TRANSDERMAL DELIVERY OF PROSTAGLANDINS

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in the
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University of Saskatchewan
Saskatoon

by
Cosmas J. N. Oguejiofor

Fall 1996

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SUMMARY OF DISSERTATION
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DEGREE OF DOCTOR OF PHILOSOPHY

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ABSTRACT

Transdermal delivery of Prostaglandin E₁ (PGE₁) would represent a significant improvement over the currently available injectable dosage forms in terms of reduced side effects and improved patient acceptance and compliance with therapy. PGE₁ is a vasodilator and smooth muscle relaxer which is used in the treatment of erectile dysfunction and peripheral arterial occlusive disease. Human skin presents a significant barrier to most drugs which must be overcome for transdermal delivery to be achieved. Liposomes are novel drug delivery vehicles which can enhance transdermal delivery. The specific objectives of the thesis were therefore to develop a stable liposomal transdermal PGE₁ formulation and to evaluate the potential for transdermal delivery of PGE₁ by liposome encapsulation.

Formulation optimization studies were carried out using nonliposomal and liposomal vehicles containing varying proportions of natural and synthetic lipids, viscosity enhancers, and skin penetration enhancers. Transdermal delivery profiles of the test formulations were determined using an in vitro flow-through diffusion cell model. Physical stability analyses of the liposomal formulations were obtained by carrying out organoleptic, microscopic, and encapsulation efficiency/leakage studies. Chemical stability analyses of PGE₁ were conducted at 4°C and 37°C using novel high pressure liquid chromatography assays that were developed in the course of the thesis work. The in vitro tissue homogenate model was used to determine the metabolism of PGE₁ in human foreskin, human placenta and rabbit lung being positive controls. In vivo transdermal PGE₁ delivery studies were conducted by using (i) laser Doppler flowmetry in healthy volunteers and (ii) color Doppler ultrasonography/clinical trials in patients with erectile dysfunction, to monitor pharmacological and/or clinical effects following topical application of PGE₁.

The in vitro and in vivo transdermal delivery studies confirmed that PGE₁ can be delivered through the skin using liposomes. Metabolism of PGE₁ in human foreskin is
low and is not likely to limit the transdermal delivery of PGE$_1$. More research is warranted in order to translate the thesis findings into a commercial transdermal PGE$_1$ product.
ACKNOWLEDGEMENTS

I wish to acknowledge with gratitude the contributions of my supervisor, Dr. M. Foldvari, and other members of my advisory committee, Dr. T. Wilson, Dr. S. Wallace, Dr. D. Gorecki, Dr. S. Afridi, Dr. E. Hawes, and Dr. T. Kudel. Their enthusiasm and willingness to lend their expertise during the various stages of the research were crucial to the successful completion of this thesis.

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I wish to acknowledge my parents, Omeile and Mary Oguejiofor, and siblings, Osita, Emeka, Okehruku, Ogonna, and Ngozi. Their love and support through the years have been invaluable to me and I dedicate this thesis to them.

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<tr>
<td>ACN</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>AHH</td>
<td>Aryl hydrocarbon hydroxylase</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>&quot;AUC&quot;</td>
<td>Area under the response-time curve</td>
</tr>
<tr>
<td>A.U.F.S.</td>
<td>Absorbance units full scale</td>
</tr>
<tr>
<td>C.V.</td>
<td>Coefficient of Variation</td>
</tr>
<tr>
<td>D</td>
<td>Diffusion coefficient</td>
</tr>
<tr>
<td>DHA</td>
<td>Dehydroepiandrosterone</td>
</tr>
<tr>
<td>DMA</td>
<td>Dimethylacetamide</td>
</tr>
<tr>
<td>DMF</td>
<td>Dimethylformamide</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
</tr>
<tr>
<td>DPPC</td>
<td>Dipalmitoylphosphatidylcholine</td>
</tr>
<tr>
<td>ED</td>
<td>Erectile Dysfunction</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene diamine tetraacetic acid</td>
</tr>
<tr>
<td>EIVs</td>
<td>Ether injection vesicles</td>
</tr>
<tr>
<td>15-OHPGDH</td>
<td>15-hydroxyprostaglandin dehydrogenase</td>
</tr>
<tr>
<td>FPVs</td>
<td>French press vesicles</td>
</tr>
<tr>
<td>GIT</td>
<td>Gastrointestinal tract</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric acid</td>
</tr>
<tr>
<td>HSH</td>
<td>Human skin homogenate</td>
</tr>
<tr>
<td>HPH</td>
<td>Human placenta homogenate</td>
</tr>
<tr>
<td>HPLC</td>
<td>High pressure liquid chromatography</td>
</tr>
<tr>
<td>H₂O</td>
<td>Water</td>
</tr>
<tr>
<td>H₂O₂</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>H₂PGE₁</td>
<td>13, 14-dihydroprostaglandin E₁</td>
</tr>
<tr>
<td>I.D.</td>
<td>Internal diameter</td>
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Ka  
Dissociation constant

KM  
Substrate concentration at half $V_{\text{max}}$

KPGE$_1$  
15-keto-prostaglandin E$_1$

KH$_2$PGE$_1$  
15-keto-13,14-dihydroprostaglandin E$_1$

Kp  
Partition coefficient

L  
Lag time

LSC  
Liquid scintillation cocktail

LUVs  
Large unilamellar vesicles

MLVs  
Multilamellar vesicles

mp  
Melting point

mV  
millivolts

MW  
Molecular weight

NaCl  
Sodium chloride

NAD$^+$  
Nicotinamide adenine dinucleotide (oxidized form)

NADH  
Nicotinamide adenine dinucleotide (reduced form)

NADP$^+$  
Nicotinamide adenine dinucleotide phosphate (oxidized form)

Na$_2$H$_2$PO$_4$  
Monosodium phosphate

Na$_2$HPO$_4$  
Disodium phosphate

N.L.  
Nonliposomal formulation

NMP  
N-methyl-2-pyrrolidone

OsO$_4$  
Osmium tetroxide

P  
Permeability coefficient

PBS  
Phosphate buffered saline

PG  
Prostaglandin (PGE = Prostaglandin E series)

PGA$_1$  
Prostaglandin A$_1$

PGA$_2$  
Prostaglandin A$_2$

PGB$_1$  
Prostaglandin B$_1$
PGB₂  Prostaglandin B₂  
PGE₁  Prostaglandin E₁  
PGE₂  Prostaglandin E₂  
PGF₂ₐ  Prostaglandin F₂ₐ  
2P  2-pyrrolidone  
RCFs  Relative centrifugal forces  
REVs  Reversed phase vesicles  
RLH  Rabbit lung homogenate  
RP  Reversed phase  
dpm  Disintegrations per minute  
rpm  Revolutions per minute  
SB  Stratum basale  
SBF  Skin blood flow  
SC  Stratum corneum  
SG  Stratum granulosum  
SS  Stratum spinosum  
SUVs  Small unilamellar vesicles  
TEM  Transmission electron microscopy  
TFA  Trifluoroacetic acid  
³H  Tritium radioactive label  
Tₘ  Phase transition temperature  
u.v.  Ultraviolet  
Vₘₐₓ  maximum velocity of enzyme reaction  
VSS  Visual sensory stimulation  
v/v  volume/volume ratio
Chapter One

INTRODUCTION

1.1 Skin

The skin is the largest and most external organ of the body and hence provides a large surface area for drug application. In most adults, the skin weighs approximately 2 kg and has a surface area of about 2 m².

1.1.1 Skin Structure

The skin is composed of two main layers - the epidermis and the dermis - supported in most cases on a subcutaneous fatty layer. Interspersed in these layers are skin appendages such as hair follicles and their associated sebaceous glands, and the sweat glands (eccrine and/or apocrine).

The epidermis is the avascular outer layer of the skin and is made up of four different cell types - keratinocytes, melanocytes, Langerhans cells, and Merkel cells. The keratinocytes are the most numerous, accounting for approximately 80% of the epidermis, and contain fibrous water-insoluble proteins (keratins). The epidermis is divided into five layers - stratum basale, stratum spinosum, stratum granulosum, stratum lucidum and stratum corneum. The division is based on the distinguishing characteristics of the keratinocytes in each layer. The stratum basale and stratum spinosum are grouped together and termed the malphigian layer by some authors. The stratum basale (stratum germinativum) makes contact with the dermis and is the layer from which keratinization is initiated. The terminal differentiation of the keratinocytes leads to the production of the
stratum corneum. The stratum corneum is the outer, horny layer of the epidermis consisting of dead keratinized cells (corneocytes). The corneocytes are usually stacked in layers that are 10 to 25 cells thick and are separated by ceramides, free fatty acids, cholesterol and other lipids. In general, the water content of the stratum corneum at normal relative humidities is believed to be about 20% (Roberts and Walker, 1993). The stratum corneum in its native state is capable of absorbing up to three times its weight of water. The outer stratum corneum layers are continuously sloughed off (desquamation) and replenished by cells from the lower layers. It is estimated that the turnover time for stratum corneum cells is about 14 days. The stratum corneum is generally considered to be the primary barrier of the skin (Marzulli, 1962; Kligman, 1983; Noonan and Wester, 1989; Landman, 1988; Magee, 1991). The stratum corneum is approximately 10 to 20 μm thick while the viable epidermis is about 100 μm thick. There is considerable variation in the thickness of the epidermis over various regions of the body and also between individuals. The melanocytes are responsible for the production of melanin which determines skin color, the Langerhans cells are involved in immunogenesis, and the Merkel cells are presumed to function as mechanoreceptors (Leeson et al., 1985).

The dermis is usually between 3 to 5 mm in thickness. Unlike the epidermis, it is vascularized and is largely acellular. It is composed of dense connective tissue made up of structural fibrous proteins (collagen and elastin) encased in a mucopolysaccharide ground substance. Collagen is the major component, accounting for 75% of the dermis (Flynn, 1990). The dermis may be divided into an upper thin papillary layer which interfaces with the epidermis, and a lower reticular layer which interfaces with the subcutis. The cell types seen in the dermis include the fibroblasts which are responsible for generating the fibrous proteins and ground substance, and the mast cells and macrophages which are involved in inflammatory reactions (Franz et al., 1992).

The appendages are embedded in the ground substance of the dermis and consist of the sweat glands (eccrine and apocrine) and the hair follicles which are associated with
sebaceous glands. The eccrine glands are the major sweat glands and occur practically all over the body while the apocrine sweat glands are limited to a few areas of the body; e.g., axilla and anogenital areas. The sebaceous glands produce sebum which aids in moisturizing/plasticizing the skin. The skin appendages account for only about 0.1% of the total skin surface area and are therefore considered to be relatively unimportant as portals of diffusion for most drugs. In addition, the pilosebaceous glands and sweat glands are often actively secreting substances making significant passive drug transport through them even more improbable.

Skin characteristics vary greatly between persons and at different sites in the same individual. The age of the individual, environmental conditions, chemical exposure, and clinical status of the skin (diseased or healthy) all influence the skin properties.

1.1.2 Nature and Importance of Skin Lipids

The skin contains a variety of lipid substances which account largely for its structural and barrier integrity. Changes occur in the lipid composition of the epidermis as the cells undergo progressive keratinization. In general, polar lipids such as the phospholipids predominate at the lower layers while the neutral lipids, ceramides, and free fatty acids predominate at the upper layers (Landmann, 1988; Wertz, 1992; Franz et al., 1992). This differential distribution of lipids in the skin explains why lipophilic drugs can more easily penetrate the stratum corneum since it is characterized by the virtual absence of the polar phospholipids and consists of the more lipophilic ceramides, 40 - 50%; cholesterol, 20 - 27%; and free fatty acids, 10 - 25% of total lipids (Wertz, 1992). The fatty acids in the epidermis are mostly C16 and C18 compounds although C12 to C24 compounds are also present (Franz et al., 1992). Phospholipids which have been identified in the epidermis include phosphatidylcholine (lecithin), phosphatidylethanolamine, sphingomyelin, lysolecithin, phosphatidylserine, phosphatidylinositol, phosphatidic acid, lysophosphatidylethanolamine, cardiolipin, and plasmalogens (Franz et al., 1992; Wertz,
1992). Cholesterol sulfate is only a minor component of the stratum corneum but it has been suggested to be important in corneocyte cohesion (Ziboh and Chapkin, 1988).

1.1.3 Skin Functions

The primary function of the skin is as a protective covering for the internal organs of the body, limiting the entry of potentially harmful substances from the exterior while ensuring body fluids and tissues are conserved. This barrier function of the skin was once considered to be absolute but the skin is now recognized to be permeable to some degree (Scheuplein and Blank, 1971; Kligman, 1983). With the realization that the barrier properties of the skin can be breached, came the conceptualization of the skin as a route of drug delivery for the treatment of systemic diseases. Based on this concept of transdermal drug delivery, several drug products are already available commercially; e.g., nitroglycerine, scopolamine, nicotine, estradiol, fentanyl, isosorbide dinitrate and clonidine.

The skin also has an excretory function. Various waste substances are eliminated from the body via the skin appendages. Other functions of the skin include the regulation of body temperature; detection of external stimuli such as pain; synthesis and metabolism of both endogenous and exogenous substrates.

1.1.4 Skin Metabolism

1.1.4.1 Role of Skin Metabolism

Contrary to some studies which tended to treat drug absorption into and through the skin as a purely physicochemical, diffusion process (Scheuplein, 1965; Scheuplein, 1976; Bronaugh et al., 1982; Franz, 1983), skin metabolism is now considered an important contributory factor to the extent of drug penetration into the skin for many drugs (Pannatier et al., 1978; Mukhtar and Bickers, 1981; Kappus, 1989; Tauber, 1989; Nicolau and Yacobi, 1989 - 90, Ademola et al., 1992). In general, the liver is considered the most
important organ for metabolism of drugs (Pannatier et al., 1978; Kappus, 1989). Previous estimates of some enzyme activity levels in the skin have shown these to be relatively low when compared to the liver (Noonan and Wester, 1989). Although skin enzymatic activity levels are relatively low, the extensive surface area and higher weight of the skin when compared to the liver imply that skin metabolism may play an important role particularly for topically applied drugs or drugs with target sites in the skin. Most cutaneous enzyme activity studies are done with whole skin homogenates and considering that the epidermis makes up only about 2.5 to 3% of the whole skin, actual enzyme activities in the epidermal layer may be grossly underestimated and in some cases may approach or exceed hepatic enzyme activities; e.g., N-acetyltransferase (Noonan and Wester, 1989; Bronaugh and Collier, 1991).

Metabolism in the skin influences the amount and rate of penetration of topically applied drugs. The skin is capable of metabolically altering the drug and/or components of the vehicle used in delivering the drug to the skin. Metabolism in the skin can play a role in limiting the systemic toxicity of topically applied drugs; e.g., benzoyl peroxide, a topical antiacne agent is metabolized to benzoic acid in the skin (Nachter et al., 1981). In the design of prodrugs; i.e., inactive drugs which are bioactivated upon administration, metabolism in the skin can be used to generate the active moiety; e.g., viprostol, a synthetic prostaglandin E₂ (PGE₂) analogue, is hydrolyzed by skin esterases to the active moiety (Nicolau and Yacobi, 1989-90). Hence, drug metabolism in the skin may enhance or reduce the pharmacologic and/or toxicologic effect of the applied drug while a lack of drug metabolism in the skin will ensure that transdermal delivery is purely a diffusional process depending primarily on drug absorption across the stratum corneum. Knowledge of the metabolic profile of a drug within the skin is invaluable in product design in order to ensure therapeutic success.
1.1.4.2 Metabolic Capacity of the Skin

The biotransformation reactions possible in the skin include phase I (functionalization) and phase II (conjugation) reactions as outlined in Table 1.1.

**Table 1.1** Metabolic reactions in the skin (Tauber, 1989; Kao and Carver, 1991).

<table>
<thead>
<tr>
<th>PHASE I REACTIONS</th>
<th>PHASE II</th>
</tr>
</thead>
<tbody>
<tr>
<td>OXIDATION</td>
<td>CONJUGATIONS</td>
</tr>
<tr>
<td>of alcohols</td>
<td>Glucuronide formation</td>
</tr>
<tr>
<td>of aliphatic C atoms</td>
<td></td>
</tr>
<tr>
<td>of alicyclic C atoms</td>
<td>Sulfate formation</td>
</tr>
<tr>
<td>of aromatic rings</td>
<td></td>
</tr>
<tr>
<td>deamination</td>
<td>Methylation</td>
</tr>
<tr>
<td>dealkylation</td>
<td></td>
</tr>
<tr>
<td>REDUCTION</td>
<td>Glutathione conjugation</td>
</tr>
<tr>
<td>carbonyl reduction</td>
<td></td>
</tr>
<tr>
<td>C = C reduction</td>
<td></td>
</tr>
<tr>
<td>HYDROLYSIS</td>
<td></td>
</tr>
<tr>
<td>ester hydrolysis</td>
<td></td>
</tr>
<tr>
<td>epoxide hydrolysis</td>
<td></td>
</tr>
</tbody>
</table>
The skin is capable of metabolizing the alcohol group in many drugs; e.g., hydrocortisone can be oxidized to cortisone, testosterone to \( \Delta^4 \)-androstene-3,17-dione and estradiol to estrone (Wotiz et al., 1956; Weinstein et al., 1968; Pannatier et al., 1978). The enzyme responsible for these oxidations is 17\( \beta \)-hydroxysteroid dehydrogenase (Noonan and Wester, 1989). Prostaglandins have been shown to be metabolized in rat skin via a cytosolic enzyme, 15-hydroxyprostaglandin dehydrogenase (Camp and Greaves, 1980; Fincham and Camp, 1983). Metabolism of dibenz(a)anthracene in skin occurs via hydroxylation of an aliphatic carbon atom (Pannatier et al., 1978). Faredin et al. (1969) demonstrated 7-hydroxylation of dehydroepiandrosterone (DHA) on incubation with human skin slices. Benzo(a)pyrene, a polycyclic aromatic hydrocarbon, is oxidized in the skin to three main classes of compounds - phenols, quinones and dihydrodiols (Pannatier et al., 1978) due mainly to the action of the enzyme aryl hydrocarbon hydroxylase (AHH). Polycyclic aromatic hydrocarbons induce skin carcinomas and elucidation of the skin metabolic pathway may prove useful in isolating the actual carcinogen (Noonan and Wester, 1989). Examples of deamination reactions in the skin include the oxidation of norepinephrine to dehydroxymandelic acid, catalyzed by monoamine oxidase, and oxidation of vidarabine to 9\( \alpha \)-d-arabinofuranosylhypoxanthine, catalyzed by adenosine deaminase (Ando et al., 1977; Noonan and Wester, 1989). Dealkylation of 7-ethoxycoumarin to hydroxycoumarin, catalyzed by a mixed function oxidase, has been demonstrated in mouse skin (Pohl et al., 1976; Kappus, 1989).

Carbonyl reduction is employed in the metabolic pathway of various drugs in the skin; e.g., 7-ketoDHA may be reduced to 7\( \alpha \)-hydroxyDHA and the 20-oxo group of hydrocortisone to a secondary alcohol (Pannatier et al., 1978; Noonan and Wester, 1989). The allodihydrocortisol metabolite of hydrocortisone is obtained via C = C reduction. Testosterone is also reduced to 5\( \alpha \)-dihydrotestosterone by a similar mechanism (Pannatier et al., 1978).
Diflucortolone valerate is hydrolyzed rapidly in human skin to diflucortolone and valeric acid (Noonan and Wester, 1989). Amethocaine is hydrolyzed by skin esterases to p-(butylamino) benzoic acid and dimethylaminoethanol (Woolfson et al., 1990). Ester hydrolysis is implicated in the metabolism of most topically administered corticosteroids: 17-esters are resistant while 21-esters are quite easily hydrolyzed by skin esterases (Tauber, 1989). Epoxide hydrolase is responsible for the metabolism of benzo(a)pyrene 4,5-oxide to benzo(a)pyrene 4,5-diol.

The skin contains UDP-glucuronyl transferase, the enzyme required for glucuronidation. As earlier noted, benzo(a)pyrene is oxidized by skin tissues via AHH. The hydroxylated derivative is then conjugated with glucuronic acid yielding a mixture of glucuronide-conjugated benzpyrenols (Noonan and Wester, 1989). Glucuronidases, enzymes which could split glucuronide conjugates, are also present in the skin (Kappus, 1989).

Glutathione-S-transferase, the enzyme required for glutathione conjugation is also present in the skin and has been implicated in the metabolism of polycyclic aromatic hydrocarbons. Sulfate conjugation, catalyzed by sulfotransferase, has been demonstrated in human skin although this appears to be a relatively minor metabolic route in the skin (Noonan and Wester, 1989; Kappus, 1989). Catechol-O-methyltransferase catalyzes the methylation of norepinephrine to normetanephrine in skin tissues (Pannatier, 1978).

### 1.1.4.3 Factors Influencing Skin Metabolism

The metabolic capacity of the skin is influenced by the degree of exposure of the substrate to the enzymes, the residence time of substrate in the skin and any factor which may affect the levels of enzymes in the skin; e.g., sex, age, skin condition, race, enzyme inducers and inhibitors.

The activity of AHH is reduced in the skin of psoriatics (Chapman et al., 1977) and skin metabolism of testosterone is increased in acne patients (Sansone and Reisner, 1971).
A number of topical agents are capable of inducing activity of certain enzymes; e.g., the application of 3-methylcholanthrene increases skin concentration of cytochrome P450 (Pohl et al., 1976), benz(a)anthracene induces AHH activity in human skin (Pannatier et al., 1978), and benzo(a)pyrene increases UDP-glucuronyltransferase activity in the skin (Dutton and Stevenson, 1962). On the other hand, 7,8-benzoflavone inhibits AHH activity (Noonan and Wester, 1989). Epoxide hydrolase and glutathione-S-transferase do not appear to be inducible in the skin to any great extent (Kappus, 1989). The activation and detoxification of drugs in the skin can thus be affected by a drug metabolizing enzyme inducer or inhibitor. The degree of exposure of a drug to enzymes in the skin is in part determined by how long it remains in the skin and this is a function of the drug structure, partition coefficient and properties of the vehicle in which the drug is formulated. Encapsulation of a drug into special delivery vehicles like liposomes could protect the drug from metabolism in the skin by limiting its exposure to enzymes in the skin.

1.1.4.4 Distribution of Enzymes in the Skin

The viable epidermis is considered to be the most metabolically active layer of the skin containing the highest concentration of enzymes (Laerum, 1969; Magee, 1991). The stratum corneum cells are not viable and little or no enzymatic activity occurs in this layer. The contribution of the dermis to cutaneous metabolism is considered minor for most compounds since it is expected that drugs which penetrate through to the dermis are rapidly taken up by the microcirculation. This may not however be true for lipophilic compounds where residence time in the aqueous dermal matrix may be prolonged and especially if the target site for the drug is the deeper layers of the skin rather than the systemic circulation. It is also important to note that despite its lower concentration of enzymes, the greater size of the dermis in relation to the viable epidermis may make metabolism in the dermis significant. Finnen et al. (1985) reported that monoxygenase activity was highest in the dermis. The contribution of the skin appendages to cutaneous metabolism of drugs has not
been rigorously characterized but it would be safe to assume that it is minor considering that they make up only about 0.1% of the total skin area and are known to play very little role in the transdermal delivery of most drugs.

In terms of subcellular localization, enzymes are present in the various subcellular fractions of the skin; e.g., the microsomes, cytosol, etc. (Camp and Greaves, 1980; Finnen et al., 1985).

1.1.4.5 Microbial Metabolism in the Skin

A myriad of microbes can be found on the skin but the most commonly encountered microbes include the *Brevibacterium*, *Corynebacterium*, *Propionibacterium*, *Micrococcus*, *Pityrosporum*, and *Staphylococcus* spp. (Denyer and McNabb, 1989). A wide range of enzymes are associated with the skin microflora; e.g., the lipases (esterases) produced by the Micrococcaceae and Staphylococci can result in ester hydrolysis of the applied drug product. Cutaneous microbes can metabolize topically applied nitroglycerin (Kao and Carver, 1991). Microbial metabolism does not, however, appear to be a significant factor in transdermal drug delivery if the microbial population is within normal limits.

1.2 Transdermal Delivery

1.2.1 Definition and Comparison with other Drug Delivery Routes

Transdermal delivery is the transport of drugs (e.g. prostaglandins) through the skin to elicit systemic effects via uptake by the skin microcirculation and lymphatics and local effects on target sites directly beneath the skin. This is in contrast to topical drug delivery where the target site is the surface and/or deeper layers of the skin although the mode of administration; i.e., application of the drug product to the skin surface is the same.

An ideal drug delivery system should be capable of delivering the drug in therapeutic amounts to the target site(s) only. Unfortunately, no such system is currently available because the available drug delivery systems - oral, injectable, inhalation, topical, and
transdermal are limited to some degree in their ability to deliver the drug effectively and selectively. For disease conditions in which the target site is directly below the skin, a transdermal delivery system is advantageous because delivery to other sites in the body and therefore the incidence of adverse effects is minimized. Transdermal drug delivery is noninvasive and can be employed to provide a steady concentration in the blood stream thus eliminating the need for more frequent dosing and improving patient compliance and therapy outcomes in chronic conditions. One major advantage of transdermal delivery is the avoidance of first pass metabolism in the liver or in the gastrointestinal tract (GIT). Cutaneous first pass metabolism is often not significant but, as mentioned earlier, this possibility cannot be ignored in transdermal drug delivery. The ability to overcome the considerable barrier properties of the skin effectively and safely is the major obstacle to transdermal drug delivery.

1.2.2 Factors Affecting Transdermal Drug Delivery

Factors influencing transdermal drug delivery can be divided into three general categories: (a) drug related factors (b) skin related factors and (c) vehicle related factors (Table 1.2).

Most drug compounds are solid in nature but crystalline or particulate material is very poorly absorbed through the skin. Hence, every transdermal product consists of the active drug(s) and a vehicle; i.e., the medium in which the drug is packaged and administered and which stabilizes the drug during storage, improves patient acceptability, and optimizes drug delivery through the skin.

1.2.2.1 Drug Related Factors Affecting Transdermal Drug Delivery

Transdermal drug delivery is dependent primarily on the properties of the drug. Particle size distribution, polymorphism, and solubility in the chosen vehicle are important since in order to be absorbed, the drug must be presented to the skin surface in molecular
dimensions or less. Only the soluble drug fraction diffuses within and out of a vehicle. Dissolution of suspended drug particles must occur prior to absorption into the skin. The dissolution rate, \( \frac{dC}{dt} \), is largely determined by the drug solubility and particle size (Eq. 1.1).

Table 1.2 Factors affecting transdermal drug delivery

<table>
<thead>
<tr>
<th>(a) Drug Related Factors:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solubility, Melting point, Stability</td>
</tr>
<tr>
<td>Partition coefficient (Kp), Diffusion coefficient (D)</td>
</tr>
<tr>
<td>Ionization state, Polymorphism</td>
</tr>
<tr>
<td>Potency, Applied dose</td>
</tr>
<tr>
<td>Molecular size, Particle size</td>
</tr>
<tr>
<td>Skin sensitization/irritation</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>(b) Skin Related Factors:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metabolism</td>
</tr>
<tr>
<td>Barrier damage, Occlusion</td>
</tr>
<tr>
<td>Biological variations:</td>
</tr>
<tr>
<td>- race, sex, age, anatomic region</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>(c) Vehicle Related Factors:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Drug release from vehicle</td>
</tr>
<tr>
<td>Use of penetration enhancers:</td>
</tr>
<tr>
<td>- chemical enhancers</td>
</tr>
<tr>
<td>- physical enhancers</td>
</tr>
<tr>
<td>- novel dosage forms</td>
</tr>
</tbody>
</table>

\[
\frac{dC}{dt} = KA (C_s - C_l)
\]  

where

- \( A \) = surface area of the particles
- \( C_s \) = solubility; i.e., concentration of a saturated solution
- \( C_l \) = concentration of drug in solution at time, \( t \)
- \( K \) = dissolution rate constant
Reduction in particle size leads to increased surface area and enhanced dissolution rate.

The diffusion of drugs through the skin is inversely related to molecular size and it has been suggested that the upper molecular size limit for transdermal absorption is about 1000 daltons with a few exceptions (Brown and Langer, 1988). Certainly, the use of novel transdermal delivery tools such as liposomes, electroporation, iontophoresis and sonophoresis will increase the possibility of transdermal delivery of relatively large molecules including proteins. In the case of ionic compounds, the nonionized species is preferably absorbed through the skin hence the dissociation constant (Ka) of the drug and pH of the vehicle are critical. The partition coefficient is a measure of the relative solubilities of a drug in hydrophilic vs hydrophobic media and is usually determined experimentally using water and octanol solvents. Since the stratum corneum is predominantly lipophilic while the lower skin layers are aqueous, a transdermal drug delivery candidate should possess intermediate solubility in water or oil. This will enable the drug to adequately penetrate the stratum corneum and also be able to partition into the dermis. In general, lipophilic drugs penetrate the skin better (Cooper, 1984). The diffusion coefficient (D) is a measure of the rate of drug diffusing per unit area of absorbing surface and may be calculated from Eq. 1.2 below (Krowczynski, 1987).

\[
D = \frac{m^2 \cdot \pi}{t \cdot (2C_0)^2 \cdot A^2} \quad [\text{cm}^2 / \text{sec}]
\]  (1.2)

where m = amount of the diffused drug (moles)
\( t \) = time(s); \( A \) = diffusion surface (cm²)
\( C_0 = q \cdot x/M \) (mol/cm³) :- q = dosage form density (g/cm³);
\( x = \) amount of drug (g/g) and \( M = \) relative molecular mass.

Transdermal drug delivery is contraindicated for those drugs with significant skin irritation potential. Despite the relatively large surface area of the skin, transdermal drug application is usually to a limited area of the skin because of cosmetic reasons, patient
acceptability/convenience, and the minimization of side effects. The potency of the drug is a crucial factor in determining its suitability for transdermal delivery since drug absorption is directly proportional to the area of application and it is unlikely that adequate transport of a low potency drug can be achieved through a limited area of the skin without permanently altering the skin barrier layer.

1.2.2.2 Skin Related Factors Affecting Transdermal Drug Delivery

Skin metabolism can be limiting to transdermal drug delivery. Any factor which alters the condition of the skin can affect transdermal drug delivery. Occlusion of the skin with its consequent increase in skin hydration levels has been shown to increase the permeation of drugs; e.g., steroids into the skin (Mckenzie, 1962; Mckenzie and Stoughton, 1962). Diseases such as psoriasis and ichthyosis can increase the permeability of the skin. Environmental insults; e.g., extreme temperatures, physical and chemical damage can also affect transdermal delivery. Interracial differences in skin permeability have been reported (Weingand et al., 1974; Wester and Maibach, 1992). Differences in skin permeability are known to exist at different body sites in the same individual (Wester and Maibach, 1989). Sex and age are considered to be relatively unimportant factors in transdermal delivery except for the preterm neonate where the skin barrier properties are underdeveloped.

1.2.2.3 Vehicle Related Factors Affecting Transdermal Drug Delivery

Vehicles affect transdermal delivery by optimizing the rate of drug delivery to the skin via solubilization, altering the barrier layer of the skin, and/or stabilizing the drug during storage and in some cases during transport through the skin. Several authors have reported the effects of vehicle composition on the transport of drugs through human and animal skin using in vitro and in vivo techniques (Fullerton et al., 1988; Seki et al., 1991; Woolfson et al., 1992; Naik et al., 1993; Roy et al., 1995).
1.2.2.3.1 Drug Release From Vehicle

The effective drug concentration (or thermodynamic activity) in the vehicle determines the rate and amount of drug released from the vehicle onto the skin in an absorbable form. Increasing the concentration of the drug in solution in the vehicle can increase transdermal delivery from dosage forms. The thermodynamic activity of a drug in the vehicle is highest in saturated solution or suspension formulations. Higuchi (1960, 1961) developed equations to describe drug release from suspensions (Eqs. 1.3 and 1.4) and solutions (Eq. 1.5).

\[ Q = \sqrt{2ADCs} t \]  
\[ \frac{dQ}{dt} = \sqrt{\frac{A\ D\ Cs}{2t}} \]  

where \( Q \) = amount of drug released at time \( t \) per unit area
\( dQ/dt \) = rate of drug release
\( A \) = total drug concentration
\( D \) = diffusivity of the drug (cm²/s)
\( Cs \) = drug solubility

\[ Q = hC_0 \left[ 1 - \sum_{m=0}^{\infty} \frac{8}{\pi^2} \frac{1}{(2m + 1)^2} e^{-\frac{D(2m + 1)^2 \pi^2 t}{4h^2}} \right] \]  

where \( Q \) = amount of drug released / unit area of application
\( h \) = thickness of the applied phase
\( C_0 \) = initial drug concentration
\( D \) = drug diffusivity in the vehicle
\( t \) = elapsed time after application
\( m \) = integer with limits of 0 to \( \infty \)
When $Q \leq 30\%$ of initial drug content, Eq. 1.5 can be reduced to Eqs. 1.6 and 1.7 (Katz and Poulsen, 1972).

\[ Q = 2C_0 \sqrt{\frac{Dt}{\pi}} \quad (1.6) \]

and \[ \frac{dQ}{dt} = C_0 \sqrt{\frac{D}{\pi t}} \quad (1.7) \]

From the mathematical relationships above, it is evident that drug release from transdermal formulations is dependent on the drug concentration, solubility, and diffusivity. Surfactants, complexing agents, cosolvents, and pH are variables that can modify the solubility profile of the drug. Drug diffusivity in the vehicle is directly proportional to the temperature and inversely proportional to viscosity of the medium and the molecular size of the particles. It is important to note that although several assumptions are implicit in Eqs. 1.3 to 1.7, some of which may not always be valid, these are relatively simple equations that help to characterize the drug delivery process. Stability of the active ingredient in the vehicle is essential to the maintenance of optimum drug concentration gradients.

1.2.2.3.2 Chemical Enhancers

Chemical enhancers are substances which act on the skin to lower its barrier properties and enhance the penetration of topically applied products. In order to understand the mechanisms of action of skin penetration enhancers, it is important to define the routes of transport through the skin. The two possible routes of drug transport across the skin are through the bulk of the stratum corneum and through the appendages. The first route involves transport across the corneocytes and surrounding lipid channels and is the primary route of transdermal drug delivery. The transappendageal route is subdivided into the
transfollicular and transeccrine routes and is ordinarily not significant in transdermal
delivery. As indicated earlier, the stratum corneum is considered to be the primary barrier
layer for transdermal delivery. The prevailing schematic model for the stratum corneum is
the brick and mortar model (Elías, 1983) where the keratinocytes - the bricks - are stacked
in layers surrounded by a lipid 'mortar'. Based on this model there are two major
pathways through which drugs penetrate the skin namely (i) the transcellular pathway; i.e.,
through the keratinocytes and lipid portions in series and (ii) the intercellular pathway; i.e.,
through the lipid or nonpolar region. Since the keratinocytes are polar, the two pathways
can be defined as polar/nonpolar and nonpolar respectively. Elías (1983) was able to prove
that the lipoidal pathway is the major barrier to transdermal delivery. This validates the
accepted view that lipophilic substances generally penetrate the skin better.

Penetration enhancers can increase transdermal delivery by acting on the polar and/or
nonpolar pathways in the skin. The various mechanisms by which chemical enhancers act
include (i) hydration of the skin (ii) fluidization of the lipid bilayer (iii) dissolution of skin
lipids (iv) denaturation/solvation of skin proteins and (v) increased partitioning of drug into
skin due to the solvent action of a chemical enhancer as it penetrates the skin. These
mechanisms are summarized in the lipid protein partitioning theory (Barry, 1988) which
characterizes enhancer effect on lipid/protein components of the skin and partitioning of the
drug into the skin.

Several natural and synthetic chemical agents have been evaluated for use as penetration
enhancers. These include water; alcohols such as ethanol, propylene glycol, glycerol;
dimethylsulfoxide (DMSO); dimethylformamide (DMF); dimethylacetamide (DMA);
pyrrolidones such as N-methyl-2-pyrrolidone (NMP) and 2-pyrrolidone (2P); surfactants
such as sodium lauryl sulfate; fatty acids and alcohols such as oleic acid and oleyl alcohol;
terpene derivatives such as 1, 8-cineole (eucalyptol); urea and urea analogues such as 1, 3-
didodecylurea; and calcium thioglycolate (Barry, 1993).
An ideal enhancer should be effective, cosmetically acceptable, pharmaceutically compatible with other formulation ingredients, nontoxic to the skin, and alter skin permeability in a unidirectional way; i.e., enhance transdermal drug delivery but leave the containment functions of the skin essentially intact. These stringent requirements have effectively constrained some enhancers to the realm of research only; e.g., dimethylsulfoxide (DMSO) is toxic to the skin and produces an odorous metabolite despite its efficacy as an enhancer. Azone has been shown to cause significant irritation in mouse skin (Lashmar et al., 1989) although human clinical studies suggest an acceptable safety profile (Hadgraft et al., 1993). Urea readily oxidizes to ammonia (offensive odor) and carbon dioxide. Unsaturated fatty acids (e.g. oleic acid) are also quite susceptible to oxidation and may pose significant formulation challenges. One enhancer which has made it to the market place is ethanol which is used in transdermal estradiol (Estraderm®) and fentanyl (Duragesic®) as a sorption promoter. Ethanol may act primarily by extraction (dissolution) of skin lipids and increased drug partitioning in the epidermis (Yum et al., 1994). The efficacy of an enhancer is not an intrinsic feature but is dependent on other components of the formulation. In general, a combination of enhancers with different mechanisms of action will lead to enhanced transdermal delivery. Synergistic effects have been reported with the combined use of two or more enhancers; e.g., the use of propylene glycol and oleic acid increased cutaneous delivery of salicylic acid compared to the use of either enhancer alone (Cooper, 1984). Propylene glycol is believed to act by its ability to hydrate the skin and solvent action whereas oleic acid fluidizes the lipid bilayer by intercalating its 'kinked' structure amongst the relatively ordered fatty acid chains of the bilayer. Most enhancers are believed to act through more than one mechanism and the exact mechanisms of action for most enhancers are yet to be elucidated.
1.2.2.3.3 Physical Enhancers

The enhancement of transdermal delivery by the use of chemical enhancers is limited by the dearth of adequately characterized, safe and effective chemical enhancers. For the delivery of large, charged molecules and with the increasing development of new biotechnology products, most of which are proteins and peptides, more effective skin enhancer techniques are needed. The physical enhancer techniques may fulfill this need. The two major physical enhancer methods are iontophoresis and sonophoresis.

Iontophoresis can be defined as a process by which an electrical current is employed as the driving force to transport a drug through a barrier membrane; e.g., the skin. It basically consists of two electrodes (positive and negative) placed in contact with the skin and connected to a power source. One electrode is attached to a drug reservoir while the other is attached to an inactive reservoir. Once the current is turned on, the drug is driven through the skin by the current as the circuit is completed. Drug may be delivered from either the positive electrode (anode) or negative electrode (cathode) depending on its charge and the configuration of the electrical circuit. Iontophoresis is not unlike electrophoresis which also employs an electrical current to drive charged molecules through a medium except that electrophoresis employs much higher current and electrophoresis is used mainly for separation purposes. Iontophoresis is commonly associated with the transport of charged molecules since these are primarily affected by the electrical gradient but can also be used to transport neutral molecules via a phenomenon known as electroosmosis. Electroosmosis however requires the presence of an electrolyte in the system so that as the relevant ion is transported across the skin, it draws along water and the neutral drug molecule. Similar to diffusional transport, iontophoretic transport is primarily limited by the stratum corneum. The appendages and especially the sweat glands are presumed to be the primary routes of iontophoretic transport (Grimnes, 1984). The advantages of iontophoresis include the potential to noninvasively deliver large charged molecules across the skin and the greater control over delivery rates including the possibility of pulsatile
delivery. These advantages are offset however by the cost and expertise required. In addition, the optimal current dosing regimens and the long term effects of iontophoresis on the skin are yet to be elucidated. Excellent reviews on iontophoresis are available (Burnette, 1989; Green et al., 1993; Wong, 1994).

Sonophoresis (phonophoresis, ultrasonophoresis) is a technique which employs ultrasound energy as the driving force for transdermal drug delivery. Ultrasound is defined as any sound with frequency beyond 20 kHz and is beyond the auditory range of humans. The exact mechanism by which sonophoresis enhances transdermal delivery is unclear but may be due to mechanical and thermal stresses on the skin induced by ultrasound energy (McElnay et al., 1993; Sun and Liu, 1994). Sonophoresis may be applied (via an ultrasonic probe) before or concurrently with the transdermal product. The latter is limited by the efficacy of the transdermal product as a coupling agent to ensure intimate contact between the ultrasound probe and the skin since ultrasound transmission is inhibited in air and by creams and ointments which contain a lot of solid matter (McElnay et al., 1993). Sonophoresis is probably not as efficient as iontophoresis and presents even more challenges with regards to practicality (Sun and Liu, 1994). Sonophoresis can be used to transport ionic and nonionic compounds and also has the advantage of greater control over delivery rates including the possibility of pulsatile delivery. Although the enhancer capability of sonophoresis is not in doubt, further research is required to determine optimal parameters for its use and its long term effect on the skin. In addition, some drugs may be degraded by the high energy stresses associated with sonophoresis.

Electroporation is a new physical enhancer method which employs high intensity electric field pulses to temporarily increase skin permeability (Vanbever et al., 1994). Electroporation is similar to sonophoresis in the sense that the primary effect is on the skin rather than the drug and similar to iontophoresis in the sense that an electric field is required. Electroporation is still very much in infancy and its efficacy / safety profile is yet to be established. Other physical enhancer techniques that have been employed include
stripping of the stratum corneum and application of thermal energy but these have potentially serious consequences and are not commonly used.

1.2.2.3.4 Novel dosage forms

Novel dosage forms such as liposomes have been shown to enhance drug transport in the skin (Mezei and Gulasekharam, 1980; Armann et al, 1990; Gehring et al., 1992; Michel et al, 1992).

1.3 Liposomes

1.3.1 Characterization and Preparation of Liposomes

Liposomes are microscopic vesicles consisting of lipid bilayers separated by aqueous compartments. Due to the amphiphilic nature of liposomes, water-soluble or lipid-soluble drugs may be encapsulated in the aqueous or lipid compartments, respectively. The partition coefficient of the drug between the lipid bilayer and aqueous phase determines the location of the drug in the liposome and the encapsulation efficiency is dependent on the relative solubility of the drug in each phase. Margalit et al. (1991) proposed lipid concentration and partition coefficient of the drug as the prime factors influencing drug encapsulation into liposomes.

Liposomes have several characteristics which contribute to their increasing use as drug delivery devices; namely:

i) They are biodegradable and biocompatible.

ii) Site specific drug delivery can be achieved.

iii) Liposomes can protect the encapsulated drug from metabolic degradation and reduce the systemic toxicity of the drug.

iv) Liposomes can be employed as sustained release vehicles.

v) Liposomes can enhance cutaneous/percutaneous drug absorption.

vi) Liposomes can be administered via practically all routes of drug delivery.
Liposomes are usually classified as a function of the number of bilayers and/or size; e.g., multilamellar vesicles, MLVs (0.5 - 15 μm); small unilamellar vesicles, SUVs (25 - 100 nm) and large unilamellar vesicles, LUVs (100 - 500 nm). Liposomes may also be classified based on the method of preparation; e.g., reversed phase vesicles, REVs; french press vesicles, FPVs; and ether injection vesicles, EIVs (Weiner et al., 1989).

The choice of liposome type depends on the drug and the intended use. MLVs have a greater proportion of lipid and hence show relatively higher encapsulation efficiencies with lipophilic drugs than SUVs and LUVs. MLVs are ideal for lipophilic drugs because of the high encapsulation efficiencies obtained and the improved stability of the encapsulated drug. Drug/lipid molar ratios of 1:10 are readily achieved with MLVs without compromising bilayer integrity (Poznansky and Juliano, 1984). MLVs tend to be taken up more efficiently by the reticuloendothelial system upon i.v. administration because of their larger size. MLV systems are usually heterogeneous in size and often contain some unilamellar liposomes also (Cullis et al., 1987). Because of the large entrapped aqueous phase relative to the lipid phase, LUVs are the most efficient liposomes for encapsulation of hydrophilic drugs but are relatively unstable and difficult to prepare. SUVs are also relatively unstable and possess low encapsulation efficiencies for both hydrophilic and lipophilic molecules. Drugs with intermediate partition coefficients; i.e., significant solubility in lipid and aqueous phases are relatively unstable in liposomes (Weiner et al., 1989).

Liposomal lipids are mostly phosphatidates; e.g., phosphatidylcholine (lecithin), phosphatidylserine, phosphatidic acid, phosphatidylinositol, and phosphatidylglycerol (Fig. 1.1). Sphingomyelin and cardiolipin also have the propensity to form bilayer structures (liposomes) upon contact with water. It is important to recognize that water is the driving force for the formation of liposomes. Liposome formation essentially serves to maintain the lipids in the most favorable thermodynamic state by limiting contact of their hydrophobic fatty acid ‘tails’ with water. Cholesterol is often included in liposomes
because it stabilizes the bilayer and greatly reduces leakage of encapsulated drug. The usual maximum cholesterol:phospholipid ratio that can be achieved in liposomes is 1:1 (Tyrrell et al., 1976).

\[
\begin{align*}
\text{CH}_2 \quad \text{O} \quad \text{C} \quad R_1 \\
\text{R}_2 \quad \text{C} \quad \text{O} \quad \text{CH} \\
\text{CH}_2 \quad \text{O} \quad \text{P} \quad \text{O} \quad R_3 \\
\text{O}^- \\
\end{align*}
\]

\[R_1, R_2 = \text{Long Chain Fatty Acids}\]

**Phosphatidic Acid:** \[R_3 = \text{H}\]

**Phosphatidylcholine:** \[R_3 = \text{CH}_2\text{CH}_2\text{N}^+(\text{CH}_3)_3\]

**Phosphatidylserine:** \[R_3 = \text{CH}_2\text{CHNH}_3^+\]

\[\text{COO}^-\]

**Phosphatidylglycerol:** \[R_3 = \text{CH}_2\text{CHOHCH}_2\text{OH}\]

**Phosphatidylinositol:** \[R_3 = \]

![Phosphatidylinositol structure](image)

**Figure 1.1** Chemical structures of common liposome forming lipids

Incorporating charged lipids in the bilayer decreases the rate of liposome aggregation in aqueous media and increases interlamellar distance in MLVs, resulting in greater trapping
efficiency. The use of negatively charged lipids to increase the encapsulation efficiency of lipophilic cationic drugs has been reported (Cullis et al., 1987). Positively charged lipids such as stearylamine and dodecylamine may increase the efficiency of encapsulation of negatively charged drugs such as PGE₁. However, the rate of release of PGE₁ from such a liposomal formulation could also be slower.

There are various methods available for the preparation of liposomes and these are reviewed in detail by Weiner et al. (1989) and Woodle and Papahadjopoulos (1989). MLVs are the simplest liposomes to make and often form spontaneously when the dry lipid is hydrated. Perhaps the most commonly used method for preparing MLVs is the solvent evaporation method (Bangham et al., 1974). This involves dissolving the lipids in organic solvent(s) in a round bottom flask and subsequently removing the solvent(s) by rotary evaporation under reduced pressure so that the lipid is deposited as a thin film on the bottom of the flask. Mezei and Nugent (1984) increased the surface area available for formation of the lipid film by placing glass beads in the round bottom flask. The lipid film is then hydrated with aqueous buffer at a temperature exceeding the phase transition temperature (Tₘ). Tₘ is the temperature above which the lipid changes from the more ordered gel-like state to a liquid crystalline state in which the fatty acyl chains are fluid and thus capable of reorientation to form bilayer structures. A synthetic phospholipid such as dipalmitoylphosphatidylcholine (DPPC) has a Tₘ of 41°C while egg phosphatidylcholine has a Tₘ of -15°C (Tyrrell et al., 1976). DPPC liposomes are therefore relatively stable at room temperature while cholesterol and/or other lipids have to be incorporated into phosphatidylcholine liposomes to improve their stability at room temperature. The thickness of the lipid film, time and method of hydration have been identified as variables critical to the reproducibility of the liposomes formed (Weiner et al., 1989). SUVs are commonly prepared from MLVs by sonication (Saunders et al., 1962; Barenholz et al., 1977) or using a French Pressure Cell which involves high pressure extrusion of MLVs through a small orifice (Hamilton et al., 1980). The ethanol injection method may also be
used to prepare SUVs and involves the rapid injection of ethanolic solution of the lipids into an aqueous buffer and subsequent removal of the ethanol from the formed liposomes (Batzri and Korn, 1973). LUVs are commonly produced by the reversed phase evaporation method which involves the prior emulsification of both lipid and aqueous phases in excess organic solvent and subsequent removal of the solvent under vacuum (Szoka and Papahadjopoulos, 1978).

The major limitations to the use of liposomes as drug delivery systems include the difficulty in reproducibly preparing liposomes with well defined characteristics, the difficulty in industrial scale up of currently available liposome preparation methods, and liposome stability problems. With the increasing data base on liposomes, these problems are quite surmountable. Commercial liposomal dosage forms of a topical antifungal (econazole) and systemic antifungal (amphotericin) are already available and many more liposomal products are in late stages of clinical trials.

1.3.2 Stability of Liposomal Formulations

The stabilizing effect of liposomes on drugs (Yotsuyanagi and Ikeda, 1980; Habib and Rogers, 1987; Pejaver and Notari, 1987; Habib and Rogers, 1988; Vigo and Lang, 1988) is mostly attributed to the limited exposure of the encapsulated drug to degradative stresses. Certain drugs however are not stabilized by liposomes and their encapsulation into liposomes may actually enhance their degradation although this is usually a function of the liposomal components used rather than an absolute characteristic of the drugs (D'Silva and Notari, 1982; Pejaver and Notari, 1985).

The stability of liposomal formulations can be broadly categorized into (a) physical stability which is determined by monitoring such factors as phase separation, liposomal size and drug retention; and (b) chemical stability which refers to lipid hydrolysis and oxidation as well as drug degradation (Cullis et al., 1987).
Degradation of liposomal phospholipids by hydrolysis (of ester groups) and oxidation (of unsaturated acyl chains) can accelerate degradation of the encapsulated drug as well as result in breakdown of the formulation. Saturated and unsaturated phospholipids in liposomes can be hydrolyzed to lysophospholipids, glycerophospholipids and fatty acids. Temperature, pH, and buffer composition can influence the rate of hydrolysis of phospholipids (Crommelin et al., 1994). The pH of maximum stability for phosphatidylcholine and distearoylphosphatidylcholine is 6.5 (Frokjaer et al., 1982; Grit et al., 1993).

The rate of drug release from liposomes depends on the permeability and/or stability of the lipid bilayers which in turn is a function of the type and concentration of lipid(s); nature of the encapsulated drug and other formulation excipients; the method of preparation: liposomal size, lamellarity, and surface charge; and environmental factors; e.g., pH, electrolytes, etc. (de Gier et al., 1968; Bangham et al., 1974; Lichtenberg, 1988). Some of these parameters can be controlled to obtain a desired release profile. Drug release from MLVs is traditionally slower compared to LUVs and SUVs because of the greater number of bilayers but it can be optimized.

The ‘proliposome’ concept; i.e., the production of dry liposome products which are hydrated just before administration (Crommelin et al., 1994) has been developed because of the potential long term stability problems of liposomes. The fact that the hydration technique has been shown to influence the characteristics of the liposomes that are formed may limit the application of the proliposome concept.
1.3.3 Liposomes as Penetration Enhancers

The mechanism of transdermal liposomal drug delivery is still controversial. Some researchers contend that liposomes do not penetrate the skin but release the encapsulated drug on the skin surface and that the enhanced drug penetration is a consequence of alteration of skin permeability induced by liposome-skin interaction (Ganesan et al., 1984; Ho et al., 1986; Hope and Kitson, 1993; Weiner et al., 1993). Other reports (Foldvari et al., 1990; Mezei, 1993) suggest that liposomes may also penetrate the skin intact and form a depot from which the drug is gradually released (Fig. 1.2).

1.3.4 Interaction of Liposomes with Cells

In order for liposomes to fulfill their drug delivery function, they should be capable of delivering the encapsulated drug to the target site which in most cases is intracellular. The four principal mechanisms by which liposomes interact with cells are binding (adsorption) to the cell surface, fusion, endocytosis, and lipid exchange between the liposome and cell (Poznansky and Juliano, 1984; Scherphof, 1986; Foldvari et al., 1992). Binding of the liposome to the cell surface is the initial interaction. The liposome may subsequently remain passively adsorbed, release the encapsulated drug at the cell surface, undergo fusion, endocytosis and/or lipid exchange with the cell. Endocytosis is believed to be the most important mechanism for liposomal drug delivery into the cell (Scherphof, 1986) and involves internalization of the liposome into the cell. After the liposome is internalized (as an endocytic vesicle or endosome), it may fuse with lysosomes and is subsequently degraded with gradual drug release into the cytoplasm from the lysosomes via diffusion across the lysosomal membrane or exocytosis. Since lysosomes have a low pH and contain hydrolytic enzymes, the encapsulated drug should be relatively stable to these stresses for drug delivery to the cytoplasm to be successful unless the target site is the lysosomes or liposomal drug delivery to the cytoplasm can be achieved by alternative pathways which bypass the lysosomal milieu; e.g., fusion, lipid exchange, and diffusion
Figure 1.2 Proposed mechanisms for transdermal delivery by liposomes
[Reproduced with permission from Foldvari et. al., 1990]

(A) Adsorption of liposome at the skin surface; (B) Rupture of liposomal vesicle at the skin surface with subsequent release of encapsulated materials which may penetrate into the skin via the intracellular or transcellular pathway (1) and the intercellular pathway (2); (C) Penetration of intact unilamellar liposomes via the intercellular pathway; (D) Penetration of multilamellar liposomes via the intercellular pathway with consequent loss of one or more outer lipid bilayers during the transport of the vesicles to the dermis.
across the cell membrane. Fusion of the liposome and the cell membrane involves complete mixing of liposomal and plasma membrane lipids with loss of liposome integrity and direct release of liposomal contents into the cytoplasm (New et al., 1992). Lipid exchange involves intermembrane transfer of liposomal and plasma membrane lipids with subsequent transfer of lipid phase components into the cell membrane and ultimately the cytoplasm. It is possible for the liposome to retain its structural integrity and effectively prevent the egress of components of the aqueous phase during lipid exchange (New et al., 1992).

1.4 Prostaglandins

1.4.1 Properties of Prostaglandins

Prostaglandins are cyclic derivatives of certain polyunsaturated fatty acids and are termed eicosanoids because of their distinctive 20-carbon skeleton and source. Prostaglandins were originally isolated from prostate secretions but are now known to exist in practically all mammalian tissues. A theoretical substance, prostanoic acid, represents the parent structure of prostaglandins (Fig. 1.3).

![Figure 1.3 Prostanoic acid chemical structure](image)

All prostaglandins have an hydroxyl (-OH) group on position 15 and a 13, 14-trans double bond. Prostaglandins containing 2 or 3 double bonds have the second and third double bonds, both of cis configuration, at the 5, 6- and 17,18- positions. The degree of
unsaturation within each series is indicated by a subscript in the trivial name; e.g., prostaglandin E\textsubscript{1} (PGE\textsubscript{1}; C\textsubscript{20} H\textsubscript{34}O\textsubscript{5}) contains only 1 double bond in the side chains (Fig. 1.4).

![Prostaglandin E\textsubscript{1} chemical structure](image)

**Figure 1.4** Prostaglandin E\textsubscript{1} chemical structure

Differences in the cyclopentane ring of prostanoic acid are used in the classification of the major series of prostaglandins as A, B, C, D, E, etc. (Fig. 1.5).

The naturally occurring prostaglandins; e.g., PGE\textsubscript{1}, PGE\textsubscript{2}, and PGF\textsubscript{2α} can be crystallized from purified biological extracts or synthesized chemically (The Merck Index, 1989). Prostaglandins are only slightly soluble in water but dissolve readily in organic solvents; e.g., methanol and chloroform.

PGE\textsubscript{1} (alprostadil) occurs as odorless, white to off-white crystals with a melting point of about 115°C, a molecular weight (MW) of 354.49, and a pKa of 6.3 in 60% ethanol (Upjohn Product Monograph, 1991; USP DI, 1992). Teagarden et al. (1989) reported a pKa of 4.95 for PGE\textsubscript{1} in aqueous solution and an upward shift to 6.84 in a lipid emulsion formulation. The aqueous solubility of PGE\textsubscript{1} is 0.075 mg/mL at pH 4.0 and greater than 200 mg/mL at pH 8.0 (Upjohn DIS).
Figure 1.5 Basic structures of the major classes of prostaglandins

1.4.2 Pharmacological Role of Prostaglandins

The pharmacological effects of prostaglandins in human or animal subjects vary widely depending on the prostaglandins or tissue being studied. Most of the effects of prostaglandins derive from their ability to either contract or relax smooth muscle. In general, the PGE series and PGF series cause relaxation and contraction of smooth muscle respectively.
Prostaglandins have been implicated in various biological processes such as inflammation, gastric acid secretion, reproduction, menstruation, lipolysis, platelet aggregation, blood pressure regulation, and relaxation or constriction of vascular, bronchial, and corporeal smooth muscle (Oesterling, 1972; Curtis-Prior, 1976; DATTA, 1991).

1.4.3 Therapeutic Uses of Prostaglandins

Due to their potent pharmacological effects, some prostaglandins, especially the PGE series, have been used therapeutically in conditions such as peripheral arterial occlusive disease (Mizushima et al., 1983; Peskar et al., 1991), maintenance of a patent ductus arteriosus in congenitally deformed infants (Heymann, 1981; Roehl and Townsend, 1982), gastric hyperacidity (Wallace, 1992), induction of menstruation, abortion, and labor (Karim and Rao, 1975), and in impotence (Ishii et al., 1986; Virag and Adaikan, 1987; Schramek et al., 1990).

The primary indication for PGE₁ is for the temporary maintenance of the ductus arteriosus in pediatrics with congenital heart defects until corrective surgery can be performed. Intracavernous PGE₁ has become an established therapy option for impotence. The mechanism of action of PGE₁ is primarily due to its ability to relax vascular and other smooth muscles.

1.4.3.1 Erectile Dysfunction

Erectile dysfunction (ED) or impotence is the partial or complete inability of the male to obtain and maintain an erection of sufficient rigidity to perform the sexual act satisfactorily and affects approximately 10 million males in the U.S. and 10% of all men (Nellans et al., 1987; Halter, 1990; Rajfer et al., 1992). ED may be due to congenital defects such as microphallus and spina bifida or may occur as a result of psychogenic, neurogenic, endocrine and vascular disorders in the adult male. ED may also result from drug therapy
or abuse. Antihypertensives (e.g. methyldopa), anticholinergics (e.g. benztropine), antihistamines (e.g. hydroxyzine), antipsychotics (e.g. thioridazine), antidepressants (e.g. amitriptyline), narcotic analgesics, sedative hypnotics, alcohol, and metoclopramide may cause ED (White and Campbell, 1990, The Medical Letter, 1992).

The penis can be subdivided into the shaft and glans penis. The main structural components of the shaft are three longitudinal erectile bodies - the paired corpora cavernosa and the corpus spongiosum. The corpus spongiosum lies ventrally in a median groove created by the paired corpora cavernosa which are arranged side by side in the dorsal part of the penis (Melman et al., 1994). Distally, the corpus spongiosum enlarges to form the glans penis. The corpora cavernosa are covered by the tunica albuginea, a fibrous sheath about 2 to 4 mm in thickness while the glans penis has no tunica albuginea (Wagner, 1981; Lue and Tanagho, 1988). The corpus spongiosum is surrounded by a thin outer coat and is traversed over its entire length by the urethra. Buck’s (deep) fascia, a thin layer of tissue, surrounds the three erectile bodies (Martin, 1985; Fitzgerald et al., 1992). The skin covering the penis is very thin, free of adipose tissue, and largely hairless. The skin forms a double layer over the glans penis known as the prepuce or foreskin. Removal of the prepuce is known as circumcision and is a commonly performed surgical procedure in male infants.

Penile erection is a complex physiological phenomenon governed by vascular, endocrine and neurologic mechanisms. Penile erection is a direct consequence of engorgement of the erectile tissues by blood. Blood flow into the penis occurs primarily through the paired internal pudendal arteries. Each internal pudendal artery continues as the penile artery after giving off bulbar, urethral, and perineal branches (Quam et al., 1989). The penile artery in turn divides into the cavernosal and dorsal arteries (Quam et al., 1989). The cavernosal or deep penile artery supplies the corpora cavernosa while the dorsal artery supplies the skin, subcutaneous tissues, and glans (Fitzgerald et al., 1992). The urethral artery supplies the corpus spongiosum (Melman et al., 1994). Venous drainage of the
corpora cavernosa occurs mostly via the deep dorsal vein (Fitzgerald et al., 1992). Erection can be induced by (i) reflexogenic means which involve the spinal cord and are consequent to tactile stimulation of the penis, and/or (ii) by psychogenic means which involve higher brain centers. In both cases, the flow of blood into the penis follows the release of neurotransmitters which cause relaxation of vascular and corporal smooth muscle. Relaxation of the corporal smooth muscle coupled with engorgement of the sinusoidal spaces with blood results in occlusion of the emissary veins as they become compressed against the inflexible tunica albuginea. An erection occurs since blood is effectively trapped in the penis. Putative neurotransmitters believed to be involved in erection include acetylcholine (Ach), nitric oxide (NO), vasoactive intestinal peptide (VIP), PGE₁, and calcitonin gene-related peptide (CGRP). NO is currently considered to be the major neurotransmitter involved in erection and acetylcholine probably acts by stimulating NO release from the corporal endothelium (Rajfer et al., 1992; Fallon, 1995). Detumescence and penile flaccidity are believed to be largely dependent on the sympathetic nervous system via release of norepinephrine which causes constriction of vascular and corporal smooth muscle (Christ, 1995; Fallon, 1995).

Intracavernous injection of a vasoactive drug(s) can bypass psychological, neurological, and hormonal influences on penile erection and induce erection by either directly relaxing vascular and corporeal smooth muscle and/or blocking α-adrenergic induced tone (Lue and Tanagho, 1987; Virag et al., 1991). PGE₁ acts via both mechanisms. An intact penile vasculature is essential for maximum efficacy with intracavernous agents though they have been found adequately effective where mild degree of penile vascular damage is present.

Injections of papaverine, a smooth muscle relaxant, or a combination of papaverine and phentolamine (an α-adrenergic blocker) directly into the corpus cavernosum have been shown to be effective in impotence (DATTA, 1990; Keogh et al., 1989) but intracavernosal PGE₁ has a better therapeutic and side effect profile (Virag and Adaikan, 1987; Waldhauser
and Schramek, 1988; Stackl et al., 1988; Hwang et al., 1989; Ishii et al., 1989; Lee et al., 1989; Levine et al., 1989; Sarosdy et al., 1989; Bernard and Lue, 1990; Earle et al., 1990; Schramek et al., 1990; Liu et al., 1991; Godschalk et al., 1994; Chen et al., 1995). The effect of PGE₁ on erectile dysfunction is known to be dose dependent (Schramek and Waldhauser, 1989; von Heyden et al., 1993; Godschalk et al. 1994; Chen et al., 1995). Recent reports indicate PGE₁ may act synergistically with papaverine allowing a reduced dosage of both agents to achieve an equivalent response (Floth and Schramek, 1991; Govier et al., 1993).

Other agents that may be useful in impotence therapy include nitric oxide donors (Wang et al., 1994), bromocriptine (Bommer et al., 1979; March, 1979), intraurethral prostaglandin E₂ (Wolfson et al., 1993), transdermal testosterone (McClure et al., 1991), topically applied nitroglycerin (Morales et al., 1988; Nunez and Anderson, 1993), trazodone (Adaikan et al., 1991), and yohimbine (Morales et al., 1988). Topical minoxidil was shown recently to have no effect on penile erection in contrast to an earlier study (Radomski et al., 1994). Nonpharmacologic treatments for impotence include psychotherapy, microsurgery to repair damaged blood vessels, vacuum devices, and implantation of a penile prosthesis.

1.4.4 Metabolism of Prostaglandins

The catabolism of prostaglandins in vivo can be divided into four main stages:

1. Oxidation of the -OH group on C-15 to a ketone, catalyzed by 15-hydroxyprostaglandin dehydrogenase.
2. Reduction of the C-13 double bond by Δ¹³ - prostaglandin reductase.
3. β-Oxidation of the carboxyl side chain to di- or tetranor- prostaglandin derivatives.
4. α-Oxidation of the alkyl side chain to a dioic acid which is the principal urinary metabolite.
The metabolic scheme for PGE₁ is shown in Fig. 1.6.

15-Hydroxyprostaglandin dehydrogenase (15-OHPGDH) is a cytosolic enzyme which catalyzes the primary, relatively rapid, step in prostaglandin metabolism. Two types exist namely the NAD⁺-dependent and NADP⁺-dependent enzymes (Chang et al., 1991; Mibe et al., 1992). There is a higher concentration of NAD⁺ than NADP⁺ in the cytoplasm hence NAD⁺-dependent dehydrogenation is presumably the major pathway. Fatty acids have been implicated as endogenous inhibitors of 15-OHPGDH (Bergolte and Okita, 1986; Mibe et al., 1992).
Figure 1.6 Enzymatic catabolism of PGE₁

15-OHPGDH has been isolated from various human tissues; e.g., placenta, spleen, lung, erythrocytes, fetal tissue, and tissues of the lower genitourinary tract (Oesterling et al., 1972; Curtis-Prior, 1976; Chang et al., 1991; Mibe et al., 1992). Human whole blood rapidly metabolizes PGA₁ and PGA₂ but does not metabolize PGE₁, PGB₁, and PGB₂.
(Golub et al., 1974). The prostaglandin E series are primarily metabolized in human lung while the prostaglandin A series are metabolized by various organs including the liver and kidney (Golub et al., 1975). The estimated half life of intravenously administered PGE₁ (Prostin VR™) is less than 10 minutes and 68% of the administered dose is metabolized in one pass through the lungs (Upjohn Product Monograph, 1991).

Δ¹³-Prostaglandin reductase is also a cytosolic enzyme and catalyzes reduction of the 15-ketoprostaglandin E₁ metabolite to 15-keto-13,14-dihydroprostaglandin E₁ (KH₂PGE₁), the major prostaglandin metabolite in the circulation (Peskar et al., 1991). The formation of 13, 14-dihydroprostaglandin E₁ from KH₂PGE₁ was initially believed to occur only in vitro (Hesse et al., 1990) but was recently demonstrated to also occur in vivo (Peskar et al., 1991), perhaps because of the higher sensitivity (< 1 pg) of the latter study.

β-Oxidation of the carboxyl side chain occurs in the mitochondria and is akin to classical β-oxidation of long chain saturated or unsaturated fatty acids (Stryer, 1988). Di- or tetranor-prostaglandin derivatives are produced depending on if 1 or 2 cycles of β-oxidation occur respectively.

ω-Oxidation of the terminal methyl group of prostaglandins is a 2-step process involving hydroxylation to an alcohol and subsequent oxidation of the alcohol moiety to an acid. Hence, both side chains of the prostaglandin metabolite now end in a carboxyl group and this polar dicarboxylic acid is the primary urinary metabolite of PGE₁ in man. Enzymes catalyzing ω-oxidation have been isolated from human seminal plasma and liver microsomes (Curtis-Prior, 1976).

The catabolic enzymes are widely distributed in the body and are present in the spleen, kidney, adipose tissue, intestine, liver, testicle and lung. The major site for side chain oxidation is probably the liver (Samuelsson et al. 1975). Studies on skin metabolism of topically applied PGE₁ or the distribution of prostaglandin metabolizing enzymes in the skin are lacking.
Nonenzymatic degradation of PGE₁ can occur. PGE₁ is thermolabile and is especially sensitive to pH variation. In general, PGE₁ is dehydrated to PGA₁ in acidic or basic conditions. PGA₁ is further isomerized to PGB₁ under basic conditions. Epimerization at C-15 or isomerization at C-8 of the PGE₁, PGA₁, and PGB₁ may also occur (Lee and DeLuca, 1991) but these are considered to be minor degradation pathways. The nonenzymatic degradation pathway for PGE₁ is shown in Fig. 1.7. Oesterling et al. (1972) reported maximum stability, at room temperature, of aqueous solutions of PGE₁ and PGE₂ to be within the pH range 6 to 7 while PGF₁α and PGF₂α were most stable at pH range 5 to 11. More recent reports, though at different temperatures of 60°C (Monkhouse et al., 1973) and 35°C (Teagarden et al., 1989) indicate an optimum pH range of 3 to 5 for aqueous solutions of PGE₁.

The intrinsic regulatory mechanisms for prostaglandin catabolism in vivo remain unclear. These mechanisms may involve modulation of cellular levels of the prostaglandin metabolizing enzymes. Exogenous fatty acids have been shown to inhibit 15-OHPGDH in tissue homogenate experiments and an endogenous inhibitor consisting of various fatty acids; e.g., palmitic, stearic and oleic acids, has been isolated from human placenta (Mibe et al., 1992). The relevance of these in vitro results to endogenous regulation of prostaglandin catabolism is yet to be established. Cigarette smoking causes increased plasma free fatty acids which may explain its reported inhibition of 15-OHPGDH in the lung (Bergolte and Okita, 1986). Other reported inhibitors of 15-OHPGDH include NADH and aspirin-like drugs (Hansen, 1976).

Ethanol at concentrations of less than 2% (Roy et al., 1989) and 10% (Schlegel et al., 1974) have been employed in tissue homogenate experiments with no apparent inhibitory effect on 15-OHPGDH. NAD⁺ has been found to inhibit Δ¹³-prostaglandin reductase in vitro (Schlegel et al., 1975).
Figure 1.7 Nomenzymatic degradation pathway for PGE₁
1.5 Methods in Transdermal Drug Delivery Research

1.5.1 Experimental Models

The experimental models for studying transdermal drug delivery are based on choice of the following variables (i) \textit{in vivo} or \textit{in vitro} setting and (ii) tissue or barrier membrane source.

1.5.1.1 \textit{In Vivo} Models

\textit{In vivo} methods for studying transdermal drug delivery are summarized in Fig. 1.8. Human, animal or skin flap models may be employed (Shah et al., 1991).

\begin{center}
\textbf{IN VIVO METHODS}
\end{center}

\begin{tabular}{ccc}
Animal Model & Skin Flap Model & Human Model \\
\hline
Pharmacological Effects & Pharmacokinetic Data evaluation & \\
Skin Blanching Assay & Cutaneous Drug Concentration & \\
Laser Doppler velocimetry & Systemic Drug Concentration & \\
Pinprick Tests & Drug Concentration in excreta & \\
Lesion Scores & Drug Concentration in Venous Blood draining Application Site & \\
Other & Residual Drug Analysis & \\
\end{tabular}

\textbf{Figure 1.8} \textit{In vivo} methods for transdermal drug delivery research
The skin flap model (Reifenrath et al., 1984; Wojciechowski et al., 1987; Pershing and Krueger, 1989; Williams et al., 1990) involves surgically grafting viable human or other animal skin onto an animal species; e.g., the athymic nude mouse, or isolating a section of skin on an animal in such a manner that the blood vessels entering and exiting the tissue can be sampled. This model requires considerable expertise and may require the use of immunosuppressant drugs or antibiotics which may influence the experimental results. Claims to the effect that animal models such as the weanling pig (Reifenrath et al., 1984), hairless guinea pig, and rhesus monkey possess skin permeability characteristics similar to human are suspect considering that skin permeability is dependent on the drug, vehicle, study conditions, skin source, metabolism, etc. Considering that skin permeability even amongst humans is highly variable, it is imprudent to ascribe similar skin permeability characteristics to an animal model solely on the basis of few studies involving a limited number of model penetrants and in which the effect of the various factors influencing skin permeability have not been rigorously characterized. Indeed, a universally acceptable animal model for human skin is probably not feasible. Animal models are however very useful in toxicology and mechanistic studies but extrapolation of the results to human in vivo situation should be done with utmost caution. In vivo human experiments are the most accurate means of assessing transdermal delivery as the drug can be tested under conditions in which it will eventually be used. In vivo human studies are, however, often beset with experimental and ethical problems. Systemic levels of topically applied drugs are often very low and may require radiotracer techniques for accurate quantitation. In addition, the target site of the drug may be the deeper layers of the skin or sites other than the systemic circulation and these areas may not be accessible for sampling. Monitoring drug concentration in the skin is also more difficult with human in vivo studies. Residual drug analysis is an indirect method for evaluating transdermal drug delivery which involves monitoring the amount of drug left on the skin surface after one or more time points. It is inexpensive and suitable for human in vivo studies but its reliability is questionable since
drug loss from the surface of the skin does not necessarily equate to transdermal delivery. Transdermal delivery \textit{in vivo} is therefore mostly monitored by indirect means such as assaying a pharmacodynamic or clinical response; e.g., skin blanching assay (corticosteroids), laser Doppler velocimetry (vasodilator drugs), pinprick tests (local anesthetics), lesion scores (psoriasis) and blood pressure (clonidine, nitroglycerin). Laser Doppler velocimetry is based on the Doppler shift in frequency when monochromatic laser light beamed onto the skin is randomly scattered by mobile red blood cells in the dermis. Some of the reflected light is collected by receiving optical fibres, transmitted back to the instrument, and is converted into flow, volume, and/or velocity measurements based on digital technology (Holloway and Watkins, 1977; Meyer, 1991; Vasamedics Inc., 1992). Laser Doppler velocimetry or flowmetry has been used to evaluate changes in cutaneous microcirculation following topical application of vasoactive agents such as methyl nicotinate (Wilkin et al., 1985; Guy et al., 1985; Poelman et al., 1989) and minoxidil (Wester et al., 1984).

In the course of transdermal product development, it is not feasible to subject each candidate to the relatively time consuming, cumbersome and expensive requirements of \textit{in vivo} human experimentation hence these are commonly reserved for the latter part of the product development schedule.

1.5.1.2 \textit{In Vitro} Models

\textit{In vitro} studies can be done with human skin, animal skin and artificial membranes. Fig. 1.9 depicts the various protocols available. Factors which influence the choice of a particular protocol include relevance, cost and availability. Artificial membranes; e.g., polydimethylsiloxane, cellulose acetate and polyurethane, are readily available and can be used to investigate drug release from the formulation or as a quality control tool to ensure batch to batch drug release equivalency. However, drug permeation through these simple homogenous monolayer membranes does not reflect transdermal delivery where transport
through the complex heterogeneous multilayered skin must be achieved. In addition, the effect of formulation excipients; e.g., skin penetration enhancers, and metabolism(binding in the skin cannot be determined by these membranes. The recent development of artificial skin membranes more closely related to human skin and with some metabolic potential may overcome some of the limitations of earlier artificial membranes (Ernesti et al., 1992; Lamb et al., 1994).

**IN VITRO METHODS**

Artificial Membrane → Animal Skin → Human Skin

- Non viable
- Viable

Whole Skin → Skin Section

Static diffusion cell design → Flow - through diffusion cell design

Percutaneous Flux Determination → Cutaneous Drug Analysis → Residual Drug Analysis

**Figure 1.9** *In vitro* models for transdermal drug delivery research
The limitations of animal models as discussed in the preceding section also apply to the \textit{in vitro} setting. Human skin \textit{in vitro} models are most reliable but may be difficult to conduct due to scarcity of human skin and the effects of anatomical site, gender, age, and race on the skin permeability (Behl et al., 1990). Since skin permeability is normally variable, \textit{in vitro} skin penetration determinations can be highly variable. Ideally, all comparative studies should be done with skin from the same body site and subject but where this is not feasible, efforts should be made to standardize anatomical site, skin preparation and storage conditions.

It is known that storage conditions may profoundly affect barrier properties of excised skin (Harrison et al., 1984; Swarbrick et al., 1982; Hawkins and Reifenrath, 1986). Human skin can be stored in the frozen state without deterioration of the barrier properties (Franz, 1975; Harrison et al., 1984). \textit{In vitro} human experimentation may be considered the backbone of transdermal drug delivery research as it combines the validity of human skin tissue with the convenience and reproducibility of the \textit{in vitro} setting. As noted earlier, the stratum corneum is the rate limiting membrane for transdermal drug delivery and since it is made of keratinized dead cells, it is accepted that \textit{in vitro} permeation results with nonviable skin show very good correlation with the \textit{in vivo} situation (Ostrenga et al., 1971; Franz, 1975; Reifenrath et al., 1984). Although physical diffusion is considered to be the primary determinant modulating skin absorption, cutaneous metabolism and the metabolic status of the skin may also play a significant role (Ando et al., 1977) and in these cases, viable skin samples can be employed by using fresh skin samples maintained in appropriate buffers (Collier et al., 1989) for the duration of the permeation study.

Diffusion cells are used for \textit{in vitro} percutaneous absorption studies. There are two basic cell designs available; namely, the static cell (e.g., Franz cell (Franz, 1975)) and the flow-through cell (Braunough and Stewart, 1985). The two diffusion cell types consist of a donor and receptor compartment between which the skin or artificial membrane is placed. In static diffusion cells, the receptor compartment is filled with 'immobile' medium which
is manually sampled for permeated drug at defined time intervals. Usually, a magnetic stirrer bar or some other form of agitation of the receptor medium is used in order to ensure uniformity of concentration of the permeated drug and to avoid build up of drug on the layer directly below the membrane which would retard further diffusion across the membrane. With flow-through diffusion cells, the receptor medium is pumped continually though the receptor compartment picking up any permeated drug and is then collected into time fractions automatically. The flow-through cells provide in vitro conditions which closely simulate those in vivo, where permeated drug is often rapidly taken up by the dermal vasculature into the systemic circulation. Several variables affect diffusion cell studies and have to be optimized; e.g., receptor medium composition and volume, temperature, flow rate, applied dose, skin viability, effective diffusion area, and analytical technique (Bronaugh, 1993). Transdermal drug delivery efficacy in diffusion cell studies is evaluated by monitoring (i) drug concentrations in the receptor; i.e., percutaneous flux determinations (ii) drug concentration in the skin (cutaneous drug analysis) and/or (iii) residual drug; i.e., drug remaining on the surface of the skin. Radiotracer technique is mostly used in diffusion cell studies because of the very high sensitivity and relative ease of analysis although it is compromised by a lack of molecular specificity. Since drug clearance in vitro may be different from the in vivo setting, cutaneous drug concentrations could be important in predicting transdermal drug delivery efficacy. Cutaneous drug analysis typically involves determining drug concentrations in the whole skin or skin sections after topical application; e.g., stratum corneum concentrations are monitored by tape-stripping the skin at least 10 times and analyzing the strips. Cutaneous and residual drug analyses results are dependent on the method used in cleansing the skin at the end of the diffusion cell experiment. Several cleansing methods have been employed ranging from the use of strong solvents such as alcohols or surfactants to simply wiping the skin. There is a need to standardize the cleansing technique although for comparative studies, it may not be critical if a consistent reproducible method is followed.
1.5.2 Tissue Homogenate Metabolism Studies

Metabolism studies in the skin are often conducted via the tissue homogenate model (Mukhtar and Bickers, 1981; Nicolau and Yacobi, 1989-90; Guzek et al., 1989; Woolfson et al., 1990; Bronaugh and Collier, 1991) because of the difficulties in separating cutaneous from systemic metabolism with in vivo experimental models. In vitro flow-through metabolism experiments (with viable skin samples) may be employed but are limited because (i) drug partitioning into the receptor medium may be less than optimum thus exposing the drug to skin metabolizing enzymes for a longer period than would occur in vivo; (ii) sampling at short time intervals may not be possible because of inadequate drug penetration; (iii) drug metabolism may occur in the receptor fluid after absorption; and (iv) sources of skin supply are usually unpredictable and may be limiting if fresh skin samples are required per metabolism experiment. Fresh human tissue samples can be obtained readily from surgical procedures, and if processed immediately and stored, under appropriate conditions for the particular enzyme(s), the enzyme activity is maintained. The use of high salt concentrations, thiol compounds, low temperatures, NAD+, and glycerol have been shown to stabilize 15-OHPGDH in tissue homogenate preparations for storage periods of up to one year (Hansen, 1976). Metal ions can inhibit 15-OHPGDH (Hansen, 1976) hence a metal ion chelator such as ethylene diamine tetraacetic acid (EDTA) is often included in 15-OHPGDH extraction/activity studies (Wright et al., 1976; Chang et al., 1991).

Tissue homogenates are prepared by mincing the tissue (or crushing the tissue under liquid nitrogen), suspending it in a physiologically based medium; e.g., phosphate buffered saline, and homogenizing with a tissue homogenizer. Subsequent sonication and/or centrifugation steps are used to obtain subcellular fractions containing the drug metabolizing enzymes. Relative centrifugal forces (RCFs) of up to 120,000 x g (Camp and Greaves, 1980) can be used since the major catabolic enzymes implicated in prostaglandin
metabolism are located in the cytosol. The use of RCFs of 10,000 x g or less (Nakano et al., 1971; Nicolau and Yacobi, 1989-90; Nicolau et al., 1989) should provide adequate removal of cellular debris and nuclei while ensuring the retention of the cytosolic, mitochondrial, and microsomal (endoplasmic reticulum) fractions. After centrifugation, the supernatant is used for drug metabolism experiments by incubating it with a known drug aliquot at 37°C. Samples are withdrawn at intervals for analysis of parent drug and metabolite(s).

Skin homogenate experiments are largely empirical in terms of estimating drug/homogenate ratios that approximate actual drug/enzyme activities in vivo. Standardization of enzyme content of tissue homogenate or whole tissue sample is often not done in prostaglandin metabolism studies and may account for difficulties in reproducibly quantifying the extent of substrate metabolism in various tissues; e.g., the inability of Sullivan et al. (1992) to confirm the metabolism of PGE₂ to PGF₂α in intact fetal membranes. However, the advantages of rigorous standardization of tissue enzyme content has to be weighed against the limitations of cost and time constraints and the utility of such information to the research being conducted. Tissue homogenate enzyme assays involving the use of purified enzyme standard concentration curves may not be representative of in vivo enzyme activity because significant increases in enzyme activity after purification, attributed to the removal of an endogenous enzyme inhibitor, has been reported (Bergolte and Okita, 1986). Purified enzyme systems are necessary for the determination of enzyme kinetics. Enzyme kinetics are described by the Michaelis-Menten equation (Eq. 1.8) and its reciprocal, the Lineweaver-Burk equation (Eq. 1.9).

\[
V_0 = \frac{V_{max} [S]}{K_M + [S]} \tag{1.8}
\]
\[
\frac{1}{v_0} = \frac{K_M}{V_{\text{max}}} \frac{1}{[S]} + \frac{1}{V_{\text{max}}}
\]  

(1.9)

where

\[v_0\] = initial velocity of the reaction  
\[V_{\text{max}}\] = maximum attainable velocity of the reaction  
\[[S]\] = drug or substrate concentration  
\[K_M\] = drug or substrate concentration at half \(V_{\text{max}}\)

\(K_M\), also known as the Michaelis or affinity constant, describes the affinity of the substrate for the enzyme; e.g., a large \(K_M\) indicates weak interaction between the enzyme and substrate while a small \(K_M\) indicates the opposite. It is common in tissue homogenate metabolism studies to ensure that concentrations of substrate(s) and cofactor(s) are not limiting by using values higher than two times the \(K_M\). It should, however, be borne in mind while interpreting such \textit{in vitro} tissue homogenate metabolism results that saturating levels of the substrate and cofactor may not be reached \textit{in vivo}.

Previous enzyme kinetic studies on 15-OHPGDH have reported \(K_M\) values ranging from 1.1 to 60.1 µM for PGE\(_1\) and 30 to 800 µM for NAD\(^+\) (Hansen, 1976; Roy et al., 1989). These widely varying results emphasize the dependence of enzyme kinetic parameters on several experimental variables such as tissue source, enzyme purity, presence of inhibitors, substrate and cofactor concentration, pH, temperature, and ionic strength.

1.5.3 Skin Ultrastructure and Localization Studies

In order to establish the drug delivery potential of transdermal liposomes, the fate of the liposomal vesicles and the encapsulated drug after administration must be understood. The localization of the drug or liposomes in the skin can shed light on the mechanism and
efficacy of transdermal liposomal drug delivery while visualization of skin structure changes can account for toxicity or lack thereof of the liposomal formulation.

Skin ultrastructure and localization studies are done via transmission electron microscopy (TEM). The very high resolution of TEM (< 0.2 nm) and its almost limitless magnifying power (up to 1,000,000x) makes it an effective tool to monitor structural changes in the skin following topical treatment as well as to determine the subcellular localization of the applied drug or vehicle components in the skin. TEM consists of eight basic procedures; namely: tissue excision, fixation, dehydration, infiltration/embedding, sectioning, staining, viewing/photographing under a transmission electron microscope, and development and printing of electron micrographs. These procedures have to be optimized in order to enhance the quality of the electron micrographs obtained. Light microscopy sections are prepared initially in order to determine skin structure at this level as well as to provide orientation for obtaining electron microscope sections from the desired area(s) of the skin. Texts by Weakley (1981) and Hayat (1989) discuss the basic theory and practical use of the TEM including general optimization techniques.

A suitable electron dense marker has to be attached to the vehicle or drug in order to enable their identification by TEM. This is especially important for liposomes which resemble several endogenous cellular structures. Liposomes, without a suitable marker, would be indistinguishable from other bilayer and vesicular structures in the skin. Liposome markers which have been used previously include colloidal gold, ferritin, percoll, potassium dichromate, horseradish peroxidase, nitroblue tetrazolium, and colloidal iron (Foldvari et al., 1988; Foldvari et al., 1992; New et al., 1992). Colloidal iron has been shown to be an optimum electron dense marker for topical liposomes (Foldvari et al., 1990).
Chapter Two

RATIONALE AND OBJECTIVES

2.1 Rationale

PGE₁ is a likely candidate for transdermal delivery because it is (i) a potent drug requiring normal dosage levels of 20 μg or less in impotence, when administered by intracavernous injection (von Heyden et al., 1993) (ii) subject to extensive first-pass metabolism when administered orally, and (iii) very rapidly metabolized in the systemic circulation when administered intravascularly and must be administered locally near the target site or by continuous intravenous infusion in order to achieve therapeutic concentrations. Intracavernous injection of PGE₁ is widely used in the treatment of impotence, a typically chronic disease with a prevalence of about 10% in men. PGE₁ is usually self-injected into the corpus cavernosum through the lateral aspect of the shaft of the penis. This mode of administration is limited by penile discomfort, pain at the injection site, a need for manual dexterity, potential development of priapism or fibrosis, and the inconvenience of taking an injection just prior to intercourse. The consequences of these limitations of intracavernous PGE₁ therapy are high dropout and noncompliance rates in patients. A transdermal PGE₁ formulation could significantly improve patient acceptance and compliance with therapy by delivering the drug more conveniently to the target site (corpora cavernosa) while minimizing local and systemic side effects.

Once considered an improbable option, transdermal drug delivery is now an established dosage route and six drugs are already commercially available in Canada in transdermal dosage forms (Table 2.1).
<table>
<thead>
<tr>
<th>Generic Name</th>
<th>Brand Name(s)</th>
<th>Delivery Rate/Application Frequency</th>
<th>Indication(s)</th>
<th>Properties</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estradiol</td>
<td>Estraderm</td>
<td>25-100 µg/24h twice weekly</td>
<td>Estrogen deficiency, Postmenopausal syndrome</td>
<td>MW 272.37, mp 173-179 °C Contains alcohol as enhancer.</td>
</tr>
<tr>
<td></td>
<td>Vivelle</td>
<td>37.5-100 µg/24h twice weekly</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Estradiol/Nor-</td>
<td>Estracomb</td>
<td>Estradiol: 50 µg/24h twice weekly</td>
<td>Estrogen deficiency, Postmenopausal syndrome</td>
<td>Contains Estraderm (estradiol) and Estragest (estradiol and NETA) patches</td>
</tr>
<tr>
<td>ethindrone acetate</td>
<td></td>
<td>NETA: 250 µg/24h twice weekly</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(NETA)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fentanyl</td>
<td>Duragesic</td>
<td>25-100 µg/h applied every 72h</td>
<td>Chronic cancer pain</td>
<td>MW 336.46, mp 83-84 °C Contains alcohol as enhancer.</td>
</tr>
<tr>
<td>Nicotine</td>
<td>Nicoderm</td>
<td>7-21 mg/24h applied once daily</td>
<td>Smoking cessation</td>
<td>MW 162.23, liquid in native state.</td>
</tr>
<tr>
<td></td>
<td>Nicotrol</td>
<td>5-15 mg/16h applied once daily</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Habitrol</td>
<td>7-14 mg/24h applied once daily</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Prostep</td>
<td>11-22 mg/24h applied once daily</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nitroglycerin</td>
<td>Nitrodur Transderm-Nitro</td>
<td>0.2-0.8 mg/h applied once daily</td>
<td>Angina</td>
<td>MW 227.09, liquid in native state.</td>
</tr>
<tr>
<td></td>
<td>Minitran</td>
<td>0.2-0.8 mg/h applied once daily</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Nitrol</td>
<td>0.2-0.8 mg/h applied once daily</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Variable. 15-30 mg ointment applied q8h and at bedtime.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Scopolamine</td>
<td>Transderm-V</td>
<td>1 mg/72h applied every 72h</td>
<td>Motion sickness</td>
<td>MW 303.35, liquid in native state.</td>
</tr>
</tbody>
</table>
The antihypertensive drug, clonidine (MW 230.10, mp 130°C) is commercially available as a transdermal dosage form (Catapres-TTS) in the U.S. Several other transdermal drug products are in the developmental or investigational stage for the treatment of impotence and other diseases; e.g., testosterone (McClure et al., 1991), isosorbide dinitrate, stearyl-norleucine-vasoactive intestinal peptide (Gozes et al., 1994), minoxidil (Cavallini, 1991), timolol (McCrea et al., 1990), triprolidine (Miles et al., 1990) and ketorolac (Roy et al., 1995).

With its relative lipophilicity, low molecular weight and melting point, PGE\(_1\) possesses the required properties for transdermal delivery. PGE\(_1\) has, however, been shown to be poorly absorbed through human skin *in vitro* (Watkinson et al., 1990; 1991) and is chemically and metabolically unstable.

In order to develop a transdermal PGE\(_1\) product, a suitable vehicle must be used which can significantly enhance percutaneous PGE\(_1\) absorption as well as protect the drug from degradative and metabolic stresses during storage and administration, respectively. The use of penetration enhancers and complexation of PGE\(_1\) with carboxymethyl-ethyl-β-cyclodextrin was shown recently to enhance transdermal absorption of PGE\(_1\) in hairless mice (Uekama et al., 1992) but the effect in human skin is yet to be established. Liposomes provide unique advantages as transdermal drug delivery vehicles because of their versatility, biocompatibility/biodegradability, and their ability to enhance percutaneous absorption as well as stabilize the encapsulated drug. Encapsulation into liposomes has been shown to enhance the stability of PGE\(_1\) relative to the drug in solution (Vigo and Lang, 1988). The effect of skin and formulation (vehicle) related factors have to be analyzed and optimized since they largely determine transdermal delivery efficacy. The fate of topically applied liposomes is controversial (see Section 1.3.3). The study of liposome-skin interaction by electron microscopy can predict the fate of liposomes and encapsulated drug *in vivo* and the effects of topical liposome application on skin ultrastructure.
Transdermal PGE\(_1\) dosage form development may not be feasible if the drug is extensively metabolized in the skin. Previous research has shown PGE\(_1\) to be extensively metabolized in the lung and placenta (Schlegel et al., 1974; Golub et al., 1975; Schlegel and Greep, 1975); however, its metabolism in human skin has not been investigated previously.

In order to conduct percutaneous absorption, stability and metabolism studies of novel transdermal formulations, appropriate assays have to be developed if not already available. Prostaglandins are generally analyzed by reversed phase HPLC with spectrophotometric (ultraviolet) detection. Because the absorption maxima for PGE\(_1\) (193 nm) is very low and several compounds, including most chromatographic solvents, absorb strongly at this wavelength, it is often derivatized prior to HPLC and then monitored at a higher wavelength of 254 nm (Zoutendam et al., 1984). Although this is the USP XXII assay for PGE\(_1\), the derivatization steps introduce additional sources of error as the thermolabile drug could undergo some degradation during derivatization. In addition, the reproducibility or completeness of derivatization has to be confirmed since it is the derivatized product not the parent drug that is eventually analyzed. Several investigators have tried to analyze underivatized PGE\(_1\) samples either by employing special chromatographic equipment that enables u.v. detection at about 200 nm (Terragno et al., 1981; Yamamura and Yotsuyanagi, 1984; Lee and DeLuca, 1991; Adachi et al., 1992) or using radioactive PGE\(_1\) samples and monitoring the radioactivity in the eluate (Hesse et al., 1990). To the best of the author’s knowledge, there are no published assays for the simultaneous detection of underivatized PGE\(_1\) and its major degradation/metabolic products or for the specific assay of PGE\(_1\) in liposomal dosage forms. Such assays would be invaluable in the proposed PGE\(_1\) metabolism and stability studies.

Clinical trials are required for validation of the transdermal delivery potential of PGE\(_1\) since these involve monitoring the therapeutic response in patients under actual use conditions. Therapeutic response in impotence can often be monitored only by subjective
patient reports on product efficacy. It is probably unreasonable to expect that full erections can be reproducibly observed in the laboratory setting because sensory stimulation and psychological factors are often critical to the erectile process. While the use of visual sensory stimulation (VSS) is beneficial in the laboratory setting (Lee et al., 1993), it is often limited by institutional and patient biases. A pharmacodynamic (laboratory) assay would however be beneficial in order to control for possible bias in patient reports on product efficacy in the home setting. PGE₁ causes relaxation of vascular smooth muscle. Changes in penile hemodynamics following transdermal PGE₁ application in impotent patients can be evaluated by laser Doppler ultrasonography and used to estimate transdermal delivery efficacy. Laser Doppler flowmetry is a pharmacodynamic assay which may be used to monitor changes in cutaneous microcirculation following topical application of PGE₁ to healthy patients and may predict the clinical efficacy of transdermal PGE₁ formulations in peripheral arterial occlusive diseases.

The development of a safe, effective, transdermal PGE₁ formulation for treatment of impotence and (potentially of) peripheral arterial occlusive disorders would represent a significant addition to the therapeutic armamentarium.

2.2 Objectives

The specific objectives of this project are:

a) To formulate a stable, liposome encapsulated transdermal PGE₁ preparation with
   in vitro transdermal flux characteristics which will be satisfactory for in vivo application.

b) To characterize the effect of formulation variables on transdermal delivery of
   PGE₁ using in vitro percutaneous absorption (foreskin) model.

c) To characterize the effect of skin metabolism on transdermal delivery of PGE₁.

d) To develop a sensitive specific assay for PGE₁ in solution, liposomal and conventional cream dosage forms.
e) To develop a HPLC assay for the simultaneous detection of PGE\textsubscript{1} and its major degradation and metabolic products.

f) To evaluate transdermal delivery of liposome encapsulated PGE\textsubscript{1} preparation \textit{in vivo} by clinical and pharmacological response monitoring in patients with erectile dysfunction.

g) To evaluate transdermal delivery efficacy of liposome encapsulated PGE\textsubscript{1} in healthy subjects by means of a pharmacodynamic assay.

The overall goal of the project is to evaluate the potential for transdermal delivery of PGE\textsubscript{1}, with the use of liposome encapsulation, for the treatment of erectile dysfunction.
Chapter Three

EXPERIMENTAL

3.1 Transdermal Liposome Formulation Studies

Multilamellar liposomes for transdermal delivery of PGE₁ were prepared by either the solvent evaporation method of Mezei and Nugent (1984) or a 'proliposome' method previously developed in our laboratory (Foldvari M, Patent Pending). The solvent evaporation method involved the dissolution of the lipid phase in chloroform:methanol (2:1 v/v) in a round bottomed flask containing glass beads. The solvents were subsequently removed by rotary evaporation (Buchii RE 111 Rotavapor, Buchii Laboratoriums, AG Flawil/Sheiz, Switzerland) so that a thin lipid film was formed on the flask wall and surface of the beads. The thin lipid film was then hydrated at 55°C with the aqueous phase by initially shaking vigorously, in a circular motion, by hand for 2-3 min and then with the aid of a mechanical shaker for 25 minutes to complete liposome formation. The 'proliposome' method avoids the use of organic solvents and hydration with the aqueous phase can be done at 55°C by vortexing, propeller mixing or homogenization depending on the product batch size.

Liposome formulation was optimized by the use in varying proportions of natural and synthetic lipids, cholesterol, viscosity enhancers; e.g., methylcellulose, preservatives (parabens), antioxidants (ascorbyl palmitate, α-tocopherol), and skin penetration enhancers. The penetration enhancers used included fatty acids (stearic acid, oleic acid and lauric acid), alcohols (ethanol, cetyl alcohol), Ajidew N50® (DL-pyrrolidone-5-carboxylate), methyl salicylate, propylene glycol, glycerol, glyceryl stearate, cineole,
calculated thioglycolate, and α-tocopherol. Fifty four liposome formulations were made and evaluated for transdermal delivery potential. The major phospholipid used in making the liposomes, Phospholipon 90H®, was obtained from Natterman Phospholipid GmbH, Cologne, Germany. Other sources of liposome materials include: Centrollex P® (Central Soya - Fort Wayne, IN, U.S.A.), N-lauroyl-l-lysine or MLL (Canamino Inc. Ottawa, ON), cholesterol, α-tocopherol, ascorbyl palmitate, dodecylamine, lauric acid, and papaverine (Sigma Chemical Co. St. Louis, MO, U.S.A.), stearic acid and methylparaben (J. T. Baker Chemical Co. Phillipsburg, NJ, U.S.A.), triethanolamine, propylene glycol, glycerol, glyceryl monostearate, and propylparaben (BDH Inc. Toronto, ON), calcium thioglycolate (Fluka Biochemika, Buchs, Switzerland), cetyl alcohol (Wiler Ltd. London, ON), Ajidew N50® (Ajinomoto USA Inc. Teaneck, NJ, U.S.A.), and methylcellulose (Aldrich Chemical Co. Milwaukee, WI, U.S.A.).

3.2 Transdermal PGE₁ Formulations

Transdermal liposomal PGE₁ formulations were prepared by incorporating the drug in the lipid phase component prior to hydration with aqueous phase as described in section 3.1. PGE₁ was obtained from Sigma and Pharmatech International Inc., West Orange, NJ, U.S.A. Radiolabelled [5, 6 (n) -3H] PGE₁ was obtained from Amersham (Oakville, ON).Nonliposomal transdermal PGE₁ formulations were prepared by incorporating the drug into Dermabase™ (Professional Pharmaceutical Corp. Lachine, Quebec) using vigorous mixing at 55°C. Dermabase™ is an oil-in-water emulsion base containing purified water, petrolatum, cetostearyl alcohol, propylene glycol, sodium lauryl sulfate, mineral oil, isopropyl palmitate, imidazolidinyl urea and parabens.
3.2.1 pH Determination of Transdermal PGE\textsubscript{1} Formulations

The pH of each preparation was determined using a Horiba Cardy compact pH meter (Horiba Instruments, CA, U.S.A.) With this pH meter, only a small aliquot of the formulation is required for the determination and the possibility of formulation contamination upon insertion of a conventional pH probe is avoided.

3.2.2 Physical Stability Analyses of Transdermal PGE\textsubscript{1} Formulations

The physical stability of the liposomal formulations was assessed by organoleptic and microscopic studies and determination of the drug encapsulation efficiency and leakage from liposomes.

3.2.2.1 Organoleptic Studies

Parameters such as color, odor, texture (tackiness), and consistency were monitored for 1 year. Each formulation was given a visual stability rating based on a scale developed by Hanna (1989).

3.2.2.2 Microscopic Studies

Each liposome formulation was examined under the light microscope (Microstar optical microscope, Cambridge Instruments Canada Ltd., Montreal, Quebec) for vesicle size distribution (with the aid of a calibrated ocular micrometer) and the presence of liposome instability indicators such as undissolved particles, crystals, and fusion/aggregation products.

3.2.2.3 Encapsulation Efficiency and Leakage Studies

About 35 mg aliquot of each radiolabelled (\textsuperscript{3}H-PGE\textsubscript{1}) liposomal PGE\textsubscript{1} formulation was placed into a Beckman polycarbonate centrifuge tube with 3 volumes of diluent (normal saline, aqueous phase or phosphate buffered saline) and subsequently centrifuged in a
Beckman L8-55 Ultracentrifuge at 50,000 rpm, 4°C, for one hour. The supernatant was separated from the pellet containing liposomes and the encapsulation efficiency of PGE₁ into the liposomes was determined by liquid scintillation counting using a water-compatible liquid scintillation cocktail, LSC, (Beckman Ready Value™) and a Packard Tri-Carb 2000 CA liquid scintillation analyzer.

\[
\text{Encapsulation efficiency} = \frac{\text{Amount of PGE₁ in pellet}}{\text{Amount of PGE₁ in pellet and supernatant}}
\]

Leakage of encapsulated drug was estimated from repeated encapsulation efficiency studies after prolonged storage at 4°C. The effect of diluent used on encapsulation efficiency determinations was analyzed.

3.2.3 Chemical Stability Analyses of Transdermal PGE₁ Formulations

Chemical stability analyses were conducted at 4°C and 37°C. Transdermal PGE₁ formulations stored at 4°C were analyzed at 0, 1, 2, 3, 4 and 5 month(s) for PGE₁ content and the presence of the major degradation products PGA₁ and PGB₁. The stability of PGE₁ under in vitro diffusion cell experimental conditions was evaluated by incubating free or liposome encapsulated drug with denatured human skin homogenate (HSH) and/or phosphate buffered saline (PBS) at 37°C for 24 hours and sampling at intervals of 0, 1, 2, 3, 4, 5 and 24 h. The analytical assays developed for the chemical stability assays are described below.

3.2.3.1 Reagents and Chemicals

Prostaglandin A₁ (PGA₁) and Prostaglandin B₁ (PGB₁) were obtained from Sigma. All the solvents were of HPLC grade. Acetonitrile (ACN), methanol, trifluoroacetic acid (TFA) and ethyl acetate were obtained from BDH. 1, 4-Dioxane was purchased from Aldrich Inc. (Milwaukee, WI, U.S.A).
3.2.3.2 HPLC Method 1 (coupled with Radioactivity Monitoring)

A \( \mu \text{Bondapak C18} \) Column (30 cm x 3.9 mm I.D., 10 \( \mu \)) equipped with a LiChroCART\textsuperscript{TM} Guard Column (Si 100 RP-8, 4 mm x 4 mm I.D., 5 \( \mu \)) was used. The guard column was encased in a Universal Guard Column Holder (BDH). The mobile phase was an ACN-H\textsubscript{2}O-TFA (50:50:0.01 v/v) mixture; flow rate, 1 mL/min (LKB-Bromma 2150 HPLC Pump); recorder speed, 20 cm/h; and Pen sensitivity, 1 mV (Canlab Chart Recorder Model 255). The eluate was monitored at a wavelength of 205 nm and 0.1000 A.U.F.S. using a Waters LC Spectrophotometric detector (Lambda-Max Model 481).

\(^3\)H-PGE\textsubscript{1} liposome or Dermabase\textsuperscript{TM} formulation (1 \( \mu \text{Ci}, 0.1 \text{g} \)) was acidified with 20 \( \mu \text{L} \) 0.1 M HCl and extracted with 1 mL dioxane (2x) as follows: The acidified preparation was mixed with the extraction solvent by vortexing. The mixture was centrifuged at 4,000 x g for 5 minutes (Fisher Centrifuge Model 59) and the supernatant was filtered through 0.45 \( \mu \)m Millipore filter. The filtrate was dried (LabConco Freeze Dryer - Lyph.Lock 6) and the solid residue was reextracted with 1 mL ethyl acetate (2x) by vortexing and filtration steps only. The resultant filtrate was dried and the residue reconstituted with mobile phase to give a theoretical PGE\textsubscript{1} concentration of 0.125 \( \mu \text{g}, 0.25 \mu \text{g}, \) or 1.25 \( \mu \text{g}/10 \mu \text{L} \) corresponding to the 0.05%, 0.1%, and 0.5% PGE\textsubscript{1} formulations respectively.

Aliquots (10 \( \mu \text{L} \)) of each extract were analyzed by HPLC with three replicates per experiment. The retention times for PGE\textsubscript{1} and its major degradation products PGA\textsubscript{1} and PGB\textsubscript{1} were determined using the standard solutions (in mobile phase) and u.v. detection at 205 nm. The fractions corresponding to the PGE\textsubscript{1} peak were determined by correlating the spectrophotometric derived chromatogram with the \(^3\)H radioactivity elution profile. Fractions were collected at 1 min intervals for 0 to 3 min; 0.5 min intervals for 3.5 to 10 min; and again at 1 min intervals for 11 to 15 min. The fractions were mixed with water-
compatible liquid scintillation cocktail (Beckman Ready Value\textsuperscript{TM}) and counted in a Liquid Scintillation Analyzer (Packard Tri-Carb 2000 CA).

The eluate radioactivity associated with the PGE\textsubscript{1} peak was determined, and expressed as a percentage of the total eluate radioactivity as follows:

\[
\% \text{ PGE}_1 = \frac{\sum \text{ dpm (PGE}_1 \text{ Fractions)}}{\sum \text{ dpm (Total Fractions)}} \times 100
\]

3.2.3.3 HPLC Method 2 (Nonradioactive Assay)

A LiChroCART\textsuperscript{TM} guard column (Si 100 RP-8, 4 mm x 4 mm I.D., 5 \(\mu\)m) or Ultrasphere\textsuperscript{TM} C18 precolumn (4.5 cm x 4.6 mm I.D., 5 \(\mu\)m) connected in series with Ultrasphere\textsuperscript{TM} C18 analytical column (15 cm x 4.6 mm I.D., 5 \(\mu\)m) were used for separation. A Beckman System Gold HPLC (Programmable Solvent Module 126, Diode Array Detector Module 168) and a Dell UltraScan 486P microcomputer were used for analysis and data handling. The mobile phase composition, flow rate and wavelength were the same as in Method 1. Unlike Method 1 above, PGE\textsubscript{1} was recovered from the samples by dissolution in methanol and subsequent filtration through 0.45 \(\mu\)m Millipore filter (Type HVHP) prior to injection. PGE\textsubscript{1} was quantified using external standard calibration curves.

3.3 In Vitro Flow-Through Diffusion Cell Studies

Some liposomal formulations were selected, based on initial physical stability evaluations, for in vitro skin penetration experiments. Nonliposomal (Dermabase\textsuperscript{TM}) formulations were also included as controls.

Teflon\textsuperscript{R} Flow-Thru Diffusion Cells (Crown Glass, Somerville, NJ, U.S.A.) with a surface area for diffusion of 0.32 cm\textsuperscript{2} (Bronaugh and Stewart, 1985) were used. Human foreskin specimens were obtained from several healthy adults at circumcision. Each skin specimen was immediately placed in ice-cold PBS and processed within 2 to 3 hours for storage as follows. The specimen was rinsed thoroughly with PBS, mopped lightly on paper towels to remove excess buffer and spread on a Petri dish. The skin specimen was
then covered with a plastic wrap and stored at -20°C until required. Prior to the start of each experiment, the frozen skin was cut to appropriate sized cubes and thawed before being mounted in the diffusion cell. In order to minimize the effect of interperson skin variability, formulation comparisons were done in pairs using the same skin specimens. At least three replicates were obtained per formulation. The better formulation was then compared with another test formulation. Additional replicates were obtained for formulations which showed relatively good in vitro diffusion and long-term stability results. Each skin piece was mounted on a porous support screen inside the diffusion cell and held in place by the open-ended cap. The application of approximately 20 mg of test formulation, containing 3H-PGE₁ as tracer, through the open-ended cap marked the beginning of each experiment. The diffusion cells are designed such that fluid may be continuously pumped through them in order to maintain sink conditions. A PBS solution (7.5 mM Na₂HPO₄, 2.5 mM NaH₂PO₄, 141.2 mM NaCl) maintained at 37°C, isotonic with body fluids, and having a pH of 7.2 was used as the perfusion fluid. The diffusion cells were mounted in a PostiBloc™ Diffusion Cell Heater (Crown Glass) and maintained at 32°C (nominal temperature of the skin surface) by a circulating water bath. Each cell was connected to a fraction collector. The flow rate was 3 mL per hour and fractions were collected at hourly intervals over a period of 24 hours. Fraction aliquots (1 mL) were each mixed with 10 mL LSC (Beckman Ready Value™) and counted in a Liquid Scintillation Analyzer (Packard Tri-Carb 2000 CA). At the end of fraction collection, each skin piece was removed from the diffusion cell, rinsed thoroughly with PBS and blotted dry. The skin was cut into two halves. The epidermal surface of one half was stripped ten times with commercial tape (Highland™, St. Paul, MN, U.S.A.) to remove the stratum corneum. Each strip was placed into a vial, mixed with LSC and analyzed separately. The stripped skin half and the whole skin half were processed separately for scintillation counting as follows. The weight of each half was determined. At least 1 mL (or six times the wet tissue weight) of Soluene® 350 Tissue Solubilizer (Packard Inc., IL) was added to each
tissue and swirled gently to mix. The tissue samples were then placed in the oven at 50°C until digested. After cooling, the samples were decolorized by mixing gently with 300 μL H₂O₂ (30%) and left to stand for a few hours before neutralization with 30 μL glacial acetic acid. The tissue samples were finally mixed with 10 mL LSC each and analyzed to determine cutaneous drug concentrations in the whole skin and viable epidermis/dermis. Plots of transdermal flux and cumulative amount vs time were made from the results of fraction analysis. The cumulative amount vs time plot was used in computing skin penetration parameters; i.e., permeability coefficient, P, diffusion coefficient, D, and lag time, L, as shown in equations 3.1 and 3.2 below.

\[
P = \frac{J_s}{\Delta C} \tag{3.1}
\]

where

\( P \) = permeability coefficient (cm/h)

\( J_s \) = slope of the linear \( r^2 \geq 0.90 \) portion of cumulative amount vs time plot

\( \mu g \ cm^{-2} \ h^{-1} \)

\( \Delta C \) = concentration of PGE₁ in the donor chamber (μg cm⁻³)

\[
D = \frac{h^2}{6L} \tag{3.2}
\]

where

\( D \) = diffusion coefficient

\( h \) = approximate thickness of the barrier layer (0.1 cm)

\( L \) = lag time (s) = the intercept on the time axis of extrapolation of the linear portion of the cumulative amount vs time plot.
3.4 Tissue Homogenate Metabolism Studies

3.4.1 Tissue Sources and Preparation of Homogenates

Three tissue types; namely, human adult foreskin (obtained post-circumcision), human placenta (obtained postpartum), and rabbit lung (harvested from female New Zealand white rabbits, 3-4 kg in weight, Charles River Laboratories, Quebec), were employed. Three specimens of each tissue type were obtained within 1 to 2 h of surgery and processed immediately as follows. Each tissue specimen was washed thoroughly with ice-cold PBS [pH 7.2, containing 1 mM EDTA (Fisher Scientific, NJ, U.S.A.) and 0.05% 2-mercaptoethanol (Sigma)] and the wet weight was determined. The tissue was then crushed under liquid nitrogen (human skin) or minced (human placenta and rabbit lung) and subsequently homogenized, intermittently, for 2 minutes, in 5 volumes (of wet weight) ice-cold PBS using a Brinkmann Polytron® homogenizer (Brinkmann Instruments, ON). Each homogenate was centrifuged at 10,000 x g in a Beckman L8-55 Ultracentrifuge and the supernatants were stored in several small aliquots at -70°C and used within 3 months.

3.4.2 Protein Determinations

The protein content of each tissue homogenate was determined by a modified Lowry assay (Lowry et. al., 1951). Lowry Reagent was prepared by mixing 1 mL each of 1% w/v copper sulfate (Wiler Ltd., London, ON) and 2% w/v sodium potassium tartrate (BDH) and then adding 100 mL of 2% w/v sodium carbonate containing 0.1 N sodium hydroxide (BDH). Each assay was conducted by mixing tissue homogenate sample or bovine serum albumin, BSA, standard (Sigma) with 1 mL Lowry Reagent and adding 0.1 mL Folin & Ciocalteau’s Phenol Reagent (BDH) after 10 minutes. The solutions were then left for 1 hour to allow optimum color development and then absorbance readings were taken at 750 nm (Shimadzu UV 265 uv-visible recording spectrophotometer). Five or six point standard calibration curves ranging from 5 to 120 µg protein were made. Sample
concentrations were determined from the standard calibration curves by regression analysis.

3.4.3 Metabolism Experiments

Metabolism experiments were carried out by incubating PGE₁ (in liposomes or in solution) with tissue homogenate at 37 ± 2°C in the presence of NAD⁺ cofactor. The total incubation volume per experiment was 2 mL and contained 705 μM PGE₁, 750 μM NAD⁺ (except for experiments monitoring the effect of NAD⁺ concentration), 1 mL tissue homogenate and buffer (sufficient quantity). The concentration of 705 μM PGE₁ used for all the experiments represents an excess of substrate since this is much higher than the highest reported Kₘ of 60.1 μM for PGE₁. Control experiments were done with heat denatured homogenate or buffer and in the absence of PGE₁. Sample aliquots (200 μL each) were collected at 0, 1, 2, 3, 4, 5, and 24 h, mixed with 300 μL methanol and filtered through Millipore 0.45 μm hydrophobic (Type HVHP) filter. Twenty five μL volumes of filtrate were immediately analyzed by HPLC Method 2 (see section 3.2.3.3) for PGE₁ and its metabolic/degradation products. The HPLC standards, 15-ketoPGE₁ (KPGE₁), 13,14-dihydroPGE₁ (H₂PGE₁), and 15-keto-13,14-dihydroPGE₁ (KH₂PGE₁), were obtained from Cayman Chemical (Ann Arbor, MI, U.S.A.) while PGA₁, PGB₁, and PGE₁ were obtained from Sigma.

3.5 Electron Microscopy Studies

3.5.1 Preparation of Colloidal Iron Solution

Colloidal iron solution was prepared by the condensation method using the protocol of Foldvari et al. (1988) with slight modifications. A 0.5 M ferric chloride solution was prepared by dissolving 6.75 g of FeCl₃·6H₂O in 50 mL distilled, deionized water (Hayat, 1989). An aliquot (16.6 mL) of this solution was then added dropwise to 200 mL of boiling distilled water and the resulting colloidal iron solution was dialyzed against ten
volumes of distilled water for 5 days with 3 changes of water per day. The final solution was diluted 1:1 with PBS before use.

3.5.2 Preparation of Colloidal Iron-labelled Liposomes

A heterogeneous population of unilamellar and multilamellar liposomes containing colloidal iron particles in their aqueous compartments was prepared by the solvent evaporation method using the lipid phase of liposome formula #29E and an aqueous phase comprising colloidal iron-PBS solution.

3.5.3 Treatment of Guinea Pig Skin

The hair was carefully removed from the back of a Hartley-outbred guinea pig (approximately 400 g in weight obtained from Charles River, St. Constant, Quebec) using an Oster electric clipper, at least 12 hours before liposome application so as to allow for skin nicks, if any, to heal. The colloidal iron-labelled liposomal cream (0.1 g) was applied to a 4 cm² area test site on the back of the guinea pig. The site was then covered with a non-occlusive wrap and left for 12 hours. After the treatment period, the skin was wiped clean with a cotton swab prior to sampling.

3.5.4 Tissue Sampling and Processing for Light and Electron Microscopy

The animal was sacrificed by carbon dioxide asphyxiation in a plastic chamber. The skin from the treatment site was quickly excised and spread out on glutaraldehyde moistened filter paper in a Petri dish. The tissue samples were subsequently trimmed down to ≤ 1 mm³ sizes and immersed in 2.5% Glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.3) at 4°C for 2 h. The glutaraldehyde was rinsed out with the cacodylate buffer 3 times (for 10 min each). Secondary fixation was done with 1% OsO₄ in cacodylate buffer at 4°C for 2 h and the OsO₄ was rinsed out with cacodylate buffer 2 times (15 min each).
The dehydration was done using a graded series of acetone:water mixtures as shown below:

- 50% acetone at 4°C x 10 min
- 70% acetone at 4°C x 10 min (2X)
- 95% acetone at 4°C x 10 min
- 95% acetone at room temp. x 10 min
- 100% acetone at room temp. x 10 min (3X)

Infiltration of the tissue with Spurr resin (4-vinylcyclohexene dioxide 5 g, D.E.R. Resin 3 g, nonenylsuccinic anhydride 13g, and dimethylaminoethanol 0.4 g) was carried out under vacuum as follows:

<table>
<thead>
<tr>
<th>100% Acetone</th>
<th>Spurr Resin</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>1</td>
<td>2h</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>2h</td>
</tr>
<tr>
<td>1</td>
<td>2</td>
<td>2h</td>
</tr>
<tr>
<td>0</td>
<td>1</td>
<td>Overnight</td>
</tr>
</tbody>
</table>

The tissue samples were then placed in flat moulds and cured at 60°C for 48 hours. Thin (0.5 μm) and ultrathin (60 to 90 nm) sections were cut on an ultramicrotome [LKB - Ulrotome III] using freshly made glass knives [LKB Knifemaker Type 7801B]. The thin sections were stained with 1% toluidine blue in 1% borax solution and viewed/photographed on a standard Zeiss microscope. The ultrathin sections were stained primarily with 2% uranyl acetate solution for 30 minutes. The secondary lead citrate stain (5 min) was used only for those sections in which ultrastructural studies were paramount as lead citrate stain can mask the colloidal iron particles (Foldvari et al., 1988). The ultrathin sections were viewed/photographed on a transmission electron microscope (Philips EM 410; accelerating voltage, 60 kV).

Both the light and electron microscope negatives were developed and printed according to standard protocols.
3.6 Clinical Studies

3.6.1 Color Doppler Ultrasonography Studies

A double-blind liposome placebo-controlled crossover design was used. Penile blood flow was evaluated by color Doppler ultrasonography (Ultramark 9 Ultrasound System with L10-5 MHz Transducer, Advanced Technology Laboratories, Ontario, CA) before and after application of test formulations. Three active liposomal formulas, #18A, #28 and #29 (0.05% PGE₁ each), and a liposomal placebo formulation were tested. Five patients were enrolled in the trial and randomly assigned to receive 1.5 g of each formulation at intervals of one week or more. No visual sexual stimulation was employed and the same investigator conducted all the Doppler measurements. After initial sonographic examination to determine blood flow in the flaccid penis, the test formulation was applied on the shaft and glans penis which was subsequently covered with an occlusive wrap. The peak systolic flow velocity in the deep cavernous arteries was monitored at 15 minute intervals for one hour.

3.6.2 Pilot Clinical Trials (Home Setting)

All studies involving human subjects were approved by the Ethics Committee on Human Experimentation at the University of Saskatchewan.

3.6.2.1 Pilot Clinical Trial I

A double blind, randomized, placebo-controlled crossover design was used to test two active (0.05% PGE₁) formulations of PGE₁ in nonliposomal (Dermabase™) and liposomal (Formula #10) vehicles. Ten patients were enrolled in the trial. Each patient was given two properly coded, identical formulations (1.5g each in a collapsible tube) at a time and requested to use the formulations at least one week apart, in a relaxed atmosphere as close to normal as possible. Prior to use, a small amount of each preparation was applied to the volar skin of the forearm for 60 minutes in the morning of the day of the test. If there was
no adverse reaction, the patient proceeded to apply the same preparation to the shaft and
glans of the penis. The cream was left on for up to one hour unless erection occurred
earlier and then washed off prior to intercourse. Each patient kept a log of formulation
effects including adverse reactions on the response sheets provided. Patients were
requested to rank formulation effects from 0 to 4 as shown below.

<table>
<thead>
<tr>
<th>Response Ranking</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td>3</td>
</tr>
<tr>
<td>4</td>
</tr>
</tbody>
</table>

3.6.2.2 Pilot Clinical Trial II

The same trial design as in section 3.6.2.1 was used to investigate the efficacy of active
liposomal preparation #29D (0.1% PGE₁) in 6 impotent patients.

3.6.3 Laser Doppler Flowmetry Studies

Nine healthy volunteers participated in the study. The volunteers were of Caucasian [1],
African [3] and Asian [5] descent. Four formulations were tested:

- #1 Empty liposomes (no PGE₁); i.e., placebo control
- #2 Nonliposomal (0.5% PGE₁); i.e., PGE₁ in a conventional cream, Dermabase™
- #3 Liposomal formula #29E (0.5% PGE₁)
- #4 Liposomal formula #40B (0.5% PGE₁)

Each subject was acclimatized to the environment (20 ± 2°C) for ten minutes prior to
the study. Each test preparation (0.1 g) was applied to a 4 cm² area on the subject’s
forearm. The application area was then covered with non-occlusive Tegaderm™
transparent dressing (3M Canada Inc. London, ON.) except for the area of probe contact.

The Laserflo BPM² Blood Perfusion Monitor (Vasamedics Inc., St. Paul, MN.) with
its Model P-430 Skin Probe was used for evaluating skin blood perfusion in the subjects
before and after the application of the test preparations. The probe was attached to the
treatment site by means of double-sided adhesive tape. The parameter monitored was the
skin blood 'flow' measured in units of mL/D/min/100g. This parameter is theoretically
derived, based on the Laserflo digital and opto-electronic technology and the speeds of
randomly mobile red blood cells, rather than an indicator of net flux in a specific direction.
Readings were taken at 2 minute intervals for (i) 16 minutes before application of the
transdermal formulation so as to establish a baseline flow curve, (ii) 60 minutes with the
formulation in situ, and (iii) 16 minutes after the formulation had been gently wiped off.

The following statistical parameters were determined and used in testing for significant
differences between formulations (1) average maximum blood flow per formulation
computed using the steady state inclusion criteria of Saville et al. (1989) and (2) area under
the response-time curve ("AUC") calculated using the trapezoidal rule. Statistical tests
were done with the nonparametric Kruskal-Wallis and multiple comparison tests for
unequal sample sizes (Zar, 1984). The p value for significance was < 0.05.
Chapter Four

RESULTS AND DISCUSSION

4.1 *In Vitro* Flow-Through Diffusion Cell Studies

The transdermal delivery of PGE\textsubscript{1} from liposomal and nonliposomal formulations was evaluated using excised human foreskin and the *in vitro* flow through diffusion cell model. In humans, the skin covering the penile shaft continues as a retractable fold (the foreskin or prepuce) over the glans penis. The choice of human foreskin as the model membrane was made because of its anatomical similarity to penile skin (Martin, 1985; Moore, 1985; Van De Graaff, 1988; Hall-Craggs, 1990; Frick et al., 1991).

Fourteen liposomal and two non-liposomal formulations were tested. The non-liposomal formulations differed only in PGE\textsubscript{1} content; i.e., 0.05\% and 0.5\% respectively. The liposomal formulations differed in PGE\textsubscript{1} content (0.05\%, 0.1\%, and 0.5\%) as well as lipid phase and aqueous phase composition. Table 4.1 shows the cumulative amount of PGE\textsubscript{1} that penetrated through the skin per formulation over the duration of the experiment (24 hours). There are three formulation pairs (N.L.-0.05\%, N.L.-0.5\%; #29-0.05\%, #29C-0.1\%; #29D-0.1\%, #29E-0.5\%) in Table 4.1, where the only difference in composition between each pair is the drug concentration. Based on the results obtained with these three formulation pairs, it is evident that transdermal PGE\textsubscript{1} delivery from the formulations increased with increase in the drug concentration regardless of the vehicle employed. The highest transdermal PGE\textsubscript{1} delivery was achieved with a liposomal PGE\textsubscript{1} formulation (#40B). At equivalent PGE\textsubscript{1} concentration of 0.05\%, liposome formulations
Table 4.1  *In vitro* transdermal delivery of PGE₁ through human foreskin after 24 h.

<table>
<thead>
<tr>
<th>Formulation # (%) PGE₁, pH</th>
<th>μg / cm² ± S.E.</th>
<th>% of Total Applied ± S.E.</th>
<th>Number of Replicates</th>
</tr>
</thead>
<tbody>
<tr>
<td>N.L. (0.05%, pH 5)</td>
<td>0.38 ± 0.30</td>
<td>1.03 ± 0.95</td>
<td>3</td>
</tr>
<tr>
<td>10 (0.05%, pH 7)</td>
<td>0.96 ± 0.51</td>
<td>2.14 ± 1.12</td>
<td>3</td>
</tr>
<tr>
<td>18A (0.05%, pH 7)</td>
<td>1.39 ± 0.55</td>
<td>3.87 ± 1.53</td>
<td>8</td>
</tr>
<tr>
<td>21 (0.05%, pH 7)</td>
<td>0.06 ± 0.02</td>
<td>0.17 ± 0.07</td>
<td>3</td>
</tr>
<tr>
<td>22 (0.05%, pH 7)</td>
<td>0.21 ± 0.19</td>
<td>0.58 ± 0.52</td>
<td>6</td>
</tr>
<tr>
<td>23C (0.05%, pH 7)</td>
<td>0.24 ± 0.20</td>
<td>0.67 ± 0.55</td>
<td>3</td>
</tr>
<tr>
<td>27 (0.05%, pH 7)</td>
<td>0.34 ± 0.32</td>
<td>0.90 ± 0.83</td>
<td>3</td>
</tr>
<tr>
<td>28 (0.05%, pH 8)</td>
<td>0.16 ± 0.06</td>
<td>0.42 ± 0.17</td>
<td>3</td>
</tr>
<tr>
<td>29 (0.05%, pH 7)</td>
<td>3.51 ± 1.14</td>
<td>9.93 ± 3.24</td>
<td>6</td>
</tr>
<tr>
<td>29C (0.1%, pH 7)</td>
<td>6.73 ± 1.62</td>
<td>8.74 ± 2.10</td>
<td>6</td>
</tr>
<tr>
<td>29D (0.1%, pH 5)</td>
<td>5.60 ± 2.23</td>
<td>7.61 ± 2.79</td>
<td>5</td>
</tr>
<tr>
<td>37 (0.1%, pH 5)</td>
<td>4.64 ± 2.68</td>
<td>5.14 ± 2.31</td>
<td>3</td>
</tr>
<tr>
<td>38 (0.1%, pH 5)</td>
<td>0.41 ± 0.16</td>
<td>0.56 ± 0.28</td>
<td>3</td>
</tr>
<tr>
<td>N.L. (0.5%, pH 5)</td>
<td>8.50 ± 3.93</td>
<td>2.57 ± 1.19</td>
<td>6</td>
</tr>
<tr>
<td>29E (0.5%, pH 5)</td>
<td>15.92 ± 5.11</td>
<td>4.04 ± 1.29</td>
<td>6</td>
</tr>
<tr>
<td>40B (0.5%, pH 5)</td>
<td>24.95 ± 8.35</td>
<td>6.25 ± 2.09</td>
<td>8</td>
</tr>
</tbody>
</table>

Radiolabelled nonliposomal (N.L.) and liposomal PGE₁ formulations were applied to human foreskin specimens using the flow-through diffusion cell model. Transdermal PGE₁ delivery was evaluated by liquid scintillation analyses of the receptor fractions.
#18A and #29 gave approximately 4-fold and 9-fold higher delivery rates when compared to the nonliposomal formulation. Similarly, at 0.5% PGE1 concentration, the liposomal formulations #29E and #40B resulted in up to 3-fold higher transdermal delivery rates compared to the nonliposomal formulation.

*In vitro* estimates of cumulative percutaneous absorption over 24 hours may not be relevant to clinical indications such as in impotence where drug action is expected in a much shorter time. Cumulative absorption profiles over 3 hours should be more indicative of product efficacy in impotence. Table 4.2 shows estimates of *in vivo* absorption of PGE1 from the test formulations based on the *in vitro* cumulative amounts penetrating in 3 hours through the projected mean area of application (80 cm²) *in vivo*.

Liposomal formulations #29E and #40B resulted in *in vivo* transdermal delivery estimates of 32.14 μg and 23.79 μg, per 3 h, respectively. These are within the therapeutic range of 5 to 40 μg PGE1 needed for the treatment of impotence. Despite the higher value of 32.14 μg seen with the liposomal formulation #29E, the liposomal formulation #40B estimate is more reliable due to its relatively low variation. Similar to the 24 h data, increase in the drug concentration in the test formulations generally resulted in increased transdermal PGE1 delivery after 3 h.

It is generally necessary to conduct *in vitro* diffusion cell experiments over long periods of time so as to ensure that steady state conditions are reached and valid percutaneous absorption parameters such as lag time, steady state flux, diffusion coefficient, and permeability coefficient can be calculated. Oftentimes however, the time to steady state is too long, approaching several days. In these instances, percutaneous absorption parameters can be calculated over a shorter period but the results must be cautiously interpreted since true steady state conditions are not reached. Use of the flow-through diffusion cell technique and conducting the experiments over 24 hours ensured that the potential problem of inadequate partitioning of diffused drug into the receptor medium was minimized.
Table 4.2  Estimates of *in vivo* absorption of PGE₁ through human penile skin based on the *in vitro* transdermal delivery of PGE₁ through human foreskin in 3 h.

<table>
<thead>
<tr>
<th>Formulation</th>
<th><em>In Vitro</em> Transdermal Delivery after 3 h (µg cm⁻² x 10⁻²) ± SE</th>
<th><em>In Vivo</em> Transdermal Delivery Estimate (µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N.L. (0.05% PGE₁)</td>
<td>0.10 ± 0.02</td>
<td>0.08</td>
</tr>
<tr>
<td>10 (0.05% PGE₁)</td>
<td>2.55 ± 2.25</td>
<td>2.04</td>
</tr>
<tr>
<td>18A (0.05% PGE₁)</td>
<td>1.25 ± 0.49</td>
<td>1.00</td>
</tr>
<tr>
<td>21 (0.05% PGE₁)</td>
<td>0.08 ± 0.01</td>
<td>0.06</td>
</tr>
<tr>
<td>22 (0.05% PGE₁)</td>
<td>0.07 ± 0.04</td>
<td>0.06</td>
</tr>
<tr>
<td>23C (0.05% PGE₁)</td>
<td>0.06 ± 0.06</td>
<td>0.05</td>
</tr>
<tr>
<td>27 (0.05% PGE₁)</td>
<td>0.12 ± 0.05</td>
<td>0.10</td>
</tr>
<tr>
<td>28 (0.05% PGE₁)</td>
<td>0.43 ± 0.20</td>
<td>0.34</td>
</tr>
<tr>
<td>29 (0.05% PGE₁)</td>
<td>4.56 ± 1.83</td>
<td>3.65</td>
</tr>
<tr>
<td>29C (0.1% PGE₁)</td>
<td>2.17 ± 0.67</td>
<td>1.74</td>
</tr>
<tr>
<td>29D (0.1% PGE₁)</td>
<td>5.53 ± 2.34</td>
<td>4.42</td>
</tr>
<tr>
<td>37 (0.1% PGE₁)</td>
<td>4.73 ± 3.93</td>
<td>3.78</td>
</tr>
<tr>
<td>38 (0.1% PGE₁)</td>
<td>0.29 ± 0.07</td>
<td>0.23</td>
</tr>
<tr>
<td>N.L. (0.5% PGE₁)</td>
<td>10.99 ± 5.94</td>
<td>8.79</td>
</tr>
<tr>
<td>29E (0.5% PGE₁)</td>
<td>40.17 ± 28.17</td>
<td>32.14</td>
</tr>
<tr>
<td>40B (0.5% PGE₁)</td>
<td>29.74 ± 7.80</td>
<td>23.79</td>
</tr>
</tbody>
</table>

Radiolabelled nonliposomal (N.L.) and liposomal PGE₁ formulations were applied to human foreskin specimens using the flow-through diffusion cell model. *In vitro* transdermal PGE₁ delivery after 3 hours was determined by liquid scintillation analyses of the receptor fractions. The *in vivo* estimate was obtained by multiplying the *in vitro* data by 80 cm² (which represents the projected mean area of application *in vivo*). See Table 4.1 for the number of replicates per formulation.
The receptor medium flow rate of 3 mL/h allowed for complete removal of contents of each diffusion cell receptor per sampling interval. This is because the receptor cell volume is 0.13 mL and the recommended flow rates sufficient to achieve complete flushing out of the receptor during each sample collection interval are only about 5 to 10 times the receptor cell volume (Crown Glass Company, Inc. Catalog No. PA-1-85; Bronaugh, 1991).

Fig. 4.1 shows the cumulative transdermal absorption profiles of the nonliposomal formulations (0.05% and 0.5% PGE₁), and two liposomal formulations #29 (0.05% PGE₁) and #40B (0.5% PGE₁). The liposomal formulations #29 and #40B delivered higher amounts of the drug than the nonliposomal formulations of similar drug concentrations.

![Cumulative transdermal absorption profiles](image)

**Figure 4.1** Cumulative transdermal absorption profiles of selected nonliposomal and liposomal PGE₁ formulations. Two nonliposomal (N.L.) preparations containing 0.05% and 0.5% PGE₁ respectively and two liposomal preparations of similar drug concentrations; i.e., #29 (0.05% PGE₁) and #40B (0.5% PGE₁) were evaluated using the flow-through diffusion cell model. The data are reported as mean ± SE. See Table 4.1 for the number of replicates per formulation.
The cumulative amount of PGE\textsubscript{1} penetrating through the skin in 24 hours ranged from 0.06 ± 0.02 to 24.95 ± 8.35 μg cm\textsuperscript{-2} for the 16 formulations (Table 4.1). Liposome formulations of higher PGE\textsubscript{1} concentration gave higher transdermal delivery values. Liposome formula #29C (0.1% PGE\textsubscript{1}) resulted in approximately a two-fold increase in transdermal PGE\textsubscript{1} delivery when compared to #29 (0.05% PGE\textsubscript{1}). Liposome formula #29E (0.5% PGE\textsubscript{1}) increased transdermal delivery approximately three-fold when compared to the essentially similar formula #29D (0.1% PGE\textsubscript{1}). The less than linear increase in transdermal PGE\textsubscript{1} delivery seen with the 0.5% formulations (#29E and #40B) when compared to the lower concentration 0.1% formulations (#29C and #29D) indicates that further increases in drug concentration only is unlikely to yield proportional increases in transdermal PGE\textsubscript{1} delivery from these liposome formulations. Increasing PGE\textsubscript{1} concentration in the transdermal formulations would also make the transdermal dosage form less cost effective when compared to the intracavernous dosage form. The lag time method (Barry, 1983) was used to determine the in vitro skin penetration parameters (Table 4.3) for all the formulations. The steady state transdermal flux from the various liposomal formulations ranged from 0.007 ± 0.006 to 1.709 ± 0.503 μg cm\textsuperscript{-2} h\textsuperscript{-1}.

The in vitro transdermal delivery profiles of all the formulations (pooled data) are shown in Appendix 1.

The steady state flux and the cumulative amount penetrating per 3 h and 24 h were found to be the most consistent parameters for evaluating the transdermal delivery potential of the formulations. Parameters such as the diffusion and partition coefficients are derived from lag time and steady state values and are more prone to error. Although the lag time method is limited by the need to achieve steady state flux, the difficulty in ascertaining which portion of the plot conforms to steady state and the exact barrier thickness (Shah, 1993), it provides for simpler data interpretation and is a useful tool for selecting an optimum formulation in transdermal drug development.
Table 4.3  *In vitro* skin penetration parameters for selected liposomal and nonliposomal PGE\(_1\) formulations.

<table>
<thead>
<tr>
<th>Formula # (%) PGE(_1)</th>
<th>LAG TIME (L) [h]</th>
<th>STEADY STATE FLUX (J(_s)) [(\mu g) cm(^{-2}) h(^{-1})]</th>
<th>DIFFUSION COEFFICIENT (D) [cm(^2) s(^{-1}) x 10(^{-8})]</th>
<th>PERMEABILITY COEFFICIENT (P) [cm h(^{-1}) 10(^{-4})]</th>
</tr>
</thead>
<tbody>
<tr>
<td>N.L. (0.05%)</td>
<td>15.1±0.5</td>
<td>0.056±0.021</td>
<td>3.08±1.78</td>
<td>0.72±0.42</td>
</tr>
<tr>
<td>10 (0.05%)</td>
<td>9.2±1.3</td>
<td>0.049±0.034</td>
<td>5.23±3.02</td>
<td>1.18±0.69</td>
</tr>
<tr>
<td>18A (0.05%)</td>
<td>8.8±1.6</td>
<td>0.091±0.030</td>
<td>5.26±0.77</td>
<td>1.82±0.59</td>
</tr>
<tr>
<td>21 (0.05%)</td>
<td>10.4±0.6</td>
<td>0.003±0.002</td>
<td>4.46±2.58</td>
<td>0.08±0.05</td>
</tr>
<tr>
<td>22 (0.05%)</td>
<td>10.5±4.2</td>
<td>0.017±0.014</td>
<td>3.40±0.73</td>
<td>0.34±0.29</td>
</tr>
<tr>
<td>23C (0.05%)</td>
<td>11.8±0.9</td>
<td>0.023±0.010</td>
<td>3.96±2.29</td>
<td>0.34±0.20</td>
</tr>
<tr>
<td>27 (0.05%)</td>
<td>11.0±2.2</td>
<td>0.043±0.016</td>
<td>4.67±2.70</td>
<td>0.54±0.31</td>
</tr>
<tr>
<td>28 (0.05%)</td>
<td>9.6±1.5</td>
<td>0.007±0.006</td>
<td>5.02±2.90</td>
<td>0.26±0.12</td>
</tr>
<tr>
<td>29 (0.05%)</td>
<td>6.0±1.5</td>
<td>0.210±0.073</td>
<td>7.76±1.63</td>
<td>4.20±1.45</td>
</tr>
<tr>
<td>29C (0.1%)</td>
<td>8.5±1.7</td>
<td>0.462±0.108</td>
<td>4.91±0.53</td>
<td>4.62±1.08</td>
</tr>
<tr>
<td>29D (0.1%)</td>
<td>5.6±1.6</td>
<td>0.261±0.113</td>
<td>6.76±0.99</td>
<td>2.61±1.13</td>
</tr>
<tr>
<td>37 (0.1%)</td>
<td>7.5±1.6</td>
<td>0.236±0.147</td>
<td>6.97±4.02</td>
<td>2.54±1.47</td>
</tr>
<tr>
<td>38 (0.1%)</td>
<td>12.5±0</td>
<td>0.030±0.020</td>
<td>3.69±2.13</td>
<td>0.34±0.20</td>
</tr>
<tr>
<td>N.L. (0.5%)</td>
<td>7.9±2.1</td>
<td>0.648±0.324</td>
<td>9.60±5.31</td>
<td>1.30±0.65</td>
</tr>
<tr>
<td>29E (0.5%)</td>
<td>6.5±1.6</td>
<td>0.888±0.221</td>
<td>8.04±2.53</td>
<td>1.78±0.44</td>
</tr>
<tr>
<td>40B (0.5%)</td>
<td>9.1±1.6</td>
<td>1.709±0.503</td>
<td>4.85±0.48</td>
<td>3.42±1.01</td>
</tr>
</tbody>
</table>

The skin penetration parameters were determined from cumulative amount vs time plots of the various formulations as described in section 3.3. Lag time = the intercept on the time axis of extrapolation of the linear portion (r\(^2\) ≥ 0.90) of the cumulative amount vs time plot; J\(_s\) = slope of the linear portion of the plot; D = h\(^2\)/6L where h is the approximate thickness of the barrier layer in cm and L is the lag time in seconds; P = J\(_s\)/ΔC where ΔC is the concentration of PGE\(_1\) (\(\mu g\) cm\(^{-2}\)) in the donor chamber of the diffusion cell. The data are reported as mean ± SE. See Table 4.1 for the number of replicates per formulation.
Although the optimum liposome formulation, #40B, showed much higher transdermal PGE₁ delivery than the nonliposomal formulations, the fact that some liposomal formulations (e.g. #28) resulted in lower transdermal PGE₁ delivery underscores the versatility of liposomal drug delivery systems. Liposomal delivery systems can either enhance or retard transdermal delivery depending on the composition. The lower transdermal delivery profile seen with liposomal formula #28 may be due to the ionization state of the drug in the formulation and the decreased stability of PGE₁ at higher pH levels. The optimum pH range for PGE₁ is 3 to 5 (Teagarden et al., 1989) while the pH of the liposomal formula #28 is 8. PGE₁, being an acidic drug with a pKa of about 5.5, will be mostly ionized in the basic milieu of the liposomal formula #28 (pH 8). Ionized drug molecules penetrate the skin very poorly in contrast to the unionized species.

Table 4.4 shows the cutaneous uptake of PGE₁ from the various liposomal and nonliposomal formulations. Cutaneous uptake refers to drug localization in the skin after topical administration. The cutaneous uptake results mirrored the efficacy profiles seen with the transdermal delivery results in that increase in drug concentration generally resulted in increased cutaneous PGE₁ delivery and the liposomal formula #40B was the optimum formulation. The in vitro cutaneous uptake data may therefore be useful as a predictive tool for transdermal delivery efficacy. The validity of cutaneous uptake (particularly stratum corneum) data as a predictive tool in percutaneous drug bioavailability studies has been demonstrated previously by Dupuis et al. (1984), Rougier (1990), and Rougier and Lotte (1993). Drug concentrations in the skin are often high enough that nonradioactive, specific assay methods such as HPLC can be employed. Cutaneous drug analysis can be adapted for in vivo human experiments and eliminate the nonspecificity, expense and ethical considerations associated with the use of radioactivity assays in these experiments.
Table 4.4  *In vitro* cutaneous uptake of PGE₁ from liposomal and nonliposomal formulations (24 h).

<table>
<thead>
<tr>
<th>Formula #</th>
<th>Whole Skin</th>
<th>Stratum Corneum</th>
<th>Viable epidermis + dermis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µg/cm²</td>
<td>%</td>
<td>µg/cm²</td>
</tr>
<tr>
<td>N.L. 0.05%</td>
<td>6.05±1.20</td>
<td>18.18±5.87</td>
<td>0.51±0.19</td>
</tr>
<tr>
<td>10</td>
<td>7.11±1.52</td>
<td>15.98±2.23</td>
<td>0.85±0.49</td>
</tr>
<tr>
<td>18A</td>
<td>4.15±0.93</td>
<td>11.98±2.66</td>
<td>0.24±0.05</td>
</tr>
<tr>
<td>21</td>
<td>1.71±0.48</td>
<td>4.85±1.00</td>
<td>0.17±0.05</td>
</tr>
<tr>
<td>22</td>
<td>2.63±0.81</td>
<td>7.66±2.73</td>
<td>0.11±0.04</td>
</tr>
<tr>
<td>23C</td>
<td>2.33±1.11</td>
<td>6.40±2.88</td>
<td>0.16±0.04</td>
</tr>
<tr>
<td>27</td>
<td>5.83±2.44</td>
<td>14.89±5.88</td>
<td>0.14±0.03</td>
</tr>
<tr>
<td>28</td>
<td>1.31±0.15</td>
<td>3.38±0.37</td>
<td>0.28±0.10</td>
</tr>
<tr>
<td>29</td>
<td>4.97±0.88</td>
<td>14.19±2.58</td>
<td>0.32±0.08</td>
</tr>
<tr>
<td>29C 0.1%</td>
<td>20.25±3.04</td>
<td>26.16±3.39</td>
<td>0.54±0.09</td>
</tr>
<tr>
<td>29D</td>
<td>9.10±2.91</td>
<td>12.38±3.79</td>
<td>0.65±0.16</td>
</tr>
<tr>
<td>37</td>
<td>11.75±3.74</td>
<td>14.90±6.70</td>
<td>0.51±0.22</td>
</tr>
<tr>
<td>38</td>
<td>2.24±0.49</td>
<td>3.02±0.60</td>
<td>1.04±0.18</td>
</tr>
<tr>
<td>N.L. 0.5%</td>
<td>32.53±6.86</td>
<td>10.42±2.25</td>
<td>11.00±1.72</td>
</tr>
<tr>
<td>29E</td>
<td>40.29±4.08</td>
<td>11.61±1.61</td>
<td>5.05±1.10</td>
</tr>
<tr>
<td>40B</td>
<td>44.52±5.34</td>
<td>11.47±1.72</td>
<td>14.01±2.29</td>
</tr>
</tbody>
</table>

Following application of the liposomal and nonliposomal (N.L.) formulations to foreskin specimens in the flow-through diffusion cell for 24 hours, each skin specimen was analyzed for PGE₁ content as follows. The skin was thoroughly rinsed with PBS to remove unabsorbed drug and cut into two halves. One half was tape stripped 10 times to remove the stratum corneum. The weight of the skin fractions and stratum corneum were determined. The tape strips (stratum corneum), the remaining skin after tape stripping (viable epidermis + dermis) and the whole skin piece were then analyzed separately by liquid scintillation counting to determine PGE₁ content. See Table 4.1 for the number of replicates per formulation. The data are reported as mean ± SE.
The results from the present investigation clearly show, however, that the use of cutaneous uptake data as the sole predictor of transdermal PGE1 delivery may not be adequate to determine the differences in transdermal delivery which exist between some test formulations; e.g., the liposomal formula #29 (0.05% PGE1) showed a transdermal delivery efficacy about 9 times greater than the nonliposomal (0.05% PGE1) formulation but the cutaneous absorption data suggests the two formulations are equivalent. It may be argued however that the difference between the two formulations may not be clinically significant as the actual percutaneous absorption values are relatively low (< 4 μg cm⁻² 24h⁻¹). Based on the drug concentrations in the stratum corneum after 24 h, one can predict that the liposomal formula #40B (0.5% PGE1) should have the highest transdermal delivery efficacy of all the test formulations. The percutaneous absorption data confirmed this since with formula #40B, transdermal PGE1 delivery is increased 7-fold and 65-fold when compared to the liposomal #29 and the nonliposomal (0.05% PGE1) formulations, respectively. One important limitation of cutaneous drug analyses is that they typically involve single time point determinations (i.e. analyses of drug content in the skin after a predefined time) and hence preclude determination of the rate of drug absorption.

Analysis of PGE1 delivery into the different layers of the skin showed that lower drug levels were present in the stratum corneum when compared to the rest of the skin; i.e., the viable epidermis and dermis. This is a favorable profile for transdermal delivery where drug delivery across the skin is the goal. Increased drug delivery across the stratum corneum (which is the primary barrier layer) and into the deeper layers of the skin from which drug uptake in vivo is relatively facile is desirable in transdermal drug delivery.

Fig. 4.2 shows the concentration of PGE1 in the stratum corneum layers following topical application of liposomal formula #40B (0.5% PGE1). Analysis of PGE1 content in the various layers of the stratum corneum following topical application of all the test formulations for 24 h showed that with each formulation, PGE1 levels were highest in the outermost layer and then gradually decreased. This is consistent with the maintenance of a
high concentration gradient between the formulation on the surface of the skin and the lower layers of the stratum corneum. This high concentration gradient is necessary to maintain the driving force for drug diffusion out of the formulation and into the skin.

![Graph showing PGE1 levels in stratum corneum layers](image)

**Figure 4.2** Analysis of PGE$_1$ levels in stratum corneum layers following *in vitro* application of transdermal liposomal PGE$_1$ formula #40B for 24 h. The stratum corneum layers were removed by successive tape stripping and each tape strip was analyzed separately for radiolabelled PGE$_1$ content. The data are reported as mean ± SE.

The validity of extrapolating *in vitro* skin permeation data to the *in vivo* situation may be questioned considering that several variables in the *in vitro* experimental set-up may significantly modify the results obtained (Behl et al., 1993; Kou et al., 1993; Sclafani et al., 1993). However, a well designed *in vitro* model can provide valuable data not only for comparing formulations but also for predicting clinical efficacy. As noted earlier, all comparative studies should ideally be done with the skin from the same body site and subject. In addition, several skin specimens should be used in order to obtain results more representative of the general population. In transdermal formulation development studies,
practical considerations may limit the extent of replicates and skin specimens employed especially for formulations showing less than optimum properties. It was not feasible to use one skin specimen for all the formulations in our study but the effects of inter- and intrapatient skin variability were minimized by standardizing skin source, method of preparation and storage conditions, and conducting replicate experiments with skin samples from two or more subjects.

Drug concentration and liposome composition were the most important factors influencing in vitro transdermal delivery of PGE\(_1\). Based on the in vitro flow-through diffusion cell data, using a physiological receptor fluid and human foreskin samples, it was estimated that a transdermal liposomal PGE\(_1\) product capable of delivering therapeutic amounts of PGE\(_1\) in vivo is feasible. Clinical studies are required, however, to confirm the clinical efficacy of transdermal liposomal PGE\(_1\).

4.2 Clinical Studies

4.2.1 Color Doppler Ultrasonography Studies

Color Doppler ultrasonography was used to monitor the effect of topically applied liposomal PGE\(_1\) on penile arterial blood flow in patients with erectile dysfunction. Increase in penile arterial blood flow is a prerequisite for erection to occur and therefore serves as an objective parameter for evaluating transdermal PGE\(_1\) delivery and therapeutic efficacy in impotence.

The results of color Doppler ultrasonography measurements following topical application of a liposomal placebo and liposomal PGE\(_1\) formulations #18A, #28 and #29 (containing 0.05% PGE\(_1\) each) are shown in Table 4.5. In general, peak systolic velocity in the deep cavernosal arteries increased with time after application for all the formulations. Amongst the liposomal PGE\(_1\) formulations, #29 resulted in the highest mean peak systolic flow velocity (14.2 cm/s at 45 min) followed by #18A and #28 respectively. This order of efficacy parallels the in vitro transdermal delivery of these formulations as seen in the
diffusion cell studies (section 4.1). The maximum increase in mean peak systolic flow velocity from preapplication values were 7-fold and 2-fold for #29 and #18A respectively. The liposomal placebo formulation also resulted in a modest increase in peak systolic flow which indicates that the liposome vehicle may enhance the pharmacological action of PGE\textsubscript{1} in vivo.

**Table 4.5** Color Doppler ultrasonography: mean peak systolic flow velocity (cm/s) in the deep cavernosal artery of impotent patients after transdermal PGE\textsubscript{1} application.

<table>
<thead>
<tr>
<th>Liposomal Formula</th>
<th>Preapplication</th>
<th>15 min</th>
<th>30 min</th>
<th>45 min</th>
<th>60 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placebo (no PGE\textsubscript{1})</td>
<td>0.0 ± 0.0</td>
<td>4.3 ± 3.8</td>
<td>6.3 ± 2.3</td>
<td>7.7 ± 6.8</td>
<td>9.0 ± 2.1</td>
</tr>
<tr>
<td>n = 3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>#18A (0.05% PGE\textsubscript{1})</td>
<td>5.5 ± 2.6</td>
<td>7.0 ± 3.6</td>
<td>7.8 ± 3.1</td>
<td>12.3 ± 1.1</td>
<td>13.5 ± 3.3</td>
</tr>
<tr>
<td>n = 4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>#28 (0.05% PGE\textsubscript{1})</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>3.3 ± 1.9</td>
<td>1.3 ± 0.8</td>
<td>3.0 ± 1.0</td>
</tr>
<tr>
<td>n = 3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>#29 (0.05% PGE\textsubscript{1})</td>
<td>2.0 ± 1.9</td>
<td>5.6 ± 3.4</td>
<td>8.4 ± 3.2</td>
<td>14.2 ± 4.4</td>
<td>10.6 ± 2.8</td>
</tr>
<tr>
<td>n = 5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The effect of three liposomal PGE\textsubscript{1} and one liposomal placebo formulation on penile blood flow in patients with erectile dysfunction was evaluated by color Doppler ultrasonography using a double-blind crossover design. The data are reported as mean ± SE.

Preapplication mean peak systolic flow velocities were variable ranging from 0 to 5.5 cm/s. This variability is normal and can be attributed to several factors such as measuring variables; e.g., Doppler angle differences can influence the detection of flow in the cavernosal arteries, and differences in patient physiology (normal and/or pathologic) and state of mind have direct implications on penile vascular tone. Previous reports of baseline
systolic blood flow velocities in the cavernous arteries have ranged from 0 cm/s (Schwartz et al., 1991) to 28 cm/s (von Heyden et al., 1993). A baseline value of 28 cm/s is suspect considering that in the nonaroused state, a predominant sympathetic tone exists in the penile tissue and penile arterial blood flow should be relatively low. However, differences in Doppler equipment and measurement technique may explain such high baseline values. The peak systolic blood flow in the deep cavernosal arteries following administration of vasoactive agents such as PGE\textsubscript{1} and papaverine is a major diagnostic criterion for assessing erectile dysfunction. In general, a threshold of greater than 25 cm/s velocity in each cavernous artery is considered as normal (Lue et al., 1985; Quam et al., 1989; Fitzgerald et al., 1992) although some authors (Lee et al., 1993; Allen et al., 1994) consider a threshold of 30 cm/s to be more accurate. The maximum peak systolic flow velocity observed was 32 cm/s following administration of liposomal PGE\textsubscript{1} formula #29 to one of the patients (Fig. 4.3A) and represented a 3-fold increase compared to preapplication values (Fig. 4.3B). Although the velocity of 32 cm/s suggests normal penile arterial function, a full erection was not observed with this patient. Lack of an erection despite adequate penile arterial blood flow in patients with erectile dysfunction could be due to factors such as patient anxiety or the existence of a venous leak. Patient anxiety and the subsequent increased sympathetic discharge associated with the laboratory setting may have decreased the clinical response. Similar results have been reported by Owen et al. (1989) who showed that the increased penile blood flow in Doppler ultrasonography studies following topical nitroglycerin correlated poorly with increased tumescence. Even with intracavernous vasoactive formulations, the adverse effect of anxiety and lack of visual sexual stimulation on the quality and duration of the erection seen in the laboratory setting is established (Schwartz et al., 1991; Meuleman et al., 1992). The existence of a venous leak can prevent the complete tumescence of the penis despite adequate arterial blood flow into the corporeal spaces. It is unlikely however that any of the patients in the study had significant veno-occlusive erectile dysfunction since they had previously been shown to
Figure 4.3 Color Doppler ultrasonographic measurement of peak systolic flow velocities in the cavernosal arteries of a patient with erectile dysfunction following administration of transdermal liposomal PGE1 formula #29. (A) = baseline (preapplication) flow (B) = flow after 45 minutes.
respond well to intracavernous injections of PGE₁. In addition, none of the patients had an end diastolic flow velocity (EDFV) of greater than 5 cm/s in the cavernosal arteries upon Doppler ultrasonography. Cavernosal artery EDFV of greater than 5 cm/s has been shown to be highly indicative of the existence of venous leaks (Quam et al. 1989; Hattery et al., 1991; Fitzgerald et al., 1992).

Erectile dysfunction due to minor cases of venous leak may be ameliorated by placing a tight rubber band at the base of the penis after administration of the vasoactive agent so as to prevent egress of blood from the penis.

Recently, topical treatment of patients with erectile dysfunction primarily due to spinal cord injuries was attempted with PGE₁ (Kim and McVary, 1995) and papaverine (Kim et al., 1995) gels. Using color Doppler ultrasonography, these researchers were able to show significant increase in mean peak systolic flow velocity in the cavernous arteries following topical PGE₁ (0.04% in a lipophilic polyethylene glycol gel) while the effect of topical papaverine on penile blood flow was not significant.

In contrast to the less than 2-fold increase in mean peak systolic flow velocity following application of topical PGE₁ gel as reported by Kim and McVary (1995), the liposomal PGE₁ formula #29 used in this study gave a 7-fold increase. In addition, the study by Kim and McVary (1995) did not have a placebo control.

The transdermal liposomal PGE₁ formulations were well accepted by all the patients. No adverse reactions were reported or observed throughout the study.

4.2.2 Pilot Clinical Trials (Home Setting)

The clinical efficacy of an earlier liposomal #10 (0.05% PGE₁), nonliposomal (0.05% PGE₁), and liposomal #29D (0.1% PGE₁) transdermal formulations was tested in patients with erectile dysfunction. Of the ten patients enrolled in the first clinical trial, three reported improved erectile response following application of transdermal PGE₁ (0.05%) formulations (Table 4.6). One patient had an erection satisfactory for intercourse while the
other two patients had distinct but unsustained erections. Seven patients reported no effect. All ten patients were free of side effects throughout the duration of the trial. No difference in efficacy was seen between the liposomal (#10) and nonliposomal (N.L. 0.05%) PGE₁ formulations tested. This correlates well with the in vitro diffusion cell results in which the two formulations exhibited relatively poor and similar transdermal delivery profiles.

Table 4.6 Clinical trial of liposomal formula #10 (0.05% PGE₁) and nonliposomal PGE₁ (0.05%) formulations in patients with erectile dysfunction.

<table>
<thead>
<tr>
<th>Patient #</th>
<th>Nonliposomal Placebo</th>
<th>Nonliposomal PGE₁</th>
<th>Liposomal Placebo</th>
<th>Liposomal PGE₁ formula #10</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>4</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>9</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>3</td>
</tr>
</tbody>
</table>

A double blind, randomized, placebo-controlled crossover trial design was used to test the clinical efficacy of two active (0.05% PGE₁) liposomal and nonliposomal formulations in patients with erectile dysfunction. The responses are ranked on a 0 to 4 scale (see Section 3.6.2.1).
In the second clinical trial involving an optimized liposomal PGE$_1$ formulation (#29D), four of the six patients tested reported increased penile engorgement following transdermal liposomal PGE$_1$ application when compared to liposome placebo (Table 4.7).

**Table 4.7** Clinical trial of liposomal #29D (0.1% PGE$_1$) formulation in patients with erectile dysfunction.

<table>
<thead>
<tr>
<th>Patient #</th>
<th>Liposomal PGE$_1$ formula #29D</th>
<th>Liposomal Placebo</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

A double blind, randomized, placebo-controlled crossover trial design was used to test the clinical efficacy of liposomal formula #29D (0.1% PGE$_1$) in six patients with erectile dysfunction. The responses are ranked on a 0 to 4 scale (see Section 3.6.2).

The increased clinical response seen with liposomal PGE$_1$ formula #29D relative to the liposomal #10 and nonliposomal (N.L. 0.05%) PGE$_1$ formulations used in the first clinical trial was also predicted by the *in vitro* transdermal delivery results. There were also no reports of adverse effects with patients in the second clinical trial despite the increased drug dose.
4.2.3 Laser Doppler Flowmetry Studies

Laser Doppler flowmetry was used to monitor the effect of topically applied liposomal and nonliposomal PGE₁ (0.5%) formulations in nine healthy volunteers. These studies were carried out because PGE₁ relaxes vascular smooth muscle and would cause increased skin blood flow if it is able to cross the stratum corneum and reach the blood vessels in the deeper layers of the skin. Hence, the relative efficacy of transdermal PGE₁ delivery from test formulations can be assessed by laser Doppler monitoring of their effects on skin blood flow. Another rationale for conducting the laser Doppler flowmetry studies was because of the difficulties associated with the color Doppler ultrasonography model such as the adverse effects of anxiety and lack of visual sexual stimulation on patient response.

The results of skin blood flow (SBF) measurements in the nine subjects prior to transdermal PGE₁ application are shown in Fig. 4.4. The baseline blood flows in the nine volunteers (#1-#9) ranged from about 0.5 to 2 mL/min/100g.

Factors which may influence SBF include exercise, psychological state, ingestion of vasoactive agents, and temperature (Bircher and Maibach, 1989). The influence of these variables were minimized by carefully selecting the subjects and maintaining a relatively uniform ambient room temperature for the study. The BPM² blood perfusion monitor that was used can be operated under environmental conditions of 5 to 40°C and 5 to 85% relative humidity. Probe and/or vehicle effects may also significantly affect blood flow measurements by laser Doppler flowmetry (Guessant et al., 1993). Appropriate controls - probe and liposomal vehicle - were thus included in the study by obtaining baseline and liposomal placebo readings.
Figure 4.4 Baseline (preapplication) skin blood flow values in nine volunteers before topical application of liposomal and nonliposomal PGE₁ formulations. The readings were obtained in an ambient environment (20 ± 2°C) using the Laserflo BPM² Blood Perfusion Monitor.

The two liposomal PGE₁ formulations (#29E and #40B) increased SBF relative to the non-liposomal PGE₁ formulation and liposomal placebo (Fig. 4.5). The placebo formulation (Lip. Placebo) did not result in any increase in skin blood flow when compared to the mean baseline (preapplication) values.

The liposomal placebo SBF values were lower than the preapplication mean values. This may be attributed to probe effects as the effective penetration distance of the probe’s incident laser beam may be modified by the presence of an additional layer (of formulation) on the skin surface.
Figure 4.5 Laserflo mean skin blood flow values in nine volunteers following application of transdermal liposomal and nonliposomal PGE$_1$ (0.5%) formulations. Two liposomal PGE$_1$ (0.5%) formulations, #29E and #40B; a nonliposomal PGE$_1$ (0.5%) formulation; and a liposomal placebo were tested. Measurements were taken at 2 min intervals with the formulations in situ for 1 h and are reported as mean ± SE.

Each formulation was gently wiped off after one hour and further readings taken with the probe in intimate contact with the skin showed liposomal placebo values that approximated preapplication baseline values (Fig. 4.6). It is however possible that the slight rubbing action used in removing the formulation from the skin may have induced increased SBF (as seen with formulations #29E and N.L.0.5%) directly or via increased absorption of the vehicle and/or drug.
Statistical comparisons of the various formulations were based on the mean SBF within one hour after topical application (Fig. 4.5). Table 4.8 shows the 'peak' blood flows per formulation per volunteer. These 'peak' values represent the highest blood flow values observed. In SBF measurements, this parameter is particularly prone to error due to possible artefactual spikes and frequency of sampling effects. It is also obvious that the mean 'peak' blood flow can be calculated in one of two ways; i.e., using the average of the individual 'peak' flows as shown in Table 4.8 or the 'peak' of the mean blood flow curve (Fig. 4.5). The average maximum blood flow parameter which incorporates all flow values within a 99% confidence interval of the mean (Saville, 1989) represents the 'peak' of the mean blood flow curve.

**Figure 4.6** Laserflo mean skin blood flow values in nine volunteers after transdermal liposomal and nonliposomal PGE₁ formulations had been applied for 1 h and then gently wiped off. Measurements were taken at 2 min intervals for 16 mins and are reported as mean ± SE.
Table 4.8 Laserflo 'peak' skin blood flow (mL\text{LD}/min/100 g) values in nine volunteers following application of transdermal liposomal and nonliposomal PGE$_1$ (0.5%) formulations.

<table>
<thead>
<tr>
<th>Volunteer</th>
<th>Liposomal Placebo</th>
<th>Nonliposomal PGE$_1$</th>
<th>Liposomal formula #29E</th>
<th>Liposomal formula #40B</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.56</td>
<td>n.a.</td>
<td>2.72</td>
<td>3.26</td>
</tr>
<tr>
<td>2</td>
<td>0.32</td>
<td>0.48</td>
<td>2.58</td>
<td>12.80</td>
</tr>
<tr>
<td>3</td>
<td>0.41</td>
<td>0.27</td>
<td>0.85</td>
<td>3.81</td>
</tr>
<tr>
<td>4</td>
<td>0.69</td>
<td>4.87</td>
<td>1.64</td>
<td>15.90</td>
</tr>
<tr>
<td>5</td>
<td>0.79</td>
<td>0.47</td>
<td>8.80</td>
<td>3.34</td>
</tr>
<tr>
<td>6</td>
<td>0.59</td>
<td>0.34</td>
<td>1.32</td>
<td>3.97</td>
</tr>
<tr>
<td>7</td>
<td>n.a.</td>
<td>n.a.</td>
<td>1.66</td>
<td>4.77</td>
</tr>
<tr>
<td>8</td>
<td>0.54</td>
<td>0.34</td>
<td>2.85</td>
<td>2.67</td>
</tr>
<tr>
<td>9</td>
<td>0.74</td>
<td>0.29</td>
<td>2.06</td>
<td>5.70</td>
</tr>
<tr>
<td>Mean Peak</td>
<td>0.58±0.06</td>
<td>0.53±0.64</td>
<td>2.72±0.79</td>
<td>6.25±1.58</td>
</tr>
<tr>
<td>Flow ± SE</td>
<td></td>
<td></td>
<td></td>
<td>Kruskal Wallis test: p = 0.0014</td>
</tr>
</tbody>
</table>

| Avg. Max. Flow ± SE | 0.39 ± 0.01 | 0.91 ± 0.01 | 2.10 ± 0.07 | 5.06 ± 0.05 |
| Kruskal Wallis test: p ≤ 0.0001 |

The 'peak' values represent the highest blood flow values observed. However, these values are more prone to error and a computed average maximum blood flow parameter (Avg. Max. Flow) was used, in addition to the mean 'peak' values, for statistical comparisons.
The average maximum blood flow (Fig. 4.7), the mean 'peak' flow (Table 4.8), and the area under the response curve "AUC" (Table 4.9, Fig. 4.8) parameters were used for comparing the formulations. The "AUC" parameter is invaluable for bioequivalency testing since it effectively compares the magnitude of the induced pharmacodynamic response over a clinically relevant time frame. Some investigators have preferred to use the area between the response curve and the physiological baseline (Poelman et al., 1989) or vehicle-control baseline (Wilkin et al., 1985) instead of the entire "AUC". The latter was chosen in this study because the possibility of probe effects exists. In addition, the relationship between a vehicle - drug combination may be synergistic rather than additive; i.e., one cannot assume that the effect of the vehicle in a drug formulation remains essentially the same as that of the vehicle when used alone.

The Kruskal-Wallis test on the average maximum blood flow parameter (Table 4.8; Fig. 4.7) showed a significant difference existed between at least two of the four formulations tested ($p \leq 0.0001$). The subsequent nonparametric multiple comparison test (Zar, 1984) showed that liposome formula #40B was significantly different from the nonliposomal PGE$_1$ and placebo formulations. The Kruskal-Wallis test on the mean 'peak' flow parameter (Table 4.8) showed a significant difference existed between at least two of the four formulations tested ($p = 0.0014$). The subsequent nonparametric multiple comparison test (Zar, 1984) also showed that liposome formula #40B was significantly different from the nonliposomal PGE$_1$ and placebo formulations. The Kruskal-Wallis test on the "AUC" parameter (Table 4.9; Fig. 4.8) gave a $p$ value of 0.0007 and the subsequent multiple comparison test also showed liposomal PGE$_1$ formulation #40B to be significantly different from the nonliposomal and placebo formulations. No statistical difference between either the nonliposomal PGE$_1$ and placebo formulations or the liposomal PGE$_1$ formulas #29E and #40B was observed.
Figure 4.7 Laserflo average maximum skin blood flow per formulation in nine volunteers following application of transdermal liposomal and nonliposomal PGE$_1$ (0.5%) formulations. These values were computed using mean skin blood flow values (Fig. 4.5) and the steady state inclusion criteria of Saville et al. (1989). The data are reported as mean ± SE. Kruskal Wallis test: $p \leq 0.0001$. 
Table 4.9 Laserflo area under the response curve (mL_{LOD}/100 g) following application of transdermal liposomal and nonliposomal PGE\textsubscript{1} (0.05\%) formulations.

<table>
<thead>
<tr>
<th>Volunteer</th>
<th>Liposomal Placebo</th>
<th>Nonliposomal PGE\textsubscript{1}</th>
<th>Liposomal formula #29E</th>
<th>Liposomal formula #40B</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>21.25</td>
<td>n.a.</td>
<td>120.09</td>
<td>109.69</td>
</tr>
<tr>
<td>2</td>
<td>14.76</td>
<td>21.82</td>
<td>92.74</td>
<td>471.19</td>
</tr>
<tr>
<td>3</td>
<td>18.27</td>
<td>11.18</td>
<td>30.35</td>
<td>152.04</td>
</tr>
<tr>
<td>4</td>
<td>29.30</td>
<td>189.91</td>
<td>61.55</td>
<td>683.83</td>
</tr>
<tr>
<td>5</td>
<td>15.84</td>
<td>15.61</td>
<td>218.58</td>
<td>121.96</td>
</tr>
<tr>
<td>6</td>
<td>22.04</td>
<td>17.41</td>
<td>45.52</td>
<td>151.21</td>
</tr>
<tr>
<td>7</td>
<td>n.a.</td>
<td>n.a.</td>
<td>64.63</td>
<td>194.31</td>
</tr>
<tr>
<td>8</td>
<td>23.35</td>
<td>12.05</td>
<td>99.00</td>
<td>125.18</td>
</tr>
<tr>
<td>9</td>
<td>26.53</td>
<td>15.90</td>
<td>72.73</td>
<td>269.59</td>
</tr>
<tr>
<td>Mean ± SE</td>
<td>21.42 ± 1.78</td>
<td>40.55 ± 24.93</td>
<td>89.47 ± 18.56</td>
<td>253.22 ± 65.78</td>
</tr>
</tbody>
</table>

Kruskal Wallis test: p = 0.0007

n.a. = results not available due to subject drop-out from the study.
**Figure 4.8** LaserFlo mean area under the response curve, "AUC", per formulation following application of transdermal liposomal and nonliposomal PGE$_1$ (0.5%) formulations in nine volunteers. The data are reported as mean ± SE. Kruskal Wallis test: $p = 0.0007$. 
In conclusion, liposome encapsulated transdermal PGE\textsubscript{1} formulations caused significantly higher increase in SBF than a nonliposomal PGE\textsubscript{1} dosage form or liposomal placebo. The results show that laser Doppler flowmetry can be used as an effective noninvasive pharmacodynamic testing tool to monitor cutaneous microcirculation changes following transdermal PGE\textsubscript{1} application. The ability to detect changes in SBF has numerous potential applications since peripheral vascular disease, skin tissue trauma, diabetic microangiopathy and central circulation disorders result in alterations to SBF. SBF can be used as an objective parameter to evaluate the severity of these diseases (Holloway, 1980; Rosenberg et al., 1982; Kristensen et al., 1983) and/or the efficacy of therapeutic interventions. It is therefore conceivable that laser Doppler flowmetry can be used to establish a therapeutic window or threshold (in terms of SBF levels) for peripheral arterial occlusive disease. This should assist in the development of even more appropriate parameters for assessing the bioequivalence and therapeutic validity of transdermal PGE\textsubscript{1} formulations.

4.3 Electron Microscopy Studies

The interactions of liposomes with guinea pig skin were investigated in order to assess (1) the efficacy of topically applied liposomes in overcoming the natural barrier layer of the skin, (2) the localization of the liposomes and/or the encapsulated colloidal iron marker in the skin, and (3) the effect of the applied liposomes on skin ultrastructure.

4.3.1 Light Micrographs

The effect of topically applied liposomes on guinea pig skin structure at the light microscopic level was determined by obtaining thin (light microscopy) sections from the dorsum of the guinea pig after pretreatment with liposomes for 12 hours. Fig. 4.9 is a light micrograph of guinea pig skin after treatment with colloidal iron containing liposomes. The structural integrity of the epidermal layer was maintained.
Figure 4.9 Light micrograph of liposome treated guinea pig skin. Colloidal iron-labelled liposomal cream was applied to the back of the guinea pig for 12 h. Thin sections of the liposome treated skin were prepared and photographed on a standard Zeiss microscope as described in section 3.5.4. The subcellular structures identified are the stratum corneum (SC), stratum granulosum (SG), stratum spinosum (SS), stratum basale (SB), dermis (Dm) and the hair follicle (Hf).
Consistent with current knowledge, the stratum corneum is seen to be made up of anucleated flattened cells stacked in rows with the hair follicle traversing the layer. The epidermal layers - stratum basale (SB), stratum spinosum (SS), stratum granulosum (SG), and stratum corneum (SC) were quite evident. The stratum basale is the deepest layer of the epidermis and consists of cuboidal or columnar, actively dividing cells. It is bordered by the dermis on one side and the stratum spinosum on the other. In sections where the SB and SS are not clearly distinguishable they are sometimes grouped together as the malphigian layer by some authors (Leeson et al., 1985). Both layers contain nucleated cells but the fact that the SB is one cell thick while the SS is several cells thick often aids identification at the light microscope level. The stratum granulosum was identified as a 2-cell thick layer filled with keratohyalin granules. The light micrograph shows that the epidermis was much better preserved relative to the dermis. This may be due to inadequate fixation although thin tissue blocks ($\leq 1$ mm$^3$) and standard double fixation technique was used.

The maintenance of the basic structure of the epidermal layer following topical liposome application reflects the biocompatibility and safety of liposomes.

### 4.3.2 Electron Micrographs

Ultrathin sections were taken from the well preserved epidermis in order to study the ultrastructure and also to obtain possible evidence of breaching of the stratum corneum barrier layer by the liposome preparation.

Fig. 4.10 is an electron micrograph of liposome treated guinea pig skin section after double staining with uranyl acetate and lead citrate. This micrograph is of the stratum basale layer and shows well preserved ultrastructure in terms of the fine granular cytoplasm and the facile identification of cellular organelles such as the nucleus and mitochondria and the presence of numerous desmosomes which bind the cells together.
Figure 4.10 Electron micrograph of stratum basale layer of liposome treated guinea pig skin (double staining of skin section). Following the application of colloidal iron-labelled liposomal cream to the back of the guinea pig for 12 h, ultrathin sections of the liposome treated skin were prepared and photographed on a transmission electron microscope as described in section 3.5.4. The skin section shown in this micrograph was double stained with uranyl acetate and lead citrate so as to improve contrast and aid visualization of the subcellular structures such as mitochondria (M), desmosomes (De), nucleus (N) and intercellular spaces (Is).
The ultrastructure in the stratum basale is essentially similar to a previous report by Rhodin (1975) although he used rat skin. These results show that topically applied liposomes do not cause damage to guinea pig skin even at the ultrastructural level and emphasizes the safety and relative lack of toxicity which are associated with liposomal dosage forms. Double staining was used in order to obtain more contrast and better visualization of the ultrastructure but the use of the secondary lead citrate stain may have precluded visualization of the colloidal iron liposome marker.

Figs. 4.11 and 4.12 are electron micrographs of liposome treated guinea pig skin sections after single staining with uranyl acetate only. Due to the fact that lead citrate secondary staining was omitted, the negatives were of very low contrast and thus had to be printed with very high contrast paper (Kodak Grade #5). The ultrastructure was well preserved when compared to earlier reports by Rhodin (1975) and Leeson et al. (1985). In addition to the identification of subcellular structures such as the nucleus, and desmosomes, colloidal iron particles and aggregates were identified in the stratum basale (Fig. 4.11) and stratum spinosum (Fig. 4.12). In the stratum basale, colloidal iron was seen both as single units and as aggregates associated with collagen in the neighboring dermal tissue. This association of colloidal iron marker with collagen fibres after topical liposome application has also been reported recently (Foldvari et al., 1990). Melanin pigments within the keratinocytes of the stratum spinosum and stratum basale may resemble colloidal iron particles but traditionally they migrate through the dendritic processes of the melanocytes to get to these keratinocytes and upon doing so, tend to accumulate above the nucleus and are closely associated with lysosomes (Leeson et al., 1985). There was no evidence of this in the micrographs. Fig. 4.12 shows colloidal iron particles in the stratum spinosum layer of the epidermis and the size range of these particles (150 - 300 nm) suggest they could represent large unilamellar liposomes (100 - 500 nm). A higher magnification (61,000x) micrograph confirmed the particles are not associated with any cellular organelle.
Figure 4.11 Electron micrograph of stratum basale layer of liposome treated guinea pig skin (single staining of skin section). The skin section was stained with uranyl acetate only so as to ensure that the colloidal iron (Fe) is not masked by the secondary lead citrate stain. [De = desmosome, N = nucleus].
Figure 4.12 Electron micrograph of stratum spinosum layer of liposome treated guinea pig skin (single staining of skin section). The skin section was stained with uranyl acetate only so as to ensure that the colloidal iron (Fe) is not masked by the secondary lead citrate stain. [De = desmosome, Is = intercellular space, N = nucleus, M = mitochondria].
Colloidal iron liposome marker was effective in overcoming the natural barrier layer of guinea pig skin and was localized in the deeper layers of the epidermis, and associated with collagen fibres in the dermis.

The colloidal iron marker identified in the skin may represent intact large unilamellar liposomes but this could not be conclusively proven from the micrographs although the previous report of Foldvari et al. (1990) would support this. In contrast to their findings, however, multilamellar liposomes could not be identified within the skin. Given this finding and the fact that Foldvari et al (1990) also noted a size limitation (≤ 0.7 μm approximately), it appears unlikely that multilamellar liposomes (0.5 - 15 μm) can cross the skin intact. They can however be used to deliver an entrapped marker through the skin with virtually no alteration of skin ultrastructure.

4.4 Stability Studies

4.4.1 Physical Stability of Liposomal PGE₁ Formulations

Transdermal skin products, in general, have to be physically stable in order to ensure the maintenance of an aesthetic homogenous appearance which is critical to patient acceptance and confidence in the product. Liposomal PGE₁ formulations have an even greater need to be physically stable since drug leakage from the liposomes would alter the dosage form and may compromise efficacy and chemical stability of the drug.

Table 4.10 shows the schematic composition and pH of selected liposomal PGE₁ formulations. All the formulations had a visual stability rating of ≥ 8 (Table 4.11). There were no significant changes in the organoleptic properties of the formulations within one year of storage. Fig. 4.13 is a light micrograph of a typical liposome formulation. Under the light microscope, all the liposomal PGE₁ formulations contained numerous multilamellar liposomes ranging in size from 1 - 6 μm. There was no increase in fusion or aggregation products upon storage but evidence of slight crystal growth was noticed with Formula #29E after 48 hours storage at room temperature. The pH of the liposomal
preparations ranged from 5.1 to 8.4 while the pH of the Dermabase formulation was 4.5. Considering that skin pH is about 5.5 and PGE₁ pH optimum in various other vehicles has been shown to be in the range of 3 - 5, the selection of these pH ranges ensured that the effect of pH could be monitored with formulations which could, theoretically, be tolerated by the skin.

The effect of the diluent used on encapsulation efficiency determinations of various liposomal PGE₁ formulations is shown in Table 4.12. There was a significant effect with the low pH formulations #29D and #38.

**Table 4.10** Schematic composition of selected liposomal PGE₁ formulations showing differences in pH, drug concentration, lipid phase, aqueous phase, and drug/lipid ratio.

<table>
<thead>
<tr>
<th>Formula # (pH)</th>
<th>PGE₁ (%)</th>
<th>Lipid Phase</th>
<th>Aqueous Phase</th>
<th>Drug/Lipid Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>18A (7.3)</td>
<td>0.05</td>
<td>B</td>
<td>1</td>
<td>1/300</td>
</tr>
<tr>
<td>28 (8.4)</td>
<td>0.05</td>
<td>B</td>
<td>2</td>
<td>1/300</td>
</tr>
<tr>
<td>29 (7.3)</td>
<td>0.05</td>
<td>C</td>
<td>1</td>
<td>1/300</td>
</tr>
<tr>
<td>38 (5.3)</td>
<td>0.10</td>
<td>E</td>
<td>3</td>
<td>1/150</td>
</tr>
<tr>
<td>29D (5.2)</td>
<td>0.10</td>
<td>C</td>
<td>3</td>
<td>1/150</td>
</tr>
<tr>
<td>29E (5.2)</td>
<td>0.50</td>
<td>C</td>
<td>3</td>
<td>1/30</td>
</tr>
<tr>
<td>40B (5.1)</td>
<td>0.50</td>
<td>D</td>
<td>4</td>
<td>1/10</td>
</tr>
</tbody>
</table>

Drug/Lipid Ratios were calculated based on Phospholipon 90H content.
Table 4.11  Visual stability rating for disperse systems (Hanna, 1989).

<table>
<thead>
<tr>
<th>Rating</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>No visual separation, completely homogenous</td>
</tr>
<tr>
<td>8</td>
<td>No visual separation, virtually homogenous</td>
</tr>
<tr>
<td>7</td>
<td>Very indistinct separation, no clear layer at bottom or top</td>
</tr>
<tr>
<td>6</td>
<td>Indistinct separation, no clear layer at bottom or top</td>
</tr>
<tr>
<td>5</td>
<td>Distinct separation, no clear layer at bottom or top</td>
</tr>
<tr>
<td>4</td>
<td>Homogenous top or bottom layer, clear layer at bottom or top</td>
</tr>
<tr>
<td>3</td>
<td>Distinct separation, clear layer at bottom or top with no coalescence</td>
</tr>
<tr>
<td>2</td>
<td>Distinct separation with slight coalescence</td>
</tr>
<tr>
<td>1</td>
<td>Distinct separation with strong coalescence</td>
</tr>
<tr>
<td>0</td>
<td>Complete separation and complete coalescence</td>
</tr>
</tbody>
</table>

Each liposome formulation was evaluated and given a visual stability rating based on the scale.

Table 4.12  Effect of diluent used on liposome encapsulation efficiency determinations.

<table>
<thead>
<tr>
<th>Formula # (pH)</th>
<th>ENCAPSULATION EFFICIENCY (n = 4, ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PBS</td>
</tr>
<tr>
<td>18A (7.3)</td>
<td>80 ± 4%</td>
</tr>
<tr>
<td>28 (8.4)</td>
<td>61 ± 4%</td>
</tr>
<tr>
<td>29 (7.3)</td>
<td>31 ± 2%</td>
</tr>
<tr>
<td>29D (5.2)</td>
<td>26 ± 2%</td>
</tr>
<tr>
<td>38 (5.3)</td>
<td>38 ± 4%</td>
</tr>
</tbody>
</table>

Each liposome aliquot containing radiolabelled PGE₁ was diluted with 3 volumes of diluent and centrifuged at 50,000 rpm, 4°C, for one hour. Encapsulation efficiency was determined by dividing the amount of PGE₁ in the pellet by the amount of PGE₁ in both pellet and supernatant after centrifugation.
Figure 4.13 Light micrograph of a typical liposome formulation.
An ideal diluent should be isoosmotic with and should not alter the pH of the liposome formulation. Of the diluents tested, normal saline is probably closest to the ideal since it is not buffered and is isotonic. The process involved in ensuring that an ideal diluent is used for encapsulation efficiency studies is not warranted so long as encapsulation efficiency results are understood to be relative. Some researchers opt to include the term “apparent” in reporting encapsulation efficiency results in order to emphasize their relativity. A normal saline diluent was routinely used in encapsulation efficiency determinations for the various liposomal PGE$_1$ formulations. Formulas #28 and #29 exhibited relatively low encapsulation efficiencies of 66% and 26% respectively while the rest of the formulations had encapsulation efficiencies greater than 74%. Leakage of encapsulated drug from liposomes was not significant even after prolonged storage; e.g., Formula #18A with an initial encapsulation efficiency of $78 \pm 2\%$, showed virtually no change in encapsulation efficiency after 2 weeks storage at either 4°C (82%) or room temperature (80%); #29E with initial encapsulation efficiency of $87 \pm 2\%$ yielded an encapsulation efficiency determination of $91 \pm 1\%$ after 2 months storage at 4°C while #29D with an initial encapsulation efficiency of $81 \pm 6\%$ gave an encapsulation efficiency result of $85 \pm 2\%$ after 6 months storage at 4°C.

### 4.4.2 Chemical Stability of Liposomal PGE$_1$ Formulations

It was important to study the chemical stability of the liposomal PGE$_1$ formulations since this determines their efficacy and projected shelf lives (expiry dates). A formulation that is clinically effective but very unstable would require a very short expiry dating and this can very well preclude its commercial feasibility. HPLC assay techniques - with radioactivity ($^{3}$H) and nonradioactivity (uv) quantitation were developed for the stability studies. The HPLC-radioactivity assay was used routinely for the stability studies since it eliminates the problem of interferences from other liposome components and can be applied universally to investigational liposomal PGE$_1$ formulations. The radioactivity assay is
however limited by the need to use radiolabelled PGE₁ samples and this precludes its use for the analysis of commercial PGE₁ formulations. The nonradioactive assay was therefore developed and used for the analysis of the optimized liposomal PGE₁ formulation #40B.

Fig. 4.14 is a radiochromatogram of standard PGE₁ solution. The retention time was 5.0 min. The counting efficiency for the tritium label was 37% and this was used in calculating the disintegrations per minute (Dpm) data. The percentage purity of 97% corresponded to the stock ³H-PGE₁ specification and was adjusted for in the formulation assays.

Figure 4.14 HPLC radiochromatogram of PGE₁ standard solution. Radiolabelled PGE₁ and the HPLC Method 1 (see section 3.2.3.2) were used. The radioactivity in the eluate fractions was analyzed by liquid scintillation counting.
Using the nonradioactive HPLC assay method 2, standard solutions of PGE\textsubscript{1}, and its major degradation and metabolic products showed good resolution (Fig. 4.15). The retention times were 3.17 min (PGE\textsubscript{1}), 6.43 min (PGA\textsubscript{1}), 6.85 min (PGB\textsubscript{1}), 3.69 min (13,14-dihydroPGE\textsubscript{1}), 4.17 min (15-ketoPGE\textsubscript{1}), and 4.98 min (15-keto-13,14-dihydroPGE\textsubscript{1}). The total time required for resolution of mixtures of PGE\textsubscript{1}, PGA\textsubscript{1} and PGB\textsubscript{1} is less than 9 minutes. This represents more than 50% reduction in total run time when compared to an earlier HPLC assay (Lee and DeLuca, 1991).

The limit of detection, defined as the lowest amount of analyte which could be reproducibly detected by the assay method, was determined using the signal to noise (3:1) ratio method. The results obtained were 10 ng (PGE\textsubscript{1}), 8 ng (PGA\textsubscript{1}) and 20 ng (PGB\textsubscript{1}), 10 ng (15-ketoPGE\textsubscript{1}), 250 ng (13,14-dihydroPGE\textsubscript{1}), and 500 ng (15-keto-13,14-dihydroPGE\textsubscript{1}).

The limit of quantitation, defined as the lowest amount of analyte which could be quantitated with acceptable accuracy and precision, was determined by analyzing progressively lower concentrations of each analyte. The results obtained were 40 ng (PGE\textsubscript{1}), 20 ng (PGA\textsubscript{1}), 40 ng (PGB\textsubscript{1}), and 200 ng (15-ketoPGE\textsubscript{1}). The acceptance criteria were not more than 10% coefficient of variation, C.V., for precision (HPB-Drugs Directorate Guidelines, 1992) and not greater than 10% deviation from the nominal value for accuracy. Accuracy and precision values at the limits of quantitation, with 6 replicate assays apiece, were as follows: PGE\textsubscript{1} (90.1%, 1.7%); PGA\textsubscript{1} (94.8%, 2.3%); PGB\textsubscript{1} (96.9%, 4.0%); KPGE\textsubscript{1} (93.9%, 2.5%). The relatively low requirement for accuracy is justified by the fact that measurements at or near the limit of quantitation is more prone to error. Seven replicate assays of 2.5 µg PGE\textsubscript{1} gave an accuracy value of 100.9% with a coefficient of variation of 1.4%.

The nonradioactive HPLC assay validation data are presented in greater detail in Appendix E.
Figure 4.15 HPLC chromatogram of PGE₁ and its major degradation and metabolic products. Nonradioactive PGE₁, PGA₁, PGB₁, H₂PGE₁, KPGE₁, and KH₂PGE₁ standards were dissolved in ethanol and chromatographed using the HPLC Method 2 (see section 3.2.3.3).
The standard calibration curves exhibited good linearity (coefficient of determination, \( r^2 > 0.999 \)) at analyte ranges of 40 ng to 4 \( \mu \text{g} \) (PGE\(_1\)), 250 ng to 2.5 \( \mu \text{g} \) (15-ketoPGE\(_1\)), and 40 ng to 2.5 \( \mu \text{g} \) (PGA\(_1\) and PGB\(_1\)). Spectral scan studies showed \( \lambda_{\text{max}} \) values of 194 nm (PGE\(_1\)), 220 nm (PGA\(_1\)), 280 nm (PGB\(_1\)), 231 nm (15-ketoPGE\(_1\)), and 193 nm (13,14-dihydroPGE\(_1\) and 15-keto-13,14-dihydroPGE\(_1\)). Contrary to suggestions by Lee and DeLuca (1991) that a monitoring wavelength of under 200 nm should be favored for simultaneous detection of PGE\(_1\), PGA\(_1\) and PGB\(_1\), a wavelength of 205 nm was adequate and minimized solvent and/or matrix effects which could be limiting at wavelengths under 200 nm.

Table 4.13 shows the chemical stability profiles of the various liposomal formulations during storage at 4°C for 5 months. The drug remained essentially intact in liposomal formulations of low pH and high encapsulation efficiency (#29D, #29E and #40B) while 17% of the initial PGE\(_1\) content had degraded in the nonliposomal formula of similar pH after 5 months. The radiochromatograms of #29E and nonliposomal formulations (Figs. 4.16 - 4.17) clearly show the enhanced stability of PGE\(_1\) in the liposomal dosage form. Assay of initial PGE\(_1\) content in all the formulations gave recovery values of between 91% and 99% except for #28 (75%). The relatively low initial PGE\(_1\) content of #28 indicates a much faster rate of degradation than the other formulations and that significant degradation occurs during its formulation. This higher susceptibility to degradation may be due to its high pH and low encapsulation efficiency. Degradation of PGE\(_1\) from #29 liposome formulation which also has a low encapsulation efficiency and a pH of 7.3 was 25% after 5 months.
Table 4.13 Chemical stability of liposomal PGE$_1$ formulations (stored at 4°C).

<table>
<thead>
<tr>
<th>Formula #</th>
<th>Encapsulation Efficiency (%) (n = 4)</th>
<th>% PGE$_1$ Remaining After</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.5 mo (n = 3)</td>
<td>1 mo (n = 3)</td>
</tr>
<tr>
<td>18A</td>
<td>78 ± 2</td>
<td>100 ± 0</td>
</tr>
<tr>
<td>28</td>
<td>66 ± 2</td>
<td>57 ± 3</td>
</tr>
<tr>
<td>29</td>
<td>26 ± 3</td>
<td>101 ± 0</td>
</tr>
<tr>
<td>29D</td>
<td>81 ± 6</td>
<td>102 ± 2</td>
</tr>
<tr>
<td>29E</td>
<td>87 ± 1</td>
<td>102 ± 1</td>
</tr>
<tr>
<td>40B*</td>
<td>83 ± 1</td>
<td>94 ± 4</td>
</tr>
</tbody>
</table>

Nonliposomal PGE$_1$ formula: 83 ± 1% PGE$_1$ remained after 5 mo.

HPLC-radioactivity assay was used for all the formulations except #40B. * = HPLC nonradioactive assay method. Normal saline was used as the diluent for the encapsulation efficiency determinations. The data are reported as mean ± SE.
Figure 4.16 HPLC radiochromatograms of #29E liposomal PGE₁ formulation showing the effect of storage at 4°C on PGE₁ stability. B = Fresh preparation; C = after 5 months storage at 4°C.
Figure 4.17  HPLC radiochromatograms of nonliposomal PGE₁ (0.05%) formulation showing the effect of storage at 4°C on PGE₁ stability. D = Fresh preparation; E = after 5 months storage at 4°C.
4.4.3 Stability of PGE₁ Under In Vitro Flow-Through Diffusion Cell Conditions

In the *in vitro* diffusion cell experiments, radioactivity quantitation was used because of its high sensitivity and the speed of assay development. This assay technique is however limited by the lack of molecular specificity in the measured samples which may contain some degradation products. It was therefore necessary to study the stability of PGE₁ under the *in vitro* flow-through diffusion cell study conditions in order to confirm that the percutaneous absorption data obtained actually represent PGE₁ levels and not PGE₁ degradation products.

Table 4.14 shows the chemical stability profiles of liposomal and free PGE₁ incubated with denatured human skin homogenate (HSH) or phosphate buffered saline (PBS) at 37°C for 24 hours. The drug was stable (<10% degradation) for up to 5 hours in all the experiments. The liposomal formula #40B (0.5% PGE₁) remained stable throughout the incubation period while only 16% of initial PGE₁ content was degraded from the free drug incubation in denatured HSH after 24 h. The degradation occurred via dehydration of PGE₁ to PGA₁ (Figs. 4.18 - 4.19). No PGB₁ was detected. PGA₁ is the major degradation product of PGE₁ and can in turn be degraded to PGB₁. There were more peaks seen in the liposomal chromatograms (Fig. 4.19) because of the more complex liposome matrix. These liposome matrix peaks did not interfere with either PGE₁ or PGA₁ detection and would not have prevented the detection of PGB₁ if present. The main liposome matrix peak had a retention time greater than 7 min compared to a retention time of 6.85 min for PGB₁. All PGE₁ peaks were shown to be homogeneous by the photodiode array peak homogeneity criterion thus indicating the selectivity of the HPLC assay method 2.
Table 4.14  Chemical stability of PGE$_1$ in liposome encapsulated and solution (free) form incubated in denatured human skin homogenate (HSH) or in phosphate buffered saline (PBS) at 37°C.

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Medium</th>
<th>1h</th>
<th>2h</th>
<th>3h</th>
<th>4h</th>
<th>5h</th>
<th>24h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liposomal PGE$_1$ #40B (n = 6)</td>
<td>Denatured HSH</td>
<td>106 ± 1</td>
<td>99 ± 5</td>
<td>99 ± 3</td>
<td>97 ± 2</td>
<td>98 ± 5</td>
<td>92 ± 2</td>
</tr>
<tr>
<td>Free PGE$_1$ 0.5% (n = 5)</td>
<td>Denatured HSH</td>
<td>100 ± 1</td>
<td>103 ± 1</td>
<td>98 ± 0</td>
<td>98 ± 2</td>
<td>91 ± 0</td>
<td>84 ± 2</td>
</tr>
<tr>
<td>Free PGE$_1$ 0.5% (n = 6)</td>
<td>PBS</td>
<td>97 ± 1</td>
<td>99 ± 1</td>
<td>94 ± 1</td>
<td>95 ± 2</td>
<td>94 ± 3</td>
<td>75 ± 0</td>
</tr>
<tr>
<td>Free PGE$_1$ 0.5% (n = 2)*</td>
<td>PBS</td>
<td>97</td>
<td>96</td>
<td>95</td>
<td>95</td>
<td>94</td>
<td>77</td>
</tr>
</tbody>
</table>

The chemical stability of the 0.5% PGE$_1$ formulations in liposomal vehicle (#40B) or solution (Free PGE$_1$) was monitored by assaying for the percentage of initial PGE$_1$ content remaining at predetermined time intervals using the nonradioactive and radioactive (*) HPLC assays described in Section 3.2.3. The data are reported as mean ± SE.
Figure 4.18 HPLC chromatograms of PGE₁ solution incubated in denatured human skin homogenate at 37°C. (A) after 0 h incubation (B) after 24 h incubation. The HPLC nonradioactive assay method was employed [Beckman System Gold HPLC with RP-C18 columns, ACN-H₂O-TFA (50:50:0.01 v/v) mobile phase, flow rate of 1 mL/min, and monitoring at 205 nm]. The identified peaks are PGE₁ and its major degradation product, PGA₁.
Figure 4.19 HPLC chromatograms of liposomal PGE$_1$ #40B incubated in denatured human skin homogenate at 37 °C. (A) after 0 h incubation (B) after 24 h incubation. The HPLC nonradioactive assay method was employed (see Figure 4.18). The identified peaks are PGE$_1$ (I), its major degradation product, PGA$_1$ (II), and the liposome matrix peak (III).
There was little evidence of decomposition of the liposome matrix during incubation of the liposomal PGE\textsubscript{1} formulation with denatured HSH (Fig. 4.19). Incubation of free PGE\textsubscript{1} in PBS only resulted in slightly higher degradation (25\%) after 24 h incubation when compared to free drug incubation in denatured HSH (16\%). Younger and Szabo (1986) studied the stability of PGE\textsubscript{1} in physiological phosphate buffered water (pH 7.4) at 37°C and reported similar results of approximately 25\% degradation after 24 hours. Previous stability studies of PGE\textsubscript{1} have shown that it is more stable in slightly acidic conditions and at lower temperatures (Monkhouse et al., 1973; Stehle and Oesterling, 1977; Teagarden et al., 1989; Shulman and Fyfe, 1995). No previous study, however, has investigated the stability of PGE\textsubscript{1} in human skin at physiological temperatures. This study shows that nonenzymatic degradation of PGE\textsubscript{1} in human skin at physiological temperature is not significant for at least 5 hours.

Both the HPLC-radioactivity assay Method 1 and the nonradioactive HPLC assay Method 2 were used to analyze samples from the incubation of PGE\textsubscript{1} in PBS. The results from both assay methods showed very good correlation (Table 4.14).

Radiolabelled PGE\textsubscript{1} was used in the in vitro diffusion cell studies and quantitation was by liquid scintillation counting which is not molecule specific. The stability results above show that the degradation of PGE\textsubscript{1} to PGA\textsubscript{1} was not significant with the liposomal formulation and was only 16\% with the free drug, after 24 h incubation at 37°C. PGE\textsubscript{1} is therefore relatively stable under conditions similar to those of the in vitro diffusion cell experiments; i.e., presence of PGE\textsubscript{1} in free and/or encapsulated form in nonviable human skin and PBS receptor medium at 37°C. The radioactive assays obtained in the in vitro diffusion cell studies are therefore representative of actual PGE\textsubscript{1} levels.
4.5 Tissue Homogenate Metabolism Studies

4.5.1 Protein Determinations

The results of protein assays of the various tissue homogenate specimens are shown in Table 4.15. Statistical comparison of all the protein determinations using the Kruskal-Wallis nonparametric one-way analysis of variance (ANOVA) revealed the existence of significant differences between at least one pair of means (p ≤ 0.0001). Subsequent nonparametric multiple comparison tests (Zar, 1984) showed that these differences related to tissue type only. There was no significant difference in protein concentration among specimens of the same tissue type. However, this does not imply that relative uniformity in enzyme activity may be expected from these specimens as actual enzyme concentrations may differ. The determination of total protein ensured that standardized replicates of each tissue type are used in the metabolism experiments and enables the determination of specific enzyme activities.

Table 4.15 Lowry protein assays of tissue homogenates.

<table>
<thead>
<tr>
<th>Tissue Type</th>
<th>Specimen 1 (mg/mL)</th>
<th>Specimen 2 (mg/mL)</th>
<th>Specimen 3 (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human Skin Homogenate (HSH)</td>
<td>2.47 ± 0.17 (n=5)</td>
<td>4.03 ± 0.18 (n=4)</td>
<td>4.70 ± 0.62 (n=5)</td>
</tr>
<tr>
<td>Rabbit Lung Homogenate (RLH)</td>
<td>13.78 ± 0.48 (n=4)</td>
<td>12.75 ± 0.05 (n=4)</td>
<td>13.45 ± 0.29 (n=4)</td>
</tr>
<tr>
<td>Human Placenta Homogenate (HPH)</td>
<td>8.22 ± 0.55 (n=6)</td>
<td>8.52 ± 0.51 (n=6)</td>
<td>7.37 ± 0.66 (n=6)</td>
</tr>
</tbody>
</table>

Three specimens of each tissue type were assayed, by the Lowry method, for protein content using BSA protein standard and absorbance measurements at 750 nm (see section 3.4.2). The data are reported as mean ± SE.
4.5.2 Metabolism of PGE₁ in Human Skin Homogenate (HSH)

The metabolism of PGE₁ in the body represents a bio-inactivation since its major metabolites have little or no biological activity (Anggard, 1966; Anggard and Larsson, 1971). The relative lack of propensity to cause priapism observed with intracavernous PGE₁ therapy for impotence is considered to be due to its rapid metabolism locally in the cavernous tissue (Roy et al., 1989; van Ahlen et al., 1994). It is therefore critical to study metabolism of PGE₁ in human skin as this could be limiting to transdermal PGE₁ delivery.

4.5.2.1 Effect of NAD⁺ on PGE₁ Metabolism in HSH

The effect of NAD⁺ (cosubstrate) concentration on the metabolism of PGE₁ in human skin was investigated using a PGE₁ solution and three concentration levels of NAD⁺ (10 μM, 750 μM and 3000 μM). This investigation was necessary because of the relatively wide range of K_M values reported for NAD⁺ in the literature and the need to ensure that NAD⁺ concentrations are not limiting. Increase in NAD⁺ concentration generally resulted in increased metabolism of PGE₁ (Table 4.16).

At the lowest concentration of NAD⁺ tested; i.e., 10 μM, no significant degradation of PGE₁ occurred in 5 hours and after 24 hours incubation, less than 10% of the initial drug content had been degraded. With NAD⁺ concentrations of 750 μM and 3000 μM, 8% each of the initial PGE₁ contents was lost after 5 hours of incubation and at 24 hours, 16% and 15% PGE₁ had been degraded respectively. The degradation of PGE₁ occurred primarily through metabolism to 15-ketoPGE₁ and nonenzymatic degradation to PGA₁ (Fig. 4.20). No other metabolites or degradation products were detected. Increasing NAD⁺ concentrations beyond 750 μM did not result in higher initial metabolic rates for PGE₁ in HSH.
Table 4.16 Effect of NAD$^+$ concentration on PGE$_1$ metabolism in human skin homogenate (HSH).

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>% of PGE$_1$ remaining after incubation of PGE$_1$ solution in HSH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10 µM NAD$^+$ (n = 6)</td>
</tr>
<tr>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>1</td>
<td>99 ± 2</td>
</tr>
<tr>
<td>2</td>
<td>96 ± 2</td>
</tr>
<tr>
<td>3</td>
<td>97 ± 2</td>
</tr>
<tr>
<td>4</td>
<td>100 ± 1</td>
</tr>
<tr>
<td>5</td>
<td>99 ± 2</td>
</tr>
<tr>
<td>24</td>
<td>92 ± 3</td>
</tr>
</tbody>
</table>

The total incubation volume was 2 mL and each mL of incubation mixture contained 705 µM PGE$_1$, 500 µL HSH, varying levels of NAD$^+$ (10, 750 or 3000 µM) and PBS (sufficient quantity to make up to volume). 200 µL sample aliquots were collected at the predetermined time intervals and analyzed by the nonradioactive HPLC assay method for PGE$_1$. The data are reported as mean ± SE.
Figure 4.20 HPLC chromatograms of PGE$_1$ solution incubated in human skin homogenate (HSH) and 750 $\mu$M NAD$^+$. (A) 0 h assay; inset is a chromatogram of blank HSH (B) 24 h assay. The HPLC nonradioactive assay method was employed (see Figure 4.18). The peaks identified are PGE$_1$, 15-ketoPGE$_1$ (KPGE$_1$), and PGA$_1$. 
There was essentially no difference in the percentages of 15-ketoPGE$_1$ produced within 5 hours at NAD$^+$ concentrations of 750 µM and 3000 µM. 5 ± 2% and 5 ± 0% 15-ketoPGE$_1$, respectively, was produced with 750 µM and 3000 µM NAD$^+$ after 5 h and after 24 h, the values were 9 ± 1% and 21 ± 0% respectively. Despite the higher levels of metabolite seen at the 24 h time point with 3000 µM NAD$^+$, the % loss of parent drug, PGE$_1$ at this time point was not significantly different from the 750 µM NAD$^+$ experiment (85 ± 3% vs 84 ± 3% respectively). The production of 15-ketoPGE$_1$ was inversely related to PGA$_1$; i.e., higher levels of 15-ketoPGE$_1$ were associated with lower levels of PGA$_1$. This negative correlation suggests that the metabolic pathway inhibits the nonenzymatic pathway to some extent and explains the relative similarity in total PGE$_1$ loss values despite differences in total metabolite production levels. Control experiments using heat-denatured human skin homogenate and 750 µM NAD$^+$ resulted in similar levels of PGE$_1$ degradation: 84 ± 2% of initial drug content remained after 24 h (see Table 4.14) and drug loss could be traced to nonenzymatic degradation to PGA$_1$ only (see Fig. 4.18).

In all the metabolism experiments of free PGE$_1$ in HSH, 15-ketoPGE$_1$ could not be detected in the first hour and could only be quantitated from ≥ 2 h after incubation. In the clinical administration of transdermal PGE$_1$ for impotence, it is therefore not likely that metabolism of the free drug in the skin will be the cause of nonoptimal therapeutic effects within one hour, particularly because NAD$^+$ concentrations in vivo may be much lower. Approximately 0.05, 3.75, and 15 nanomoles of NAD$^+$, respectively, were added per mg wet tissue weight in the metabolism experiments (10 µM, 750 µM, and 3000 µM NAD$^+$ cofactor levels) whereas Im and Hoopes (1970) reported that the concentration of NAD$^+$ in human abdominal and inguinal skin was only approximately 1 nanomole per mg dry tissue weight.
The relatively low amount of PGE₁ degradation within 24 h in the human skin homogenate experiments (≤ 20%) indicates that skin metabolism should also not be limiting in transdermal PGE₁ treatment for peripheral arterial occlusive diseases where onset and duration of therapeutic effect may be much longer.

4.5.2.2 Effect of Liposome Encapsulation on PGE₁ Metabolism in Human Skin Homogenate

Two optimum liposome formulations from the stability and diffusion studies were used in these experiments namely liposome formulas #29E and #40B. Liposomal formulations (0.5% PGE₁) were incubated with active human skin homogenate and 750 μM NAD⁺ as described in Section 3.4.3. The results are summarized in Table 4.17 which also includes data from the PGE₁ standard solution experiment for comparison. Metabolism of PGE₁, followed by monitoring loss of initial drug content, was not significant (≤ 10%) over 5 h of incubation. After 24 h incubation, less than 20% of initial PGE₁ content was metabolized regardless of whether the drug was encapsulated in liposomes or present in solution.

Chromatograms of blank liposome formula #40B in HSH revealed liposome matrix peaks with retention times of approximately 4.0 min and 7.2 min but no potential interfering peaks with respect to PGE₁ quantitation were seen (Fig. 4.21A). Incubation of blank liposomes #40B in human skin homogenate resulted in metabolism of component(s) of the liposome matrix. The matrix derived metabolite spanned a range of approximately 3.5 to 6.0 minutes on the chromatogram at 24 h (Fig. 4.21B).
Table 4.17 Metabolism of liposomal and free PGE₁ incubated in human skin homogenate (HSH) and 750 μM NAD⁺.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>% of PGE₁ remaining after incubation in HSH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Liposomal PGE₁ formula #40B (n = 4)</td>
</tr>
<tr>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>1</td>
<td>101 ± 2</td>
</tr>
<tr>
<td>2</td>
<td>92 ± 3</td>
</tr>
<tr>
<td>3</td>
<td>95 ± 1</td>
</tr>
<tr>
<td>4</td>
<td>91 ± 2</td>
</tr>
<tr>
<td>5</td>
<td>96 ± 1</td>
</tr>
<tr>
<td>24</td>
<td>83 ± 1</td>
</tr>
</tbody>
</table>

The total incubation volume was 2 mL and each mL of incubation mixture contained 705 μM PGE₁, 500 μL HSH, 750 μM NAD⁺, and PBS (sufficient quantity to make up to volume). 200 μL sample aliquots were collected at the predetermined time intervals and analyzed by the nonradioactive HPLC assay method for PGE₁. The data are reported as mean ± SE.
Figure 4.21 HPLC chromatograms of blank (no PGE$_1$) liposomal formula #40B incubated in HSH. (A) 0 h assay (B) 24 h assay. The HPLC nonradioactive assay method was employed (see Figure 4.18). The identified peaks are liposome matrix (II) and liposome matrix metabolite(s) (I).
Metabolism of matrix component(s) was also seen with the liposomal PGE\textsubscript{1} (Formulas #40B and #29E) incubations in HSH. These liposome-matrix derived metabolites occurred in the area of the chromatogram where the PGE\textsubscript{1} metabolites typically appear and thus preclude their quantitation (Figs. 4.22 - 4.23). PGE\textsubscript{1} and PGA\textsubscript{1} peaks were not affected and could be quantitated using peak height or area measurements. Nonenzymatic degradation of PGE\textsubscript{1} in the liposomal formulations occurred through conversion to PGA\textsubscript{1} only (Figs. 4.22 - 4.23). Therefore, the extent of metabolism in the liposomal PGE\textsubscript{1} experiments may be estimated by calculating the percentage of initial PGE\textsubscript{1} content that is degraded to PGA\textsubscript{1} and comparing this value to the total loss of PGE\textsubscript{1}. With liposome formula #29E, 10 ± 0\% (n = 4) of the initial PGE\textsubscript{1} content was degraded to PGA\textsubscript{1} after 24 h incubation indicating that practically no metabolism occurs in the skin with this formulation since 92 ± 2\% PGE\textsubscript{1} remained after 24 h (Table 4.17). In contrast, with liposome formula #40B, 83 ± 1\% PGE\textsubscript{1} remained after 24 h indicating a total loss of approximately 17\% while only 8 ± 0\% (n = 4) of the initial PGE\textsubscript{1} content was converted to PGA\textsubscript{1}. The difference of about 11\% may be attributed to metabolism. This estimate of about 11\% metabolism in HSH with liposomal PGE\textsubscript{1} formula #40B is supported by results from a control experiment where the formulation was incubated with heat-denatured HSH. Only 8\% of the initial PGE\textsubscript{1} content was lost after 24 h in the control experiment and was accounted for by the nonenzymatic production of PGA\textsubscript{1} only. When compared to the 17\% loss seen with the active homogenate this represents a difference of 9\% which can be attributed to metabolism.
Figure 4.22  HPLC chromatograms of liposomal PGE₁ formula #40B incubated in HSH. (A) 0 h assay (B) 24 h assay. The HPLC nonradioactive assay method was employed (see Figure 4.18). The identified peaks are PGE₁ (I), PGA₁ (II), and liposome matrix (III).
Figure 4.23  HPLC chromatograms of liposomal PGE₁ formula #29E incubated in HSH. (A) blank liposomes #29E in HSH (B) liposomal PGE₁ #29E in HSH at zero time (C) liposomal PGE₁ #29E in HSH after 24 h. The identified peaks are PGE₁ (I), PGA₁ (II), and liposome matrix (III).
Figure 4.23 continued
Investigations into the actual identities of the liposome matrix peaks (retention times approximately 4.0 and 7.2 minutes) indicated that they are largely due to methyl salicylate (Fig. 4.24). The metabolite peak arising from the liposome matrix peak had the same retention time (4.0 min) as salicylic acid. Methyl salicylate which is an ester is presumably metabolized by esterases in the human skin homogenate to salicylic acid. The presence of salicylic acid (retention time, 4.0 min) in the stock methyl salicylate solution (Fig. 4.24) may be the result of nonenzymatic hydrolysis of methyl salicylate. Nonenzymatic hydrolysis of methyl salicylate does not appear to play a major role in the active homogenate metabolism experiments since minimal degradation of the methyl salicylate peak was seen when liposomal PGE₁ formula #40B was incubated in denatured HSH (see Fig. 4.19 in Stability Section 4.4). These results are in agreement with those from another study (Boehnlein et al., 1994) which also found significantly increased metabolism of methyl salicylate in viable skin when compared to nonviable skin.

4.5.3 Metabolism of PGE₁ in Rabbit Lung Homogenate (RLH)

4.5.3.1 Effect of NAD⁺ on PGE₁ Metabolism in RLH

The effect of NAD⁺ concentration on the metabolism of PGE₁ in rabbit lung was investigated using a PGE₁ solution and four concentration levels of NAD⁺ (10 μM, 750 μM, 1500 μM, and 3000 μM). The metabolism of PGE₁ in RLH was directly proportional to the concentration of NAD⁺ cofactor used (Table 4.18; Fig. 4.25).

The degradation of PGE₁ in RLH occurred primarily through metabolism to 15-ketoPGE₁ and nonenzymatic degradation to PGB₁. At 10 μM NAD⁺ concentration, very slight metabolism occurred with the detection of 15-ketoPGE₁ as the only metabolite but the amounts were below the system limit of quantitation.
Figure 4.24 HPLC of methyl salicylate standard solution. The HPLC nonradioactive assay method was employed (see Figure 4.18). The identified peaks are methyl salicylate (I) and salicylic acid (II).
Table 4.18  Effect of NAD\(^+\) concentration on PGE\(_1\) metabolism in rabbit lung homogenate (RLH).

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>% of PGE(_1) remaining after incubation of PGE(_1) solution in RLH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10 (\mu)M NAD(^+) ((n = 4))</td>
</tr>
<tr>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>1</td>
<td>108 ± 1</td>
</tr>
<tr>
<td>2</td>
<td>107 ± 3</td>
</tr>
<tr>
<td>3</td>
<td>101 ± 2</td>
</tr>
<tr>
<td>4</td>
<td>100 ± 3</td>
</tr>
<tr>
<td>5</td>
<td>95 ± 3</td>
</tr>
<tr>
<td>24</td>
<td>79 ± 2</td>
</tr>
</tbody>
</table>

The total incubation volume was 2 mL and each mL of incubation mixture contained 705 \(\mu\)M PGE\(_1\), 500 \(\mu\)L RLH, varying levels of NAD\(^+\) (10, 750, 1500 or 3000 \(\mu\)M) and PBS (sufficient quantity to make up to volume). 200 \(\mu\)L sample aliquots were collected at the predetermined time intervals and analyzed by the nonradioactive HPLC assay method for PGE\(_1\). The data are reported as mean ± SE.
Figure 4.25 Effect of NAD$^+$ concentration on PGE$_1$ metabolism in rabbit lung homogenate. n = 6 per concentration level of NAD$^+$. The data are reported as mean ± SE.

PGB$_1$ was the major nonenzymatic degradation product and was observed at only the 24 h time point. This is in contrast to the human skin homogenate (HSH) experiments where the major nonenzymatic degradation product was PGA$_1$. Just as with the HSH experiments, the metabolic pathway inhibited the nonenzymatic pathway in the RLH experiments. The amount of PGB$_1$ produced was highest with 10 μM NAD$^+$ where metabolism was the lowest while with 1500 μM and 3000 μM NAD$^+$, no PGA$_1$ or PGB$_1$ could be detected indicating complete inhibition of the nonenzymatic pathway. The loss of parent drug was paralleled by the production of 15-ketoPGE$_1$. With 3000 μM NAD$^+$, the initial PGE$_1$ content was completely metabolized to 15-ketoPGE$_1$ in 2 hours while with 750 μM NAD$^+$, about 30% of initial PGE$_1$ content was metabolized to 15-ketoPGE$_1$ in 2
hours (Table 4.18, Fig. 4.25). In contrast, less than 3% substrate conversion to 15-ketoPGE$_1$ occurred in 2 hours when PGE$_1$ was incubated in human skin homogenate containing either 750 μM or 3000 μM NAD$^+$. Unlike the HSH experiments where 15-ketoPGE$_1$ was the only metabolite identified, there was evidence of other unidentified metabolites with retention times of 5.8 min and 6.2 min respectively (Fig. 4.26) in the RLH experiments. These unidentified metabolites are derived presumably from the major metabolite, 15-ketoPGE$_1$, since their production correlated positively with the decrease in 15-ketoPGE$_1$ levels seen at the latter time points. The further metabolism of 15-ketoPGE$_1$ in RLH underlines the importance of conducting the drug metabolism studies by following the loss of parent drug in addition to monitoring the production of 15-ketoPGE$_1$. In the basic metabolism scheme for PGE$_1$, further metabolism of its primary metabolite, 15-keto PGE$_1$, usually occurs via Δ$^{13}$-prostaglandin reductase (Anggard and Larsson, 1971). There was no evidence of Δ$^{13}$-prostaglandin reductase activity in the RLH experiments since its product, 15-keto-13,14-dihydroprostaglandin E$_1$ (KH$_2$PGE$_1$), was not detected. Schlegel and Greep (1975) reported that NAD$^+$ has an inhibitory action on Δ$^{13}$-prostaglandin reductase which could explain the apparent lack of Δ$^{13}$-prostaglandin reductase activity seen in the RLH experiments. It is possible, however, that Δ$^{13}$-prostaglandin reductase activity occurred but the amount of KH$_2$PGE$_1$ produced was below the detection limits due to low total production and/or a very fast rate of degradation of this secondary metabolite.
Figure 4.26 HPLC chromatograms of PGE₁ solution incubated in rabbit lung homogenate (RLH) and 750 μM NAD⁺: (A) after 0 h incubation; inset is a chromatogram of blank RLH (B) after 24 h incubation. The HPLC nonradioactive assay method was employed (see Figure 4.18). The identified peaks are PGE₁, 15-ketoPGE₁ (KPGE₁), and PGB₁.
Control experiments using heat-denatured rabbit lung homogenate and 750 μM NAD⁺ resulted in conversion of PGE₁ primarily by nonenzymatic degradation to PGA₁ only. PGB₁ was not identified throughout the course of the incubation. 94 ± 1% of initial PGE₁ content remained after 5h incubation in denatured RLH and no metabolite peaks were identified within this time period. After 24 hours incubation in denatured RLH, 77 ± 1% of initial PGE₁ content remained compared to 60 ± 3% seen in the active RLH experiment. Although PGA₁ was the major product, KPGE₁ (2 ± 0%) was also detected at the 24 h time point (Fig. 4.27). This KPGE₁ production is probably a result of residual metabolic activity in the denatured RLH since nonenzymatic production of KPGE₁ from PGE₁ has not been shown to occur previously.
Figure 4.27  HPLC chromatogram of PGE₁ solution incubated in denatured RLH.  
(A) 0 h assay  (B) 24 h assay. The HPLC nonradioactive assay method was employed (see 
Figure 4.18). The identified peaks are PGE₁ (I), 15-ketoPGE₁ (II), and PGA₁ (III).
4.5.4 Metabolism of PGE\textsubscript{1} in Human Placenta Homogenate (HPH)

The rate and extent of degradation of standard PGE\textsubscript{1} solution incubated in HPH containing 750 \(\mu\text{M} \text{NAD}^+\) was most rapid when compared to equivalent HSH and RLH experiments. The degradation of PGE\textsubscript{1} in HPH occurred primarily through metabolism to 15-ketoPGE\textsubscript{1} (Fig. 4.28).

![Graph showing metabolism of PGE\textsubscript{1} and 15-ketoPGE\textsubscript{1} over time](image)

**Figure 4.28** Metabolism of PGE\textsubscript{1} in human placenta homogenate (HPH). 15-ketoPGE\textsubscript{1} (KPGE) values are reported as a percentage of its maximum expected value i.e. assuming PGE\textsubscript{1} is completely metabolized to 15-ketoPGE\textsubscript{1}. PGE\textsubscript{1} (PGE) values are reported as a percentage of the initial PGE\textsubscript{1} content in the incubation mixture. \(n = 9\) (3 specimens x 3 replicates each). The data are reported as mean ± SE.
After 2 hours incubation in HPH, 59 ± 4% of the initial PGE₁ content was converted to 15-ketoPGE₁ compared to 29 ± 3% and less than 1% in RLH and HSH respectively. At the end of the 24 h incubation period in HPH, only 5 ± 3% of initial PGE₁ content remained. There was no evidence of nonenzymatic degradation to PGA₁ or PGB₁ confirming the earlier observations with HSH and RLH experiments that extensive metabolism inhibits the degradative pathway. Similar to the RLH experiments, other metabolites in addition to 15-ketoPGE₁ were observed at the latter time points and are most probably due to further metabolism of 15-ketoPGE₁ since their production correlated positively with the decrease in 15-ketoPGE₁ levels seen from ≥ 2 h. In addition to the peaks with retention times of 5.8 min and 6.2 min seen with the RLH experiments, an additional metabolite peak with a retention time of 3.3 min was seen (Fig. 4.29). The hypothesis that these unknown metabolite peaks originate from 15-ketoPGE₁ was confirmed by incubating 15-ketoPGE₁ standard in HPH (Fig. 4.30).

Control experiments using heat-denatured HPH and 750 μM NAD⁺ resulted in conversion of PGE₁ primarily by nonenzymatic degradation to PGA₁. Practically no substrate conversion occurred in 5 h as 98 ± 2% of the initial PGE₁ content remained after 5 h incubation in denatured HPH. However, after 24 h incubation, 80 ± 1% of the initial PGE₁ content remained compared to 5 ± 3% seen in the active HPH experiment. Similar to the denatured RLH experiment, 15-ketoPGE₁ (5 ± 0%) was detected after 24 h incubation of PGE₁ in denatured HPH and is likely due to residual enzyme activity. No other degradation products apart from PGA₁ and 15-ketoPGE₁ were seen (Fig. 4.31).
Figure 4.29 HPLC chromatograms of PGE₁ solution incubated in human placenta homogenate (HPH) and 750 μM NAD⁺. (A) after 0 h incubation; inset is a chromatogram of blank HPH (B) after 24 h incubation. The HPLC nonradioactive assay method was employed (see Figure 4.18). The peaks identified are PGE₁, 15-ketoPGE₁ (KPGE₁), and 13,14-dihydroPGE₁ (H₂PGE₁).
Figure 4.30 HPLC chromatograms of 15-ketoPGE\textsubscript{1} solution incubated in human placenta homogenate (HPH) and 750 µM NAD\textsuperscript{+}: (A) after 0 h incubation (B) after 5 h incubation. Inset is 15-ketoPGE\textsubscript{1} standard solution. The peaks identified are 15-ketoPGE\textsubscript{1} (I), and 13,14-dihydroPGE\textsubscript{1} (II).
Figure 4.31 HPLC chromatograms of PGE₁ solution incubated in denatured human placenta homogenate (HPH) and 750 μM NAD⁺: (A) after 0 h incubation (B) after 24 h incubation. The peaks identified are PGE₁ (I), 15-ketoPGE₁ (II), and PGA₁ (III).
4.5.5 Relative 15-Hydroxyprostaglandin Dehydrogenase Activity in HSH, RLH, and HPH

The activity of 15-hydroxyprostaglandin dehydrogenase (15-OHPGDH) in HSH, RLH, or HPH under conditions of physiological pH and temperature, 750 μM NAD⁺ and excess substrate concentration is expressed as nanomoles of product (KPGE₁) generated in one hour. The results are summarized in Table 4.19. 15-OHPGDH activity was lowest in HSH. The enzyme activities in RLH and HPH were approximately 37x and 71x higher respectively when compared to HSH enzyme activity. 15-OHPGDH activity was not always proportional to the total protein content. Although HSH with the lowest total protein content had the lowest enzyme activity, HPH showed the highest activity despite having a lower total protein content than RLH. This underscores the invalidity of using total protein content as a measure of actual enzyme activities in tissue homogenates.

Table 4.19 Relative activity of 15-OHPGDH in HSH, RLH, and HPH.

<table>
<thead>
<tr>
<th>Tissue Type</th>
<th>Total Protein Content (mg)</th>
<th>Enzyme Activity (nmoles h⁻¹)</th>
<th>Specific Enzyme Activity (nmoles h⁻¹ mg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human Skin Homogenate (n = 6)</td>
<td>0.37 ± 0.07</td>
<td>0.08 ± 0.03</td>
<td>0.22</td>
</tr>
<tr>
<td>Rabbit Lung Homogenate (n = 6)</td>
<td>1.33 ± 0.03</td>
<td>2.20 ± 0.19</td>
<td>1.65</td>
</tr>
<tr>
<td>Human Placenta Homogenate (n = 9)</td>
<td>0.80 ± 0.03</td>
<td>4.23 ± 0.36</td>
<td>5.29</td>
</tr>
</tbody>
</table>

The enzyme activity is expressed as the amount of 15-ketoPGE₁ produced from PGE₁ per 0.2 mL tissue homogenate incubation mixture. Total protein content represents the amount of protein in 0.2 mL incubation mixture. Each mL of incubation mixture contained 705 μM PGE₁, 500 μL tissue homogenate, 750 μM NAD⁺, and PBS (sufficient quantity to make up to volume). The data are reported as mean ± SE.
This study is to the best of the author's knowledge the only one to have compared the activities of 15-OHPGDH in human skin, rabbit lung, and human placenta. The results indicate that human skin is much less capable of metabolizing PGE$_1$ than rabbit lung and human placenta. Camp and Greaves (1980) compared the activities of 15-OHPGDH in rat skin and rat lung homogenates in order to evaluate the relevance of skin metabolism of prostaglandins and their results also showed much greater (5-fold) enzyme activity in the lung compared to the skin.
Chapter Five

CONCLUSIONS AND RESEARCH PERSPECTIVE

The goal of the thesis was primarily to analyze the transdermal delivery potential of prostaglandins using PGE$_1$ as a model prostaglandin. Transdermal delivery in this case means drug delivery through the skin but not for systemic effect. The development of a transdermal PGE$_1$ dosage form would provide clinical advantages over the commercially available injectable PGE$_1$ dosage form. Transdermal drug delivery, TDD, has the following features (i) it is noninvasive (ii) hepatic and/or gastrointestinal metabolism of the drug can be avoided (iii) systemic side effects can be minimized due to better drug targeting and (iv) site-specific drug delivery and sustained release formulations can be achieved.

These features make TDD an attractive concept when compared to other more traditional drug delivery routes such as the oral and intravenous routes. TDD is limited by the fact that the skin poses a considerable barrier to most drugs and is affected by numerous factors which have to be optimized. The focus of the thesis was therefore to optimize these factors with respect to the transdermal delivery of PGE$_1$.

PGE$_1$ is a model drug for TDD since it is (i) potent (ii) lipophilic (iii) subject to extensive systemic metabolism (iv) has a low molecular weight and melting point and (v) is used for the treatment of impotence and peripheral arterial occlusive disease which are both diseases for which site-specific drug delivery would be beneficial.

Liposomes were chosen as the drug delivery vehicle because they can enhance TDD, stabilize PGE$_1$, are biocompatible/biodegradable and provide versatility in composition.
TDD research can be conducted via *in vitro* and/or *in vivo* techniques. *In vitro* techniques provide greater flexibility and reproducibility and are invaluable in formulation development studies while *in vivo* techniques are required for ultimate proof of product efficacy in the target population. *In vitro* and *in vivo* techniques were employed for this thesis and studies were conducted mostly with human models in order to minimize the problem of extrapolating animal derived data to humans.

### 5.1 Transdermal Delivery of PGE$_1$ *In Vitro*

Research was conducted with the goal of developing a liposome encapsulated PGE$_1$ formulation with *in vitro* transdermal flux characteristics which would be satisfactory for clinical application. Sixteen formulations (two nonliposomal and fourteen liposomal) were selected, based on results of physical stability analyses, from a total of fifty six test formulations of PGE$_1$. The effect of formulation variables (vehicle type, drug concentration, drug/lipid ratio, apparent encapsulation efficiency, pH, and penetration enhancers) were assessed using the *in vitro* flow-through diffusion cell technique, radiolabelled PGE$_1$, and full thickness human foreskin.

The steady state transdermal flux for the various liposomal formulations ranged from $0.007 \pm 0.006$ to $1.709 \pm 0.503$ (µg cm$^{-2}$ h$^{-1}$) while for the nonliposomal formulations, the range was $0.056 \pm 0.021$ to $0.648 \pm 0.324$ (µg cm$^{-2}$ h$^{-1}$). The extent of transdermal PGE$_1$ delivery in 24 hours ranged from $0.16 \pm 0.06$ to $24.95 \pm 8.35$ (µg cm$^{-2}$) for the liposomal formulations and $0.38 \pm 0.30$ to $8.50 \pm 3.93$ for the nonliposomal formulations. At equivalent drug concentrations, the optimum liposome formulation delivered at least three times more PGE$_1$ through the skin in 3 and 24 hours when compared to the nonliposomal formulation. Encapsulation of PGE$_1$ into liposomes of suitable composition was shown to enhance transdermal PGE$_1$ delivery. Increasing the drug concentration in the liposomal and nonliposomal formulations, from 0.05% to 0.5%, resulted in increased transdermal PGE$_1$ delivery. Transdermal PGE$_1$ delivery *in vitro* was optimum from a
liposome formulation of pH 5, encapsulation efficiency of 83%, drug concentration of 0.5% and a drug/lipid ratio of 1/10. This optimum liposome formulation (#40B) contained various skin penetration enhancers.

An estimate of in vivo transdermal PGE\textsubscript{1} delivery from the liposome formula #40B, based on the in vitro results and the projected mean area of application in vivo, showed that it can hypothetically deliver enough PGE\textsubscript{1} to elicit therapeutic effects in patients with erectile dysfunction.

5.2 Transdermal Delivery of PGE\textsubscript{1} In Vivo

The transdermal delivery of PGE\textsubscript{1} from liposomal and nonliposomal formulations was evaluated in healthy volunteers and in patients with erectile dysfunction. Cutaneous microcirculation changes were monitored in the healthy volunteers while changes in cavernous artery blood flow and/or clinical effects were monitored in the patients.

In nine healthy volunteers, laser Doppler flowmetry technique was used to monitor blood flow changes in the skin following application of liposomal placebo, a nonliposomal (0.5%), and liposomal (0.5%) PGE\textsubscript{1} formulations. The optimum liposomal PGE\textsubscript{1} formulation, #40B, significantly increased skin blood flow when compared to the placebo and nonliposomal formulations. Laser Doppler flowmetry was proven to be an effective noninvasive pharmacodynamic test that can be employed routinely in transdermal PGE\textsubscript{1} product development.

In five patients with erectile dysfunction, topical application of liposomal PGE\textsubscript{1} formulations (#18A, #28, #29) resulted in increased mean peak systolic flow velocity in the deep cavernous arteries. The order of in vivo efficacy for the three liposomal formulations tested mirrored the results obtained in vitro and supports the validity of in vitro flow-thru diffusion cell technique as a tool for TDD research. In a clinical trial involving six patients with erectile dysfunction, increased clinical efficacy was seen with transdermal liposomal PGE\textsubscript{1} formula #29D relative to placebo.
The *in vivo* studies showed that PGE₁ can be delivered through the skin to elicit pharmacological and/or clinical effects. It is conceivable that the tunica albuginea, which lies below the penile skin and covers the erectile corpora cavernosa tissue, may be a second barrier to the transdermal delivery of PGE₁. The tunica albuginea is made up of fibrous connective tissue (Martin, 1985) and ranges in thickness from 0.8 to 2.2 mm depending on the location in the penis (Hsu et. al., 1994). The dermis which is a thicker layer and is also made up, largely, of fibrous connective tissue, does not constitute a major barrier to transdermal drug delivery. It is, therefore, likely that the tunica albuginea is not a significant barrier to transdermal PGE₁ delivery. In addition, the topical application of nitroglycerin (Morales et al., 1988; Owen et al., 1989) and PGE₁ (results from this thesis: Kim and McVary, 1995) have resulted in penile erection, which indicates that the applied drugs can traverse both penile skin and the underlying tunica albuginea. In this research, the application of PGE₁ to both the entire penile body (which has varying tunica albuginea layer thickness) and the glans penis (which has no tunica albuginea) decreases the possibility of the tunica albuginea layer being limiting to transdermal PGE₁ delivery.

5.3 Stability and Metabolism Studies of PGE₁

Stability analyses of PGE₁ formulations were conducted in order to ensure that (i) the formulations remained stable over the course of the transdermal delivery experiments and (ii) the optimum formulation has a shelf life that is feasible for clinical use. Metabolism of PGE₁ in human skin was studied since it could be potentially limiting to transdermal PGE₁ delivery. Novel HPLC assays with radioactivity quantitation or photodiode array u.v. detection were developed and used to conduct the chemical stability and metabolism studies of PGE₁.

PGE₁ encapsulated in liposomes or free in solution remained stable for at least 5 h when incubated with denatured human skin homogenate (HSH) or phosphate buffered saline at 37°C. Chemical degradation of PGE₁ *in vivo* during clinical use is therefore not
likely. The optimum liposome formulation, #40B, was stable for at least 5 months or 24 hours when stored at 4°C or 37°C respectively. Liposome encapsulation enhanced the stability of PGE₁. The stabilization of PGE₁ by liposomes was dependent on the pH, encapsulation efficiency and composition of the liposomes, but was independent of the drug concentration. Acidic pH and high encapsulation efficiency enhanced the stability of PGE₁ in liposomes.

Metabolism of PGE₁ in human skin was minimal (less than 10% after incubation at 37°C in HSH for 5 h). In contrast, metabolism of PGE₁ in rabbit lung and human placenta homogenates were considerably higher (31% and 60% in 1 h, respectively). These novel metabolism results indicate that cutaneous metabolism of PGE₁ is not likely to be an important factor in its transdermal delivery.

5.4 Research Perspective

The results from this thesis demonstrate that transdermal delivery of PGE₁ is practicable and that more research is needed in order to translate the thesis findings into a commercial transdermal PGE₁ product.

One question that has never been resolved unequivocally is whether topically applied PGE₁ can effectively penetrate the tunica albuginea and reach the corpora cavernosa in concentrations adequate for clinical use in the vast majority of patients with erectile dysfunction. In order to resolve this issue, assay of PGE₁ levels in the corpora cavernosa following transdermal PGE₁ therapy needs to be done. Such an assay is fraught with ethical and experimental constraints but is not impossible given the right conditions. The use of radiotracer assay methodology appears necessary considering that a recent study (van Ahlen et al., 1994) which monitored PGE₁ concentrations in the cavernous vein after intracavernous injection of a 20 μg bolus dose showed peak drug concentrations of only 300 ng/mL and a very rapid decline to baseline values within one hour. The use of animal (primate) models may provide some mechanistic data and avoid the problems associated
with conducting radioactivity experimentation on humans but would still leave the question of clinical relevance unanswered. Kligman (1983) in his review of previous comparative studies involving animal skin models highlighted the fact that a reliable universal animal model for the human skin is yet to be found. In addition, metabolic, physiologic, and/or anatomic differences between the animal model and human may also be significant. The ability of PGE₁ to penetrate the tunica albuginea may be studied in vitro using the flow-through diffusion cell technique and samples of human tunica albuginea obtained from surgical procedures. Large clinical trials of transdermal PGE₁ formulations are needed to demonstrate proof of its clinical efficacy conclusively. A new approach to transdermal PGE₁ delivery was recently proposed by Borges (1994) and involved applying the drug to an area of the penis which had been surgically altered to create a 1 cm² window in the tunica albuginea and Buck's fascia. This new approach may require more investigation especially if it is proven that the tunica albuginea is the rate limiting barrier to transdermal PGE₁ delivery since it effectively circumvents this layer.
LIST OF REFERENCES


APPENDIX A

*In Vitro* Transdermal Delivery Profiles of Liposomal and Nonliposomal PGE$_1$ Formulations

The data are presented as flux (i.e. amount of PGE$_1$ penetrating through the skin per cm$^2$ per h) and cumulative amount (which are derived from the flux values) vs time plots. Error bars are included only on the primary, flux, data for the sake of clarity. The PGE$_1$ concentration (%) and the number of replicates per formulation are included in each graph. The scales for the graph axes have been selected to emphasize the differences in flux between formulations.
#29D (0.1%; n=5; ± SE)

- Flux
- Cum. Amount

Cumulative Amount µg/sq.cm

Time (hr)

#37 (0.1%; n=3; ± SE)

- Flux
- Cum. Amount

Cumulative Amount µg/sq.cm

Time (hr)
APPENDIX B

Consent Form for Color Doppler Ultrasonography Studies

[TOPEF Research Group, University of Saskatchewan]
Title: Topical PGE₁ for impotence

Purpose and Objectives:

Your problem in attaining and maintaining an erection has been found to respond to injections into the penis of prostaglandin E₁ (PGE₁). We believe we can deliver a similar amount of PGE₁ into the penis using a special skin delivery system called liposomes. These are small packets that help drugs penetrate the skin and underlying tissues. We wish to compare PGE₁ containing liposomes with an inactive liposome cream (placebo).

Possible Benefits:

We hope to develop a treatment for impotence that does not require injecting drugs with a needle.

Procedure:

Stop injections of PGE₁ (or other substance) one week before the first test and for the duration of the study. Avoid alcohol on days of the test.

Prior to the test, some preparation will be made available to you to determine any allergic reaction to the test formulation. Apply a little of this preparation (the size of a dime) to a non-hairy part of your arm and cover it with occlusive wrap. If no redness or irritation, severe enough to cause distress, is observed in 60 minutes, you may proceed to the actual test.
We will test four liposome preparations (including one control). You will receive the products in random order and will not know the identity of the product. We will supply you with a small container of each product. Apply the cream vigorously on the entire penis (including the glans) and cover the penis with occlusive wrap. Penile blood flow profile will be determined using Doppler ultrasonography at 15 minute intervals for 1 hour.

Wash off the cream and if an erection occurs later or is sustained for over 6 hours contact Dr. S.K. Afridi at 652-5757 or Dr. T. Kudel at 655-5146. You may require injection of an antidote to terminate the erection. Keep a record of your response on the sheet we provide you.

**Risks**:

1. Any topical preparation can cause redness and irritation of the skin. This is why we want you to try it on your arm first.

2. Injections of PGE₁ into the penis are sometimes painful. We hope this topical preparation will be less so.

3. PGE₁ entering the body can cause headache, flushing, palpitations and changes in blood pressure. Such reactions are short-lived due to the rapid metabolism of PGE₁ in the lungs.

4. As with all research studies there may be unforeseen risks.

You are free to withdraw from this study at any time and such withdrawal will not affect your future medical care.
All data will be kept confidential. Publication of the results will not require identifying you.

For further information you may contact:

Dr. S.K. Afridi 652-5757 or Dr. T.W. Wilson 966-7976 or Dr. M. Foldvari 966-6338

If, during the course of the study, new information becomes available which could have a bearing on your decision to participate, you will be notified.

You have been advised of alternate forms of treatment including injections, vacuum assist devices and surgical implants.

I, ____________________________ of ________________________________ have read the above and understand the nature, benefits of risks ____________________ (initial). I have a copy of this consent form.

_________________________________________  ______________________________
  Signature                           Date

_________________________________________
  Witness
APPENDIX C

Patient Selection Criteria for Pilot Clinical Trials

[TOPEF Research Group, University of Saskatchewan]
INCLUSION CRITERIA

1. Patients must be males, ≥ 18 years old.

2. Patients previously diagnosed with organic erectile failure due to vascular impairment as defined by history, physical examination and Color Doppler Ultrasonography.

3. Peak systolic flow velocity in the deep cavernosal arteries, following intracavernosal PGE₁ at a 10 to 20 μg dose, must be higher than 25 cm/s but lower than 50 cm/s.

4. Adequate response (sufficient for penetration) to intracavernosal PGE₁ in the home setting.

5. Partner for sexual intercourse must be available.

6. Patients must give informed (written) consent.
EXCLUSION CRITERIA

1. Patients with a history of hemoglobinopathy, bleeding diathesis, severe systemic disease (unstable angina, transient ischemic attacks), Peyronie's disease, idiopathic priapism and patients on anticoagulation therapy.

2. Patients with other than vascular causes of sexual dysfunction (diabetes requiring drug or insulin therapy, hypogonadism, major pelvic surgery).

3. Patients with history of alcoholism or abuse of chemical substances within 12 months of study.

4. Lack of informed consent from either party.

5. Contraindication to PGE₁ in subject or partner.

6. Patients whose partner is pregnant or breast feeding.

7. Patients who received any investigational medication within the past 30 days.

8. Patients deemed uncooperative or noncompliant.
APPENDIX D

Procedure and Result Sheet for Pilot Clinical Trials

[TOPEF Research Group, University of Saskatchewan]
PROCEDURE:

1. Wash the penis with mild soap and dry it carefully.

2. Apply ALL of the cream to the penis as earlier demonstrated. Cover the penis carefully with occlusive wrap for at least 1 hour unless erection occurs earlier.

3. Wash cream off prior to intercourse.

4. Record the results on the result sheet below.

RESULT SHEET:

Time of Application: ......................... Date: .........................

Length of Occlusion Time: .........................

Response Ranking (from Table 1 below): .........................

Table 1

<table>
<thead>
<tr>
<th></th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No effect whatsoever observed</td>
</tr>
<tr>
<td>1</td>
<td>Slight effect i.e. slight enlargement of the penis. No erection</td>
</tr>
<tr>
<td>2</td>
<td>Erection obtained but not sufficient for intercourse</td>
</tr>
<tr>
<td>3</td>
<td>Good erection obtained. Intercourse attempted but not satisfactory</td>
</tr>
<tr>
<td>4</td>
<td>Good erection obtained. Satisfactory for intercourse.</td>
</tr>
</tbody>
</table>

Name: .............................................. Trial #: .................

Remarks: 
APPENDIX E

HPLC Validation Data
**INTRAASSAY VARIATION FOR PGE₁**

Nominal Concentration of PGE₁ standard = 40 ng:

<table>
<thead>
<tr>
<th>Assay Results, n = 6 (ng)</th>
</tr>
</thead>
<tbody>
<tr>
<td>37.4</td>
</tr>
</tbody>
</table>

Mean = 36.0 ng; SE = 0.6 ng; C.V. = 1.7%; Accuracy = 90.1%

Nominal Concentration of PGE₁ standard = 2.5 µg:

<table>
<thead>
<tr>
<th>Assay Results, n = 7 (µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5281</td>
</tr>
</tbody>
</table>

Mean = 2.5226 µg; SE = 0.0129 µg; C.V. = 1.4%; Accuracy = 100.9%

**INTERASSAY VARIATION FOR PGE₁**

Nominal Concentration of PGE₁ standard = 1.25 µg

<table>
<thead>
<tr>
<th>Assay Results, n = 10 (µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
</tr>
<tr>
<td>1.2232</td>
</tr>
</tbody>
</table>

Mean = 1.2388 µg; SE = 0.0242 µg; C.V. = 6.2%; Accuracy = 99.1%
## INTRAASSAY VARIATION FOR PGA₁

Nominal Concentration of PGA₁ standard = 20 ng:

<table>
<thead>
<tr>
<th>Assay Results, n = 6 (ng)</th>
</tr>
</thead>
<tbody>
<tr>
<td>18.5</td>
</tr>
</tbody>
</table>

Mean = 19.0 ng; SE = 0.2 ng; C.V. = 2.3%; Accuracy = 94.8%

## INTRAASSAY VARIATION FOR PGB₁

Nominal Concentration of PGB₁ standard = 40 ng:

<table>
<thead>
<tr>
<th>Assay Results, n = 6 (ng)</th>
</tr>
</thead>
<tbody>
<tr>
<td>38.1</td>
</tr>
</tbody>
</table>

Mean = 38.8 ng; SE = 0.6 ng; C.V. = 4.0%; Accuracy = 96.9%

## INTRAASSAY VARIATION FOR KPGE₁

Nominal Concentration of KPGE₁ standard = 200 ng:

<table>
<thead>
<tr>
<th>Assay Results, n = 6 (ng)</th>
</tr>
</thead>
<tbody>
<tr>
<td>186.7</td>
</tr>
</tbody>
</table>

Mean = 187.8 ng; SE = 1.9 ng; C.V. = 2.5%; Accuracy = 93.9%
Typical standard calibration curve for PGE₁ using the HPLC nonradioactive assay. Seven concentration levels (µg) were employed with three replicates each.
Typical standard calibration curve for PGA₁ using the HPLC nonradioactive assay. Six concentration levels (µg) were employed with three replicates each.
Typical standard calibration curve for PGB1 using the HPLC nonradioactive assay. Six concentration levels (µg) were employed with three replicates each.
Typical standard calibration curve for 15-ketoPGE₁ using the HPLC nonradioactive assay.

Six concentration levels (µg) were employed with three replicates each.