant. The sample was shaken at 25°C for 72 h. The supernatant was then filtered through a 0.45 µm membrane filter and the concentration of progesterone and dutasteride were determined by HPLC.

#### 2.3.5 Melting Point Determination (MP)

The thermal behaviour of progesterone and dutasteride was investigated using a DSC instrument (TA instrument, DSC Q1000, Wilmington, DE) coupled with the Thermal Advantage software (Thermal Advantage 2.2.0.248). The MP was measured by heating 10 mg ± 0.01 mg of each sample at 10°C/min from 10°C to 200°C (progesterone) or 300°C (dutasteride). To induce amorphous progesterone and dutasteride, the samples were rapidly cooled at 50°C/min to -10°C and then reheated at 10°C/min to 200°C or 300°C. For all
analysis, nitrogen was used as the purge gas and a crimped empty pan was used as a reference. Sample measurements were carried out in closed pans.
### Chapter 2: Preformulation Studies

#### Table 2.1: MP, logP, LSER parameters, experimental and calculated solubilities of reference steroids

| Compound                        | logS<sub>W</sub> | GSE  | LSER  | ALOGPs  | logP  | MP   | Σα<sub>2</sub>H | Σβ<sub>1</sub>H | Π<sub>1</sub>H | R<sub>2</sub> | V<sub>x</sub> |
|--------------------------------|-----------------|------|-------|---------|-------|------|----------------|----------------|------------|-----------|-----------|-----------|
| Beclomethasone                 | -4.02           | -4.23| -6.61 | -5.40   | 3.80  | 118.50| 0.80          | 2.00          | 3.09      | 2.30      | 3.02      |
| Cholesterol                    | -7.17           | -8.12| -10.68| -7.14   | 7.40  | 146.50| 0.00          | 0.87          | 1.89      | 1.22      | 3.79      |
| Clobetasol                     | -5.08           | -4.71| -5.94 | -5.05   | 3.50  | 196.25| 0.56          | 1.69          | 2.93      | 2.03      | 2.98      |
| Desoximetasone                 | -3.95           | -3.77| -5.43 | -4.08   | 2.35  | 217.00| 0.56          | 1.69          | 2.84      | 1.94      | 2.85      |
| Dexamethasone                  | -3.64           | -3.59| -6.12 | -3.89   | 1.83  | 262.00| 0.80          | 1.97          | 2.95      | 2.07      | 2.91      |
| Estradiol                      | -4.88           | -4.51| -5.14 | -4.11   | 3.50  | 176.00| 0.81          | 0.95          | 2.30      | 1.85      | 2.20      |
| Estradiol                      | -4.96           | -4.52| -5.40 | -3.38   | 2.45  | 282.00| 1.04          | 1.26          | 2.55      | 2.06      | 2.26      |
| Estrone                        | -4.00           | -3.69| -4.47 | -4.84   | 3.13  | 131.00| 0.50          | 0.95          | 2.53      | 1.85      | 2.16      |
| Ethynyl Estradiol              | -4.42           | -4.75| -6.05 | -4.64   | 3.67  | 183.00| 0.90          | 1.02          | 2.43      | 2.07      | 2.39      |
| Finasteride                    | -4.50           | -4.71| -6.00 | -5.72   | 3.03  | 243.00| 0.51          | 1.60          | 3.23      | 1.77      | 3.10      |
| Fludrocortisone                | -3.43           | -3.53| -5.77 | -3.23   | 1.67  | 261.00| 0.80          | 1.88          | 2.86      | 1.91      | 2.82      |
| Flumethasone Pivalate          | -6.10           | -5.30| -6.13 | -4.67   | 3.86  | 219.00| 0.80          | 1.96          | 2.93      | 1.98      | 2.93      |
| Fluocinolone Acetonide         | -5.02           | -5.53| -6.13 | -4.47   | 3.19  | 309.00| 0.56          | 1.96          | 3.11      | 2.09      | 3.16      |
| Fluocinonide                   | -3.70           | -4.33| -5.56 | -3.87   | 2.38  | 270.00| 0.38          | 2.13          | 3.46      | 2.03      | 3.46      |
| Fluoxymesterone                | -5.99           | -6.04| -5.34 | -4.64   | 4.07  | 272.50| 0.70          | 2.19          | 2.42      | 1.61      | 2.60      |
| Hydrocortisone                 | -3.05           | -3.06| -5.65 | -3.26   | 1.61  | 220.00| 0.73          | 1.29          | 2.92      | 2.04      | 2.80      |
| Lotepeprednol Etabonate        | -4.97           | -4.51| -5.46 | -4.83   | 3.04  | 222.00| 0.31          | 1.90          | 3.32      | 1.91      | 3.37      |
| Medroxyprogesterone            | -4.19           | -5.07| -5.45 | -4.35   | 3.61  | 221.75| 0.17          | 1.93          | 2.49      | 1.67      | 2.82      |
| Megestrol                      | -5.23           | -4.56| -5.17 | -4.52   | 3.12  | 219.00| 0.00          | 1.35          | 2.89      | 1.73      | 3.08      |
| Norethindrone                  | -4.63           | -4.26| -5.07 | -4.65   | 2.97  | 203.50| 0.40          | 1.46          | 2.44      | 1.81      | 2.44      |
| Prednisone                     | -3.06           | -3.05| -5.17 | -3.51   | 1.46  | 234.00| 0.41          | 1.07          | 3.25      | 2.19      | 2.71      |
| Progesterone                   | -4.55           | -4.38| -3.46 | -4.76   | 3.87  | 126.00| 0.00          | 1.97          | 2.49      | 1.56      | 2.62      |
| Testosterone                   | -4.09           | -4.13| -5.17 | -3.94   | 3.32  | 155.50| 0.31          | 1.04          | 2.27      | 1.55      | 2.38      |
| Testosterone Propionate        | -5.53           | -5.15| -4.89 | -4.84   | 4.69  | 118.123| 0.00         | 1.01          | 2.40      | 1.41      | 2.82      |
2.4 Results and Discussion

2.4.1 High Performance Liquid Chromatography (HPLC)

2.4.1.1 Method Development

Progesterone

The UV spectrum of progesterone, shown in Figure 2.1, revealed a single maximum wavelength at 241 nm. This value was selected for the HPLC assay. Stress studies, performed to develop a stability indicating assay, did not reveal any degradation products in acid (0.2M HCl) and H$_2$O$_2$ at room temperature for 24 h. Subsequent heating of the samples, at 70°C for 24 h, resulted in mild degradation in acid and complete degradation in H$_2$O$_2$. Progesterone was highly unstable in alkaline conditions, decomposing rapidly within 1 h at room temperature. Exposure to heat (70°C) or light for 24 h did not induce any degradation of progesterone. Figure 2.2 shows the chromatogram of progesterone under various stress conditions.

For the initial trial runs, acetonitrile and water (75:25 %v/v) were used as the mobile phase. At this composition, a single sharp peak was observed at 6.8-7 min. When the stressed samples were injected, the resolution of progesterone with the nearest degradation peak was acceptable (>1.5), with the peak purity spectrum demonstrating no other degradation products coeluting at the same time as progesterone (Figure 2.2).

Dutasteride

The absorption spectrum of dutasteride showed three maximum wavelengths in the region of 210 nm, 244 nm and 305 nm (Figure 2.1). At the lower wavelength, absorption of solvents and impurities may interfere with the analytical method, whilst at 305 nm the sensitivity of the analytical method is reduced due to the low absorbance of dutasteride. Thus, 244 nm was selected as the optimum wavelength for quantification of dutasteride. Figure 2.3 shows the chromatogram of progesterone under various stress conditions. Stress studies showed that
dutasteride was stable under all conditions for up to 24 h. Degradation was only induced in alkaline (1 M NaOH) and acidic (1 M HCL) conditions, when the samples were heated at 70°C for up to 1 week.

The mobile phase composition for the initial runs was acetonitrile and water (60: 40% v/v). At this composition, a single sharp peak was observed at ~ 9 min, however, dutasteride was poorly resolved from its degradation products. Increasing the acetonitrile concentration up to 95% resulted in peak tailing and reduced resolution (0.92). When acetonitrile was substituted with methanol (60% v/v), the peak resolution slightly improved, while the retention time increased to 13 min. Optimal resolution (>1.5) and retention times (5.9-6.1 min) were observed in the mobile phase containing acetonitrile, methanol and water (55: 25: 20% v/v).

![Figure 2.1: UV spectrum of dutasteride (A), and progesterone (B) showing their maximum absorption wavelengths.](image)
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0.2 M HCl, 70°C, 24h
Peak purity spectrum of the peak at 7 min

0.2 M NaOH, Room temperature, 1h
UV spectrum of the peak at 7 min

3% H₂O₂, 70°C, 24h
Peak purity spectrum of the peak at 5 min

Figure 2.2: Chromatogram of progesterone under stress conditions.
Note that the results are only shown for those systems in which degradation is observed. The UV spectrums (shown on the right hand side) of the peak observed at 7 min resemble the spectrum of progesterone. Peaks A-D represent the unknown degradation products of progesterone.
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1 M HCL, 70ºC, 1 week

Peak purity spectrum of the peak at 6 min

Figure 2.3: Chromatogram of dutasteride under stress conditions.
Note that the results are only shown for those systems in which degradation is observed. The UV spectrums (shown on the right hand side) of the peak observed at 6 min resemble the spectrum of dutasteride. Peaks A-I represent the unknown degradation products of dutasteride.

2.4.1.2 Method Validation

Linearity and Range

Progesterone: The LOD and LOQ of this method was 300 and 500 ng/ml respectively. The method was linear over the concentration range of 1-100 µg/ml showing excellent correlation
between the peak area and the concentration of progesterone. The data for the regression analysis is summarised in Table 2.2.

Dutasteride: The developed HPLC method was linear over the concentration range of 4-100 µg/ml ($R^2>0.999$) with a LOD and LOQ of 1 and 3 µg/ml respectively. The regression equation and detection limits are summarised in Table 2.2.

Table 2.2: Regression data, LOD and LOQ for dutasteride and progesterone HPLC methods

<table>
<thead>
<tr>
<th>Method</th>
<th>Slope</th>
<th>Intercept</th>
<th>$R^2$</th>
<th>Linearity Range (µg/ml)</th>
<th>LOD (µg/ml)</th>
<th>LOQ (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dutasteride</td>
<td>20.63±0.36</td>
<td>5.60±0.99</td>
<td>0.9993</td>
<td>4-100</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Progesterone</td>
<td>57.13±0.23</td>
<td>18.48±12.64</td>
<td>0.9998</td>
<td>1-100</td>
<td>0.30</td>
<td>0.50</td>
</tr>
</tbody>
</table>

Results are presented as mean ±SD, n=3

Precision

The repeatability or intra-assay precision was determined by analysing three repeated injections of the standard solution at three concentrations in the lower, middle and upper range of the calibration curve. For the intermediate precision, these concentrations were analysed over three consecutive days. The results for the repeatability and intermediate precision are shown in Table 2.3. Both HPLC methods were reproducible with Relative Standard Deviation (RSD) <1.3%. 

66
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**Table 2.3:** Intermediate precision and repeatability for dutasteride and progesterone HPLC methods

<table>
<thead>
<tr>
<th>Validation Parameter</th>
<th>Compound</th>
<th>Concentration (µg/ml)</th>
<th>AUC</th>
<th>Variability (%RSD)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Intermediate Precision</strong></td>
<td>Dutasteride</td>
<td>8</td>
<td>162.53±2.20</td>
<td>1.36</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50</td>
<td>1023±7.00</td>
<td>0.64</td>
</tr>
<tr>
<td></td>
<td></td>
<td>90</td>
<td>1848±4.00</td>
<td>0.20</td>
</tr>
<tr>
<td></td>
<td>Progesterone</td>
<td>5</td>
<td>296.28±2.26</td>
<td>0.88</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50</td>
<td>2939±14.20</td>
<td>0.48</td>
</tr>
<tr>
<td></td>
<td></td>
<td>95</td>
<td>5773.84±1.06</td>
<td>0.02</td>
</tr>
<tr>
<td><strong>Repeatability</strong></td>
<td>Dutasteride</td>
<td>8</td>
<td>158.39±3.12</td>
<td>1.96</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50</td>
<td>1016±7.21</td>
<td>0.70</td>
</tr>
<tr>
<td></td>
<td></td>
<td>90</td>
<td>1833.69±5.82</td>
<td>0.31</td>
</tr>
<tr>
<td></td>
<td>Progesterone</td>
<td>5</td>
<td>293.44±0.21</td>
<td>0.23</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50</td>
<td>2935.32±6.6</td>
<td>0.23</td>
</tr>
<tr>
<td></td>
<td></td>
<td>95</td>
<td>5725.22±14.60</td>
<td>0.25</td>
</tr>
</tbody>
</table>

Results are presented as mean ±SD, n=3

**Accuracy**

The accuracy of the analytical methods was tested within 50-150% of the test concentration, which was set as 50 µg/ml for dutasteride and progesterone. The recovery data shown in Table 2.4 comply with ICH guideline that states that the recovery of the analyte should be within 98%-102% of the actual values [232].

**Table 2.4:** Recovery data for dutasteride and progesterone

<table>
<thead>
<tr>
<th>Compound</th>
<th>Actual Concentration (µg/ml)</th>
<th>Theoretical Concentration (µg/ml)</th>
<th>Variability (%RSD)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dutasteride</td>
<td>40</td>
<td>40.53±0.80</td>
<td>0.94</td>
<td>99.00</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>50.19±0.48</td>
<td>0.96</td>
<td>99.06</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>60.21±0.70</td>
<td>1.16</td>
<td>99.28</td>
</tr>
<tr>
<td>Progesterone</td>
<td>25</td>
<td>25.94±0.25</td>
<td>0.94</td>
<td>103.70</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>49.40±0.20</td>
<td>0.40</td>
<td>98.80</td>
</tr>
<tr>
<td></td>
<td>75</td>
<td>75.22±0.1</td>
<td>0.14</td>
<td>100.30</td>
</tr>
</tbody>
</table>

Results are presented as mean ±SD, n=3
Specificity

Specificity is the ability of the analytical method to measure the analytes response in the presence of its potential impurities or degradation products [274]. Both analytical methods are specific as they were optimised such that dutasteride and progesterone could be separated from their degradation products with a resolution of >1.5.

2.4.2 Octanol Water Partition Coefficient (P) Determination

RP-HPLC is commonly performed on stationary phases packed with C8 or C18 hydrocarbons chemically bounded to silica. In preliminary runs, a standard C8 column was used. However, poor correlation was obtained for all investigated methods. This is possibly due to hydrogen bonding of free silanol groups on the stationary phase with the analyte. In order to mimic the Shake Flask method, pure hydrophobic interactions are desired. Subsequently, some authors have used small amounts of n-decylamine in the aqueous phase to avoid hydrogen bonding of the analyte to the stationary phase [247]. In the present study, a SUPELCOSIL LC-AZB column was used. The alkyl chains on this column contain a polar embedded amino group, which protects the silanol from polar compounds and do not affect the retention time of the analyte. Furthermore, a high degree of orientation of alkaline chains can aid hydrophobic interaction of the stationary phase with the solute [275]. Thus, there is no need to add any other additives to the mobile phase, which may influence the retention time of the analyte. Despite its excellent reproducibility and accuracy, the column resulted in long retention times for some steroids and hence it was necessary to use high flow rates (4ml/min).

Figure 2.4 A-C represents the regression plot of logK\textsuperscript{w}, CH\textsubscript{Ii} and CH\textsubscript{Ig} against the experimental logP values. The regression analysis of these plots are described though Equation 2.8-Equation 2.10. The aforementioned parameters and the experimental logP values are presented in Table 2.5. The parameters of regression in Equation 2.8-Equation 2.10 were also used to calculate a theoretical logP for each model using the LOO method. The summary of the regression data obtained from this analysis is listed in Table 2.6.
Isocratic retention time measurements of selected steroids were performed at various methanol concentrations ranging from 60% to 70% (v/v). In the case of stigmasterol and cholesterol, a higher percentage of methanol was required to yield a reasonable retention time. Equation 2.1 was used to calculate $K'$. A strong linear relationship ($R^2 > 0.99$) was observed between log$K'$ of reference compounds and the volume fraction of methanol (Table 2.5). For stigmasterol and cholesterol, the linearity was reduced, possibly due to the high percentage of methanol employed. Linear regression was used to extrapolate log$K'_w$. The slope and intercept of Equation 2.2 were subsequently used to calculate the CHI$_i$ values.

Figure 2.4 A and B show that a linear correlation exists between log$P$ and calculated CHI$_i$ or log$K'_w$. This correlation can be described by the following equations:

$$\log K'_w = 1.108(\pm 0.072) \log P + 0.246(\pm 0.339)$$

$\text{Equation 2.8}$

$n = 13, R = 0.978, R^2 = 0.956, \text{SE} = 0.58, F = 236.39$

$$CHI_i = 0.057(\pm 0.002) \log P + 0.547(\pm 0.009)$$

$\text{Equation 2.9}$

$n = 13, R = 0.994, R^2 = 0.989, \text{SE} = 0.015, F = 967.33$

where, $n$ is the number of compounds, $R$ and $R^2$ are the correlation coefficients, $\text{SE}$ is the standard error of prediction, and $F$ is the Fishers test value. The standard deviation of the slope and intercepts are shown in brackets. From the data presented, it can be stated that a poor linear correlation exists between log$P$ and log$K'_w$ as compared to CHI$_i$. Under ideal conditions, the extrapolated log$K'_w$ for reference compounds must be similar to log$P$. The slope in Equation 2.8 must approach unity and the intercept should be close to zero. Some authors have directly used the extrapolated log$K'_w$ as a measure of the partition coefficient [276]. However, in most cases, a standard set of reference compounds are required in order to calibrate the chromatographic system [250]. In the current study, it was found that the extrapolated log$K'_w$ and the reported literature log$P$ values did not have a 1:1 ratio, the slope did not approach unity, and the intercept was not close to zero. The slope of unity is important as it indicates that the free energies of the two processes are comparable [246]. Furthermore, only a moderate correlation was observed between log$P$ and log$K'_w$ ($R^2 = 0.956$). Given the lack of correlation, some researchers have suggested that extrapolation to 30% water is a better representation of the octanol water system [277]. However, this was not
applicable to the reference steroids used in this study. Hayward et al. also reached the same conclusion for highly hydrophobic compounds [278]. Based on these findings, it can be stated that simple extrapolation techniques are not suitable for measuring the logP of the selected steroids.

Figure 2.4: Plot of experimental logP versus $\log K'_w$ (A), CHI ($\text{I}$) (B) and CHI ($\text{g}$) (C).
Table 2.5: Summary of linear and isocratic data obtained for the selected steroids

<table>
<thead>
<tr>
<th>Compound</th>
<th>logP</th>
<th>logP_{KOWWIN}</th>
<th>Slope (a)</th>
<th>logK'_{w} Intercept (a)</th>
<th>R^2 (a)</th>
<th>CHI_i</th>
<th>CHI_g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrocortisone</td>
<td>1.61</td>
<td>1.62</td>
<td>-4.27</td>
<td>2.65</td>
<td>0.99</td>
<td>0.62</td>
<td>0.68</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>1.83</td>
<td>1.72</td>
<td>-4.71</td>
<td>3.06</td>
<td>0.99</td>
<td>0.65</td>
<td>0.68</td>
</tr>
<tr>
<td>Estriol</td>
<td>2.45</td>
<td>2.81</td>
<td>-4.37</td>
<td>2.97</td>
<td>0.99</td>
<td>0.68</td>
<td>0.69</td>
</tr>
<tr>
<td>Nandroline</td>
<td>2.62</td>
<td>4.23</td>
<td>-5.15</td>
<td>3.66</td>
<td>0.99</td>
<td>0.71</td>
<td>0.71</td>
</tr>
<tr>
<td>Finasteride</td>
<td>3.03</td>
<td>3.2</td>
<td>5.18</td>
<td>3.73</td>
<td>0.99</td>
<td>0.72</td>
<td>0.72</td>
</tr>
<tr>
<td>Testosterone</td>
<td>3.32</td>
<td>3.27</td>
<td>4.73</td>
<td>3.50</td>
<td>0.99</td>
<td>0.74</td>
<td>0.72</td>
</tr>
<tr>
<td>MPA</td>
<td>3.50</td>
<td>3.5</td>
<td>5.32</td>
<td>3.99</td>
<td>0.99</td>
<td>0.75</td>
<td>0.73</td>
</tr>
<tr>
<td>Progesterone</td>
<td>3.87</td>
<td>3.65</td>
<td>4.95</td>
<td>3.86</td>
<td>0.99</td>
<td>0.78</td>
<td>0.74</td>
</tr>
<tr>
<td>Estradiol</td>
<td>4.01</td>
<td>3.94</td>
<td>5.45</td>
<td>4.31</td>
<td>0.99</td>
<td>0.79</td>
<td>0.78</td>
</tr>
<tr>
<td>TP^</td>
<td>4.69</td>
<td>4.77</td>
<td>6.07</td>
<td>5.04</td>
<td>1.00</td>
<td>0.83</td>
<td>0.81</td>
</tr>
<tr>
<td>Clostebol</td>
<td>4.94</td>
<td>-</td>
<td>5.6</td>
<td>4.70</td>
<td>1.00</td>
<td>0.83</td>
<td>0.79</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>-</td>
<td>8.50</td>
<td>9.48</td>
<td>10.05</td>
<td>0.94</td>
<td>1.06</td>
<td>1.07</td>
</tr>
<tr>
<td>Stigmasterol</td>
<td>-</td>
<td>9.30</td>
<td>10.06</td>
<td>11.13</td>
<td>0.89</td>
<td>1.05</td>
<td>1.07</td>
</tr>
</tbody>
</table>

# Testosterone propionate

(a) Values were calculated from Equation 2.2

Figure 2.4 C demonstrates that a moderate linear relationship exists between the experimental logP values and the CHI_g values. This relationship can be described by Equation 2.10.

\[
CHI_g = 0.057(\pm0.002)\log P + 0.547(\pm0.009)
\]

Equation 2.10

\[n = 13, R = 0.98, R^2 = 0.97, SE = 0.02, F = 353.43\]

Despite improvement of linearity over the logK’_w method, the error of prediction of the CHI_g is almost twice that of CHI_i model. The error associated with CHI_g was lower than those reported for neutral form of 52 molecules [251]. This improvement in linearity may be attributed to the choice of acetonitrile versus methanol that was used in the current study. Methanol is known to produce higher linearity in plots of logK’ versus \(\varphi\) when compared to other HPLC solvents [279]. This may be because the solubility parameter of methanol is closer to water than any other compound. Moreover, absorption of methanol (and hence water) into the stationary phase can further prevent its hydrogen bond donating ability [280].

Often a gradient run is used in order to decrease the run time through gradual increase of the mobile phase strength during the chromatographic run. Quarry et al. have demonstrated that a minimum of two gradient runs can be used to successfully predict precise isocratic retention.
data [281]. However, the CHI$_g$ model developed by Valko et al. requires both linear and gradient retention times, which would result in longer analysis time. Thus, considering the larger error of estimate of the CHI$_g$, its long analysis time, and lack of correlation with experimental logP values, CHI$_g$ is also not a suitable model for accurate measurement of logP.

As shown in Table 2.6, logP values of test compounds, calculated using CHI$_i$, are in good agreement with their experimentally determined values with an AAE of only 0.02 log units. This is further confirmed by the high $R^2$ (0.98) and F (528) values listed in Table 2.6. Even for the highly hydrophobic compound clostebol, logP could be calculated to within 0.06 log units of its experimental value. Previously CHI$_i$ has been employed to structurally unrelated compounds [251]. Here the model was proven invaluable for estimating the logP of selected steroids. The advantage of such a model is that the CHI$_i$ can be accurately determined as it falls within a measurable range of values, compared to logK’$_w$, which needs to be extrapolated to outside the measuring range of retention factor [251]. In addition, the CHI$_i$ is independent of the column length, flow rate and volume fraction of organic modifier. Comparison of CHI$_i$ of seven reference steroid, measured on a ZORBAX SB-C8 column with the current CHI$_i$ values, supports this theory (Equation 2.11). When comparing all three models, CHI$_i$ is the most accurate method for calculating log P of steroids in the range of 1-5.

$$CHI_{\text{ZORBAX}} = 1.256(\pm 0.056)\times CHI_{\text{SUPELCO}} - 2.11(\pm 0.069)$$

$N = 7, R = 0.99, R^2 = 0.99, SE = 0.02, F = 334.00$  

Equation 2.11

None of the models was suitable for calculation of logP of stigmasterol and cholesterol. In general, the error of prediction was greater than 0.5 log units. This is to be expected as the validity of HPLC based models only hold true for compounds with logP of up to 7. Hence, the calculated values for these compounds were not included in the final regression analysis of the test compounds (Table 2.6). Overall, the prediction accuracy of the models was in the following order: CHI$_i$ > CHI$_g$ > logK’$_w$. A similar trend was observed when logP$_{KOWWIN}$ were used in the regression analysis, although the accuracy of prediction is reduced (Table 2.6).
Table 2.6: Regression parameters of logP calculated using selected HPLC based models

<table>
<thead>
<tr>
<th>Method</th>
<th>Slope</th>
<th>Intercept</th>
<th>R</th>
<th>R²</th>
<th>SE</th>
<th>F</th>
<th>AAE*</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>logK’w</td>
<td>1.47±0.25^</td>
<td>0.53±0.07</td>
<td>0.92</td>
<td>0.85</td>
<td>0.25</td>
<td>51.78</td>
<td>-0.08</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>0.73±0.06#</td>
<td>0.99±0.34</td>
<td>0.96</td>
<td>0.92</td>
<td>0.67</td>
<td>127.73</td>
<td>0.25</td>
<td></td>
</tr>
<tr>
<td>CHI_i</td>
<td>1.11±0.05^</td>
<td>0.34±0.17</td>
<td>0.99</td>
<td>0.98</td>
<td>0.17</td>
<td>528.76</td>
<td>0.02</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>0.95±0.07#</td>
<td>0.23±0.32</td>
<td>0.97</td>
<td>0.95</td>
<td>0.54</td>
<td>204.74</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>CHI_g</td>
<td>1.03±0.26^</td>
<td>0.67±0.08</td>
<td>0.947</td>
<td>0.90</td>
<td>0.29</td>
<td>77.81</td>
<td>-0.06</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>0.96±0.77#</td>
<td>0.18±0.37</td>
<td>0.96</td>
<td>0.93</td>
<td>0.61</td>
<td>156.62</td>
<td>-0.01</td>
<td></td>
</tr>
</tbody>
</table>

*Average Absolute Error
^ Regression analysis using experimental logP values
# Regression analysis using calculated logP values (logP<sub>KOWWIN</sub>)

The above experimental models were used to calculate the logP value of dutasteride. The values are represented in Table 2.7. Based on the CHI<sub>i</sub> model, dutasteride has a logP value of 4.21. This indicates that dutasteride is highly lipophilic and thus is likely to accumulate in the skin, particularly in the lipid rich domains of the SC. Accumulation in the viable epidermis and dermis might be beneficial for optimum local inhibitory effects. However, high lipophilicity may also limit its permeation through the SC. Therefore, novel lipid or surfactant based delivery systems, which contain permeation enhancers, might be suitable for transdermal delivery of dutasteride.

Table 2.7: Linear and gradient experimental logP values for dutasteride and progesterone.

<table>
<thead>
<tr>
<th>Compound</th>
<th>logK’w</th>
<th>CHI&lt;sub&gt;i&lt;/sub&gt;</th>
<th>CHI&lt;sub&gt;g&lt;/sub&gt;</th>
<th>logP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dutasteride</td>
<td>4.34</td>
<td>4.21</td>
<td>4.66</td>
<td>-</td>
</tr>
<tr>
<td>Progesterone</td>
<td>3.35</td>
<td>3.99</td>
<td>3.40</td>
<td>3.87</td>
</tr>
</tbody>
</table>
2.4.3 Solubility Studies

Aqueous solubility

The logSw of dutasteride could not be determined accurately as its value was less than the LOD of the developed HPLC method. Thus, three models for estimating this parameter were compared using 25 reference steroids.

Figure 2.5 represents the regression plot of logSw calculated using GSE, LSER and AlogPs against the experimental logSw values. The regression analysis of these plots is summarised in Table 2.8. As shown in Table 2.8 logSw of test compounds calculated using GSE are in agreement with their experimentally determined logSw values, with an AAE of only 0.04 log units and $R^2$ value of 0.81. Conversely, the prediction abilities of the AlogPs and LSER were poor. The slope and the $R^2$ of the regression analysis (Table 2.8) deviate from unity. The large positive AAE of LSER also implies that for the investigated steroids, logSw is significantly overestimated.

**Table 2.8:** Regression parameters of logSw calculated using selected solubility models

<table>
<thead>
<tr>
<th>Model</th>
<th>Slope</th>
<th>Intercept</th>
<th>$R^2$</th>
<th>SE</th>
<th>F</th>
<th>AAE</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSE</td>
<td>0.84±0.08</td>
<td>-0.74±0.39</td>
<td>0.81</td>
<td>0.42</td>
<td>100.47</td>
<td>-0.04</td>
<td>25</td>
</tr>
<tr>
<td>AlogPs</td>
<td>0.78±0.18</td>
<td>-1.15±0.79</td>
<td>0.46</td>
<td>0.72</td>
<td>19.39</td>
<td>-0.12</td>
<td>25</td>
</tr>
<tr>
<td>LSER</td>
<td>0.59±0.24</td>
<td>-2.98±1.12</td>
<td>0.22</td>
<td>1.12</td>
<td>6.06</td>
<td>1.09</td>
<td>25</td>
</tr>
</tbody>
</table>
Figure 2.5: Plot of experimental log$S_w$ versus log$K_w$ calculated using A: GSE; B: AlogPs; and C: LSER.
This study shows that LSER developed by Abraham and Le is not useful for predicting logSw of steroids. On average the solubility was overestimated by about 12.30 mol/l (1.09 log unit), which is a significant difference, especially for poorly water-soluble steroids. LSER is associated with a number of limitations. To calculate the coefficient values in Equation 2.6, a large training set covering a wide range of compounds with known experimental solubilities is required. Abraham and LE only used a 665 training set that included 13 steroids. For these steroids, a poor correlation is also observed between the calculated and experimental logSw values ($R^2=0.001$). For the current analysis, the computer software ABSOLVE was used to calculate the descriptors ($\Pi2H, \Sigma\alpha2H, R2 \Sigma\beta2H, Vx$) listed in Equation 2.6 from the steroid structure. When using these values, logSw could not be calculated with a high degree of accuracy. Therefore, these descriptors may need to be experimentally determined in order to obtain reliable results. Although the prediction ability of AlogPs was improved over LSER, it was also not effective for calculating logSw of steroids. In this model, artificial neural network is used to estimate logSw based on E-state index and molecular weight. Similar to LSER, a large set of training compounds with reliable experimental logSw values are required to obtain a correlation between logSw and E-state index. Moreover, the predictability of AlogPs decreases with increase in the number of non-hydrogen atoms present in the analysed structure [261]. Despite using only two input parameters and a small set of test compounds, GSE demonstrated satisfactory correlation with the experimental solubility values ($R^2=0.81$). The accuracy of GSE relies on the availability of experimental MP and logP data. In general, logP can be calculated using various models with a high degree of accuracy [272]. Models for prediction of MP have also been reported [282, 283], although according to Yang et al. these are not reliable [272]. Overall, GSE can be considered a rapid and reliable method for estimating the logSw of the tested steroids. Using this model the aqueous solubility of progesterone and dutasteride were calculated (Table 2.9).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Aqueous Solubility (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Experimental</td>
</tr>
<tr>
<td>Dutasteride</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Progesterone</td>
<td>8.86</td>
</tr>
</tbody>
</table>
Based on the biopharmaceutical classification system, progesterone and dutasteride are class IV compounds with low permeability and low aqueous solubility. The high lipophilicity of these compounds (logP >3) also makes their transdermal drug delivery challenging due to low permeation rates and possible accumulation within the lipid rich layers of the skin. As discussed in Chapter 1, the permeability across the SC can be enhanced by either increasing drug concentration or disordered the SC lipids. These mechanisms are explored in Chapter 4.

Solubility in Oils and Surfactants

The saturation solubilities of progesterone and dutasteride, measured in a series of non-ionic surfactants, solvents and oils, are shown in Figure 2.6 and Figure 2.7. Surfactants increased the solubility of both drugs by > 500 times with progesterone displaying higher solubilities. In general, the solubility was related to the lipophilicity or Hydrophilic-Lipophilic Balance (HLB) of the surfactant molecules, with the highest solubility observed in Transcutol P, which has a low HLB value (HLB=4.2). In oils, the solubility of progesterone and dutasteride ranged from 15-22 mg/ml and 0.5-1.3 mg/ml respectively with the highest solubility observed in Myritol 318 and EPO. Enhanced solubility in oils and surfactants indicates that progesterone and dutasteride can be formulated in lipid-based delivery systems such as microemulsions and liposomes. By employing oils and surfactants with large solubilisation capacity for these compounds, the concentration gradient and hence the permeation rate across the skin could increase.
Chapter 2 - Preformulation Studies

**Figure 2.6**: Saturation solubility of progesterone and dutasteride in solvents and surfactants.

**Figure 2.7**: Saturation solubility of progesterone and dutasteride in oils.

2.4.4 Melting Point Determination (MP)

The MP of progesterone and dutasteride were obtained from DSC thermograms shown in Figure 2.8 and Figure 2.9 respectively. In the first melting cycle, a single peak was observed
around 129.68°C, which corresponds to the MP of form I progesterone [264]. In the second melting cycle, which followed a rapid cooling at 50°C/min, a glass transition and an exothermic transition corresponding to the conversion of the amorphous form to the crystalline form was observed at 11.86°C and 48.86°C respectively. Upon reheating the sample, the MP of progesterone reduced to 122.78°C. This phenomenon can be attributed to the changes in the packing conformation of progesterone crystals and the possible formation of type II polymorphic form due to recrystallisation upon cooling [284]. The Glass transition temperature observed in this chapter was slightly higher than the value reported by Kerc and Srcic (~6) [285]. This might be attributed to the heating and cooling rates used in the present study. Studies investigating the effect of heating rate on the glass transition temperature have demonstrated that this parameter increases as the heating rate is increased [286, 287].

**Figure 2.8:** DSC thermogram showing the MP at 129.68°C and the behaviour of progesterone during heating and cooling cycles.
The MP of dutasteride was 248.75°C, in line with previously reported range of 242-250°C [288]. The second heating cycle shows that dutasteride maintained its amorphus structure following the rapid quenching process. When the amorphous dutasteride is heated, it softens gradually forming a liquid state. This process is presented by the small endothermic peak observed around 113.4°C. The absence of a melting peak indicates the loss of crystallinity. This is typical thermal behaviour of amorphous materials and has been reported for many polymers [289].

Figure 2.9: DSC thermogram showing the MP at 248.75°C and the behaviour of dutasteride during heating and cooling cycles.

2.5 Conclusion

Preformulation studies play a significant role in understanding formulation problems and identifying the most appropriate dosage form. In transdermal drug delivery, the need for
adequate lipophilicity and solubility is highly emphasised. Overall, the preformulation studies performed in this chapter demonstrated that progesterone has the ideal low melting point for transdermal drug delivery, although its lipophilicity is slightly higher than the optimal range required. Conversely, dutasteride is highly lipophilic, with a MP of 248°C, and thus it may accumulate within the lipophilic layers of the skin when applied transdermally. Both dutasteride and progesterone were found to be soluble to very soluble in various solvents, oils and surfactants. Enhanced formulation solubility is important when designing a transdermal delivery system. Given that the transport of drugs across the skin is via passive diffusion, increasing the drug loading dose can increase the concentration gradient across the skin and hence the permeation rate. Based on their physiochemical properties, progesterone and dutasteride could be formulated in a lipid-based delivery system with penetration enhancing properties. In the next chapter, a novel lipid based delivery system will be developed and examined for transdermal delivery of progesterone.
3 Formulation Development and Characterisation

3.1 Introduction

In the previous chapter, it was demonstrated that progesterone is a suitable candidate for a lipid-based delivery system. Lipids are defined as fatty acids, their derivatives, and structurally related compounds [290]. Small has classified lipids into polar and non-polar groups based on their ability to form a monolayer at the water-air interface and micelles in bulk water [291]. Non-polar lipids, such as unsaturated aromatic compounds and paraffins, are insoluble in bulk water and will not spread at the interface. In water, they generally appear as crystals (solid) or oil droplets (liquid). In contrast, polar lipids are able to form a monolayer at the water-air interface. These lipids are further classified into four main categories based on their behaviour in bulk water. Class I lipids contain long chain hydrocarbons or bulky aromatic structures with at least one hydrophilic group. Owing to their structure, they are insoluble in bulk water, appearing as oil droplets or crystals. Examples of these lipids include diglycerides, triglycerides, long chain alcohols, and protonated long chain fatty acids. A certain class of insoluble lipids (Class II) can swell and form liquid crystals in bulk water. This phenomenon is associated with the partial liquefaction of their hydrocarbon chain at a critical temperature and can be affected by chain length, branching and the degree of saturation. Phospholipids and 2-monoglycerides are examples of lipids belonging to this class. Ionic and non-ionic amphiphiles, or class IIIA lipids, with a lipophilic tail and a hydrophilic polar head group can form micelles above their Critical Micelle Concentration (CMC) in bulk water. At a higher surfactant concentration, they may also form liquid crystals. Class IIIB lipids behave in a very similar manner, although they do not form liquid crystals. Unfortunately, Small’s classification does not consider complex lipid systems such as those containing 2-monoglycerides and triglycerides (Labrasol) [290].

Lipid based systems for oral drug delivery have been classified into four main categories including simple digestible oils, self emulsifying and Self Microemulsifying Drug Delivery
systems (SMEDDS), and oil free surfactant mixtures [292]. SMEDDS are water free dispersions that can transform into microemulsion upon dilution with water. The formation of a microemulsion is a spontaneous process and can be described using the second law of thermodynamics [293]. Accordingly, the free energy of forming a microemulsion is described by the following equation:

\[
\text{Equation 3.1}
\]

where \( \delta \) represents the change in the surface area as the result of reduced droplet size, \( \gamma \) is the interfacial tension at the interface, \( T \) is the absolute temperature and \( \Delta S \) is the change in the system’s entropy. It is well known that when the oil or water droplets are dispersed in the continuous phase the entropy of the system will increase. At the same time, the presence of surfactants at the interface will lower the interfacial tension. The interfacial tension can further be reduced by addition of cosurfactants. The resulting microemulsions typically have a droplet size of < 200 nm. Thus, given their small size, the surface area in the above equation will also increase. The net effect of these processes is a negative free energy term, which corresponds to the spontaneous formation of microemulsions [293].

SMEDDS are commonly formulated using a mixture of oils and surfactants (Class I and Class II lipids) with or without a cosurfactant or a cosolvent. The self-emulsification process depends on the nature of the oils and surfactants employed [294]. These are in turn screened based on their toxicity profiles, the effective microemulsion area, and their solvent capacity [295]. Non-polar lipids, or class I lipids, are amongst the most commonly used oils. Examples of these oils include vegetable oil, isopropyl palmitate and ethyl oleate. The role of the surfactant is to reduce the interfacial tension and to aid in the spontaneous microemulsification. The successful formulation of SMEDDS requires the presence of highly hydrophilic surfactants [294] at concentrations >20%. Type IIA surfactants are commonly used, with non-ionic surfactants being preferred due to their low toxicity profile.

One of the main advantages of SMEDDS is their ability to overcome the stability issues associated with microemulsions. Microemulsions can undergo phase separation or phase inversion when diluted with gastrointestinal (GI) fluid or skin sweat, thus significantly affecting
drug release and permeation. Long-term stability is also of a concern and can be affected by environmental factors such as heat and humidity. In SMEDDS, microemulsification will take place upon dilution with an aqueous medium hence long-term microemulsion stability is not of a concern [290]. SMEDDS are mainly used for oral delivery of class II compounds that have a low solubility and a high lipophilicity [296]. Kemken et al. were the first group to investigate SMEDDS as a potential transdermal drug delivery matrix [297]. It was hypothesised that the uptake of water from the occluded skin can transform the SMEDDS into microemulsions. Compared to matrix patches, enhanced therapeutic activity of model β blockers were observed from SMEDDS. More recently El Maghraby found that SMEDDS significantly increased the transdermal permeation rate of indomethacin compared to other colloidal systems including microemulsions and liquid crystals [151]. It is hypothesised that the absorption of water into the SMEDDS decreases the saturation solubility of the drug. This in turn can result in the formation of a transient supersaturated solution with an enhanced thermodynamic activity and higher permeation rate [297].

Before SMEDDS and microemulsions can be used as drug delivery systems, it is necessary to characterise the internal structure of the resulting microemulsion. This is highly critical as multiple colloidal and coarse dispersions may coexist. Amongst these are microemulsions, coarse emulsions, various liquid crystalline systems (hexagonal, reverse hexagonal, lamellar, cubic) and gels. These can form upon mixing oils, surfactants and water. Moreover, as discussed in Chapter 1, microemulsions are known to have different microstructures namely o/w, w/o and bicontinuous [219]. Elucidating the internal structure of microemulsions is of great interest as the phase behaviour of these systems can influence drug solubility, stability and in vitro release. The ideal formulation must remain as a single transparent one-phase system, when diluted with up to 15-20% water. This value is based on the water content of the SC, which is about 15-20% of its dried weight [117]. The phase behaviour of microemulsions can be monitored through visual inspection, polarised light microscopy, Fourier Transform Infrared Spectroscopy (FTIR) measuring electrical conductivity, viscosity, droplets size, density determination, surface tension measurements, nuclear magnetic resonance, electron microscopy, and diffusion coefficient measurements.
3.1.1 Visual Observation and Polarised Light Microscopy

Visual observation is one of the first and foremost techniques used to establish the phase boundaries and distinguish microemulsions from coarse emulsions, liquid crystals and gels. The samples are equilibrated for a minimum of 24 h before being inspected for colour, transparency, and signs of phase separation. Temperature control is important, especially when dealing with non-ionic surfactants. Visually, microemulsions (clear, low viscosity) can be distinguished from coarse emulsions and gels based on their appearance and viscosity. Coarse emulsions are milky while gels are highly viscous and resist flow when tilted at a 90° angle. To further differentiate microemulsions from lamellar liquid crystals, one can take advantage of polarised light microscopy.

Microemulsions are isotropic in nature, having the same optical properties (such as refractive index) in all directions. They do not alter the direction of the light that passes through them. In contrast, liquid crystals, with the exception of cubic phases, are anisotropic [219], showing different optical properties in different directions. Liquid crystals can split the light along their optical axis into two rays travelling with different speeds, resulting in different refractive indices. Polarised light microscopy takes advantage of this feature to distinguish isotropic material from anisotropic liquid crystals.

The basic components of a polarised light microscope are depicted in Figure 3.1. A polarised light microscope consists of two filters located in the optical pathways over (analyser) and under the specimen (polariser) [298, 299]. Anisotropic materials convert the polarised light into ordinary and extraordinary light waves travelling at different speeds and directions [298]. Only light waves travelling perpendicular to the analyser will pass through it, providing structural information on the form of the liquid crystal present [219]. In isotropic materials, the object will appear dark given that the polarised light passes through the specimen unchanged. Polarised light microscopy is often used to identify phase boundaries. However, it may also be effective for monitoring the stability and droplet size when used in combination with other techniques.
**Figure 3.1**: Schematic representation of a polarised light microscope showing the interaction of an anisotropic material with the polarised light. Image modified from [300].

### 3.1.2 Electrical Conductivity

Measurement of the electrical conductivity is valuable for understanding the structural changes taking place within a microemulsion system. Typically, an exponential profile is observed between the electrical conductivity and the volume fraction of water ($\phi$), with the water continuous phase showing high electrical conductivity as compared to the insulating oil continuous phase [221, 301, 302]. This profile, which is shown in Figure 3.2, has been described through the concept of percolation. Initially, the attractive interactions between the conductive water molecules are weak, as they form isolated spheres dispersed in the continuous oil phase. This accounts for the low electrical conductivity observed in Figure 3.2 A. At a critical point in the system, known as the percolation threshold ($\phi_c$), the water molecules begin to contact each other forming either an assembly of water molecules (dynamic percolation model) or transient bicontinuous water channels (static percolation model), which allow for charge transfer [303, 304]. These assemblies result in a rapid increase in the electrical conductivity as shown in Figure 3.2 B followed by the formation of bicontinuous and eventually o/w microemulsions. A deviation from this model has been reported for a system employing a conductive ionic...
hydrophobic liquid as the oil phase [304]. For this system, the electrical conductivity of the oil continuous phase was greater than the conductivity of water and hence the percolation theory was not applicable.

![Graph showing variation in electrical conductivity (σ) with φ]  

**Figure 3.2:** Hypothetical graph showing variation in the electrical conductivity (σ) with φ (expressed as %w/w).  
A: w/o microemulsions; B: conversion of w/o microemulsions to bicontinuous or o/w microemulsions; C: o/w microemulsions.

### 3.1.3 Viscosity

The rheological properties of microemulsions have been used to distinguish them from gels and various types of liquid crystalline systems [305], to elucidate their internal structure [221] and to identify delivery systems with optimal viscosity [306]. Microemulsions display Newtonian flow behaviour with low viscosity while liquid crystals exhibit non-Newtonian flow and are of relatively high viscosity.

Figure 1.4 shows typical viscosity profiles of microemulsions as a function of φ. For those systems displaying a bell shape curve [307], the viscosity gradually increases with φ (Figure 3.3 A) to a threshold value followed by a steady decline, with the maximum viscosity representing the transformation of the oil continuous microemulsion to water continuous or a bicontinuous system. The sinusoidal profile [301, 308], shown in Figure 3.3 B, typically displays a maximum...
viscosity around 10-30% and 60-80% water. The viscosity of the oil continuous system increases with $\phi$, and reaches the first maximum where the bicontinuous microstructures immerge. The viscosity of these systems remains constant, until the emergence of a second maximum after which the viscosity sharply declines. This represents the transformation into a water continuous microemulsion, which in general has a lower viscosity than the oil continuous microemulsion. An exception to this behaviour was reported by Mehta et al. (Figure 3.3 C), who showed the viscosity gradually increasing with $\phi$ [221, 309]. The transformation into a bicontinuous microstructure was identified as the maximum change in the viscosity as a function of $\phi$. The viscosity of the o/w microemulsions were not reported in these studies [221].

![Figure 3.3](image)

**Figure 3.3:** Schematic representation of the various viscosity versus $\phi$ profile for microemulsions.
A: Bell shaped curve; B: Sinusoidal profile; C: Linear increase.

For the profiles described above, the increase in the viscosity of the oil continuous microemulsion corresponds to the attractive interaction and reorganisation of the water molecule at the interface. In general, for a colloidal system, the viscosity is expected to increase as the volume fraction of the dispersed phase is increased [219]. This is represented by the Einstein equation:

$$\eta = \eta_0(1+2.5\Phi)$$

**Equation 3.2**
where $\eta_0$ represents the viscosity of the dispersion medium, $\eta$ is the viscosity of the dispersion and $\phi$ is the volume fraction of colloidal particles present. However, in most cases, the increase in the viscosity deviates from this equation due to formation of transient clusters of droplets, which are held together by small intermolecular forces or the formation of nonspherical droplets.

### 3.1.4 Dynamic Light Scattering (DLS)

Dynamic Light Scattering (DLS) or Photon Correlation Spectroscopy (PCS) is a useful technique for measuring the particle size in the submicron range. In this technique, a polarised laser beam hits a molecule, inducing light radiating in all directions. Due to the Brownian motion of the molecules, the scattering intensities of the radiated light will fluctuate. The fluctuation rate is related to the diffusion of the molecules. Smaller molecules will diffuse fast, causing a fast fluctuation and hence a fast decaying of the scattering intensities. The diffusion coefficient ($D$) is used to calculate the hydrodynamic radius ($R_H$) according to Stokes-Einstein equation:

$$R_H = \frac{kT}{6\pi\eta D} \quad \text{Equation 3.3}$$

where $k$ is the Boltzmann’s constant, $T$ is the absolute temperature and $\eta$ is the viscosity of the continuous phase. The possibility of interparticulate interaction limits this technique to dilute samples. However, microemulsions undergo phase separation or phase inversion when diluted. Subsequently, Shukla et al. proposed to correct the scattering intensities for the effect of interparticulate interactions [310-312]. However, using this model, only a small change in the droplet size was observed.

The viscosity of the continuous phase is required for accurate measurement of $R_H$. The size results must be interpreted with care when the viscosity of the pure external phase is used, as the presence of surfactants can alter this value. Subsequently, the viscosity of microemulsion or SMEDDS [313] have been used. The droplet size of w/o microemulsions designed for transdermal delivery of lipophilic drugs is not critical, as the drug will partition directly from the oil continuous phase into the SC. In the present investigation, DLS is only used as a qualitative tool to monitor the phase behaviour of microemulsions.
3.1.5 Cloud Point (T\textsubscript{Cloud})

The phase behaviour of microemulsions, especially those formulated with non-ionic surfactants, can be affected by variation in the temperature. The ideal transdermal delivery system should remain as a single transparent phase above the surface temperature of the skin (32°C). Thermal stability of microemulsions can be monitored by measuring the cloud point (T\textsubscript{Cloud}), also known as the Phase Inversion Temperature (PIT) or HLB temperature. T\textsubscript{Cloud} is defined as the temperature at which the solubility of the surfactant molecule changes from water soluble to oil soluble or vice versa [314]. At this temperature, the net interfacial curvature of the surfactant molecule is changed from positive (o/w microemulsions) to negative (w/o microemulsions) by passing through a zero curvature (bicontinuous microemulsions). The T\textsubscript{Cloud} has also been described as the lowest temperature at which the sample turns cloudy or separates out into two phases [315, 316]. This definition will be used to evaluate the thermal stability of SMEDDS and microemulsions.

Phase separation of samples observed at T\textsubscript{Cloud} is associated with temperature dependent solubility of the surfactant molecules. The surfactants employed in the current study are non-ionic polyoxyethylenes. The oxyethylene group consists of a linear CH\textsubscript{2}-CH\textsubscript{2}-O group as well as a single and double C-C and C-O functional groups respectively [317]. The trans orientation of the oxygen molecules around the C-O bond generates a strong temperature dependant dipole-dipole interaction with the water molecules [318]. Increase in the temperature significantly reduces the hydrophobicity of the surfactant molecules due to a decrease in the net dipole moment of the head groups. Consequently, the solubility of the surfactant molecules in water will drastically decline as they become dehydrated. The hydration number of a polyoxyethylene surfactant (pentaethylene glycol dodecyl ether) was reported as 2 water molecules per oxyethylene group and decreased as the temperature was increased [319]. Changes in the T\textsubscript{Cloud} may also be related to the internal structure of the microemulsion. It is well known that the T\textsubscript{Cloud} may be affected by the symmetrical structure of swollen micelles, with symmetrical structures having higher T\textsubscript{Cloud} [320].
### 3.1.6 Fourier Transform Infrared Spectroscopy (FTIR)

Fourier Transform Infrared Spectroscopy (FTIR) can be used to characterise the state of water in microemulsions. The asymmetrical OH stretching of pure water is observed at approximately 3400 cm$^{-1}$ [221]. Upon the solubilisation of water into the oil/surfactant mixture, the OH stretching (3000-3400 cm$^{-1}$) frequency shifts to a higher wavenumber eventually reaching towards the OH stretching frequency of pure water. This shift has been attributed to the properties and state of water in the microemulsion system. In effect, it has been found that different kinds of water can exist in a microemulsion [321]. Different models have been used to describe the different state of water molecules in microemulsions. In the three-phase model, the peak from the broad OH stretching is resolved into three Gaussian peaks as shown in Figure 3.4 [322-324]. The high-energy band (~3500-3660 cm$^{-1}$) arises from the non-hydrogen bonded monomers trapped between the surfactant chains (trapped water). Conversely, the medium energy band reflects the water molecules, which are interacting with the surfactants head group (bound water). The free water molecules contribute to the low energy Gaussian. These are water molecules, which are fully hydrogen-bonded, and resemble the properties of pure water. According to the two phase model, the properties of the water molecules present at the interface are different from those of bulk water [321]. Although these models have generally been applied to w/o microemulsions, they may still be useful for evaluating the state of water in all types of microemulsions. Comparison of peaks arising from different kinds of water can provide information on the type of microemulsion present, and allows them to be differentiated from micelles.

![Figure 3.4: OH stretching of pure water.](image)

The coloured curves represent the different Gaussian component of the water molecules.
3.1.7 Composition of SMEDDS

In Chapter 2, the solubility of progesterone in various oils and surfactants was measured. Based on the results from these measurements, IPM and Myritol 318, with intermediate and high solubilisation capacity for progesterone, were selected for further investigation. The surfactants were screened to find potential systems with high water solubilisation capacities. Imwitor 308 (Figure 3.5) is used as the cosurfactant. The active constituent of Imwitor 308 is glycercylnonocaprylate monoester (>80%), which is manufactured through esterification and molecular distillation of caprylic acid and glycerol. In pharmaceutical formulations, Imwitor 308 is frequently used as a solubilising agent, penetration enhancer, plasticiser, and as a co-emulsifier [325]. In particular, Imwitor 308 has been used successfully as a cosurfactant to construct pseudoternary phase diagrams with large microemulsion areas [326-329].

![Chemical structure of Imwitor 308](image)

**Figure 3.5:** Chemical structure of Imwitor 308 (Glyceryl monocaprylate).

3.2 Chapter Aim and Objectives

The aim of this chapter is to formulate novel SMEDDS that can remain as a stable one-phase system when diluted with 15-10% water and at skin temperature (32°C).

The specific objectives of this chapter are to:

- identify oil and surfactant combinations that can solubilise over 15-20-% water;
- identify pseudoternary systems with a large microemulsion area; and
- investigate the phase behaviour of selected SMEDDS upon dilution with water.
3.3 Materials and Methods

3.3.1 Materials

IPM, Tween 85, Tween 80, Tween 40, Brij 30 and Span 80 were purchased from Sigma-Aldrich (USA). Myritol 318 was obtained from Cognis Care Chemicals (Germany), Labrasol and Transcutol P were kindly donated by Gattefossé (Saint Priest Cedex, France). Imwitor 308 was obtained from Sasol (Germany). Cremophor EL was supplied by BAFS (Germany). All samples were used without any further purification. Triple-distilled water was obtained in-house by reverse osmosis (MilliQ, Millipore, USA).

3.3.2 Screening of Surfactants and Cosurfactants

The aim of this study was to identify potential pseudoternary systems capable of forming a large microemulsion area. $W_{\text{max}}$ represents the maximum amount of water that can be solubilised in the oil/surfactant/cosurfactant mixture. This parameter was used to screen oil and surfactant combinations that can form the largest microemulsion area. The effect of different surfactants/cosurfactants and Km (mass ratio of surfactant to cosurfactants) on $W_{\text{max}}$ was investigated. $W_{\text{max}}$ was measured using the titration method. Mixtures of surfactants and cosurfactants were formulated at different Km ratios (4:1, 2:1, 1:1). The obtained mixtures were then mixed with the oil phase (IPM and Myritol 318) at a weight ratio of 1:1. To this mixture, water was added drop-wise under constant magnetic stirring. $W_{\text{max}}$ was determined as the point of transition from a clear, one-phase system to a two-phase system. The surfactants used were Tween 40, Tween 80, Tween 85, Labrasol, Cremophor EL, Brij 30, and Span 80. Imwitor 308 was used as a cosurfactant.

3.3.3 Construction of Pseudoternary Phase Diagrams

Pseudoternary phase diagrams were constructed using Gibbs triangle shown in Figure 3.6 and the equilibration method [219, 330]. Briefly, predetermined amounts of oil, surfactant/cosurfactant were mixed with water, at ambient temperature. Mixtures of surfactant/ cosurfactant were formulated at different Km ratios (4:1, 2:1, 1:1). The ratio of oil to the surfactant mixture was varied from 9:1 to 1:9 at 10% increments. Predetermined amounts of this oily mixture was
prepared (5%-95% w/w), and to this water (95% to 5% w/w) was added drop-wise under constant magnetic stirring. After being equilibrated overnight, visual observation and polarised light microscopy (Leica DMR, GmbH, Germany) were used to identify microemulsions and differentiate them from various liquid crystalline systems and coarse emulsions. The contribution of each system to the total area of the phase diagrams was measured by the cut and weight method [331]. The phase diagrams were printed on an A4 paper sheet. The weight of each system and the total weight of the phase diagram were determined and were then used to calculate the percentage occupied by the different regions.

![Gibbs triangle](image)

**Figure 3.6: Gibbs triangle.**

Each corner represents 100% of one component (A, B or C). The sidelines joining these corners represent a two-component mixture of AB, AC and BC. A point inside the triangle corresponds to a three-component system comprising of A, B and C. Point X is a three-component system consisting of 20% C, 20% A and 60% B.

### 3.3.4 Electrical Conductivity Measurements

The specific conductivity of double distilled or triple distilled water is approximately 0.005 µS/cm. Thus, the presence of salt (NaCl) in non-ionic microemulsions may be necessary to provide charge transfer [309]. However, the solubility of non-ionic surfactants in water can be greatly compromised (salting out effect) by the presence of non-organic salts, such as NaCl, as
the addition of electrolyte can reduce the hydration of the surfactant head group [316, 318]. In this study, the electrical conductivity was measured without the addition of any salts to the samples.

The conductivity was measured at room temperature (22-23°C) using a Seven Easy Mettler Toledo (Mettler, Switzerland) conductivity meter with a Mettler Toledo Incap® 730 electrode. All measurements were autocorrected for variation in the temperature. The range of application was between 0.01 mS/cm to 1000 mS/cm with the accuracy of ±0.5%. The preconcentrated formulations (water-free) were titrated with water. Initially 200 µl of water was added to the preconcentrated SMEDDS. The sample was then equilibrated for at least 1 min under constant magnetic stirring before a reading was taken. This was continued until a two-phase system was formed. All measurements were carried out at least in duplicate to ensure the reproducibility of the results.

3.3.5 Viscosity Measurements

The dynamic viscosities of microemulsions were measured with a Brookfield DV-III cone and plate rheometer (Brookfield Engineering Laboratories Inc., USA) fitted with a CP-40 spindle. The sample cup was connected to the circulating water bath maintained at 25°C ± 0.5°C. A sample volume of 600 µl was used. The measurements were made from 75-500 rpm and in triplicate. The data analysis was performed by Rheocalc V3.1 operating software (Brookfield Engineering Laboratories Inc., USA). All of the viscosity values were recorded at 150 rpm.

3.3.6 Dynamic Light Scattering (DLS) Studies

DLS was used to determine the effect of dilution on the phase behaviour of microemulsions. The measurements were made with a Malvern Zetasizer Nano ZS instrument (Malvern Instruments, UK). The instrument contained a 4 mW He-NE laser operating at 633 nm and non-invasive backscattering optics. The measurements were made at a detection angle of 173° and the measurement position was automatically selected by the software. The viscosity of the SMEDDS investigated was used as the viscosity of the continuous phase. Each measurement was made in triplicate at 25°C and subsequently the average droplet size (Z-Average) and Polydispersity
Index (PDI) were calculated. The PDI measures the homogeneity of the sample. A PDI value of 0.5 or higher indicates a sample of high polydispersity [306].

3.3.7 Cloud point ($T_{\text{Cloud}}$) Determination

The effect of temperature on the phase behaviour of microemulsions was evaluated through measurement of the $T_{\text{Cloud}}$. $T_{\text{Cloud}}$ was measured using a Malvern Zetasizer Nano ZS instrument (Malvern Instruments, UK) temperature controlled chamber according to the conditions set above. The size-temperature trend analysis was made at a starting temperature of 18°C to 20°C and was increased by 1°C increments. Each sample was equilibrated at the specified temperature for at least 2 min. At the end of each measurement, the samples were visually assessed for optical transparency or signs of phase separation.

3.3.8 Fourier Transform Infrared Spectroscopy (FTIR) Studies

The FTIR spectrum of selected microemulsions were recorded on a Bruker Tensor 37 FTIR spectrometer (Bruker Optik GmbH, Germany) using the OPUS software (OPUS 6.5, Germany). The Absorbance mode was used for all measurements. A clean diamond window was used to measure the background spectrum. The FTIR spectrum of each sample was then recorded at 2 cm$^{-1}$ spectral resolution with an average of 120 scans. The spectrum of water was resolved into bound water, trapped water, and free water using the least squares curve fitting (OMNIC 7.3, Thermo Electron Corporation, USA). The area under the curve (AUC) for each peak was then calculated using OMNIC software (Thermo Electron Corporation, USA). This analysis was performed for the 7:3 and 6:4 dilution line (DL) of Cremophor EL and Tween 85 pseudoternary systems, respectively.

3.4 Results and Discussion

3.4.1 Screening of Surfactants and Cosurfactants

Table 3.1 and Figure 3.7 show the effect of Km (expressed as mass ratio of surfactant to cosurfactants) on $W_{\text{max}}$ determined for systems comprising of IPM and Myritol 318. When IPM was used as the oil phase (Figure 3.7 A) Tween 40, and Tween 80 showed an increase in $W_{\text{max}}$ of
up to 95% (w/w) as the Km and HLB reduced. $W_{max}$ was similar for both surfactants at all Km values. Tween 40 (HLB=15.5) and Tween 80 (HLB=15) are polysorbates, which are derived from pegylated sorbitan esterified with fatty acids. Tween 85 is a trioleate derivative with three unsaturated hydrocarbon chains in its lipophilic tail and has a lower HLB value (HLB=11). Addition of Tween 85 at all investigated Km values resulted in a lower $W_{max}$, which was found to decrease from 40% (w/w) to 16.7% (w/w) as the Km was reduced to a weight ratio of 1:1. At a Km of 4:1, the Tween 85 system had an HLB value of 10 that reduced to 8.5 with increase in the weight fraction of Imwitor 308. This corresponded to a decrease in $W_{max}$. The more hydrophilic surfactants, Tween 40 and 80, showed higher $W_{max}$ as the HLB of the surfactant mixture approached 10.5. Similar results were observed with the Cremophor EL system, which showed a $W_{max}$ of >95% (w/w) when the HLB of its surfactant mixture reduced from 11.6 at a Km of 4:1 to 10.5 at a Km of 2:1. This trend was observed for all the surfactants investigated. The optimum HLB value at which > 95% (w/w) water could be solubilised was about 10.5-10.8. When the HLB deviates out of this range, a significant decrease in $W_{max}$ can be observed. The most effective systems were Tween 40 (Km: 1:1), Tween 80 (Km 1:1) and Cremophor EL (Km 2:1 and 1:1). A similar trend was observed when Myritol 318 was used as the oil phase (Figure 3.7 B). Maximum amount of water (70% w/w) was solubilised at an HLB of 10. For systems with HLB values ranging from 7.85 to 9.5, between 15 to 30% (w/w) water could be solubilised. The most effective surfactant was Tween 85 (Km 4:1), which could solubilise 70% (w/w) water. Solubilisation is a complex process and depends on several factors including the size and the molecular volume of the oils and surfactants, the size and packing parameter of the head groups, and the HLB value and temperature. Another important factor affecting the solubilisation capacity is the elasticity of the interfacial film [332]. For the surfactants employed in this study, optimum elasticity is likely to be achieved at an HLB value of 10-10.5 at which high $W_{max}$ was observed. The amount of water solubilised was also dependent on the HLB value of the individual surfactants. For example, $W_{max}$ was < 5% for span 80, which is highly lipophilic with an HLB of 4.3.

When IPM was used as the oil phase, Tween 40, Tween 80, Brij 30 and Cremophor EL formed liquid crystals. Furthermore, selected Myritol 318 systems were also capable of forming liquid crystals. The microscopic appearance of these liquid crystals was typical of lamellar liquid crystals with their characteristic oily streaks, maltese crosses and fan-shaped textures (Figure 3.8).
Table 3.1: Summary of the effect of Km and HLB values on the water solubilisation capacity ($W_{max}$) measured for selected oil/surfactant mixtures

<table>
<thead>
<tr>
<th>Surfactant</th>
<th>HLB Surfactant</th>
<th>HLB SCo/S*</th>
<th>Km#</th>
<th>Myritol 318 $W_{max}$ (% w/w)</th>
<th>IPM $W_{max}$ (% w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brij 30</td>
<td>9.70</td>
<td>8.96</td>
<td>4:1</td>
<td>12.28</td>
<td>25.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8.47</td>
<td>2:1</td>
<td>14.53</td>
<td>35.19</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7.85</td>
<td>1:1</td>
<td>17.36</td>
<td>25.92</td>
</tr>
<tr>
<td>Cremophor EL</td>
<td>13.00</td>
<td>11.60</td>
<td>4:1</td>
<td>13.79</td>
<td>30.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10.67</td>
<td>2:1</td>
<td>11.50</td>
<td>&gt;95.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td>9.50</td>
<td>1:1</td>
<td>9.09</td>
<td>&gt;95.00</td>
</tr>
<tr>
<td>Labrasol</td>
<td>14.00</td>
<td>12.40</td>
<td>4:1</td>
<td>0.99</td>
<td>15.10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>11.33</td>
<td>2:1</td>
<td>5.66</td>
<td>27.20</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10.00</td>
<td>1:1</td>
<td>3.85</td>
<td>5.00</td>
</tr>
<tr>
<td>Span 80</td>
<td>4.30</td>
<td>4.64</td>
<td>4:1</td>
<td>0.99</td>
<td>1.40</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4.87</td>
<td>2:1</td>
<td>2.91</td>
<td>2.40</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5.15</td>
<td>1:1</td>
<td>5.66</td>
<td>4.70</td>
</tr>
<tr>
<td>Tween 40</td>
<td>10.80</td>
<td>13.68</td>
<td>4:1</td>
<td>8.00</td>
<td>25.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12.40</td>
<td>2:1</td>
<td>8.26</td>
<td>38.60</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10.80</td>
<td>1:1</td>
<td>8.26</td>
<td>&gt;95.00</td>
</tr>
<tr>
<td>Tween 80</td>
<td>15.00</td>
<td>13.20</td>
<td>4:1</td>
<td>9.09</td>
<td>20.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12.00</td>
<td>2:1</td>
<td>9.91</td>
<td>40.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10.50</td>
<td>1:1</td>
<td>10.71</td>
<td>&gt;95.00</td>
</tr>
<tr>
<td>Tween 85</td>
<td>11.00</td>
<td>10.00</td>
<td>4:1</td>
<td>70.93</td>
<td>40.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td>9.33</td>
<td>2:1</td>
<td>30.84</td>
<td>24.50</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8.50</td>
<td>1:1</td>
<td>20.00</td>
<td>16.70</td>
</tr>
</tbody>
</table>

*SCo/S : Surfactant/cosurfactant mixture

# Km is the mass ratio of surfactants to Imwitor 308
Figure 3.7: The effect of Km on the water solubilisation capacity ($W_{\text{max}}$) measured for selected surfactants.

A: IPM is used as the oil phase.
Figure 3.7 (continued): The effect of $K_m$ on the water solubilisation capacity ($W_{\text{max}}$) measured for selected surfactants.

B: Myritol 318 is used as the oil phase.
**Figure 3.8:** Polarised light micrographs of the formed liquid crystals.
A: lamellar liquid crystals with characteristic oily streaks (IPM/Tween 40/Imwitor 308/water) at 10× magnification, B: maltese crosses (IPM/ Brij 30/Imwitor 308/water) at 10 × magnification, C: maltese crosses showing the characteristic fan-shaped texture (IPM/ Brij 30/Imwitor 308/water) at 40 × magnification.

### 3.4.2 Phase Behaviour Studies

In order to investigate the phase behaviour of the identified surfactant(s) / cosurfactant, oil(s) and water blends and identify the optimum microemulsion area, pseudoternary phase diagrams were constructed. Systems investigated comprised Myritol 318/Tween 85/Imwitor/water and IPM/Cremophor EL/Imwitor/water at different Km ratios, as these combinations could solubilise up to 70-95% (w/w) water. Figure 3.9 shows the phase diagrams of selected pseudoternary systems at different Km ratios. For the Cremophor EL and Tween 85 systems, the optimum surfactant/cosurfactant ratio was at a Km of 1:1 and 4:1, respectively. In both systems, a large liquid crystalline area could also be observed as the weight fraction of the cosurfactant decreased. Imwitor 308 is a lipophilic cosurfactant with lesser tendency to dissolve in water. At lower concentrations (Km 4:1) less Imwitor 308 is incorporated into the lipophilic tail of the surfactant and at the interface, leading to the long range order and packing of the surfactant molecule, and hence the formation of liquid crystals (Table 3.2). The high concentration of Imwitor (Km 2:1) destroyed this surfactant arrangement, which consequently reduced the size of the liquid crystalline region. No liquid crystalline region was observed at a ratio of 1:1 (Tween 85 system).
Despite the large $W_{\text{max}}$ of the Tween 85 system (Km 4:1), the microemulsion area was small (20.78%). In an attempt to further optimise the phase boundary of the Tween 85 systems, the effect of a secondary cosurfactant, Transcutol P, was investigated. At a Km of 4:1, Transcutol P significantly enlarged the microemulsion area without further affecting $W_{\text{max}}$. At higher concentrations however, $W_{\text{max}}$ was reduced, consequently destroying the microemulsion and the liquid crystalline phases. Transcutol P (diethylene glycol monoethyl ether) has a smaller molecular volume (224.48 cm$^3$/mol) compared to Imwitor 308 (613.73 cm$^3$/mol), and thus can be easily incorporated into the interface, with its lipophilic tail perturbing the long-range packing of the surfactant. In other words, it can act as a typical cosurfactant. Although Transcutol P is highly lipophilic (HLB value of 4.2), some of it can escape from the interface into the aqueous phase due to its smaller molecular volume and water solubility. Thus, at a Km of 1:1, Transcutol P partitions favourably into the aqueous phase. High concentration of Transcutol P in the aqueous phase may minimise $W_{\text{max}}$ and destroy the microemulsion area. This phenomenon might be highly important in the view point of SMEDDS and supersaturated systems. Within the aqueous phase, Transcutol P may act as a cosolvent. When water is absorbed into the saturated SMEDDS, the extent of supersaturation can be minimised leading to a lower permeation rate across the skin. Systems comprising of IPM/ Cremophor EL (50% w/w)/ Imwitor (50%w/w) and Myritol 318/ Tween 85 (64% w/w)/ Transcutol P (20%w/w)/ Imwitor (16% w/w) were selected for further characterisation. Cremophor EL pseudoternary systems were characterised across the 7:3 and 5:5 DLs, whilst Tween 85 pseudoternary systems were characterised across the 4:6 and 2:8 DLs.
Figure 3.9: Pseudoternary phase diagram of selected microemulsion systems.
A: IPM/Water/Cremophor EL (80% w/w)/Imwitor 308 (20% w/w); B: IPM/Water/Cremophor EL (67% w/w)/Imwitor 308 (33% w/w); C: IPM/Water/Cremophor EL (50% w/w)/Imwitor 308 (50% w/w); D: Myritol 318/Water/Tween 85 (80% w/w)/Imwitor 308 (20% w/w); E: Myritol 318/Water/Tween 85 (67% w/w)/Imwitor 308 (33% w/w); F: Myritol 318/Water/Tween 85 (50% w/w)/Imwitor 308 (50% w/w); G: Myritol 318/Water/Tween 85 (50% w/w)/Imwitor 308 (16% w/w), Transcutol P (20% w/w); H: Myritol 318/Water/Tween 85 (54% w/w)/Imwitor 308 (13% w/w), Transcutol P (33% w/w); I: Myritol 318/Water/Tween 85 (40% w/w)/Imwitor 308 (10% w/w), Transcutol P (50% w/w); W: Water, O: Oil, SCo/S: Surfactant/cosurfactant mixture.
Table 3.2: The percentage of different phases as a function of the total area of the phase diagram

<table>
<thead>
<tr>
<th>System</th>
<th>Microemulsion Area (% w/w)</th>
<th>Liquid Crystal Area (% w/w)</th>
<th>Coarse Emulsion Area (% w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>36.11</td>
<td>19.86</td>
<td>0</td>
</tr>
<tr>
<td>B</td>
<td>49.17</td>
<td>17.22</td>
<td>6.90</td>
</tr>
<tr>
<td>C</td>
<td>64.15</td>
<td>12.60</td>
<td>4.55</td>
</tr>
<tr>
<td>D</td>
<td>20.78</td>
<td>28.65</td>
<td>0</td>
</tr>
<tr>
<td>E</td>
<td>30.56</td>
<td>4.65</td>
<td>0</td>
</tr>
<tr>
<td>F</td>
<td>27.87</td>
<td>0</td>
<td>15.59</td>
</tr>
<tr>
<td>G</td>
<td>40.38</td>
<td>5.7</td>
<td>0</td>
</tr>
<tr>
<td>H</td>
<td>24.55</td>
<td>0</td>
<td>9.11</td>
</tr>
<tr>
<td>I</td>
<td>6.82</td>
<td>0</td>
<td>2.50</td>
</tr>
</tbody>
</table>

3.4.3 Electrical Conductivity Measurements

Figure 3.10 is a representative electrical conductivity (σ) versus φ plot for the Cremophor EL system (7:3 DL). The curve can be divided into three distinct parts. Initially the electrical conductivity is very low (< 5 µs/cm\(^{-1}\)) and increases slowly as the φ increases up to about 10% (w/w). This is followed by a steep increase in the conductivity up to 40% (w/w) water. Above this, the conductivity of the system increases slightly and reaches a plateau. In this chapter, \(\phi_C\) was determined as the maximum change (dσ/dφ) in the electrical conductivity (dσ) relative to changes in water weight fraction (dφ). As shown by the blue lines in Figure 3.11, \(\phi_C\) was observed around 21-31% (w/w) water in selected Tween 85 and Cremophor EL systems. At this point, the w/o microemulsions are transformed into bicontinuous or o/w microemulsions.

For the 7:3 DL of the Cremophor EL pseudoternary system, \(\phi_C\) was approximately 23% (w/w). This value increased to 31% (w/w) when the surfactant concentration was increased to 50% (5:5 DL). The Tween 85 microemulsions behaved similarly to the Cremophor EL, with the system undergoing percolation transition around 21% (w/w) and 25% (w/w) water at 4:6 and 2:8 DL, respectively. Such an increase in the \(\phi_C\) may be attributed to greater interaction of water molecules with the ethylene oxide head group (increase hydration of ethylene oxide groups) as the surfactant concentration is increased. In effect, it has been found that \(\phi_C\) is dependent on the
interaction of water molecules with each other and with the surfactant head group. This in turn
will affect the probability of forming water clusters or continuous channels [308]. Lower values
of $\varphi_C$ can be the direct result of greater interaction of water molecules with each other in the
system [333].

![Graph showing variation in electrical conductivity (σ) with φ (expressed as %w/w) along the 7:3 DL of Cremophor EL pseudoternary system.]

**Figure 3.10:** Variation in the electrical conductivity (σ) with φ (expressed as %w/w) along the 7:3 DL of Cremophor EL pseudoternary system.

### 3.4.4 Viscosity Measurements

The dynamic viscosity was measured for all systems as a function of φ. All microemulsion
samples showed Newtonian flow. For the Cremophor EL pseudoternary system the viscosity
ranged from 18-34 mPa.s across the 7:3 DL, and increased by a factor of two as the ratio of
surfactant to oil increased (Table 3.3). Similar trends were observed for the Tween 85
pseudoternary system, although the viscosities were much higher as compared to the Cremophor
EL system.
Table 3.3: Dynamic viscosity values for pseudoternary systems at 0% water (SMEDDS) and at $\phi_C$ (microemulsion)

<table>
<thead>
<tr>
<th>Dilution line (DL)</th>
<th>0% water (SMEDDS)</th>
<th>$\phi_C$ (microemulsion)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cremophor EL (7:3)</td>
<td>18.09 ± 0.73</td>
<td>34.87 ± 1.76</td>
</tr>
<tr>
<td>Cremophor EL (5:5)</td>
<td>36.93 ± 0.03</td>
<td>61.48 ± 0.01</td>
</tr>
<tr>
<td>Tween 85 (4:6)</td>
<td>68.01 ± 0.01</td>
<td>104.72 ± 3.60</td>
</tr>
<tr>
<td>Tween 85 (2:8)</td>
<td>80 ± 0.01</td>
<td>104.20 ± 1.57</td>
</tr>
</tbody>
</table>

The dynamic viscosity versus $\phi$ plot for the Cremophor EL and Tween 80 pseudoternary systems are shown in Figure 3.11. The plot can be divided into two parts. In Figure 3.11 A (Cremophor EL 7:3 DL) the viscosity increases from 18 mPa.s to a maximum of 34 mPa.s as the $\phi$ rises to 20% (w/w). This is followed by a gradual decrease in the dynamic viscosity as the $\phi$ further increases. The existence of a maximum at 20% (w/w) water is indicative of the presence of two possible colloidal structures, namely, the transition of oil continuous microemulsion to bicontinuous or water continuous form. The initial increase of viscosity represents the aggregation of monomeric surfactant molecules and the subsequent presence of droplets within the core of the system. Below 20% (w/w) the attractive interaction and molecular reorganisation of droplets at the interface will dominate. Above 20% (w/w) the viscosity changes are gradual, a behaviour that is typically reported for bicontinuous systems [301, 308, 334].

The above changes in the viscosity were observed for 7:3 and 5:5 DLs of the Cremophor EL pseudoternary system, as well as 4:6 and 2:8 DLs of the Tween 85 pseudoternary system, and were in agreement with the conductivity data. These data are summarised in Table 3.4. For the Tween 85 system, (2:8 DL) further increase in the viscosity was observed above 40% (w/w) water. This might indicate the presence of low concentration of liquid crystals that were not detected with polarised light microscopy. However, the viscosity results showed Newtonian flow behaviour. Alternatively, the bicontinuous microemulsions may have transformed into o/w microemulsions. The high viscosity might be due to the increased volume faction of the dispersed phase (oil).
A: Cremophor EL 7:3 DL

B: Cremophor EL 5:5 DL

C: Tween 85 4:6 DL

D: Tween 85 2:8 DL

**Figure 3.11:** Variation in dσ/dφ and dynamic viscosity (η) as the function of φ (expressed as %w/w).

The arrow points to the value of φ_c. Below φ_c, w/o microemulsions are formed. Above this value, bicontinuous or o/w microemulsions are present. dσ/dφ is the change in the electrical conductivity (dσ) relative to changes in water weight fraction (dφ). The results are presented as mean ± SD, n=3. ME: microemulsion.
3.4.5 Dynamic Light Scattering (DLS) Studies

DLS was only effective for 7:3 DL of the Cremophor EL and 4:6 DL of the Tween 85 system, for which a single monodispersed peak was obtained. At higher surfactant concentrations, DLS showed polymodal distribution with polydispersity of >0.50. As these concentrated microemulsions were diluted, a single monodispersed peak started to emerge again. The observation of a monomodal distribution corresponded with the onset of percolation observed in the viscosity and conductivity measurements (Table 3.4). Overall, clear changes in the average droplet size were observed for 7:3 DL of the Cremophor EL system and 4:6 DL of the Tween 85 system.

Figure 3.12 shows the variation in the droplet size as a function of φ for the Tween 85 (4:6 DL) and the Cremophor EL (7:3 DL) pseudoternary systems. In general, the droplet size was small, ranging from 1.5 nm to <12 nm. This size range is in line with previously published data [335-337]. The Tween 85 system displayed the characteristic swelling profile observed when the φ of the dispersed phase (water) is increased (Figure 3.12). The droplet size increased by 47% (w/w) to 3.5 nm as the φ increased to 15% (w/w). This was followed by a gradual decrease in size. Conversely, for the Cremophor EL system, a decrease in the droplet size was observed for up to 15% water after which the size remained constant. With increase in the water content, larger globules or assemblies of molecules that are very close to each other may form. This was the case for the Tween 85 system. For the Cremophor EL system, it is possible that some of the added water dissolved in the surfactant head group. A decrease in the particle size may also suggest an increased number of the droplets, rather than their size with increased φ [323].
Figure 3.12: Variation in size as a function of φ (expressed as % w/w).
Results are presented as mean± SD, n=3.

Table 3.4: Summary of the φ values (expressed as % w/w) at which the transition from w/o microemulsion to bicontinuous or o/w microemulsion was observed

<table>
<thead>
<tr>
<th>Surfactant</th>
<th>Dilution Line (DL)</th>
<th>Electrical Conductivity</th>
<th>Viscosity</th>
<th>DLS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cremophor EL</td>
<td>7:3</td>
<td>23% w/w</td>
<td>20% w/w</td>
<td>15% w/w</td>
</tr>
<tr>
<td>Cremophor EL</td>
<td>5:5</td>
<td>31% w/w</td>
<td>30% w/w</td>
<td>30% w/w</td>
</tr>
<tr>
<td>Tween 85</td>
<td>4:6</td>
<td>21% w/w</td>
<td>15% w/w</td>
<td>15% w/w</td>
</tr>
<tr>
<td>Tween 85</td>
<td>2:8</td>
<td>24% w/w</td>
<td>25% w/w</td>
<td>35% w/w</td>
</tr>
</tbody>
</table>

Note that the values shown in the table were obtained by the following techniques: electrical conductivity, viscosity and DLS measurements.
### 3.4.6 Cloud point ($T_{\text{Cloud}}$) Determination

Temperature dependent phase behaviour is one of the major problems associated with microemulsions, especially when dealing with non-ionic surfactants. An ideal formulation should remain as a one-phase transparent system at its storage temperature and at the temperature of its intended use.

$T_{\text{Cloud}}$ was measured at a constant $\phi$ (30% (w/w) water) and at a constant weight ratio of oil to surfactant across the DLs listed in Table 3.4. In general, $T_{\text{Cloud}}$ ranged from 20°C for pseudoternary systems with high percentage of water to > 100°C for pseudoternary systems with lower water content. $T_{\text{Cloud}}$ was inversely related to water content of the sample, increasing exponentially as the $\phi$ decreased. Instability or phase separation of samples observed at $T_{\text{Cloud}}$ can be related to the temperature dependent solubility of the surfactant molecules [317, 318]. Increase in the temperature may result in a decrease in the solubility of the surfactant in water, the dehydration of the head group and phase separation.

The Tween 85 pseudoternary system (at the 4:6 DL) had a slightly higher $T_{\text{Cloud}}$ compared to the Cremophor EL pseudoternary system (Figure 3.13 A). This is noticeable in w/o microemulsions at a lower water concentration and can be attributed to the greater concentration of the Tween 85, which results in increased binding of the water molecules to the polar head groups. Moreover, the amount and the molecular volume, of the oil phase may contribute to the temperature sensitivity of the microemulsions. For example, it was observed that larger molecular volume oils tend to have a higher $T_{\text{Cloud}}$ compared to smaller volume oils for o/w microemulsions [338]. Smaller molecular oils may penetrate into the interface, behaving as cosurfactant, and thus resulting in a more asymmetrical structure with lower $T_{\text{Cloud}}$. Although this explanation was applied to o/w systems, the results of the current investigation shows that w/o Cremophor EL microemulsions containing the lower molecular volume oil (IPM) have also a lower $T_{\text{Cloud}}$. However, it is acknowledged that the oil and surfactant composition of these two pseudoternary systems are variable and a direct comparison of the effect of the oil and surfactant phase is difficult.

$T_{\text{Cloud}}$ was also measured at a constant weight fraction of water (30% w/w), whilst changing the ratio of oil to the surfactant mixture (Figure 3.13 B). For the Cremophor EL pseudoternary
system, \( T_{\text{Cloud}} \) decreased up to 25% oil above which it was constant. Conversely, the Tween 85 system showed an initial decrease in \( T_{\text{Cloud}} \) for up to 20% oil, above which it increased with the oil concentration. These findings may be due to the stronger interaction of water molecules with the surfactant head group at higher fraction of surfactant and possible changes in droplet shape. Overall, the Tween 85 and Cremophor EL pseudoternary systems containing up to 35% and 25% water respectively were stable at 32°C.
Figure 3.13: Variation in the $T_{\text{cloud}}$ as a function of A: $\varphi$, B: volume fraction of the oil phase.

In Figure 3.13 A, $T_{\text{cloud}}$ is measured at a constant weight ratio of oil to surfactant, whilst changing the $\varphi$. In Figure 3.13 B, $T_{\text{cloud}}$ is measured at a constant $\varphi$ (30% w/w) whilst changing the weight ratio of oil to surfactant. The arrows mark the regions of the phase diagram investigated.
3.4.7 Fourier Transform Infrared Spectroscopy (FTIR) Studies

Table 3.5 represents the assignment of the most predominant peaks arising from various functional groups observed in the FTIR spectrum of Cremophor EL and Tween 85 microemulsions. The OH spectrum of water was observed at 1635 cm\(^{-1}\) due to the bending of water molecules, and at 3000-3400 cm\(^{-1}\) due to the symmetric and asymmetric vibrational modes [339]. Intense peaks were also observed at a frequency of 2800-3000 cm\(^{-1}\) arising from the asymmetrical and symmetrical CH stretching vibrations [324]. Upon the solubilisation of water into the oil/surfactant mixture a noticeable shift in the frequency of the OH stretching (3000-3400 cm\(^{-1}\)) was observed. In general, the intensity of the OH stretching increased and the peaks shifted to a higher frequency. Moreover, the total area of the OH stretching increased linearly (\(R^2 > 0.990\)) with increase in the water content.

**Table 3.5:** Infrared frequencies of common functional groups present in the Tween 85 and Cremophor EL microemulsions

<table>
<thead>
<tr>
<th>Frequency (cm(^{-1}))</th>
<th>Functional group</th>
</tr>
</thead>
<tbody>
<tr>
<td>3000-3800</td>
<td>OH stretching</td>
</tr>
<tr>
<td>2923</td>
<td>CH stretching</td>
</tr>
<tr>
<td>2853</td>
<td>CH Stretching</td>
</tr>
<tr>
<td>1733</td>
<td>CO Stretching</td>
</tr>
<tr>
<td>1650</td>
<td>OH stretching</td>
</tr>
<tr>
<td>1466</td>
<td>CH stretching</td>
</tr>
</tbody>
</table>

Figure 3.14 shows the percentage of each type of water as a function of total water content within the Cremophor EL microemulsion (7:3 DL). Initially the percentage of bound water was large compared to the free water or trapped water. These water molecules are likely to bind to the head group of the surfactant. As the \(\varphi\) is increased, the surfactant head group starts to become saturated. This leads to the interaction of water molecules with each other, which can result in the formation of aggregates and eventually bicontinuous water channels. This corresponds to an increase in the percentage of free water and a decrease in the percentage of bound water as shown in Figure 3.14. On the other hand, the percentage of trapped water did not change with changes in the microemulsion structure and contributed to only 10% of the total water content.
This is in line with previously reported data [324]. At 50% water, the contribution from each type of water is similar to that of pure water, with free water molecules dominating amongst the three types of water. Moreover, a downshift in the OH stretching frequency of free water component was observed. Increasing the free water molecule in the system will minimise the oscillation of the OH stretching (due to increased hydrogen bonding) leading to a downshift in the OH frequency. A similar trend was observed for the 4:6 DL of the Tween 85 system.

![Graph showing the percentage area of free water, bound water, and trapped water as a function of ϕ (%w/w) across the 7:3 DL of the Cremophor EL pseudoternary system.]

**Figure 3.14:** Percentage area of free water, bound water and trapped water as a function of ϕ (expressed as %w/w) across the 7:3 DL of the Cremophor EL pseudoternary system.

Free water represents water molecules interacting with each other, bound water represents the water molecules that are interacting with the surfactants head group, and trapped water represents the non-hydrogen bonded monomers trapped between the surfactant chains. The total water content is the sum of free water, bound water and trapped water.

### 3.5 Conclusion

In this chapter, novel Imwitor 308 based SMEDDS were developed and investigated as potential transdermal delivery matrix for progesterone. The aim was to identify a SMEDDS that can
remain as a single transparent o/w microemulsion when diluted with water and at skin temperature. Preliminary screening of oils and surfactants demonstrated that IPM/Cremophor EL (50% w/w)/Imwitor (50% w/w) and Myrtil 318/Tween 85 (64% w/w)/Transcutol P (20% w/w)/Imwitor (16% w/w) SMEDDS were capable of forming microemulsions when diluted with water. The resulting pseudoternary systems were shown to undergo phase transition (from w/o to bicontinuous or o/w microemulsion) at about 15-35% water. This was further supported by the droplet size analysis and the FTIR measurements. FTIR data showed that the percentage of bound water will dominate below $\phi_C$ where w/o microemulsions are present. Furthermore, the selected SMEDDS were found to be stable at skin temperature (32ºC) when diluted with up to 30% water. When applied to the skin (under occlusion), these systems have the potential to absorb water and form a microemulsion. Dilution with water can reduce the saturation solubility of the drug, leading to a supersaturated solution with higher thermodynamic activity. Such a formulation can remain stable upon storage, yet offer enhanced transdermal drug flux through the skin. This hypothesis was tested as described in the next chapter.
4  

**In Vitro Permeation Studies**

4.1 Introduction

The previous chapter examined the phase behaviour of SMEDDS upon dilution with water. It is hypothesised that these systems will form microemulsions upon contact with the surface of the skin. When saturated, absorption of water from the skin surface may result in the formation of a transient supersaturated system with a high thermodynamic activity and potentially a high permeation rate. Additionally, some of the oils and surfactants present in the SMEDDS may act as penetration enhancers, further increasing the transdermal flux. Transdermal permeation enhancement across the skin can be assessed through:

- evaluation of the skin barrier properties;
- quantification of the amount of the drug permeated through the skin;
- assessment of the penetration depth and penetration pathways;
- evaluation of the skin structure; and
- assessment of conformational changes in the skin lipids.

Attenuated Total Reflectance (ATR)-FTIR measures drug diffusion through the SC by monitoring the drug specific absorption intensities. This technique allows for real time assessment of the permeation rate in intact human skin [340] as well as excised skin from human or animals. Generally, a special setup whereby the conventional transmission cell is replaced by an ATR crystal is required. Moreover, the technique is only limited to IR active compounds [139]. Alternatively, the permeant concentration in SC layers removed by tape stripping can be measured. By knowing the concentration in the uppermost layer of the SC and the thickness of the removed layers, the diffusion rate across the SC is calculated. This technique is commonly used for real time assessment of the diffusion coefficient and the penetration depth in human volunteers [341]. The nature of the adhesion, the packing of the
SC cells, skin hydration, and inter-individual and regional skin variability are amongst the factors that can affect the quantity of the SC removed. Similar to ATR-FTIR, the permeation rate is only assessed within the SC.

The diffusion cell model is widely used for measuring passive skin permeation. In the standard diffusion cell, shown in Figure 4.1, the skin is mounted between a donor and a receiver compartment with the formulation applied to the surface of the skin. At specific time intervals, samples from the receiver solution are removed and analysed for drug content. Using this model the permeation rate through heat-separated epidermis, split thickness skin (200-400 µm) and full thickness skin can be rapidly and reliably measured. The permeation rate of various hydrophilic and lipophilic permeants across split thickness porcine skin was shown to correlate well with data obtained from human skin [342]. In general, for polar permeants, both full thickness and split thickness skin are used. In contrast, heat separated epidermis or split thickness skin are used for highly lipophilic permeants (logP >3-4) as the lack of blood supply to the dermis can create a rate limiting step for their permeation [139]. According to the Organisation for Economic and Cooperation and Development (OECD) full thickness skin can be used, although thickness beyond 1 mm should be avoided [343]. Maintenance of sink condition⁴ is one of the major limitations of the diffusion cell model. For hydrophilic drugs, skin conditions can be maintained by using phosphate buffer as the receptor fluid. For lipophilic compounds, addition of cosolvents or surfactants such as ethanol (30%) [151], polyethylene glycol 20 oleyl ether (6%) [344], γ-CD (0.1%) [345], and bovine serum albumin [345] is necessary to ensure sink conditions. However, the integrity of the skin could be compromised by back diffusion of the solvent into the skin. This problem can be avoided by using viscous solvents. Propylene glycol has been successfully used to study the transdermal permeation of progesterone [187, 346, 347].

Barrier properties of the skin can be monitored through a wide range of techniques including TEWL [348], electrical conductivity [349], electrical resistance [350] and impedance spectroscopy [351]. Generally, these techniques are based on monitoring the rate of water loss or monitoring the changes in the skin electrical resistance before and after applying the test formulation. A disrupt barrier allows for rapid evaporation of water from the skin surface and a lower electrical resistance due to free passage of conducting ions across the skin.

---

⁴Keeping the concentration of the drug in the receiver medium below 10% of its saturation solubility
Monitoring of cutaneous blood flow is often used to assess cutaneous skin irritation. However, Tanojo et al. successfully used this parameter to assess the penetration enhancing capacity of oleic acid [352].

![Schematic diagram of a Franz diffusion cell used to measure the transdermal permeation rate of a drug.](image)

**Figure 4.1:** Schematic diagram of a Franz diffusion cell used to measure the transdermal permeation rate of a drug.

Microscopic and spectroscopic studies can provide further information on the structure and arrangement of the SC lipids. Confocal laser scanning microscopy is used to study the pathways and depth of penetration of permeants through the SC. This technique is particularly useful for monitoring the permeation of intact liposomes through the skin. Confocal microscopy allows for the visualisation of skin layers without mechanically sectioning the tissue, although it is only limited to fluorescence tagged permeants [353]. Conversely, histological examination of the skin, scanning (SEM) and transmission (TEM) electron microscopy allow for the visualisation and characterisation of skin surface in the micrometer range [181, 354]. In SEM and TEM, analysis of different types of electrons emitted from the sample allows for the evaluation of the surface topology, texture, morphology, and crystalline structure. Conventional SEM and TEM require fixation and dehydration of the sample prior to
Chemicals such as glutaraldehyde and osmium tetroxide are used for crosslinking and immobilization of the SC proteins and lipids [181, 354]. Controlled dehydration of the tissue is highly critical to maintain structural integrity and can be achieved in a two-step process [355]. Initially the water is replaced by graded series of organic solvents such as ethanol or acetone. This is followed by critical point drying in which the organic solvent in the tissue is replaced by liquid carbon dioxide. The liquid carbon dioxide is then heated to just above its critical temperature (31.1 °C) where the density of its gas and liquid phases are identical. This process prevents the formation of a high surface tension that may damage the delicate structures of the skin tissue [356].

In contrast to the aforementioned techniques DSC and FTIR, provide information on the changes in the structural arrangement of the SC lipids at a molecular level. DSC monitors the changes in the lipid and protein endothermic transition temperatures. The melting transitions observed in a DSC thermogram correspond to the melting of the SC lipids and the denaturation of skin proteins [357]. It has been reported that these melting transitions can vary significantly with the hydration level of the SC [358]. On the other hand, FTIR provides information on the vibrational modes of the skin lipids. There are two spectral regions in the FTIR spectrum, which are of particular interest. Bands due to the asymmetric and symmetric vibrations of carbon-hydrogen (CH) bonds are found in the region of 2920 cm$^{-1}$ and 2850 cm$^{-1}$ respectively [359]. Changes in the vibrational frequency of these bands provide information on the modes of action of penetration enhancers. At the physiological temperature, the SC lipids can exist as various crystalline, gel and liquid crystalline phases. In their lowest free energy level (gel phase), the alkyl backbone arrange in a All-Trans conformation (Figure 4.2) such that the C-C bonds are maximally apart [360]. The tightly packed lipid bilayers restrict the vibrational motion of the CH bonds. In the presence of thermal or chemical stimuli, the lipid backbone will adopt a gauche conformation (Figure 4.2), which is associated with a higher energy. The atoms in these non-linear conformers are less tightly packed such that the CH bonds can vibrate freely. Consequently, the CH vibrational frequency shifts to a higher wavenumber. When the SC lipids fluidize the gel phases are converted to liquid crystal phases. A decrease in the CH vibrational frequency is observed when the SC lipids are extracted [361]. The bands in the region of ~1650 cm$^{-1}$ and ~1550 cm$^{-1}$ correspond to amide I (CONH$_2$ group) and amide II (CONHR group) stretching of the SC proteins respectively [362]. The intensity ratio of these bands can be used to study the hydration level of the SC.
FTIR allows for real time analysis of the skin in human volunteers as well as full thickness skin or SC excised from human or animals.

![Figure 4.2: Schematic representation of All-Trans and Gauche conformation in an alkyl chain.](image)

In the All-Trans conformation, the CH$_3$ groups are 180° apart. In the Gauche conformation, the CH$_3$ groups are 60° apart.

Although human skin is ideal for evaluation of transdermal permeation, it is often substituted with skin from alternative species such as mice, rabbit, rat and pig as adequate supply may not be available. However, mammalian skin varies widely in thickness, skin appendages and hair follicles density. In general, the permeability of drugs across the mammalian skin is in the following order: mice > rabbit > rat > porcine > human [363, 364]. Mice, rat and rabbit skins have a large number of appendages but no sweat glands [133]. Higher permeability in mice is attributed to the thickness of its SC which is half the thickness of the human SC [365]. Porcine skin has been found to be an excellent surrogate of human skin despite having a slightly thicker SC (~21 µm). A linear correlation has been observed in the permeation data obtained from porcine and human skin ($R^2 = 0.84$). This correlation was further improved when porcine skin was substituted with guinea pig skin ($R^2 = 0.96$). With respect to the lag time, a significant correlation was only observed between human and guinea pig skin [366]. Reconstructed skin is another possible alternative to human skin, although current models are a poor representation of human skin. In one study the transdermal flux from reconstructed skin was 900 times higher than the flux from human skin [367]. Typically, fresh skin is preferred for the diffusion studies, although frozen skin can also be used. The diffusion rate across mouse, pig, rat, rabbit and guinea pig skin is not affected when the skin is used within 6 month of its storage at -20°C. Human skin can be stored at -20°C for up to 12 month [368].

In this chapter, the Franz cell diffusion system was used to measure the permeation of
progesterone from selected formulations across porcine skin. FTIR in combination with SEM and histological examination of the skin tissue are used to elucidate the mechanism of action of SMEDDS.

4.2 Chapter Aim and Objectives

The aim of the current chapter is to investigate the potential of SMEDDS as a matrix for transdermal delivery of progesterone.

The specific objective of the current chapter is to:

- measure the permeation rate of progesterone from SEMDDS, microemulsions, aqueous solution and commercially available creams, and;
- investigate the mechanism of action of SMEDDS.

4.3 Materials and Methods

4.3.1 Materials

IPM, Tween 85, glutaraldehyde and osmium tetroxide were purchased from Sigma-Aldrich (USA). Myritol 318 was obtained from Cognis Care Chemicals (Germany). Transcutol P was kindly donated by Gatcefosé (France). Imwitor 308 was obtained from Sasol (Germany). Cremophor EL was supplied by BAFS (Germany). Disodium hydrogen phosphate (Na$_2$HPO$_4$), potassium dihydrogen phosphate (KH$_2$PO$_4$) and potassium chloride (KCL) were purchased from Scharlau Chemie S.A (Spain). NaCL and absolute ethanol were from ECP LTD (New Zealand). Acetonitrile and methanol were purchased from Merck KGaA (Germany). Pro-Gest was obtained from Emerita (USA), PCNZ cream was supplied by Pharmaceutical Compounding New Zealand, Pro-Fem was from BioZone Laboratories (USA), Serenity cream was from the Health and Science Institute (USA) and Oasis Serene was from Oasis Advanced Wellness (USA). All samples were used without any further purification. Triple-distilled water was obtained in-house by reverse osmosis (MilliQ, Millipore, USA).
4.3.2 Preparation of SMEDDS and Microemulsions

The composition of the formulations evaluated is listed in Table 4.1. SMEDDS were prepared by mixing predetermined amount of the oil component with the surfactant mixture. To this mixture, water was added drop by drop under constant magnetic stirring to formulate a microemulsion. Excess amount of progesterone was then added to each sample to create a saturated formulation with excess drug crystals. Supersaturated microemulsions (SSME) were formulated by adding the required amount of water (previously maintained at 32ºC) to the saturated SMEDDS. The water was added either immediately prior to the permeation experiment (SSME B) or gradually over the duration of 12 h (SSME A). All of the formulations were equilibrated at 32ºC in a shaking water bath for 72 h prior to the diffusion study. Saturated solution of water was used as a control.

4.3.3 Preparation of Skin Tissue

*In vitro* permeation studies were carried out using full thickness porcine skin. Fresh skin was obtained from a local abattoir. The underlying fat and cartilage were carefully removed using a size 20 scalpel. The skin was then cut into 16 cm² sections. The sections were wrapped in an aluminium foil and stored at -20ºC for no longer than 1 month.

4.3.4 Evaluation of Skin Integrity

Initially the skin sections were carefully assessed for any visible wholes or damage. The skin was then mounted on the diffusion cells with the SC side facing up. Two millilitres of MilliQ water was added to the donor compartment. The permeation of water to the empty receiver compartment was then monitored for 1 h. Skin samples that did not allow the water to permeate during this time were selected for *in vitro* permeation studies.
**Table 4.1:** Composition of different SMEDDS and microemulsions tested

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Code</th>
<th>IPM*</th>
<th>Myritol 318*</th>
<th>Cremophor EL*</th>
<th>Tween 85*</th>
<th>Imwitor 308*</th>
<th>Transcutol P*</th>
<th>Water*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cremophor EL SMEDDS (7:3 DL)</td>
<td>A</td>
<td>70</td>
<td>-</td>
<td>15</td>
<td>-</td>
<td>15</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cremophor EL SMEDDS (5:5 DL)</td>
<td>B</td>
<td>50</td>
<td>-</td>
<td>25</td>
<td>-</td>
<td>25</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cremophor EL SMEDDS (3:7 DL)</td>
<td>C</td>
<td>20</td>
<td>-</td>
<td>35</td>
<td>-</td>
<td>35</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Tween 85 SMEDDS (4:6 DL)</td>
<td>D</td>
<td>-</td>
<td>40</td>
<td>-</td>
<td>38.4</td>
<td>9.6</td>
<td>12</td>
<td>-</td>
</tr>
<tr>
<td>Tween 85 SMEDDS (2:8 DL)</td>
<td>E</td>
<td>-</td>
<td>20</td>
<td>-</td>
<td>51.2</td>
<td>12.8</td>
<td>16</td>
<td>-</td>
</tr>
<tr>
<td>Tween 85 ME (4:6 DL)</td>
<td>F</td>
<td>-</td>
<td>32</td>
<td>-</td>
<td>30.72</td>
<td>7.68</td>
<td>9.60</td>
<td>20</td>
</tr>
<tr>
<td>Cremophor EL ME (7:3 DL)</td>
<td>G</td>
<td>56</td>
<td>12</td>
<td>-</td>
<td>12</td>
<td>-</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>Cremophor EL SSME (B)</td>
<td>H</td>
<td>56</td>
<td>-</td>
<td>12</td>
<td>-</td>
<td>12</td>
<td>-</td>
<td>20</td>
</tr>
<tr>
<td>Tween 85 SSME (A)</td>
<td>I</td>
<td>-</td>
<td>32</td>
<td>-</td>
<td>30.72</td>
<td>7.68</td>
<td>9.60</td>
<td>20</td>
</tr>
<tr>
<td>Tween 85 SSME (B)</td>
<td>J</td>
<td>-</td>
<td>32</td>
<td>-</td>
<td>30.72</td>
<td>7.68</td>
<td>9.60</td>
<td>20</td>
</tr>
</tbody>
</table>

*data are presented as %w/w, ME: Microemulsion, SSME: Supersaturated Microemulsion
4.3.5 *In Vitro* Skin Permeation Studies

*In vitro* permeation studies were carried out using Franz Cell FDC-6 diffusion instrument (Logan Instrument, USA) fitted with a re-circulating water bath and water-jacketed glass cells. Each cell has an effective diffusion surface area of 1.7 cm$^2$ and a 12 ml cell volume. The receptor compartment was filled with 40% (w/w) propylene glycol, and stirred continuously at 600 rpm. This composition was selected to ensure sink conditions. The temperature of the water bath was adjusted to $37^\circ$C ± $0.5^\circ$C to maintain a skin surface temperature of $32^\circ$C. The skin samples were clamped between the donor and the receiver chamber of the diffusion cell. The samples were equilibrated with the receptor solution for 12 h prior to the diffusion experiment. Two millilitres of each formulation was applied to the surface of the skin, which was then covered with parafilm. At predetermined time intervals samples (200 µl) were withdrawn from the receptor chamber and replaced with fresh medium. The samples were then diluted and analyzed by HPLC. Each formulation was evaluated in triplicate.

At the end of the study, the skin samples were removed from the diffusion cell and carefully wiped with a cotton bud soaked in ethanol. The skin sections were then cut and added to 2 ml of methanol, which was placed in a shaking water bath for at least 24 h. At the end of this time interval, the samples were centrifuged at 1300 rpm for 10 min. The supernatant was then removed and analyzed by HPLC for progesterone content.

4.3.6 Calculation of *In Vitro* Data

The cumulative amount ($Q_t$) of progesterone permeated through excised porcine skin per unit of area was calculated from the following equation:

\[
Q_t = \frac{V_r C_t}{V_n C_n} S A
\]

Equation 4.1

where $V_r$ is the volume of the receptor solution (12 ml), $C_t$ is the drug concentration in the receptor solution at each sampling time, $V_n$ and $C_n$ are the volume and the concentration of the $n$th sample withdrawn and $S A$ is the effective diffusion surface area (1.7 cm$^2$). The cumulative amount of progesterone permeated was plotted as a function of time (h).
Regression analysis was performed on the linear section of this plot. The steady state flux \( J_{ss} \) was then calculated using the equation:

\[
\text{Equation 4.2}
\]

where \( \Delta Q_t/\Delta t \) is the slope of the linear regression equation. The intercept of the linear regression equation was used as a measure of the lag time. The relative increase in the transdermal permeation rate was expressed as the Enhancement Factor (EF), which was calculated by the following equation:

\[
\text{Equation 4.3}
\]

Where \( J_{ss} \text{ sample} \) is the steady state flux from the tested formulations and \( J_{ss} \text{ control} \) is the steady state flux from the control (saturated aqueous solution).

### 4.3.7 Saturation Solubility Studies

Saturation solubility of progesterone was measured in SMEDDS, microemulsions and SSME systems containing 0-60% water (w/w). The formulations were prepared as described in section 4.3.2. An excess amount of progesterone was added to 2 ml of each formulation. Samples were shaken at 25\(^\circ\)C or 37\(^\circ\)C for 72 h. The supernatant was then filtered through a 0.45 \( \mu \text{m} \) membrane filter and the concentration of progesterone was determined by HPLC. The solubility phase diagram was constructed by plotting the saturation solubility of microemulsions and SSME against the \( \phi \) added. The degree of saturation (DS) was calculated according to the following equation:

\[
\text{Equation 4.4}
\]

where \([\text{SSME}]\) is the concentration of progesterone in the SSME immediately after the addition of water and \([\text{ME}]\) is the saturation solubility of progesterone in the microemulsions.
4.3.8 Evaluating the Water Content of Microemulsions

The FTIR spectrum of SMEDDS and microemulsions were used to quantify the percentage of water absorbed into the SMEDDS. The FTIR spectrum of microemulsions containing 0-10% water was recorded as described in section 3.3.8. From these the AUC of the OH spectrum of water in the region of ~3400 cm⁻¹ was calculated. The AUC of the OH spectrum of SMEDDS was subsequently subtracted from the spectrum of microemulsions. The standard curve was obtained by plotting the resulting areas against φ. Linearity, accuracy, and precision were used to validate the method. The AUC of the samples removed at the end of the diffusion study was calculated using the same procedure. The regression equation obtained from the standard curve was then used to calculate the percentage of water absorbed into the SMEDDS. The calibration standards were measured in triplicate. All other samples were measured at least 6 times.

4.3.9 Histological Examination of the Skin

Cremophor EL and Tween 85 SMEDDS were applied on excised porcine skin, mounted on the diffusion cell at 37°C for 24 h. The skin was then removed, rinsed with phosphate buffer (pH 7.4) and fixed in 10% v/v buffered formalin for 24 h followed by 70% v/v ethanol for a further 24 h. Following this, the skin tissue was embedded in paraffin blocks and vertically cut into 5 µm thick sections. The sections were stained with Hematoxylin–Eosin (H&E) and examined under the light microscope (Leica DMR, GmbH, Germany). Fresh skin and skin treated with water were used as controls. For each formulation, the study was carried out in triplicate.

4.3.10 Scanning Electron Microscopy (SEM)

Porcine skin was incubated with the selected formulations using the procedure discussed in section 4.3.9. Fresh skin or skin treated with MilliQ water were used as controls. The skin samples were fixed with 2.5% glutaraldehyde (in 0.1 M phosphate buffer, pH 7.4) for 4 h at room temperature. The samples were then washed in phosphate buffer at least three times (15...
min each), and fixed with 1% osmium tetroxide (in 0.1 M phosphate buffer, pH 7.4) for a further 2 h. This was followed by drying the samples in a graded series of ethanol solutions (50%, 70%, 90%, and 100%). The specimens were then further dried by critical point drying (Polaron, E3000 Series II critical point drier, Polaron Equipment Ltd, UK). The dry samples were mounted on a specimen stub using an electrically conductive double-sided adhesive tape and sputter coated with gold (Polaron SC 7640 sputter coater, Quorum Technologies Ltd, UK). The samples were examined with an electron microscope (Philips XL30S Field Emission Gun, Netherlands) fitted with a lithium drifted silicon electron-dispersive X ray spectrometer (EDS, USA) with a super ultra thin window.

4.3.11 FTIR Spectroscopy

Porcine skin was cut with a dermatome (Davies® Gold Series, DeSoutter Medical, USA) into 200 µm thick sections. The skin sections were immersed in 2 ml of selected SMEDDS for 24 h at room temperature. The sections were then rinsed with phosphate buffer (pH 7.4) to remove the excess formulation and blotted dry with a paper towel. The infrared spectrum of the samples was measured using the FTIR spectrometer described in section 3.3.8 with the SC side facing the diamond window. Each sample was scanned 32 times at a resolution of 4 cm\(^{-1}\). Fresh skin, and skin treated with MilliQ were used as controls.

4.3.12 Statistical Analysis

The statistical analysis of the permeation data was performed by the Student's \(t\)-test with \(p<0.05\) set as the level of significance. One way ANOVA was used to compare the shifts in the CH and amide bands of the FTIR spectrum. All data are reported as mean ±SD. The data were analysed using SPSS software (SPSS 16.0, USA) and Microsoft Excel 2007 (Microsoft® Office 2007, USA).
4.4 Results and Discussion

4.4.1 In Vitro Permeation Studies

The percutaneous permeation rate of progesterone from selected formulations including saturated SMEDDS, microemulsions, SSME, and selected progesterone creams are presented in Table 4.2. The corresponding permeation profiles are shown in Figure 4.3-Figure 4.4. The statistical analysis of the flux and the lag time are shown in Table 4.4 and Table 4.5, respectively. The permeation profiles are typical of a zero-order kinetic with a lag time, which is defined as the time required to attain a constant permeation rate across the skin. Comparison of the flux values shows that all Imwitor based SMEDDS resulted in a significantly higher flux (p<0.05) compared to the saturated aqueous solution of progesterone and the available commercial products. The Cremophor EL SMEDDS (7:3 DL) with the lowest surfactant concentration (30 %w/w) provided the highest transdermal flux (7.59±2.17 µg.cm⁻².h⁻¹). Nonetheless, the flux values of SMEDDS were not significantly different from each other. Significant differences in the percentage of progesterone permeated from Tween 85 SMEDDS were noted after 8 h. Overall, the permeation rate from the SMEDDS was in the following order EL 7:3 DL> EL 5:5 DL> EL 3:7 DL > Tween 85 4:6 DL> Tween 85 2:8 DL.

Figure 4.3 C and D compare the permeation profiles of SMEDDS with microemulsions and SSME containing 20% water. SMEDDS and microemulsions were saturated formulations containing excess progesterone crystals. Supersaturation (SSME) was achieved by adding the required amount of water to the saturated SMEDDS. The water was added either immediately prior to the permeation experiment (SSME B) or gradually over the duration of 12 h (SSME A). Saturated microemulsions significantly increased (P <0.05) the transdermal flux when compared to the aqueous solution. The flux from the Cremophor EL microemulsions and SSME was lower than the SMEDDS, although this difference was not statistically significant. The Tween 85 SMEDDS had a significantly higher flux compared to its corresponding microemulsion and SSME. The transdermal flux of the SSME was independent of the preparation method and did not increase beyond 3.19 µg.cm⁻².h⁻¹.

With respect to the lag time, most formulations produced a significantly shorter lag time compared to the control (Table 4.5). In general, for SMEDDS the lag time was inversely proportional to the transdermal flux. The shorter the lag time the greater the flux across the
skin. The shortest lag time of 1.7 h was observed for Cremophor EL SMEDDS (5:5 DL) with the transdermal flux of 6.33 µg.cm\(^{-2}\).h\(^{-1}\).

**Table 4.2:** *In vitro* permeation parameters of progesterone obtained from control and various Cremophor EL and Tween 85 based formulations

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Flux (µg.cm(^{-2}).h(^{-1}))</th>
<th>Lag Time (h)</th>
<th>Enhancement Factor</th>
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<tbody>
<tr>
<td>Control (saturated water)</td>
<td>1.15±0.43</td>
<td>5.47±2.78</td>
<td>1.00</td>
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<tr>
<td>Cremophor EL SMEDDS (7:3 DL)</td>
<td>7.59±2.17</td>
<td>3.4±0.53</td>
<td>6.60</td>
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<tr>
<td>Cremophor EL SMEDDS (5:5 DL)</td>
<td>6.33±1.27</td>
<td>1.70±0.64</td>
<td>5.50</td>
</tr>
<tr>
<td>Cremophor EL SMEDDS (3:7 DL)</td>
<td>5.18±1.30</td>
<td>1.97±0.47</td>
<td>4.50</td>
</tr>
<tr>
<td>Tween 85 SMEDDS (4:6 DL)</td>
<td>4.73±0.073</td>
<td>2.94±0.11</td>
<td>4.11</td>
</tr>
<tr>
<td>Tween 85 SMEDDS (2:8 DL)</td>
<td>4.08±0.58</td>
<td>3.77±0.76</td>
<td>3.52</td>
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<tr>
<td>Tween 85 Microemulsion (4:6 DL)</td>
<td>3.58±0.26</td>
<td>3.60±0.50</td>
<td>3.11</td>
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<tr>
<td>Cremophor EL Microemulsion (7:3 DL)</td>
<td>4.69±0.49</td>
<td>3.10±0.30</td>
<td>4.07</td>
</tr>
<tr>
<td>Cremophor EL SSME (A)</td>
<td>4.86±0.87</td>
<td>2.29±0.74</td>
<td>4.22</td>
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<tr>
<td>Tween 85 SSME (A)</td>
<td>3.12±0.87</td>
<td>2.95±1.07</td>
<td>2.70</td>
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<tr>
<td>Tween 85 SSME (B)</td>
<td>3.19±0.81</td>
<td>2.65±0.66</td>
<td>2.77</td>
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</table>

Results are presented as mean ± SD, n=3
Figure 4.3: Permeation profiles of progesterone through full thickness porcine skin. A: Cremophor EL SMEDDS, B: Tween 85 SMEDDS. Results are presented as mean ± SD, n=3.
Figure 4.3 (continued): Permeation profiles of progesterone through full thickness porcine skin.

C: Cremophor EL systems, D: Tween 85 systems. Results are presented as mean ± SD, n=3.
Figure 4.4 compares the permeation profiles of various commercially available transdermal progesterone creams. These formulations contain variable percentages of progesterone ranging from 1.6%–4% (w/w). Generally, the transdermal flux was independent of the progesterone concentration. The transdermal flux from PCNZ cream (4% w/w) was 3% lower than the Natural Women cream, which has a lower progesterone concentration. The transdermal flux from Serenity cream is also 57% lower than the Natural Women cream, despite having a higher progesterone concentration. Overall, the permeation rate from selected progesterone formulations was in the following order: Natural Women > Pro-Fem > PCNZ > Pro-Gest > Serenity. With the exception of Pro-Gest and Serenity all other creams resulted in significantly higher flux compared to the control.

Figure 4.4: Permeation profiles of progesterone from selected commercial creams. Results are presented as mean ± SD, n=4.
Chapter 4 - In Vitro Penetration Studies

**Table 4.3:** *In vitro* permeation parameters of progesterone obtained from various commercially available formulations.

<table>
<thead>
<tr>
<th>Product</th>
<th>Abbreviation</th>
<th>Progesterone Content (% w/w)</th>
<th>Flux (µg.cm(^{-2}).h(^{-1}))</th>
<th>Lag Time (h)</th>
<th>Enhancement Factor</th>
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<tr>
<td>Pro-Fem</td>
<td>PF</td>
<td>2.50</td>
<td>2.19±0.29</td>
<td>2.13±0.76</td>
<td>1.90</td>
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<tr>
<td>Pro-Gest</td>
<td>PG</td>
<td>1.60</td>
<td>1.30±0.59</td>
<td>1.69±1.04</td>
<td>1.13</td>
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<tr>
<td>PCNZ</td>
<td>PN</td>
<td>4.00</td>
<td>2.18±0.39</td>
<td>2.29±1.69</td>
<td>1.89</td>
</tr>
<tr>
<td>Serenity for Women</td>
<td>SW</td>
<td>2.10</td>
<td>0.96±0.26</td>
<td>1.71±1.57</td>
<td>0.83</td>
</tr>
<tr>
<td>Natural Women</td>
<td>NW</td>
<td>1.70</td>
<td>2.24±0.25</td>
<td>3.37±0.25</td>
<td>1.94</td>
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</table>

Results are presented as mean ± SD, n=4
**Table 4.4:** Statistical analysis of the transdermal flux data

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NS: Not Significant (p >0.05, Student's t-test), S: Significant (p <0.05, Student's t-test)
**Table 4.5: Statistical analysis of the lag time data**

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NS: Not Significant (p >0.05, Student's t-test), S: Significant (p <0.05, Student's t-test)
4.4.2 Evaluating the Water Content of Microemulsions

As described previously, it is hypothesised that SMEDDS can form SSME or microemulsions when in contact with the surface of the skin. FTIR spectroscopy was used to investigate this hypothesis. Initially, the method was validated in order to ensure that it is accurate and reliable. Equation 4.5 demonstrates the linearity of the method. The calibration curve was found to be linear over a concentration range of 0-10% water, with an $R^2$ of 0.9996.

Equation 4.5

The accuracy and precision of the results were obtained at three concentration levels (3, 5, 7% w/w). The calculated percentage of water absorbed was within 98%-103% of the actual $\varphi$ in the microemulsions with the RSD of <10% indicating that the method is accurate and reproducible.

Table 4.6 shows the percentage of water absorbed into the SMEDDS following their exposure to the skin surface for 24 h. The results illustrate that SSME or microemulsions can be formed when SMEDDS are exposed to the skin surface. However, the amount of water absorbed is low and highly variable. No noticeable differences could be observed between the amount of water absorbed into the Tween 85 and Cremophor EL SMEDDS. Overall, up to 11% water was absorbed into the formulations. Based on this and the characterisation results from the previous chapter, it can be concluded that SMEDDS can undergo transition into w/o microemulsions when applied to the skin surface under occlusion.
Chapter 4 - *In Vitro* Penetration Studies

**Table 4.6:** φ (expressed as %w/w) absorbed into the SMEDDS following their exposure to the excised porcine skin for 24 h

<table>
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<th>φ (Range)</th>
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<td>1.12-4.08</td>
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<td>Cremophor EL (5:5 DL)</td>
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<td>1.24-4.85</td>
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<td>Cremophor EL (3:7 DL)</td>
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<td>2.12-10.99</td>
<td>9</td>
</tr>
<tr>
<td>Tween 85 (4:6 DL)</td>
<td>3.88</td>
<td>1.49-5.79</td>
<td>10</td>
</tr>
<tr>
<td>Tween 85 (2:8 DL)</td>
<td>2.26</td>
<td>0.72-3.12</td>
<td>10</td>
</tr>
</tbody>
</table>

### 4.4.3 Saturation Solubility Studies

In section 4.4.2, FTIR spectroscopy was used to demonstrate that SMEDDS could form microemulsion when they are exposed to the surface of the skin. It is also important to evaluate the effect of water absorbed on the thermodynamic potential of the resulting microemulsions. The saturation solubility of progesterone (at 25°C) in Tween 85 SMEDDS (4:6 DL) is 42.25 mg/ml. When 20% water was added to this system, the solubility reduced exponentially to 24.05 mg/ml. Such change in the solubility demonstrates that SMEDDS have the potential to form supersaturated formulations with higher thermodynamic activity. To investigate this hypothesis, the saturation solubility of progesterone in Tween 85 (4:6 DL) and Cremophor EL (7:3 DL) SMEDDS, SSME, and microemulsions was measured.
Figure 4.5 is a representative plot of changes in the saturation solubility of progesterone with increase in $\varphi$ for the Tween 85 system (4:6 DL). The blue line represents the saturation solubility of progesterone in Tween 85 microemulsions containing 0-60% water. The red line represents the solubility in saturated SMEDDS that have been diluted with 0-60% water. The ratio of the two concentrations is the degree of saturation, (Figure 4.6). The degree of saturation of 1 indicates that saturated SMEDDS or microemulsions are formed. Values above 1 suggest that the thermodynamic activity has increased to beyond unity leading to the formation of SSME. The Tween 85 systems were able to form transient SSME in the range of 10-40% water (degree of saturation>1). Generally, the degree of saturation was low and did not surpass 1.5. Maximum increase in the thermodynamic activity was observed at 20% water. In contrast, for the Cremophor EL system (7:3 DL) the degree of saturation did not surpass 1 in the range of 0-60% water (data presented in Appendix I).
Figure 4.5: Solubility versus φ (expressed as %w/w) for Tween 85 microemulsions (ME) and SSME.

- represents the saturation solubility curve of progesterone in microemulsions.
- represents the solubility curve for SSME produced by mixing saturated SMEDDS with water. The ratio of the solubility in SSME to solubility in microemulsion is the degree of saturation. Data are presented as mean ± SD, n=3.
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Figure 4.6: Degree of saturation as a function of φ (expressed as %w/w).
The degree of saturation of 1 represents the formation of a saturated SMEDDS or microemulsion. Values above 1 (as shown by the dashed red line) indicate the formation of a SSME. Data are presented as mean ± SD, n=3.

4.4.4 Histological Examination of the Skin

Histological examination of porcine skin was performed in order to evaluate the effect of SMEDDDS on the integrity of the skin. Micrographs of the skin sections exposed to water (negative control) and SMEDDDS are represented in Figure 4.7. Fresh skin was used as a control. Sections of the control skin revealed the presence of well-defined dermal and epidermal layers as shown in Figure 4.7 A. The dermis contains a large number of cells embedded within the collagen fibres and is devoid of any skin appendages and hair follicles. The water treated skin shows separation of collagen fibres due to dermal edema (Figure 4.7 B). Epidermal degradation in small region of the epidermis is also observed. Dermal edema and epidermal degradation can also be seen in the skin sections treated with SMEDDDS. Overall, no clear differences could be observed between the micrographs of skin treated with SMEDDDS and water.
Figure 4.7: Micrographs of skin sections showing the untreated skin, skin treated with water and skin treated with SMEDDS.

The micrographs are magnified 40 times. Ep: Epidermis, Dr: Dermis, DE: Dermal Edema, Fr: Epidermal Fragmentation.
**Figure 4.7** (continued): Micrographs of skin sections showing the skin treated with SMEDDS.

The micrographs are magnified 40 times. DE: Dermal Edema, Fr: Epidermal Fragmentation.
Figure 4.7 (continued): Micrographs of skin sections showing the skin treated with SMEDDS.
The micrographs are magnified 40 times. DE: Dermal Edema, Fr: Epidermal Fragmentation.

4.4.5 Scanning Electron Microscopy (SEM) Studies

Morphological changes of the SC surface treated with SMEDDS and water were examined with SEM. The scanning electron micrographs generated are presented in Figure 4.8. At a low magnification (200-250×), the untreated SC surface (A1) appears smooth and flat. At a higher magnification (A2), the SC surface resembles close assembly of tightly packed bilayers. In contrast, water and SMEDDS treated skin appears rough and swollen. This effect seems to be less pronounced with SMEDDS (C1, D1). Closer examination of the water treated skin reveals loosening of the SC lipids, while maintaining their parallel conformation (B2). Conversely, a greater degree of disorder is observed in the Cremophor EL treated skin, with the lipid bilayers no longer maintaining their linear conformation (C2). Tween 85 system resulted in the cleavage of SC lipids, although some degree of disorder was also observed (D2).
Figure 4.8: Scanning electron micrographs of excised porcine skin.
A: Untreated skin; B: skin treated with water; C: skin treated with Cremophor EL SMEDDS (7:3 DL); D: skin treated with Tween 85 SMEDDS (4:6 DL). Micrographs on the right hand side are magnified 200-350 times and micrographs on the left hand side are magnified 2500-3500 times.
4.4.6 FTIR Spectroscopy Studies

A representative FTIR spectrum of porcine skin is shown in Figure 4.9. The spectrum is typical of a hydrated SC with two absorbance regions of interest. The peaks at 2920 cm\(^{-1}\) and 2850 cm\(^{-1}\) arise from asymmetric and symmetric CH stretching, respectively. The peaks in the region of 1640 cm\(^{-1}\) and 1540 cm\(^{-1}\) correspond to amide I and amide II stretching.

The CH and amide stretching frequencies following the treatment of the porcine skin with selected SMEDDS and controls are listed in Table 4.7-Table 4.8. The CH stretching is also shown in Figure 4.10. The asymmetric and symmetric absorbance regions were highly reproducible for untreated skin as well as skin treated with water. No significant shift (p > 0.05) in the CH stretching wavenumber was noted when the skin was treated with these solvents. Conversely, treatment with SMEDDS significantly shifted the CH stretching towards a higher wavenumber. The extent of this shift was similar for all the formulations tested.

The amide I and II stretching were less reproducible than the CH stretching region. Significant shift (p <0.05) in the amide I stretching region was only observed in the skin sections treated with Tween 85 SMEDDS. Tween 85 (4:6 DL) also shifted the amide II stretching towards a higher wavenumber, indicating possible change in the SC secondary protein structure.

Overall, the FTIR data suggest that SMEDDS can act as penetration enhancers when in contact with the skin surface. All SMEDDS changed the packing arrangement of the SC lipids at a molecular level.
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![FTIR Spectrum](image)

**Figure 4.9:** The FTIR spectrum of fresh porcine skin.

**Table 4.7:** Summary of asymmetric and symmetric CH peak positions following the treatment of porcine skin with SMEDDS and controls for 24 h

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Asymmetric CH Stretching (cm⁻¹)</th>
<th>Symmetric CH Stretching (cm⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native Skin</td>
<td>2919.95±0.14</td>
<td>2851.26±0.06</td>
</tr>
<tr>
<td>Control (Water)</td>
<td>2920.09±0.62</td>
<td>2851.38±0.27</td>
</tr>
<tr>
<td>Cremophor EL (7:3 DL)</td>
<td>2923.96±0.35*</td>
<td>2854.10±0.16*</td>
</tr>
<tr>
<td>Cremophor EL (5:5 DL)</td>
<td>2924.11±0.36*</td>
<td>2854.33±0.11*</td>
</tr>
<tr>
<td>Cremophor EL (3:7 DL)</td>
<td>2924.31±0.33*</td>
<td>2854.80±0.06*</td>
</tr>
<tr>
<td>Tween 85 (4:6 DL)</td>
<td>2923.722±0.25*</td>
<td>2854.90±0.73*</td>
</tr>
<tr>
<td>Tween 85 (2:8 DL)</td>
<td>2924.01±0.41*</td>
<td>2855.16±0.50*</td>
</tr>
</tbody>
</table>

* represents significant shift in the wavenumber. Data are presented as mean ± SD, n=3
Chapter 4 - *In Vitro* Penetration Studies

**Table 4.8:** Summary of amide I and amid II peak positions following the treatment of porcine skin with SMEDDS and controls for 24 h

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Amid I Stretching (cm$^{-1}$)</th>
<th>Amid II Stretching (cm$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native Skin</td>
<td>1640.67±1.83</td>
<td>1540.37±0.92</td>
</tr>
<tr>
<td>Control ( Water)</td>
<td>1642.47±0.87</td>
<td>1541.62±0.53</td>
</tr>
<tr>
<td>Cremophor EL (7:3 DL)</td>
<td>1642.91±1.28</td>
<td>1542.83±0.27</td>
</tr>
<tr>
<td>Cremophor EL (5:5 DL)</td>
<td>1641.28±1.30</td>
<td>1542.73±0.27</td>
</tr>
<tr>
<td>Cremophor EL (3:7 DL)</td>
<td>1642.15±2.37</td>
<td>1542.77±0.18</td>
</tr>
<tr>
<td>Tween 85 (4:6 DL)</td>
<td>1644.86±1.28*</td>
<td>1543.58±0.71*</td>
</tr>
<tr>
<td>Tween 85 (2:8 DL)</td>
<td>1643.07±1.54</td>
<td>1543.58±.38*</td>
</tr>
</tbody>
</table>

* represents significant shift in the wavenumber. Data are presented as mean ± SD, n=3

**Figure 4.10:** FTIR spectrum of porcine skin in the CH stretching region following the treatment with SMEDDS and controls for 24 h.

A: Tween 85 (2:8 DL); B: Tween 85 (4:6 DL); C: Cremophor EL (3:7 DL); D: Cremophor EL (5:5 DL); E: Cremophor EL (7:3 DL); G: water (control); H: native skin (control).
4.4.7 Discussion

As discussed in Chapter 1, passive transdermal penetration enhancement can be achieved either by increasing the drug concentration across the skin, or by diminishing the diffusional resistance of the skin [143]. SMEDDS may enhance transdermal drug delivery by both of these mechanisms.

Cremophor EL and Tween 85 SMEDDS significantly increased the permeation rate of progesterone (p< 0.05) compared to the control and selected commercially available creams. The permeation rate of progesterone was inversely proportional to the surfactant concentration. Cremophor EL (7:3 DL), with the lowest surfactant concentration, resulted in a 6.6 fold increase in the transdermal flux. This finding has been previously reported for various colloidal delivery systems [369, 370]. An inverse relationship between the transdermal flux and the surfactant concentration was observed in hydrogen thickened microemulsions used for topical delivery of antitumorigenic agent, triptolide [369]. In this study, increase in the thermodynamic activity was suggested as the main contributing factor for enhanced transdermal flux across the skin [369]. However, the thermodynamic potential of progesterone in the SMEDDS investigated equals unity, as all formulations were saturated. Other factors that may influence the flux across the skin include drug mobility and diffusion within the formulation and partitioning into the SC. Mobility and diffusion can be affected by the solubility of the drug and the viscosity of the formulation in accordance with Stokes-Einstein equation (Equation 3.3)[370]. An exponential increase in the viscosity of the SMEDDS was observed with increase in the concentration of the surfactant/cosurfactant mixture as shown in Table 4.8 and Figure 4.11. Such increase in the viscosity may prohibit the free diffusion and mobility of progesterone in the formulation, leading to a decrease in the transdermal flux [371]. Furthermore, SMEDDS with higher saturation solubility displayed a lower transdermal flux. High solubility may limit the escaping tendency of progesterone from these formulations, thus preventing its partitioning into the SC. As discussed previously, the logP of progesterone (3.87) is slightly higher than the optimal lipophilicity requirement for transdermal drug delivery (logP=1-3). As such, it may be anticipated for progesterone to form a depot within various layers of the skin. However, as shown in Table 4.9, the progesterone content in the skin was low and did not surpass 157 µg/ml. Compared to the control, SMEDDS constituents can increase the transdermal diffusivity of progesterone, thus preventing it from accumulating in the skin.
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The SMEDDS investigated in this chapter were optimised in order to increase the saturation solubility of progesterone. Compared to the aqueous solution, the solubility of progesterone was increased by over 3100 times in selected SMEDDS. In the Cremophor EL SMEDDS, the saturation solubility of progesterone increased as the surfactant concentration increased. The reverse was observed for the Tween 85 SMEDDS, although progesterone displayed higher solubility in these systems. High concentration of progesterone in these formulations is the main driving force for enhanced transdermal permeation. In SMEDDS and the resulting w/o microemulsion, progesterone will be in direct contact with the skin, providing a high concentration gradient and consequently a higher flux across the skin. Some of the commercially available creams such as PCNZ have a higher progesterone content compared to SMEDDS. Despite this, the transdermal permeation rate from these formulations was low, indicating that other additional mechanisms might interfere with the permeation of progesterone through the skin. One possibility may be due to the presence of viscosity enhancing agents such as aloe vera gel and Xanthan gum, which can slow down the diffusion of progesterone from these formulations.

![Graph showing the effect of viscosity on the permeation rate (flux) of progesterone across porcine skin.](image)

**Figure 4.11:** The effect of viscosity on the permeation rate (flux) of progesterone across porcine skin.

The blue line represents the flux from Cremophor EL SMEDDS as the function of viscosity and the red line represents the flux from Tween 85 SMEDDS and microemulsions.
Table 4.9: The solubility, viscosity and skin progesterone content of SMEDDS

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Solubility (mg/ml)(\text{32}^\circ\text{C})</th>
<th>Viscosity (mPa.s)(\text{25}^\circ)</th>
<th>Skin Progesterone Content (µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (water)</td>
<td>8.86*</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cremophor EL SMEDDS (7:3 DL)</td>
<td>25.91±3.14</td>
<td>18.09±0.74</td>
<td>95.53±27.085</td>
</tr>
<tr>
<td>Cremophor EL SMEDDS (5:5 DL)</td>
<td>30.05±0.84</td>
<td>36.93±0.03</td>
<td>157.90±40.58</td>
</tr>
<tr>
<td>Cremophor EL SMEDDS (3:7 DL)</td>
<td>36.93±0.87</td>
<td>79.41±0.005</td>
<td>116.88±48.55</td>
</tr>
<tr>
<td>Tween 85 SMEDDS (4:6 DL)</td>
<td>52.06±2.30</td>
<td>68.01±0.01</td>
<td>57.57±5.77</td>
</tr>
<tr>
<td>Tween 85 SMEDDS (2:8 DL)</td>
<td>40.26±40.06</td>
<td>80.00±0.01</td>
<td>62.50±9.47</td>
</tr>
</tbody>
</table>

*values were measured at room temperature, Data are presented as mean ± SD, n=3

Another important mechanism, which might promote the permeation of progesterone across the skin, is the formation of a SSME. Supersaturated Self Emulsifying Drug Delivery Systems (SSEDDS) have been investigated for oral delivery of poorly water soluble drugs [372, 373]. SSEDDS containing hydroxypropyl methylcellulose resulted in a significant improvement in paclitaxel bioavailability, which was 5 fold higher than the self-emulsifying delivery systems and a commercially available formulation. El Maghraby was the first to explore the potential of supersaturated SMEDDS for transdermal drug delivery [151]. The formulations investigated were based on ethyl oleate as the oil component and the surfactant blend of Tween 80 and Span 20. Transdermal flux of indomethacin from SMEDDS was compared against the flux from microemulsions, liquid crystals and coarse emulsions. Of these formulations, SMEDDS resulted in the highest permeation rate. Moreover, SSME containing 10% water produced permeation rates similar to that of SMEDDS. It was concluded that SMEDDS could form SSME upon contact with the hydrated skin. The current in vitro studies demonstrated that SMEDDS could absorb up to 11% water when they are exposed to the occluded skin. In vivo this value can be influenced by the hydration state of the skin, perspiration and sweating. Subsequent studies showed that the flux from SSME were similar to that of microemulsions. Furthermore, only Tween 85 SMEDDS (4:6 DL) were able to form SSME, although the degree of supersaturation was low and did not surpass 1.5. Upon dilution
of SMEDDS with water, progesterone rapidly crystallised thus preventing the formation of SSME. It is possible to reduce the crystallisation rate of progesterone through the addition of antinucleant polymers. However, as most commonly used antinucleant polymers are water soluble, it is challenging to identify antinucleant polymers with lipophilic components. Overall, the concept of SSME cannot be used to explain the supremacy of SMEDDS over microemulsions and other commercially available products.

Histological examination of the skin cross sections together with microscopic and FTIR studies indicated that the SMEDDS could reduce the diffusional resistance of the SC. Increased swelling was observed in the skin sections treated with water. However, at a molecular level the SC lipid conformation was not affected. Conversely, SMEDDS resulted in the detachment of the epidermis from the dermis. SEM studies also depicted a high degree of disorder on the SC surface. This was further supported by the FTIR studies, which showed a blue shift in the CH vibrational frequency. These observations are a direct consequence of the fluidisation of the SC lipids brought about by the partitioning of the oils and surfactants into this layer. The Tween 85 based SMEDDS (2:8 DL) also resulted in a significant shift in the amide I and II bands. Shifts in these bands are attributed to changes in the secondary structures of keratins. Red shifts in the amide stretching suggests the conversion of the α spiral structures into β sheets [374]. The reverse is observed when the amide band shifts to a higher wavenumber. Therefore, Tween 85 SMEDDS may increase the amount of β folding in the keratin structure.

Previous studies have shown that the lag time from SMEDDS and microemulsions is higher than the control [151, 375]. For the SMEDDS, the delay in the lag time was attributed to the time required to convert them into microemulsion. For the microemulsions, the delay in the lag time was related to the lack of penetration enhancing effect of the cosurfactant employed. In the present study, SMEDDS had a longer lag time compared to microemulsions. This might be attributed to the time required to form microemulsions as described above. Nonetheless, the lag time was shorter than the control. Barrier properties of the skin can be reduced due to the partitioning of oils and surfactant into the SC allowing for a faster diffusion of the drug through the depth of the skin and hence a shorter lag time. This, together

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5 Shortening of the wavelength and increase in frequency (increase in wavenumber)
6 Lengthening of the wavelength and decrease in frequency (decrease in wavenumber)
with the aforementioned studies, suggests that the constituent of SMEDDS can act as penetration enhancers. Enhanced penetration rate can be achieved because of an increased drug partitioning and solubility into the skin or due to increased diffusivity through the SC lipids. Transcutol P can increase the flux by both of these mechanisms [117, 143]. It has been reported that Transcutol P can increase the flux and partitioning by shifting the solubility parameter ($\delta_s$) of the skin lipids closer to the solubility parameter of the permeant ($\delta_i$) [117]. Transdermal bioavailability of poorly water soluble drug celecoxib increased by 3.6 times when Cremophor EL (17.5% w/w) was used in combination with Transcutol P (17.5% w/w) in nanoemulsions and gel based formulations [376]. The penetration enhancing mechanism of IPM is via liquefying and dissolving the cholesterol and lamellar gel phases of the SC lipids [377]. Tween 85 has also shown to enhance epidermal permeability through alteration of the SC lipid conformation [378]. In fact, Myritol 318 is the only component that has not previously demonstrated penetration enhancing properties [379]. Overall, the increase diffusivity through the SC lipids is achieved via synergic effect of the oil and surfactant constituents.

*In vitro* permeation studies were carried out with the skin occluded with parafilm. Occlusion is often used to increase the transdermal permeation rate across the skin. Increase in the transdermal flux by occlusion is primarily attributed to the hydration of the SC and prevention of TEWL [380]. Moreover, occlusion can delay the recovery of the SC lipid barrier, which is compromised by penetration enhancers. Ahn et al. showed that the SC lipid barrier recovery was hindered when mice skin was occluded for 3 days following the application of surfactants and tape stripping [381]. This effect was not observed with non-occluded skin. The SC lipid bilayer steadily recovered following the removal of the occlusion. Therefore, the occlusion of the skin can further enhance the transdermal flux of progesterone.

### 4.5 Conclusion

In the current chapter, SMEDDS were investigated as a novel transdermal delivery matrix for bioidentical progesterone. The advantage of using SMEDDS is their superior stability and high drug solubilisation capacity. The transdermal diffusion studies showed that SMEDDS could increase the permeation rate of progesterone across the skin. Subsequent studies also demonstrated that SMEDDS could form microemulsion upon contact with the skin, although
the amount of water absorbed was low. The absorption of water can modify the viscosity of the formulation, offering a great flexibility in selecting formulations with optimal viscosity. The increased transdermal flux was predominantly associated with the high saturation solubility of progesterone and the penetration enhancing effect of the SMEDDS constituents. Conversely, supersaturation did not play any role in enhanced drug delivery across the skin. SMEDDS are effective for enhancing the transdermal permeation of progesterone across the skin. It is acknowledged that such permeation enhancement alone may not be associated with increased transdermal bioavailability, as progesterone could be metabolised by the dermal and epidermal enzymes. The metabolism of progesterone by the 5α-reductase enzymes will be explored in the next chapter.
5 Transdermal Metabolism of Progesterone: A Pilot Clinical Study

5.1 Introduction

The previous chapters focused on the development of a novel delivery system that can enhance the transdermal permeation of progesterone through modifying the SC barrier. In addition to the physical barrier of the skin, metabolic barriers may also limit the transdermal distribution of drugs into the systemic circulation. The microflora present on the skin surface can metabolise the drug or alternatively it can be degraded by the enzymatic barriers present in the viable component of the skin [382]. The biotransformation reactions taking place in the skin include phase I (oxidation, reduction, hydrolysis) and phase II (glucuronidation, sulfation, methylation and glutathione conjugation) reactions [383]. Table 5.1 lists the skin's major metabolising enzymes, which are involved in such reactions. In comparison to the liver enzymes, which are confined to a small region, the skin enzymes in adults are distributed over a large surface area of at least 2 m² [382]. Thus, when considering delivery to a confined area, transdermal drug metabolism will not have the same impact on the bioavailability as the first pass hepatic metabolism. Nonetheless, transdermal metabolism might be the rate-limiting step in the percutaneous absorption of some drugs [384]. Hydrocortisone, testosterone, 17β-estradiol, theophylline esters and metronidazole esters are examples of compounds metabolised in the skin.

Generally, the transdermal metabolism is a complex process and can be affected by many factors including the rate of permeation, formulation additives, the nature of the permeant, physiological and pathological status of the skin, age, sex, race and hormonal status. For highly hydrophilic drugs such as acyclovir valerate, transdermal metabolism did not have a significant impact on the total amount of drug permeated as permeation through the SC was the rate-limiting step for its absorption [385]. In contrast, the metabolite concentration of
lipophilic propylparaben and butylparaben significantly increased following their permeation through full thickness abdominal skin [386]. Several studies have demonstrated that chemical penetration enhancers such as ethanol [387] and l-menthol [388] may inhibit transdermal enzymes thus allowing for enhanced drug penetration. A slow absorption across the skin may also extensively expose the permeant to the skin enzymes, subsequently leading to a lower bioavailability [384].

The viable epidermis is considered the main site of localisation and activity of the skin enzymes. Conversely, short transient time in the dermis and rapid clearance of drugs by the circulation can limit the metabolism in this layer. The metabolism by the hair follicles, sebaceous glands and sweat glands is also limited, although significant activity of certain enzymes such as 5α-reductase have been observed in sebaceous and sweat glands [389].

Table 5.1: Skin enzymes and their corresponding biotransformation reactions

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mixed Function Oxidases</td>
<td>Phase I Oxidation Reactions</td>
</tr>
<tr>
<td>Hydroxylases</td>
<td>Aliphatic and Alicyclic C-Atoms, Aromatic Rings</td>
</tr>
<tr>
<td>Dehydrogenases</td>
<td>Aromatic Rings</td>
</tr>
<tr>
<td>Monoamine Oxidases</td>
<td>Alcohols</td>
</tr>
<tr>
<td>Deethyalaes, Demethylases</td>
<td>Deamination</td>
</tr>
<tr>
<td></td>
<td>Phase I Reduction Reactions</td>
</tr>
<tr>
<td>Ketoreductase</td>
<td>Carbonyl Groups</td>
</tr>
<tr>
<td>5α-Reductase</td>
<td>C=C Double Bonds</td>
</tr>
<tr>
<td></td>
<td>Phase I Hydrolysis Reactions</td>
</tr>
<tr>
<td>Esterase</td>
<td>Ester Bonds</td>
</tr>
<tr>
<td>Epoxide Hydrase</td>
<td>Epoxides</td>
</tr>
<tr>
<td></td>
<td>Phase II Conjugation Reactions</td>
</tr>
<tr>
<td>UDPG-Transferases</td>
<td>Glucuronidation</td>
</tr>
<tr>
<td>Sulpho-Transferases</td>
<td>Sulphation</td>
</tr>
<tr>
<td>Catechol-0-Methyl-Transferases</td>
<td>Methylation</td>
</tr>
<tr>
<td>Glutathione-S-Transferases</td>
<td>Glutathione</td>
</tr>
</tbody>
</table>

Adapted from [382, 383]
5α-reductases are membrane bounded steroid enzymes, which catalyse the nicotinamide adenine dinucleotide phosphate (NADPH) dependant reduction of steroids containing a 3 keto, 4-5 double bond in their A ring [390]. The outcome of such reduction is variable and significantly depends on the substrate involved and the site of action. In the liver, these enzymes play a catabolic role reducing C19 and C21 steroids to their inactive metabolites, while in the androgen target tissue such as the prostate, enzymatic catalysis may result in the formation of potent metabolites. Conversion of testosterone to dihydrotestosterone is an example of this later reaction. Glucocorticoids, mineralocorticoids, progestogens, and androgens are examples of steroids that are susceptible to 5α reduction.

Two isoenzymes of 5α-reductase have been identified within the body. The type I isoenzyme is predominately found in the skin and the type II isoenzyme is found in the prostate [391]. Both of these are relatively hydrophobic, with only 50% similarities in their amino acid sequence [391]. The gene encoding the type I isoenzyme is located on the P15 band of the distal arm of chromosome 5 [392]. This isoenzyme possesses optimum activity in the natural and alkaline pH ranges (6.8-8), with moderate affinity for steroid substrates [390, 392]. Within the skin, type I isoenzyme is mainly localised in the sebaceous glands, epidermis, sweat glands, and hair follicles. The enzyme activity is lower in sebaceous glands from non-acne prone areas compared to the scalp and face [393]. In vitro studies have also detected type I isoenzyme in cytoplasm of human cells such as hair dermal papilla, keratinocytes, sebocytes fibroblasts, and epithelial cells in various tissues of the body [394-396]. Type II isoenzyme possesses optimum activity in weak acidic conditions (pH 5-6) and has high affinity for steroid substrates [392]. This isoenzyme has been extensively localised in the testes, prostate, outer and inner root sheath of hair follicles, and sebaceous ducts [391, 397, 398]. The activity of type II isoenzyme was reported to be 3 fold higher than type I in beard dermal papilla [399, 400]. Moreover, compared to type II isoenzyme, which is present in the skin from the time of birth, type I isoenzyme is only found in the skin around puberty [401].

Transdermal progesterone is a substrate for steroid 5α-reductase enzyme [402, 403]. Peripheral metabolism of progesterone to 5α-reduced derivatives could significantly affect its
transdermal bioavailability. Table 5.2 shows the 5α-reduced metabolites of progesterone formed in the skin [404]. In vivo studies have also shown high concentration of urinary 5α-pregnane-3α,20α-diol, following the topical administration of radiolabeled progesterone [403]. More recently, Cooper et al. and Lewis et al. reported the presence of pregnanediol-3α-glucuronide, another urinary metabolite, following the topical administration of progesterone cream [99, 105].

**Table 5.2:** 5α-reduced metabolites of progesterone formed in the skin

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Chemical Structure</th>
<th>Metabolite</th>
<th>Chemical Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>5α-Pregnane-3,20-dione</td>
<td><img src="image1" alt="Chemical Structure" /></td>
<td>5α-Pregnan-3β-ol-20-one</td>
<td><img src="image2" alt="Chemical Structure" /></td>
</tr>
<tr>
<td>4.3% (w/w) *</td>
<td></td>
<td>16% (w/w) *</td>
<td></td>
</tr>
<tr>
<td>5α-Pregnan-3α-ol-20-one</td>
<td><img src="image3" alt="Chemical Structure" /></td>
<td>5α-Pregnane-3α,20α-diol</td>
<td><img src="image4" alt="Chemical Structure" /></td>
</tr>
<tr>
<td>1.6% (w/w) *</td>
<td></td>
<td>0.5% (w/w) *</td>
<td></td>
</tr>
<tr>
<td>5α-Pregnane-3β,20α-diol</td>
<td><img src="image5" alt="Chemical Structure" /></td>
<td>Unknown Metabolite</td>
<td><img src="image6" alt="Chemical Structure" /></td>
</tr>
<tr>
<td>0.5% (w/w) *</td>
<td></td>
<td>9.7% (w/w) *</td>
<td></td>
</tr>
</tbody>
</table>

The * represents the percentage of metabolite formed.
5.1.2 Steroid 5α-Reductase Inhibitors

Over the past two decades, many different natural and synthetic 5α-reductase inhibitors have been synthesized and investigated. Permixon is a lipid sterol extract of *Serenoa repens*, commonly known as saw palmetto. This agent has been used for treatment of benign prostatic hyperplasia, a condition characterised by increased 5α-reductase activity. Although its therapeutic efficacy has been controversial, *in vitro* studies have shown that Permixon is a non-competitive dual inhibitor of 5α-reductase enzymes [405]. *Sabal serrulate* is another example of a plant belonging to the same family as saw palmetto with 5α-reductase inhibitory effects. Weisser et al. showed that *Sabal serrulate* fruit extracts induce a dose dependant non-selective inhibitory effect on these enzymes, with a mean inhibition of 38% and 39% observed in stroma and epithelial cells respectively [406]. The same study also showed that the fatty acids of the extract also possess 5α-reductase inhibitory effects. Maximum inhibition was observed for myristic acid. The inhibitory effect of polyunsaturated fatty acids has also been demonstrated in other studies. The relative inhibitory effect of unsaturated fatty acids are in the following order: myristoleic acid < oleic acid < palmitoleic acid < arachidonic acid < cis-6, 9, 12, 15-octatetraenoic acid < linoleic acid < a-linolenic acid < y-linolenic acid [407].

Zinc sulphate and azelaic acid can inhibit the 5α-reductase enzymes in the skin. *In vitro* assays of zinc sulphate and azelaic acid with radiolabeled testosterone showed that these agents can completely inhibit the enzyme at concentrations of 9 mmol/l and 3 mmol/l respectively [408]. Moreover, the addition of vitamin B6 (0.025%) to 1.5 or 3 mmol/l of zinc sulphate further reduced the enzymatic activity. Zinc regulates the conversion of testosterone to dihydrotestosterone. At a low concentration it has shown to have stimulatory effects on the 5α-reductase enzyme, while at a high concentration the enzyme is inhibited [409].

4-Azasteroids such as dutasteride (Figure 5.1) and finasteride are FDA approved 5α-reductase inhibitors used for management of prostate hyperplasia. Both agents are time dependant, irreversible inhibitors of the type II isoenzyme. Dutasteride is more potent than finasteride as it is a dual inhibitor of both isoenzymes. Many other 5α-reductase inhibitors such as bexlosteride and turosteride have been developed but never marketed by their manufacturers.
Figure 5.1: The chemical structure of dutasteride.

Lewis et al. [99] were the first group to evaluate the effect of enzyme inhibition on the plasma concentration of progesterone. One subject was given 5 mg of oral finasteride, for 9 days, followed by 80 mg (40 mg twice a day) of progesterone cream. Although salivary progesterone levels peaked 1 h after the first dose of progesterone, no change in the plasma or red cell progesterone concentrations was noted. The authors concluded that transdermal metabolism of progesterone is unlikely to affect its plasma concentrations. Finasteride inhibits the type II 5α-reductase enzymes, which have a low distribution in the skin. To prevent the transdermal metabolism of progesterone, an inhibitor with a strong affinity for the type I isoenzymes is required. Currently, dutasteride is the only FDA approved non-selective 5α-reductase inhibitor available. Dutasteride has been shown to inhibit the conversion of progesterone to 5α-dihydroprogesterone in tumorigenic breast cell lines [410].

5.1.3 Salivary Distribution of Progesterone

In premenopausal women the salivary progesterone concentration is about 19.6 – 86.5 pg/ml during the follicular phase of the menstrual cycle, and increases to 99.1 – 332.6 pg/ml in the luteal phase. In line with its serum levels, the salivary progesterone reduces to 6-56 pg/ml in postmenopausal women. Clinical studies have shown that salivary progesterone concentration can rise rapidly following the transdermal application of progesterone. In one study the salivary concentration peaked to about 18 ng/ml within 4 h of treatment with progesterone cream (64 mg) [411]. In comparison to the control and premenopausal women, the salivary levels were increased by 150-230 times [99].
Progesterone circulates the blood mainly bound to the Corticosteroid Binding Globulin (CBG), serum albumin and $\alpha_1$-acid glycoprotein [412]. Only 2-10% of the progesterone circulates as a free hormone. Transport of hormones to the saliva is governed by three main mechanisms, intracellular diffusion, ultrafiltration and active transport (Figure 5.2) [413]. Unbound substances that are soluble in the lipid membrane of the salivary glands acinar cells can freely diffuse across the cells into the saliva. Alternatively, water and smaller polar molecules (< 1900Da) will diffuse through the tight junctions of the acinar cells. Salivary transport of endogenous progesterone is an example of the former mechanism. Progesterone leaks into the saliva down its concentration gradient from a region of high concentration (serum) to a region of low concentration (saliva). The high molecular weight of albumin and CBG does not allow the bound fraction of the hormone to diffuse into the saliva [414]. Therefore, unconjugated salivary concentration reflects the free concentration in the serum.

Figure 5.2: Schematic of drug transport from the systemic circulation to the salivary glands.

To study the effect of 5α-reductase inhibition, it is necessary to monitor both serum and salivary levels of progesterone. The simple and non-invasive nature of saliva collection makes it ideal for evaluating concentrations of progesterone in this biological fluid. When measured properly, salivary levels reflect the unbounded biologically active markers in the circulation. The human saliva is mainly produced by the parotid, submandibular, and sublingual glands, although buccal glands and gingival fluid may also contribute to the salivary content. The salivary hormone level can be affected by many factors. Bleeding from the gums can lead to the contamination of saliva by blood exudate, a major limiting factor specially for those drugs
that have a saliva plasma ratio of < 1% [415]. The flow rate and the pH of the saliva can further affect the concentration of some drugs in saliva. For example, the salivary concentration of dehydroepiandrosterone sulphate dropped significantly when saliva was collected at a higher flow rate [416]. In contrast, increase in the salivary flow rate did not affect its progesterone or cortisol concentration [416, 417]. The pH of the saliva ranges from 6-8. Variations in this pH may selectively attract or exclude drugs with a pKa ranging from 5-9 [415]. The normal salivary flow rate is about 0.05 ml/min and could be further increased to 1-3 ml/ min by chewing paraffin wax, rubber bands and unflavoured chewing gum or by placing 0.1-0.2 mol/l of citric acid on the tongue [418]. When measuring the hormone levels, sampling of whole or mixed saliva is generally preferred. Whole saliva can be collected by using novel cotton based devices, direct spitting or passive drooling. The former method has the potential to interfere with the assay results. Specifically, it a has been found that the salivary concentration of progesterone was significantly higher when cotton absorbent materials were used [419]. In the present study, passive drooling was used to collect whole, non-stimulated saliva.

5.2 Chapter Aim and Objectives

The aim of the current study is to investigate the effect of 5α-reductase metabolism on the bioavailability of transdermal progesterone in menopausal women.

The specific objectives of this chapter are to:

- evaluate the serum and salivary concentration of transdermal progesterone in menopausal women,
- inhibit the transdermal metabolism of progesterone across the skin,
- investigate the effect of 5α-reductase enzyme inhibitor dutasteride on the serum and salivary concentration of transdermal progesterone, and;
- investigate the effect of dutasteride on serum concentration of selected hormones.
5.3 Experimental Method

5.3.1 Pilot Clinical Study Protocol

This study was a randomised, double blind, single-centre pilot study comparing the effects of topical dutasteride (2 mg/g) on the bioavailability of transdermally delivered bioidentical progesterone. Dutasteride and progesterone cream were extemporaneously compounded in a non-ionic cream base (PCNZ, New Zealand). Ethics approval was obtained from Tehran University of Medical Sciences, Iran (#9414). All participants were given written informed consent before the start of the study. Twenty-three menopausal women aged between 40 to 65 years were recruited to take part in the study. None had taken any hormones for at least 6 months, smoked > 5 cigarettes per day, or had any skin disease. All women were postmenopausal for at least 1 year with a follicle stimulating hormone (FSH) of greater than 40 IU/L. All women were required to have normal liver and renal function to be eligible for the study. The women undertook a full medical check-up, which included measurement of serum progesterone, estradiol, testosterone, and cortisol in addition to the assessment of liver, renal and thyroid function prior to the start of the study.

Eligible subjects were randomised to treatment groups using computer generated numbers (Random Allocation Software, Isfahan, Iran) in opaque sealed envelopes. Subjects in each group applied 500 mg of either non-ionic cream or dutasteride cream (2 mg/g) to the same area of the right arm for 15 days. This was followed by applying either 500 mg of progesterone cream (80 mg/g) or combined cream (80 mg/g progesterone, 2 mg/g dutasteride) for a further 15 days (Figure 5.3). The creams were packed in calibrated syringes each containing 43.8 ± 1.50 mg of progesterone with or without 1.10 ± 0.04 mg of dutasteride. The average surface area of application was 239.60 ± 22.50 cm². Participants were instructed not to wash their right arm for at least 2 h following the application of progesterone. Blood and saliva samples were collected on day 30 at 0, 2, 4, 6, and 12 h following the final application of the cream. Blood samples were centrifuged at 3000 rpm and the serum was separated and stored at -20°C until analysis by enzyme immunoassay.

Steroids containing a 4-5 double bond in their A ring are a substrate for 5α-reductase enzymes [390]. Inhibition of these enzymes could potentially increase the endogenous level of hormones such as testosterone and cortisol. Dutasteride has also been shown to significantly
increase the TSH levels [420]. Therefore, the effect of dutasteride on the serum concentration of other hormones including testosterone, estradiol, cortisol, total triiodothyronine (T3), thyroxin (T4), and thyroid stimulating hormone (TSH) was also monitored during the study. Due to the diurnal variation in the concentration of cortisol, baseline samples were always collected at exactly 8 am. Compliance was monitored via daily phone calls to the participants.

**Figure 5.3:** Schematic representation of the study protocol from day 0-30.

### 5.3.2 Sampling of Saliva

A specific protocol was adopted for sampling of saliva. The subjects were instructed not to brush their teeth for at least 12 h prior to sampling. Samples were taken in accordance with the passive drooling method [421, 422]. The head was tilted down allowing the whole saliva to accumulate in the mouth and dribble into a glass test tube via a glass straw. The samples were collected over the duration of 5 minutes. The first sample was collected after an overnight fasting whilst for the other samples food and drinks were prohibited 1 h prior to sampling. The subjects were placed on a strict diet where by the intake of all dairy products, meat, coffee and juice was forbidden on day 29 and 30 of the study. This was critical as food and drinks may directly or indirectly (via changes in salivary pH and flow rate) interfere with the concentration of drugs in saliva [423]. The participants were instructed to rinse their mouth with warm water 30 min prior to sampling. After the collection of saliva, the samples were stored at -20°C until the time of analysis.
5.3.3 Enzyme-Linked Immunosorbent Assay (ELISA)

Serum progesterone was measured using IBL progesterone ELISA kit (RE52231, IBL International, Germany). Salivary progesterone was measured using Demeditec progesterone ELISA kit (DESLV2931, Demeditec Diagnosis, GmbH, Germany). Serum testosterone (EIA-1559), estradiol (EIA-2693), and cortisol (EIA-1887) were measured using DRG ELISA kits (DRG instruments, GmbH, Germany). EUAgen ELISA kit (Adaltis Italia S.p.A, Casalecchio di Reno, Italy) was used for the measurement of total serum T3 (LI4019B/ LI4026), total T4 (LI4020/ LI4027) and TSH (LI4025K/ LI4028). Serum FSH was measured using Ideal diag ELISA kit (Ideal diag, Iran).

5.3.3.1 Assay Procedure

Initially 20 to 100 µl of standard, control, or sample was dispensed into the appropriate well. To this, 50-200 µl of enzyme conjugate was added and mixed for 10 s. The samples were incubated at room temperature for 60-120 min in accordance with the kit’s instructions. The wells were then washed 3-5 times with the diluted wash solution. After each washing step, the wells were struck against an absorbent paper towel in order to remove any residual water. Following this, 100-200 µl of the substrate solution was added to each well and the samples were incubated for a further 15-30 min at room temperature. The enzymatic reaction was stopped by adding 100 µl of stop solution. The optical density was then measured at 450 ±10 nm with a microplate reader (Biochrom Anthos 2010 Microplate Reader, United Kingdom) within 10 min.

5.3.4 Statistical Analysis

The following pharmacokinetic parameters were determined and used to study the absorption of progesterone across the skin:

\[ C_{\text{max}}: \text{Maximum progesterone concentration achieved in 12 h} \]
\[ T_{\text{max}}: \text{Time to maximum progesterone concentration} \]
Chapter 5-Transdermal Metabolism of Progesterone

AUC\(_{0-12}\): The area under the progesterone concentration vs. time curve from 0-12 h calculated using the trapezoidal rule

\(C_{\text{average}}\): Average progesterone concentration between 0 and 12 h

\(C_{\text{baseline 30}}\): Baseline progesterone concentration on day 30 prior to applying the last dose of progesterone cream

\(C_{\text{baseline}}\): Baseline progesterone concentration at the start of the study prior to applying the progesterone cream

All data are reported as median and range values. Statistical analysis was performed by the Mann-Whitney U test. \(P< 0.05\) was set as the level of significance. The Wilcoxon test was used to compare the baseline hormone levels before and after the application of progesterone/dutasteride cream. All data were analysed using SPSS software (SPSS 16.0, USA) and Microsoft Excel 2007 (Microsoft® Office 2007, USA).

## 5.4 Results and Discussion

### 5.4.1 Results

Twenty-three postmenopausal women were recruited to take part in the study. Of these women, only 20 were eligible to participate in the study. Ten women were randomised to the placebo and 10 were randomised to the dutasteride group. Demographics of the study participants are reported in Table 5.3. No significant differences were observed between the two groups. Two women from the placebo group and one woman from the dutasteride group withdrew from the study due to non-compliance and loss of follow up. The 2, 3 and 6 h blood samples of three subjects in the dutasteride group and one subject in the placebo group were haemolyzed during sampling and resulted in an abnormally high progesterone concentration (> 50ng/ml). Hence, the serum results for these subjects were eliminated. Overall serum hormone concentrations were analysed for six subjects in the dutasteride group and seven subjects in the placebo group. Analysis of the saliva data was carried out for all subjects (n=17) that completed the study.

Table 5.3: The demographics of the study subjects
Table 5.4 summarises the serum pharmacokinetics data obtained following the application of the progesterone cream for 2 weeks. The median baseline progesterone concentration on day 0 \((C_{\text{baseline}})\) was 0.1 ng/ml in both groups. This is in agreement with the normal range of progesterone reported for menopausal women [424]. After 15 days of treatment with the progesterone cream, the \(C_{\text{baseline}}\) increased significantly \((p < 0.05)\) to 1.40 ng/ml and 1.15 ng/ml in the placebo and dutasteride treated groups respectively (Figure 5.4 B). This was followed by a further increase in the serum concentration to a maximum of 5.50 ng/ml (placebo group) and 7.35 ng/ml (dutasteride group) within 6-9 h. By 12 h the serum progesterone concentration declined, although it was still significantly higher than the \(C_{\text{baseline}}\) level. The average serum concentration of progesterone over 12 h was 3.04 ng/ml in the placebo treated group and 3.14 ng/ml in the dutasteride treated group. Overall, whilst the \(C_{\text{average}}, C_{\text{max}},\) and the AUC were slightly higher in the dutasteride treated group, no statistically significant difference between the groups was noted.

The salivary pharmacokinetics data are summarised in Table 5.5 and Figure 5.4 B. On day 30, salivary \(C_{\text{baseline}}\) increased by 7 fold from 0.4 ng/ml to 2.9 ng/ml in the placebo treated group with the maximum salivary progesterone concentration observed 3-4 h after the last dose of progesterone. The \(C_{\text{max}}\) and AUC in the placebo treated group were 21.45 ng/ml and 131.27 ng.h/ml, respectively. In the dutasteride treated group these values were slightly increased to 29.00 ng/ml \((C_{\text{max}})\) and 132.60 ng.h/ml (AUC), although no statistically significant difference was noted between groups.
Chapter 5-Transdermal Metabolism of Progesterone

**Table 5.4:** Pharmacokinetic parameters of progesterone in serum

<table>
<thead>
<tr>
<th>Pharmacokinetic Parameters</th>
<th>Placebo Treated Group n=7</th>
<th>Dutasteride Treated Group n=6</th>
<th>Statistical Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>C&lt;sub&gt;max&lt;/sub&gt; (ng/ml)</td>
<td>5.50 (2.40-8.10)</td>
<td>7.35 (1.20-25.90)</td>
<td>NS</td>
</tr>
<tr>
<td>T&lt;sub&gt;max&lt;/sub&gt; (h)</td>
<td>6 (2-12)</td>
<td>9 (4-12)</td>
<td>NS</td>
</tr>
<tr>
<td>AUC&lt;sub&gt;0-12hr&lt;/sub&gt; (ng.h/ml)</td>
<td>34.70 (22.10-58.10)</td>
<td>40.70 (11.2-126.6)</td>
<td>NS</td>
</tr>
<tr>
<td>C&lt;sub&gt;average&lt;/sub&gt; (ng/ml)</td>
<td>3.04 (1.78-4.12)</td>
<td>3.14 (0.98-9.80)</td>
<td>NS</td>
</tr>
<tr>
<td>C&lt;sub&gt;baseline 30&lt;/sub&gt; (ng/ml)</td>
<td>1.40 (1-5.4)</td>
<td>1.15 (0.40-3.1)</td>
<td>NS</td>
</tr>
</tbody>
</table>

Data are represented as median (range). NS: Not Significant

**Table 5.5:** Pharmacokinetic parameters of progesterone in saliva

<table>
<thead>
<tr>
<th>Pharmacokinetic Parameters</th>
<th>Placebo Treated Group n=8</th>
<th>Dutasteride Treated Group n=9</th>
<th>Statistical Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>C&lt;sub&gt;max&lt;/sub&gt; (ng/ml)</td>
<td>21.45 (4.80-89.00)</td>
<td>29.99 (3.10-58.00)</td>
<td>NS</td>
</tr>
<tr>
<td>T&lt;sub&gt;max&lt;/sub&gt; (h)</td>
<td>4 (2-12)</td>
<td>3 (2-6)</td>
<td>NS</td>
</tr>
<tr>
<td>AUC&lt;sub&gt;0-12hr&lt;/sub&gt; (ng.h/ml)</td>
<td>131.47 (49.00-398.00)</td>
<td>132.20 (20.00-406.00)</td>
<td>NS</td>
</tr>
<tr>
<td>C&lt;sub&gt;average&lt;/sub&gt; (ng/ml)</td>
<td>9.97 (3.88-35.00)</td>
<td>12.17 (1.58-37.20)</td>
<td>NS</td>
</tr>
<tr>
<td>C&lt;sub&gt;baseline 30&lt;/sub&gt; (ng/ml)</td>
<td>2.90 (0.40-7.50)</td>
<td>1.70 (0.46-7.60)</td>
<td>NS</td>
</tr>
</tbody>
</table>

Data are represented as median (range). NS: Not Significant
Figure 5.4: Comparison of baseline serum (A) and salivary (B) progesterone levels before and after application of progesterone in the dutasteride and placebo treated groups. The data presented are the median values, error bars represent the range. The * represents significant increase in concentration, p<0.05.

Figure 5.5 shows the effect of dutasteride cream on the serum level of selected hormones. Of the hormones measured, only TSH showed a significant change following the application of dutasteride cream for 1 month (p <0.05), although its values remained within the normal range. The serum TSH level was increased by 1.70 fold, while T3 and T4 levels were slightly reduced. The aforementioned changes were in line with a previous report that showed a 1.12 fold increase in the TSH level, after 52 weeks of treatment with oral dutasteride (0.5mg/day) [420]. The larger increase in the TSH levels seen in this study may be due to the higher dose used, or due to topical administration avoiding first pass hepatic metabolism. This effect is observed for other steroids such as 17β-estradiol, which is used at 0.5-1 mg/day orally and 0.025-0.10 mg/day transdermally [425]. The underlying reason for increase in the level of TSH is not known, although animal studies have established a relationship between thyroid hormones and 5α-reductase expression [426].

Over 30 days a noticeable increase in the serum estradiol levels was observed in both treatment groups, although levels were maintained in the normal postmenopausal range (< 65 pg/ml). This increase was greater in the placebo group compared with the dutasteride group, although this difference was not statistically significant (p >0.05). In premenopausal women estrogen secretion is cyclic, where the levels rise in the mid follicular to mid luteal phase of
the cycle (~ day 7 to 25). In postmenopausal women, estrone is the predominant hormone in the serum. The peripheral conversion of estrone to estradiol may contribute to the serum level of estrogen observed in this study [427].

Figure 5.5: Comparison of the effect of dutasteride and placebo on the serum level of selected hormones. The data are presented as median plus range. The * represents significant increase in concentration.
5.4.2 Discussion

It is hypothesised that the cutaneous metabolism of progesterone may significantly reduce its transdermal bioavailability. Therefore, topical dutasteride was used to study the effect of cutaneous metabolism on the bioavailability of transdermal progesterone. This study shows that topical administration of progesterone is associated with a significant increase in the plasma progesterone level. In the placebo treated group, 15 days of treatment with topical progesterone resulted in a 14 fold increase (0.1 to 1.4 ng/ml) in the baseline progesterone level. In general, the percutaneous absorption of progesterone was low and highly variable, achieving serum $C_{\text{baseline}}$ levels usually associated with the follicular phase of the menstrual cycle. During this phase, the plasma progesterone concentration does not surpass 1.4 ng/ml. Conversely, in the luteal phase the levels sharply increase, ranging from 4-25 ng/ml. It is widely accepted that luteal progesterone levels must be attained to achieve maximum therapeutic benefits [85]. Wren et al. also found that applying 64 mg/day of progesterone does not increase the plasma concentrations beyond the levels observed during the follicular phase [84, 428]. O’Leary et al. suggest that higher doses (>64 mg/day) of transdermal progesterone cream should used in order to obtain higher plasma levels [411]. Nonetheless, luteal plasma levels were not obtained even when progesterone was applied at a dose of 80 mg/day, two to four times its recommended dose [105]. Age, hydration, and skin condition are amongst the many factors that affect absorption of drugs across the skin, leading to a large inter-individual variability. It has been reported that the permeation of drug molecules across the skin can vary by up to 45% amongst subjects [429]. The serum pharmacokinetic data are in line with the permeation data obtained in the previous chapter. Low permeation of progesterone from the PCNZ cream can reduce its transdermal bioavailability.

This study further showed that dutasteride was not able to increase the bioavailability of transdermally administered progesterone. Although serum and salivary $C_{\text{max}}$ and AUC of progesterone were slightly higher in the dutasteride treated group ($p > 0.05$), the overall $C_{\text{average}}$ was similar to the placebo treated group (Table 5.4). Frost et al. studied the extent of progesterone metabolism in various tissues within the body including the vaginal mucosa, neonatal foreskin, and abdominal skin. The number and percentage of metabolites formed were different at various tissues. Relatively less 5α-reduced metabolites were formed in the abdominal skin (27%) compared to the foreskin (53%) and vaginal mucosa (63%). It was suggested that three other enzymatic pathways might contribute to the metabolism of
progesterone in the skin. These enzymes include 20α-ol-dehydrogenase, 3α-ol- and 3β-ol-dehydrogenases. In another study, a significant amount of urinary 5α-pregnane-3α,20α-diol was reported following the percutaneous administration of progesterone [403], although other 5α-pregnan derivatives previously reported by Frost et al. were not detected. Therefore, it appears that the metabolism of progesterone across the skin is a complex process involving possibly multiple enzymatic pathways. Moreover, the rate and the extent of 5α-reductase metabolism across the skin is variable and depends on the site of application. The current data suggest that peripheral metabolism by the 5α-reductase enzymes is unlikely to significantly affect salivary or serum concentrations of progesterone. Dutasteride slightly enhanced the serum and salivary Cmax, although this was not sufficient to achieve the Cbaseline 30 and Caverage observed during the luteal phase of the menstrual cycle.

A low serum progesterone level can originate from short time of exposure in the systemic circulation and rapid distribution into the saliva. From the saliva, progesterone can enter the gastrointestinal tract and distribute back to the systemic circulation. Extensive metabolism could take place during such transport process. In the current study, the salivary Cbaseline 30 was 30 times the level of progesterone in menopausal women. The salivary Cbaseline 30 increased to 2.90 ng/ml, which was twice the corresponding value in the serum (1.40ng/ml). In premenopausal women salivary progesterone levels have shown to correlate well with total and free serum progesterone levels, with both levels increasing during the second half of the menstrual cycle [430, 431]. As mentioned earlier, the unconjugated salivary progesterone concentration reflects the free hormone in the serum. The transport of progesterone to the saliva is via passive diffusion and is concentration gradient driven [432]. In the present investigation, maximum salivary progesterone concentrations were four times higher than the total serum levels. The median Cmax was 21.45 ng/ml in the saliva as opposed to 5.50 ng/ml in the serum. Furthermore, no correlation was observed between the serum and salivary concentrations of progesterone. Progesterone cannot move against its concentration gradient unless an active transport process is involved. However, no such transporters have been reported [433]. The mechanism for transport of progesterone to the saliva is debatable and not fully understood. Lee et al. have proposed that the transport of progesterone from the blood to the saliva is via binding to the cytocellular and membrane associated components of red cells [101]. However, Koefoed et al. demonstrated that only 15-30% of the total hormone content present in the whole blood is confined to the red blood cells [434]. Moreover, Lewis et al. observed a large intersubject variability in the red cell bound progesterone, with only a
maximum of 0.27 ng/ml binding to the red cell as opposed to 1.1 ng/ml measured in the plasma after 8 weeks of treatment with progesterone cream (40mg daily) [99]. Hermann et al. were the first group to measure the concentration of progesterone in whole blood following the application of the transdermal cream for 12 days [435]. At steady state, maximum whole blood level was < 1 ng/ml. Therefore, the level of progesterone bound to the red blood cell is too low to account for high levels of salivary progesterone observed in the current study. Further studies are required to investigate the mechanism for transport of progesterone to the saliva. Due to high variability in salivary progesterone levels and lack of correlation with serum levels, it has been suggested that salivary progesterone levels cannot be used for monitoring hormone therapy [436-438].

It is hypothesised that the metabolism in the saliva may further contribute to its low plasma levels. The saliva is a mixture of leucocytes, microorganisms, exfoliated epithelial cells and gingival fluid [439]. These components may play a significant role in the metabolism of steroids in the saliva. Elattar has found that progesterone is metabolised to 5α and 5β metabolites in patients with inflamed gingiva [440]. Conversely, the rate of progesterone metabolism in healthy human saliva is low. In effect, the rate of progesterone metabolism is about 9.3 pmol/ml/h in young subjects and decreases to 6.3 pmol/ml/h in menopausal women [439]. Thus, metabolism of progesterone in saliva is also unlikely to influence its plasma concentration.

5.5 Conclusion

Transdermal delivery of progesterone was capable of reaching serum levels ($C_{baseline\ 30}$) usually observed during the follicular phase of the menstrual cycle. However, previous studies have shown that such levels are not adequate to induce secretory changes on proliferative endometrium. No long term studies have been conducted to compare the effect of transdermal progesterone cream on vasomotor symptoms with its plasma and salivary concentrations. The present study suggests that transdermal metabolism of progesterone by 5α-reductase is unlikely to influence its serum or salivary concentrations. Low plasma progesterone levels may be attributed to poor permeation across the skin and rapid distribution of progesterone to the saliva, although no correlation was found between the serum and salivary progesterone levels. Overall, the transdermal absorption of progesterone across the skin is low and highly
variable. While some progesterone was successfully delivered transdermally, in order to achieve higher serum levels, further studies are required to gain a better insight into the mechanism of progesterone delivery across the skin.
6 General Discussion and Future Directions

6.1 General Discussion and Future Directions

The low levels of estrogen and progesterone during the menopausal transition are associated with a wide range of symptoms of which vasomotor hot flushes and night sweat are the most common and troublesome. About 80% of women will experience vasomotor symptoms. Whilst in most women these symptoms will subside within one to two years, for some the symptoms may last for more than three decades. The pathophysiology of hot flushes is not fully understood, although it has been directly linked to the reduction of the thermoregulatory set point due to estrogen deficiency and increase in the central nervous system’s neurotransmitters noradrenaline and serotonin.

The available therapeutic options for management of vasomotor symptoms are classified into three main categories: HRT, non-hormonal therapy, and natural products. Generally, these products are selected on the bases of severity of symptoms and risk benefit assessment. ERT, HRT, and progestins are the cornerstone for management of hot flushes. However, it is now well established that treatment with these agents particularly for > 5 years is associated with increased risk of endometrial cancer, breast cancer and heart disease. Other therapeutic options such as non-hormonal therapy or natural products are either associated with severe adverse effects or lack of therapeutic efficacy.

Bioidentical or natural micronised progesterone is considered as an alternative to its synthetic counterparts due to milder and tolerable side effects. It is manufactured from diosgenin, a compound commonly extracted from Mexican wild yam. The efficacy of progesterone for management of vasomotor symptoms and estrogen induced endometrial hyperplasia has been demonstrated in clinical trials. When taken orally, high doses of up to 300-400 mg/day are required to achieve optimum therapeutic response, as progesterone is extensively metabolised.
in the gut wall and the liver. Transdermal formulations will not only bypass first pass metabolism, but will also allow for reduced drug dose, controlled drug delivery and improved patient compliance.

Although the available clinical data about the efficacy of transdermal progesterone are controversial, it is established that the bioavailability of progesterone cream is low, achieving plasma levels usually observed during the follicular phase of the menstrual cycle. In addition to the physiochemical properties and environmental factors discussed in Chapter 1, the low bioavailability of progesterone is also attributed to the physical (SC) and chemical (5α-reductase enzymes) barriers that it will encounter during its passage to the systemic circulations. By overcoming these barriers, the transdermal bioavailability of progesterone could be significantly improved.

In Chapter 2, it was demonstrated that progesterone displays optimum physiochemical properties for transdermal drug delivery, including a low melting point, although its partition coefficient is slightly higher than the required optimal value of 2-3. High solubility in oils and surfactants further indicated that this drug could be formulated as a lipid based delivery system. To overcome the first transdermal barrier, i.e. the SC, a formulation approach was considered. SMEDDS were investigated for the transdermal delivery of progesterone due to their high dissolving capacity. These systems are described as water free oil and surfactant mixtures, which have the potential to form microemulsions with enhanced thermodynamic activity upon contact with the surface of the skin. The oils and cosurfactant investigated for the development of SMEDDS included Myritol 318, IPM, and Imwitor 308. The oils were selected based on their high solubility for progesterone. Using a series of non-ionic surfactants, it was demonstrated that systems comprising Myritol 318/Tween 85/Imwitor 308/Transcutol P and IPM/Cremophor EL/Imwitor 308 were able to solubilise over 70% water at an oil-surfactant and surfactant-cosurfactant ratio of 1:1. Pseudoternary phase diagrams were then constructed to identify the optimal Km value at which the largest microemulsion area forms. Systems with the largest microemulsion area were then characterised in terms of their internal structure. SMEDDS are known to form o/w, w/o or bicontinuous microemulsions depending on the volume fraction of the aqueous phase. Typically, the release and permeation rate of a lipophilic drug such as progesterone is slower from o/w microemulsions compared to w/o microemulsions, as the drug must first partition in the aqueous phase before overcoming the SC barrier. Therefore, the ideal SMEDDS were
determined as the ones that were able to form w/o microemulsion when diluted with up 15-20% water whilst remaining as a single transparent phase at the skin temperature (32°C). The maximum dilution capacity of the w/o microemulsions was selected based on the water content of the skin, which is about 15-20% of its dried weight. Electrical conductivity and viscosity, together with droplet size analysis and PIT measurements, depict formation of stable transparent w/o microemulsions for up to 15-35% water and at 32°C. It is acknowledged that the water content of the skin is variable and can be affected by age, disease and external environment. Sweating and perspiration may further affect the phase behaviour of the SMEDDS. Overall, there were two limitations in this part of the study. Firstly, characterisation studies were only performed for drug free microemulsions. Incorporation of progesterone into the SMEDDS could influence the phase behaviour of the resulting microemulsions. However, given the lipophilic nature of progesterone and its low aqueous solubility, it is more likely for progesterone to accumulate in the bulk of the formulation rather than the interface. Moreover, several authors have previously investigated the effect of loading dose on the phase behaviour of microemulsions [221, 301, 302, 306]. Whilst slight changes in the electrical conductivity and viscosity were reported, no change in the microemulsion structure or the percolation threshold was noted. Additionally, the phase behaviour of the drug loaded SMEDDS was only considered critical if microemulsions could be formed upon contact with the surface of the skin. This phenomenon was investigated in Chapter 4. The second limitation of this part is that the SMEDDS were not optimised for the effect of dilution with sweat. Although the sweat is mainly composed of water, it may also contain lactate, mineral and urea. The mineral content varies amongst individuals and can be affected by stress and exercise. Such composition could potentially interfere with the phase behaviour of SMEDDS and the percolation threshold. Again, this phenomenon is only considered critical when microemulsions form upon the exposure of SMEDDS to the skin. The microemulsion formation ability of SMEDDS should be further investigated in human volunteers. It would also be interesting to evaluate the effect regional skin variability on the amount of water that could be absorbed into the SMEDDS.

In Chapter 4, the permeation rate of progesterone from SMEDDS, selected commercial formulations, and saturated aqueous solution was investigated. Porcine skin was used in these studies due to its availability and similarities with human skin. Compared to the aqueous solution, SMEDDS increased the permeation rate of progesterone by about 3 to 7 fold. The increased permeation rate can be attributed to two possible mechanisms, the enhanced
thermodynamic activity due to formation of SSME or as the result of oil and surfactant constituents acting as penetration enhancers. Whilst FTIR studies showed that the SMEDDS were able to absorb water from the surface of the skin, subsequent solubility studies indicated that the degree of saturation in the resulting SSME is too low to account for high permeation rate of progesterone from SMEDDS. Moreover, the permeation rate of progesterone from SSME was similar to its corresponding microemulsion. On the other hand, the histological examination of the skin surface indicated that the enhanced permeation rate of progesterone is attributed to the penetration enhancing effects of the oils and surfactants employed. Microscopic evaluation of the skin showed that the effect of SMEDDS on the integrity of the skin is somewhat comparable to that of the aqueous solution. Thus, the selected SMEDDS were deemed to be non-toxic to the skin, although this fact should be further investigated and confirmed in animal models.

SMEDDS can be either directly applied to the surface of the skin covered with non-absorbent dressing or be incorporated into a liquid reservoir patch. This later system may act as a rate limiting membrane to permeation of progesterone and thus its permeation rates should be reassessed. It is also important to monitor the stability of progesterone in SMEDDS in accordance with the ICH guidelines. Given that the progesterone content of the SMEDDS is close to its saturation solubility, changes in the environmental conditions may lead to the precipitation of progesterone. Therefore, it is important to establish the maximum progesterone concentration at which optimal stability over a range of environmental temperatures is achieved.

The primary objective of this thesis was to enhance the transdermal permeation rate of progesterone through overcoming the physical barrier of the skin. Although SMEDDS showed to successfully achieve this objective, the formulations could be optimised to further enhance the permeation rate of progesterone. From the saturation solubility studies, it is known that progesterone will rapidly precipitate when water is added to the SMEDDS. This precipitation can be prevented or delayed by addition of antinucleating polymers, which result in a formation of a transient SSME. It would be also interesting to investigate the effect of supersaturation by the solvent evaporation method on the thermodynamic activity and permeation rate. This method could be particularly useful when the water content of the skin is low and variable.
The second objective of this thesis was to evaluate the effect of transdermal progesterone metabolism on its bioavailability. Due to the variability of the skin enzymes and the lack of suitable techniques for collection of saliva from animal models, the pharmacokinetic studies were directly carried out in postmenopausal women. Dutasteride was selected for the inhibition studies as it is the only FDA approved dual 5α-reductase inhibitor. In the first 15 days of the study, topical dutasteride or a non-ionic cream were applied to the inner right arm. This was followed by application of progesterone (40 mg/day) with or without dutasteride (1mg/day) for a further 15 days. The duration of this study was selected on the bases of the maximum inhibitory effect of dutasteride, which is typically observed after 2 weeks. Subsequently, ELIZA was used to measure the serum and salivary concentration of progesterone in the dutasteride and placebo treated groups before and after the application of the progesterone cream.

The study showed that whilst some progesterone could be delivered transdermally, the bioavailability of progesterone cream was low and variable, achieving serum levels usually associated with the follicular phase of the menstrual cycle. It was also shown that transdermal metabolism of progesterone is unlikely to account for its low plasma levels. The maximum salivary progesterone concentration was four times the serum levels, although no correlations were observed between the two values. Based on these results and results from previous publications, it was concluded that transport of progesterone by red blood cells could not account for the high salivary progesterone concentration.

One of the main limitations of the clinical study was the number of the participants recruited. The initial sample size evaluation indicated that when the sample size in each group was nine a mean difference of 1.5 ng/ml could be detected in the serum. Based on, the standard deviations obtained from the initial pilot study, the sample size was recalculated. It was demonstrated that the sample size of at least 41 subjects per group is required to observe the same mean difference of 1.5 ng/ml. The sample size should further increase to 91 subjects per group in order to detect smaller statistically significant differences. Based on the sample size used for the pilot study, only significant difference of > 3 ng/ml could be detected. This level of detection is considered acceptable for the current study as the aim was to identify if transdermal progesterone metabolism has a major impact on its bioavailability. Furthermore, the pharmacokinetics data only showed minimal differences between the average and baseline serum and salivary concentration of progesterone in the two treatment groups.
Whilst the clinical study only evaluated the serum concentration of progesterone, it would be interesting to monitor the urinary metabolites of progesterone as well as the amount of progesterone bounded to the red blood cells. This, together with analysis of progesterone content in the whole blood, would clarify the mechanism of progesterone transport to the saliva and extent of involvement of red blood cells in this process. No long-term studies have been conducted to compare the effect of transdermal progesterone cream on vasomotor symptoms with its plasma and salivary concentrations. It is not known whether the plasma concentration of progesterone observed in this study displays any therapeutic efficacy. Thus, it would be interesting to evaluate the optimum serum and salivary progesterone levels at which maximum relief from hot flushes is observed. The effect of regional skin variability on the extent and rate of progesterone metabolism could also be further investigated.

### 6.2 Conclusion

From all of the studies performed, it can be stated that SMEDDS can enhance the transdermal permeation rate of progesterone through modification of SC barrier of the skin. On the other hand, the enzymatic barriers of the skin did not significantly influence the transdermal bioavailability of progesterone. Specifically, these studies showed that:

- Imwitor 308 based SMEDDS can form stable transparent w/o microemulsions when diluted with up 20-35% water and at 32°C,

- SMEDDS can increase the permeation rate of progesterone across the skin by up to 7 fold,

- The primary mechanism of action of SMEDDS is due to their oils and surfactants acting as permeation enhancers, and;

- Transdermal metabolism of progesterone by the 5α-reductase enzymes does not account for its low bioavailability.
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Figure A.1: Variation in the electrical conductivity ($\sigma$) with $\phi$ (expressed as %w/w) along the 5:5 DL of Cremophor EL pseudoternary system.
Figure A.2: Variation in the electrical conductivity (σ) with φ (expressed as %w/w) along the 4:6 DL of Tween 85 pseudoternary system.
**Figure A.3**: Variation in the electrical conductivity ($\sigma$) with $\phi$ (expressed as %w/w) along the 4:6 DL of Tween 85 pseudoternary system.
Figure A.4: Percentage area of free water, bound water and trapped water as a function of φ (expressed as %w/w) across the 4:6 DL of the Tween 85 pseudoternary system. Free water represents water molecules interacting with each other, bound water represents the water molecules, which are interacting with the surfactants head group and trapped water represents the non-hydrogen bonded monomers trapped between the surfactant chains. The total water content is the sum of free water, bound water and trapped water.
Figure A.5: Solubility versus φ (expressed as %w/w) for Cremophor EL microemulsions (ME) and SSME.

- represents the saturation solubility curve of progesterone in microemulsions. represents the solubility curve for SSME produced by mixing saturated SMEDDS with water. The ratio of the solubility in SSME to solubility in microemulsion is the degree of saturation. Data are presented as mean ± SD, n=3.