# EVALUATION OF SAFETY OF TRANSDERMAL DRUG DELIVERY USING ELECTROPORATION BY In Vitro AND In vivo STUDIES



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# Evaluation of Safety of Transdermal Drug Delivery using Electroporation by *In Vitro* and *In vivo* Studies.

by

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## Abstract

**Introduction:** Transdermal electroporation involves the application of high voltage electrical pulses for microsecond to millisecond duration to produce reversible increase in permeability of skin. It can provide an alternative route to intravenous injection for the fast delivery of macromolecules molecules such as proteins and peptide drugs in clinically effective amounts for the patient. However, the mechanism of electroporation and its safety are unclear. Hence, electrical parameters for delivery of individual drugs have to be chosen empirically or by careful optimization.

**Objectives:** To carry out optimization of electrical parameters using fuzzy rat skin tissue *in vitro* for delivery of terazosin hydrochloride (TRZ), followed by use of these parameters in live fuzzy rats *(in vivo)*, to study their safety and effectiveness. To design *in vitro* and *in vivo* tests to predict the safety of this technique.

**Materials:** Side-by-side diffusion cells were used for *in vitro* and *in vivo* studies. Ag/AgCl electrodes of different areas were used to deliver the exponentially decaying electroporation pulses from a Gene Pulser® (BioRad Laboratories, USA). Fuzzy rats and freshly excised full thickness skin from fuzzy rats were used for *in vivo* and *in vitro* studies respectively.

**Methods:** Pulse length and rate of pulsing were evaluated with respect to their ability to reverse the increased permeability caused by electroporation. The correlation between TRZ concentration and increase in electroporative delivery was

studied. Based on above studies optimal parameters were chosen to deliver TRZ *in vivo.* Their safety and effectiveness were compared to delivery without electroporation (control). Pharmacokinetic parameters were estimated by giving drug intravenously and subcutaneously. *In vivo* impedance recovery of skin after electroporation to pre-electroporated state was studied to predict safety. Similarly, uptake of glucose by skin with or without electroporation was studied to predict change in viability (damage) *in vitro.* Finally, electrodes of different area were characterized *in vitro* with respect to the electrical parameters. These parameters in conjunction with the *in vitro* and *in vivo* drug delivery and safety studies would throw some light on the mechanism of electroporation and the effect of electrode area on drug delivery and safety by electroporation.

**Results and discussion:** If electroporation is completely reversible then the rate of transport of drug through the skin after stopping electroporation pulses should be the same as that through non-pulsed skin. Using this method an applied voltage ($U_{electrode,0}$) of 400V, a pulse length of 20 millisecond and a rate of pulsing of 10 pulse per minute (ppm) were found to be relatively safe and delivered significantly higher drug compared to passive drug delivery. Increased donor concentration gave higher delivery and may help in reducing exposure to higher electrical conditions to produce same amount of drug delivery. *In vivo* studies showed that TRZ can be delivered safely and effectively with electroporation. However, the effect of electrode area needs to be studied further. Pharmacokinetic studies indicated depot

formation within skin after electroporation and this could be due to limited blood flow to the skin. *In vitro* biochemical studies showed a lag time in lactate production when a very high voltage electroporation pulse was used and there was a general stimulation of lactate production (as a result of glucose utilization) after electroporation compared to non-electroporated skin. The lag time may be used to predict damage due to electroporation. *In vitro* electrode characterization studies gave considerable insight into the observed drug delivery profiles and the differences in safety profiles between the different electrodes. *In vivo* impedance studies showed that complete recovery of skin impedance after electroporation might take hours to days. Recovery was faster with shorter pulse lengths and lower number of pulses.

**Conclusions:** An applied voltage of 400 V, 20 pulses of 20 millisecond at the rate of 10 pulses per minute with 10% TRZ in contact with skin and using a small area electrode was found to be relatively safe and effective for delivery of TRZ *in vitro* and *in vivo*, with higher delivery for higher electrode area as predicted. The *in vitro* electrode characterization experiments could explain some of the observed differences between the drug delivery by different electrodes and *in vitro* viability studies could predict damaging electrical conditions. *In vivo* impedance studies showed that the parameters which can cause appreciable recovery of impedance after electroporation, are those that have not shown to deliver appreciable amount of drug *in vitro*, at least by electroporation alone. New electrode designs or new

methods will have to be devised to increase safety of electroporation before electroporation can be considered useful or tested on humans.

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Sincerely

Deepak P. Kini

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**Kini D.P.**, Kara M., Smith F.R., Krishnan T.R., "Parameters affecting transdermal drug delivery by electroporation" at "Millenial world congress", April 16-20, 2000, San Fransico, CA, USA.

**Kini D.P.**, Kara M., Smith F.R., Krishnan T.R., "Principles of electroporation for enhancing transdermal drug delivery demonstrated using a model drug in hairless rats" "Aldrich Interdisciplinary Conference", Feb 22, 2000, St. John's, NF, Canada.

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## 1.0 Introduction

### 1.1Drug Delivery Systems :

Advances in biological sciences have provided newer insights in various physiological and pathological processes. This has led to the discovery of large number of new drug molecules to mitigate, prevent or treat diseases. The biotechnology area has been growing fast too, resulting in the introduction of new generation of therapeutic moieties viz: proteins and peptides. The recent completion of the human genome project will likely lead to identification of genetic links to many diseases. Indirectly this will fuel the discovery of many new functional proteins and peptides which could then be used to treat the condition (Baba, 2001). Enzyme susceptibility is one of the challenges of these therapeutic entities that make conventional dosage forms unsuitable for their delivery. Presently, parentral route is the main delivery system for the peptide and protein moieties but they are painful, invasive, and expensive. Thus, many companies and researchers have been investing in research for alternative drug delivery systems (Cleland, 2001).

Combined efforts of many scientists has led to the development of a vast array of non-invasive drug delivery systems capable of delivering proteins and peptides. Among the many drug delivery systems which have made to the market or are in clinical trials, are the transdermal drug delivery systems. In this thesis effort is directed towards optimizing transdermal drug delivery using electroporation as a physical means of enhancement of drug delivery.

## 1.2 Transdermal drug delivery systems :

This route of drug delivery offers many advantages over conventional dosage forms. It is easily accessible, non-invasive, avoids the hepatic first-pass metabolism, provides better rate control and might also lead to fewer side-effects due to the elimination of peaks and valleys of the conventional dosage forms. It also provides a less harmful environment for enzyme and pH susceptible drugs like proteins and peptides compared to the conventional dosage forms. This might lead to increase in bioavailability of some of the drugs. The skin behaves like a depot especially for the lipophilic drugs. Thus, some drugs having short half-lives can be delivered for extended periods like an IV infusion.

Local and systemic delivery of drugs by topical route have been used for decades. However, the present popularity of this route for controlled clinically effective systemic delivery of drugs was triggered by the introduction of transdermal scopolamine patches for the treatment of motion sickness in 1981 followed by introduction of the transdermal nitroglycerine patches in 1982 (Chien, 1987). Since then a number of transdermal products have made it to the market (Flynn, 2002).

Presently, molecules upto 178 KDa have been delivered with transdermal drug delivery systems (TDDS) (Prausznitz, 1997). Macromolecules pose a real challenge for TDDS. However, this has only aggravated the need for better techniques and delivery enhancers. Many physical and chemical enhancers have been utilized to breach the formidable barrier of the skin's outermost layer- stratum

corneum (SC). Improved understanding of the SC's barrier structure and function, and the ability to breach it has led to better success rates in TDDS (Prausnitz, 1997 and Barry 2001).

### 1.3 Skin Structure and Composition :

Skin of an average adult covers approximately 2 $m^2$ and receives one third of the blood circulating through the body and as such provides an excellent portal for delivery of drugs (Jacob, 1970). However, the barrier to percutaneous delivery lies within the SC - the outermost 10-15 μm thick layer of skin. It serves to keep foreign substances out and water in.

With regards to drug delivery, it is to be noted that the barrier properties of skin vary greatly with anatomical site, age, phenotype of individual cells, the number and type of glands, the sebaceous secretions and the degree of vascularization. The following is a general discussion on structure and composition of the skin which will help in the better understanding of the skin barrier.

#### 1.3.1 Anatomy of skin as relevant to drug delivery :

The skin is divided into the following major layers: the epidermis, dermis and hypodermis, located below the dermis. The epidermis and dermis are separated by the basement membrane. The epidermis consists of viable tissue supplied by blood vessels but devoid of nerves, a feature which is still debated by many

scientists (Hillinges,1995). The dermis which lies below the epidermis also contains living cells, in addition nerves and blood vessels.

The epidermis consists of five layers. The bottom-most layer consists of fast dividing keratinocytes and is called basal layer. These cells then migrate upwards to mature into spinous cells forming spinous layer. Then into granular cells forming the granular layer. Then form the stratum lucidum and finally undergo edge-to-edge fusion to form the sheet like layer made up of the flattened, keratinized and dead cells of the SC. As the cells mature from the basal layer to the SC, a number of existing cellular components have to be degraded, e.g. proteins or lipids, by the lytic enzymes. These enzymes can also degrade topically applied drugs. Thus, the SC represents the physical barrier to TDDS, whereas the viable epidermis the enzymatic barrier. The dermal-epidermal junction is convoluted and forms papillae which enormously increase the surface area of contact between these two layers. It is relatively permeable to molecules upto 40 KDa (Schaefer, 1996)

The dermis consists of loose connective tissue and contains collagen and elastic fibers as well as various cells including fibroblasts, mast cells, macrophages and lymphocytes. The blood vessels and lymphatics are also located here. Finally, the epidermal appendages such as hair follicles, sweat glands and sebaceous glands are also embedded in this tissue.

### 1.3.2 Structure and composition of SC :

The importance of SC as a barrier cannot be over-emphasized. Physically stripping off the outmost layer of skin increases water and drug permeability by several fold (Scheuplein, 1971 and Schaefer, 1982) and this simple experiment can be used as a proof of SC as a barrier layer. In another study, it was shown that the transepidermal water loss (TEWL) increased with the sequential removal of SC (van der Valk, 1990). It is imperative that the SC structure and composition be understood to achieve better success rates by the transdermal route.

Improved visualization techniques such as atomic force microscopy, freeze fracture and electron microscopy, attenuated total reflectance - Fourier transform infra-red spectroscopy (ATR-FTIR), differential scanning calorimetry (DSC)have led to a better understanding of the structure of epidermis in general and more specifically the SC (Turner,1997 and Jadoul,1999,). Advances in various other scientific fields have helped characterize the chemical composition and the physical nature of this layer.

Structurally the SC is likened to the "brick and mortar" with the bricks made-up of flattened keratinocyte cells, also called corneocytes, filled with protein - keratin, which provides strength and chemical resistance. These cells are surrounded by intercellular lipid layers which form the "mortar". The keratinocyte bulk is considered to be relatively permeable compared to the highly impermeable lipid layers surrounding it. This inter-cellular layer can be resolved into further

alternating lipid and aqueous domains. There are about 15-20 keratinocyte layers and thus about 75-100 lipid bilayers in the SC. Typically each of these layers has 4-5 lipid layers alternating with 4-5 aqueous layers as revealed by freeze-fracture electron microscopy (Van Hal,1996). These aqueous layers reduce the diffusional pathlength for hydrophilic molecules within the SC which explains the increased permeability due to occlusion.

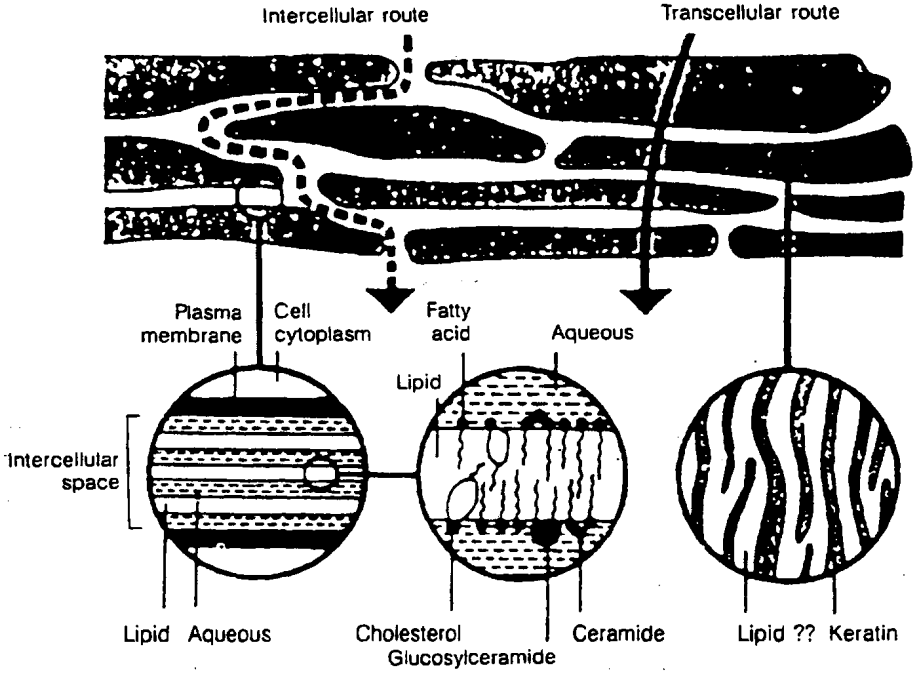



**Figure 1.1:**Structure of SC and its components. (Reproduced with permission from -Skin barrier principles of percutaneous absorption by Schaefer H, Redelmeier TE, Karger and Basel, 1996, pp78)

Although, the keratinocytes provide the bulk of the SC, the intercellular lipids form the only continuous domain within the SC, following a tortuous path around

the keratinocytes. This feature of the lipid domain makes it the most important component for providing the barrier function. Selective removal of SC lipids by using organic solvents like acetone has the same effect as selective tape stripping (Potts,1991) which provides additional proof for the importance of SC lipids in providing barrier property to the skin. However, several pathological conditions which alter the structural proteins within the corneocytes, also increase dermal permeability. Care has to be taken when interpreting the determinant of SC barrier function.

### 1.3.3 Chemical composition of the SC barrier layer:

Just as the SC evolves from the epidermis as a distinct layer, the cells within the SC also undergo changes as they move up the SC during the desquamation process. The uppermost 3-4 layers are called stratum disjunctum and the lowermost 3-4, stratum compactum. These layers differ in their composition and morphology of the lipids and hence an analysis of the average chemical and material properties of the SC would be an oversimplification.

The SC is a rather dry layer compared to other physiological membranes with an approximate water content of 15 %, proteins 70 %, and 15 % lipids. This is considerably different from the rest of the epidermis which has approximately 70% water, 15 % protein, 5 % nucleic acid and 5 % lipids. The decreased water content in the SC is attributed to the hydrolysis and degradation of nucleic acids,

proteins, and phospholipids leading to a decrease in the number of chemical groups that can bind to water. Though most of the water in the SC is present within the bulk corneocytes, the intercellular lipid domains also contain some water. Artificially hydrating the SC to more than the physiological levels (above 30-40%) leads to swelling of the corneocytes (Blank, 1985 and Mak, 1991) and progressive loss of barrier function (Bodde, 1990 and Barry, 1983). The corneocytes contain many low molecular weight polar compounds (amino acids, sugars etc) collectively referred to as the natural moisturizing factors (NMF) which bind to water.

A significant gradient of phosphorus and potassium exists within the SC, decreasing from the stratum compactum as it reaches the stratum disjunctum where it is the lowest. Similarly, there exists a gradient of calcium too, however, the calcium concentration is the highest in the outermost stratum disjunctum and reduces in the deeper layers of SC. Mauro et al (1998), have shown that barrier perturbation abolishes the gradients of calcium and potassium within the SC. The phenomenon responsible for the presence of this gradient or its absence with the barrier perturbation is unclear. However, several processes during the epidermal maturation require calcium ions viz: conversion of profilaggrin to filaggrin, activation of serine proteases and transglutaminases for programmed cell death (Mauro, 1998).

The corneocytes contain the bulk of the proteins within the SC. Mainly, they contain keratin associated with some filaggrin, surrounded by a cornified envelope.

This envelope is stable to chemical treatment. The keratins have an endothermic transition temperature of 95°C (Golden, 1986). The cornified envelope is made up of equimolar concentration of high molecular weight proteins (80% by mass) and lipids. The proteins which evolve from degradation of organelles, as well as the proteins and lipids of the viable epidermal cells are resistant to proteases. These proteins are cross-linked by three types of bonds which make them highly resistant to lysis by proteases. The lipids within the cornified envelope are covalently bound to the proteins and may interact with intercellular lipids to decrease discontinuities or provide barrier function to the corneocytes. Ceramides are the major lipids of the cornified envelope. Corneodesmosomes are proteins which cross-link the corneocytes and provide mechanical strength to the SC. Their gradient decreases in the upper layers of SC, emphasizing their importance in desquamation. Hydration can influence the corneodesmosome degradation and therefore increase permeability (Rawlings, 1995).

The intercellular lipids within the SC have a different composition and morphology compared to that of cellular membranes. They also differ from the lipids present within the viable epidermis in their composition. The intercellular lipids contain equimolar concentrations of ceramides, free fatty acids and cholesterol. These lipids differ from membrane lipids in that, they have very 'small' polar head groups which can potentially bind to water, thus, the low water content of SC. These lipids are also predominantly neutral in nature in contrast to cellular lipids which are

predominantly anionic. The SC also contains lipids derived from sebaceous secretions, however, these are present mostly in the upper stratum disjunctum layer. Although, these lipids can influence SC lipid composition, they do not contribute to the barrier property of SC, probably because these lipids are not ampipathic and hence do not get incorporated into the lipid bilayers. However, they change the surface concentration of lipids and may get incorporated into topical applications, diluting them and thereby influencing percutaneous absorption.

The SC is shown to have four transition temperatures by differential scanning calorimetry (DSC) viz; 35, 65, 80 and 95°C (Golden 1986). As pointed out earlier, the 95°C transition has been assigned to keratins, since the removal of lipids by solvents does not abolish this transition temperature. The low temperature transition of 35°C, has recently been proposed to be due to minor lipid component or to that having a low enthalpy (Gay, 1994). The 65°C transition point was attributed to SC lipids, analyzed by selectively extracting these lipids. However, these extracted lipids did not show the 80°C transition, which has been proposed to be due to the specific morphological ordering or protein-lipid interaction within the intact SC. These phase transition temperatures differ from that of cellular membranes which have a transition at -20°C, indicating a liquid crystalline state of these membranes. The lateral diffusion of lipids within the layers increases from a solid-crystalline to gel to liquid crystalline states, so does the molecular permeability. Thus, the SC with high transition temperature can be considered in

a more ordered state or in a solid-crystalline state and contributing to its unique barrier properties. This highly ordered state also permits the study of SC using X-ray crystallography. Thus changes caused in SC can be analyzed using DSC or X-ray crystallography.

### 1.3.4. Other features - Enzymes in the SC:

The SC is not as 'dead' as it seems. Metabolic processes do go in the SC, especially at the junction of the viable epidermis and the stratum compactum. Formation of precursor lipids to the intercellular lipids requires enzymes such as sphingomyelinase, phospholipases, acid lipases, beta-glucocerebrosidases, proteases, acid hydrolases and acid phosphatases. The degradation of corneodesmosomes during desquamation process also requires the presence of proteases. Hence the enzyme activity may be present in the upper layers of SC too. Hydrolases required for hydrolysis of cholesterol esters may also be present in the *stratum disjunctum*. These proteases and hydrolases in the SC need to be considered seriously when using a transdermal system for delivering proteins and peptides. Due to the ability of skin to form depot for drugs, they might get exposed to significant levels of lytic enzymes reducing the bioavailability through skin.

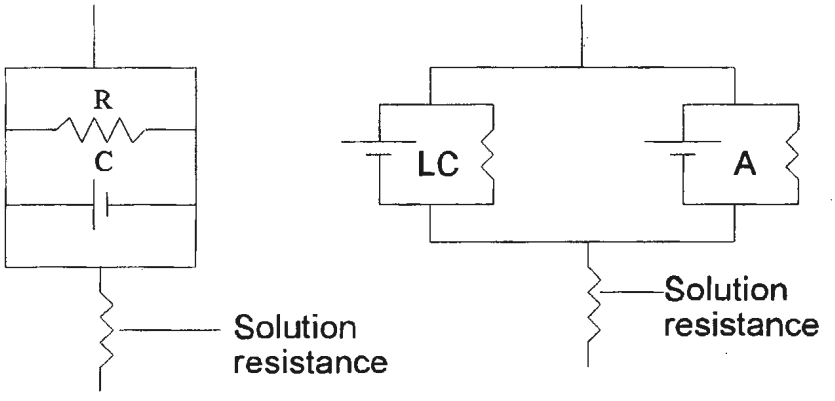
### 1.3.5 Electrical properties of the skin:

Waters is a good conductor of electricity. The current in such an electrolyte is carried by ions. Such a system provides "resistance" or obstruction to the ion flow. The skin also conducts electricity. In a complex biological tissue like skin, the current is carried by ions through the hydrophilic pathways. Under direct current the skin exhibits resistance only. However under alternating current, when the frequency of current keeps changing, the lipid milieu of skin also starts taking a significant part. The lipids within the skin act like capacitance - or parts which store current. Capacitances behave differently with varying frequency of current. Measuring the resistance of the skin in such a case will not provide the complete picture. Impedance which is a measure of the resistive and capacitative components of a circuit would be more meaningful measure. This also means that measuring the impedance of skin would give us an idea of the barrier to drug delivery (ion transport) across skin. Conversely if due to any reason there is a change in drug delivery (for example application of electroporation pulse to skin) it should be reflected by a change in the impedance component of the skin.

Impedance measurement involves application of fixed voltage with varying frequency current and measuring the potential drop across the skin. The typical impedance spectrum of skin shows the real impedance on Y-axis which represents the resistive pathways (e.g. hair follicles, sweat ducts, aqueous layers) and imaginary impedance on X-axis which represents the capacitive pathways (e.g. lipid

milieu of skin) over a range of frequency.

Since there could be various pathways, the impedance plots of skin can be interpreted in various ways. Based on impedance plots various equivalent circuits of skin have been proposed. The most simplest equivalent electrical circuit representing the electrical behavior of skin is shown in Figure 1.2A. Basically the skin is considered to be a resistor and capacitor in parallel with a resistance in series to account for the properties of the electrolyte. (Rosendahl, 1945 and Yamamoto, 1976).




**(A) Simplest model**

**(B) Includes two pathways**
LC = Lipid-Corneocyte and
A = Appendageal

**Figure 1.2 :** A simple electrical equivalent circuit for skin's electrical behavior. R = resistance and C = capacitance.

The above model is adequate for low voltages applied to skin, however, in more specific studies, two pathways for applied electrical current have been

reported, one through the lipid-corneocyte matrix and another through the appendages (Pliquett, 2000b and c). Hence, the electrical circuit would now have two parallel components each containing a resistor and capacitor in parallel (Fig 1.2B). Many such modifications can be drawn to reflect the changes in skin's electrical properties.

However, for general purposes, the resistance of the skin can be used as a predictor of skin permeability. Normal skin has a resistance in the order of 5-100 Kohm.cm$^2$ upto a temperature of 60°C. Increasing the temperature beyond that to about 60-70°C, leads to a dramatic drop in this resistance (Oh, 1993). This has been attributed to the melting or phase transition of the intercellular lipids in the SC which can lead to a dramatic increase in the permeability to ions and electrolytes. Similarly a very low resistance in an *in vitro* skin preparation indicates an obvious leak. Puncturing the skin with a hypodermic needle also can cause a dramatic drop in the skin resistance (Prausnitz, 1999). Thus, the resistance of skin cannot be taken as a single measure of barrier disruption. However, being the fastest and most convenient method, it is often used to predict the skin's permeability (DeNuzzio, 1990).

### 1.4 Pathways for Percutaneous Penetration:

Three percutaneous penetration routes have been proposed to exist: (i) appendageal, (ii) intercellular and (iii) transcellular (Figure 1.3).

(i) Appendageal route is also called as the shunt pathway. It involves the hair follicles and sweat gland ducts. Permeation through the shunts avoids the intercellular lipids of the SC. However, the area covered by the shunts is less than 1% (Scheuplein, 1971) of the total skin area. Hence, even if the permeability of this route is higher, the major pathway will be across the SC. Drug permeating through this route directly reaches the dermis, where the bases of these follicles as well as the vascular network are located. Thus, it could be considered as the shortest and fastest route to vascular delivery for topically applied compounds. Many studies have shown that this route is important in determining molecular flux in transdermal iontophoresis (Scott 1993, Cullander 1991a,b and 1992,Burnette, 1988).

(ii) Intercellular route involves the tortuous route through the intercellular lipid domains. Diffusion of molecules across a lipid bilayer would be energetically less favorable than the diffusion within the bilayer. This is one of the main reasons which favors the tortuous pathway(Devaux, 1993). Moreover, using the partition coefficient and lag time, the water penetration pathway was found to be around 50 times longer than the thickness of the SC, which strongly suggests that the tortuous pathway may dominate (Potts, 1991). Another explanation for diffusion of polar molecules is the presence of alternating lipid and aqueous layers within the intercellular domains.

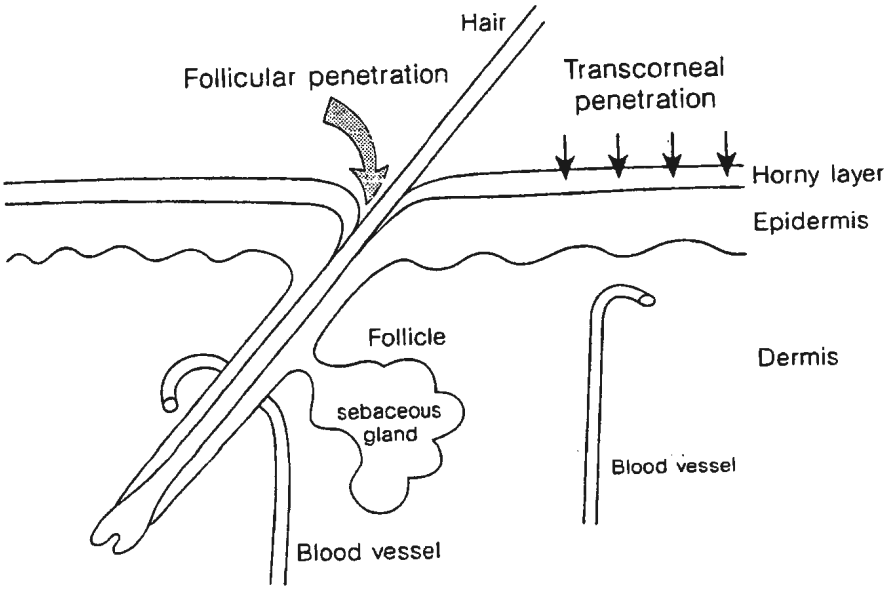



**Figure 1.3:** Pathways of transdermal delivery (Reproduced with permission from - Skin Barrier Principles of percutaneous absorption by Schaefer H, Redelmeier TE, Karger and Basel, 1996, pp78)

Such a conclusion was a combination of two observations, one that water seems to follow the tortuous path through the intercellular domains as detected by FTIR (Potts, 1990) and the second that the loss of ordering within this lipid domain leads to increased water loss. Thus, these tortuous aqueous and lipoidal pathways have been accepted as the most favorable routes for topically applied compounds.

(iii) Transcellular pathway: Although, the penetration of compounds require that they traverse the lipoidal domain, it does not exclude the possibility that they can enter

the corneocytes which contain hydrophilic proteins. Hydration or occlusion leads to swelling of the corneocytes, thus water may enter the corneocytes, increasing the permeation compounds. Even large molecules like mercury could penetrate the corneocytes in the upper *stratum disjunctum* layer of the SC suggesting that the corneocytes within this layer could be more permeable. Thus, these pathways are not mutually exclusive and permeation is determined by the area of the pathway, partitioning and diffusivity of the compound within each pathway.

**1.5 Strategies to enhance transdermal drug delivery:**

To reach the systemic circulation a topically applied drug must permeate the SC and epidermis to reach the dermis, where the vascular network is located. For compounds with a molecular weight of less than 500 daltons, the SC seems to be relatively permeable. However, as the molecular weight is increased, the partition coefficient, between the hydrophobic and hydrophilic layers of skin, becomes rate limiting. The permeability seems to increase with increase in the partition coefficient. Thus the rate-limiting barrier in the SC is lipoidal in nature. For hydrophilic drugs the partitioning into this barrier will be rate limiting, and for lipophilic drugs the partitioning out of this barrier would be rate limiting. This also presents a strong possibility of depot formation for lipophilic compounds within the SC. Thus, an enhancer can act in three ways (i) to increase the partitioning of compounds into SC, (ii) to increase diffusivity of compound from the SC or (iii) to cause a change in the

penetration pathway. Classically, strategies to enhance transdermal or topical drug delivery have been studied based on whether these enhancers cause chemical changes in skin (SC) or physical changes. This system of classification still exists and is used in the following discussion.

**1.5.1 Occlusion:** It is the application of an impermeable barrier layer to skin, which decreases TEWL. This can lead to an increased water content in the SC up to 300%. Studies have shown that this water is mainly taken up inside the corneocytes. Also, formation of small water channels has been proposed which reduces the diffusional pathlength of mainly hydrophilic compounds, and also lipophilic and amphiphilic compounds (Bucks,1989).

**1.5.2 Chemical enhancers :** These can act in any of the three ways discussed earlier to increase transdermal drug delivery. Mainly chemical enhancers such as dimethyl sulfoxide and azone can disrupt the lipids in SC. Surfactants have also been used to increase penetration of drugs. Non-ionic surfactants are preferred to ionic since these have less potential to cause irritation. Concentration of surfactants has to be regulated such that aggregation to micelles is prevented. Monomer incorporation into SC lipids would not be possible with concentrations reaching critical micelle concentration. These agents in their monomer state, are hypothesized to get incorporated in the intercellular lipid domains providing additional medium within which an agent can diffuse into, reducing the transit time of the drug across the SC (Walters, 1988 ). Similar results were also evident with

oleic acid and other fatty acids which provided alternate pathways generally for diffusion of lipophilic compounds (Mak,1990 and Ongpipattanakul,1991).

Evaporation of a chemical enhancer like ethanol might cause saturation of drug and thereby increase the thermodynamic activity of the drug, which in turn could indirectly contribute towards enhancement (Hadgraft, 1989). However, chemical enhancers are more likely to cause irreversible changes in the SC, some of them may cause irritation, and could potentially get absorbed and produce unwanted pharmacological effects themselves (Guy 1996). These factors make them very unlikely candidates for approval from United States Food and Drug Administration (USFDA) or Health Canada.

**1.5.3. Physical enhancers:**

Many methods exist to alter the physical properties of the skin or specifically the SC. Although, tape stripping can be used to selectively remove layers of SC causing a modest increase in the permeability, it does not completely eliminate the barrier properties of the skin. It has however, been used as a tool to study the mechanism of action of other enhancers.

**1.5.3.1 Iontophoresis:**

This method involves the use of electric current to deliver drugs across the SC. Briefly, a few milliamps of electric current is applied using an electrode of opposite charge to that of an ionic drug to repel the drug into the skin. However, electro-neutral water soluble drugs can also be delivered due to electro-osmosis of

water. Controlling the current can help control the delivery, hence this method has an excellent potential to be used as a controlled drug delivery method. As discussed earlier, the primary route of iontophoretic drug delivery is shown to be the appendageal route.

Peptides like calcitonin containing upto 32 amino acids have been delivered successfully (Greene, 1996 and Santi, 1997). The delivery of oligonucleotides has also been shown(Oldenburg, 1995 and Brand, 1996). Most importantly, this method has been extensively studied as an alternative delivery method for insulin. However, the success of this technique to deliver insulin remains questionable. Some of the difficulties can be attributed to the properties of insulin such as, its isoelectric point, which lies in the range of skin pH and can cause its precipitation in the SC and its ability to associate to form hexamers can cause a huge increase in the molecular weight making transdermal transport very difficult (Banga, 1999). The onset of action is not as fast as an IV injection. As a result the drug has to be given over minutes to hours to get significant rates of delivery. Nevertheless, significant progress has been made over the years. Wearable devices or miniature devices which can be attached to the body for long periods of time to provide programmed drug delivery have been developed and look promising (Banga, 1998a). Another important application of iontophoresis has been "reverse iontophoresis" for analysis of metabolites in the interstitial fluids which can act as bio-feedback mechanism for various drug delivery devices (Glikfeld, 1989). Since the first reporting of this technique, research has

progressed very fast. Recently the US-FDA has granted permission to Cygnus, Inc (Redwood city, CA) for the marketing of Glucowatch™ in the USA (FDA, 2001). This device can monitor the body glucose levels by reverse iontophoresis. The glucose here is extracted at the cathode by electroosmosis. This device can be used to regulate insulin delivery using iontophoresis.

Iontophoresis is being routinely used in the diagnosis of cystic fibrosis in hospitals (Warwick, 1986). Alza® corporation, CA, USA is also in the process of developing a transdermal iontophoresis system for the delivery of fentanyl for local anaesthesia (Gupta, 1999). While many drugs or even peptides can be delivered at therapeutic rates, iontophoresis cannot deliver very high molecular weight peptides or proteins and oligonucleotides. This has led to combining iontophoresis with other enhancers. Chemical enhancers, like ethanol pretreatment followed by iontophoresis has led to significant enhancement of insulin delivery compared to iontophoresis alone (Langkjear, 1994). Similar results were seen with leutenizing hormone releasing hormone (LHRH) using oleic acid as an enhancer along with iontophoresis (Bhatia, 1997). However, the most promising study seems to be the combination of iontophoresis with electroporation (Prausnitz, 1994). This will be discussed in the following section of electroporation.

### 1.5.3.2 Sonophoresis:

Use of high frequency sound waves - ultrasound to deliver drugs and even macromolecules has been shown in several studies (Kost, 1993). The enhancement is influenced by the frequency used ( 20 kHz to 10 MHz) and the intensity (0-3.0 $W/cm^2$) with either pulsed exposure (1:1 to 1:10) or continuous exposure (<10mins). The biggest limitation is the local heating of tissue which can be reduced by decreasing the pulse exposure. Low frequency ultrasound has been shown to have better enhancement ratios (Mitragotri,1995a). There are several proposed mechanisms to explain the enhancement of transdermal drug delivery by ultrasound which include cavitation, thermal effects, generation of convective velocities, and mechanical effects. Cavitation or formation of air bubbles within skin, has been shown to occur in the keratinocytes of the SC upon ultrasound exposure. Cavitation has been attributed to the presence of a tensile cycle (negative pressure), which is always present in ultrasound (Miller, 1996). It is hypothesized that oscillations of the cavitation bubbles induce disorder in the SC lipid bilayers, thereby enhancing transdermal transport (Mitragotri, 1995b). However, the contribution to transdermal drug delivery enhancement due to heating and ultrasound has not been clearly established.

Ultrasound has also been used for non-invasive monitoring of glucose and other biochemicals (Kost, 2000) similar to iontophoresis. Here vacuum is applied after ultrasound to extract interstitial fluid from the skin and analyze the various

biochemicals in this extract. Further, sonophoresis has also been combined with iontophoresis to deliver macromolecules like heparin (Le,2000). However, tissue damage may occur in sonophoresis (Byl,1995) and is attributed to cavitation (Bommannan,1992).

### 1.5.3.3 Microfabricated microneedles:

This is a very recent addition to transdermal drug delivery enhancers. This method utilizes micro-fabricated array of micron sized needles etched on silicon using ion etching procedure similar to the one used in manufacture of integrated circuits (Henry,1998). These can be inserted into the skin thereby creating conduits within the SC. Once past the SC these compounds can easily diffuse into the dermis and be taken up by the capillaries. Increased flux upto $10^4$ times compared to passive diffusion have been reported for calcein (Henry,1998). However, the device is in its infancy and its safety will have to be clearly proven, especially to show that the microneedles do not get broken or detached within the SC.

### 1.5.3.4 Powderject™ system:

High-velocity powder injection is a promising new drug-delivery technique that provides needle- and pain-free delivery alternative for proteins, peptides, and oligonucleotides as well as traditional and genetic vaccines (Burkoth, 1999). The energy of a transient helium gas jet accelerates fine drug particles of 20 microns-100

microns diameter to high velocities and delivers them into skin. This method was used to deliver antigens transdermally and has been shown to produce excellent immunizations (Tacket (1999), Degano(1998)).

**1.5.3.5 Photomechanical waves:**

Large molecules of upto 40 kDa of dextran molecules have been delivered using photomechanical waves (Lee, 1998a). These are produced by the ablation of a target material with a laser in a donor solution placed in contact with skin. The photomechanical waves produced due to ablation of the target get transmitted into the skin to produce changes in the skin, which cause the increase in permeability. Unlike ultrasound, photomechanical waves do not generate cavitation (Doukas, 1995). In cells the photomechanical waves seem to act on the membrane integral proteins increasing trans-cellular permeation. Similarly it is hypothesized that photomechanical waves might act on the corneodesmosomes increasing the permeability of SC (Lee, 1999). However, the mechanism of this method in increasing transdermal drug permeation is not clearly understood. The main disadvantage of this system is that the target gets ablated and can potentially get permeated in the skin along with the drug.

### 1.5.4 Electroporation:

Electroporation would also be classified as a physical enhancer. However, to provide easier readability and a detailed background for studies undertaken in this manuscript, it has been dealt with under a new heading.

#### 1.5.4.1 What and how ?

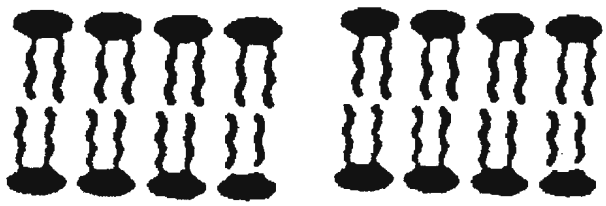
Application of high voltages to cells transiently alters their membrane potential. This phenomenon has been used to transfect cells with macromolecules like DNA, proteins and oligoneucleotides (Weaver, 1995). These voltages are believed to cause pore formation within the lipid bilayer membranes, the exact cause of which has not yet been ascertained. Similar electrically induced pore formation or electroporation has been observed in different bilayers seemingly independent of the composition. This prompted its first transdermal application in 1993 by Prausnitz et al with very promising results. The SC of the skin resembles the cellular membrane in that it has lipid bilayers, however, it is much more complex than a simple single bilayer lipid membrane. The SC has around 50-100 bilayers alternating with aqueous channels, and the presence of corneocytes further complicates the structure. Molecular rearrangement within the lipids forming aqueous channels is believed to be the cause of increased molecular transport across a cell membrane after electroporation (Weaver, 1993), similarly in the SC it is shown to be the formation of localized transport regions or LTR's (Pliquett, 1996d). Up to $10^4$ times

increase in epidermal molecular flux has been noted with calcien due to electroporation compared to passive diffusion (Prausnitz, 1993a).

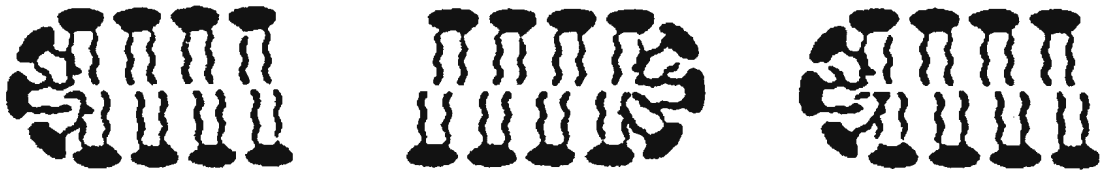
### 1.5.4.2 Changes in properties of skin after electroporation

Studies on various properties of the SC have been carried out, observing the effect of electroporation on these properties. Such studies help in understanding the mechanistic basis of electroporation. The first of such studies observed the changes in the passive electrical properties of the SC after electroporation (Pliquett, 1995). The presence of aqueous channels alternating with lipid bilayers gives the SC, properties of electrical resistance and impedance, where the resistance was attributed to the aqueous channels and the capacitance to the lipids in the bilayers. Essentially, an intact SC has been shown to have a very high resistance and impedance spectroscopy also revealed a capacitative component. After electroporation there was a dramatic drop in the resistance and an increase in the capacitative factor (Pliquett, 1995). The decrease in resistance rather than an increase in the capacitance correlated directly with increased calcein transport. This is because the transport of small ions through aqueous pathways will be indicative of pathways available for the transport of molecules that can "fit" into these pathways. In contrast, the pathways for ac current flow, i.e. involving voltage changes with higher frequency components, can involve displacement currents through capacitance elements. However, there is no actual transport of ions across

these capacitances. For this reason, the capacitance-based pathways do not contribute to ionic or molecular transport across the skin. These electrical changes were reversible within milliseconds to hours, depending on the various electrical parameters used. The reversibility was slower when pulse length or applied voltage was increased. This places a limit to the electrical parameters that can be used safely and effectively at least for chronic applications involving the same site. However, a combined study of passive electrical properties along with molecular flux



(A) Intact lipid bilayer- before electroporation



(B) Hydrophobic pore after electroporation

(C) Hydrophilic pore after electroporation

**Figure 1.4:** Schematic of the changes in lipid bilayer occurring after electroporation.

studies can be useful to screen the reversible and effective electrical parameters which can be used for a particular molecular species.

The order in which the lipid bilayers are arranged within the SC can be studied using X-ray scattering as well as differential thermal analysis (DTA). X-ray scattering studies showed an decrease of the small angle x-ray scattering (SAXS) peaks with no shift. The decrease was more pronounced with long pulses and low voltages indicating a disordering in the lipid lamellar packing as well as lipid lateral packing. Similarly, the intensity of wide angle x-ray scattering peaks (WAXS) was also decreased indicating a shift from crystalline phase to liquid phase. These changes were not significant with short pulses of high voltage and it has been suggested that such pulses could have caused localized changes which are undetectable by X-ray scattering (Jadoul, 1997). Similarly, the DTA studies show a decrease in the enthalpy indicating generalized disordering within the lipid bilayers rather than shift in the transition peaks of the SC which has been correlated to the increase in water content of SC (Jadoul, 1998). However, in this study the skin was hydrated initially for 2 hrs in both the control and electroporation treated samples, thus the shift in peak was either very small or insignificant. Studies using FTIR/ATR-FTIR have shown that there is an increase in SC hydration after iontophoresis treatment (Jadoul, 1996 and Clancy, 1994). Both, X-ray diffraction and DTA concluded that generalized changes were occurring in the lipid ordering of the skin mainly because these methods were capable of only recording bulk phenomenon.

Pliquett et al, performed confocal fluorescence microscopy to observe the electro-transport of calcein and sulforhodamine in real time using electroporation (Pliquett, 1996d). The probes followed a localized transport pathway across the SC as seen from the regions of fluorescence which were interpreted in the study as "sites where transport had taken place". These regions did not coincide with appendages. In fact sulforhodamine did not show any fluorescence associated with appendages. The intensity of calcein fluorescence associated with appendages was comparatively much less than that associated with the LTR's. Interestingly, increasing the number of pulses beyond three, at a constant voltage, did not increase the number of LTR's. Also, fluorescence was found within the keratinocyte interiors which may suggest that transcellular transport has occurred (Prausnitz, 1996c).

Other visualization methods for example electron microscopy, freeze fracture electron microscopy (FFEM) have also been used to observe the microscopic morphological changes in the SC after electroporation. A host of ultrastructural changes have been observed including generalized disordering in the lamellar structure, formation of network like structures and spherical deformation, however, no changes in the protein fine structure were observed in the cross-sections indicating that the changes are manifested mainly in the lipid ordering and not the keratinous matrix of the corneocytes.

Gallo et al, performed similar experiments with a modified freeze-fracture electron microscopy (FFEM) apparatus designed to reduce temporal resolution time

as well as to provide structural details which are beyond the scope of fluorescence imaging as performed earlier (Gallo, 1999). These images showed formation of vesicle aggregates which increased in density from 0.5 sec after the pulse to 5 sec after the pulse and thereafter decreased until 5 min after they completely disappeared. These aggregates seem to be similar to the LTR's. However, both of these phenomena do not directly correlate with the dramatic decrease in the resistance within microseconds of pulsing since the aggregate formation increased 0.5 sec after pulsing. Nevertheless, they correlated reasonably with the recovery of resistance over minutes since the number of aggregates decreased after 5 sec until 5 min whereby they were nearly absent. The temporal resolution of the fluorescence study was 0.5 sec and hence it would not be possible to directly correlate whether the LTR's were the remains of the elusive electropores. Similar aggregates at a lower density formed after heating the SC to about 60°C. Intense hydration of SC also may lead to aggregate formation (Van Hal, 1996). However, studies on dry SC have not been performed yet, hence aggregate formation cannot be clearly related to hydration.

**1.5.4.3 Explanations and mechanistic analysis** :

All the above studies have to some extent helped in understanding the electrical behavior (voltage, conductance, and capacitance of skin), mechanical behavior (rupture or recovery of skin) and to some extent molecular transport after electroporation. Nevertheless, the primary phenomenon involving the molecular changes and the final recovery of barrier function still remains unexplained.

Many theories have been proposed to explain the changes that take place after electroporation, one of the reasons include changes due to joule heating occurring within local regions of skin. The SC is not completely homogeneous, although the bulk of the lipids shows a solid-crystalline nature with a transition temperature of around 65°C. The SC has some regions (~1%) of fluidity due to lipids of lower transition temperature. Similarly, corneodesmosomes are also considered relatively permeable structures. The upper layers of the SC are relatively devoid of these structures, making them more permeable. These so called "defects" in the SC are considered as the preferred sites of electroporation (Pliquett, 1999). Electric field can force electrolyte into these regions leading to the initial fast (<10 μs) decrease in the resistance and increase in current density within this regions. This might lead to joule heating and heating of the surrounding lipids and creating new aqueous pathways which result in a further decrease in the resistance and as long as the electric field lasts the heating front is propagated. Localized heating may even exceed the transition temperature of the lipids, causing a further drop in the resistance. Experimentally such regions have been observed using liquid crystals which respond to temperature by a color change (Pliquett, 2000d). The FFEM studies also point towards a similar mechanism, although indirectly, through observation of the secondary changes i.e. lateral diffusion of water within the SC leading to creation of new pathways, as inferred in the study (Gallo, 1999).

Once these pathways are formed the molecular transport may occur due to either electrophoresis, electro-osmosis, or pressure/concentration gradients or a

combination of these phenomenon. The molecular transport after electroporation has been modeled to predict the functional dependence of transport molecules to various structural features of the SC (Chizmadzev, 1995). A straight-through pathway (transcellular) was shown to be more favorable compared to a tortuous pathway (intercellular). This is also consistent with recent studies which suggest that molecular transport is localized within the SC and is associated with the sites of electroporation. It follows a straight-through path along the bulk of the SC. This study was done with fluorescent probes, calcein and sulforhodamine, which were delivered into the skin by electroporation followed by low voltage iontophoresis. A gel was set immediately below the skin on the epidermal side to trap the probes coming out of the SC. Examination of the gel showed that the fluorescent probes were localized within the gels suggesting that they followed a localized pathway within the skin, termed as the LTR's (Pliquett, 1996d).

Recovery of the resistance starts immediately on stopping the pulse and reaches 10 - 80% of prepulse value within 30 ms of stopping the pulse depending on the pulsing protocol. The regions where the transition temperature has exceeded may not recover or exhibit a very slow recovery, thus accounting for the incomplete recovery of resistance over minutes to hours or sometimes days (Pliquett, 1995b and 1996e).

Since the SC is not as homogeneous as single bilayers, the electroporation pathways differ in their location with the applied voltage. Primarily, low voltages (5-50 V) have been shown to electroporate the appendages since these have lesser

lipid bilayers and hence the transmembrane voltage limit for electroporation can be easily reached in these regions. Several experiments have shown this phenomenon (Chizmadzev, 1998). However, as the voltage is increased (> 50) electroporation of the bulk starts becoming more prominent (Pliquett, 1996e). Theoretical fitting of molecular flux data (of Prausnitz,1993a) by Chizmadzev has shown that the transcorneal pathway gives a better fit for the observed phenomenon compared to the intercellular or appendageal pathways. Similar conclusions were obtained by Edwards et al (1995) who used actual dimensions of the SC fine structures to calculate the best available pathways for electroporative molecular transport and compared them to the molecular flux obtained by Prausnitz et al (1993a).

Electroporation had been likened to iontophoresis occurring at higher voltages. However, the idea has been successfully disproved. The molecular transport in iontophoresis utilizes the phenomenon of electrorepulsion, electro-osmosis and to some extent passive diffusion, however, most of these take place through pre-existing pathways mainly, the appendages (Cullander, 1992b). In electroporation however, new aqueous pathways have shown to be created which are not associated with the appendages (Chen, 1998). In fact, the study showed that iontophoresis did not show any molecular transport in snake skin which has no hair or hair follicles, but electroporation did show significant transport (Chen, 1998). Prausnitz, has given sufficient evidence for the differences between iontophoresis and electroporation (Prausnitz, 1996d).

### 1.5.4.4 Electroporation candidates

Many advantages of electroporation make it a versatile technique for the delivery of large variety of molecules. *In vivo* results with transdermal electroporation have shown that the drug delivery is fast, quite similar to an intravenous injection (Vanbever, 1998a). Moreover, this technique is non-invasive unlike an intravenous injection. Several *in vitro* experiments have shown that complete control over the drug delivery is possible, with large amount of drug being delivered when required (Prausnitz, 1994). These flux increases have been shown to be reversible.

Most importantly, electroporation does not place any restrictions on the physico-chemical properties or the molecules that can be delivered across the skin. Drugs with various physico-chemical properties have been delivered *in vitro* using electroporation. Prausnitz has reviewed the wide range of charged (positive or negative) and uncharged, small and large molecules, lipophilic or hydrophilic drugs. Results have been shown to produce appreciable transdermal flux enhancements by electroporation compared to passive or control samples (Prausnitz, 1999). Cyclosporine, a highly lipophilic drug, which has negligible transdermal delivery by passive diffusion, showed an insignificant enhancement of flux by electroporation alone. However, when the skin was pre-treated with a chemical enhancer like alcohol and then electroporated, the flux increased considerably (Wang, 1998).

#### 1.5.4.4.1 Chemotherapeutic agents

By electroporation of tumor cells, the accessibility of an chemotherapeutic agent into the cell can be increased, thus increasing its efficiency. One of the first

studies was done using bleomycin and the success of the study triggered a wave of new studies using different chemotherapeutic agents, so much so that they have reached stage II and III of clinical trials, using electro chemotherapy (Heller,1996).

Combined with chemotherapy, electroporation has increased efficiency of anti-cancer agents in surface tumors dramatically (Mir 1991 and Belehradek, 1993). Electro chemotherapy is a new therapeutic alternative to treat surface tumors especially of head and neck since these are difficult to remove by other modalities like surgery and chemotherapy. Several other forms of tumors can also be treated by electro chemotherapy (Hoffmann, 1999 and Mir, 1999).

**1.5.4.4.2 Macromolecules - proteins, peptides, genes, oligonucleotides**

The technique has shown potential to deliver macromolecules like insulin, enkephalin and LHRH in significantly higher quantities compared to passive or even iontophoretic delivery (Potts, 1997). This shows the potential for transdermal delivery of proteins and peptides with electroporation (Banga, 1998b). Delivery of oligoneucleotides, plasmids and genes have been shown to be possible with electroporation *in vitro* and *in vivo* (Nishi, 1996, Zhang, 1996, Rols, 1998, Regnier, 1997 and 1999). Some of these techniques involve delivery of the plasmids or genes by intra-atrial injections and then application of electroporation to increase local transfection rates.

An interesting phenomenon was observed with delivery of heparin with electroporation. The transdermal electroporative flux of smaller molecules was higher in the presence of a macromolecule like heparin and remained elevated long after the pulsing was stopped (Vanbever, 1997). It was found that only linear macromolecules produce this enhancement. This effect has been explained using a "foot-in-the-door" hypothesis, large molecules seem to prevent the resealing of the pores created during electroporation and thereby increasing the flux of smaller molecules (Weaver, 1997). Thus, this phenomenon has the potential to be used as an enhancer for transdermal electroporation, further increasing the flux of smaller molecules.

### 1.5.4.5 Electroporation with other enhancers:

#### 1.5.4.5.1. With iontophoresis

*In vitro* studies with LHRH and calcitonin have successfully shown that electroporation given before iontophoresis is more effective than iontophoresis alone (Riviere, 1995 and Bommannan, 1994). Since electroporation creates new pathways, application of iontophoresis after an electroporation pulse would provide more pathways for the current to travel and therefore increase the molecular flux. Iontophoresis and electroporation can be combined to provide complete control over delivery, where iontophoresis can be used to maintain the basal level of a drug and electroporation used to deliver high drug doses when required. Such a system could

prove very useful for the delivery of molecules like insulin which require variable dosing, with high dose required after meals followed by basal levels between meals.

**1.5.4.5.2. With ultrasound**

A combination of electroporation and sonophoresis i.e. ultrasound application to skin produced a synergistic effect (Kost, 1996). This could be due to the fact that both produce alterations in the lipid structure of the SC. Such a combination would reduce the intensity of electrical pulses or ultrasound required to produce the same drug flux, thus decreasing the chances of any harmful effect.

**1.5.4.5.3. With keratinolytics and pressure**

Electroporation has been shown to deliver drugs across the SC through the formation of LTR's. Ilic et al(1999), have devised a system to restrict these LTR's to occur in microholes (40-100 μm diameters) of a thin electrically insulating sheet placed close to the skin. As an extension to this they delivered a low-toxicity keratinolytic molecule through the LTR's into the corneocytes to create microconduits in this restricted area. Thus, a combination of new aqueous pathways through the multilamellar lipid bilayer membranes and subsequent disruption of the keratin matrix by the keratinolytic molecule is believed to result in dislodgement of entire stacks of corneocytes, creating ~50-μm diameter microconduits through the SC. This provides unrestricted pathway for any water soluble molecule to the epidermis. Weaver et al, have delivered macromolecules as well as charged beads across the SC with this method with application of slight pressure. Ultimately, this

type of system has been proposed for delivery of macromolecules or even sampling of interstitial fluids. This type of system would mimic the traditional needle system eliminating the pain. However, the barrier property of skin would be compromised for a longer time than electroporation alone, since the recovery of skin following keratinolysis would be slow. Further studies are needed on the safety and consequences of long term application of this method.

**1.5.4.5.4 Electroincorporation**

Hoffmann et al,1995 have devised a new type of electrode - The meander electrode- to restrict the electric field during electroporation only to the SC (Hoffmann, 1995). The design consists of closely spaced array of electrodes 0.2 mm thick with a gap of 0.2 mm between two electrodes. These electrodes can be placed on the skin and the electric pulses applied. The hypothesis is that since the gap between the electrodes is so small, the electric current has to travel a smaller distance within the skin, resulting in a swallow distribution of electric field. This could lead to electroporation of the SC only and since the current does not travel deeper into the epidermis and dermis it can prevent unwanted nerve stimulation. This translates into less sensations and less pain. In such a system, the electroporation would most likely occur at the sites of deformities leading to field enhancement or at structurally weak points such as existing channels. If particulates like liposomes are placed in the pathway of the electric field, there would be local field enhancements making the surface beneath them preferred sites for breakdown.

Initial pulses are believed to cause pore formation, with the following pulses causing pore enlargements. The enlargements are proportional to the follow through current and energy deposited into the pores which in turn is related to the resistivity offered by the media between the electrode and SC. The meander electrodes are directly placed on the skin without any conductive liquid between. This reduces the resistivity between the electrode and SC and thus a higher energy can be deposited into the pores formed within the SC after the first pulse. This can lead to larger pore diameters, making delivery of particulates possible. Hoffmann et al, have delivered solid latex particles with a diameter of 0.3-0.45 μm across the SC by electroincorporation. Liposomes seem to elongate under the presence of electric field and such elongated liposomes were observed within the viable epidermis using the electroincoporation technique (Hoffmann,1995).

It has to be noted however that results of studies for sensation with the Meander electrodes gave a threshold of 80 V and 20 ms on the facial skin (Zhang, 1999). Possibly the threshold on forearm could have been higher, but were not reported in the above study. The Meander type of electrodes have not been compared with conventional plate or wire electrodes. Further studies might be needed to confirm whether they have a better safety profile and comparable efficacy with regards to drug delivery.

### 1.5.4.6 Present challenges in electroporation (Statement of problem)

Since the exact molecular basis for electroporation has not yet been identified, it is difficult to predict the molecular flux with any particular set of electrical conditions and for a given drug. There exist a large number of variables. The voltage to be applied can range from 100 V to 1000 V. Similarly, the pulse length can vary between several microseconds to milliseconds. The other variables include, number of pulses, rate of pulsing and electrode design. It is difficult to assess the delivery potential of these variables in combination, making the choice empirical. This puts safety *in vivo* at question.

High voltage electric pulses are applied to the body in several clinical techniques such as, transcutaneous nerve stimulation, cardiac defibrillators, electroconvulsive therapy etc; these techniques present as precedents for use of electroporation in humans (Prausnitz, 1996b). However, the safety of electroporation has to be assessed, mainly because of the large currents generated, unlike in the other methods. These currents have the potential to cause stimulation of nerves, pain and also tissue damage. Nerve stimulation beyond a certain limit will lead to pain, which itself can be the limiting cause for the use of electroporation *in vivo*. The voltage and pulse lengths (~100 μs) used in electro chemotherapy clinical trials did not produce any pain in the patients. However, the *in vitro* transdermal drug delivery studies in the literature predict very minimal or no enhancement at the low pulse lengths used in these trials. It would be necessary to know whether patients can

tolerate higher pulse lengths, especially, since the patients in the above trials did experience involuntary muscle contraction at the low pulse lengths. If not then ways will have to be devised to make electroporation effective at low pulse lengths. One way would be to use synergetic enhancers.

Although, some *in vivo* biophysical studies with electroporation and iontophoresis have indicated no differences between the two with respect to the changes caused in the skin. The electrical pulses used in the study were chosen arbitrarily and the safety cannot be extrapolated to any other conditions. Neither were any biochemical changes in the skin studied. So far there is no biophysical or biochemical basis established for the cause of the changes if any. Such studies would be necessary to establish safety of electroporation during chronic use. Thus, only "proof of principle" studies exist on the safety and efficiency of electroporation. Many more studies are required before this technique can be utilized *in vivo*.

**1.5.4.7 Previous work in our laboratory**

Initial focus of our laboratory was to evaluate electroporation for delivery of highly lipophilic drugs. Studies with cyclosporine showed that electroporation alone was ineffective in increasing the flux. But combined with a chemical enhancer like alcohol did produce acceptable results (Wang 1997 and 1998). As a means of using electroporation for delivering peptides we first planned to study the safety and efficacy of electroporation using a model drug which would be easier to analyze quantitatively and present less formulation restraints than peptides. This led us to

terazosin hydrochloride, a moderate sized drug with fluorescence, which could be beneficial to the analysis. Terazosin is not highly hydrophilic or lipophilic hence we could eliminate formulation problems as well as prevent complications in the delivery aspect. Systematic optimization of the electrical parameters for electroporative transdermal delivery of terazosin was carried out with efficacy and safety in mind (Sharma 1999). Preliminary investigations into the safety of electroporation were also performed using electron microscopy to observe any morphological changes. To further improve the efficacy of electroporation we devised novel electrode systems and carried out *in vitro* studies to prove their efficacy (Sharma,2000a, b).

## 2.0 Objectives and Research Plan

### 2.1Objectives:

The present research plan was developed in light of the work done previously in our laboratory and the problems faced in the area of transdermal drug delivery with electroporation.

Specifically, we plan to carry out further optimization of electroporation parameters for the transdermal delivery of terazosin hydrochloride. Assess the potential of these optimized parameters to deliver the drug *in vivo*. Test our hypothesis that larger electrode area leads to better drug delivery by conducting *in vivo* studies with electrodes of different areas. Most importantly, assess the safety of these parameters in specific, and electroporation in general, through various *in vitro* and *in vivo* techniques.

## 2.2 Research Plan

a. To optimize the following electrical parameters for transdermal delivery of terazosin hydrochloride by electroporation

(i) Rate of pulsing

(ii) Pulse length

(iii) Concentration

b. To characterize three electrodes of different areas (for delivering electroporative pulses to skin) with respect to the transdermal voltage produced, current density produced etc;

c. To evaluate the above (*in vitro* optimized) electrical parameters for electroporative delivery of terazosin hydrochloride, *in vivo*, in fuzzy rats using a large area and small area electrode.

d. To carry out studies to assess the safety of electroporation *in vivo* and *in vitro*, specifically:

i) To observe the animal behavior and gross changes in skin occurring during and after electroporation pulses,

ii) To estimate the electrical parameter which might best predict the safety/recovery after electroporation *in vivo*,

iii) Conducting *in vivo* impedance measurements before and after various electroporation protocols and observe the recovery of impedance, and

iv) To observe the biochemical changes in skin occurring after electroporation, specifically to look for changes in the pattern of glucose utilization by skin before and after electroporation, thus establishing an *in vitro* method for assessing the safety of electroporation.

## 3.0 Materials and Methods :

### 3.1 Chemicals :

Terazosin hydrochloride (TRZ) [1-(4-amino-6,7-dimethoxy-2-quinazolinyl)-4-(2-tertrahydrofuroyl) piperazine hydrochloride dihydrate] was provided by Abbott Laboratories, QC, Canada (Mol. Wt. Of terazosin = 387.44). The internal standard for HPLC assay of TRZ, prazosin hydrochloride (PRZ) [1-(4-amino-6,7-dimethoxy-2-quinazolinyl)-4-(2-furoyl) piperazine hydrochloride monohydrate] was purchased from Sigma Chemicals Co., MO, USA. For biochemical studies of skin after electroporation, lactate analysis kits (No.826 UV) were procured from Sigma Diagnostics, MO, USA and Eagle's Minimum Essential Media (MEM) with Earle's Balanced Salt Solution (BSS) was purchased from Sigma Chemical Co., MO, USA. All other solvents and reagents used were of HPLC or analytical grade, respectively. Deionized water was obtained using a Barnstead® Nanopure II system (Barnstead, MA, USA). Cyanopropyl bonded silica Solid Phase Extraction (SPE) cartridges (Bond Elut®, 100mg, 1mL) were obtained from Varian Sample Preparation Products, CA, USA.

### 3.2 Choice of animals :

Ideally, human skin would be best for conducting *in vitro* investigation of any transdermal phenomenon, however the difficulty of obtaining human skin requires that animal skin be used for preliminary studies such as this. It was reported that animal skin is generally more permeable than human skin (Bronaugh,1982). The

choice of animal model in part may depend on the ease of preparation of skin for diffusion cells. Hairless animals such as fuzzy rat or hairless guinea pig provide satisfactory substitutes (Bronaugh,1991 and Marit,1995). Since hairless guinea pigs were not easily available fuzzy rats were used here.

Seven to eight week old male fuzzy rats were procured from Harlan Sprague-Dawley, IN, USA. The rats were housed in the Animal Care Center, Memorial University of Newfoundland and cared in accordance with CCAC institutional guidelines. Plasma for calibration curves was obtained from eight to ten week old in-bred male albino rats (Vivarium, Memorial University of Newfoundland, St.John's, Canada).

### 3.3 Electroporation Unit and Electrodes :

A Gene Pulser® II (Bio-Rad Laboratories, Hercules, CA, USA) was used as the source for generating single and multiple exponentially decaying electrical pulses, with a pulse length of "$\tau$" defined as, the time taken for the applied voltage ($U_{electrode,0}$) to decay to 37% of its peak value. The shocking chamber of the electroporation unit was replaced with custom made silver/silver chloride electrode. The Gene Pulser® II was equipped with a capacitance extender, a pulse controller unit and a panel that displayed the applied voltage (the actual voltage drop across the two electrodes), pulse length in milliseconds and internal resistance of system. The $U_{electrode,0}$ can be defined as the peak value of voltage of an exponentially

decaying electric field applied between anode and cathode during pulsing. Custom made Ag/AgCl delivery electrodes consisted of silver wires (0.5 mm diameter) electro-coated with AgCl. The electrodes were either straight pieces of wire (small-area electrode, 1X = 0.2061 $cm^2$) or spiraled loops of wire (large-area electrode, 5X = 1.023 $cm^2$ and 15X = 3.065 $cm^2$). Studies of *in vivo* or *in vitro* electroporation in the literature (Vanbever,1998 and 1999) have reported applied voltages ($U_{electrode,0}$) as opposed to transdermal voltages ($U_{skin,0}$). Hence, to facilitate comparison we have reported applied voltages.

### 3.4 Isolation of skin for *in vitro* drug delivery studies :

The rats were sacrificed using a carbon dioxide chamber. Full thickness abdominal and back skin were excised and used within 8 hours. Sharma,1999 has shown that, the location from where the skin is removed does not matter if the drug is estimated in the skin rather than the receiver compartment. Subcutaneous fat and muscle layers were carefully removed from the skin. The excised skin samples were hydrated by soaking for two hours in PBS (Phosphate Buffered Saline, pH 6.4, 0.1 M). Skin samples below 2 KΩ were discarded since this could be the result of damaged / leaky skin.

It is important to note that freshly excised skin was used as opposed to frozen skin since it has been shown that frozen skin loses its viability (Wester et al, 1998). Also, full thickness skin as opposed to separated epidermis was used here since

it has been shown that procedures used for separation of epidermis from dermis can damage skin viability (Wester et al, 1998). We believe that using full thickness skin would mimic *in vivo* conditions more closely than would using epidermis only.

### 3.5 Choice of Drug - Terazosin Hydrochloride :

Since the present work was a continuation of the previous study in our laboratory, the same drug used previously i.e. TRZ was used. Briefly, the drug was chosen for the ease with which it could be analyzed in skin by extraction and quantification by HPLC equipped with fluorescence detector. Since the drug allowed the use of fluorescence detection, the interference with skin lipids and enzymes during analysis can be reduced.

### 3.6 *In vitro* Drug Delivery Optimization Experiments:

#### 3.6.1. Assembly *in vitro* drug delivery optimization experiments:

Custom designed side-by side diffusion cells made of Delrin (Polymethanal, Dupont®) were used for all *in vitro* electroporation experiments. A freshly excised, full-thickness, fuzzy rat skin was sandwiched between the donor and receiver compartments, with the SC side of the skin facing the donor compartment. Multiple exponential pulses were delivered to the skin by placing the anode in the donor compartment which also contained TRZ solution (1.03 mg/mL unless stated otherwise) in phosphate buffered saline (PBS, 0.1 M NaCl, 0.02 M phosphate, pH

6.4). The receiver chamber was used as the cathode chamber and contained only PBS. The effective diffusional cross-section area was 1.3 $cm^2$. The electrodes were placed 10 mm from the skin in each compartment, with the anode on the donor side (Fig 3.1). All experiments were performed at room temperature (22 ± 1°C).

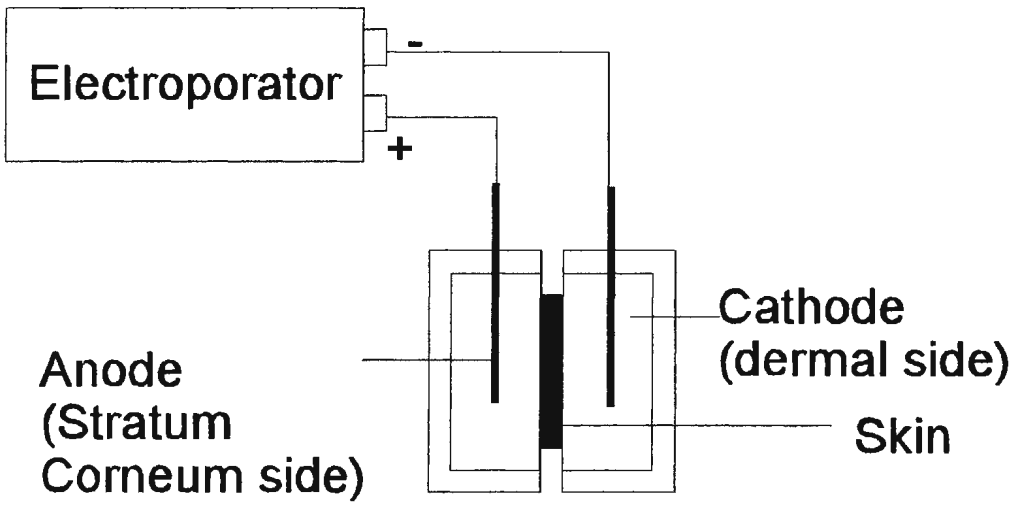



**Figure 3.1 :** Assembly for *in vitro* drug delivery optimization.

### 3.6.2 Drug delivery optimization experiments :

In continuation of our previous experiments to optimize transdermal delivery of TRZ by electroporation (Sharma,2000a) we evaluated the effects of pulse length and rate of pulsing. Three pulse lengths were evaluated viz: 400V, 1 ppm, 10 pulses

were delivered with either one of 20 ms, 30 ms or 40 ms. Likewise three rates of pulsing were evaluated viz: 400 V, 20 ms, 10 pulses delivered at 1 pulse per min (ppm), 4 ppm or 10 ppm. The experiment was divided into two parts for studying each parameter :

(i) The drug was kept in contact with the skin during pulsing and after pulsing for a total of 20 min. The control for this study was 20 min drug contact time but no electrical pulse applied (passive drug delivery). After the 20 min contact time, the skin was analyzed (ref 3.6.4) for TRZ content. The TRZ delivered under both conditions was compared to obtain the permeation enhancement by electroporation over passive delivery.

(ii) During pulsing the donor side contained only PBS with no drug in it. Within 10 seconds after pulsing the PBS was replaced with the drug solution and kept in contact for 10 min. The control for this study was 10 min drug contact time and no electrical pulse was applied (passive drug delivery). In either case after the 10 min contact time, the skin was analyzed (ref 3.6.4) for TRZ content. The amount of TRZ delivered was compared. If electroporation conditions cause complete reversibility then the permeability of skin should be the same as control (passive) and if drug was placed immediately after electroporation, the amount of drug in skin should be same as control (passive). Thus, this part of the experiment would tell us about the extent of skin recovery after the use of particular electroporation parameter(s).

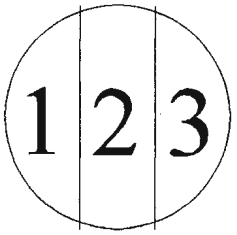
The effect of donor concentration was also studied. 400 V, 20 ms, 10 pulses at 1 ppm were delivered with either 0.1 or 1 % TRZ solution in contact with skin for a total of 20 min (10 min during pulsing and 10 min after pulsing). Control for this was a 20 min passive drug delivery with either 0.1 or 1 % TRZ solution in contact with skin. After the 20 min contact time, the skin was analyzed (ref 3.6.4) for TRZ content. The delivery with and without electroporation was compared.

### 3.6.3 *In vitro* experiments to characterize drug distribution in skin :

Previous experiments in our laboratory had revealed different current density values across the skin for different size/shape electrodes (Sharma,2000b). We wanted to investigate whether this current density was uneven causing uneven (or localized) electroporation in the skin and thus uneven drug distribution. An experiment was designed to characterize the drug distribution in the skin after electroporation.

Electroporation pulses were delivered to skin with either a small area electrode (1X, 0.2061$cm^2$) or a large area electrode[(15X), 3.065$cm^2$)] at $U_{electrode,0}$ of 400 V, pulse length 20 ms, 10 pulses at 1 ppm with TRZ (1.0 mg/mL) in contact for 20 min (10 min during pulsing and 10 min after pulsing). Control for this was a 20 min passive drug delivery with TRZ (1.0 mg/mL) in contact with skin. After the 20 min contact period, the skin was washed thrice with deionized water, stripped in a controlled manner with Scotch® tape thrice to remove the superficially adsorbed drug

(see Sec 3.6.4). Approximately 1.3 $cm^2$ of the skin corresponding to the area exposed to the drug was cut and the excess area unexposed to the drug discarded. The exposed skin piece was further cut into three equal pieces, dried with Kim® wipes and weighed (Figure 3.2). One milliliter of methanol and an appropriate amount of PRZ was added to these pieces and then extracted as described in Sec 3.6.4 and analyzed for TRZ content. The amount of TRZ in each skin piece was normalized to the weight of the piece.




Skin piece

**Figure 3. 2:** Diagramatic representation of the way skin was cut for characterization of drug distribution.

### 3.6.4 Extraction of drug from skin - sample preparation for HPLC :

After pulsing, the diffusion chambers were emptied, dismantled and skin specimens were removed. The SC side of the skin was washed with deionized water three times and Scotch® adhesive tape (3M, Ontario, Canada) was used to strip it three times to remove any superficially attached drug. It was found that the drug in the tape strips was less than 0.01% (Sharma,1999). 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 PRZ 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 TRZ content (section 3.8).

### 3.7 *In vivo* Drug Delivery Experiments :

#### 3.7.1 Assembly for *in vivo* drug delivery experiments:

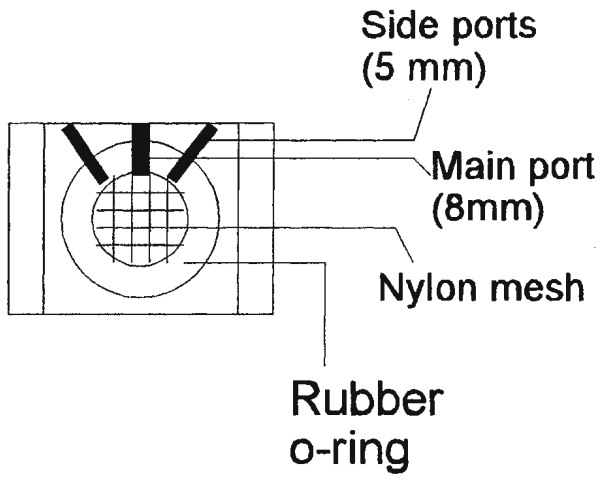



**Figure 3.3 :** Diffusion cell for *in vivo* drug delivery experiment.

A side-by-side diffusion cell unit, made of Plexiglass® (Acrylic, Johnson's Industrial Plastics Ltd, ON, Canada) was used. The rats were subjected to light halothane anaesthesia and then restrained using a cloth restrainer. The diffusion cells were then pinched together on a skin fold on the dorsal side the rat. The drug

solution (TRZ, 10.03 mg/mL) in PBS was added to both cells to permit maximum delivery. The distance between the skin and electrodes during pulsing was 8 mm. A nylon mesh was placed between the skin and the electrode to prevent the electrodes from coming into direct contact with the skin (Figures 3.3 and 3.4).

### 3.7.2 *In vivo* Drug Delivery Experiments:

The drug delivery experiments were performed using the small area electrode with $U_{electrode,0}$ of 400 V ($U_{skin,0} \cong 100$ V) and large area electrode with $U_{electrode,0}$ of 250 ($U_{skin,0} \cong 100$ V) and 400 V ($U_{skin,0} \cong 160$ V). This allowed comparison of the two electrodes at similar values of both $U_{electrode,0}$ and $U_{skin,0}$. The other electrical parameters were taken from the optimization studies, viz: 20 ms pulse length, 20 pulses at 10 pulses per minute (ppm). The electric pulses were applied and the rat was transferred back to its cage soon after the pulsing was over, the total drug contact time was 2 min. The controls consisted of passive drug delivery for either 2 min or 20 min, with the same conditions as above but no electric pulses were applied. Intravenous drug (30 µg / kg) was administered through the femoral vein. Subcutaneous injections (60 µg / kg) were given on the abdominal side. The blood samples were taken from the sino-orbital vein of the rat maintained under halothane anaesthesia. These were immediately centrifuged at 13,000 g for 6 min and the isolated plasma samples were stored at -20°C until analysis.

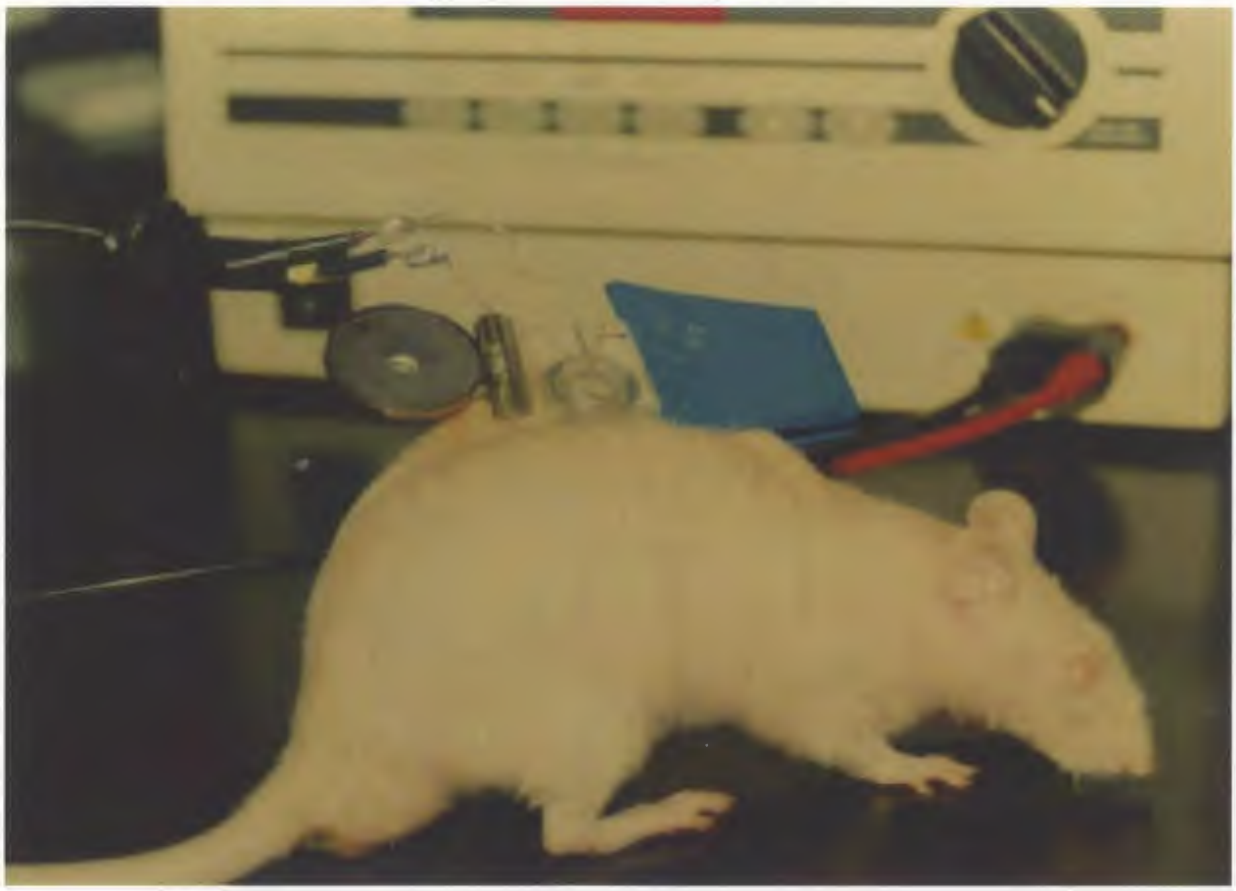


**Figure 3.4:** Photograph of the assembly for *in vivo* drug delivery with electroporation.

### 3.7.3 Extraction of drug from plasma by Solid Phase Extraction (SPE) - sample preparation for HPLC (Singh, 1995) :

Bond Elut® columns for SPE were first conditioned using 1.0 mL of methanol followed by 1.0 mL of PBS (pH 7.0). Plasma samples (0.2 mL) were spiked with 20µL of PRZ internal standard and 0.5 mL of phosphate buffer (pH 7.0), and mixed using a vortex mixer. The samples were then extracted using conditioned SPE columns with two 0.5mL portions of extraction solution consisting of tetrahydrofuran and acetonitrile in respective volume ratio of 1:3. The eluate was evaporated to dryness using nitrogen gas in a thermostated water bath (37°C). The residue was

reconstitued in 100µL of mobile phase for HPLC analysis (section 3.8). For calibration curve determination the blank plasma was spiked with internal standard, PRZ, and standard solutions of TRZ and extracted similarly.

**3.8 Drug Analysis by HPLC (Singh, 1995) :**

The HPLC system consisted of a Waters® Model 510 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 cm cartridge packed with Nova-Pak® C18 reverse phase particles, fitted with Nova-Pak® C18 guard column. Responses were recorded as peaks on a 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 0.22µm membrane filter at least twice (Millipore Corporation, MA, USA) and was pumped isocratically at a flow rate of 1.0 mL/min. Accumet® model pH meter (Fisher Scientific, PA, USA) was used to measure pH of the solutions. The *in vivo* sample extracts were quantified using the same HPLC procedure.

### 3.9 Measurements of Skin Electrical Parameters *In Vitro*:

#### 3.9.1 Assembly for measurements of skin electrical parameters *in vitro*:

A side-by-side diffusion cell made with Plexiglass® (Acrylic, Johnson's Industrial Plastics Ltd, ON, Canada) was used. The cross-section area was maintained at 1.3 $cm^2$. Three holes were made on the donor and receiver side of the cell at 5, 10 and 15 mm from the side to facilitate the insertion of electrodes. Two Ag/AgCl electrodes were placed in the nearest holes (inner electrodes) on either side (5 mm) of the cell, these electrodes were simple pieces of silver wires (d = 0.5mm) electrocoated with silver chloride. Two Ag/AgCl electrodes were placed at 10 mm (outer electrodes) and these were either simple pieces of silver wires or spiraled silver wires (to increase electrode area, ref section 5.3) electrocoated with silver chloride. The inner electrodes were connected to channel 2 of a Tectronix oscilloscope through a 1:100 differential amplifier, whereas the outer electrodes were connected to the electroporator. A 5 ohm resistance was connected on the outer circuit in series with the diffusion cell (Figure 3.5) to measure the current flowing through the circuit. The voltage drop across the resistor was measured on channel 1 of the oscilloscope and the current was calculated using Ohms law (I = V/R). The probes used to connect the amplifier were properly compensated and calibrated using a Eppley standard cell, The Eppley laboratory Inc., Newport,RI, USA. Freshly excised skin pieces were sandwiched between the diffusion cells. A fresh piece of skin was used for each pulse and all the

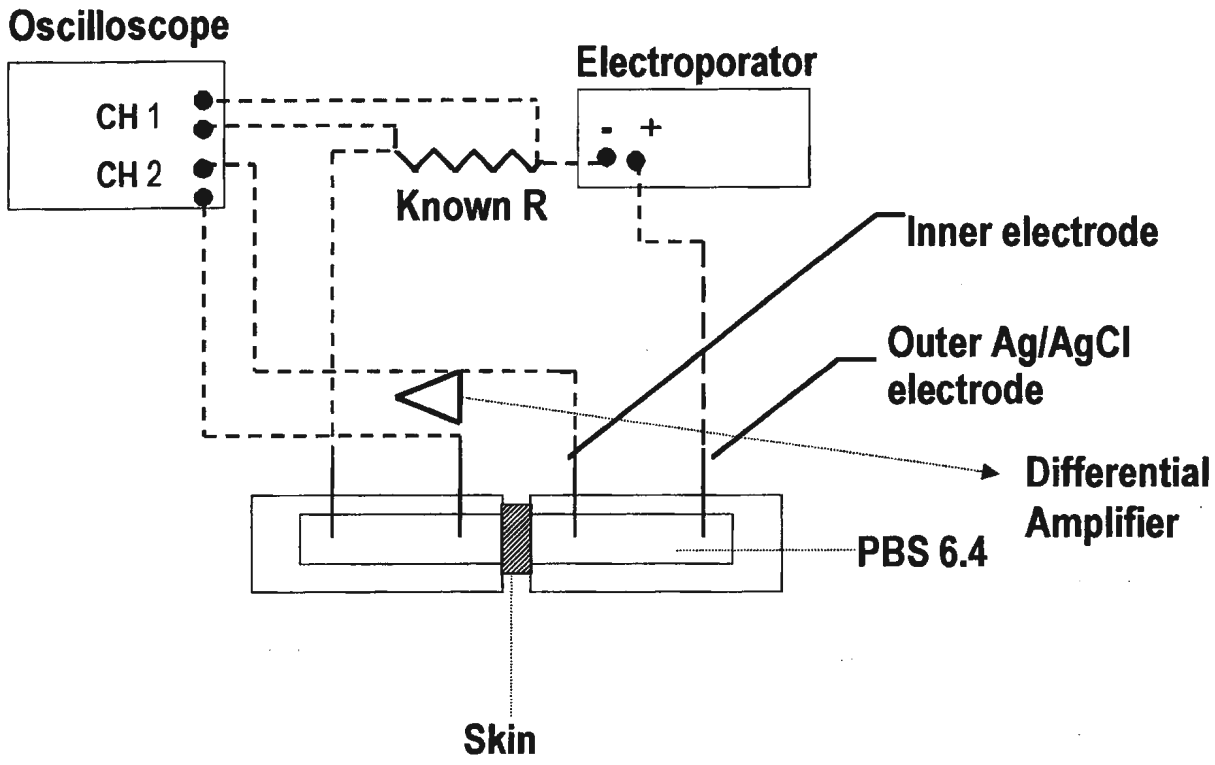



**Figure 3. 5:** Assembly for *in vitro* measurement of skin electrical parameters.

parameters recorded and then discarded. PBS (pH 6.4) was used as the electrolyte.

### 3.9.2 Experimental procedures :

Solution resistance ($R_{sol}$) was first calculated by applying a range of voltages (100 to 1000 V) using the outer electrodes (repeated for all different electrode areas) and measuring the voltage drop across the inner electrodes ($U_{inner,0}$) and current flowing through the circuit.

$$R_{sol} = U_{inner,0} / I_0 \qquad \text{.....(Eqn 3.1)}$$

To calculate the voltage drop across the skin (transdermal voltage, $U_{skin,0}$),

various combination of $U_{electrode,0}$ and pulse lengths were applied to the skin using all three electrode sizes / types. The $U_{inner,0}$ and $I_0$ during each of these pulses were recorded. From these measurements the $U_{skin,0}$ was calculated using the following equation :

$$U_{skin,0} = U_{inner,0} - (R_{sol} \times I_0) \qquad \text{.....(Eqn 3.2)}$$

Knowing the current in the circuit, pulse length and the transdermal voltage, a large number of parameters can be calculated (Sharma et al, 2000b) viz: dynamic resistance ($R_{dy}$) which is the resistance of the skin at the beginning (time = 0) of pulse, current density (current per unit area of skin), instantaneous power density to skin (Instantaneous power per unit area of skin), and energy density per pulse to the skin (energy per unit area of skin). The charge delivered to the skin (Q) can also be calculated. Since the current decreases exponentially it can be calculated as follows :

$$Charge = I_0 \int_0^{\tau} e^{-t}.dt = I_0[-e^{-t}]_0^{\tau} = I_0 - I_0 e^{-\tau} \qquad \text{....(Eqn 3.3)}$$

### 3.10 *In vivo* Impedance Measurements :

#### 3.10.1 Assembly for i*n vivo* impedance measurements :

The assembly was similar to the one described above for *in vivo* drug delivery except that each of the cells had an additional hole at 5 mm from the open end to facilitate insertion of another pair of electrodes. The outer electrodes (at 8 mm from

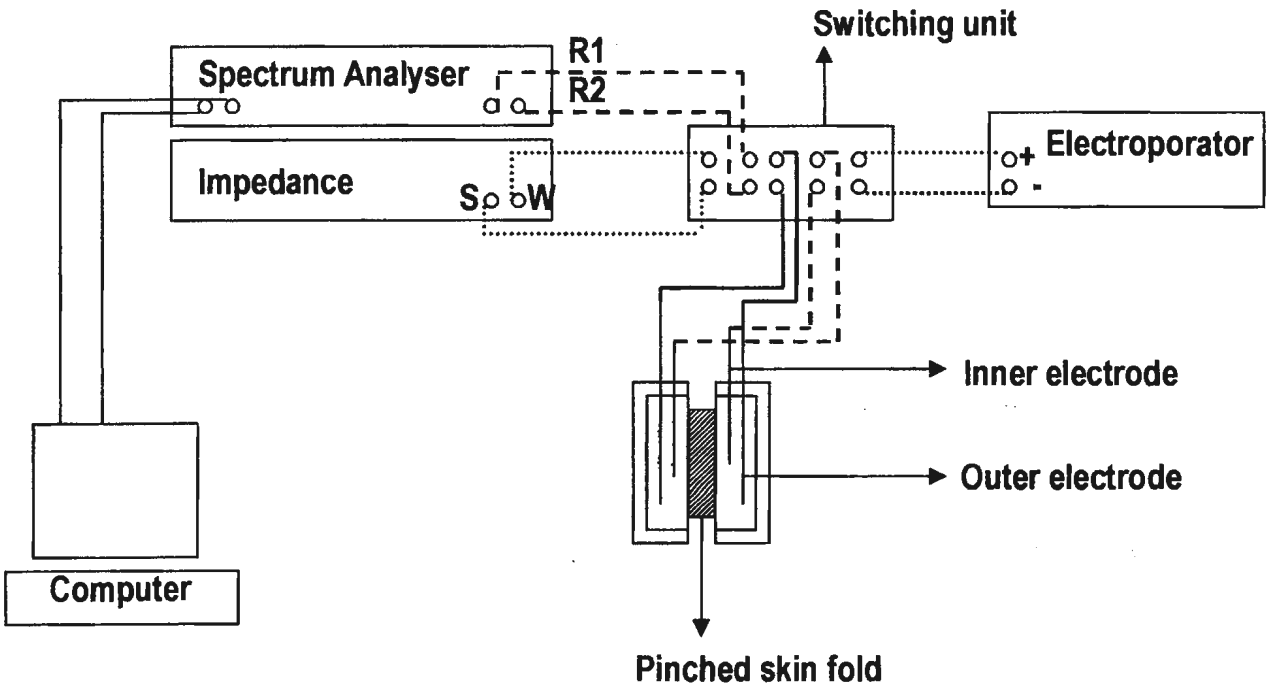



**Figure 3.6:** Assembly for *in vivo* skin impedance measurements after electroporation.

skin) used to deliver the electrical pulses were also connected to the impedance measurement equipment. The anode side was used as the working electrode and the cathode as the secondary electrode. The inner electrodes were connected to the spectrum analyzer (reference electrode1 to cathode side and reference electrode 2 to the anode side). All the electrodes were connected using a switching unit. When the switch was turned on, it connected only the outer electrodes to the electroporator and the inner electrodes were disconnected and the assembly was ready for delivery of electroporation pulses. When the switch was in the off position, the outer and

inner electrodes were connected only to the impedance measurement equipment. The impedance data were recorded and analyzed using a computer software - Z-plot© (Solartron analytical, TX, USA) (Fig 3.6).

**3.10.2. Experimental procedures for *in vivo* impedance measurements :**

The animals were anaesthetized by giving sodium pentobarbitone by i.p. (50 mg/kg) and restrained with cloth for the first two hours and released into their cages. Thereafter, they were restrained again only during each measurement. The diffusion cells were pinched on to the dorsal skin fold of the rats. The electrodes were arranged as described earlier (section 3.7.1). The real (representing the resistive component) and imaginary (representing the capacitive component) impedance (see sec 1.3.5.) was measured between 1 - 65,000 Hz with 0.0 bias. The data were captured in a software - Z plot© and a Nyquist plot was drawn using Sigma Plot© 5.0. This represented the passive impedance or control. The switch was then put "on" and electroporation pulses were delivered. The following protocols were evaluated (n=2 for each) :

(i) 400 V, 1 ms, 1 pulse with 1X electrode

(ii) 400 V 10 ms, 1 pulse with 1X electrode

(iii) 400 V, 20 ms, 20 pulses at 10 ppm with 1X electrode

(iv) 400 V, 20 ms, 1 pulse with 15X electrode

Immediately after pulsing (within 10 sec) the switch was put to the "off" position and the impedance measurement was started (0 hr). The impedance was recorded at 30

min, 1.0, 1.5, 2.5, 4.5, 7.5 and 24 hr after pulsing. The rats were put back into their cages after 2.5 hrs (or earlier if the anaesthesia wore off) and re-restrained during measurements only. The area of skin where cells had been attached was clearly marked with ink to help replace the cells again in the same spot.

### 3.11 Skin Viability Assessment Using Glucose Utilization Index :

#### 3.11.1 Assembly for skin viability assessment and experimental procedure :

Side-by-side diffusion cells with water jackets to maintain temperature at 37°C were used. The water temperature was maintained using thermostat by circulating it through a water bath. Each cell was equipped with a port for removing samples. Also, the contents of the cell were constantly stirred by using a magnetic bar and magnetic stirrer (Fig 3.7). The Minimum Essential Media with Earle's Balanced Salt Solution (MEM-BSS) was kept only on the receiver side and the dermal side, the SC side was kept empty.

Freshly excised skin either (immediately) after electroporation treatment or without treatment was sandwiched between the two diffusion cells. The following treatments were carried out:

(i) Passive - no electroporation pulses applied (n = 4)

(ii) 400 V, 20 ms, 10 pulses at 1 ppm (n = 6)

(iii) 500 V, 60 ms, 20 pulses at 1 ppm (n = 3)

(iv) 1000 V, 5.74 ms, 20 pulses at 1 ppm (n = 5)

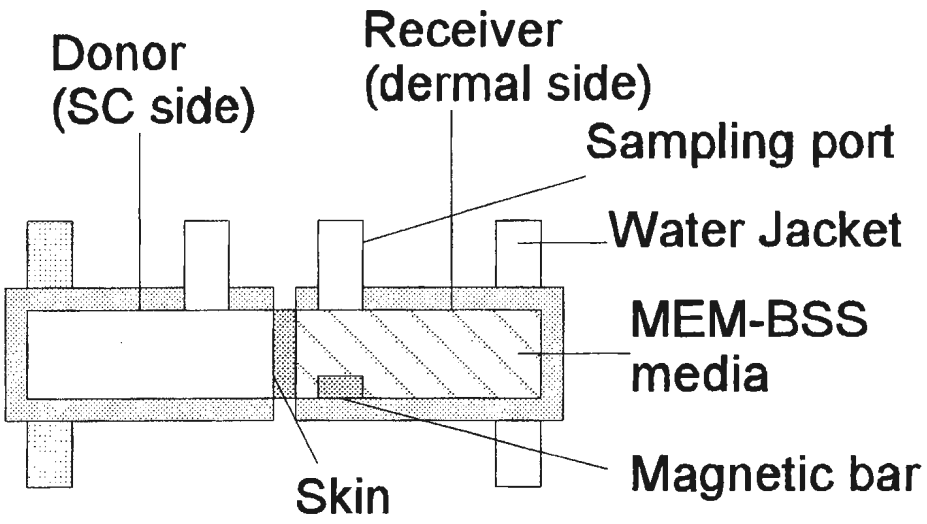



**Figure 3.7:** Assembly for *in vitro* skin viability assessment.

The electroporation pulses were delivered using the same assembly described in section 5.6.1, with PBS on both side of the skin. Immediately after pulsing (within 1min) the skin was transferred to the assembly of Figure 6 and incubated with the medium. Samples of 100 µL were withdrawn at 0 min, 2, 4, 6, 8, 12 and 24 hr and replaced with 100 µL of fresh media. The samples were analyzed immediately as described below.

**3.11.2 Background for lactate analysis :**

The ability of skin to convert glucose to lactate was used as a measure of the skin's viability (Wester, 1998), since this conversion involves the formation of adenosine triphosphate (ATP), which is the energy source of the living cells.

Glucose is one of the components of MEM-BSS media. Viable skin converts this to lactate, which is measured in this experiment. Sigma Diagnostic's, lactate analysis kit #826 UV was used. The procedure for the calibration curve and estimation of lactate was followed exactly as mentioned in the kit. The brief principle of the assay is described below:

$$\text{Pyruvate} + \text{NADH} \xleftarrow{\text{LD}} \text{Lactate} + \text{NAD}$$

Excess of nicotinamide adenine dinucleotide (NAD) is added to the lactate present in the solution in the presence of lactate dehydrogenase (LD). The pyruvate formed is trapped with hydrazine to force the above reaction to the left. The nicotinamide adenine dinucleotide dehydrogenate (NADH) formed is measured by UV spectrophotometry at $\lambda$= 340 nm.

**3.11.3 Procedure for lactate analysis :**

*Calibration curve :*The lactate standard provided in the kit was diluted with water and then mixed with the analysis reagent (see below) to prepare the following lactate standards: 0,12,36,60,96,120 mg/dL. The mixed solutions were incubated for 30 min at room temperature and then the UV absorbance at 340 nm was measured for each of the solutions. The concentration of lactate was plotted *versus* the absorbance and the regression equation was determined.

*Analysis :* 100 µl of the test solution was incubated with 2.9 mL of reagent mixture

for 30 min at room temperature and the UV absorbance measured at 340 nm. The concentration in the test solution was calculated using the regression equation of the standards.

The analysis reagent mixture contained glycine buffer (0.6 mol/L) and hydrazine, pH 9.2, NAD, lactate dehydrogenase, and distilled water.

**3.12 Data Analysis :**

All experiments were performed a minimum of three times and statistical calculations were done using Sigma Stat® 2.0 software. Group data were compared using one way ANOVA followed by pairwise multiple comparison procedures (Tuckey test).

## 4.0 Results and Discussion:

### 4.1 *In vitro* Electroporative Drug Delivery Studies :

#### 4.1.1 Drug delivery optimization :

The difference between the amount of TRZ delivered to the skin when TRZ solution was brought in contact with the skin soon after delivering an electroporation pulse and the passive diffusion gives an idea of the degree of reversal of the skin's permeability. A drug delivery higher than passive diffusion indicates that the barrier property of skin has not completely recovered. Previously, Sharma et al used the reversibility of skin's permeability barrier as an index to evaluate safety of electroporation (Sharma, 2000b). Here the concept of reversibility was used to optimize the electrical parameters for drug delivery by electroporation. Possibly reversibility of the permeability barrier could have totally different mechanisms under *in vitro* and *in vivo* conditions. However, the long term safety of electroporation would require that the changes in skin permeability caused by electroporation be reversed as soon as possible. If not, the electroporation technique may be considered as invasive as an injection, defeating the original purpose of developing electroporation as a non-invasive delivery system.

**A) Effect of pulse length :** Twenty pulses of 20 ms at $U_{electrode,0}$ of 400V, were found optimal for TRZ delivery (Sharma, 2000a). The optimization was based on the efficiency of drug delivery and observation of gross changes in the skin. In our study three pulse lengths were investigated using reversibility of the barrier property.

Figure 4.1 shows the results of TRZ delivery obtained with ten pulses, 1ppm and of 20, 30 or 40 ms at $U_{electrode,0}$ of 400 V. Amount of drug delivered increased with increase in pulse length. Figure 4.2 compares the passive delivery of TRZ obtained without subjecting the skin to any electroporation pulse with those obtained after subjecting the skin to 20, 30 and 40 ms lengths of electroporation pulses, and then introducing the drug to the already pulsed skin as stated above. The higher TRZ amount with the post-pulse passive diffusion indicates that the permeability of skin was irreversibly increased. Increasing pulse length from 20 ms to 30 ms caused a significant increase ($p<0.005$) in delivery. However, there was no significant difference between the TRZ levels obtained after subjecting the skin to 30 and 40 ms pulses. Decreasing pulse length below 20 ms to 10 ms, greatly decreased the delivery of TRZ (Sharma, 2000a). Thus, to minimize any damage to the skin and obtain reasonable drug delivery 20 ms was chosen as the optimum pulse length at $U_{electrode,0}$ 400 V to deliver TRZ.

**B) Effect of rate of pulsing:** The effect of three rates of pulsing was investigated, 1 pulse per minute (ppm), 4 ppm and 10 ppm using $U_{electrode,0}$ 400 V and 10 ms pulses. No differences were found (Figure 4.3) in the amount of TRZ delivered between the three pulse rates, 1 ppm, 4 ppm and 10 ppm ($p>0.05$). Neither was there any difference between the reversibility profiles when the three pulse rates were compared ($p>0.05$).

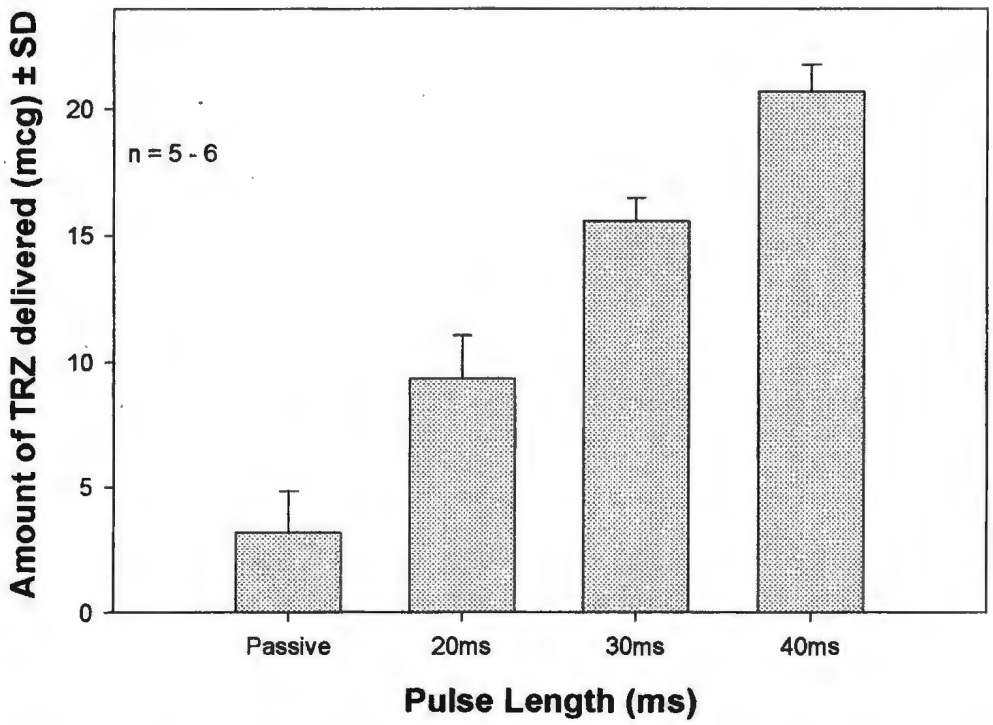



**Figure 4.1 :** Effect of increasing pulse length (x-axis) on TRZ delivery to skin (y-axis) -drug in contact with skin during and after electroporation.
Other conditions were applied voltage of 400V, 10 pulses, 1 ppm with 1% TRZ in contact with skin during and after pulsing for a total of 20 minutes. Passive was 20 minutes drug in contact with skin without pulsing.
The electroporated samples are significantly different from passive diffusion ($P<0.001$)
* The three electroporated samples are significantly different from each other ($P<0.001$).
Statistical test was one-way ANOVA followed by pairwise multiple comparison procedures (Tuckey test).

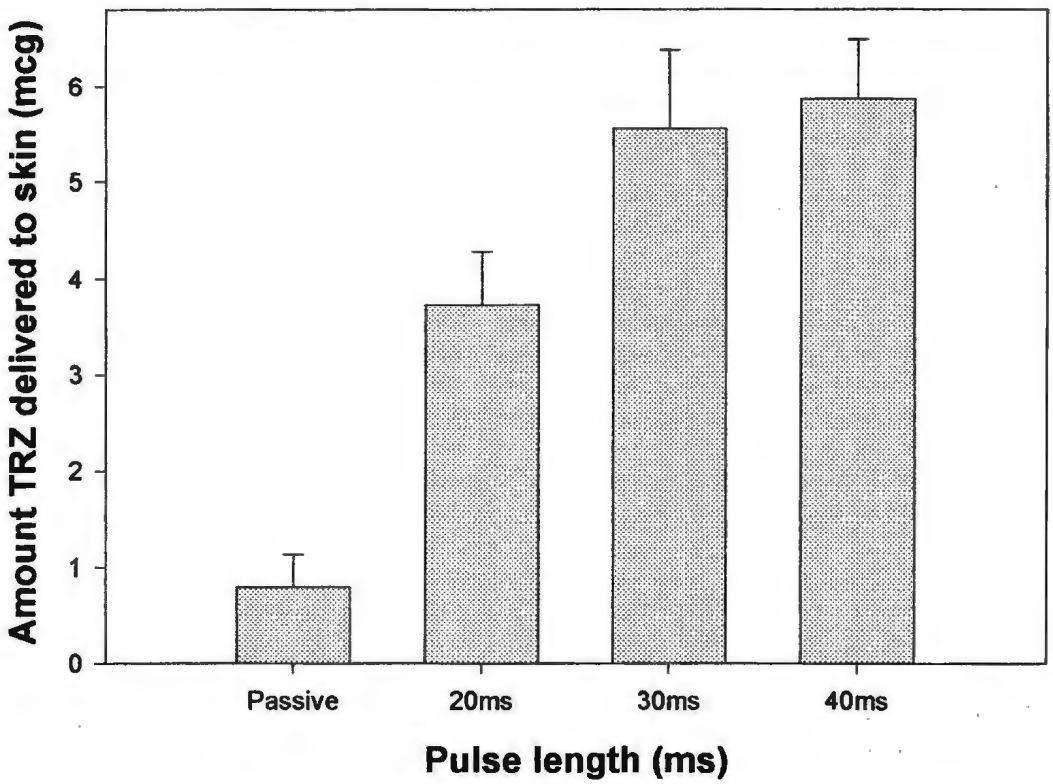



**Figure 4.2 :** Effect of pulse length (x-axis) on amount TRZ delivered to the skin (y-axis) - Drug in contact with skin only after electroporation pulses were stopped. Other conditions were: applied voltage of 400V, 10 pulses, at 1 ppm with 1% TRZ placed in contact with skin, within 10 sec of stopping pulses, for a total of 10 min. and passive was 10 min without pulsing.
Passive samples are significantly different from electroporated samples ($P<0.001$), The 20 ms samples are significantly different from 30 ms & 40 ms ($P<0.005$), The 30 ms and 40 ms samples were not significantly different from each other ($P=0.75$). Statistical test was one-way ANOVA followed by pairwise multiple comparison procedures (Tuckey test)

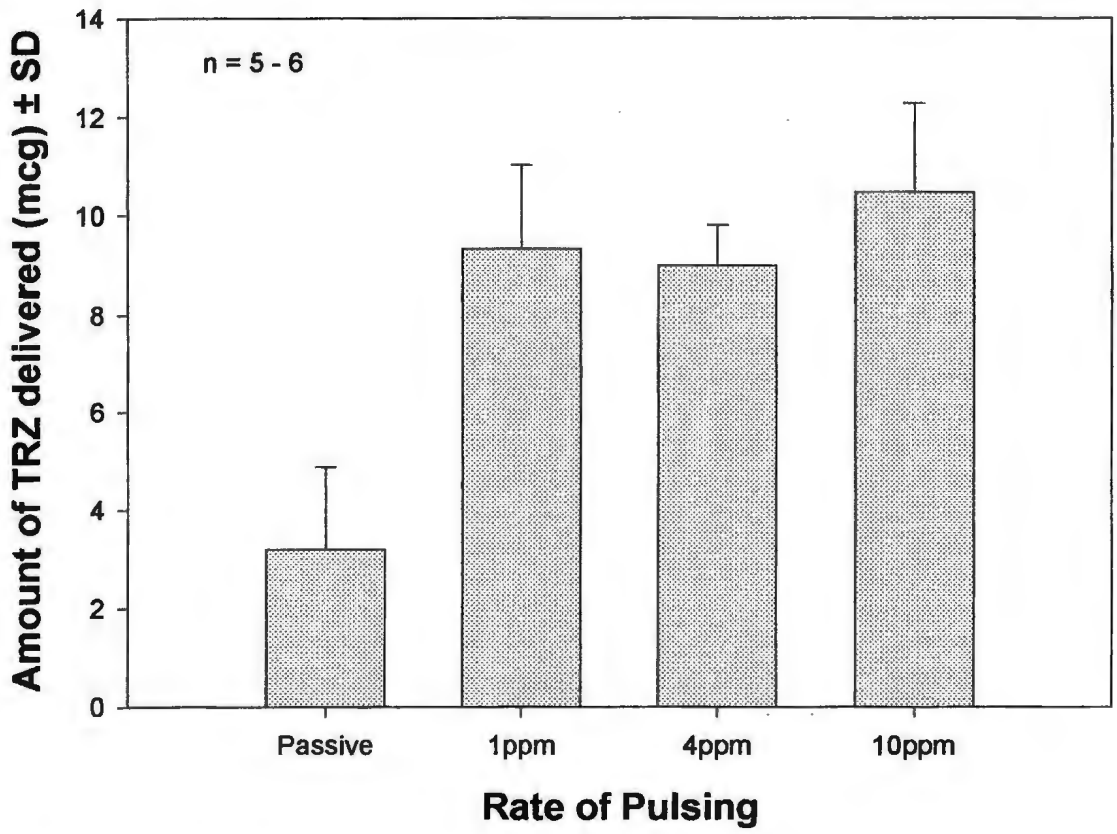



**Figure 4.3:** Effect of rate of pulsing (x-axis) on amount of TRZ delivered to the skin (y-axis). Other pulse conditions were; applied voltage of 400V, 20 ms, 10 pulses, with 1% TRZ in contact with skin during and after pulsing, for a total of 20 minutes. Passive diffusion was 20 min drug delivery in contact with skin without pulsing.

There is a significant difference between passive and electroporated samples ($P<0.001$). There is no significant difference between the three electroporation protocols ($P = 0.27$) Statistical test was one-way ANOVA followed by pairwise multiple comparison procedures (Tuckey test).

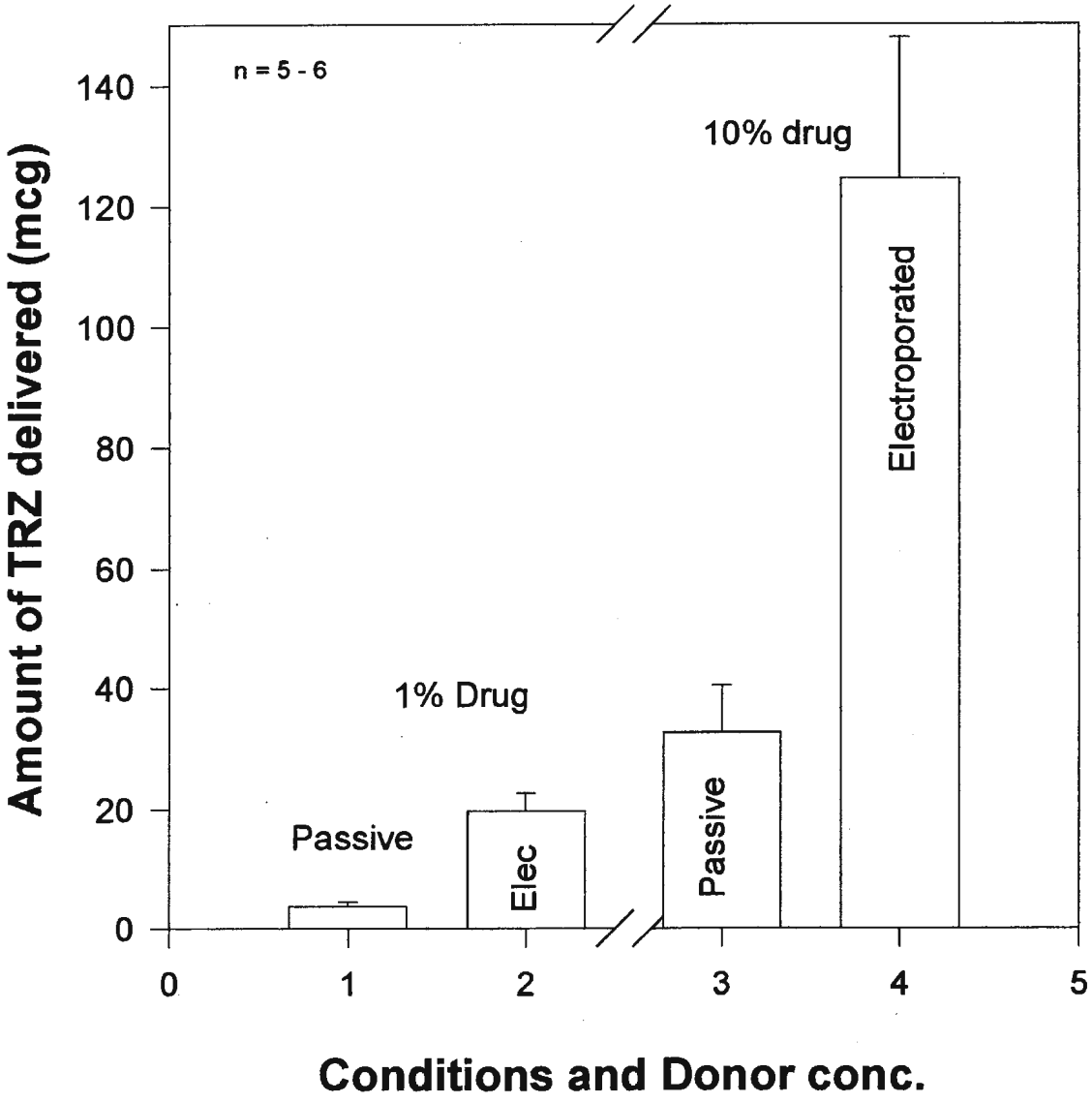



**Figure 4.4:** Effect of increasing donor TRZ concentration (x-axis) on amount of drug delivered to skin (y-axis) by electroporation and passive delivery. Pulsing conditions were 400V,20 ms, 10 pulses at 1ppm with1% or 10% w/v TRZ in contact with skin during and after pulsing for a total of 20 mins. Passive drug delivery consisted of 20 mins drug contact time with no pulses applied.

Earlier studies have pointed out that most of the drug was delivered during pulsing rather than after pulsing (Sharma, 2000b and Vanbever, 1996b). Thus, the drug could be kept in contact during pulsing only and removed soon after pulsing. Using a faster rate of pulsing would be beneficial in the *in vivo* studies, as the animals would have to be restrained for a shorter period of time and a larger number of pulses could be delivered in a relatively short period of time.

**C) Effect of drug concentration in the donor :** Increasing the drug concentration from 1% to 10% resulted in increased TRZ delivery both during passive and electroporative treatments (Figure 4.4). The amount of TRZ delivered with the use of electroporation pulse increased from 21 µg with 1% solution to 119 µg with 10% solution. The nearly six fold increase in drug delivery may not be surprising. Nevertheless, maximizing drug concentration at the donor cell is an important aspect that could be utilized to minimize the use of electroporation pulses. Less number of pulses in a short time would improve compliance.

To summarize, use of 20 pulses at 10 ppm with an $U_{electrode,0}$ of 400V and a pulse length of 20 ms were found to be optimal electroporation conditions for TRZ delivery. These electroporation conditions and the use of electrodes with varying surface areas were subsequently evaluated *in vivo* in rats.

### 4.1.2 Characterization of drug distribution in skin :

Sharma et al indicated possible non-uniformity in current density distribution with different shape/sized electrodes (Sharma, 2000b). A small area electrode which

was basically a simple piece of wire produced a distinct red lesion on the skin after electroporation, parallel to the electrode. However, a large area electrode shaped as a spiraled coil did not produce any such deleterious effects at the same electrical conditions. This phenomenon was further investigated in the following study. The hypothesis was that if the electrode produces non-uniform current density, it would cause non-uniform electroporation within the skin and thus the drug diffusion would be non-uniform.

In the study, the drug was delivered by electroporation with the above two types of electrodes after which the skin was cut into three equal pieces. Since the wire electrode produced a lesion parallel to its position, the skin was cut into three pieces of which the middle piece (subscript 2) was parallel to the wire electrode (Figure 3.2). For the large area electrode and control (passive delivery) the skin was cut in the same manner. The drug delivery was estimated in these pieces and the results were compared using one way ANOVA followed by multiple pairwise comparisons (Tuckey test). No differences in amount delivered were found between the three pieces for any electrodes (Table 4.1). It could be argued that lateral diffusion took place leading to uniform drug distribution within the skin. However, Pliquett, 1996d, and Chen, 1998a, have shown that the drug transport during electroporation occurs primarily through localized transport regions (LTR's) and that increasing the number of pulses only causes the LTR's to widen. Thus, an uneven current density during electroporation could have produced an uneven distribution

of LTR's and thus uneven drug distribution. However, this was not the case in this study.

If an electrode is touching the skin or is very close to the skin, a burn may be produced (Hoffmann,1999 and Pliquett,1999). This could have been a cause of the lesion seen with the wire electrode in the previous study (Sharma 1999). Such contact can be prevented by using a wire mesh to separate the electrode from skin and also using a conductive gel to localize the electrode rather than an electrolyte solution.

**Table 4.1:** Results of drug distribution experiment. P = Passive delivery (control), S = Small area electrode (wire electrode) L= Large area electrode (coiled electrode). Subscripts 1, 2 and 3 represent the three pieces cut out. n= 3-5 for all conditions, results are mean ± standard deviations.

| Condition | Amount of TRZ in skin ± SD (mg/g of skin) |
|---|---|
| $P_1$ | 0.018 ± 0.005 |
| $P_2$ | 0.020 ± 0.008 |
| $P_3$ | 0.031 ± 0.02 |
| $S_1$ | 0.099 ± 0.014 |
| $S_2$ | 0.146 ± 0.069 |
| $S_3$ | 0.13 ± 0.05 |
| $L_1$ | 0.184 ± 0.024 |
| $L_2$ | 0.176 ± 0.042 |
| $L_3$ | 0.157 ± 0.048 |

## 4.2 *In Vivo* Electroporation Studies :

### 4.2.1 Safety of electroporation *in vivo* :

The rats tolerated the electroporation pulses at $U_{electrode,0}$ of 400 V ($U_{skin,0} \approx$ 100 V), although muscle twitching was seen during the pulse delivery. Electroporative delivery caused a slight discoloration of the rat skin at the site of delivery with the small-area electrode, which subsided within a few minutes after stopping the pulse. This effect was not distinguishable from the discoloration produced by just pinching the diffusion cells to the rat skin. Electroporation pulses with the large area electrode at $U_{electrode,0}$ of 250 V ($U_{skin,0} \approx$ 100 V) also did not produce any visible damage. However, with the large area electrode at $U_{electrode,0}$ of 400 V ($U_{skin,0} \approx$ 150 V) erythema, more pronounced on the anode side, developed within an hour of the treatment, and lasted for nearly a day. Edema, which lasted for a day, also developed after about 2-3 hrs of pulse treatment. Two of the five rats receiving pulses with the large area electrode at $U_{electrode,0}$ of 400 V developed an eschar at around the third day after pulsing, which fell off within 20 days. Such observations were reported during electrochemotheraphy studies (Hoffmann, 1999). The damaging effects seen with the large area electrode at 400V were also observed in controls, wherein electroporation was done without the drug, ruling out any drug-related adverse effects.

Sharma et al, used a large-area electrode similar to the one used in the above study. It was shown to produce a higher drug delivery and transdermal

voltage, as well as a higher current for the same $U_{electrode,0}$, as compared to the small-area electrode (Sharma, 2000a and b). Interestingly, histological studies showed that at the same transdermal voltages the large-area electrode showed fewer morphological changes compared to the small-area electrode (Sharma, 2000b). This was suggested to be due to the more uniform current distribution of the large-area electrode. In the morphological studies the skin was frozen immediately after pulsing. Changes taking place after late periods of time were not observed. In the study conducted here, the development of the erythema and eschar was recorded hours and days after pulsing. Localized joule heating could have occurred leading to tissue necrosis. Such observations are not uncommon and are hypothesized to be a function of the electrical field strength in the tissue (Zachary et al, 1990). Further characterization of both large and small area electrodes would be necessary to clearly understand the effects produced by these electrodes. Miklavcic, 1998, used magnetic resonance current density imaging along with finite element three-dimensional analysis to evaluate two different types of electrochemotherapy electrodes. Such studies would be very useful in characterizing and designing a new electrode.

A recent review by Vanbever,1999b, suggests the use of pulse lengths shorter than 1 ms to reduce muscle twitching. However, muscle twitching was reported at 100 microseconds in electrochemotheraphy clinical trials (Hoffmann, 1999). *In vitro* studies suggest that delivery as well as damage increases linearly

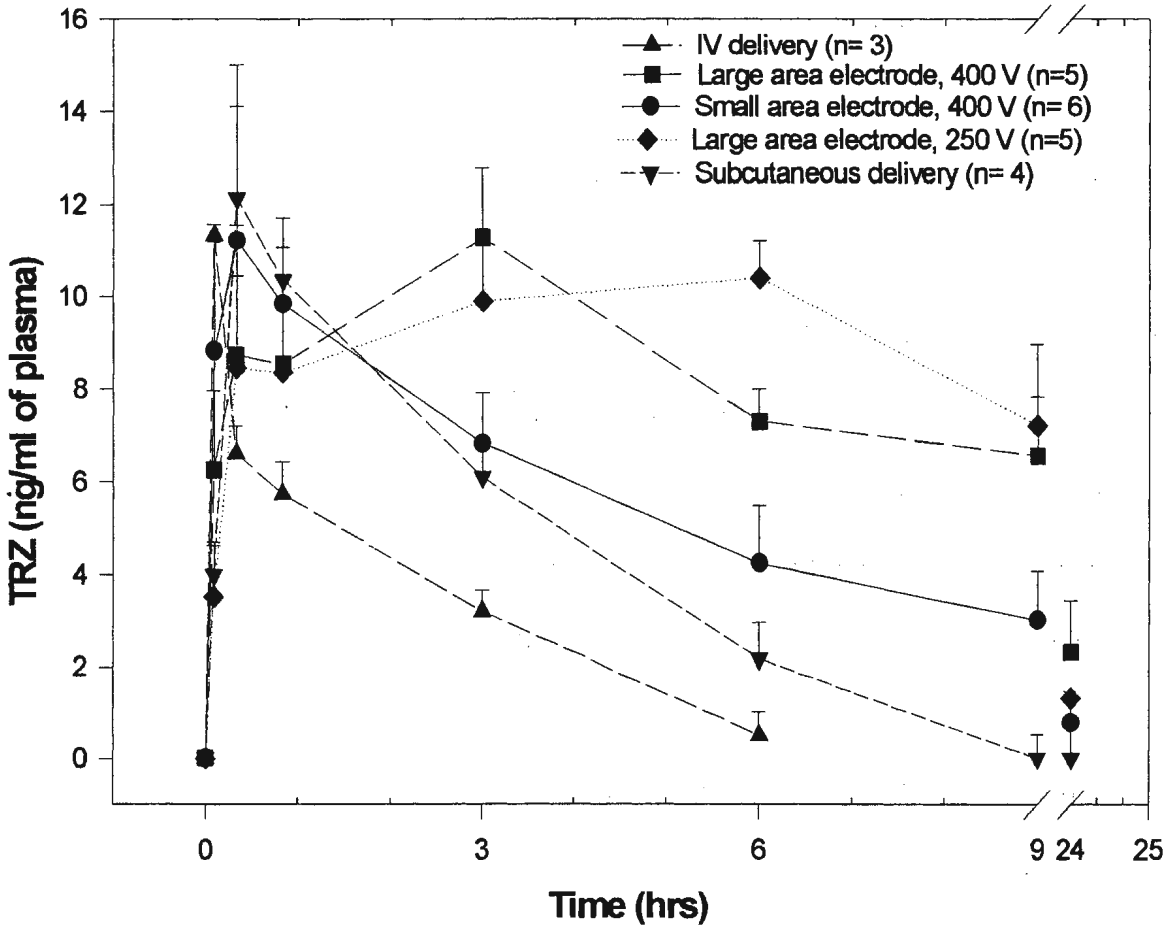



**Figure 4.5:** In vivo electroporative drug delivery in male fuzzy rats. Plasma TRZ concentration (y-axis) against time (x-axis) curves for various treatments. Passive delivery showed no detectable drug levels. Pulse conditions for both types of electrodes were: applied voltage 400 V, 20 ms pulse length, 20 pulses at 10 ppm, 10% TRZ in contact only during pulsing(2 mins).

with pulse lengths and voltages (Vanbever, 1999 and Sharma, 2000a). A study reported in a patent, for improved electrode design for use in electroporation, an $U_{electrode,0}$ of 300V and 1 ms pulse length appeared to be the upper limit of tolerance in humans (Weaver, 1996). Zhang et al,1999, also developed a new electrode design which was used to deliver vitamin C. They reported a threshold of tolerable sensation in human facial skin at an $U_{electrode,0}$ of 80 V and 20ms with a cream formulation of vitamin C, and 50 V, 2 ms with a suspension formulation. The improvement in drug delivery over passive delivery with these conditions was only marginal. Even if these parameters are taken as rough indicators of acceptable limits, with the small amount of drug delivered it would be difficult to make electroporation acceptable as an alternative means of drug delivery. We are not aware of any other studies which comment on the tolerable human thresholds for the various electrical parameters with the traditional wire and plate type electrodes used in electroporation.

### 4.2.2 *In Vivo* Drug Delivery Studies :

Figure 4.5 shows the results of the *in vivo* studies. The passive drug delivery (2 min to 20 min exposure) showed no measurable drug delivery. The electroporation treatment showed significant delivery. Nearly two thirds of the peak drug concentration ($C_{max}$) was reached within the first 5 min. Thus, reasonably high drug delivery can be achieved using electroporation within a short period of time, just as with an intravenous injection. The larger area electrode showed higher

delivery compared to the small-area electrode. Although the $C_{max}$ in each case was similar, the time ($t_{max}$ ) to reach $C_{max}$ was 20 min for the small-area electrode compared to 6 hours for the large-area electrode at similar $U_{skin,0}$. The half lives of drug delivered by electroporation were also higher than that of drug delivered by IV or SC. This is a case exhibiting flip-flop kinetics wherein the half-life is influenced more by the absorption phase than the elimination phase, a common phenomenon in sustained release drug delivery systems.

Longer half-lives and $t_{max}$ indicate drug depot formation within the skin after electroporation enhanced delivery. This observation is in agreement with previous reports (Prausnitz,1993a and Vanbever,1998a). Depot formation has been reported for many drugs delivered by the transdermal route (Guy,1989 and Singh,1996). One suggested mechanism is limitation of dermal blood perfusion, which could decrease the rate of drug absorption (Benowitz, 1992). If it is assumed that in electroporation the drug is delivered just below the SC of the epidermis and vascular dermis at a much faster rate than in passive delivery (Vanbever, 1996b and Lombry 2000), and if the blood flow to skin is not the limiting factor a higher delivery should produce a higher $C_{max}$ with the same $t_{max}$. The large area electrode delivered a larger quantity of drug compared to small area electrode. Therefore, it should have recorded a higher $C_{max}$ but this was not seen in our study, suggesting that blood flow could be another factor affecting drug uptake. TRZ is a peripheral vasodilator, thus it may increase the blood flow to the skin. However, we cannot rule out a perfusion barrier

to its uptake, since one could argue that it may not increase the blood flow enough to eliminate perfusion limited drug uptake.

Such a scenario was seen in iontophoretic drug delivery, which also provided higher levels of drug than passive diffusion in the skin for vascular uptake (Riviere, 1991). Thus there could be two events happening in depot formation, initially a perfusion-limited drug uptake could be taking place and then as the drug level in the skin gets reduced, diffusion through the epidermis could be taking over which could be a rate limiting step. Further studies will be needed to elucidate the exact mechanisms involved in depot formation.

Ultrasound (3.5 MHz, <1 W/cm$^2$) may block follicular routes by causing a flow of sebum from the sebaceous glands into the follicle. Similar effects can be seen if the hair follicle is heated, i.e., by electric current either by low or high voltage application (Meiden, 1998). Thus, even blocking of newly created pathways, as in electroporation, is possible. This may also lead to localized heating and thus damage as observed with the large area electrode at $U_{electrode,0}$ of 400 V. Also this could explain the lower drug delivery seen here with the large area electrode at $U_{electrode,0}$ of 400 V rather than 250 V.

### 4.2.3 Estimation of pharmacokinetic parameters :

To estimate the pharmacokinetic parameters, TRZ was given by IV and subcutaneous routes. Table 4.2 shows the various estimated pharmacokinetic

parameters. Area Under the Curves (AUC's) were calculated by the trapezoidal rule and half-lives were calculated from the terminal portions of the logarithm of drug delivered *versus* time curves for the four treatments. The terminal half-lives for both the small-area and large-area electrodes, were higher than that of IV or subcutaneous injections. As explained previously, these half-lives indicate drug depot formation within the skin. Bioavailability for the subcutaneous injection was calculated from the AUC of IV and subcutaneous injections. Similarly the amount of drug delivered was calculated for the two electrodes using the AUC's. Around

**Table 4.2:** Estimated pharmacokinetic parameters from *in vivo* data (± SD).

| **Delivery conditions** | $t_{1/2}$ **(hrs)** | $t_{max}$ **(hrs)** | $C_{max}$ **(ng/ml of plasma)** | $AUC_0^\infty$ | **Amount delivered (µg)** |
|---|---|---|---|---|---|
| IV injection (30µg/kg) | 2.6 ± 0.35 | 0.083 | 11.3 ± 0.3 | 24 ± 5.24 | 14.5 ± 1 |
| Subcutaneous injection (60µg/kg) | 2.3 ± 1.08 | 0.33 ± 0.19 | 12.1 ± 2.94 | 41 ± 10.35 | 25 ± 6.13 |
| Small area electrode,400V | 6.6 ± 2.15 | 0.33 ± 0.29 | 11.2 ± 4.48 | 90 ± 53.03 | 55 ± 17.96 |
| Large area electrode, 400V | 10.5 ± 3.6 | 3.0 ± 1.16 | 11.3 ± 2.62 | 178 ± 57.96 | 109 ± 18.28 |
| Large area electrode, 250V | 6.0 ± 1.83 | 6.0 ± 1.83 | 10.4 ± 1.14 | 207 ± 40.16 | 125 ± 23.77 |

96% of the drug recovery was seen in the subcutaneous route, which rules out any significant drug metabolism in the subcutaneous tissue. In a previous *in vitro* study matching $U_{skin,0}$ voltages gave similar deliveries irrespective of electrode area (Sharma, 2000a). However, *in vivo* the average amount of drug delivered by the large-area electrode was nearly twice that of the small-area electrode with similar $U_{skin,0}$. This needs further studies for clear understanding.

**4.3 Measurements of Electrical Parameters of Skin During Electroporation - Effect of Electrode Area :**

It was seen that the $U_{skin,0}$ increased with increase in applied voltage keeping pulse length constant (Figure 4.6a). However, it decreased with an increase in pulse length when the applied voltage was kept constant (Figure 4.6b).

The current density increased with increase in either applied voltage (Figure 4.7a) or pulse length (Figure 4.7b) - with the other parameter kept constant. Instantaneous power density showed an increase with applied voltage when the pulse length was constant (Figure 4.8) and remained nearly constant with increase in pulse length . The $U_{skin,0}$, initial current density and instantaneous power density showed an increasing trend with increasing electrode area at any given applied voltage and/or pulse length conditions

However, the small-area electrode showed a lower dynamic resistance than the 5 or 15X coil electrodes with either increasing applied voltage and constant pulse length(Figure 4.9) or increasing pulse length with constant applied voltage

(Figure 4.10). This can be explained by disproportionate increase in $U_{skin,0}$ relative to current as the applied voltage and electrode area are both increased. Nevertheless, a smaller dynamic resistance could mean lower skin resistance or "more aqueous pathways". This should actually cause an increase in drug delivery, but no increase was observed. This could be explained by an increased electrophoretic component in case of the larger-area electrodes as indicated by the increase in instantaneous power and current density when electrode area was increased, since TRZ is ionized at pH 6.4. If this electrophoretic component was removed, drug transport with the small-area electrode could be higher or at least comparable with the large-area electrodes. It would be interesting to observe the drug transport for all three electrodes if the electrophoretic component of the transport was removed by making the drug unionized in the electrolyte.

The energy density delivered to skin also increased with increasing electrode area at the same applied voltage (Figure 4.11). Pliquett et al (1996b) have proposed the formation and expansion of localized dissipation regions (LDR's) in skin during electroporation. The expansion of these LDR's was suggested to be because of localized joule heating. If this heat exceeds the transition temperature of the lipids it could lead to irreversible damage. Increasing the amount of energy delivered could only worsen the situation. Thus, a large-area electrode may cause more damage to skin than a small-area electrode at the same applied voltage. This was also observed in the *in vivo* studies.

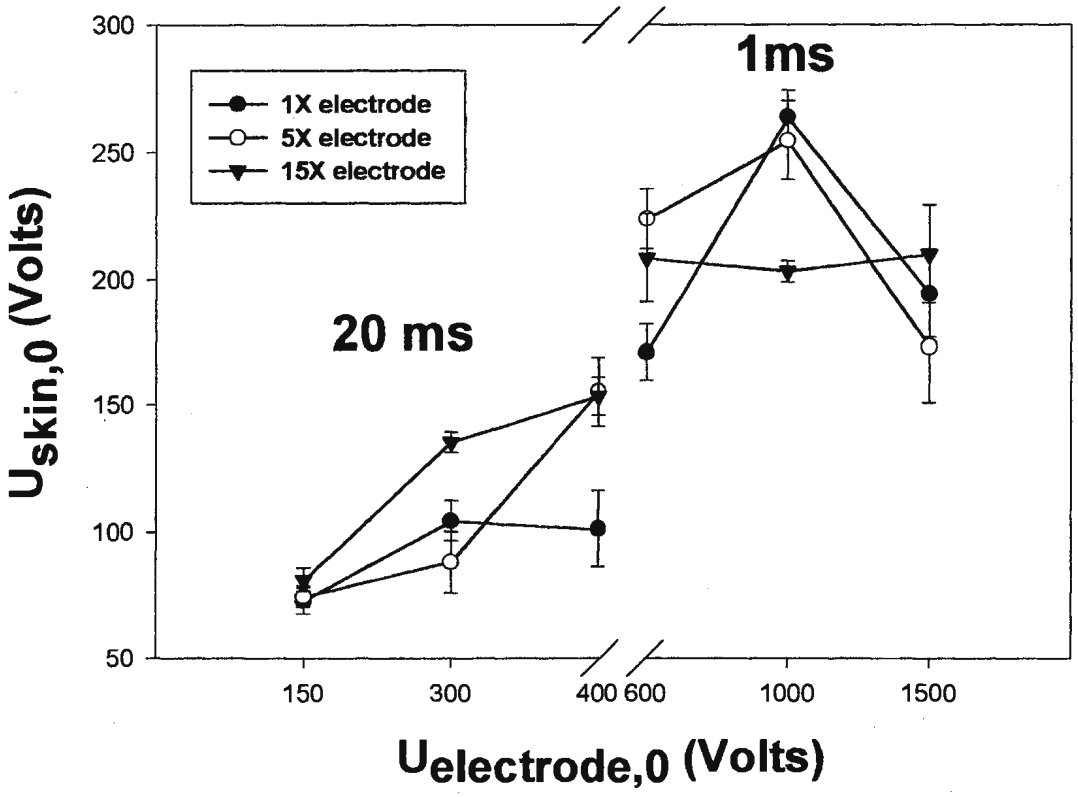



**Figure 4.6a:** $U_{skin,0}$ in relation to the applied voltage ($U_{electrode,0}$) as the pulse length is kept constant.

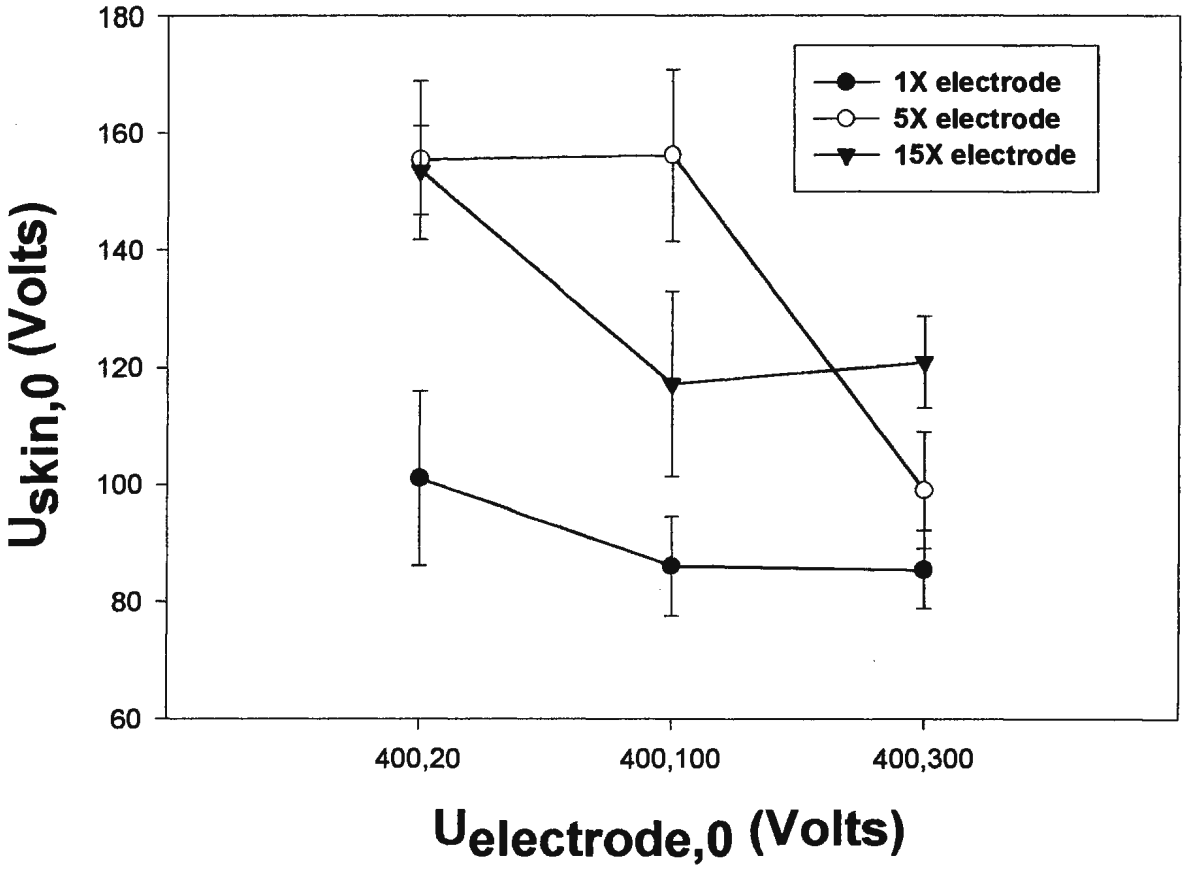



Figure 4.6b : Uskin,0 in relation to the pulse length when the applied voltage ($U_{electrode,0}$) is kept constant.

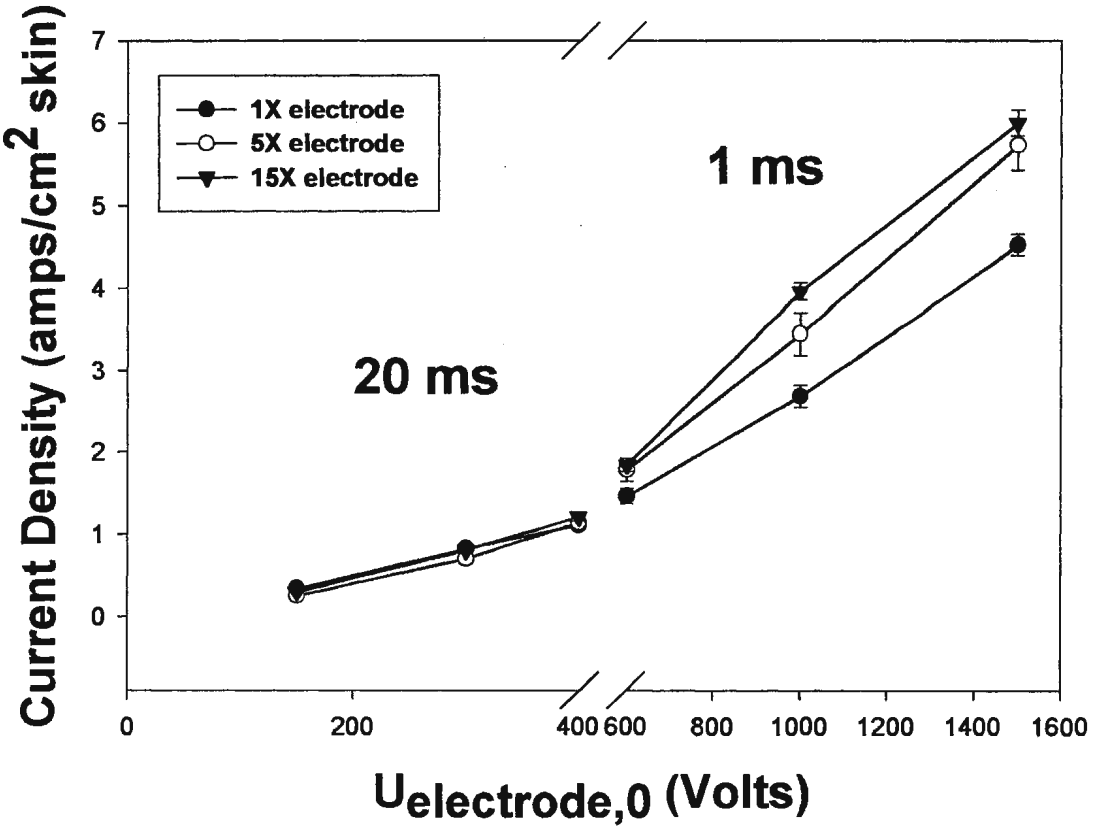



**Figure 4.7a:** Instantaneous current density in relation to the applied voltage ($U_{electrode,0}$) with the pulse length kept constant.

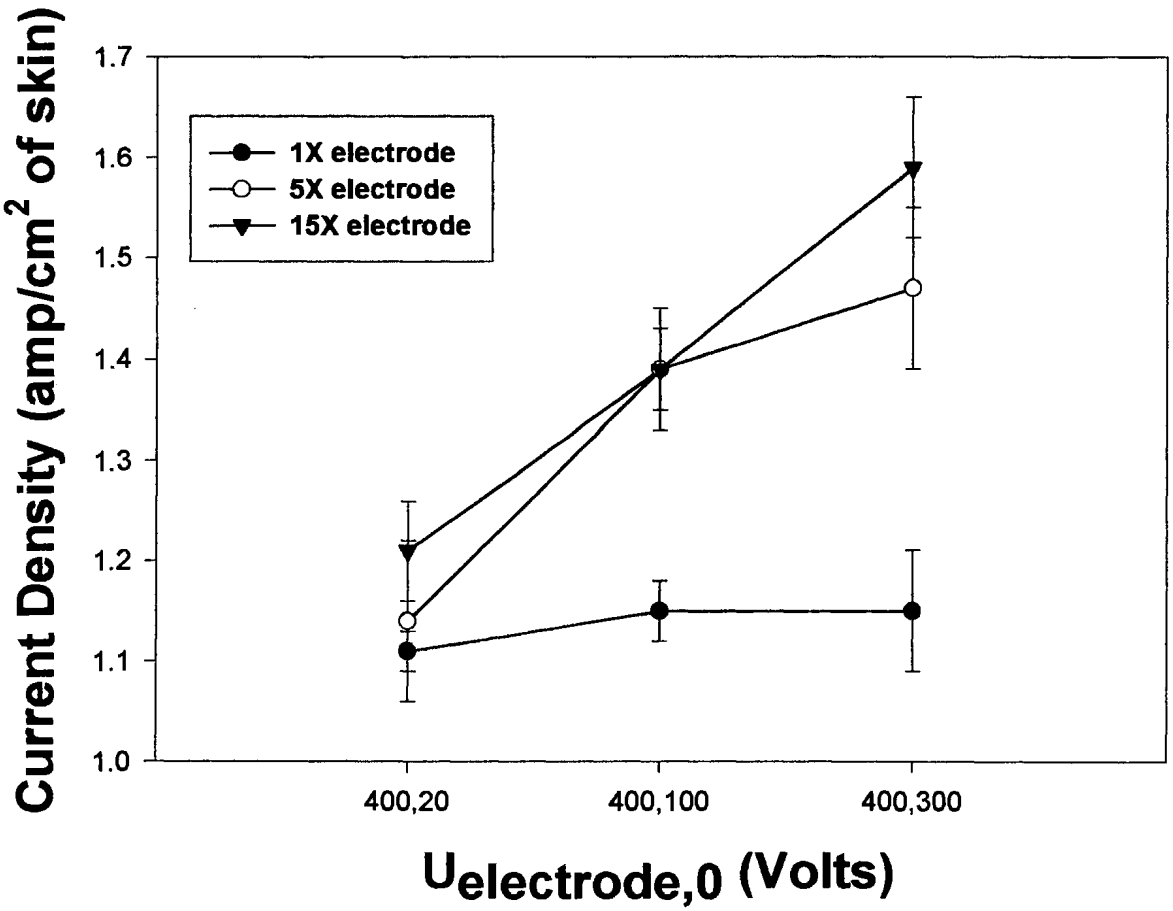



**Figure 4.7b:** Instantaneous current density in relation to the pulse length when the applied voltage ($U_{electrode,0}$) was kept constant.

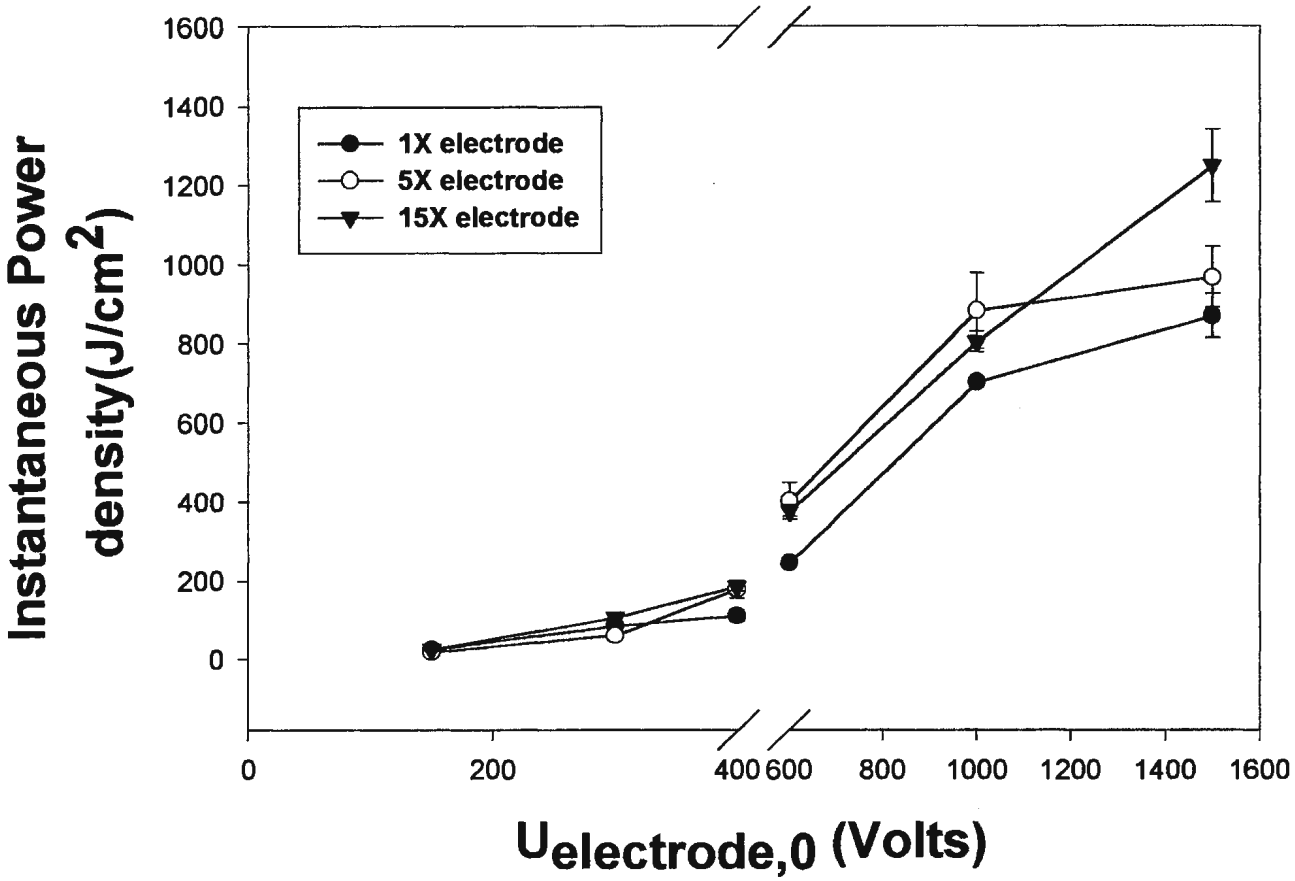



**Figure 4.8:** Instantaneous power density in relation to the applied voltage ($U_{electrode,0}$) with the pulse length kept constant.

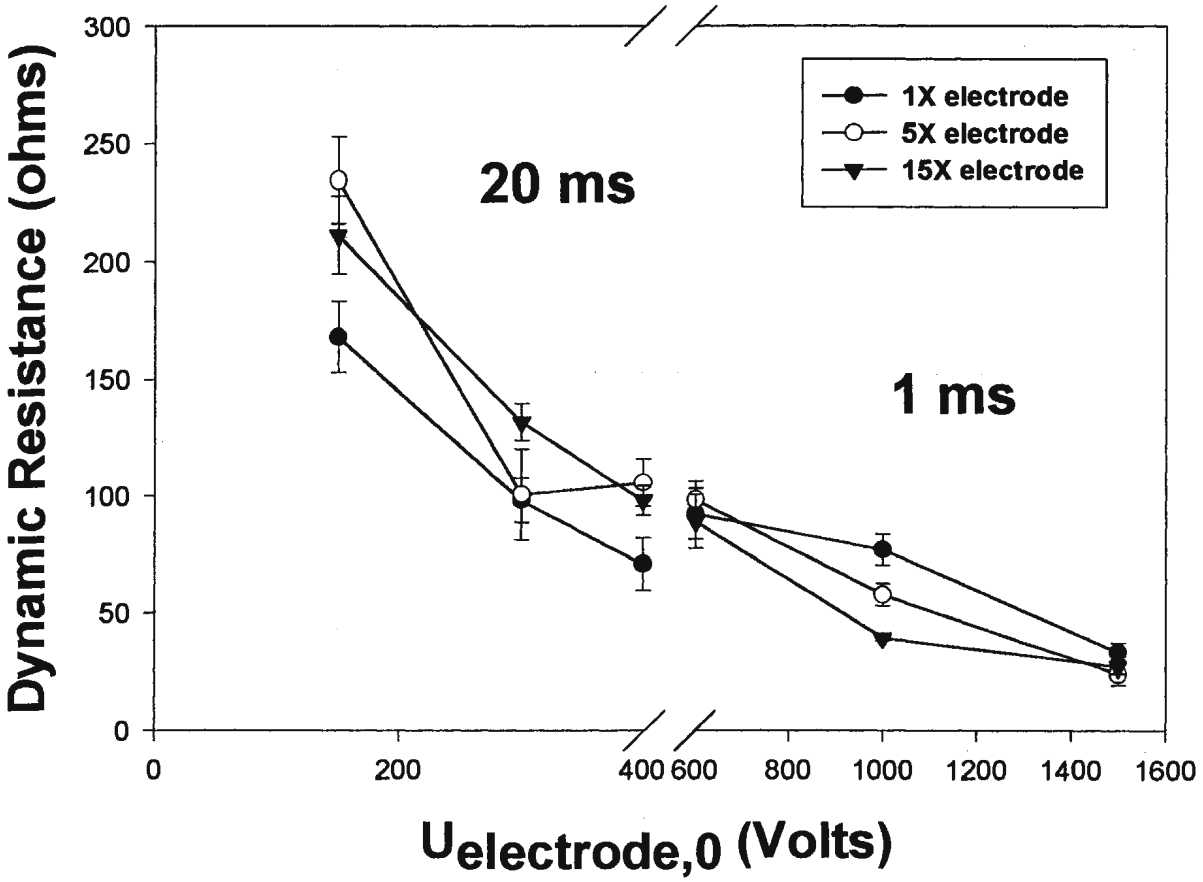



**Figure 4.9:** Dynamic resistance in relation to the applied voltage ($U_{electrode,0}$) with the pulse length kept constant.

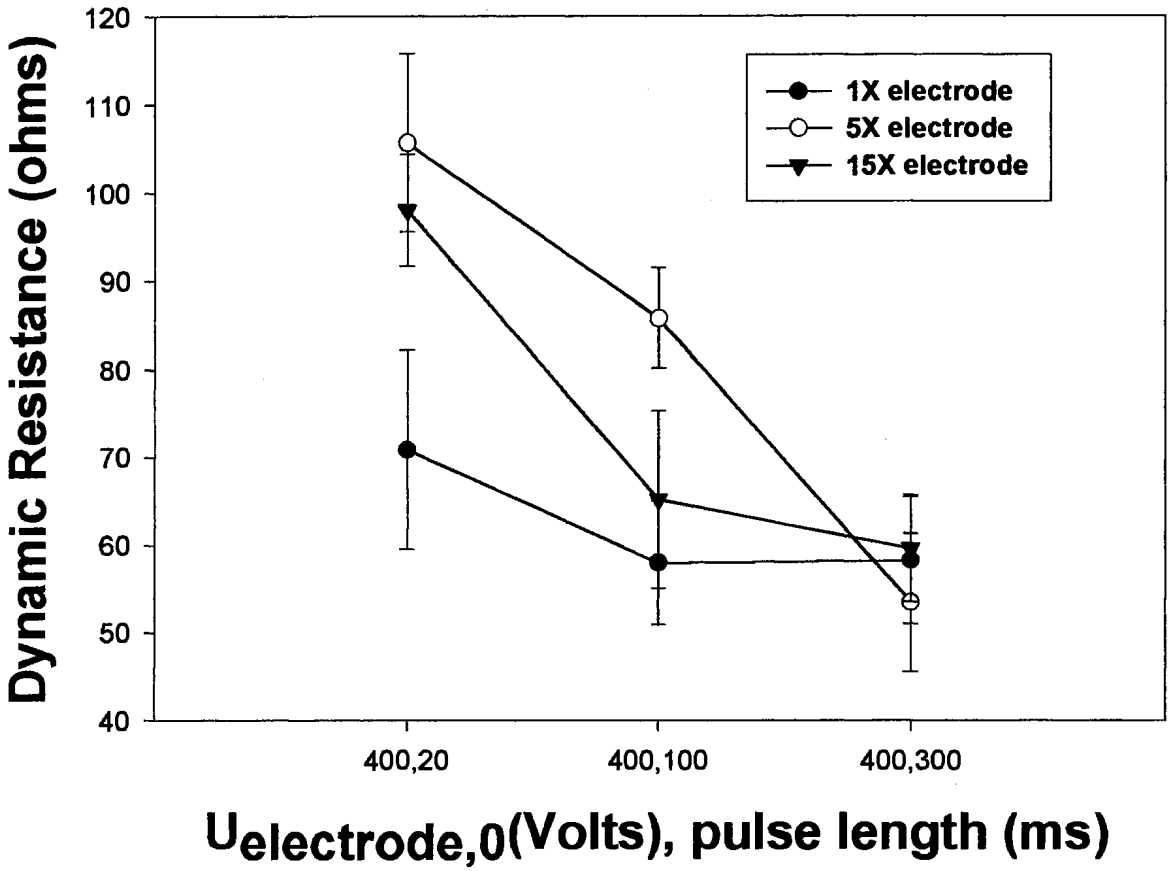



**Figure 4.10:** Dynamic resistance in relation to the pulse length with the applied voltage ($U_{electrode,0}$) kept constant.

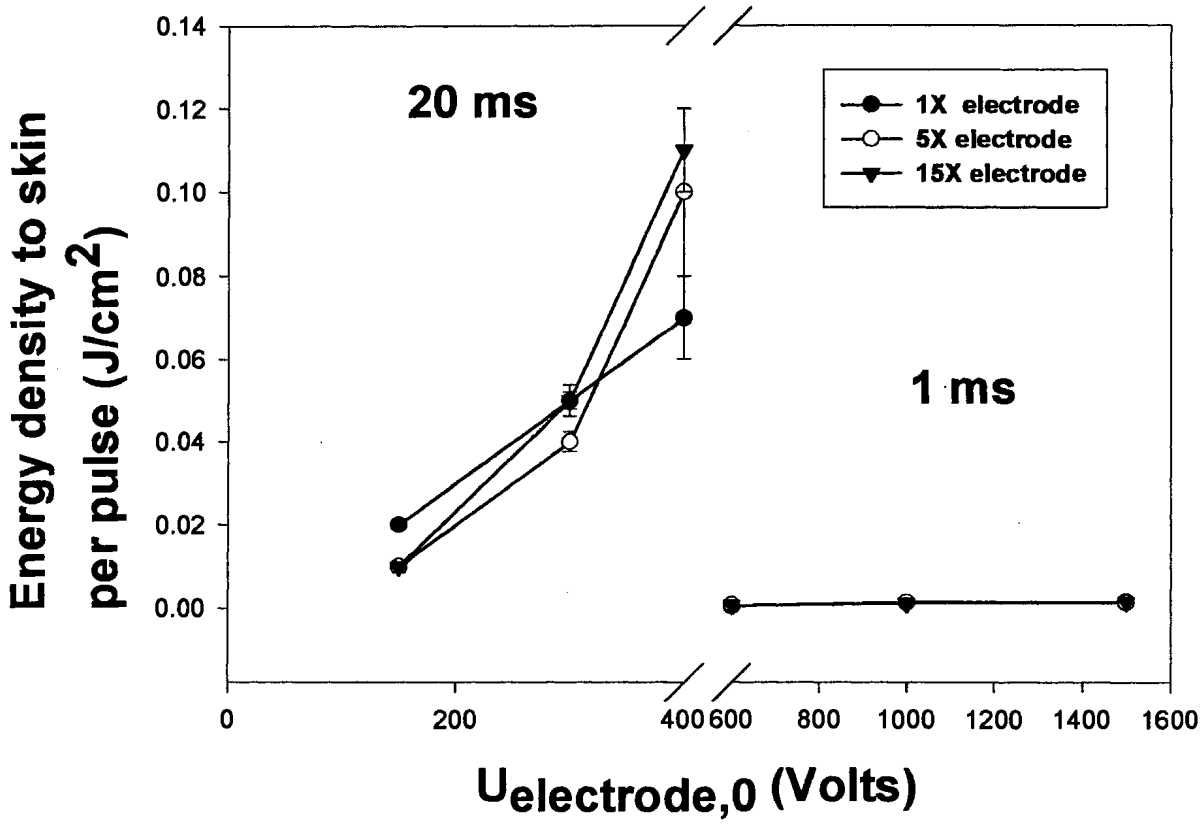



**Figure 4.11:** Energy density to the skin per pulse ($J/cm^2$) with increasing $U_{electrode,0}$ and pulse lenght kept constant.

### 4.4 *In Vivo* Skin Impedance Measurements :

Measuring passive impedance of skin before and after the application of electroporation would give an idea of changes taking place in the skin. The change in real (resistive pathways) and imaginary (capacitive pathways) impedance of the new plot can help in developing alternate equivalent circuits for the skin for example: if an additional resistance element explains the new impedance plot of skin (after electroporation), it would mean that additional aqueous pathways had been created by electroporation. However, such measurements have to be done within microseconds after the electroporation pulse application since electroporation causes skin changes which are reversible in milliseconds to seconds (Pliquett, 1995). Some such studies have already been done (Pliquett, 1995b) and no attempt was made here to reproduce them. However, in the above situation, if the electroporation conditions are 'harsh', they may cause irreversible changes to occur in skin. Thus the impedance plot after electroporation would remain altered from its pre-electroporation state. A return from this altered state to the pre-electroporated state would indicate recovery of skin, which was tested in the following experiments.

The aim of the study was to investigate whether the parameters which were arrived at by *in vitro* optimization experiments would cause reversible changes to skin. And if not, what electroporative conditions would produce a complete recovery of skin ? Results indicated that the optimized parameters viz: twenty pulses of 20 ms at 10 ppm, $U_{electrode,0}$ of 400 V using the small area electrode did not show any

appreciable recovery at all, even after 24 hrs (Figure 4.12). Thus, the number of pulses were drastically reduced from 20 to 1 pulse and the recovery measured. However, even under these conditions no recovery was observed (Figure 4.13) . Therefore, a reduction in pulse length was carried out from 20 ms to 10 ms. This condition showed a modest 50% recovery at the end of 24 hrs (Figure 4.14). To evaluate whether a further reduction in pulse length would cause complete recovery or not, a single electroporation pulse of 1 ms with $U_{electrode,0}$ of 400 V was delivered. The recovery was not 100%, but was similar to that following the 10 ms electroporation pulse i.e close to 50%, Figure 4.15. The large area electrode was also evaluated under $U_{electrode,0}$ of 400 V, 20 ms and single pulse. It did not show any greater recovery than with the small-area electrode under the same electrical conditions.

Thus an appreciable recovery occurs only with one pulse of 1 to 10 ms, of $U_{electrode,0}$ of 400 V. If we take the results of the *in vitro* study of TRZ delivery by electroporation (Sharma,2000a) into consideration, the above conditions would not produce any useful drug delivery. However, it would be premature to draw such conclusions without doing the impedance recovery studies at lower $U_{electrode,0}$ and longer pulse lengths and so on. Also it is important to elucidate the exact relationship between impedance recovery and *in vivo* safety of electroporation. Even puncturing skin with a hypodermic needle can cause a dramatic reduction in resistance of skin (Prausnitz,1999). But it is accepted that such a procedure is safe.

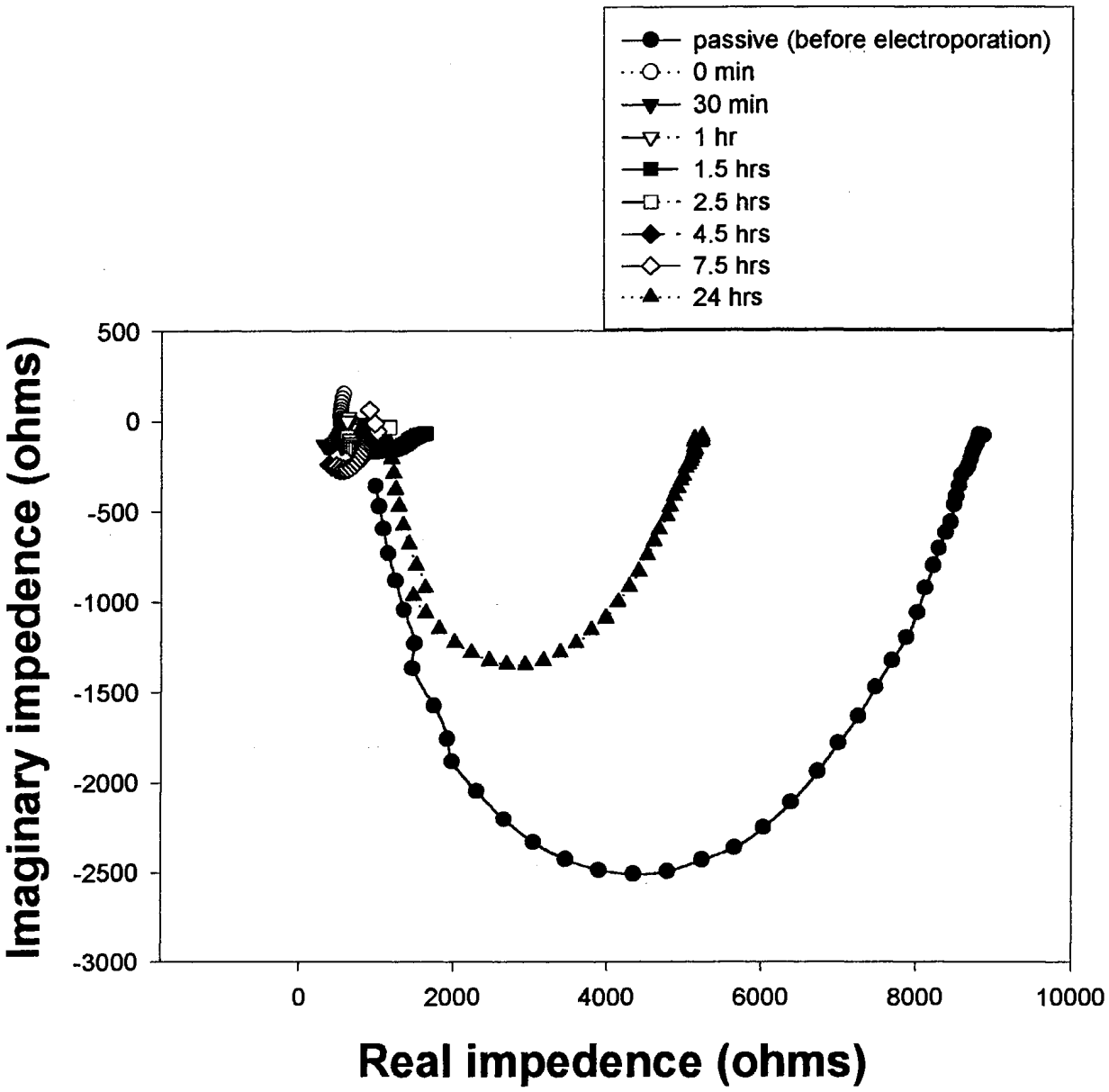



**Figure 4.12:** *In vivo* recovery of impedence
(400 V, 20 pulses of 20 ms @ 10 ppm, small area electrode)

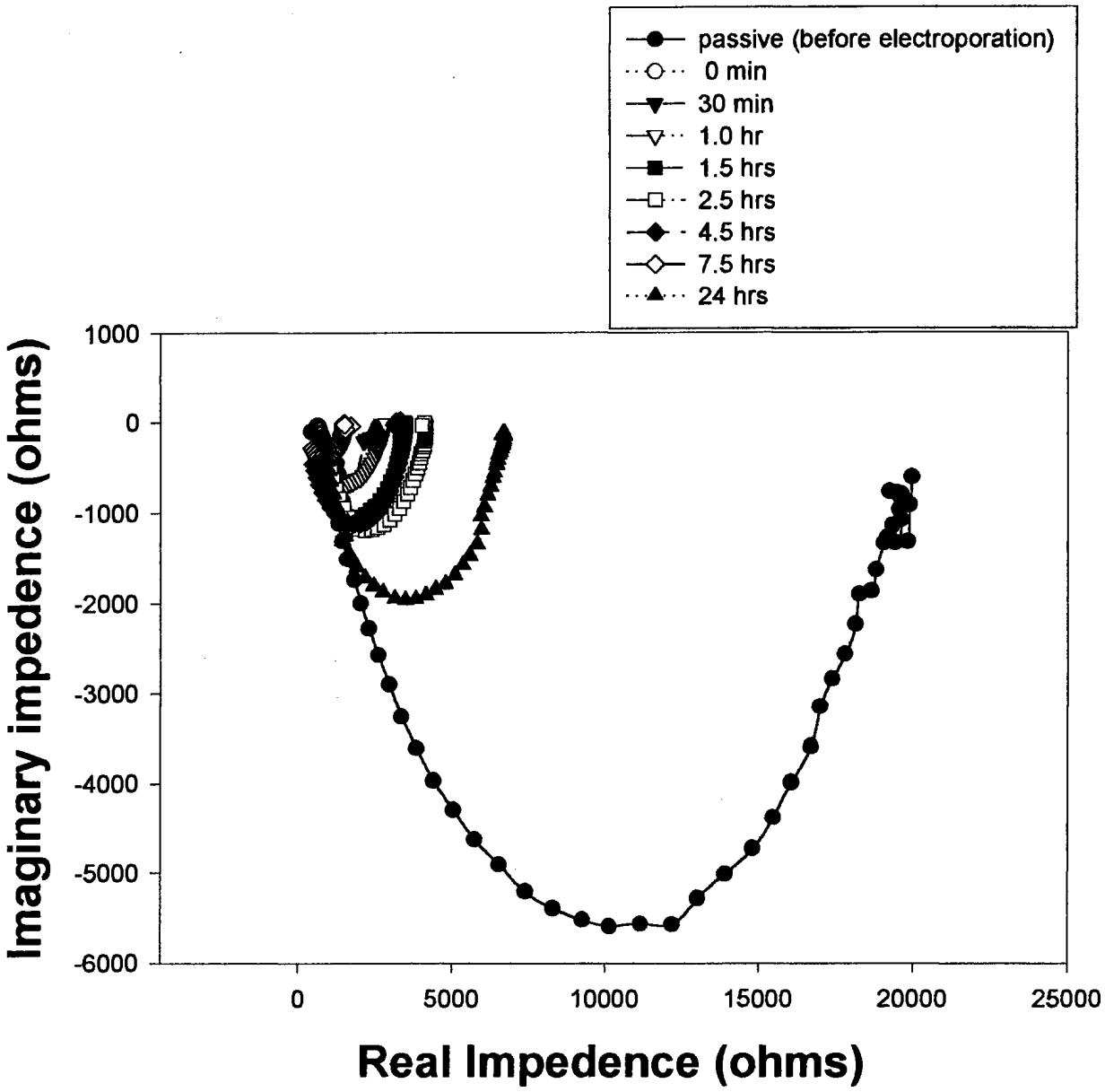



**Figure 4.13:** *In vivo* recovery of impedence
(400 V, 1 pulse of 17.3 ms with coil 15X electrode)

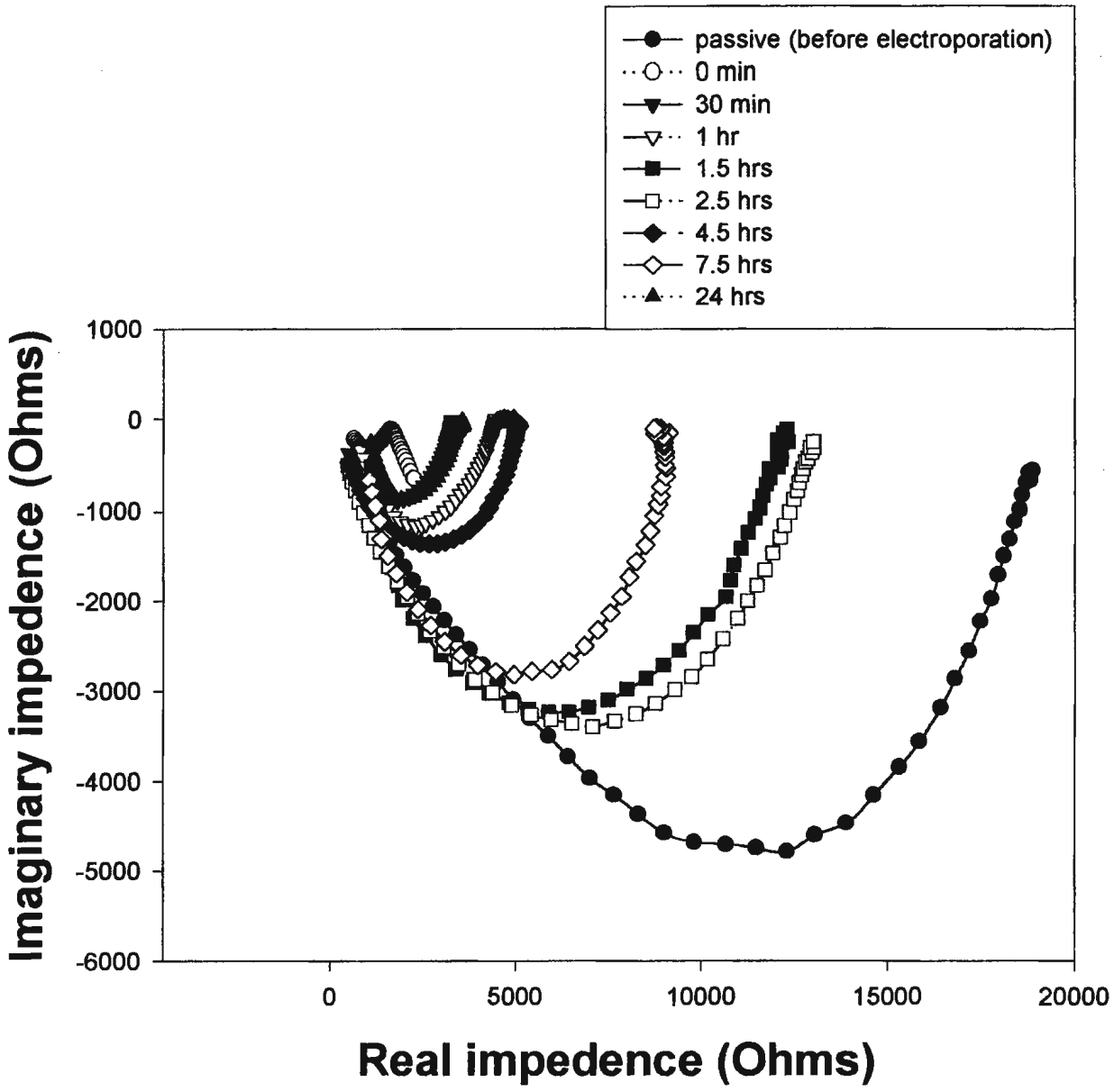



**Figure 4.14:** *In vivo* recovery of impedence
(400 V, 1 pulse of 10.4 ms, small area electrode)

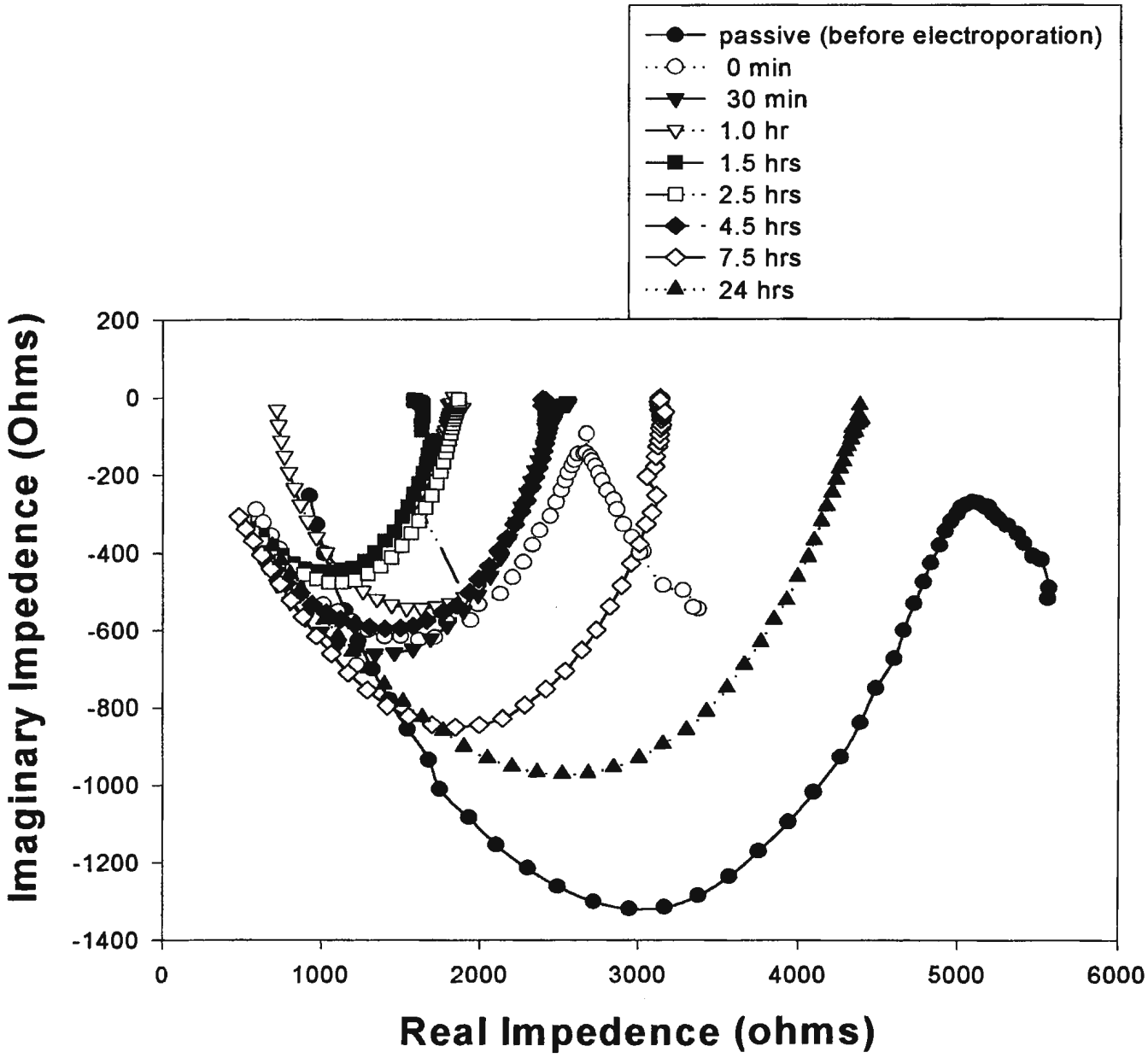



**Figure 4.15:** *In vivo* recovery of impedence
(400 V, 1 pulse of 1.4 ms, with small area electrode)

**4.5 Skin Viability Assessment :**

Three electroporative conditions were tested. The results of the study were quite informative. As can be seen from the Figure 4.16, after electroporation with the one pulse of 60 ms at $U_{electrode,0}$ of 500 V, and one pulse of 4 ms at $U_{electrode,0}$ of 1000 V, the initial skin's glucose consumption was disrupted (lag phase, no lactate production) and then began suddenly to increase (increased lactate formation) compared to the control (no electroporation pulse applied). One pulse of 20 ms at $U_{electrode,0}$ of 400 V showed no lag phase, however, these conditions also produced a dramatic increase in the glucose consumption (higher lactate formation). Overall, electrical pulses seemed to 'stimulate' glucose consumption.

The primary means of glucose uptake could be through the microvasculature in the excised skin. Using extreme electrical conditions might cause electroporation of the nerves in the dermis causing a secretion of epinephrine and leading to vasoconstriction of the micro-vasculature. This may hinder glucose uptake, which possibly causes the observed lag phase in lactate formation using very 'harsh' electroporative conditions. The permeabilization of cell membrane is an important mechanism of skeletal muscle and nerve injury in electrical shock (Lee,1995).

In the *in vivo* impedance studies, an incomplete recovery of skin after 24 hrs was observed. Even with the "mildest" possible electrical conditions, skin recovery continued after 24 hrs. Similar observations were made in an *in vitro* experiment by Pliquett,1995. Probably even the "mildest" electroporation condition causes some

damage to the lipid milieu in the skin. As a consequence of this the skin begins its repair process and this requires energy, ATP, which is produced by converting glucose to lactose. This could explain the increase in lactate production after application of electroporation pulses.

Interestingly, calcium levels have been shown to be important for epidermal differentiation (Yuspa,1989) and low extracellular levels of calcium are very important to trigger the repair process in the epidermis (Lee, 1992 and 1994). This was further demonstrated using sonophoresis to disrupt the calcium gradients within the epidermis by Menon et al, 1994. During electroporation the extracellular calcium could have been driven out of the epidermis due to the current passing through it. Such an event has been shown to occur with iontophoresis (Lee, 1998b). This might lead to triggering of the repair process which involves secretion of the lamellar bodies requiring various synthetic processes to start in the epidermis, which in turn require energy supplied by the glucose utilization method. Thus electroporation may lead to a decrease in extracellular levels of calcium in the epidermis or disruption in the epidermal calcium gradient leading to a cascade of events which cause increase in glucose utilization in the epidermis. This could be used as an alternative explanation to explain the increase in lactate levels of electroporated samples beyond that of the control samples. However, this hypothesis needs to be further investigated. A method to selectively disrupt the lipids only without affecting the calcium gradients and another method to selectively disrupt calcium gradients

without damaging the lipids and measuring glucose utilization in either case would possibly provide some insight into the observed trends in glucose utilization after electroporation. Such an experiment could also help in explaining the mechanism of wound healing by use of electrical stimuli (Fleischli, 1997).

Lee et al (1995), have suggested the denaturation of ion-channel proteins due to electroporation of cell membranes in electric shock, leading to a loss of ion gradient across the membrane. This would lead to higher work by the electroporated cells to maintain the ion gradient and use more ATP, which in turn would result in increased utilization of glucose and formation of higher lactate.

The damage could be occurring in two steps, initially, at lower electric field strengths only the extracellular calcium levels could be affected but as the field strength increases the viable cells below the SC could get electroporated leading to the damage to ion-channel proteins and thus increase in energy utilization. The extent of damage may determine the time taken for recovery of the skin.

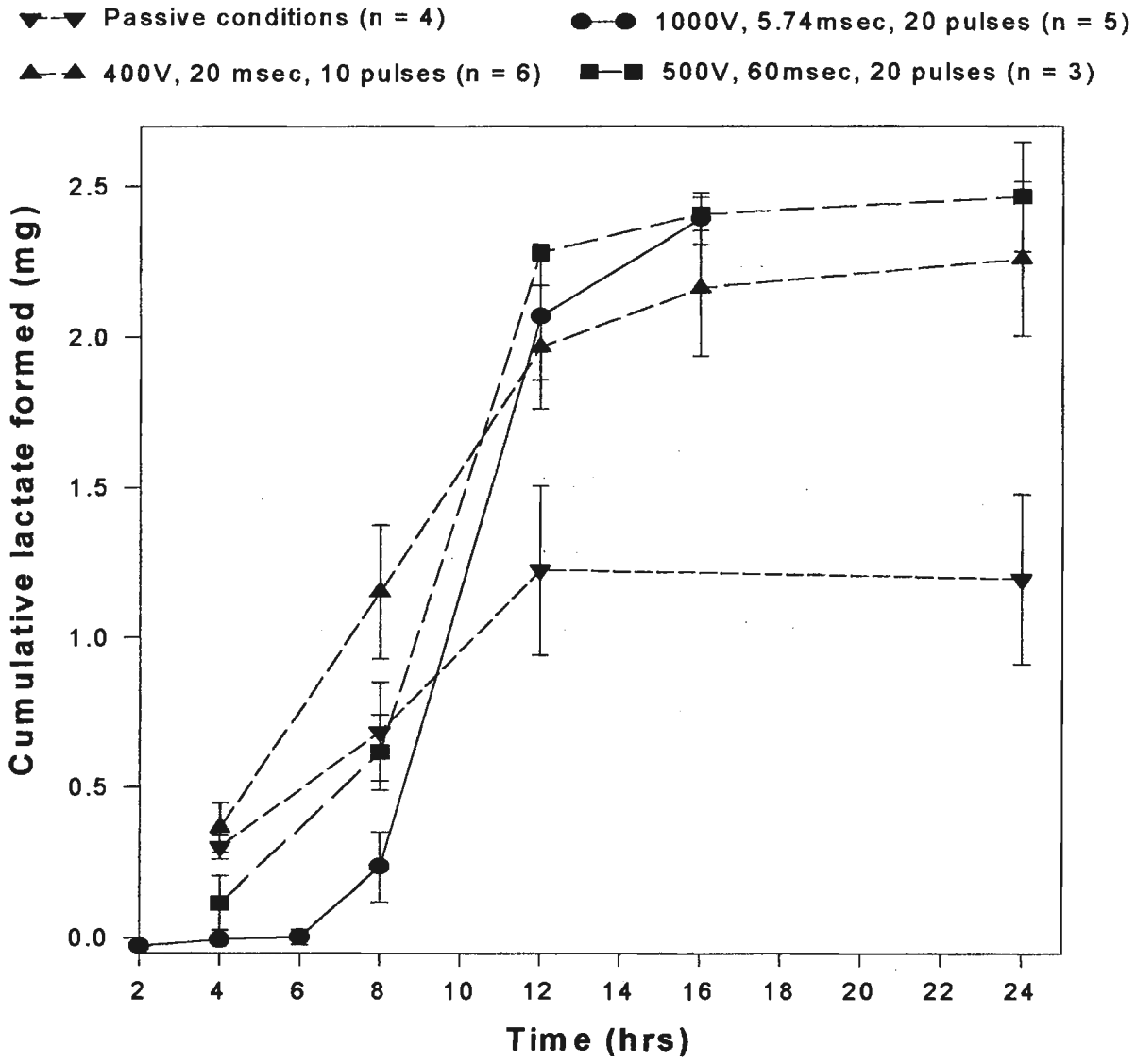



**Figure 4.16:** Cumulative lactate formed as a measure of viability of skin under various electroporation conditions

## 5.0 Conclusions :

The electrical parameters selected by the *in vitro* optimization experiments for the electroporative delivery of TRZ were, $U_{electrode,0}$ 400 V , 20 ms, 20 pulses at the rate of 10 ppm. The *in vivo* studies demonstrated that electroporation mediated drug delivery was advantageous compared to passive diffusion and the parameters chosen from *in vitro* optimization were safe and effective. At the same $U_{skin,0}$ the large area electrode showed a higher TRZ delivery than the small area electrode conclusively proving that the large area electrode had better *in vivo* drug delivery compared to small area electrode, consistent with the *in vitro* studies.

The pulse length used in the above *in vivo* study, 20 ms, was considerably less than 300 ms used by Vanbever et al 1998a, but it was much higher than the pulse length (~ 1ms) used by Prausnitz et al 1993a . However, muscle twitching and vocalization were observed in all studies and under all electrical conditions. Increasing the $U_{electrode,0}$ to 400 V with the larger area electrode caused unacceptable skin damage. Edema and later eschar formation were observed. The reasons could be attributed to thermal and non-thermal effects of electrical trauma.

The drug distribution in skin after electroporation study did not show a non-uniformity of drug delivery in the three different pieces of the exposed skin with either a straight wire electrode or a coiled wire electrode. If the assumption that "drug distribution within the skin is proportional to the electric field it is exposed to", is correct, then it could be concluded that the electric field produced by both types

of electrodes is uniformly distributed on the exposed skin.

In the *in vivo* recovery of impedance studies complete recovery at 24 hrs was not observed under any pulsing conditions. Skins impedance recovery at 24 hrs after pulsing improved with decreasing pulse lengths and lesser number of pulses. These observations are consistent with the previous observations. In the previous studies higher applied voltages and longer pulse lengths led to a irreversible or prolonged increase in drug delivery even after pulsing was stopped. However at lower applied voltages and shorter pulses the drug delivery returned back to the pre-pulsing rate very quickly. Meaning therefore, that recovery of skin was faster with lower applied voltages and shorter pulses. By comparison *in vivo* impedance gave similar results and hence, could be considered a useful tool to gauge the safety of electroporation.

Using higher $U_{electrode,0}$ to pulse the skin caused a delay in initial glucose utilization, due to possible permeabilization of the nerves releasing epinephrine and causing vasoconstriction. All the pulsed skin samples showed higher cumulative lactate formation (glucose utilization) at the end of 24 hrs. Electroporation could lead to the disruption/destruction of lipids and changes in extracellular calcium gradient which triggered the cells to increase glucose uptake giving energy to the cells to replace/restore the lipid/lipid ordering and to restore the calcium gradient. Combined together we could infer that electroporation does cause unwanted changes in the viable epidermis and dermis.

## 6.0 Future Perspectives:

The results of this study conclusively prove that electroporation is a feasible method to deliver drugs systemically. Moreover, using various combinations of electrical parameters might be possible to alter the drug levels delivered.

Muscle twitching and vocalization were observed at all electrical pulse conditions used. Using side-by-side type of electrodes in electroincorporation studies (Sec 1.5.4.5.4) has been suggested to reduce sensations, however, further *in vivo* studies need to be done. Ultimately, electroporation technique would be less likely successful as a simple alternative to the hypodermic needle if the pulsing causes discomfort or unacceptable sensations like those of needle puncture.

No gross morphological changes were observed with $U_{skin,0}$ of ~100V. However, at higher $U_{skin,0}$ delayed edema and eschar formation were observed. *In vivo* impedance recovery experiments showed that the conditions which deliver the drug effectively, do not cause recovery of impedance within 24 hrs. The significance and cause of these long lasting and delayed changes would have to be determined in future studies.

The glucose utilization studies showed that electroporation does cause biochemical changes within the skin as noticed by increased lactate production in all electroporated samples. With certain "harsh" electrical pulse conditions a lag phase was also observed. It is speculated that electroporation causes the changes in two stages. Initially the changes in the lipid milieu and calcium gradients occurring

result in increased glucose utilization for repair or replacement of the damaged SC lipids. In the second stage when the electrical conditions are strong enough to permeabilize the cells and nerves in the lower epidermal and dermal layers, the increased glucose utilization would occur for maintaining the ion gradient within the cells. Experiments to evaluate changes in calcium gradient and changes in the cell membrane proteins on certain electroporation conditions, need to be designed to prove the above hypothesis.

The cause of delayed reactions in the skin after electroporation has to be found and methods to avoid it have to be established. Further innovation in electrode design and/or combination of enhancement techniques need to be evaluated to reduce physical discomfort during the drug delivery. These factors could directly affect the tolerability of electroporation pulses and thus its acceptability as a non-invasive and safe method for drug delivery in humans.

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# Appendix I

## Calculation of area of three electrodes

Diameter of silver wire = d = 0.5 mm = 0.05 cm

∴ Radius of wire used for all three electrodes = (r) = 0.025 cm

L = length of the wire used

1. Small area electrode (1X)

( L = 1.3 cm)

Area $= 2\pi rL + \pi r^2$

$= 0.2042 + 0.0019635$

$= 0.2061 cm^2$

2. Large area electrode (5X)

(L = 6.5 cm)

Area $= 2\pi rL + \pi r^2$

$= 1.021 + 0.0019635$

$= 1.023\ cm^2$

3. Large area electrode (15X)

(L = 19.5 cm)

Area $= 2\pi rL + \pi r^2$

$= 3.063 + 0.0019635$

$= 3.065$ cm