ELECTROPORATIVE TRANSDERMAL DRUG DELIVERY: OPTIMIZATION AND SAFETY

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by

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ABSTRACT

The major hurdle in transdermal drug delivery is the barrier property of stratum corneum. This barrier can be overcome by using electroporation. Electroporation involves the application of exponentially decaying high voltage pulses of short duration, which results in a transient increase in skin's permeability. This application of electroporation in transdermal drug delivery is at its early stages of development and needs to be studied in detail.

Objectives:

1) To optimize the electroporative transdermal drug delivery by investigating the following:

i) relationship between $U_{electrode}$ (voltage drop applied across the pulse delivery electrodes) and U_{skin} (voltage drop across skin).

ii) effect of electrode design on U_{skin}.

iii) influence of $U_{electrode}$, pulse length (τ), and number of pulses on the transdermal delivery of a model flourescent molecule, i.e., terazosin hydrochloride (TRZ).

2) To investigate safety issues related to electroporative delivery by studying the following:

i) reversibility of skin's enhanced permeability

ii) histological changes in the skin due to electroporation

In-vitro experiments were performed using freshly excised skin from hairless rats, assembled in a custom-designed cells. Gene Pulser® II (Bio Rad, CA, USA) fitted

with custom designed Ag/AgCl electrodes was used for generating exponentially decaying electroporation pulses. Tektronix® - 5111 storage oscilloscope (Oregon, USA) was used for measuring the voltage drop across the skin (U_{skin}) and the current flowing across the skin. TRZ was detected using HPLC with fluorescent detector.

Results: Use of electroporation significantly increased the transdermal delivery of TRZ. For example, electroporative delivery with 20 pulses of $U_{electrode}$ = 500 V, τ = 20ms resulted in 15 times increased transdermal delivery of TRZ, as compared to passive diffusion. A $U_{electrode}$ of 300 V (corresponding U_{skin} 66 V) seemed to be the minimum voltage required to make a significant enhancement compared to the control. The delivery of TRZ increased with an increase in $U_{electrode}$, pulse length and number of pulses. However, in order to maintain skin safety while achieving a reasonable increase in the permeability of skin the upper limit for $U_{electrode}$ and pulse length seem to be 500V (U_{skin} 88 V)and 20 ms, respectively. At $U_{electrode}$ of 600 V and at pulse length 60 ms the damage to the skin seems to be very apparent. The U_{skin} values were about 1/2 to 1/5 that of $U_{electrode}$ depending on the voltage used. Electrode design and area also had profound effects on transdermal drug delivery. The pH, macroscopic, and microscopic changes associated with the skin after pulsing indicate that the use of a large electrode would minimize the damage to the skin in comparison to a small electrode.

Conclusions: Electroporative transdermal delivery of TRZ was markedly influenced by $U_{electrode}$, pulse length, number of pulses and electrode design. These factors can be controlled to make this technique efficient and safe.

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GLOSSARY OF ABBREVIATIONS AND SYMBOLS

TEWL	Transepidermal water loss
ms	Millisecond
Ω	Ohms
k Ω	Kilo-ohms
π	Pi
τ	Pulse length
U_{skin}	Voltage drop across the skin
$U_{electrode}$	Voltage drop across the electrode
V	Volts
A	Amperes
h	Hours
SE	Standard error
SD	Standard deviation
r	Radius
R^2	Squared correlation coefficient of regression
PBS	Phosphate buffered saline
GIT	Gastro-intestinal tract
HPLC	High Performance Liquid Chromatography
UV	Ultra-violet
vs.	Versus
LTR	localized transport regions
CV	Coefficient of Variation
p	probability value

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Think the world...but not of her or him. Think the world....not of 'us' but as 'us'.

o my late grandfather Mr. H. L. Sharma, my inspiration forever.

I. Introduction

1.0 Drug delivery systems

The principal objective of any drug delivery system is to promptly attain a predictable therapeutic quantity of drug at a proper site in the body and maintain a desired concentration. The optimized drug delivery system is intended to produce maximum simultaneous safety, effectiveness, and reliability. Additionally, it should be possible to manufacture the drug delivery system on a large scale with reproducible product quality (York 1992). Reproducible product quality requires physical and chemical stability, any necessary preservation against microbial contamination, uniform dosage, acceptability to users which includes both the patients and the prescribers, suitable packaging and labelling. The need for novel, advanced drug delivery systems is justified by the need to optimize therapy or to circumvent problems in drug absorption or metabolism. The sustained-release products were first marketed during the late 1940s and early 1950s as a major new class of pharmaceutical product in which the product design was intended to modify and improve the drug performance by increased drug duration of action as well as reduced dosing frequency. Following this, the term "controlled drug delivery" came into existence in the mid- to late- 1960s to describe a new concept of dosage form design which also involved controlling and retarding drug dissolution from the dosage form with the additional objectives of sustained drug action, improved safety, efficiency, and enhanced bioavailability. In the 1970s a new concept of drug product design and administration appeared called the

"therapeutic system". Three types of therapeutic systems have been proposed: passive preprogrammed therapeutic systems, active externally programmed or controlled therapeutic systems, and active self-programmed therapeutic systems. Amongst these three systems the first one which contains a logic element programmed to a predetermined delivery pattern (mostly constant zero order release) are already in use. These systems are independent of all the physical, chemical and biological processes. The second type of systems consists of logic elements capable of receiving and converting signals external to the body to control and properly modulate the drug release from the device within the body. The third type of systems have sensory elements which modulates the drug delivery corresponding to the information they gather from the biological environment (Banker 1990). A tremendous amount of work is presently being done to develop newer dosage forms and perfect the various existing drug delivery systems in order to meet the growing clinical demand. A popular and very acceptable delivery system is the transdermal drug delivery system.

1.1 Transdermal drug delivery systems

There are a variety of routes available for drug delivery corresponding to a number of biological membranes in the human body: buccal, nasal, and vaginal mucosa, the gastrointestinal tract including rectum and colon, the eye and the skin. Further to this there also exists parenteral delivery, including implants and targeted

delivery. Among all these routes, the transdermal route has received considerable attention in the recent times due to its convenience, accessibility, and simplicity in comparison to other routes. The practise of application of drugs to the skin for systemic absorption and action is an age old approach but has caught more attention with the introduction and the success of the transdermal nitroglycerin ointment products in the market.

Transdermal delivery (TDD) can be defined as the delivery of drugs through intact skin to reach the systemic circulation in quantities sufficient to achieve a therapeutic dose. Some of the primary dosage forms associated with transdermal route are: ointments, creams, pastes, plasters, powders, aerosols, lotions, transdermal patches, solutions, and iontophoretic devices (Ansel 1995). With products like creams and ointments it is more difficult to control the drug dosage, and there is an additional disadvantage of smearing of the preparation. In contrast, the transdermal patches can not only lead to a controlled release of the drug, but are also relatively carefree with improved patient acceptability over the other conventional transdermal dosage forms such as those mentioned above. ALZA corporation developed the first transdermal drug delivery patch in 1980 for the systemic delivery of scopolamine to control nausea, and vomiting associated with motion sickness. Since then TDD has been recognised as a viable and attractive dosage form, whose success and acceptability can be measured by the numerous transdermal products in the market (table 1).

Table 1. Examples of currently marketed transdermal drug delivery systems

Drug	Company	Design type
clonidine	Boehringer-Ingelheim	four layered solid-state laminate structure
estradiol	Ciba-Geigy	liquid form, fill and seal laminate structure
etofenamate	Bayer	semi-solid amorphous (cream, lotion etc.) structure
nicotine	Lederle	peripheral adhesive laminate structure
nitroglycerine	3M Riker	Two layered solid-state laminate structure

(Cleary 1996)

1.2 Clinically perceived benefits of transdermal drug delivery

There are several clinical benefits associated with TDD. It is often associated with improved patient acceptability over some conventional dosage forms due to the ease of application and removal of the dosage form, non-invasiveness, and reduced incidences of side effects. This non-invasiveness also leads to a reduction in the infection hazards associated with injection needles. The reduced incidences of side effects may be attributed to the fact that with TDD the plasma levels of the drugs can be maintained at a certain desired level rather than the peaks and valleys seen with the conventional oral dosage forms. TDD can be tailored to meet the requirements of individual patients. It also results in the improved bio-availability of some of the orally administered drugs due to elimination of variables like hepatic first-pass metabolism, degradation of drugs caused by the: GIT (Gastro-intestinal tract) pH, enzymes, etc. Despite all the advantages mentioned above, TDD delivery is feasible for only a few drugs due to the barrier function of skin.

1.3 The skin and its barrier function

The skin is one of the most extensive and readily accessible organs of the human body. Although it provides a great degree of physical protection and integrity, the principal role of the skin is to keep harmful agents out and water in. The skin consists of the following main layers: epidermis, dermis and hypodermis (figure 1).

Most of the skin's barrier resides in the stratum corneum (horny layer): a very thin (10-15 μm in thickness) outermost layer of skin. It consists of approximately fifteen cell layers. The upper 3-5 layers are constantly undergoing desquamation and are called stratum disjunctum. The lower layers of stratum corneum (stratum compactum) are thicker, more regularly and densely packed and have resemblance to the lower viable epidermis. Stratum compactum has higher density of corneodesmosomes (polar structures interconnecting the corneocytes of the stratum corneum and contributing to stratum corneum cohesion). These structures need to be proteolysed for desquamation (Schaefar et al. 1996).

Stratum corneum was earlier described as a heterogenous two compartment -"brick and mortar"model, in which the keratin rich cells (corneocytes or horny cells) are embedded in lipid-laden intercellular domains (Elias 1983). This layer was considered essentially inert. Later findings showed that Stratum corneum is catabolically active and that the intercellular domains are basically a heterogenous mixture of lipids, enzymes, and membrane glycoconjugates, etc. having both hydrophilic and hydrophobic lamellae. These findings show that stratum corneum is not inert (Elias 1991). The intercellular lipids with their unique arrangement as ordered multilayers have an important role in the barrier's impermeability (Potts et al. 1991). These lipids form the only continuous domain in the stratum corneum and follow a

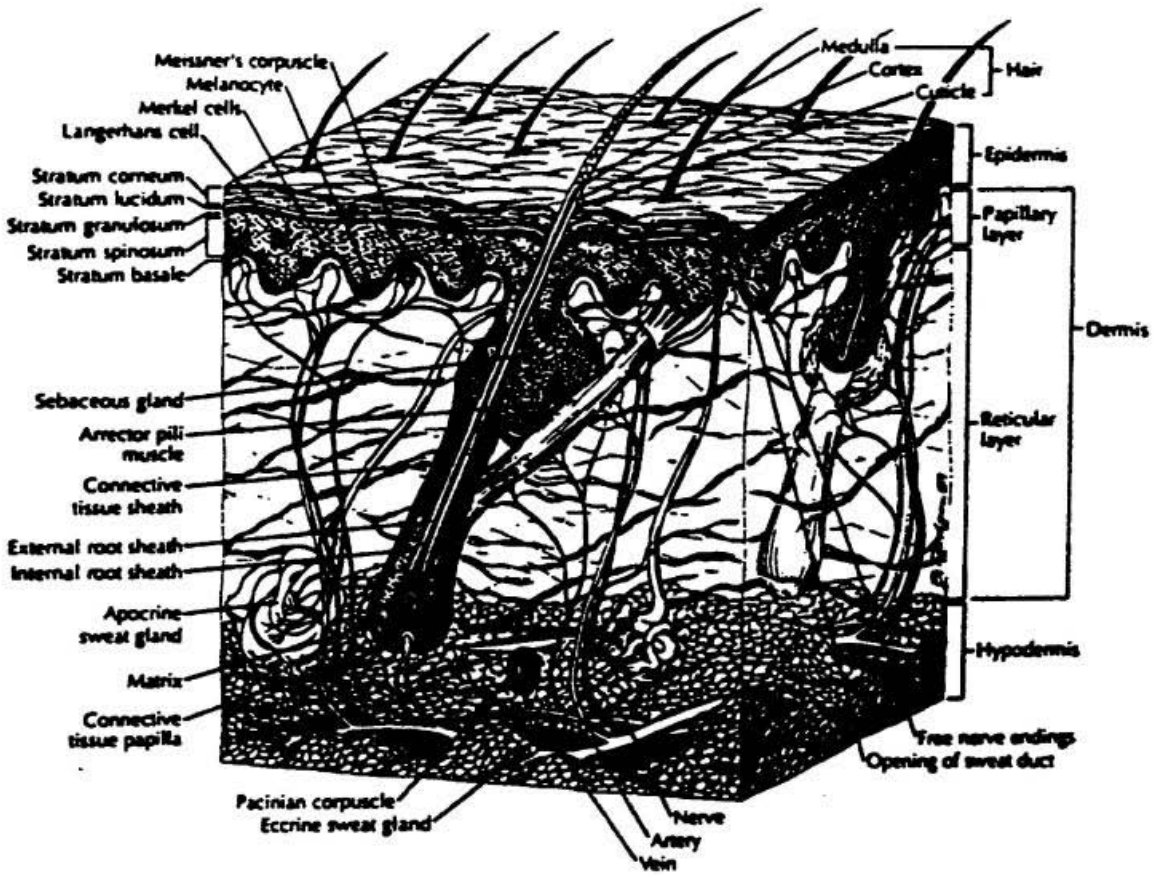


Figure 1. Representation of the anatomical structures found in mammalian skin. (The stratum corneum, the outermost layer, provides the primary transdermal transport barrier of most compounds. The epidermis is avascular and hence the drugs must reach the circulation in the dermis for systemic administration.)

tortuous path that penetrates the stratum corneum, a structural feature which contributes to the barrier properties of the skin. They are mainly derived from exocytosis of the keratinocytes. In the granular layer of the epidermis, the lamellar granules move to the periphery of the keratinocytes and fuse with the cell membrane. Subsequently, the content of these lamellar granules gets discharged into the intercellular space. The discharged material in the granular layer retains the short disk like structure which it had in the lamellar granules. However, these disks start to undergo edge-to-edge fusion and result in the broad, and multilamellar sheet like structure, that forms a continuous domain, by the time they reach the stratum corneum (Madison et al. 1987). Sebaceous secretions also contribute to the formation of the intercellular lipids although their contribution is much lesser than that derived from the exocytosis of keratinocytes.

The 100 μm thick viable epidermis (directly below stratum corneum) is responsible for the synthesis of the stratum corneum. As the cells move from the basal layer to the horny layer there is a shift from the polar lipids to neutral lipids and almost complete loss of phospholipids. The conversion into the neutral lipids is a step toward the barrier formation. The lipid content of the stratum corneum is unique in comparison to the cellular membranes and other epidermal structures. The stratum corneum has 15% water, 70% protein and 15% lipid. Among lipids, it has neutral lipids

(70-80%), ceramides (15-20%), cholesterol sulfate (2-5%) and has virtually no phospholipids (Franz et al. 1992). Feingold and workers have shown that barrier perturbation results in an increased biosynthesis of sterol and fatty acid in the epidermis, leading to the lipid replenishment in the stratum corneum. Transepidermal water loss appears to be the critical regulator of this entire process (Grubauer et al. 1989). There is a sequential increase in transepidermal water loss as the stratum corneum is slowly stripped showing that all of the stratum corneum is involved in providing barrier to diffusion (Bommannan et al. 1990).

The inhibition of the enzymes involved in the synthesis of ceramide, cholesterol, and free fatty acids leads to an impaired barrier function (Feingold et al. 1990, and Holleran et al. 1993). Hence, the lipid composition of the stratum corneum appears to be a key determinant of the biological action of this membrane. Recent work has shown that the aqueous dispersion of ceramide, cholesterol, and palmitic acid (representing the lipid portion of the stratum corneum) have a complex phase behaviour as a function of temperature and pH (Bouwstra et al. 1997). Such ceramide dispersions, even in the presence of a considerable mole fraction of cholesterol, can form crystalline lamellar structures between room temperature and 40 °C. The majority of the crystalline cholesterol is not in a separate phase. Above 40 °C a fluid lamellar phase results which is completely reversible with temperature. The precursor

lipids for the stratum corneum (determined by the change in the lipid composition in the mammalian epidermis), consist of more typical membrane lipid classes such as phosphoglycerolipid and sphingolipids (specially sphingomyelin and monoglycosylceramides). Sphingomyelin under the similar dispersion conditions as mentioned for ceramides was found to form a fluid lamellar phase. Hence, the existence of the crystalline structures may be important for unique physical properties such as epidermal barrier function.

Although the intercellular lipids have a major role in the stratum corneum's impermeability, their role in the tissue cohesion is limited (Bisset et al. 1987, and Chapman et al. 1991). The tissue cohesion of the horny layer is contributed mostly to the intercellular junctions and associated corneocytes (Skerrow et al. 1989 and Lundström et al. 1990). The stratum corneum acts as the rate controlling barrier to the transdermal permeation of both lipophilic and hydrophilic drugs. Both proteins and lipids in the stratum corneum could resist drug permeation. The protein and lipid regions in the stratum corneum form polar and nonpolar pathways, respectively. The permeation through the lipid pathway occurs by the dissolution and diffusion of the drug in the lipids of the stratum corneum. Correspondingly, the permeation through the polar pathway takes place by partitioning into protein regions swollen by water (Franz et al. 1992). Earlier, Barry had proposed that both the polar as well as

nonpolar pathways for drug permeation reside within the intercellular lipids. According to him, the dominant pathway of the polar molecules resides in the aqueous region of the lipids, with the nonpolar pathway residing within the lipid chains of the hydrophobic region (Barry 1987). Recently, using tracers (hydrophilic and hydrophobic) for normal, undisturbed skin and/or after permeation-enhancement (with occlusion, vehicle enhancers, a lipid synthesis inhibitor, sonophoresis, and iontophoresis) the pore permeation pathway for molecules was studied (Menon 1997). It has been found that regardless of their polarity or the permeation-enhancement method, tracers got localised to discrete lacunar domains embedded within the extracellular lamellar membrane system. The discontinuous microdomains, or lacunar dilatations, arise at sites of desmosomal dissolution within the extracellular lamellae (Hou et al. 1991). These lacunar domains were discontinuous under basal conditions but appeared to gain structural continuity with permeation-enhancement by lateral expansion. It has been proposed that permeabilization results in transient coalescence into an interconnected network across the stratum corneum. Hence, the extracellular lacunar domains appear to comprise the pore pathway for permeation of molecules across the stratum corneum.

The stratum corneum is considered to have a molecular size constraint on penetration. As the size of the permeant increases over 800-1000 Daltons the

penetration of molecules decreases dramatically (Wester et al. 1985). The epidermis has gap (Salomon et al. 1988, and Brissette et al. 1994) and adherence junctions (Kaiser et al. 1993). Gap junctions, though not prevalent, facilitate the intercellular diffusion of low molecular weight substances which suggests that there is a functional interconnection between the keratinocytes. There are no tight junctions found in the stratified epidermis that could prevent the diffusion of molecules. The desmosomes and adherence junctions form a barrier to the transcellular diffusion of very large molecules within the epidermis.

Besides the stratum corneum, the epidermis has three other layers, i.e., stratum basale, stratum spinosum and stratum granulosum. These layers represent different stages of the keratinocytes differentiation and result in the formation of the stratum corneum. The dermal-epidermal junction is not uniform and in fact, it undulates to form papillae which increase the surface area between these two layers. This junction is made up of four sub-layers: intra-epidermal plane, lamina lucida, lamina densa, and sublamina densa. The lamina densa consists of a dense network of interconnecting proteins. The lamina densa is attached to the basal keratinocytes with an interacting meshwork of laminin, entactin/nidogen and fibronectin (Martin et al. 1987, Yurchenco et al. 1990) and to the dermis by larger anchoring fibrils composed of type VII collagen. This dense network of interconnecting proteins reduces the flux of large

(>40,000 Daltons) but not small molecules. Hence, the dermal-epidermal junction does not represent the primary barrier to the diffusion of compounds. Below the dermal-epidermal junction is the dermis. This layer forms the bulk of the skin and determines its tensile strength, elasticity, and provides physical support for extensive nerve and vascular networks. Appendages that originate from the dermis often penetrate into the horny layer and represent the sites for penetration of drugs into the body. The appendages like hair follicles and sweat glands are sites of physical discontinuity in the stratum corneum which serve as a penetration pathway. These appendages account for only 0.1-1% of the surface area of the skin and only 0.01-0.1% of the skin volume. These shunt pathways are believed to be important for molecules which have a slower percutaneous absorption and are believed to play an important role in iontophoretic transdermal transport. The vascular network of the skin does not reach the epidermis. As a result, the drugs need to reach the vasculature in the dermis for systemic absorption. Underlying the dermis is the hypodermis which is basically a layer of adipocytes arranged in lobules. The intracellular fat droplets here may act as a depot for hydrophobic compounds that permeate the stratum corneum and pass the vascular network in the dermis without getting absorbed. However, the hypodermis has an extensive vascular network and hence molecules reaching it can be considered as getting distributed throughout the body.

Skin has a relatively lipophilic stratum corneum and a relatively hydrophilic viable skin (epidermis and dermis). Hence, partitioning of a hydrophilic drug into the stratum corneum and partitioning of a lipophilic drug out of the stratum corneum and into the dermis are the rate determining steps for skin penetration. Besides the stratum corneum, proteolytic enzymes in skin form the other major transdermal delivery barrier which could inactivate some peptide drugs like enkephalins, insulin, etc. Proteolytic enzyme activity including leucine aminopeptidases, and endopeptidases is present in the Triton X-100 extracts of human skin fibroblasts (Homsy et al. 1988). Amidon and coworkers reported that at neutral pH, the enzymatic activity in the skin was so strong that they observed no flux of leucine-enkephalin in the receptor compartment, while the donor concentration also decreased very rapidly (Choi et al. 1990). This suggests that the enzymatic barrier of the skin cannot be completely undermined as such while planning transdermal delivery for peptide and protein drugs.

1.4 Transdermal drug delivery enhancement

TDD delivery is only feasible for a few drugs due to the barrier function of skin. Generally small, potent, uncharged and lipophilic molecules, but with a certain degree of water solubility, are good models for transdermal delivery. To extend the spectrum of drugs that can be delivered transdermally, a number of enhancement techniques have been tried out:

1.4.1 Chemical enhancers

Franz et al. have defined chemical enhancers as compounds which by their presence in the stratum corneum are able to alter the flux of the desired drug either by alteration of the stratum corneum structure or by increasing the drug concentration in the skin (Franz et al. 1992). In addition to enhancing the delivery of the drug, the enhancer needs to be systemically nontoxic, nonirritating and should not produce any pharmacological responses. Its actions are expected to be reversible, prompt, and with a predictable duration of action.

First and foremost, hydration of the stratum corneum such as that caused by the occlusive transdermal patches has been shown to result in the decrease in the barrier function in a majority of cases. This has been well demonstrated in the case of many penetrants, including esters of salicylic acid (Wurster et al. 1961), and corticosteroids (McKenzie et al. 1962). Additionally, sodium pyrrolidone carboxylate which is believed to be the principal humectant in its role as a natural moisturizing factor for the skin (Barry 1983) has been tried out for its possible role in enhancing the skin transport of molecules. Although the effectiveness of this molecule in transdermal delivery enhancement has not been as encouraging, its analogs i.e., 2-pyrrolidone and N-methyl-2-pyrrolidone have shown to increase the transport of steroids (Bennett et al. 1984) and aspirin (Southwell et al. 1984). Besides hydration, a number of solvents,

detergents, oils etc have been tested for their enhancement property. Surfactants like decylmethyl sulfoxide are known to act preferentially as enhancers on the polar pathways (Cooper 1982). Azone (1-dodecylazacycloheptan-2-one; laurocapram) has been shown to be a very effective enhancer of skin permeability. In fact this molecule had been designed and synthesized specifically for this purpose. Azone is believed to act by an ion-pairing mechanism for the permeants and a single dose of azone is capable of enhancing the subsequent doses of permeant for at least 5 days. This molecule is effective enhancer of a number of hydrophilic and hydrophobic molecules (Stoughton et al. 1983). Additionally, unsaturated fatty acids like *cis*-9- and *cis*-11–octadecenoic acids (Golden et al. 1987) and short chain alcohols like ethanol (Ghenem et al. 1987) generally result in the enhanced permeation of lipophilic solutes. The usefulness of ethanol as a permeation enhancer has been demonstrated by its co-administration with estradiol (Campbell et al 1984). In fact, due to fears of toxicity, only ethanol has been approved as a chemical enhancer by the Health Protection Branch (HPB) till now. The chemical enhancers are believed to mainly act by disrupting the stratum corneum lipid structure (Hadgraft et al. 1989 and Smith et al. 1995). Interestingly, the transdermal delivery of lipophilic molecules has also been shown to be enhanced by incorporation in liposomes. The topical application of the drug-loaded liposomes promotes delivery of the active ingredient to local tissues and, may also reduce systemic drug levels. The result of the topical application of liposomes was first

reported by Mezei and Gulasekharam (1980) in which triamcinolone acetonide entrapped within dipalmitoylphosphatidylcholine-cholesterol multilamellar vesicles resulted in significantly enhanced drug levels in the rabbit dermis and epidermis along with reduced blood and urine levels. Similarly, lidocaine applied on the forearm of human volunteers has been found to produce greater local anaesthetic effect in the liposomal form than in the cream form (Foldvari et al. 1990). A number of parenteral-liposomal formulations are presently in clinical trials. The rapid progress in liposome technology that has been made in the past few years suggests that topical liposomal products have a promising future.

1.4.2 Physical enhancers

Some of the physical enhancement techniques like iontophoresis, phonophoresis, and electroporation have also been tested for delivery enhancement. Another physical enhancing method which was also tested earlier involves the controlled removal of the stratum corneum using pulsed laser light. The stratum corneum is removed without a significant damage to the underlying epidermis, with the use of a proper wavelength, pulse-length energy, and pulse rate (Nelson 1990).

1.4.2.a Iontophoretic transdermal drug delivery

A lot of work has been done on the transdermal drug delivery enhancement

using iontophoresis. Iontophoresis has been defined as the "process or technique which involves the transport of ionic (charged) molecules into the tissue by the passage of a direct electric current through an electrolyte solution containing the ionic molecules to be delivered using an appropriate electrode polarity" (Banga et al. 1988). Iontophoresis has the advantage that it can be used to deliver ionic drugs, which have a poor passive transdermal absorption and require chemical enhancers which can be irritating or sensitizing to the immune system. The driving forces for the iontophoresis of charged molecules are believed to be electrorepulsion and electroosmosis. Electrorepulsion involves a charged drug dissolved in an electrolyte solution surrounding an electrode of similar polarity and when electromotive force is applied, molecules get repelled into the subjacent tissue. The iontophoretic delivery of neutral molecules is intrinsically linked with electroosmotic water flux (Gangarosa et al. 1980, and Srinivasan et al. 1989). The major pathways for iontophoretic drug delivery are believed to be appendageal pores including the sweat ducts and hair follicles (Burnette 1988). A dot-like pattern over the sweat gland openings has been observed following the iontophoresis of a charged dye in human skin, showing the contribution of the sweat glands in iontophoretic drug delivery (Abramson et al. 1940). Lee and coworkers observed that in case of cultured skin models, which have no appendages, the paracellular route provides the path of least resistance to ions traversing the skin (Lee et al. 1996). However, they also found that the appendageal routes form the

major transport pathways in hairless mouse skin. Iontophoresis is used in the treatment of hyperhydrosis (Grice et al. 1972, and Sloan et al. 1986) and for the diagnosis of cystic fibrosis (Gibson et al. 1959). Iontocaine®, an iontophoretic system for the transdermal delivery of lidocaine has been recently patented by Iomed Inc. Iontophoresis is being presently studied for transdermal delivery of a number of small drug molecules for commercial use. However, the success of the iontophoretic delivery of large-molecular-weight compounds such as peptides remains elusive because the enhancement produced by iontophoresis is generally insufficient for macromolecules.

1.4.2.b Phonophoretic transdermal drug delivery

Phonophoresis (sonophoresis) involves the application of ultrasound energy for enhancing transdermal drug delivery. The mechanical energy used in phonophoresis is obtained by passing alternating current through a piezoelectric crystal, which causes it to vibrate. Physical therapists use ultrasound for delivering topical anti-inflammatory or local anaesthetic agents, and for lithotripsy procedure (Barnett et al. 1994, Stewart et al. 1983, and NCRP Report No. 113). Clinically it is used at dose intensities below 2 W/cm^2, treatment duration ranging between 5 and 20 min and with frequencies usually lesser than 3 MHz. Phonophoresis has been used for the past fifty years, however recent studies, that demonstrated therapeutically relevant delivery of macromolecules with phonophoresis, have sparked a renewed interest (Mitragotri et

al. 1995a). Ultrasound produces two kinds of effects on tissues - heating and cavitation. In order to avoid over-heating, ultrasound is applied under therapeutic conditions. When used at lesser frequency or greater intensity than therapeutic, ultrasound leads to excessive generation of gas bubbles often referred to as the 'cavitation effect'. The cavitation effects can lead to significant changes in the biological tissues such as the skin. There is no agreement on the possible mechanism of phonophoretic enhancement. It is generally believed that the thermal contribution is limited. However, evidence of increased convection (e.g., acoustic mixing) and cavitation mediated effects are possible explanations (Levy et al. 1989, and Mitragotri et al. 1995).

1.4.2.c Electroporative transdermal drug delivery

The application of electroporation in transdermal delivery has been very recent (Prausnitz et al. 1993). Electroporation has been defined as a method for reversible permeabilization of lipid bilayers, involving the creation of transient aqueous pores, by the application of an electric pulse. Permeability and the electrical conductance of the lipid bilayers (including living cells, artificial spherical and planar systems) is increased by many orders of magnitude. Electroporation appears to be universal in lipid bilayers, with onset independent of their exact composition or structure. In fact, prior to the recent (5 years ago) transdermal application, electroporation was already being used

for a number of purposes including the enhancement of the uptake of molecules by cells and tissues. For instance, electroporation is being widely used in molecular biology as a method for gene transfection by introducing DNA into cells (Neumann et al. 1989, and Chang et al. 1992). The transdermal work by Prausnitz and co-workers (1993) was the first application of electroporation to a multilamellar and non-phospholipid system.

There exists a clinical precedent for safely applying electrical pulses of hundreds of volts with a duration of up to milliseconds to the skin. There are a number of diagnostic and therapeutic applications, which while causing nerve stimulation also lead to electroporation. These include: somatosensory-evoked-potential testing, electromyography, functional electrical stimulation, and transcutaneous electrical nerve stimulation (Prausnitz 1996b). Electroporation has also been used in combination with chemotherapy (electrochemotherapy) for potentiation of the antitumour effects of intramuscularly administered bleomycin in nude mice with subcutaneously transplanted tumours (Mir et al. 1991) and in C3H/Bi mice with spontaneous mammary carcinomas (Belehradek et al. 1991). In the subsequent clinical phase I-II trials, electrical pulses were applied to permeation nodules of head and neck squamous cell carcinomas in patients to increase the uptake of the intravenously administered bolus doses of bleomycin (Belehradek et al. 1993). The absence of toxicity, good tolerance by

patients, and net antitumour effects (57% nodules had a complete clinical response) encouraged further work. Recently, intralesional bleomycin mediated electrochemotherapy was tried out in 20 patients with basal cell carcinoma (Glass et al. 1997). In all, electrical pulses were delivered to 54 tumours. Complete responses were observed in 53 (98%), and in majority of these (94%) after a single treatment with no recurrences with around 18 months of observation.

Basis for the application of electroporation in transdermal drug delivery

The stratum corneum is a very thin layer with a very high resistance (resistivity in the range of 100-5000 $k\Omega.cm^2$) with the underlying skin exhibiting a much lower resistance (resistivity around 0.1- 1.0 $k\Omega.cm^2$) (Chien et al. 1993). This difference in the resistivity is expected to result in the concentration of the applied electric field in the stratum corneum, with the other viable tissues exposed to much lower fields. Hence, a field that is sufficient to cause electroporation in the stratum corneum may be present, while a significantly lower field exists in the viable tissue which is insufficient to electroporate them. Thus, it would seem that a natural targeting mechanism is present where the greatest electric fields and perturbation occurs in the layers where the greatest resistivities exist, and at the same time the already permeable and viable parts of the skin would be affected very little.

The electroporation threshold for a single bilayer is in the order of 1 V (Tsong 1991 and Weaver 1993). Prausnitz and co-workers note that the presence of a few hundred bilayers in the stratum corneum should then result in a threshold of a few hundred volts, up to which long lasting changes in the skin should not be observed (Prausnitz et al. 1993). Their *in-vitro* data suggests that pulses at or below 100 V (transdermal voltage) caused no long lasting changes in the skin. Whereas voltages higher than that result in changes in the skin that do not go away even after 18-24 hours. Additionally, their *in-vivo* work has shown that the fluxes did not increase with increasing voltage, suggesting that the rate-limiting step is not affected (or influenced) by applied voltage. This is in contrast to the in-vitro data, where the transdermal drug delivery is seen to keep increasing with an increase in the applied voltage.

Vanbever and co-workers have shown in their earlier *in-vitro* work that it is possible to enhance the transdermal delivery of metoprolol using electroporation (Vanbever et al. 1994). They found that the delivery of metoprolol is affected by the voltage, pulse length (τ), and number of pulses. A small number of low voltage long duration pulses were more efficient than a large number of high voltage-short duration pulses (Vanbever et al. 1995). Similarly, work has been done on the transdermal delivery of fentanyl which has shown that the wave form of the electric pulses is very important (Vanbever et al. 1996). At the same applied energy, the fentanyl

permeation-enhancement was greater with exponentially decaying pulses than the square-wave pulses. Vanbever and co-workers reported that electrophoresis and diffusion through highly permeabilized skin led to a rapid transport during pulsing and according to them no electroosmosis occurs during electroporation (Vanbever et al. 1996a). If electrophoresis is assumed to be involved in electroporative transdermal drug delivery then the physicochemical properties of the molecule being transported as well as the solution would be very important in drug delivery. Hence, care needs to be taken in choosing the formulation used for electroporation. Recent *in-vivo* delivery studies done by the same group have shown the potential of skin electroporation in rapidly achieving significant plasma levels of fentanyl in rats (Vanbever et al. 1998). The onset of analgesia was expedited to a few minutes by high voltage pulsing compared with the several hours onset time seen with passive delivery. The onset of analgesia seen after electroporative transdermal fentanyl delivery was even more rapid than by iontophoretic delivery. The skin tolerance was examined visually and scoring was done after pulsing (voltage values reported being $U_{electrode}$) at 15 pulses of 100 V-500 ms, 15 pulses of 250 V-200 ms and 60 pulses of 500 V-1.3 ms. 250 V appeared to cause a slightly increased redness while neither iontophoresis nor pulsing at 100 and 500 V seemed to induce erythema. It is very important to note here that the absence of erythema at 500 V is not surprising looking at the very small pulse length used compared to the prolonged pulse length used for 100 and 250 V. It has

been recently reported that the application of long electroporation pulses led to a decrease in the intensity of the lipid peaks observed in SAXS (Small Angle X-ray scattering) and WAXS (Wide Angle X-ray scattering) as opposed to short pulses, indicating that both interlamellar and intralamellar structures were being affected (Jadoul et al. 1997). Correspondingly, 60 pulses of 500 V-1 ms caused only a slight decrease in the intensity values observed with SAXS and WAXS. In a separate study done using pig skin, it has been shown that when a single pulse with a $U_{electrode}$ of 0 (control), 500, or 1000 V was administered before a current density of either 0.4 or 0.8 mA/cm^2 was applied for 30 minutes, erythema and edema (most severe at 0.8 mA/cm^2) were observed at all pulse voltage values (Potts et al. 1995). Gross and light microscopic evaluation showed that electroporation did not cause any skin damage that has not been seen with iontophoresis alone. The only dermal alterations that were seen with electroporation are mild intraepidermal vacuolization and a transient erythema.

Prausnitz and coworkers have shown that electroporation leads to a rapid transdermal transport involving steady state lag times and onset time of minutes which indicates that rapid temporal control of transport is possible (Prausnitz et al. 1994). Transdermal delivery enhancement of drugs through the skin has been shown to occur with each pulse (Chen et al. 1998).

1.5 Enhancement techniques used in conjunction with electroporation

The permeability barrier of the skin is very high for a number of drugs. To overcome this barrier, a strong physical force such as electroporation needs to be used. The strongest of these forces that can be used is likely to be limited by their adverse physiological effects. For instance, electroporative pulsing conditions such as voltage, pulse length and pulse number can only be increased for transdermal delivery enhancement to a point, beyond which it may be harmful. This forms the limitation of electroporation in transdermal drug delivery. Many of the transdermal drug delivery enhancement techniques have a different route of action and hence if used in synergism may be more effective, while being safe. Some of the different techniques tried out in conjunction are described below:

1.5.1 Iontophoresis and electroporation

Although both iontophoresis and electroporation involve electric fields, their basic mode of action differs (Prausnitz et al. 1993). Iontophoresis basically acts on the drug and moves the molecules across the skin surface by electrophoresis and/or electroosmosis. Any changes that occur in the skin structure occur only as a secondary effect (Hadgraft et al. 1989). However, electroporation causes large changes in the permeability and the conductance of the skin. Hence, for electroporation the electric field is believed to act by creating aqueous pores in the skin

and molecules are speculated to move through these pores by diffusion, electrophoresis and/or electroosmosis. It has been recently shown that there is no iontophoretic enhancement of drug delivery when black rat snake skin was used in the *in-vitro* studies as opposed to the human skin (Chen et al. 1998). However, the use of electroporation caused an enhanced drug delivery with both snake and human skin samples in the same study. Snake skin lacks hair follicles while iontophoresis is known to cause transport through hair follicles and sweat ducts. This explains the absence of iontophoretic delivery in the snake skin. This also shows that iontophoresis and electroporation have different routes of drug delivery. As has been mentioned earlier, the route for iontophoretic drug delivery is believed to be appendageal. Recently, it has been shown that electroporative transdermal delivery occurs through localized transport regions (LTR) in the epidermis which do not correspond to the appendages (Pliquett et al. 1996). Interestingly, the LTR's have been found to be localized in the area of the heat-stripped skin corresponding to the dermal papillae (troughs of the epidermis) and no LTR's appeared in the Rete pegs (crests of the epidermis). After applying lower voltage pulses (transdermal voltage < 80 V) the edges show more fluorescence, than the interiors of the keratinocytes in the LTR's. However, after high voltage pulsing (transdermal voltage > 160 V) the fluorescence of the edges and interiors of the keratinocytes are indistinguishable. This shows that pulsing at high voltage was found to result in the creation of the aqueous pathways that pass through

the keratinocytes. The LTR's were found to occur at and above the transdermal voltages of 75 V. The number of LTR's were found to increase with the increase in the applied voltage (between 75 V and 160 V) but in spite of the voltage used, their apparent initial diameter was 10 μm. An increase in the number of pulses increased the diameter of the LTR's to approximately 40-80 μm. It is important to note that the increase in the number of LTR's created after the third pulse at a constant voltage was very limited.

Work has been done to compare the *in-vitro* transdermal delivery obtained by iontophoresis alone, with the delivery seen with iontophoresis following a single electroporation pulse (Tamada et al. 1993). The latter had a significantly increased delivery as compared to iontophoresis alone. This shows the potential of combining these two techniques for drug delivery enhancement.

1.5.2 Chemical enhancers and electroporation

It has been found that the transdermal electroporative delivery of a highly-charged macromolecule (heparin) remained partially elevated for several hours after high voltage pulsing (Prausnitz et al. 1995). Most heparin molecules are long enough to span the five to six lipid bilayer membranes that separate corneocytes within the stratum corneum. Hence it is quite possible for a linear macromolecule such as

heparin to enter the aqueous pathways created by the high voltage pulsing and alter the flux of the smaller, co-transported ions and molecules by either increasing the pathway life time or providing charge within the pathway that can attract/repel co-transported species (Weaver et al. 1997). This "foot in the door" hypothesis has been proposed even for some single bilayer membranes such as those involved in the cell membrane electroporation (Weaver 1993 and Sukharev et al. 1992). The possibility of heparin getting entrapped in the transport pathways while further enhancing transport delivery comes from the fact that heparin did not enhance the passive or iontophoretic drug delivery.

In the work mentioned above, heparin is enhancing its own transdermal electroporative drug delivery due to its "foot in the door" action. Besides heparin, a number of other macromolecules i.e., dextran-sulfate, neutral sulfate, and poly-lysine have also been tried out as electroporative transdermal transport enhancers for mannitol (Vanbever et al. 1997). Electroporation by itself enhanced the transdermal delivery of mannitol by approximately two orders of magnitude while the presence of a macromolecule enhanced the delivery of mannitol up to five fold. Macromolecules with greater charge and size have been found to be more effective in enhancing transdermal transport. These macromolecular chemical enhancers differ from the ones that are utilized in the passive delivery. The traditionally used chemical enhancers like

ethanol, azone, DMSO etc are believed to work by disrupting the stratum corneum lipid structure. However, the macromolecules act mainly by stabilizing the transient disruptions already caused by the electroporation of the skin. Ethanol, a traditionally used enhancer has been tried out for enhancing the transdermal delivery of cyclosporin-A (CSA) in combination with iontophoresis and electroporation (Wang et al. 1998). It was found that enhancement of CSA's transdermal permeation, both by ethanol alone and in combination with iontophoresis was inadequate for transdermal delivery. However, in the presence of ethanol, multiple electroporative pulses (>10) resulted in a sixty fold increased delivery of cyclosporin in the skin. Hence, use of chemical enhancers in combination with electroporation appears to hold a potential for higher transdermal drug delivery.

1.5.3 Ultrasound and electroporation

The application of ultrasound in the presence of electroporative pulsing has been found to enhance the transdermal flux of calcein and sulphorhodamine by 2 and 3 times respectively, as compared to delivery by electric pulsing alone (Kost et al. 1996). The lag time for drug delivery is also reduced by this combination. Ultrasound application also reduced the threshold voltage required for the onset of calcein and sulphorhodamine transport in the presence of electric fields. Application of ultrasound induces cavitation in the keratinocytes of the stratum corneum. The oscillation of the

cavitation bubbles has been shown to induce a partial disorder in the skin lipid bilayer (Mitragotri et al. 1995a). The electrical resistance of the disordered bilayers is less than that of the normal bilayers hence the applied electric field would preferentially concentrate across the normal bilayer. This decreases the threshold electroporating voltage for transdermal transport of compounds. Hence, the combination of ultrasound and electroporation is another viable option for higher transdermal drug delivery.

1.5.4 Electroincorporation

Electroincorporation has been defined as the transport of substances by mass transport of microcarriers through a biomembrane (Hofmann et al. 1995). Carriers such as vesicles can migrate through the stratum corneum as a whole by electro-incorporation. Hence, the drug that needs to be delivered is encapsulated into a microcarrier. These microcarriers are brought in close contact with the skin. A field with components perpendicular to the stratum corneum is created by the electrodes on top of the microcarriers which results in the transport. The encapsulation of the drug in the microcarriers is supposed to protect the sensitive drugs from enzymatic breakdown and hence result in an improved drug delivery.

II. RESEARCH PLAN

The application of electroporation in the field of transdermal drug delivery enhancement has only recently been explored. Electroporation involves the application of high voltage pulses of a few ms duration to the skin. Although the knowledge in this field is rapidly expanding there are still many unanswered, or incompletely answered questions. The influence of electrode design has not yet been studied yet as a parameter influencing electroporative drug delivery. There also exists a need to study the macroscopic or microscopic changes in the skin due to electroporation and to establish the safety of the technique for any such application. Hence, the **overall objective** of this project was to study the parameters involved in the electroporative transdermal drug delivery technique so as to optimize its efficiency and safety. The **specific objectives** of the research were to:

1. optimize the basic parameters of electroporative transdermal delivery using a fluorescent drug terazosin hydrochloride (TRZ) as a probe, under varying conditions of applied electrode voltage ($U_{electrode}$) & voltage drop across the skin (U_{skin}), pulse lengths and number of pulses and electrode area.

2. study the safety of the technique by:

a) studying the recovery of skin's barrier property at the different electroporative conditions.

b) studying the histological changes in the skin after using different electroporation

pulsing conditions.

c) determining the energy delivered to the skin at various electroporative conditions so as to get an insight to the processes and mechanisms. This would be done by measuring various electrical properties of the skin during pulsing.

This research project was based on the following hypotheses.

1. Use of electroporation would enhance the transdermal delivery of the model drug TRZ as compared to its passive delivery.
2. The efficiency and safety of the technique would be dependent on electroporation parameters such as voltage, pulse length, number of pulses and electrode area.
3. By altering the above parameters it would be possible to achieve an effective transdermal delivery with minimum damage to skin, thus ensuring safety of the technique.

V. MATERIALS AND METHODS

5.0 Materials

5.1 *Chemicals*

Terazosin hydrochloride [1-(4-amino-6,7-dimethoxy-2-quinazolinyl)-4-(2-tetrahydrofuroyl)piperazine hydrochloride dihydrate] was provided by Abbot Laboratories, QC, Canada (Mol. Wt of Terazosin: 387.44). Prazosin hydrochloride [(1-(4-amino-6,7-dimethoxy-2-quinazolinyl)-4-(2-furoyl) piperazine hydrochloride monohydrate] was used as an internal standard and was obtained from Sigma Chemicals Co., MO, USA. All other solvents and reagents used were of HPLC and analytical grade. Purified water was obtained using the Barnstead Nanopure II system (Barnstead, MA, USA).

5.2 *Electroporation set-up*

Gene Pulser® II (Bio Rad, CA, USA) fitted with Ag/AgCl electrodes was used as the source for generating single and multiple exponentially decaying electrical pulses for electroporation. Custom made Ag/AgCl electrodes were used to replace the shocking chamber that comes attached to Gene Pulser® II. The Gene Pulser® II unit was equipped with a capacitance extender, a pulse controller unit and a panel that displayed the values of applied voltage ($U_{electrode}$), pulse length (τ), capacitance, and

resistance (sample + system). $U_{electrode}$ can be defined as the peak value of voltage of an exponentially decaying electric field applied between anode and cathode during pulsing. Similarly, τ can be defined as the time required for the applied voltage to reduce to 37% of its original value. The voltages generated from the electroporator were verified using a Tektronix® -5111 storage oscilloscope (Oregon, USA) for determining the accuracy of the output pulses in comparison to the values displayed on screen. The storage oscilloscope was also utilized for measurement of the voltage drop (U_{skin}) across and through the skin. Probes-10X (Fluke®-Phillips) were used to assist in these measurements, by reducing the output 10 times, as the transdermal voltage drop was in some cases beyond the normal range of the Tektronix® -5111 storage oscilloscope. The probes appended to the oscilloscope were calibrated using an Eppley Standard Cell to ensure that they accurately reduced and transmitted the output voltage to the oscilloscope.

5.3 *Electrodes*

Ag/AgCl electrodes, made from a silver wire (1.0 cm in diameter, purity: 99.99+ %, Aldrich® Chemical Co., WIS, USA) by electrocoating with AgCl on the surface, were used as both anode and cathode in electroporative TRZ delivery experiments. The influence of the electrode area on the values of U_{skin} was studied by replacing the normally used small electrode (area = 0.56 cm^2) with a large electrode (area = 2.74

cm^2) and measuring the values of U_{skin} achieved after pulsing. The silver wire was spiralled to form a loop for increasing the surface area of electrodes. For all these experiments an identical silver wire with a diameter of 0.5 mm was used.

5.4 *HPLC analysis*

The analysis of the TRZ samples was done using an HPLC system that consisted of a Waters® Model 510 HPLC pump (Waters associates, MA, USA), a Shimadzu SIL-9A autoinjector and RF-535 Fluorescence HPLC monitor (Shimadzu Corporation, Kyoto, Japan). The fluorescence detector was set at an excitation wavelength of 345 nm and an emission wavelength of 400 nm. The analytical column was a Waters® RCM 8 x 10 cartridge packed with Nova-Pak C18 reverse phase particles, fitted with a Nova-Pak C18 guard column. Responses were recorded as peaks on an LKB 2210 (Bromma, Sweden) recorder operated at the speed of 10 mm/min. The mobile phase consisted of phosphate buffer (pH 7.0), acetonitrile and tetrahydrofuran in the respective volume ratio of 76:18:6. The mobile phase was clarified and degassed by filtering through a 0.22 µm membrane filter, at least twice (Millipore Corporation, MA, USA) and was pumped isocratically at a flow rate of 1.0 mL/min. Accumet® - model 10 pH meter (Fisher Scientific, PA, USA) was used to measure pH of the solutions.

5.5 *Animals*

Seven to eight weeks old hairless rats were initially procured from Charles River Laboratories, QC, Canada. However, at a later stage for reasons beyond our control the animal supplier source had to be changed to Harlan Sprague Dawley, IN, USA. The excised skin from the two sources were compared with regard to electrical resistance and were found to be similar. However, in order to maintain uniformity of skin source all the drug transport experiments were performed using the Charles River Laboratories rats and all the experiments for measuring U_{skin} were performed using Harlan Sprague Dawley rats.

5.6 *Diffusion cell assembly*

For the drug delivery studies, a custom designed side-by-side diffusion unit (figure 2) made of Delrin (Polymethanal, Dupont®) was used. The distance between the two electrodes during pulsing was 10 mm. The effective diffusional cross section-area of the cell was 1.3 cm^2. Isolated skin was sandwiched between the donor and receiver compartments with the stratum corneum side of the skin facing the donor side. The anode was inserted in the donor compartment which contained TRZ solution (1.03 mg/mL) in phosphate buffered saline (PBS, pH 6.4). The cathode was placed in the receiver compartment during pulsing and contained the PBS. All experiments were performed at room temperature (22 ± 1 ° C).

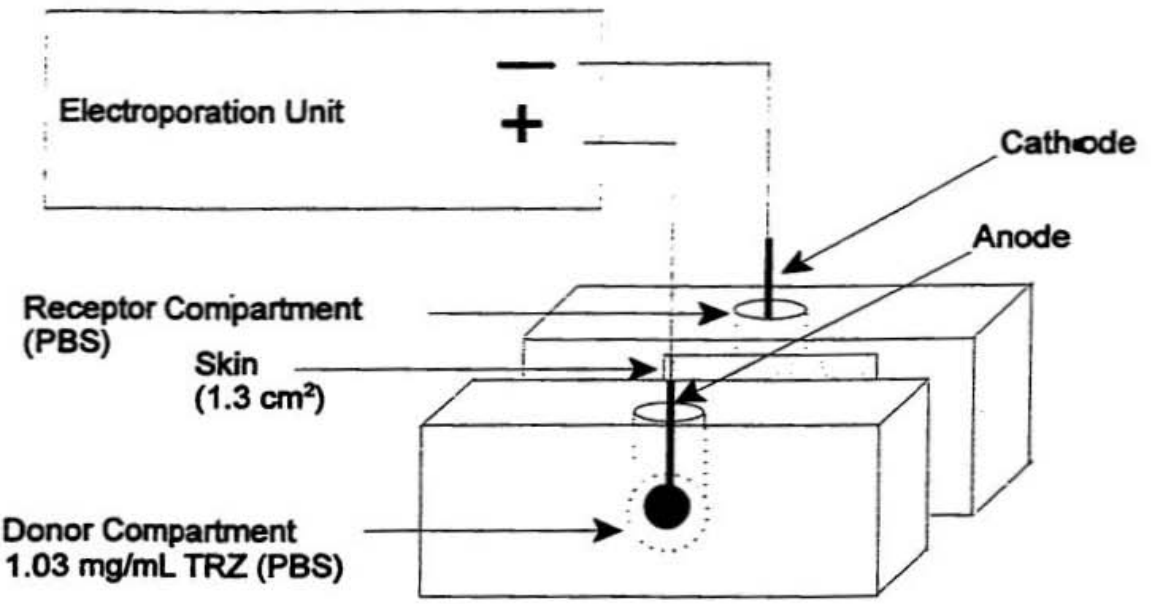


Figure 2: Side-by-side cell for drug delivery

5.7 *Four electrode assembly for U_{skin} determination*

For determining the $U_{electrode}$ vs U_{skin} relationship, custom designed side-by-side cells (figure 3) made of Plexiglass® (Rohm and Haas Co., PA, USA) were used. This material has the advantage that it is transparent and allows easy viewing of the contents. These cells had the same cross-sectional area as the diffusion cells used for drug delivery, viz. 1.3 cm^2. The cells had three holes for the placement of the pulsing electrodes, so the distance between the two pulsing electrodes could be varied between 10 to 20 or 30 mm. Besides having holes for placing the delivery electrodes, each of the chambers had holes very close to the open end of the chamber (2 mm from the skin when assembled), through which L-shaped Teflon® tubes (Haber-Luggin capillaries) were inserted. The open end of the tube just touched the skin in the assembled unit. The Teflon® tube (0.03" Internal Diameter and 1/16" Outer Diameter) was filled with agar-KCl gel and Ag/AgCl electrodes were inserted in the gel. These two inner Ag/AgCl electrodes were connected to the oscilloscope to record U_{skin}, while electroporation pulses were delivered from the outer electrodes.

5.8 *Choice of TRZ as the model drug*

One of the challenges in transdermal drug delivery research is the development of a suitable analytical method to accurately quantify drugs in the skin. Organic substances such as lipids and enzymes from the skin interfere with drug separation

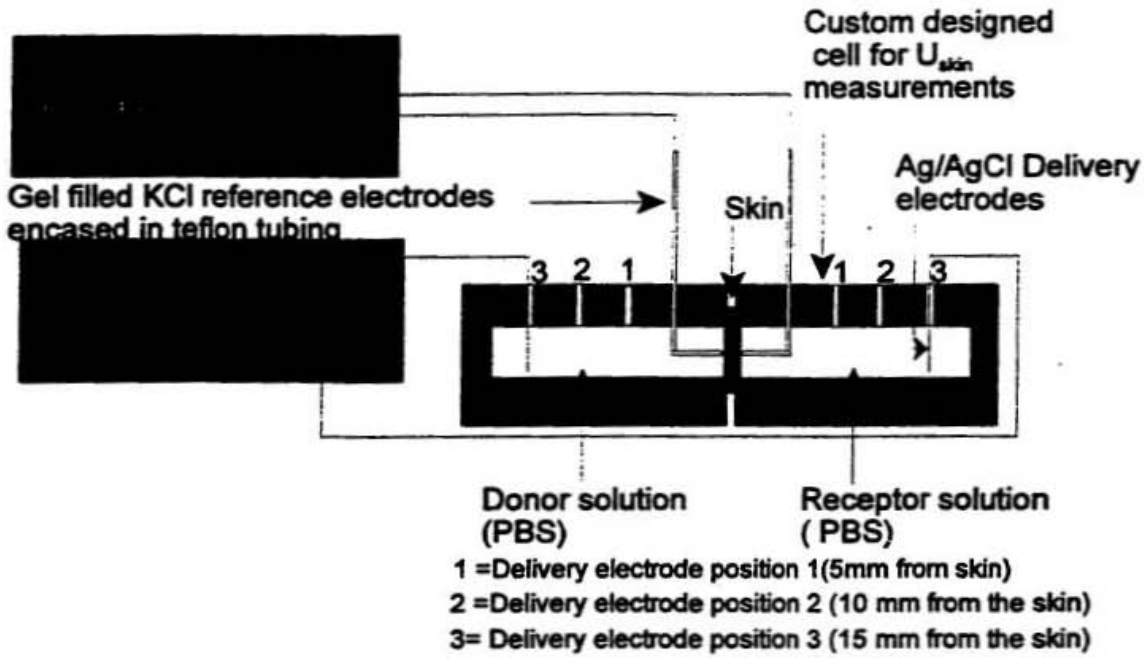


Figure 3: Cell for the measurement of transdermal voltage drop

and analysis. Selective extraction of a drug is often complicated and so is the analysis of its extract using HPLC with a UV detector. This problem could be best solved by selecting a radiolabeled or a fluorescent drug molecule that could be detected using an HPLC system coupled with a radiometric or fluorescent detector. TRZ, a fluorescent molecule, was chosen as the model compound for the initial optimization experiments. TRZ can be easily and accurately analyzed using HPLC equipped with a fluorescence detector (Singh et al. 1996). Use of TRZ as a model probe served the purpose to conduct optimization experiments and to get a better understanding of permeabilization of skin using electroporation.

5.9 *Control*

The control study consisted of the passive diffusional delivery of TRZ. All the conditions for control delivery, including the donor and receiver solution composition were kept the same as that in the electroporation experiments. In the pulsed samples, electroporative pulses under specified conditions were used for enhancing the delivery of the drug whereas in the case of the control drug delivery was by passive diffusion and therefore unassisted. Control experiments were performed with each set of electroporation experiments.

5.10 *Electroporation experiments*

The rats were sacrificed using a carbon dioxide chamber. Full thickness abdominal and back skins were excised and used within 8 hours. Subcutaneous fat and muscle layers were carefully removed from the skin. The excised skin samples were hydrated by soaking for 2 hours in PBS. Skin samples were sandwiched in the diffusion cell (figure 2) between the donor and receiver compartments. The stratum corneum side of the skin faced the donor chamber. Skin samples having resistance below 20 KΩ were discarded. The lowered resistance could be due to any damage inherently present in the rat skin or inflicted to the skin during its preparation, which may have rendered the skin 'leaky', resulting in the low resistance. Exponentially decaying electroporation pulses were delivered using the Gene Pulser® II unit with Ag/AgCl electrodes. Multiple pulses were delivered with one minute interval between each pulse. The pulses were characterised by the applied electrode voltage ($U_{electode}$), the number of pulses, and the decay time constant of the applied exponential pulse, i.e., the pulse length (τ).

5.10a *TRZ delivery across the skin over 8 h period*

Samples of 100 µl were withdrawn from the receiver chambers at different times, viz., 0, 15, 30, 60, 120, 180, 240, 300, 360, 420, and 480 minutes. Immediately after withdrawing samples, an equal amount of PBS was added to the receiver side to

replenish the volume loss. Samples were analysed using HPLC. The decrease in the concentration of TRZ in the receiver side due to the sampling was accounted for while calculating the amount of TRZ recovered in the subsequent samples.

5.10b. *TRZ delivery in the skin tissue*

The amount of TRZ delivered into the skin soon after delivery of the pulses was quantified in a separate set of experiments performed using a similar set-up as described above. After pulsing, samples were withdrawn from the receiver side. The cells were emptied, dismantled, and skin specimens were removed. The stratum corneum side of the skin was washed with deionized water 3 times and Scotch® adhesive tape (3M, Ontario, Canada) was used to strip it 3 times to remove any superficially attached drug. The Scotch® tape strippings were collected in methanol solution, and extracted. The extract was analysed to account for the amount of drug removed in the strips. A biopsy punch was used to cut out 0.87 cm^2 of the skin sample to quantify the TRZ delivered in the skin. This sample was stored in 1 mL of methanol. An appropriate amount of prazosin solution was added to the solution, and the skin sample was homogenized using a Brinkmann homogenizer (Polytron®, Switzerland). It was further extracted using 3 x 1 mL of methanol and centrifuged. The combined extracts were centrifuged and analyzed using HPLC for the TRZ content. The amount

of drug delivered into the receiver side was also analysed. The influence of various pulsing parameters like $U_{electrode}$, τ, and number of pulses on the transdermal delivery of TRZ were studied to determine the optimal pulsing conditions.

5.10c *Reversal of the permeability enhancement caused by electroporation*

This experiment was designed to assess the reversal of the electroporation-induced permeability-enhancement in skin. The contact time of the drug with the skin was maintained as constant (20 minutes) unless otherwise mentioned. PBS was present in the receiver side throughout. The contents of the donor and receiver compartments were varied to assess the time required for the reversal of the permeability of the skin samples in the cells. The various treatments were as follows:

i. The skin present in the delivery cell was pulsed in the regular fashion. The drug solution was present in the donor side and 10 pulses of 20 ms each at a voltage of 400 V were applied. After pulsing, the solution was left in contact with the skin for further 10 minutes for a total contact time of 20 minutes. The cells were then emptied, dismantled and the amount of TRZ in the skin was determined using HPLC. The amount of drug recovered from this treatment is a measure of the permeation-enhancement caused by electroporation when compared to the control samples.

ii. TRZ solution was present in the donor chamber. Electroporative pulsing was as in #i. After 10 minutes of pulsing, the TRZ solution present in the donor side was removed and the chamber was washed 3 times with PBS solution. The donor chamber was refilled with PBS, left undisturbed for another 10 minutes and then processed as in treatment #i. The amount of drug delivered is that delivered during the pulsing period alone.

iii. PBS was present in the donor side. Electroporative pulsing was as in #1. Immediately after the 10 minutes of pulsing the buffer present in the donor side was replaced with the TRZ donor solution. The diffusion cell was left undisturbed for a further 20 minutes and then processed as in treatment #i. This experiment determines the change in permeability of the skin immediately after pulsing.

iv. Cells were treated in a manner similar to treatment #iii except that the PBS was replaced with the TRZ donor solution 5 minutes after the pulsing was over. The diffusion cell was left undisturbed for a further 20 minutes post pulsing and then processed as in treatment #i. This experiment determines the residual change in permeability of the skin samples 5 minutes after pulsing.

v. Diffusion cells were treated in a manner similar to treatment #iii except that the PBS was replaced with the TRZ donor solution 1 hour after the pulsing was over. The diffusion cell was left undisturbed for a further 20 minutes and then processed as in treatment #i. This experiment determines the residual change in permeability of the skin samples 1 hour after pulsing.

5.10d *Studies on $U_{electrode}$ vs. U_{skin} relationship*

Isolated skin was sandwiched in the diffusion cell (figure 3) between the donor and receiver chambers. The stratum corneum side of the skin faced the donor side of the diffusion chamber. The donor and the receiver sides of the cell were filled with PBS. Exponentially decaying electroporation pulses were delivered using an electroporation unit with Ag/AgCl electrodes. Two sets of electrodes were introduced into the cell. For the outer pulsing electrodes, the anode was placed in the donor compartment and cathode in the receiver compartment. On the other hand, an inner set of agar-KCl gel electrodes was connected to a storage oscilloscope using 10X probes. The lower end of the agar-KCl electrodes were in touch with the skin surface in the diffusion cell to study the voltage drop occurring across the skin surface (U_{skin}). As the pulses were passed using the outer set of pulsing electrodes, the inner set of electrodes displayed U_{skin} as an exponentially decaying curve on the screen of the storage oscilloscope. The values of U_{skin} for varying values of $U_{electrode}$ and pulse length

were determined. There were 3 holes made on each side of the chamber for the placement of the pulsing electrodes at a distance of 5, 10 and 15 mm from the skin surface. The influence of varying the distance between the pulsing electrodes on the values of U_{skin} for a given $U_{electrode}$ value was studied. Additionally, the influence of varying the area of the pulsing electrodes, while they were placed 5 mm away from the skin, on the values of U_{skin} was studied. The values of U_{skin} for a small electrode (area = 0.56 cm^2) were compared with that of a large electrode (area=2.74 cm^2).

5.10e *Determination of current passing through skin during delivery of the electroporation pulse*

The amount of current passing through the skin during pulsing was assessed by measuring the voltage drop occurring across a resistance placed in series with the delivery electrodes (see figure 4) and was recorded in a storage oscilloscope (Tektronix®-5111, OR, USA). The value of the resistance was varied between 0.1 to 5 Ω depending on the electrode area and the $U_{electrode}$ used for pulsing. This was done to overcome the limitation of storage oscilloscope. For an electrode, at any given value of $U_{electrode}$, the oscilloscope would sometimes either fail to trigger or overshoot when a certain amount of resistance was attached in series. Hence, the experiment had to be repeated with a new skin sample, with a new value of resistance attached in series with the electroporation cell. The amount of current passing through the skin during

pulsing was calculated using *Ohm's law (R= E/I, where I is the current in amperes, E is the voltage drop across skin (U_{skin}, in volts), and R is the resistance in Ω).* This experiment was performed with both small and large electrodes using a single pulse ranging between 2-5 ms, at $U_{electrode}$ 100, 200, 300, 400, and 500 V. For each determination a fresh piece of skin was used.

5.10f *Studies of the macro- and microscopic changes in the skin due to pulsing*

After determining the optimal pulsing conditions from the drug delivery studies done earlier, studies were conducted on the changes in the skin due to pulsing. In separate experiments, 20 pulses of 500 V each and at varying pulse lengths (20, 30, 40, and 60 ms) were passed to the skin samples. Similarly, the influence of using small and large electrodes on the changes in the skin was studied after applying 20 pulses of 60 ms each at 500 V. Duplicate samples were taken in each case. The pH of the donor and receiver solutions were recorded before and after pulsing using non-bleeding colorpHast® (E. Merck Science, NJ, USA) pH indicator strips. Immediately after electroporation, the skin samples were fixed in glutaraldehyde fixative (0.1 M cacodylate buffer, pH 7.2). Photographs of these post-pulsing skin surfaces (stratum corneum and dermal side) were taken to record any superficial effects of pulsing like burns etc. For microscopy, 0.5-1.0 µm sections of skin were cut on a Cambridge Huxley Ultramicrotom. The samples were stained for 2 minutes at 70°C in 1% toluidine

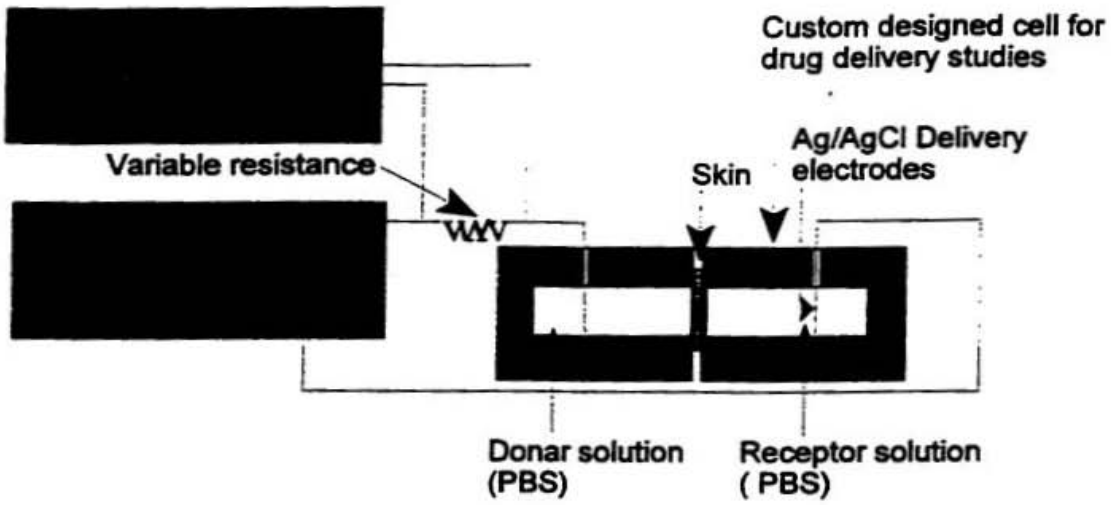


Figure 4: Experimental set-up used for the determination of the current passing through skin during delivery of the electroporation pulse.

blue in 1% sodium borate. The slides were viewed under a Phase contrast microscope (Zeiss® IM-35). The samples were subject to a 400-times magnification. Photographs of the slides were taken on a 35 mm Pentax® camera using a T-Max® (Kodak, CA, USA) b/w 400 ASA film.

5.11 *Data Analysis*

The experiments were performed at least five times (unless specified otherwise) and the means ± S.E. are reported. All the statistical analysis was performed using the Sigma-stat® statistical software (Jandel Scientific, CA, USA). Skin samples are known to exhibit a wide variation making it difficult to assume a normal distribution. So non-parametric analysis of the variance was performed.

VI. RESULTS AND DISCUSSION

6.I The choice of a skin model for transdermal drug delivery studies

The characteristics of skin are different in different mammals. These include differences in horny layer thickness, sweat gland, and hair follicle densities. The sweating ability as well as the capillary blood supply vary between humans and common laboratory animals. These factors can affect resistance, route and amount of percutaneous absorption between different species. In view of such differences human skin would be most desirable for *in-vitro* studies. However, it is interesting to note that the human skin viability, as determined by anaerobic metabolism where glucose is converted to lactose, is maximal for the first 18 hours after death of donors (Wester et al. 1998). When properly preserved, human skin samples retain their integrity for a maximum of 8 days. Heat-treated/separated epidermis and dermis lose viability. Fresh human skin samples are not easy to acquire and the availability may vary depending upon a number of factors such as population, demography etc. On the other hand, animals can be easily acquired and employed for *in-vivo*/*in-vitro* transdermal delivery studies. Porcine skin flaps from the caudal abdominal, inguinal, and ear flaps are recommended models for studying percutaneous absorption (Carver et al. 1989, de Lange et al. 1994, and Reifenrath 1984). Pig skin is known to slightly overestimate

lipophilic compound absorption and slightly underestimate hydrophilic compound absorption (Schaefer et al. 1996). The total amount of drug delivered tends to give a close estimate and hence pig skin is accepted as a good substitute for human skin samples. Mouse skin has a considerably higher percutaneous absorption rate and hence, is not recommended for transdermal studies (Schaefer et al. 1996). For studying drug absorption, an alternate approach is to use reconstituted skin in which the keratinocytes are seeded on dermal substrate (Bell et al. 1991)/de-epidermized dermis (Rheinwald 1989, and Régnier et al. 1990) and grown in standard tissue culture medium. It is also possible to graft human skin onto nude rat skin flaps with the associated microvasculature to study the percutaneous absorption in *in-vivo* conditions (Wojciechowski et al. 1987). Although the last two techniques are very attractive, they are also expensive, time consuming, tedious, and hence are not followed very commonly for percutaneous absorption studies. If human or pig skins are not easily available, the other type of animal skin recommended and very commonly used is rat skin (Bronaugh et al. 1982). Hairless/fuzzy rat skin has been used in our transdermal delivery studies. Normal (hairy) rat skin needs to be shaved for such studies. This can damage the stratum corneum making the results ambiguous. In any case, after optimizing the electroporative transdermal drug delivery conditions in animal models, delivery can be tested on viable human skin samples.

6.2 Electrical measurements in the skin

6.2.1 *Studies on the relationship between $U_{electrode}$ vs U_{skin}*

The values of U_{skin} achieved for the various applied values of $U_{electrode}$ using the dual electrode system are given in table 2 and figure 5. It was observed that all the U_{skin} values achieved from the first pulses were always higher than those obtained from subsequent pulses applied to the same skin sample, even if the subsequent pulses were applied 8 hours later. After 3-4 pulses the U_{skin} more or less stabilized at a value lower then the initial values. The electroporator, Gene Pulser® is designed to display the total resistance that includes its internal resistance and the test sample's resistance (designated as the 'base value' prior to pulsing). This displayed resistance also drops after the very first applied pulse and does not rise back to the base values. Since the machine's internal resistance remains constant, the reduction in the displayed resistance could be attributed to changes occurring in the skin. Hence, for all the $U_{electrode}$ vs U_{skin} relationship experiments a skin sample was used only once to determine the changes occurring with the first pulse. For replicate determinations a fresh skin sample was used.

Table 2: The relationship between $U_{electrode}$ and U_{skin} for a small electrode

$U_{electrode}$ (V)	U_{skin} Mean ± SE (V)
100	32.4 ± 1.7
200	43.6 ± 2.9
300	65.8 ± 0.8
400	83.6 ± 2.5
500	88.3 ± 2.3
600	254 ± 4.2

Table 3: The amount of current passing through the skin during pulsing with small electrode

$U_{electrode}$ (V)	U_{skin} (V)	Current passing through the skin (amperes)	n
100	32.4	0.4 ± 0.04	3
200	43.6	1.0 ± 0.01	3
300	65.8	1.8 ± 0.1	3
400	83.6	2.5 ± 0.1	3
500	88.3	3.1 ± 0.1	3

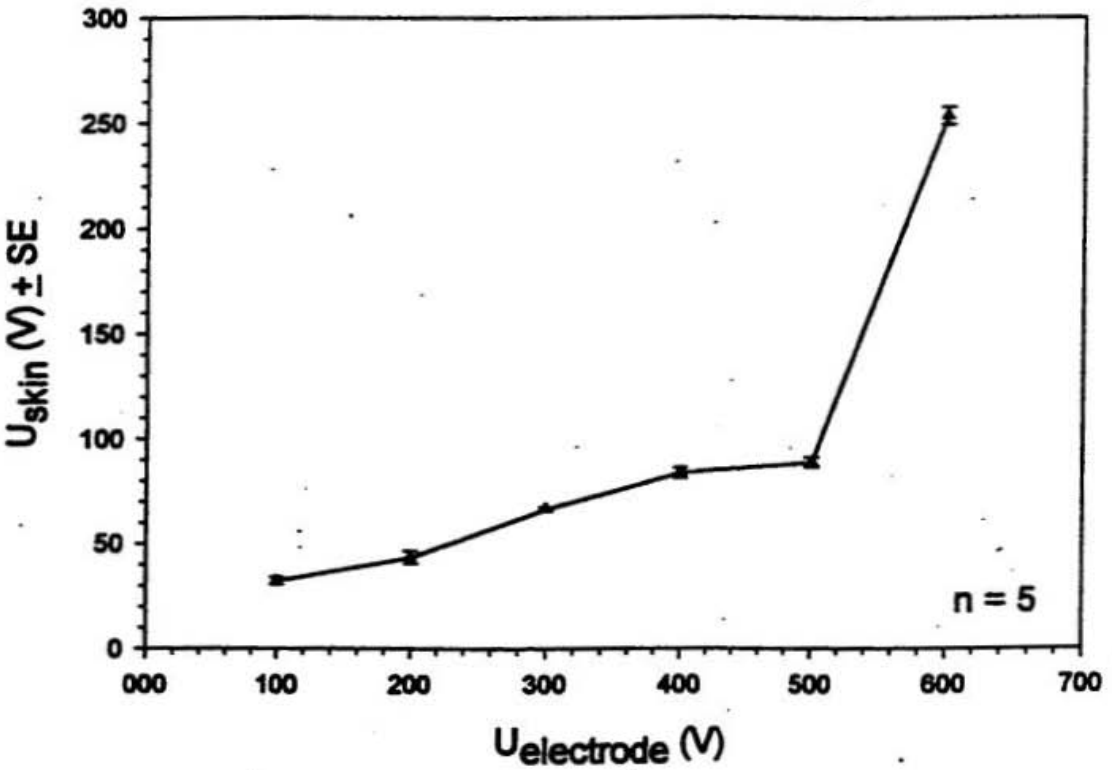


Figure 5: Relationship between $U_{electrode}$ and U_{skin}

The observations of a drop in the value of the U_{skin} and the electroporator's indicated circuit resistance after the initial pulsing point towards the possibility of irreversible changes being caused by the electroporation pulses. It is envisaged that this situation would be different in an *in-vivo* setup where the skin is essentially viable. Normal skin undergoes continuous repair and replacement process in response to damage and the natural exfoliation processes. With the decrease in the resistance, and the decrease in the observed voltage drop across the skin, an increase in the permeability of the skin is indicated.

As the voltage drop applied across the delivery electrodes was increased the voltage drop occurring across the skin sample also increased. As Fig 5 shows U_{skin} increased roughly linearly with $U_{electrode}$, being one third to one fifth of the latter up to 500 V. A large rise in U_{skin} occurred for $U_{electrode}$ of 600 V while measurements at $U_{electrode}$ = 700 V was not possible. Due to this reason we restricted our transdermal work to voltages of 500 V and below. Thus, these results show that the actual voltage to which the skin is exposed at the time of pulsing is much lower than the applied voltage and in addition pulsing effects the resistance of the skin. It should be noted here that the values of U_{skin} quoted are the maximum values observed at the beginning of the exponential decay of $U_{electrode}$. It was observed that varying the pulse length did not affect the U_{skin} values for a given $U_{electrode}$ (observations were noted but the data is not shown in any tabular form).

6.2.2 The amount of current passing through the skin during pulsing

The values of the current passing through the skin at various applied values of the $U_{electrode}$ are given in table 3 and figure 6. The values of current are those obtained with the small electrode. The values for current obtained with the larger electrode are mentioned elsewhere in this thesis (please see table 14 on page 84). The amount of current passing through skin increased linearly with an increase in the value of $U_{electrode}$.

6.3 HPLC analysis

TRZ was quantified using a method already established in our laboratory (Singh et al. 1995). A typical chromatogram from the analysis is shown in figure 7. The first peak represents the amount of TRZ and the second peak represents the amount of prazosin (PRZ) the internal standard, in the chromatogram of the injected sample.

Calibration curves were produced on each day of analysis using stock solutions with different concentrations of TRZ. These calibration curves resulted in statistically well correlated straight lines. The mean calibration curve is shown in figure 8. The regression equation for the mean curve was:

Peak height ratio = 17.06 (TRZ concentration) - 0.02

(where R^2=0.99, n = 3, Interday Coefficient of Variation (CV) = 5.30%,and linear range = 0.76 -7.56 µg/mL.)

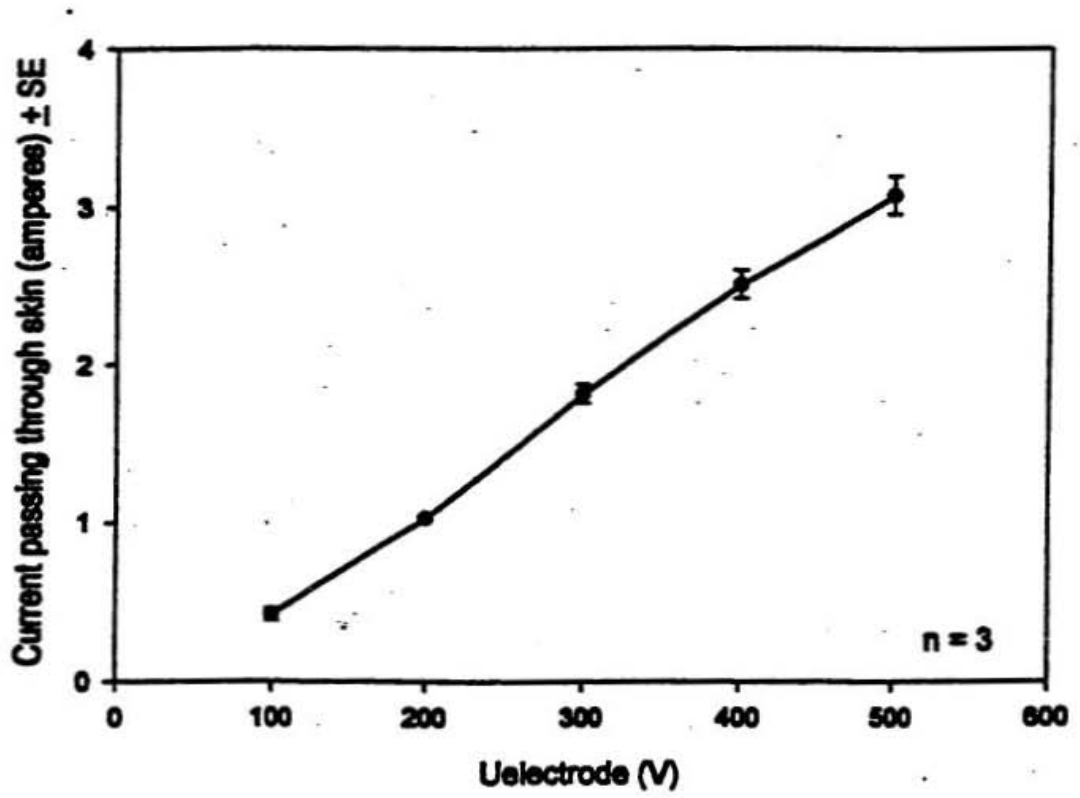


Figure 6: $U_{electrode}$ vs current passing through skin.

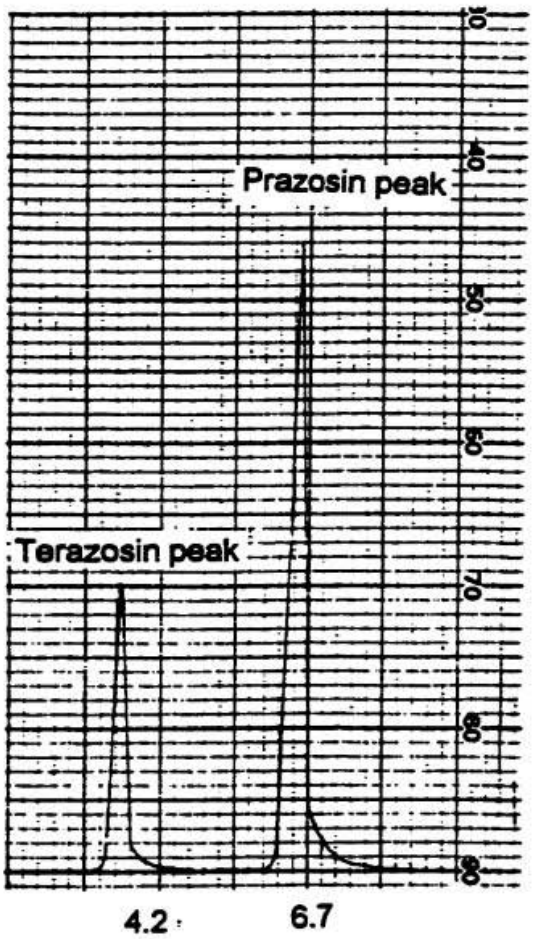


Figure 7: A typical chromatogram obtained from the analysis of terazo-sin.

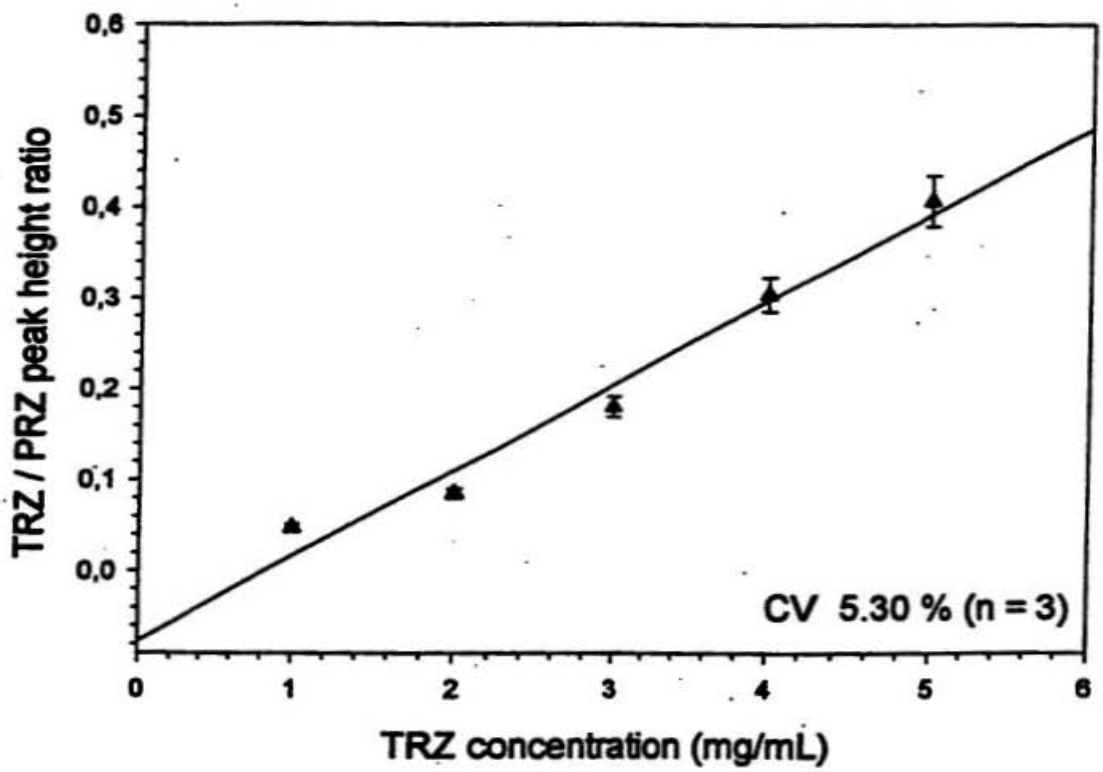


Figure 8: Calibration Curve for TRZ analysis by HPLC

6.4 Eight hour transport studies

Figure 9 shows the profile obtained for TRZ delivery into the receiver using 20 pulses at $U_{electrode}$ = 400 V, τ = 5 ms. There was a phase lag of one hour before a measurable quantity of TRZ was detected in the receiver.

The cumulative amount of TRZ delivered in the receiver in 8 hours using 5 pulses of 5 ms each at different $U_{electrode}$ voltages are given in figure 10 and table 4. There was no statistically significant difference ($p > 0.05$) between the amount of TRZ delivered with control and with 200 V. With 300 V there was a marginal increase in the TRZ delivered. With $U_{electrode}$ of 400 and 500 V there was a significant increase in the amount of TRZ delivery ($p < 0.05$) compared to control. However, at 400 and 500 V there was no significant ($p > 0.05$) difference. Further study to determine the influence of pulse length and number of pulses, was done at $U_{electrode}$ 500 V. The results are shown in table 5. Increasing the number of pulses from 5 to 20 at $U_{electrode}$ 500 V (τ = 5 ms) increased the cumulative TRZ delivery by four times. Doubling the pulse length from 5 ms to 10 ms (at 500 V, 20 pulses) had much greater impact and the TRZ delivery increased by about five times more.

Table 4: Cumulative amount of TRZ delivered in 8 hours in the receiver using 5 pulses of 5 ms each at different $U_{electrode}$ voltages

$U_{electrode}$ (V)	Amount of TRZ delivered in receiver Mean (µg) ± SE	n
Control (0)	0.1 ± 0.02	5
200	0.1 ± 0.02	5
300	0.2 ± 0.1	5
400	0.6 ± 0.2	5
500	0.9 ± 0.3	5

Table 5: Cumulative amount of TRZ delivered in 8 hours in the receiver using $U_{electrode}$ = 500 V at different pulsing conditions

Treatment	Amount of TRZ delivered in receiver Mean (µg) ± SE	n
Control (0 pulses)	0.04 ± 0.02	4
5 pulses X 5 ms	0.7 ± 0.02	4
20 pulses X 5 ms	3.3 ± 1.3	4
20 pulses X 10 ms	14.6 ± 3	4

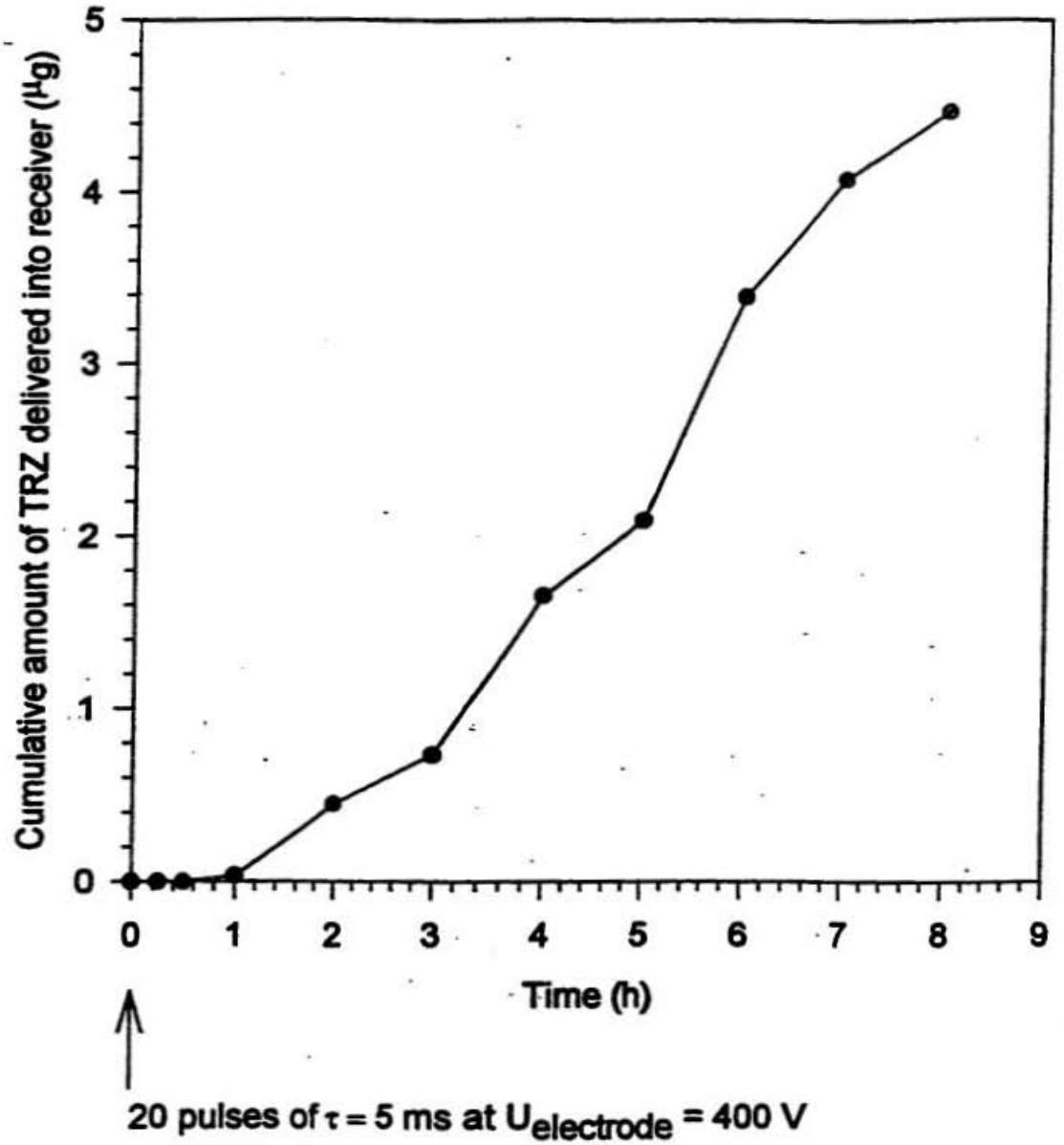


Figure 9: Typical 8 h TRZ delivery profile into receiver

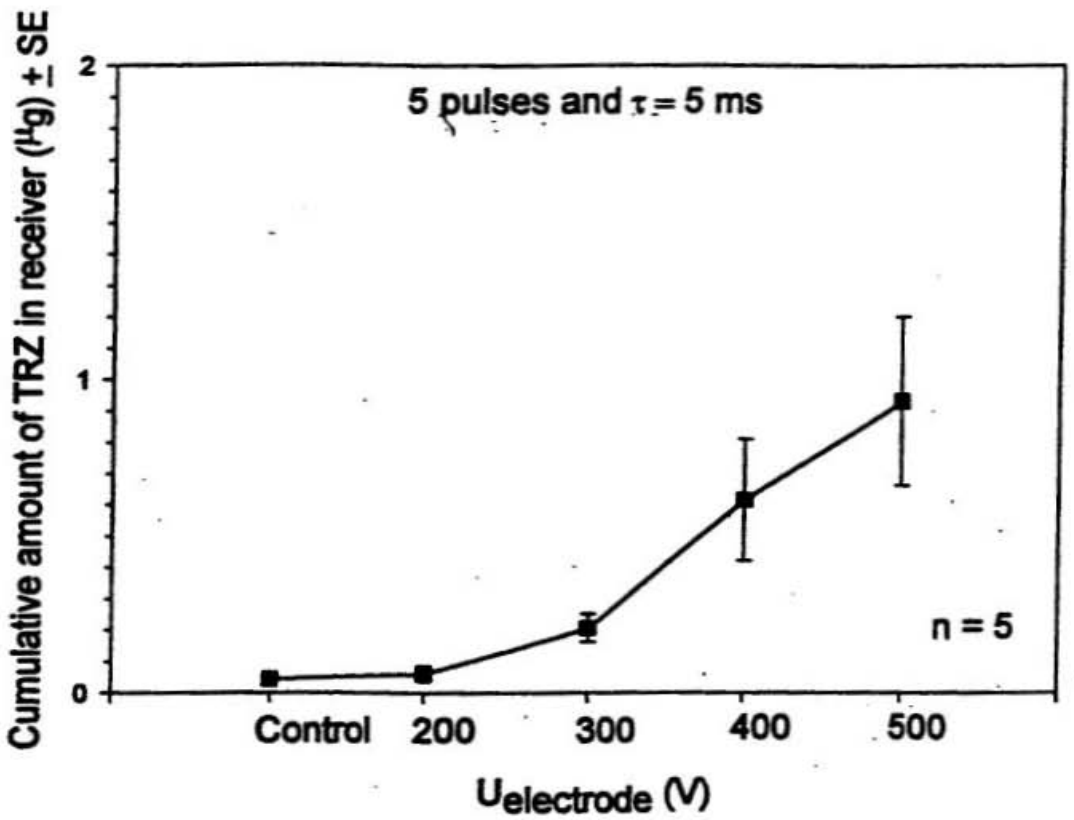


Figure 10: Effect of $U_{electrode}$ on TRZ delivery

(8 hours study)

In all the above experimental results, it was seen that TRZ transport continued for a long time (at least 8 hours) after the delivery of the electroporation pulses. This could be attributed to a 'depot-effect'. Apparently, TRZ depot forms in the skin as soon as the electroporation pulse is delivered and then the drug diffuses out slowly over a long period of time. Calculation of the cumulative TRZ delivered in the 8-hour study (see table 4) exhibited large standard deviations. It is envisaged that these large deviations in the results were introduced due to two problems in the experimental design. Firstly, although freshly excised skin was used in our work, its viability deteriorated over the period of 8 hours. Secondly, the differences amongst the skin samples, especially abdominal and back skin introduced additional variations in the results. To elucidate this further skin samples from different regions of the same animal were subjected to TRZ delivery using 20 pulses of 10 ms each at 500 V. From the results (table 6) it appeared that the abdominal skin was five times more permeable than the back skin. To further investigate the differences in skin type, the amount of TRZ delivered in the skin was quantified. In this study TRZ delivery in the skin immediately after the delivery of 20 pulses, 10 ms at 500 V was quantified. Interestingly, there was no significant difference ($p > 0.05$) in the amount of TRZ delivered in the skin (table 7). From this it may be inferred that the total amount of TRZ delivered in the skin during pulsing is the same and, in this respect there is no difference between the abdominal and back skin. The

Table 6: Influence of skin type on the cumulative amount of TRZ delivered in 8 hours in receiver after 20 pulses, τ = 10 ms at $U_{electrode}$ = 500 V.

Skin type	Amount of TRZ delivered in receiver Mean (µg) ± SE	n
abdominal skin	24.3 ± 5.3	4
back skin	4.9 ± 1.1	4
control	0.05 ± 0.02	4

Table 7: Influence of skin type on the amount of TRZ delivered in skin after 20 pulses, τ = 10 ms, $U_{electrode}$ = 500 V.

Skin Type	Amount of TRZ delivered in skin Mean (µg) ± SE	n
abdominal skin	31.9 ± 3.6	5
back skin	32.9 ± 2.9	5
control	4.03 ± 0.34	5

difference between abdominal and back skins seen in the 8-hour transport study (table 6) is due to anatomical differences in the two skin types mentioned. Rat abdominal and back skins have been seen to vary in stratum corneum thickness as well as the percutaneous absorption rate of compounds (Bronaugh et al. 1983). Hence, the diffusion of TRZ through different thicknesses of the skin varied. Based on the above findings, it was decided to conduct all transdermal electroporation experiments by quantifying TRZ in the skin and the receiver after pulsing. This also allowed us to use the entire skin of the hairless rat without differentiating into abdominal and back skins.

6.5 Effect of electroporation parameters on the delivery of TRZ into skin

Electroporative transdermal delivery is primarily governed by the voltage, pulse length, and number of pulses used (Wang et al. 1997). Although there are few papers published in this specific area of research by different workers including us, the work is far from complete.

The U_{skin} changed after the 1st pulse and subsequent pulses (see above). The $U_{electrode}$ is the voltage set on the electroporator. To maintain constant U_{skin} would require a sophisticated electronic set-up which has not yet been devised. Hence, in all our discussions reference has been made only to $U_{electrode}$.

6.5.1 Influence of $U_{electrode}$

TRZ delivered into the skin using 20 pulses of 20 ms each at different $U_{electrode}$ values ranging between 100 and 500 V are shown in table 8 and figure 11. Control indicates TRZ that was delivered by passive diffusion alone. Using an $U_{electrode}$ of 200 V did not produce any significant TRZ delivery enhancement over the control. At 300 and 400 V the TRZ delivery was seven and thirteen times higher than the control, respectively. Although a further increase in $U_{electrode}$ to 500 V produced fourteen times increased delivery of TRZ compared to control, there was no statistically significant difference ($p > 0.05$) between the deliveries achieved with 400 and 500 V. This is visualized from figure 11, where there is a linear relationship between $U_{electrode}$ and TRZ delivery in the 200 to 400 V range.

Our findings are consistent with those reported by Pliquett et al. (1996). They have reported that a minimum U_{skin} of 75 V has to be exceeded to observe a significant enhancement in the permeability of the epidermis. From our experimental results it was found that for the $U_{electrode}$ 200, 300, 400, and 500 V the corresponding U_{skin} were 43.6, 68.7, 83.6, and 88.3 V respectively. Thus for our experimental set-up it required a U_{skin} of 68.7 V to observe a five times enhanced delivery of TRZ compared to the control. The marginal increase in TRZ delivery from 13 to 14 times that of control, with the increase in $U_{electrode}$ values from 400 to 500 V is consistent with the nominal expected

Table 8: Amount of TRZ delivered in skin using 20 pulses of τ-20 ms each using a small electrode at different $U_{electrode}$ voltages.

$U_{electrode}$ (V)	Amount of TRZ delivered in skin Mean (µg) ± SE	n
0	4 ± 0.5	5
200	2.8 ± 1.0	3
300	28.1 ± 1.2	5
400	53.5 ± 1.7	5
500	58.7 ± 5.6	5

Table 9: Amount of TRZ delivered in skin by applying 20 pulses of $U_{electrode}$ 500 V using a small electrode at different pulse lengths (τ)

Pulse length (τ) ms	Amount of TRZ delivered in skin Mean (µg) ± SE	n
0	4 ± 0.5	5
10	28 ± 1.8	5
20	58.7 ± 5.6	5
30	79.3 ± 1.5	5
40	151.7 ± 16.3	5

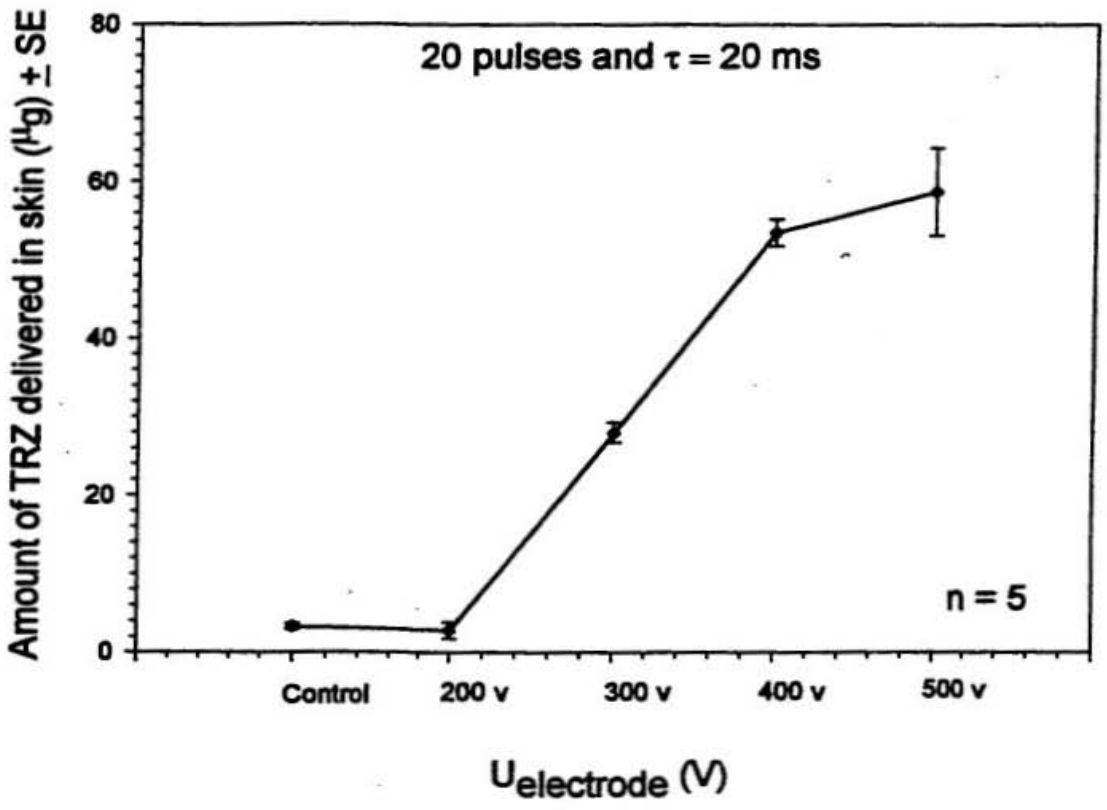


Figure 11: Effect of $U_{electrode}$ on TRZ delivery

increase in the corresponding U_{skin} values from 83.6 V to 88.3 V. It is important to note that increasing the $U_{electrode}$ to 600 V caused a sudden jump in the U_{skin} to 254 V. This was accompanied with visible damage to the skin manifested as deep burn marks. Hence, drug delivery experiments at 600 V were not attempted. From these observations it can be concluded that with regard to U_{skin} values, $U_{electrode}$ values of 300 to 500 V appear to be a useful operating range. Below 300 V there is minimal enhancement in the permeability of skin and above 500 V it may be detrimental to the safety of the skin.

6.5.2 Effect of pulse length

Pulse length had a profound effect in increasing the permeability of skin. Based on our experimental results it is the second most important electroporative parameter, after $U_{electrode}$. TRZ delivery obtained using 20 pulses at 500 V ($U_{electrode}$) at four different pulse lengths viz, 10, 20, 30, and 40 ms are shown in table 9 and figure 12. Similar to the effect of $U_{electrode}$, here also there seemed to be a linear relationship between the pulse length and TRZ delivered up to 30 ms. Use of pulse lengths of 10, 20 and 30 ms enhanced TRZ delivery, compared to control, by 7, 14.5 and 20 times, respectively. However, use of 40 ms pulses enhanced drug delivery by about 38 times, as compared to control. Damage to the skin seemed to appear with the use of pulse lengths of 30 and

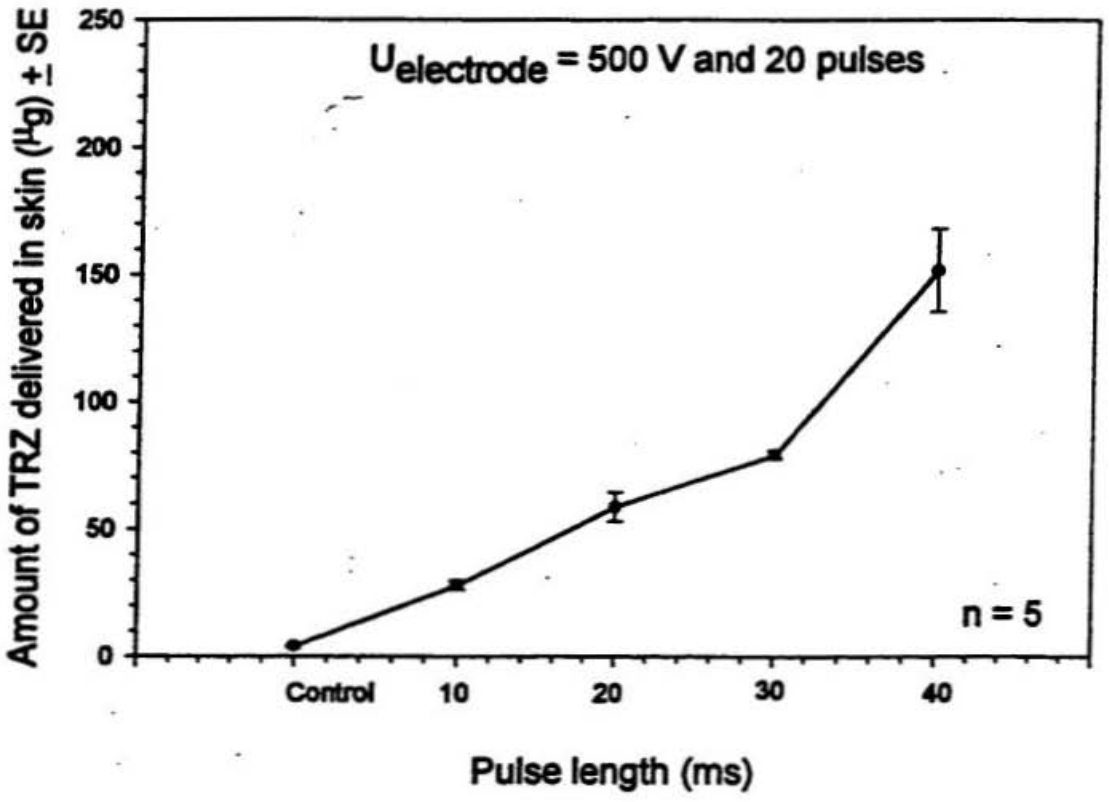


Figure 12: Effect of pulse length on TRZ delivery

40 ms. To further ascertain these findings, the histological details of the pulsed skin were studied under a light microscope. The details of this study are given in section 6.7.2.

6.5.3 Effect of the number of pulses

The number of pulses is the third most important parameter, after $U_{electrode}$ and pulse length. From the previous experiments pulse lengths of 30 and 40 ms seemed detrimental to skin's safety but these studies had been done with the use of 20 pulses. It was not known whether the skin would be damaged with fewer pulses. Since, 20 pulses of 40 ms are known to cause a visible damage to the skin, this part of the experiment was pursued to determine the effect of the number of pulses with 40 ms as pulse length.

A single electroporation pulse with 500 V ($U_{electrode}$) at 40 ms enhanced the TRZ delivery by 3.5 times. This seemed significant but not adequate. As the number of pulses were increased, the amount of TRZ delivered to the skin also increased, although not proportionately (table 10, figure 13). Use of 5 pulses increased TRZ delivery, compared to the control, by 9.9 times but use of 10 pulses increased the TRZ delivery only 10.8 times. The Use of 20 pulses gave a very sharp 38 times increase in TRZ delivery. Hence, with each applied pulse, the permeability of the skin sample keeps on increasing slightly. Upto 10 pulses, no burn marks indicative of physical damage were visible on the skin surfaces.

Table 10: Amount of TRZ delivered in skin by applying variable number of pulses of $U_{electrode}$ = 500v, τ = 40 ms using a small electrode.

Number of pulses	Amount of TRZ delivered Mean (µg) ± SE	n
0	4 ± 0.5	5
1	11.8 ± 1.8	5
5	39.3 ± 1.4	5
10	43 ± 3.6	5
20	151.7 ± 16.3	5

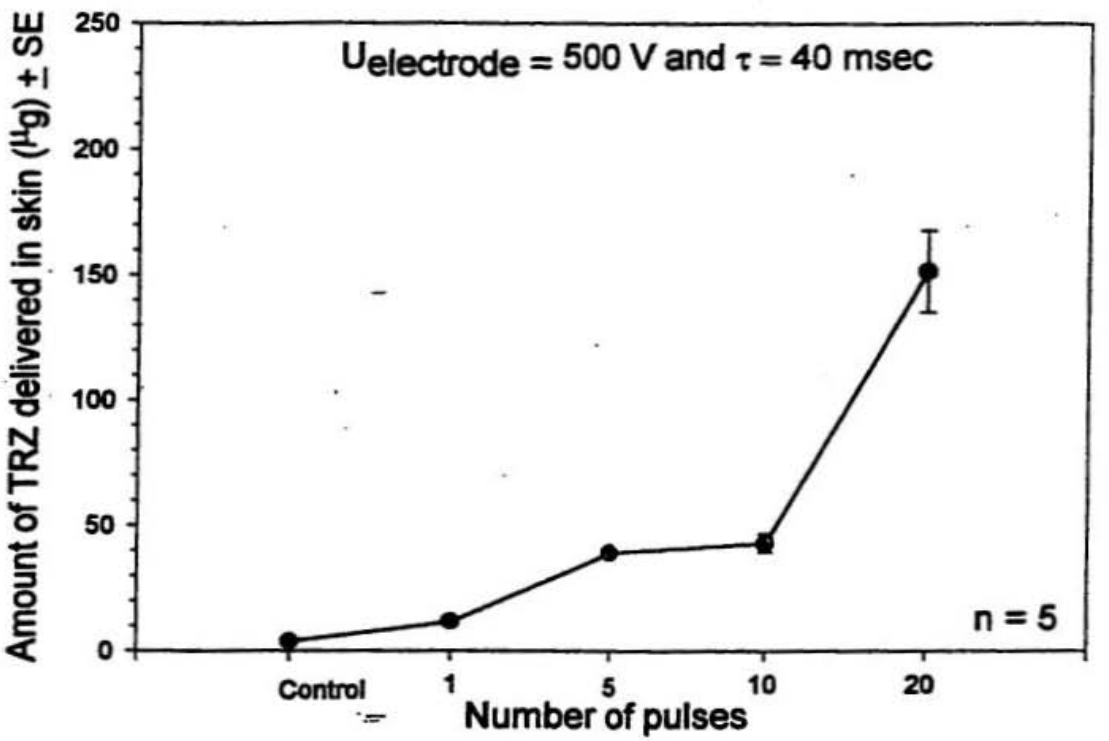


Figure 13: Effect of number of pulses on TRZ delivery

6.6 Electrode design

6.6.1 Effect of distance between delivery electrode and skin

The diffusion cell designed for the U_{skin} studies had three bores in both donor and receiver sides for placement of the delivery electrodes. Due to this arrangement the delivery electrodes could be placed at 5 mm, 10 mm, and 15 mm from the skin surface to study the influence of distance between electrode and skin on the values of U_{skin} which are shown in the figure 14 and table 11. Changing the distance between the skin and the delivery electrodes appeared to have a trend that suggested that skin-delivery electrode distance did affect the U_{skin} values. The U_{skin} value appeared to decrease as the delivery electrodes were moved away from the skin surface. However, no statistically significant differences were found between the values, with these small number of samples.

6.6.2 Effect of 'electrode area' on values of U_{skin} and drug delivery

The effect of electrode design with particular reference of area, has not been systematically studied by any of the research groups in electroporative drug delivery research. Therefore, experiments to study the effect of electrode area on drug delivery and safety issues were performed. To increase the electrode area, a long piece of Ag / AgCl wire was spiralled to obtain a 5-fold increase in the area (2.74 cm^2). The area of the small electrode which was basically a straight wire was 0.56 cm^2. The

Table 11: Effect of distance between delivery electrode and skin.

$U_{electrode}$ (V)	U_{skin} (V) Mean ± SE	Skin-delivery electrode distance (mm)	n
200	40 ± 8	15	3
200	42 ± 6.6	10	3
200	50 ± 11	5	3
300	55 ± 4	15	3
300	61.3 ± 4.3	10	3
300	67.3 ± 5	5	3
400	65 ± 6.1	15	3
400	70 ± 5	10	3
400	71.3 ± 5.2	5	3

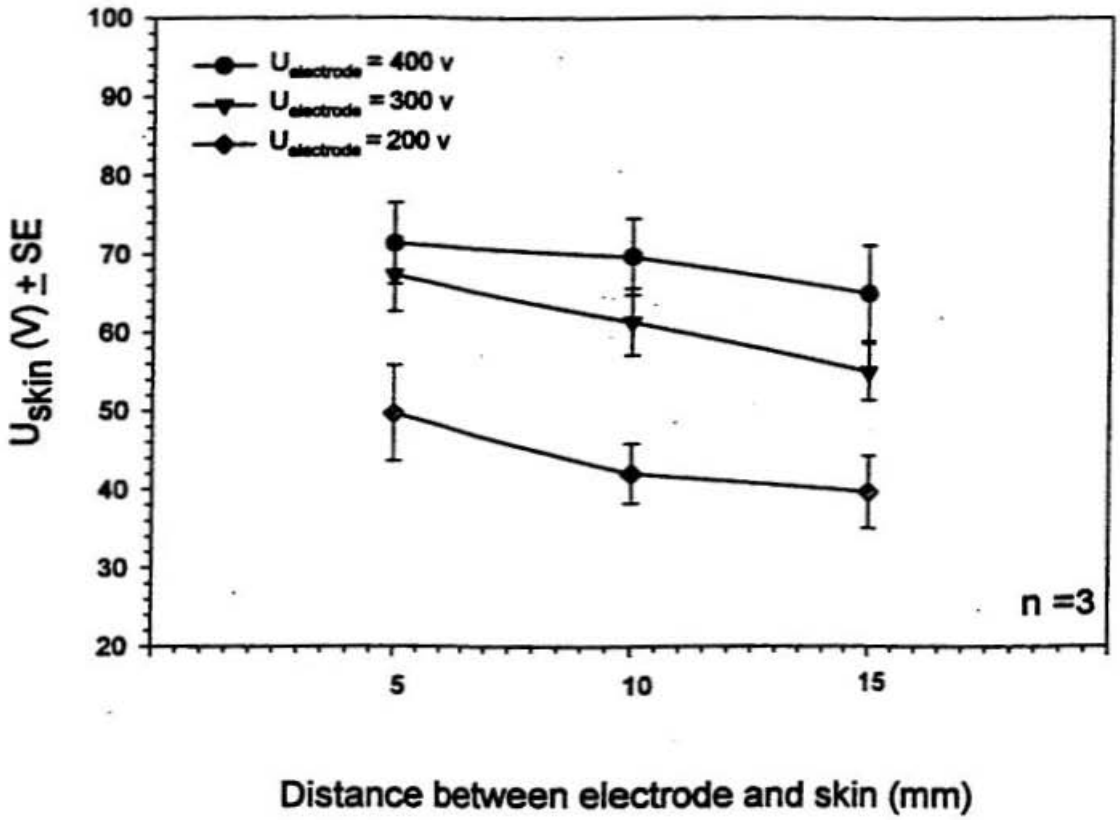


Figure 14: Effect of distance between delivery electrode and skin

area of the electrode was calculated by assuming the wire to be a cylindrical piece ($2\pi r l + \pi r^2$, where r is the radius, 0.025 cm; l is the length, 3.54 cm and 17.4 cm for small and large electrodes, respectively). The U_{skin} values determined with the large electrode gave a 30-40 % increase compared to the small electrode (table 12 and figure 15). This would mean that to obtain a certain U_{skin}, with the use of a large electrode, it would be possible to use a lower $U_{electrode}$. One obvious benefit would be the need for an electroporator with smaller capacitors. With further research in electrode design there would be a possibility of miniaturizing the electroporation unit to make it portable.

In order to determine if the increased area electrodes (that enable the use of lower $U_{electrode}$) also delivered the corresponding amount of TRZ, drug delivery experiments were performed using two conditions: 5 pulses of 10 ms and 20 ms. The $U_{electrode}$ of the small and large electrodes were chosen to obtain similar U_{skin} with the first pulse. The U_{skin} obtained with the small ($U_{electrode}$ = 500 V) and large electrodes ($U_{electrode}$ = 316 V) were the same (88.3 V). The results are shown in the table 13 and figure 16. There was no statistically significant difference in the amount of TRZ delivered with both small and large electrodes. Thus, the use of large electrodes would allow one to use smaller $U_{electrodes}$ for drug delivery. In our drug delivery studies, all of which were done using the small electrodes, pulsing at a high pulse length (higher then 20 ms at 500 V) mostly led to black marks on the skin surface indicating burns. The burn marks corresponded in shape and size to that of the electrode

Table 12: The influence of electrode area on the values of U_{skin} corresponding to the applied $U_{electrode}$.

$U_{electrode}$ (V)	U_{skin} (Small electrode) Mean ± SE (V)	U_{skin} (Large electrode) Mean ± SE (V)	Percentage Increment	n
100	32.4 ± 1.7	48 ± 1.4	48	5
200	43.6 ± 2.9	57 ± 2.4	31	5
300	65.75 ± 0.8	82 ± 1.6	25	5
400	83.6 ± 2.5	111.2 ± 2.1	33	5
500	88.3 ± 2.3	116 ± 2.2	31	5
600	254 ± 4	338 ± 5	33	5

Table 13: The effect of electrode area on drug delivery

Type of electrode (area)	$U_{electrode}$ (V)	Uskin (V)	τ (ms)	Number of pulses	Amount of TRZ delivered Mean (µg) ± SE	n
Small	500	88.3	10	5	6.5 ± 0.7	5
Large	316	88.3	10	5	6.7 ± 1.2	5
Small	500	88.3	20	5	14.7 ± 3.2	5
Large	316	88.3	20	5	13.3 ± 1.6	5

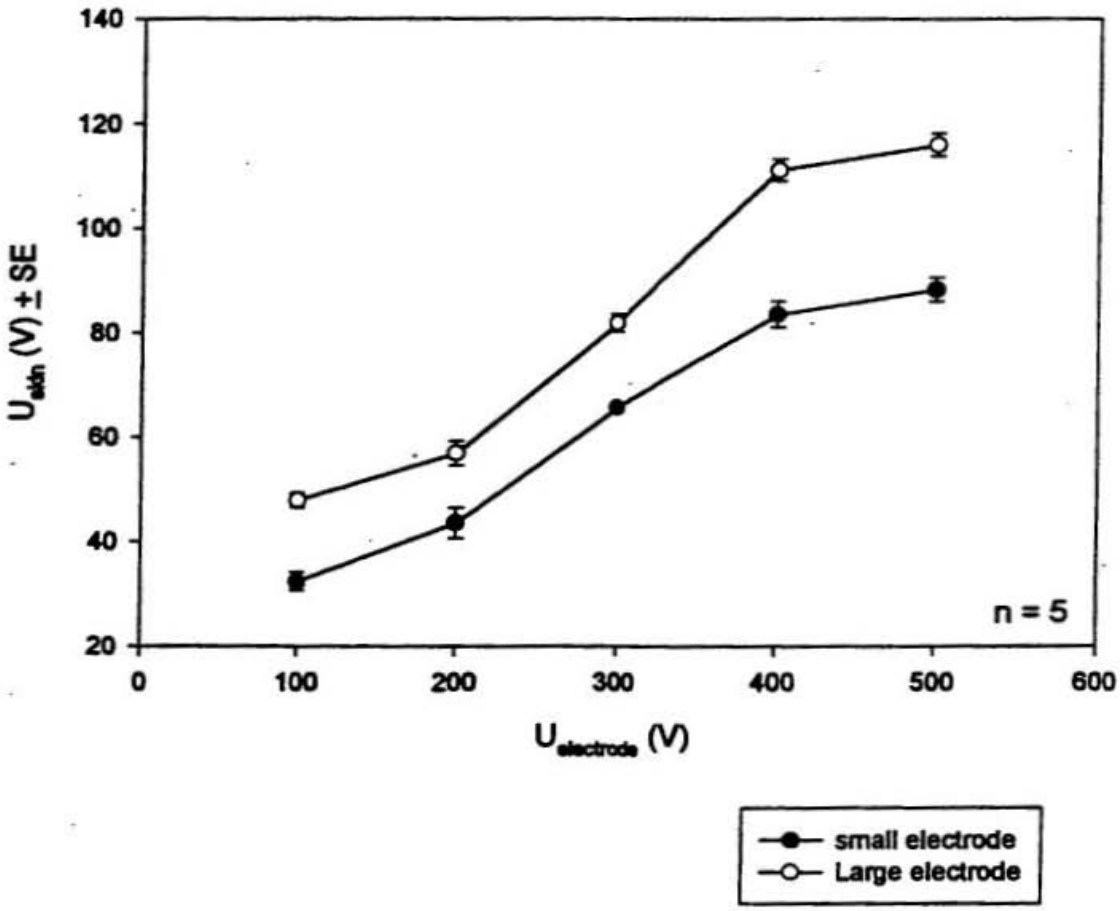


Figure 15: Influence of electrode area on U_{skin}.

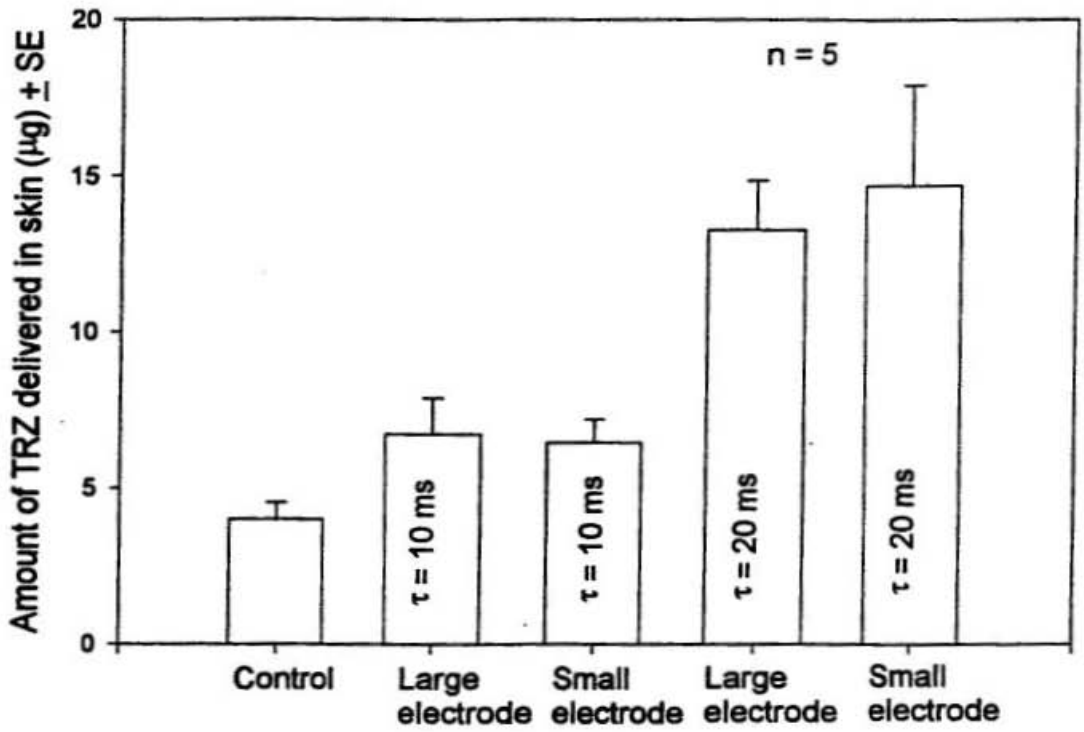


Figure 16: The effect of electrode area on drug delivery

being used for electroporation. However, the large electrodes used in our studies did not produce the burn marks unlike the small electrodes. It seemed that our large electrode design could have a similar effect in current distribution as has been mentioned by Wiley and Webster (1982) in their work on the effect of electrode design on overall and local current density as applied to electrosurgery. According to these authors there exists a significant difference between overall and local current densities. Overall current density is based on the area of the electrical contact with the skin. Local current density under an electrode is highly variable, with greater current densities along its edges. It was seen earlier that patients undergoing electrosurgery suffered from burns around the perimeter of the dispersive electrodes. Circular dispersive electrodes which produced more uniform current distributions by forcing a more effective utilization of the entire area were proposed. Applied to electroporative transdermal delivery, we saw that the use of a large electrode not only delivered the same amount of drug at a lower value of $U_{electrode}$ as compared to a small electrode, but with the additional advantage of reduced incidence of skin burns.

6.6.3 Effect of electrode area on the current passing through skin and overall current density

The effect of the area of the electrode used for pulsing on the amount of current passing through the skin was checked. An experiment was designed which was

similar to the one done earlier (section 6.2.1). It was found that the currents passing through the skin at various values of $U_{electrode}$ were about 3 to 4 times greater for the large electrode as the current passing for the small electrode (Table 14 and figure 17). The area ratio was about 5 so one might expect this ratio of currents.

The plots of U_{skin} *vs* Joules per pulse, with respect to skin area and electrode area (small and large) are shown in (table 15) figures 18 and 19, respectively. Due to technical limitations we could determine the currents only at lower pulse lengths, ranging between 2 and 5 ms. However, we believe that the basic information obtained relating energy and area of the skin and the electrode, is still meaningful and useful in explaining the results obtained by us. During each current measurement the exact pulse length was determined and used in arriving at the energy values reported in figure 18 and 19. The changes in the skin's stratum corneum, caused by electroporation pulses, can be expected to depend on the area of the skin exposed to the current and this appeared to be the case. The slopes of the plots obtained using skin area to calculate the energy per unit area per pulse, shown in figure 18, are similar. In other words, the energy delivered to the skin appears to be similar, irrespective of the area of the electrode, and seems to primarily depend on the voltage, U_{skin} and pulse length. If this were true, a burn scar would have been observed for the 60 ms electroporation pulses, not only for delivery using the

Table 14: Effect of electrode area on the values of current passing through skin

$U_{electrode}$ (V)	Current passing through skin (amperes)		n
	Small electrode (area = 0.56 cm^2)	*Large electrode* (area = 2.74 cm^2)	
100	0.42 ± 0.04	1.21 ± 0.27	3
200	1.03 ± 0.01	3.35 ± 0.47	3
300	1.82 ± 0.06	5.82 ± 0.79	3
400	2.51 ± 0.09	9.33 ± 0.79	3
500	3.08 ± 0.12	12.79 ± 0.84	3

Table 15: Energy delivered to the skin

$U_{electrode}$ (V)	Energy delivered to the skin (Joules.$pulse^{-1}cm^{-2}$)				n
	(energy/electrode area)		(energy/skin area)		
	Small electrode (Joules.$pulse^{-1}$ 0.56 cm^{-2})	*Large electrode* (Joules.$pulse^{-1}$ 2.74 cm^{-2})	*Small electrode* (Joules.$pulse^{-1}$ 1.3 cm^{-2})	*Large electrode* (Joules.$pulse^{-1}$ 1.3 cm^{-2})	
100	0.17 ± 0.02	0.08 ± 0.02	0.07 ± 0.01	0.16 ± 0.04	3
200	0.41 ± 0.0	0.17 ± 0.02	0.18 ± 0.0	0.36 ± 0.05	3
300	1.09 ± 0.04	0.41 ± 0.06	0.47 ± 0.02	0.86 ± 0.12	3
400	1.91 ± 0.07	0.81 ± 0.07	0.82 ± 0.03	1.71 ± 0.14	3
500	2.37 ± 0.09	0.95 ± 0.06	1.02 ± 0.04	2.01 ± 0.13	3

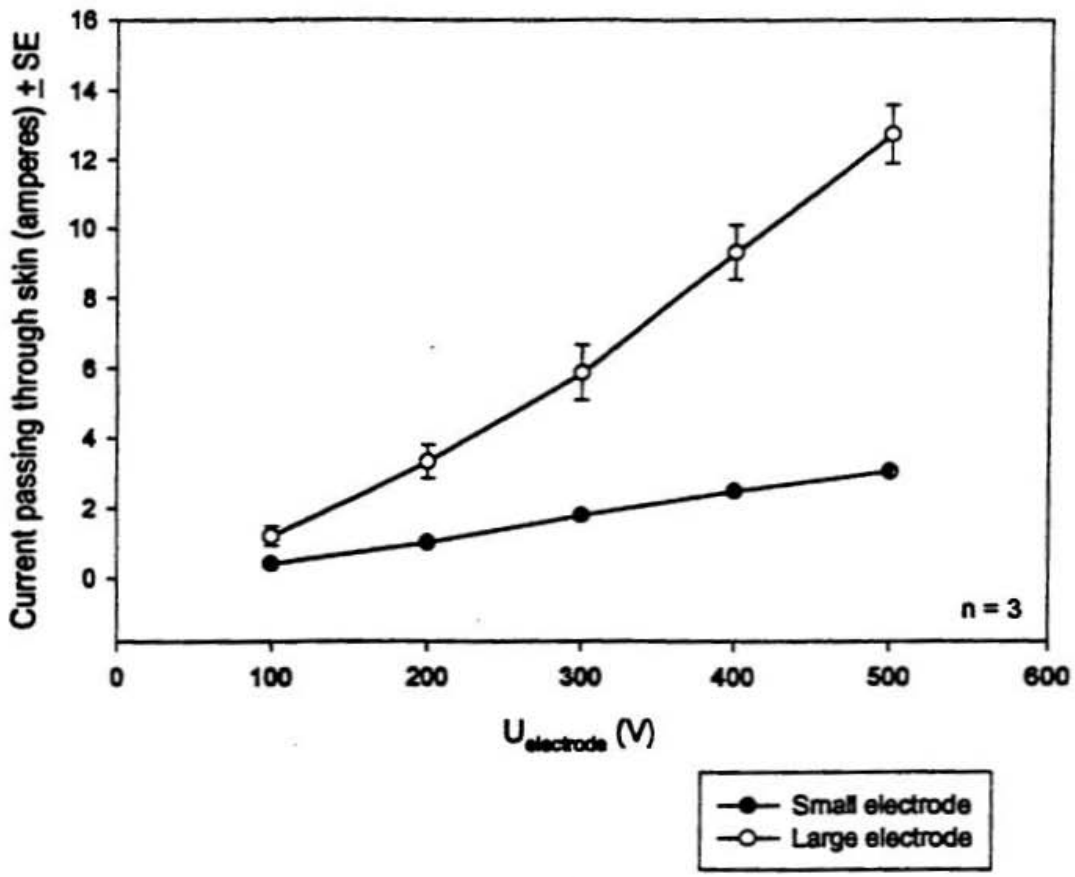


Figure 17: Electrode area vs current passing through skin

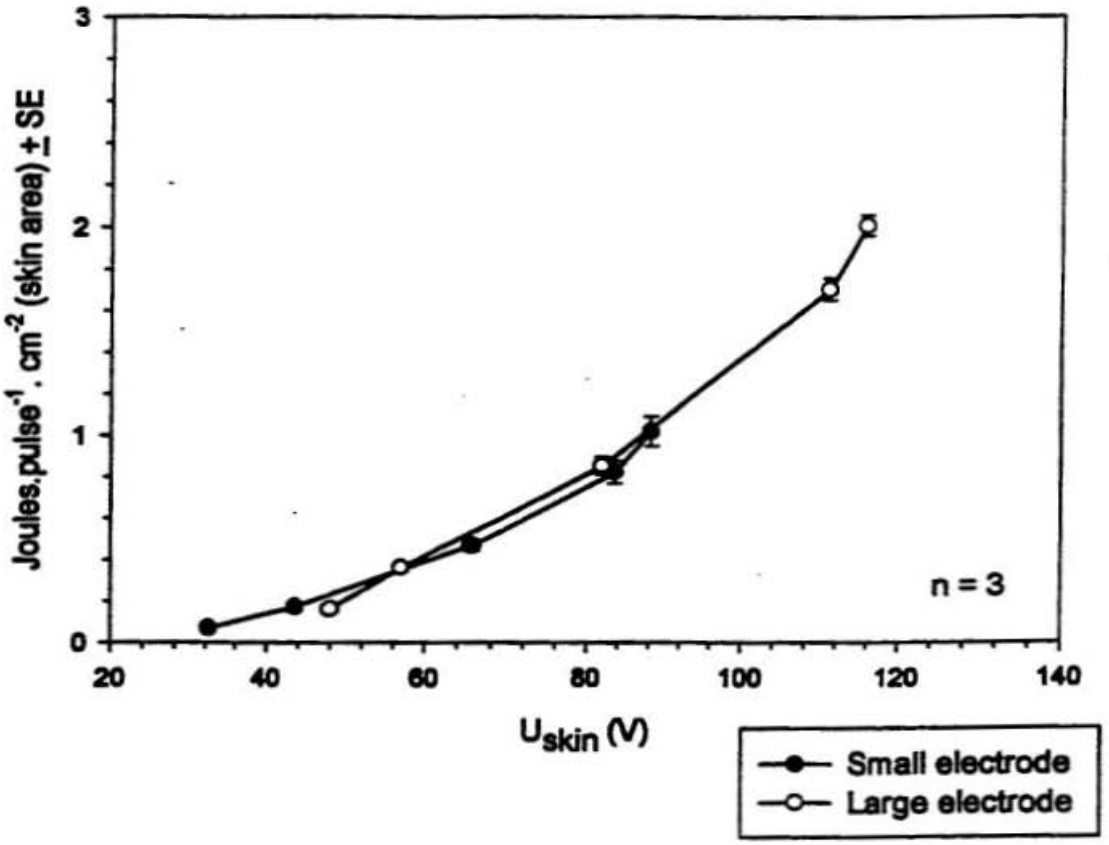


Figure 18: U_{skin} vs Joules per pulse (Skin area)

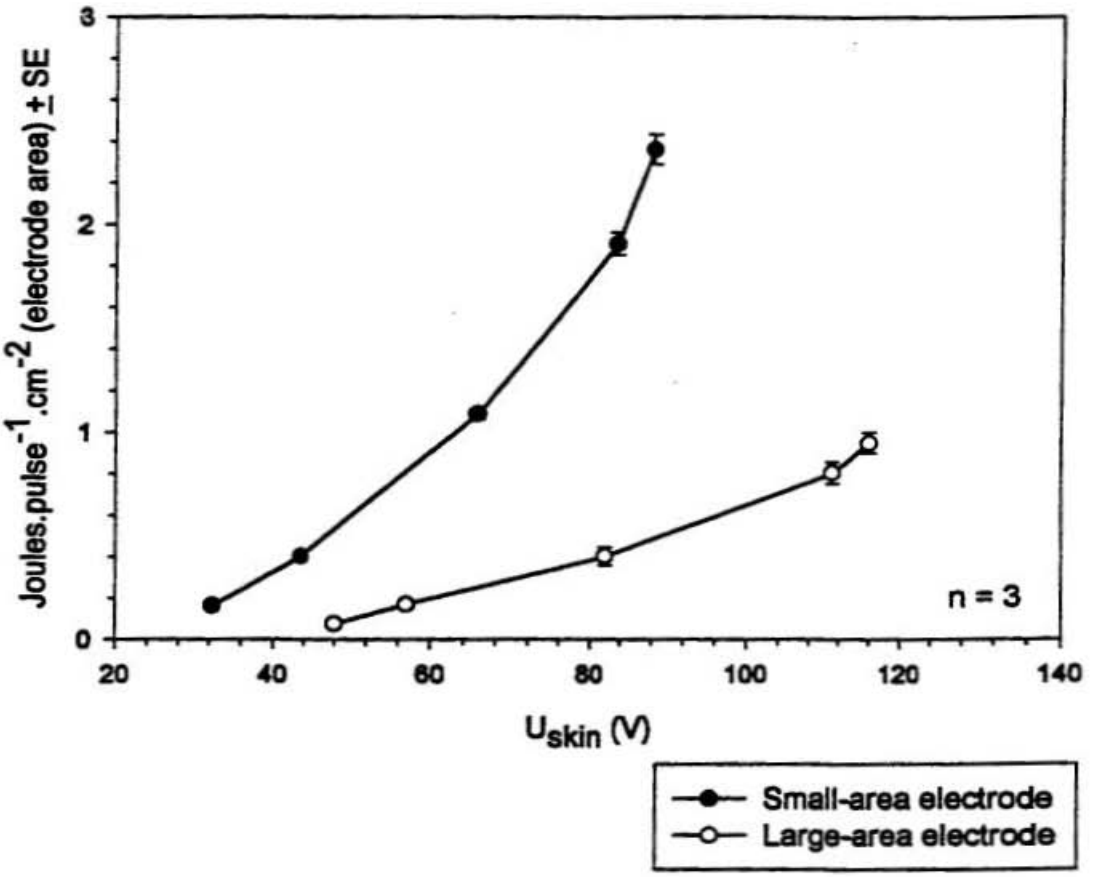


Figure 19: U_{skin} vs joules per pulse (Electrode area)

small-area electrodes but also for the large electrodes (see section 6.7.2). When energy per unit area per pulse was calculated with respect to electrode area and plotted against U_{skin}, very different profiles were obtained (figure 19). The large electrode delivered 2 - 2.7 times less energy to the skin when compared to the small-area electrode.

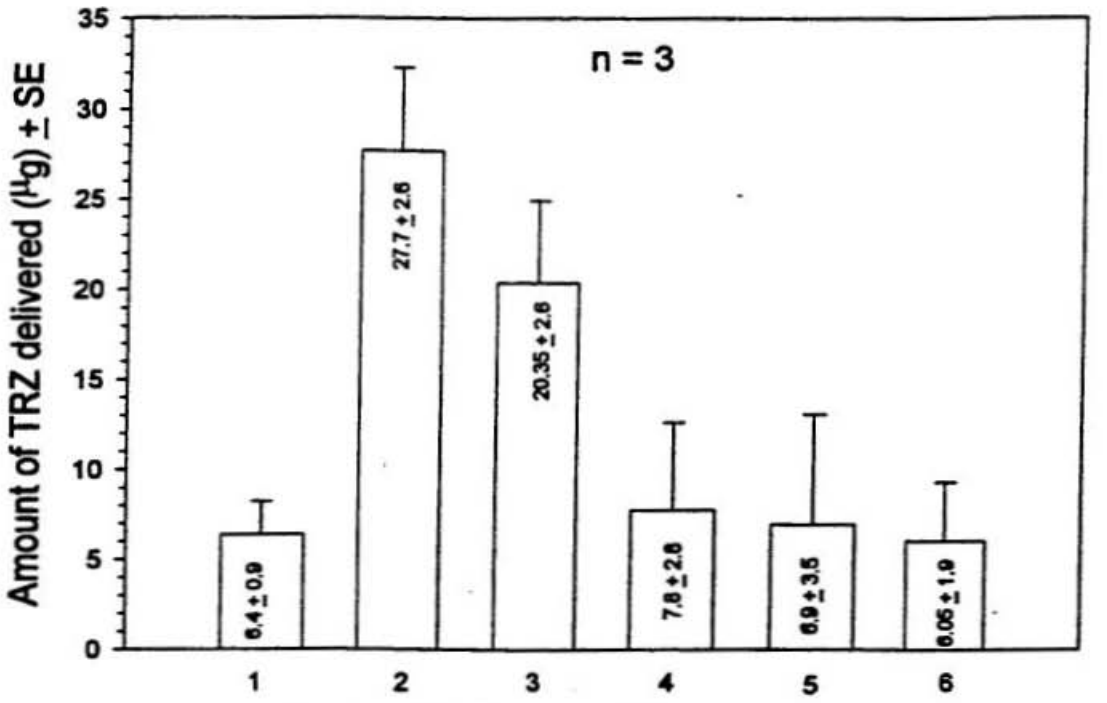
6.7 Safety of electroporation

The safety of transdermal electroporation is an important issue. Just as any other permeation-enhancement technique electroporation would be useful only when the optimized condition of use is both efficient for drug delivery, as well as safe to the skin. Although the barrier property of the skin would be broken down to achieve permeation-enhancement with electroporation, the process should be reversible and there should be no permanent and/or long term damage caused to the skin. A complete study of safety issues will involve *in-vitro* as well as *in-vivo* work. In this project *in-vitro* experiments were done to get initial information about the safety of this technique. The two *in-vitro* studies carried out were 'reversal of permeability' and 'histological examination of skin', which are described below.

6.7.1 Reversal of permeability enhancement

This experiment was specifically designed to gain an insight on how electroporative permeation-enhancement takes place in the skin and how long do the effects lasts. In other words, this study would furnish information on the safety of the

technique and provide some tips on the mechanism of the electroporative enhancement. The results are presented in the form of a histogram in figure 20. The histogram bars labeled 2 and 3, standout with TRZ quantities 27.7 µg and 20.35 µg, respectively. They represent electroporation pulse delivery with the TRZ solution in the donor chamber. In histogram 2, the TRZ solution had been left in contact with the skin during and after the pulse, while in the histogram 3, the TRZ solution was replaced by PBS immediately after delivery of the pulse. Although with the small number of samples (n = 3), there was no statistical difference ($p>0.05$) between the two deliveries (histogram 2 and 3), the mean TRZ delivered in the case where the solution was left in contact with the skin during and after the pulse, was 25% larger. The other histogram bars viz, 4, 5 and 6 (which were respectively, for TRZ added immediately after the pulse, five minutes after the pulse and one hour after the pulse) were much smaller and statistically not different ($p>0.05$) from the controls. This clearly demonstrated that the permeation-enhancement in the skin occurred primarily during the electroporation pulses. It is speculated that the permeation-enhancement is due to creation of aqueous pores in the stratum corneum (Pliquett et al. 1996a). Since adding the TRZ solution immediately after the pulse did not show any significant increase in delivery compared to the control, it appears that the pores created close very quickly. This is also in agreement with the reports of Pliquett et al. It should be noted that in this experiment the electroporation conditions (20 pulses, τ = 10 ms, $U_{electrode}$ = 400V) was carefully chosen to ensure that the permeation-enhancement was achieved with a minimal electric force. This was done to ensure that the



1 = Control - Passive diffusion, no pulsing

2 = Electroporation pulse (10 pulses at $U_{electrode} = 400$ V and $\tau = 20$ msec) applied with TRZ as donor solution. This corresponds to the normal electroporation drug delivery described in most of the preceeding sections.

3 = Same as in 1 but donor TRZ solution was replaced with PBS soon after delivery of the electroporation pulses.

4 = Electroporation pulses delivered with PBS as the donor solution. PBS was replaced with TRZ soon after delivery of the pulses.

5 = Same as in 3 except that TRZ was added 5 minutes after delivery of the pulses.

6 = Same as in 3 except that TRZ was added 1 hour after delivery of the pulses.

Figure 20: Studies on the reversibility of the skin's permeability enhancement after pulsing

reversibility of permeation was influenced primarily by time and there was little effect due to excessive electrical force that could cause skin damage. It was speculated that using a higher $U_{electrode}$ (e.g., 600 V or larger) and/or pulse length (e.g., 30 ms or larger) would make the skin behave differently. Depending on the severity of the electroporation conditions the permeation-enhancement could be much more prolonged but it may not revert to the base level at all. This information could hence be used as an indicator of safety for the electroporation technique.

6.7.2 Macroscopic and microscopic changes in skin due to electroporation pulse

Attempts were made to correlate the inferences made until now regarding the upper limits of $U_{electrode}$ and pulse length, with any macroscopic and/or microscopic changes in the skin. Since pulses with $U_{electrode}$ of 600 V produced a very steep increase in U_{skin} (figure 5) causing visually detectable damage to the skin, there didn't seem any need to prove it further. Twenty pulses with $U_{electrode}$ of 500 V having different pulse lengths were chosen for studying the histological changes in the skin. In each case the pH in the donor and receiver compartments before and after electroporation were also determined, which are given in table 16. With the control sample the pH of the donor and receiver compartments remained unchanged at pH 6.4. With the 20 ms pulse length experiment, the donor pH remained unchanged while that in receiver went up from 6.4 to 8.0. As the pulse length was increased to

Table 16: Influencing of electroporative pulsing on the pH in the receiver and donor chambers

Treatment				n	Initial donor pH	Final donor pH	Initial receiver pH	Final receiver pH
Voltage (V)	τ (ms)	# of pulses	Electrode area					
0	0	0	small	2	6.4	6.4	6.4	6.4
500	20	20	small	2	6.4	6.4	6.4	8
500	30	20	small	2	6.4	6.4	6.4	11
500	40	20	small	3	6.4	6.4	6.4	12
500	60	20	small	5	6.4	6.4	6.4	12
500	60	20	large	3	6.4	6.4	6.4	12

30 ms the pH in the receiver increased to 11. At pulse lengths above 30 ms the pH of the receiver rose to 12. Such a large increase in the pH can result in irritation/chemical damage to the skin or may even result in the degradation of the sensitive drugs. The change in pH was accompanied by a significant amount of foaming in the receiver chamber (cathode), which was absent in the donor chamber (anode). When a high current flows at the reactive electrode, Ag/AgCl in the presence of Cl^- ions, the Ag would be converted to AgCl and no foaming should be expected. However, at the cathode, the AgCl would first be reduced to metallic Ag. Upon completion of this process (or even in parallel with it) the extra current would generate hydrogen gas from water. This may also be represented using the following equations:

Donor (anode) side

$Ag\ (s) + Cl^-(\text{from PBS}) \longrightarrow AgCl\ (s) + e^-$

Receiver (cathode) side

$2H_2O\ (l) + 2e^- \longrightarrow H_2\ (g) + 2OH^-$

In the receiver chamber the hydrogen gas formed caused the foam and the hydroxyl ions formed raised the pH. Increase in the pH to 11 or 12 with pulse lengths 30 ms and above is indicative of generation of large currents that could cause damage to the skin. The reactive Ag/AgCl electrodes are normally not expected to cause the

electrolysis of water, which causes such drastic pH changes (Phipps et al. 1989, and Burnette et al. 1987). The high currents used here, even transitorily, are larger than those encountered in usual electrochemical experience and hence, the above scenario is quite probable.

In order to determine the effect of alkaline pH, the skin was exposed to dilute sodium hydroxide (pH 12) for 20 minutes. Subsequent examination of the skin did not show any damage similar to that seen with the passage of electroporation pulses of 30 and 40 ms. This indicated that the visible skin damage mentioned earlier probably did not arise solely from contact with alkaline solution.

Two sets of skin samples were chosen for observing changes due to electroporation pulses at different settings. The first set was photographed to observe any gross changes visible to naked eye. The other set of skin samples was fixed in glutaraldehyde fixative and their cross sections were prepared for observation under a phase contrast microscope. The simple photograph and the light microscopic photographs of the control skin are shown in Figures 21 and 27 respectively. Please note that the photographs show only the area of the skin which was exposed to the drug solution. In all the test conditions the $U_{electrode}$ was kept constant at 500 V and the number of pulses were kept at 20. The variables tested were pulse length and area of the electrode.

Visual examination of the skin samples indicated that there was no obvious damage at the stratum corneum or dermal side with use of 20 ms and 30 ms pulses (Figures 22 and 23). As the pulse length was increased to 40 ms, red patches were seen on the dermal side and a few dark patches were seen on the stratum corneum side (Figure 24). With the use of 60 ms pulse a dark mark was seen on the stratum corneum side (Figure 25) which looked like a burn mark. The dermal side of the skin also bore red and black patches. In all the above tests small electrodes were used. However, it was very interesting to find that when the skin was subjected to electroporation pulses of 60 ms, using the large electrode (with the loop design and U_{skin} the same as for small electrode), the visually apparent damage was much lesser (Figure 26) compared to the skin subjected to 60 ms pulses with the small electrode. When two line-electrodes (small electrodes) placed parallel to one another in an electrolyte solution are involved, current flows principally between the two wires and widens out like magnetic iron filings pattern between two point magnets (Kasper 1940). In our case, because the skin placed between the electrodes was not electrically conducting, the current flow was not uniform over the skin surface when the small-area electrodes were used, and a burn occurred at 60 ms pulse length. The burn mark corresponded to the general shape (straight piece of wire) and orientation of the electrode, which was probably the main path followed by the current. With the large electrode the current seemed to have been delivered more uniformly, thereby preventing a burn.

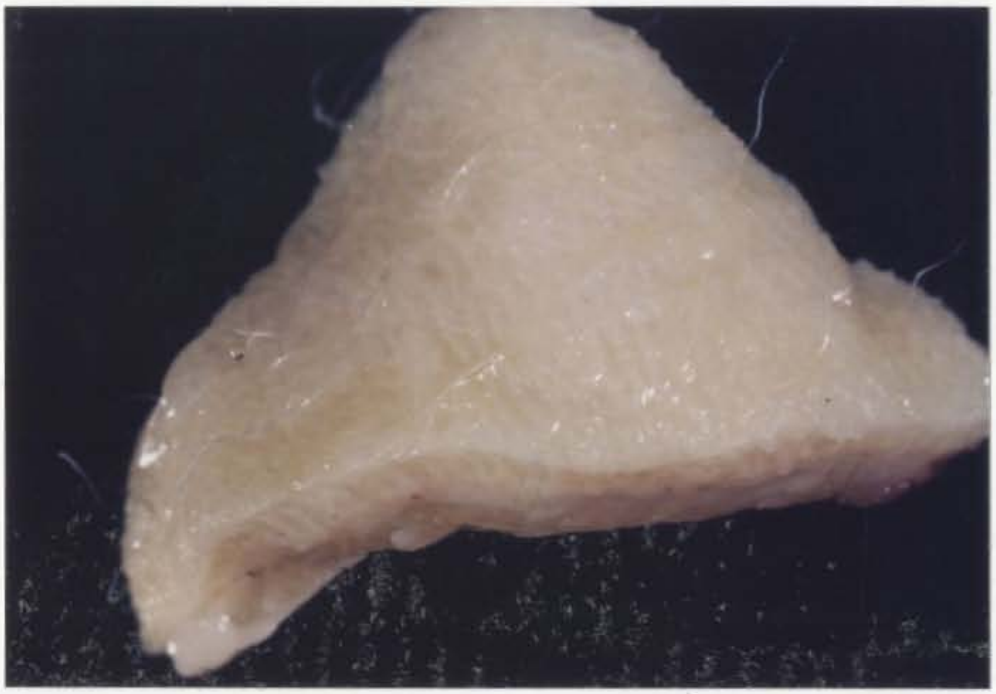


Figure 21a: Stratum corneum side of a control skin sample

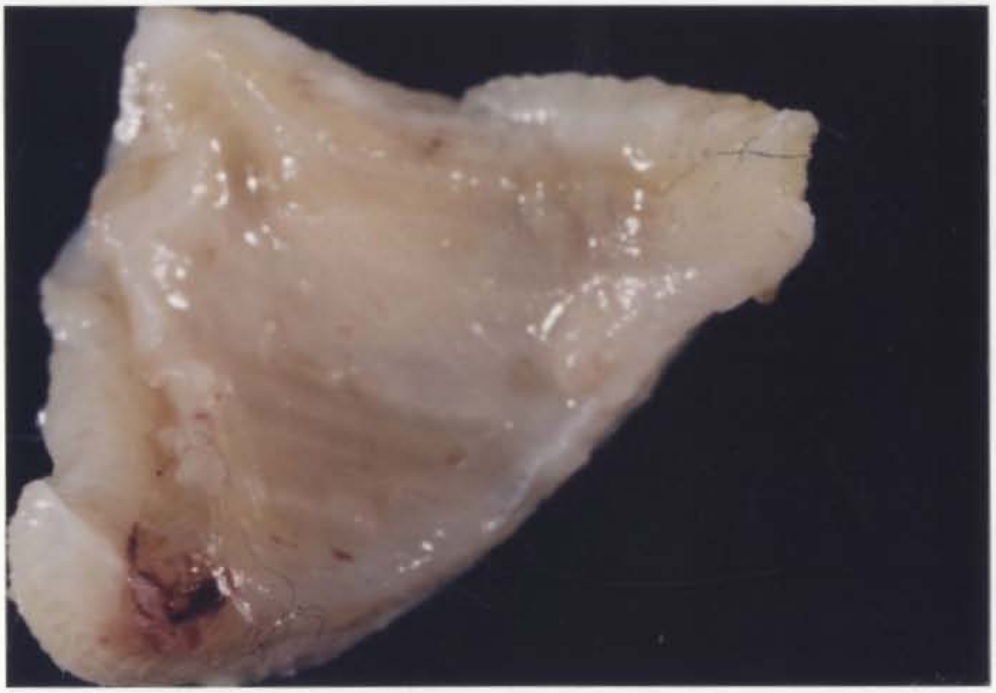


Figure 21 b: Dermal side of a control skin sample.

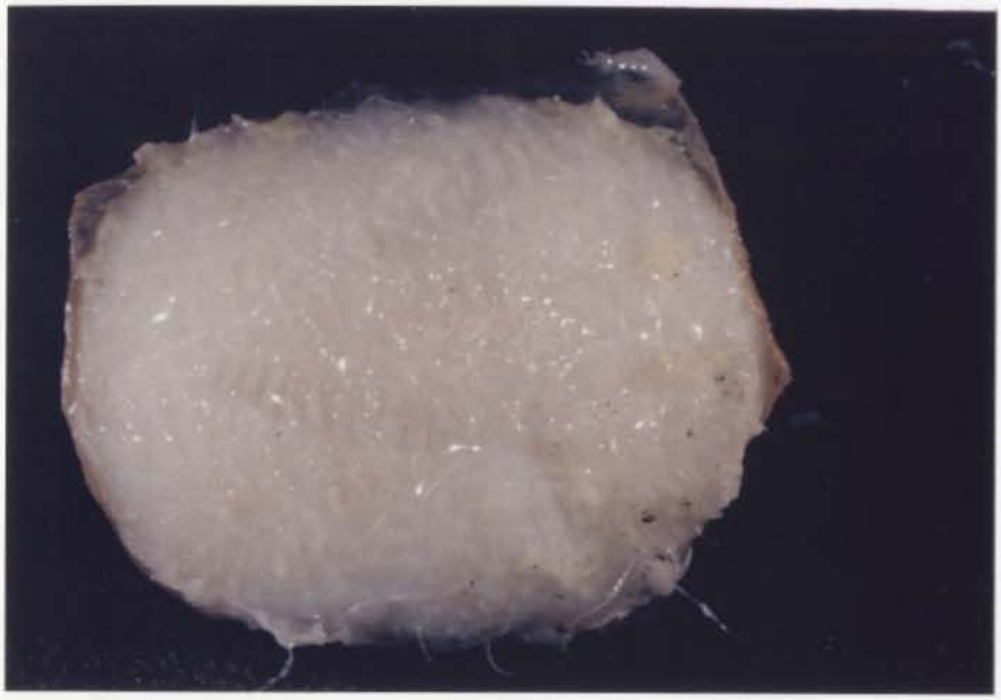


Figure 22a: Stratum corneum side of a skin sample subjected to 20 pulses of 500 V and 20 ms each using a small electrode.

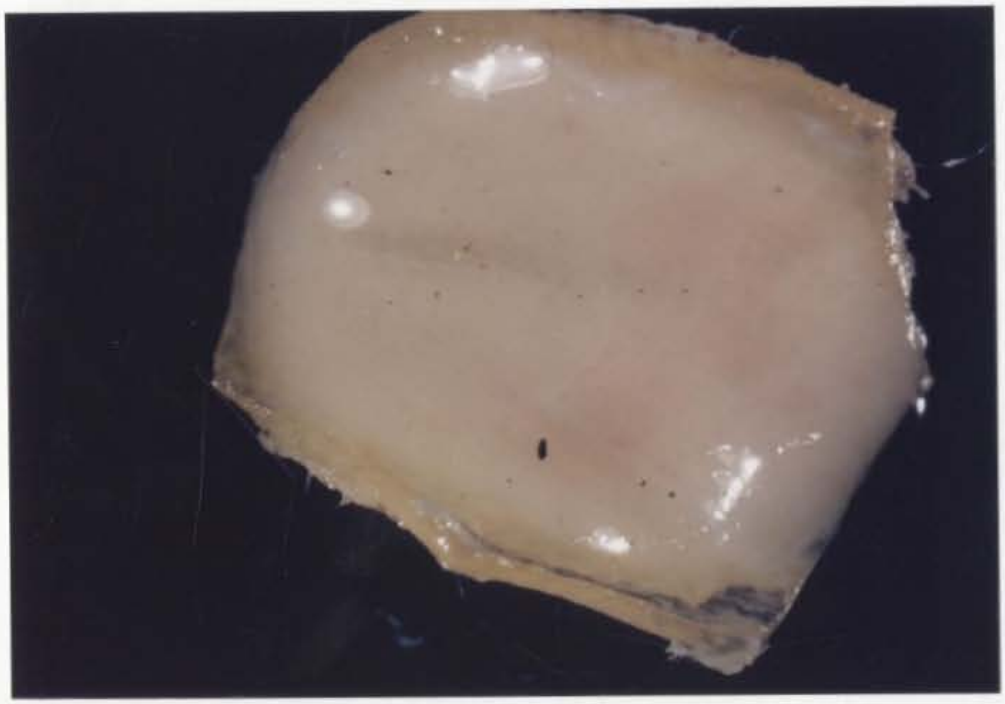


Figure 22b: Dermal side of a skin sample subjected to 20 pulses of 500 V and 20 ms each using a small electrode.

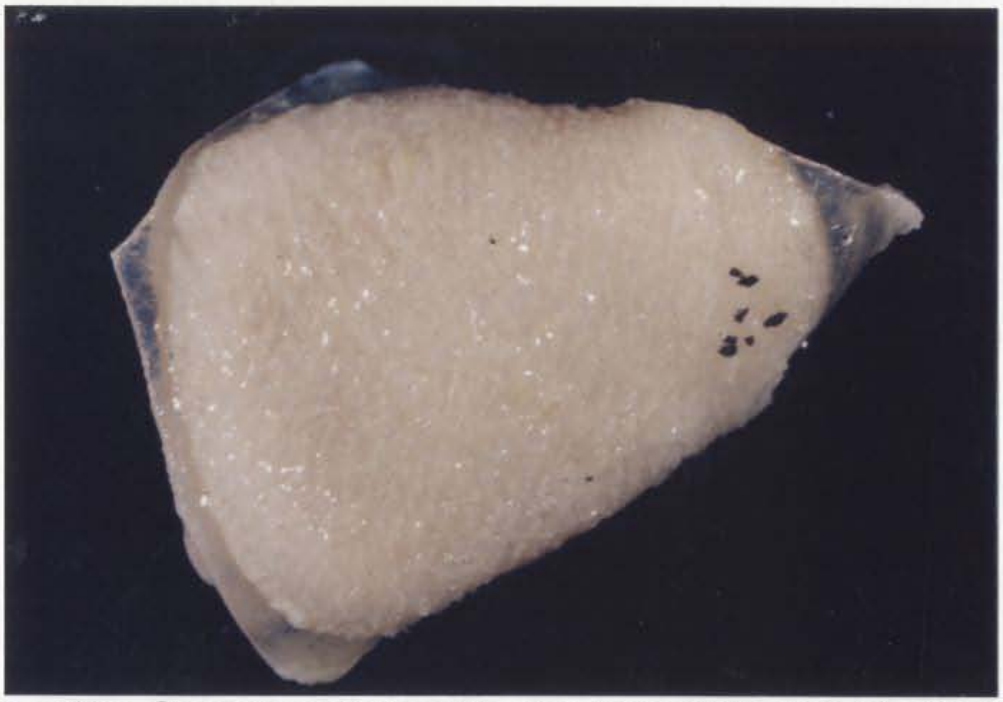


Figure 23a: Stratum corneum side of a skin sample subjected to 20 pulses of 500 V and 30 ms each using a small electrode.

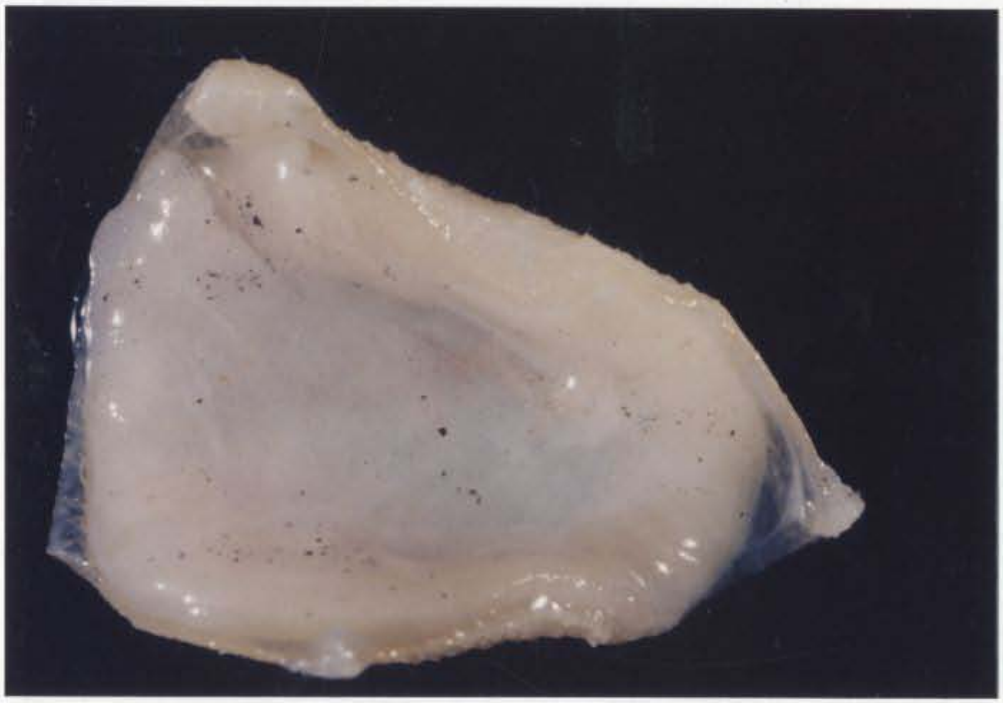


Figure 23b: Dermal side of a skin sample subjected to 20 pulses of 500 V and 30 ms each using a small electrode.

Figure 24a: Stratum corneum side of a skin sample subjected to 20 pulses of 500 V and 40 ms each using a small electrode.

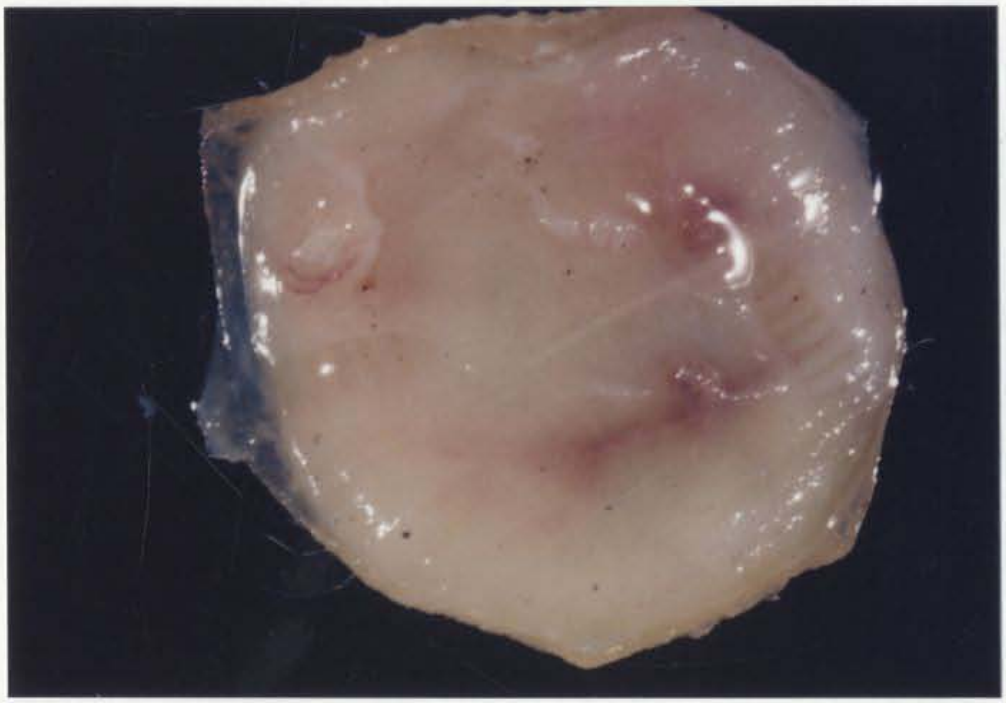


Figure 24b: Dermal side of a skin sample subjected to 20 pulses of 500 V and 40 ms each using a small electrode.

Figure 25a: Stratum corneum side of a skin sample subjected to 20 pulses of 500 V and 60 ms each using a small electrode.

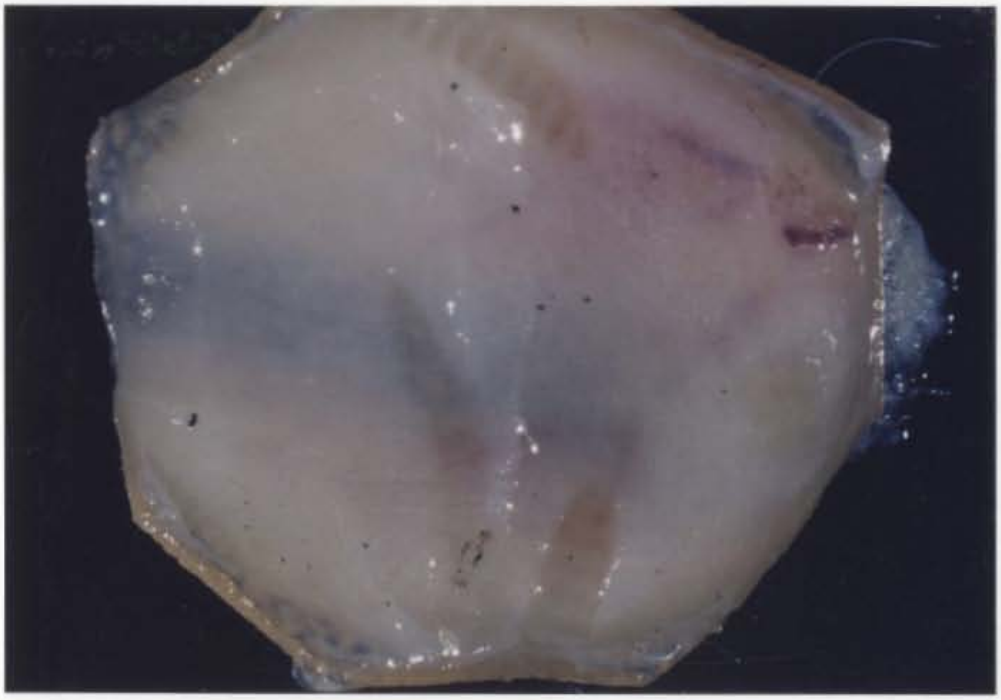


Figure 25b: Dermal side of a skin sample subjected to 20 pulses of 500 V and 60 ms each using a small electrode.

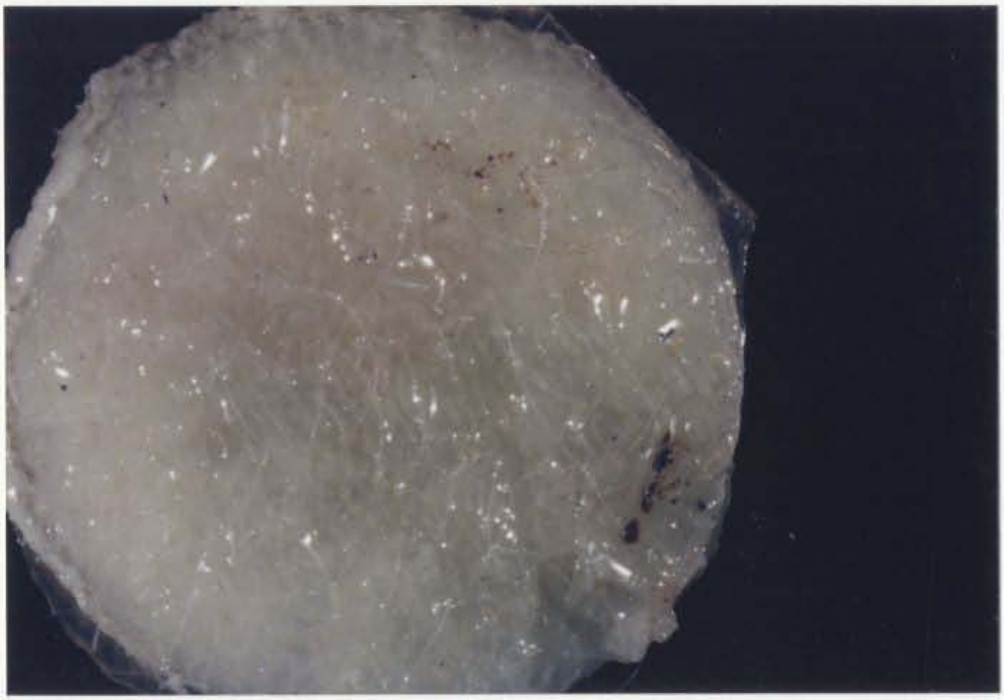


Figure 26a: Stratum corneum side of a skin sample subjected to 20 pulses of 500 V and 60 ms each using a large electrode.

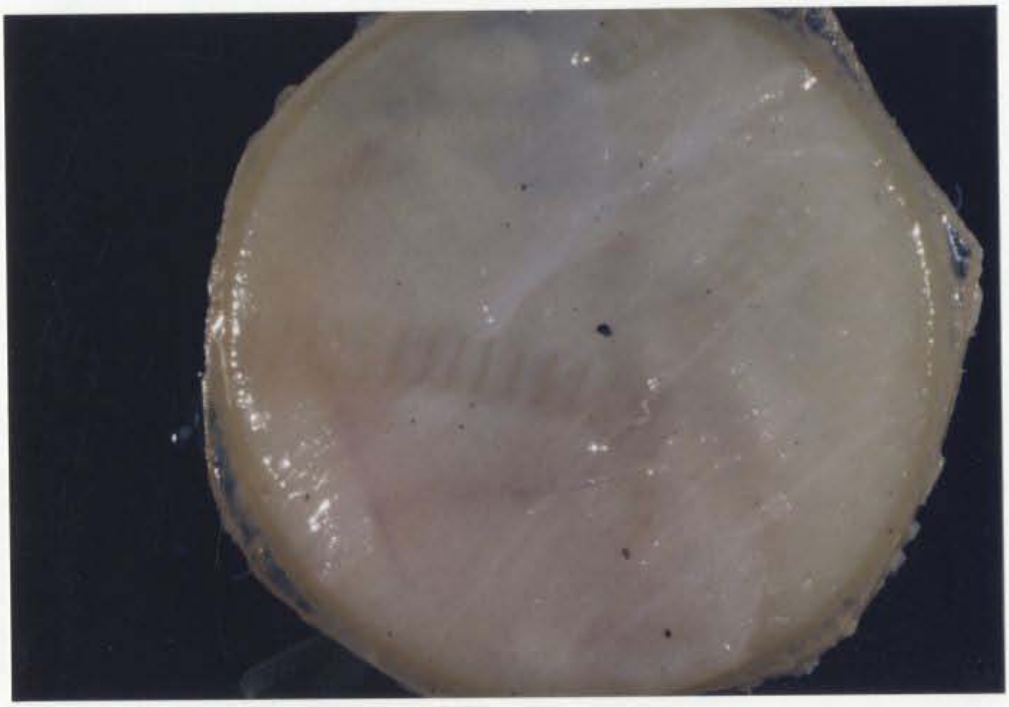


Figure 26b: Dermal side of a skin sample subjected to 20 pulses of 500 V and 60 ms each using a large electrode.

Examination of the cross sectional light microscope photographs corroborated the above findings. In the control skin sample the different layers of skin were clearly demarcated (Figure 27). For the skin samples subjected to electroporative pulsing, as the pulse lengths were increased from 20 to 60 ms, there was progressively increased damage to the skin. At one end of the spectrum with the pulse length of 20 ms, the different layers of epidermis and dermis were clearly visible, whereas at the other extreme with the pulse length of 60 ms, the different layers of dermis had gelled together (Figure 28 to 31). As the pulse length was increased gradually from 20 to 60 ms, the damage to the skin also increased progressively. The skin sample subjected to electroporation pulse with the increased area electrode did show some damage (Figure 32) but it was much lesser than that caused with the small electrode with same electroporation conditions.

Based on the above findings it may be reasonable to conclude that using 20 pulses with an $U_{electrode}$ of 500 V, and pulse length of 20 ms is relatively safe. The damage caused to the skin seems to be minimal and the skin reverts back from the enhanced permeation mode to the base level within one minute. Use of a loop electrode with larger area and hence, smaller $U_{electrode}$, also seems to be a more efficient way to use the technique.

Figure 27: Microscopic cross-section of a control skin sample.

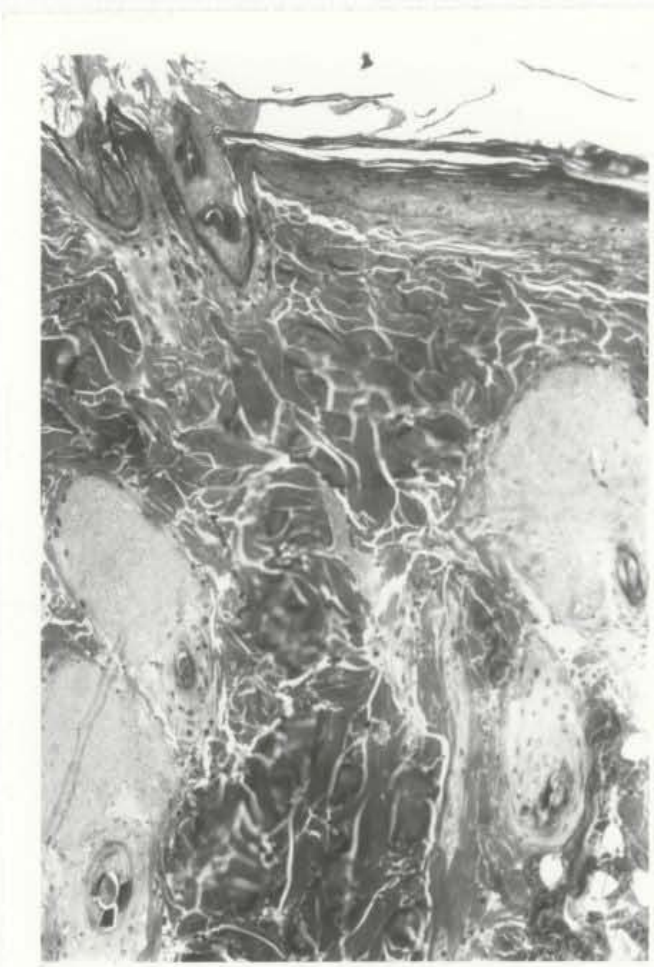


Figure 28: Microscopic cross-section of a skin sample subjected to 20 pulses of 500 V and 20 ms using a small electrode.

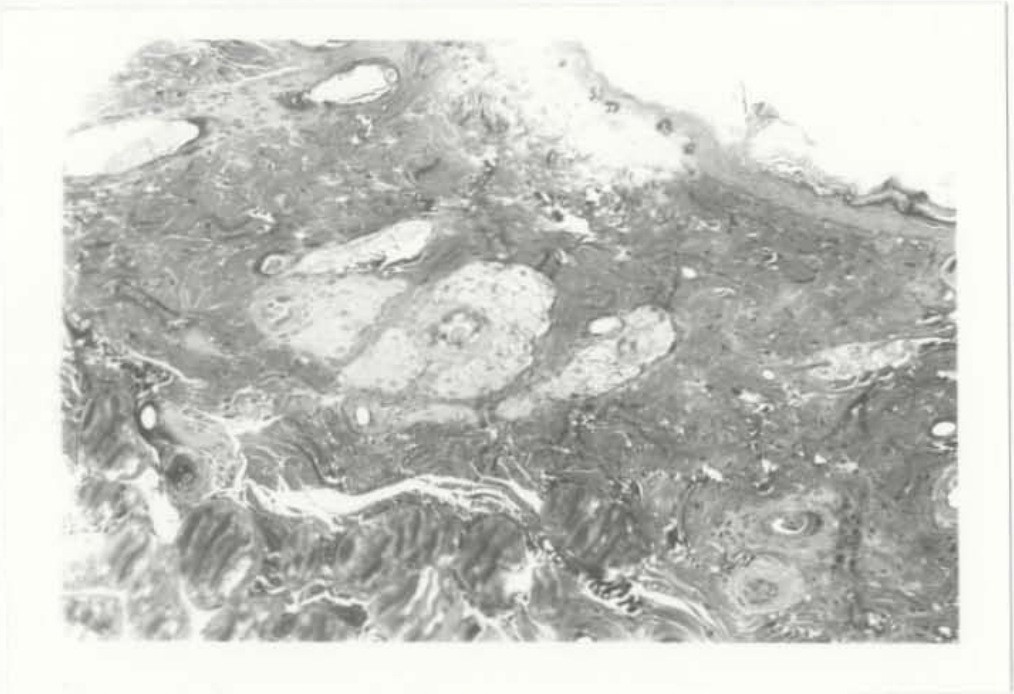


Figure 29: Microscopic cross-section of a skin sample subjected to 20 pulses of 500 V and 30 ms using a small electrode.

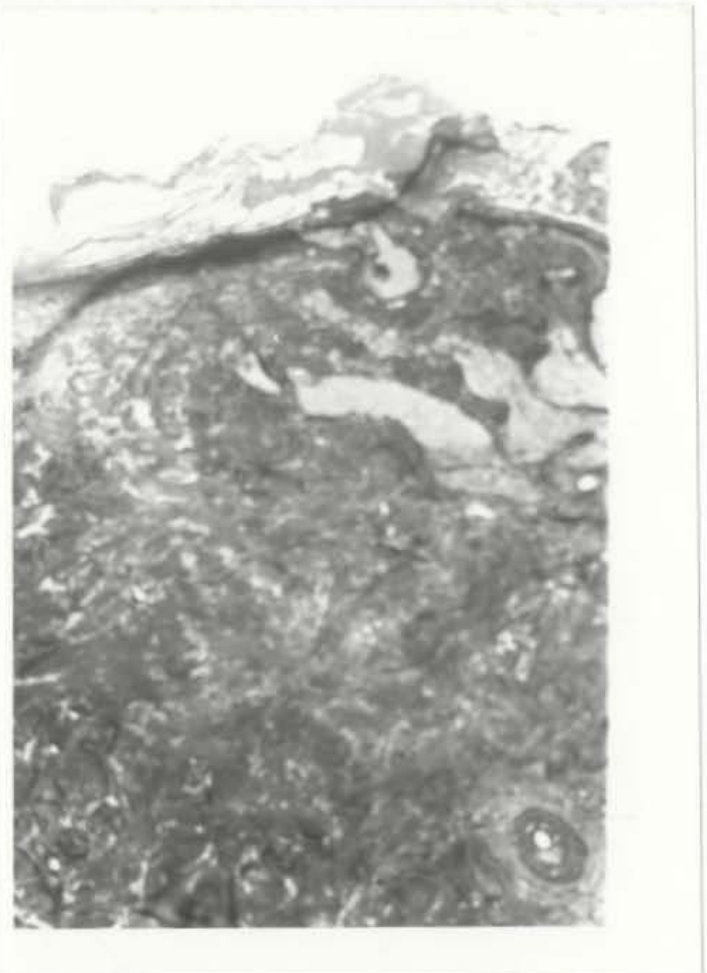


Figure 30: Microscopic cross-section of a skin sample subjected to 20 pulses of 500 V and 40 ms using a small electrode.

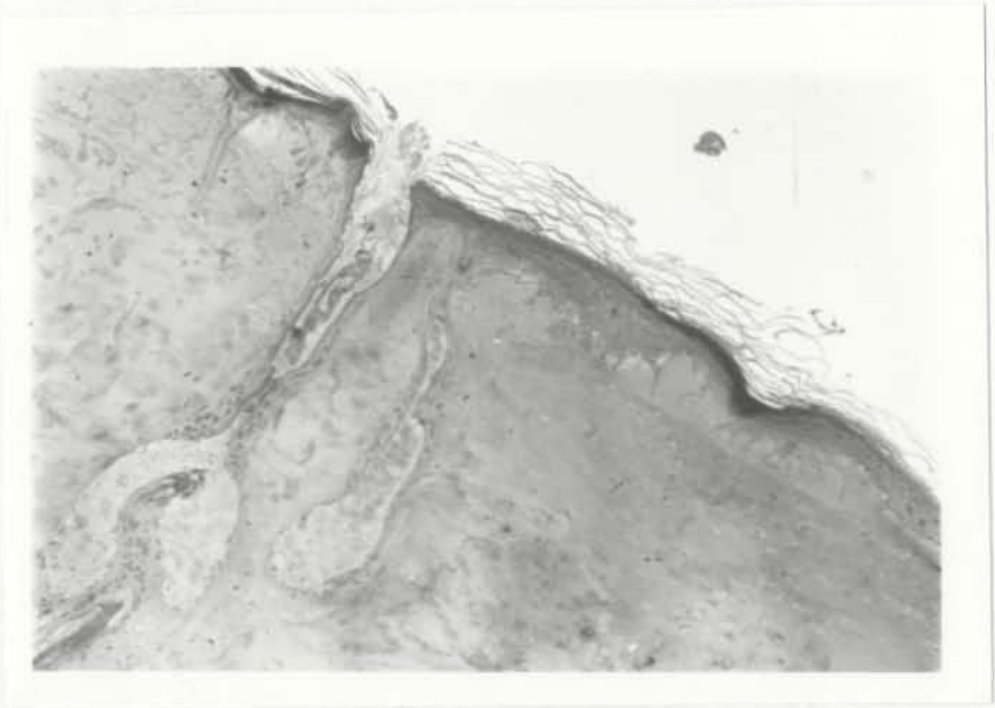


Figure 31: Microscopic cross-section of a skin sample subjected to 20 pulses of 500 V and 60 ms using a small electrode.

Figure 32: Microscopic cross-section of a skin sample subjected to 20 pulses of 500 V and 60 ms using a large electrode.

VII. CONCLUSIONS:

1. Electroporative pulsing causes a dramatic increase in the delivery of terazosin into skin. For example, electroporative delivery with 20 pulses at $U_{electrode}$ of 500 V, pulse length of 20 ms resulted in 15 times increased delivery of TRZ into skin, as compared to passive diffusion.

2. Electroporative permeation-enhancement was transient and reversible provided the conditions of pulsing were controlled.

3. Electroporative transdermal delivery was markedly influenced by:

i. U_{skin} ($U_{electrode}$): As the values of U_{skin} were increased, above a threshold (or minimum) value, there was almost a linear increase in the delivery of terazosin into the skin.

ii. Pulse length: Pulse length had a profound influence on the delivery of terazosin into the skin. The increase in the amount of Terazosin delivered into skin, corresponding to the pulse length, was almost linear.

iii. Number of pulses: Delivery of terazosin occurred with each pulse. Hence, as the number of pulses was increased, the amount delivered also increased, almost linearly.

iv. Electrode design: There was a significant change in the current density, and

voltage drop across the skin associated with the electrode design. With five times increase in the electrode area $U_{electrode}$ could be reduced by 40% to achieve a U_{skin} comparable to that obtained with an electrode of a smaller area.

It can be reasonably concluded from the results that pulsing with a $U_{electrode}$ of 500 V and a pulse length of 3.0 ms forms a borderline below which electroporation is relatively safe.

4. U_{skin}: The U_{skin} values were about one third to one fifth that of the $U_{electrode}$ depending on the voltage used. The area of the pulsing electrode also influenced the U_{skin} values. For instance, an increase in the electrode size by about 5 times increased the value of U_{skin} by 30-40%.

5. The large electrode delivered 2 - 2.7 times less energy to the skin when compared to the small electrode. An increase in the area of the delivery electrode is speculated to result in a more even distribution of the current resulting in less damages to the skin.

VIII. Prospects and Future Directions:

This project addressed issues related to the optimization and the safety of electroporation as applied to transdermal drug delivery. Studies related to the delivery of drug into the skin instead of delivery across the skin (into the receiver) were found to be more satisfactory for the *in-vitro* studies. The pulsing conditions were found to strongly influence the transdermal delivery of the model drug (TRZ). This shows that it would be ultimately possible to predict and control, to quite an extent, the transdermal delivery using electroporation. The influence of the drug concentration on the donor side is another factor that may affect drug delivery and it should be studied. The optimisation and safety issues addressed in this work form a ground work for trying out transdermal electroporative delivery *in-vivo* under the optimized conditions. Damage to the skin on the macroscopic and microscopic levels were detected after electroporative pulsing beyond certain conditions. It would be worthwhile at this stage to perceive the changes occurring in the skin at the molecular level after pulsing at different conditions using techniques such as the NMR (Kitson et al. 1994, Bowstra et al. 1997 and Barry et al. 1995), DSC (Lee et al. 1998), Small Angle X-Ray Diffraction, Wide Angle X-Ray Diffraction (Jadoul et al. 1997), and ATR-FTIR (Harrison et al. 1996) etc. The permeability barrier of the skin has been attributed to complex crystalline structures in the stratum corneum with lipid portion like ceramides and cholesterol. Work needs to be done for studying the possible

changes in the type of crystalline structures present in the stratum corneum after electroporative pulsing. Keeping in view the changes in the permeability of the skin after pulsing, transepidermal Water Loss (TEWL) can be expected to change after electroporative pulsing at different conditions and needs to be studied in detail in *in-vivo* studies. Finally, electrochemotherapy using bleomycin has been found to be very effective and useful in recent clinical trials in treating skin cancer (Glass et al. 1997). Electroporation has been speculated to increase the effectiveness of the anticancer drug molecules. The mechanism of the increased effectiveness is believed to be the increased permeability of the drug inside the already highly permeability cancer cells. As a result, bleomycin which is not conventionally used in skin cancer treatments was very effective. In the same context, it might be interesting to do similar electrochemotherapy studies using naturally occurring anti-cancer moieties such as vincristine (Knyihar-Csillik E et al. 1982) for skin cancers.

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