TRANSDERMAL DELIVERY OF CYCLOSPORIN A BY ELECTRICALLY ENHANCED PERMEATION TECHNIQUES

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by

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ABSTRACT

Transdermal drug delivery has recently received increasing attention in the face of growing challenges to deliver peptide and protein drugs. Controlled transdermal delivery is an important route for the delivery of peptides and proteins that can maintain the therapeutic effectiveness of the drug by minimizing enzymatic degradation which is a major concern in other noninvasive routes of delivery such as the oral route. Although the advantages of transdermal delivery are very desirable, the natural obstacle to drug entry imposed by the skin's barrier function makes it one of the most difficult route of administration.

Iontophoresis and electroporation have been reported to be useful as permeation enhancing techniques in the transdermal delivery of protein and peptide drugs. The objective of present study is to use the above enhancement techniques to deliver cyclosporin A (CSA) to treat psoriasis.

The *in vitro* experiments were performed using hairless rat skin as the model with Franz diffusion cells for iontophoresis and custom made diffusion cells for electroporation. The donor drug solution of CSA consisted of an aqueous solution of CSA - polymer solid dispersion, coevaporate, and/or a hydroethanolic solution of CSA. PBS was used as the receiver solution. ^{3}H labelled CSA and ^{14}C labelled ethanol were used to facilitate analysis using a liquid scintillation counter. The control experiment consisted of passive diffusion study. Silver/silver chloride

electrodes were used in all studies. In the iontophoresis experiments a constant DC current (0.5 mA/cm^2) was used. In the electroporation experiments different delivery parameters were studied: (1) applied electrode voltage ($U_{electrode}$), (2) decay time constant (τ), (3) the number of pulses delivered - single or multiple, and (4) the time of diffusive contact with drug after electroporation ('contact duration'). Compared to the passive diffusion, iontophoresis did not result in a significant increase in the amount of CSA delivered transdermally with both the CSA-polymer donor and hydroethanolic drug solutions.

With the use of electroporation there was a significant increase in the transdermal delivery, compared to passive transport. With the CSA-polymer coevaporate donor solution the increase in delivery was only about 6 fold higher whereas with the hydroethanolic solution the increase was about 60 times higher compared to passive diffusion. The 'contact duration' was an important factor and a 4-hour 'contact duration' was found to be the optimum time period required for effective transdermal delivery. Use of single pulse (τ=5.6 ms) electroporation resulted in a significant increase ($p<0.05$) in the delivery of CSA in skin (CSA_{skin}) and EtOH in receiver ($EtOH_{receiver}$). With multiple pulse (τ=10 ms, 25 pulses) the increase in CSA_{skin} was more pronounced with a 60 fold increase than compared to the passive delivery. However there was no significant increase in the other two quantities viz. $CSA_{receiver}$ and $EtOH_{receiver}$.

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GLOSSARY OF ABBREVIATIONS AND SYMBOLS

A	area
Ag/AgCl	silver-silver chloride electrodes
AC	alternative electric current
AUC	area under the curve
C	concentration of drug in vehicle
C_{CSA}	concentration of CSA in standard
C_i	concentration of permeant i
CPM	counts per minute
CSA	cyclosporin A
$CSA_{receiver}$	transport of CSA delivered into the receiver
CSA_{skin}	transport of CSA delivered into the skin
CSC	cyclosporin C
CSD	cyclosporin D
CSG	cyclosporin G
CV	coefficient of variation
D	effective diffusion coefficient of drug in SC
Da	Dalton, a unit of mass
DC	direct electric current
D_i	diffusion coefficient of permeant i

DPM	disintegrations per minute
EtOH	ethanol
$EtOH_{receiver}$	transport of EtOH delivered into the receiver
$EtOH_{skin}$	transport of EtOH delivered into the skin
F	Faraday's constant
GI	gastrointestinal
h	effective diffusion path length through the skin barrier
HPLC	high performance liquid chromatography
$H_{CSA/CSD}$	height ratio of HPLC peaks between CSA and CSD
HLB	hydrophilic-lipophilic balance
I	electrical current
J_i	steady-state flux of a permeant i
J_v	solvent velocity
K_p	partition coefficient of drug between skin and vehicle
k	Boltzmann constant
K_0	zero-order rate constant
K_1	first-order rate constant
log P_{hep}	partition coefficient measured in n-heptane/water system
log P_{oct}	partition coefficient measured in 1-octanol/water system
LSC	liquid scintillation counting

MeOH	methanol
ms	millisecond
MTX	methotrexate
M. Wt.	molecular weight
NS	not significantly different
NSB	nonspecific binding
p	probability value adjusted to Type I error
PBS	phosphate buffered saline
PUVA	psorialens and ultraviolet A light therapy
PVME/MA	polyvinyl methyl ether maleic acid copolymer
Q_t	percentage of CSA released at any time
Q_∞	percentage of CSA released at infinite time
r	radius of permeant
R	gas constant
R^2	squared correlation coefficient of regression
REB	reversible electrical breakdown
RIA	radioimmunoassay
rpm	revolution per minute
R_s	resolution of adjacent HPLC peaks
R_{skin}	transdermal electrical resistance

SC	stratum corneum
SD	standard deviation
Sig	significantly different
t_i	retention time of HPLC peaks
t_{wi}	peak width of HPLC peaks
T	absolute temperature
TEWL	transepidermal water loss
U(t)	transmembrane voltage
$U_{electrode}$	applied electrode voltage
U_{skin}	transdermal voltage
V	voltage
$\bar{x}$	mean value
Z_i	charges of permeant i
η	viscosity
σ_i	permeant's reflection coefficient at the membrane
τ	decay time constant or pulse length
ϕ	electric potential at any point x in the membrane
$\%B/B_0$	percent of specific binding to nonspecific binding
$\frac{\partial C}{\partial t}$	change in concentration with time

$\frac{\partial^2 C}{\partial x^2}$	change in the concentration gradient at the given point
$\frac{dQ}{dt}$	rate of skin penetration

List of abstracts and manuscripts published or submitted are as follows:

1) Su Wang, M. Kara and T.R. Krishnan, Transdermal delivery of cyclosporin A-coevaporate using electroporation technique, Drug Dev. Ind. Pharm., 23(7), 1-7 (1997).

2) Su Wang, M. Kara and T.R. Krishnan, Transdermal delivery of cyclosporin A using electroporation, submitted to J. Controlled Release.

3) Su Wang, M. Kara and T.R. Krishnan, Preparation and *in vitro* evaluation of cyclosporin coevaporates, Poster presentation in the AFPC conference at Montreal, May, 1995.

4) Su Wang, M. Kara and T.R. Krishnan, Pulsed electroporation for transdermal delivery, in the Controlled Release Society conference at Baltimore, August, 1996.

TO MY WIFE, SON AND PARENTS:

YOUR LOVE, ENCOURAGEMENT AND UNDERSTANDING

HAVE MADE IT WORTHWHILE

I INTRODUCTION

1. 0 Transdermal Drug Delivery System

Transdermal drug delivery has recently received increasing attention in the face of growing challenges to the delivery of peptide and protein drugs. Controlled transdermal delivery is an important route, which could maintain the therapeutic effectiveness of peptide and protein drugs and reduce their side effects. As a non-invasive method of delivery, transdermal delivery minimizes the risk of contamination and infection associated with needle penetration. The therapeutic value of many drugs could be enhanced by transdermal delivery due to the elimination of gastrointestinal (GI) irritation, avoidance of the drug's deactivation caused by hepatic "first-pass" effect or degradation in GI tract and reduction of the frequency of dosing for the drugs with short half lives. Transdermal delivery can also enhance absorption of the drug if a poor bioavailability is caused by the effect of GI pH, enzymatic degradation in GI tract or the drug interactions with food. Since peptide and protein drugs usually have very short half lives and poor absorption in the GI tract, the delivery of them through the intact skin is attractive and worth pursuing.

In transdermal drug delivery, the drug molecules pass from the surface of the skin through its various layers into the systemic circulation. In conventional topical dosage forms, such as ointments and creams, the percutaneous absorption is

controlled by the skin membrane and the amount of drug delivered is unpredictable and uncontrollable. Significant progress has been made, in the last two decades, in achieving a better control in the delivery through the skin. The first transdermal therapeutic system designed to control the delivery of drug to the skin for absorption was developed in 1980 by Alza Corporation, in which scopolamine was delivered for systemic absorption to control nausea and vomiting associated with motion sickness. Since then, a number of transdermal delivery systems have been developed and commercialized (Table 1).

Although the advantages of transdermal delivery make this route of delivering very desirable, only a selected number of drugs are suitable as candidates for transdermal delivery due to the natural obstacle to drug entry imposed by the skin's barrier function. Research in transdermal delivery has been directed to find ways and means of delivering different types of drug molecules with the use of enhancement techniques to overcome the skin's barrier function.

Table 1 **Examples of Transdermal Drug Delivery Systems** *

Therapeutic agents	Type of System	Trade Name and Manufacture
Clonidine	Four-layered Patch	Catapres-TTS(Boehringer Ingelheim)
Estradiol	Four-layered Patch	Estraderm (CIBA)
Fentanyl	Four-layered Patch	Duragesic (Janssen)
Nicotine	Multi-layered Patch	Habitrol (Ciba-Geigy)
	Multi-layered Patch	Nicoderm (Marion Merrell Dow)
	Multi-layered Patch	Nicotrol (Warner-Lambert)
	Multi-layered Patch	Prostep (Lederle)
Nitroglycerin	Matrix Plastic	Deponit (Schwarz Pharma)
	Polymer Adhesive	Minitran (3M Pharmaceuticals)
	Polymer Adhesive	Nitrodisc (Searle)
	Gel-like Matrix	Nitro-Dur (Key Pharmaceutical)
	Four-layered Patch	Transderm-Nitro (Summit)
Scopolamine	Four-layered Patch	Transderm Scop (CIBA)

* Ansel HC *et al*, 1995

1.1 Skin Barrier Function and Percutaneous Absorption

The skin is an integumentary system, which plays a major role in maintaining homeostasis, preventing the loss of body fluids and retarding the entrance of potentially toxic agents from the environment rather than being just an inert body wrapping. It probably has the lowest water permeability of any biologically produced membrane. In serving as a barrier, the skin also impedes the invasion of most microorganisms and protects the underlying tissue from many external physical stresses, such as ultraviolet radiation, electrical energy and mechanical forces. The structure of the skin consists of three distinct layers, epidermis, dermis and subcutaneous tissue, from surface downward (Figure 1).

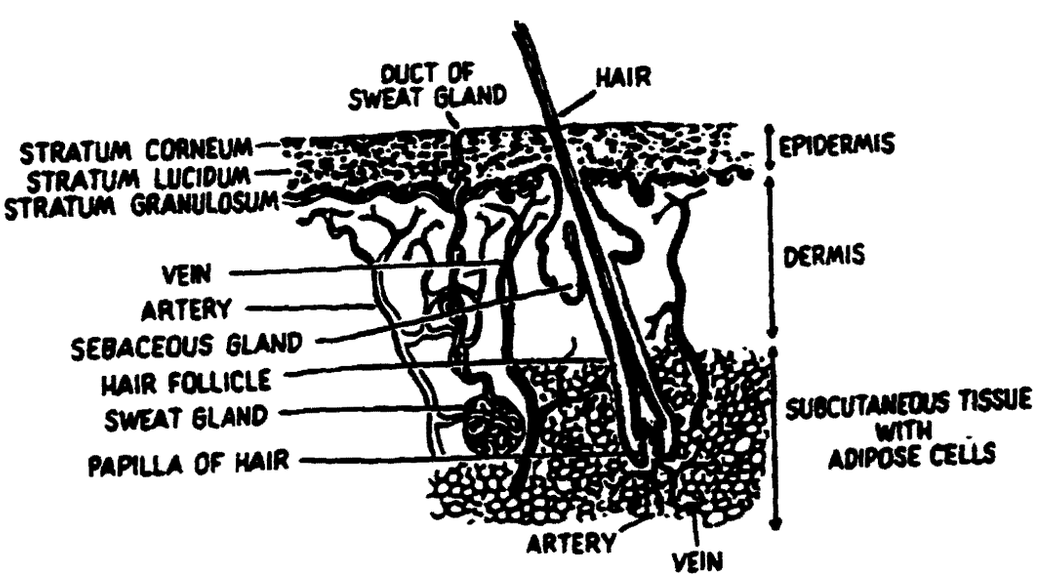


Figure 1 Structure of the Skin

The epidermis is a continually renewing stratified, squamous and cornifying epithelium layer. The majority of cells in the epidermis are the keratinocytes that keratinize and give rise to derivative structure - appendages including sweat glands and hair follicles. The epidermis varies in thickness from 0.04 to 1.5 mm, compared to 1.5 to 4 mm for the full thickness of the skin (Moschella SL *et al*, 1992).

One of the principal functions of epidermis is cornification, a specific form of cellular differentiation that results in the formation of a highly resistant cutaneous barrier, which possesses the properties of physical toughness, high electrical impedance and physicochemical barrier to water and other foreign substances. In the process of cornification, epidermal keratinocytes undergo characteristic changes, which is vertically oriented. At the end of this process, cornified cells are arranged regularly and form orderly columns, a stacking of cells that varies somewhat on different sites of the skin. Within the cornified layers, a type of fibrous protein, termed α-keratin, is formed and embedded in a sulfur-rich amorphous matrix. As the keratinocytes mature to cornified cells, plasma membranes in the upper portion of epidermis become thickened by deposits of a thick band of protein on the inner surface of the plasma membrane, which is cross-linked by disulfide bonds and transglutaminase-dependent isopeptide linkages. The thickened cornified layer, about 15 nm thick, is named stratum corneum (SC). The SC acts as the main barrier for the entry of external agents through the skin (Moschella SL

et al, 1992). The layer of the SC is composed of lipid-depleted corneocytes embedded in a complex mixture of polar and non-polar, lipid-enriched bilayer matrix in the intercellular space, presenting in the form of multiple lamellae (Jackson SM *et al.*, 1993; Wertz PW, 1992). The epidermal barrier is mediated by a series of intercellular multiple lamellae in the SC, which consist of cholesterol, ceramides (*N*-acylsphingosines) and fatty acids (Schurer NY *et al*, 1991a). The barrier function of SC to permeation is attributed not only to the interstitial lipid composition but also to its unique arrangement as ordered multilayers (Potts RO *et al*, 1991). The cells of SC in the epidermis of some mammalian species are precisely stacked in columns in a honeycomb fashion and architecturally organized parallel to the surface of the SC (Honda H *et al.*, 1996). The architecture of the SC was modeled as a "brick and mortar" structure, a two-compartment model, the protein contained in corneocytes as "brick" embedded in the neutral bilayer lipid "mortar" (Elias PM, 1983; Barry BW, 1987; Wertz PW *et al.*, 1989). The more recent findings relate the skin's remarkable barrier property to the heterogeneous morphology of the SC, in which the SC is catabolically active and the intercellular domains are not a purely lipid compartment but possess considerable heterogeneity, i.e., a mixture of lipids, enzymes and membrane glycoconjugates and perhaps other constituents as well, consisting of both hydrophilic and hydrophobic lamellae (Elias PM, 1991). The heterogeneous morphological arrangement makes the SC more complicated in

permeation than a normal diffusion model (Heisig M *et al.*, 1996).

The chemical insolubility and stability of SC derive from the presence of numerous disulfide and other highly resistant covalent bonds in proteinaceous envelope and intercellular neutral lipid structures. The lipids include ceramides, free fatty acids, cholesterol and cholesteryl sulfate that are required for permeability barrier homeostasis (Mao-Qiang M *et al.*, 1996). The chemical and enzymatic resistance and cohesiveness of SC are largely attributed to these protein and lipid envelope (Wertz PW *et al.*, 1989; Elias PM *et al*, 1991; Schurer NY *et al.*, 1991a; b).

The dermis consists mostly of relatively non-cellular connective tissue constituted of collagen, elastic fibers and ground substances, within which are embedded nerves, blood vessels, lymph vessels, muscles and follicle-sebaceous-apocrine and eccrine units. The dermis is 15-40 times thicker than epidermis, depending on the anatomic site (Moschella SL *et al*, 1992).

The subcutaneous tissue is technically not considered as part of the skin. It is primarily composed of connective tissue to attach the skin to the underlying organs and contains numerous fat cells to insulate against cold and store energy in the form of calories, rich network of blood vessels to regulate skin temperature and the complex system of nerves to register sensation.

The skin is regarded essentially as an impermeable membrane. The

permeability of the skin shows regional variation and changes with aging probably because of the variation and change of lipid content and composition in SC (Lampe MA *et al.*, 1983; Ghadially R *et al.*, 1995).

There is a molecular size constraint on penetration through the SC. The penetration decreases dramatically as molecular size of the penetrating agent rises above 800-1000 Da (Wester RC *et al*, 1985).

The permeability barrier of the skin mainly resides in the epidermis and almost exclusively in the intercellular spaces of SC (Monash S, 1957; Elias PM *et al.*, 1977; Madison KC *et al.*, 1987; Elias PM, 1991; Potts RO *et al*, 1992; Craane-van Hinsberg WHM *et al.*, 1995). The lipid content and lipid type in the SC determine the epidermal permeability barrier. A relationship was found between the total lipid content of SC and its barrier function (Grubauer G *et al.*, 1989). The SC is a rate-limiting membrane for passage of water, electrolytes and other substances between the external and internal environment of the body.

There are two anatomic routes available for percutaneous absorption through intact skin (Figure 2): transcellular route and intercellular route. Recent studies indicate that skin appendages, hair follicles and sweat ducts, act essentially as diffusion shunts in normal skin (Elias PM, 1991), which is a hydrophilic porous transport pathway and a relatively easy pathway through the skin for certain compounds, such as small ions. However, for most drug compounds, the

percutaneous absorption takes place across intercellular lipid multilayer domains of SC (Krill SL *et al.*, 1992a), which occupies a much greater surface area than the

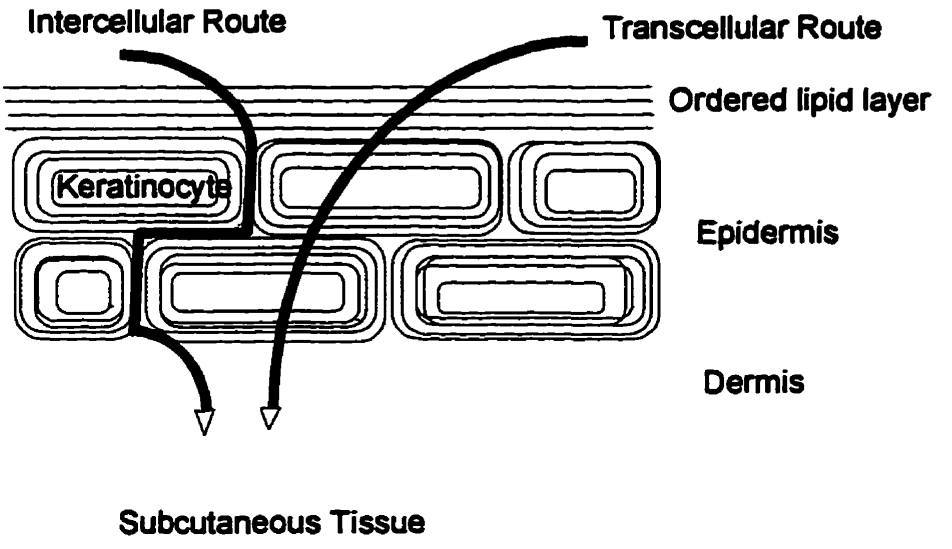


Figure 2 **Percutaneous Absorption Through Intact Skin**

appendageal diffusion shunts. For peptides, both intercellular and shunts seem to be important in penetration (Craane-van Hinsberg WHM *et al.*, 1995). Two parallel penetration mechanisms are believed to be involved in the percutaneous absorption: partitioning through lipid pathway, and diffusion through hydrophilic pore pathway (Morimoto Y *et al.*, 1992; Sznitowska M *et al.*, 1995a; b). This

suggests that the percutaneous absorption of a compound will be largely determined by its physicochemical properties. The shunt route may function as diffusional route and bypass the low diffusivity domain of SC. However, the appendages provide a small portion of skin surface area, approximately 0.1% of total area of the skin (Scheuplein II, 1967). It is unlikely that an appreciable number of continuous hydrophilic pathways exist in the SC considering the high electrical resistance of the skin (Oh *et al.*, 1993). Thus, enhancing drug penetration through the intact SC seems to be very important in achieving a successful transdermal delivery.

In abnormal skin, the permeability barrier of the SC could be changed. In psoriatic epidermis, for example, the defective permeability barrier function resulted in an increased transepidermal water loss (TEWL), which could be correlated to the severity of the disease (Ghadially R *et al.*, 1996).

Between epidermis and dermis is the dermoepidermal junction, a highly specialized attachment, which regulates the permeability across the interface between epidermis and dermis. The permeability characteristic of this layer is reported to depend on molecular charge of the penetrating species but not on their size. For a neutral or lipophilic compound, the thick dermis may offer a significant resistance to its permeation (Roy SD *et al.*, 1994).

Besides the barrier function of SC, the metabolic capacity of the skin is also

considered as a barrier for percutaneous absorption during transdermal drug delivery (Martin RJ *et al.*, 1987). Both phase I and phase II reactions occur in the skin, particularly in the epidermis. Many penetrating molecules may be metabolized in the epidermis by certain xenobiotic metabolizing enzymes, protease and esterase, which are known to be present in the outer layer of the epidermis (Menon GK *et al.*, 1986; Noonam PK *et al*, 1985; Potts RD *et al.*, 1989).

In certain disease state, for example in psoriatic skin lesions, the metabolic activity of enzymes may be subject to change that may induce changes in the xenobiotic substrate metabolism and endogenous substrate metabolism (Goerz G *et al*, 1992).

1.2 **Electrically Enhanced Permeation**

The permeability of skin can be increased by some chemical and physical means. The chemical enhancers have long been used in topical formulations. The effects of physical means are also being extensively investigated, such as increasing the permeability by use of heat energy (Krill SL *et al.*, 1992a; Prausnitz MR, 1996a) and use of ultrasound (Ueda H *et al.*, 1995; Mitragotri S *et al.*, 1995; 1996; Johnson ME *et* al., 1996). To deliver peptide and protein drugs, electrical enhancement techniques, to improve the permeability of skin, have been studied in the recent years. The most extensively investigated physical means of

enhancing transdermal delivery is iontophoresis (Srinivasan V *et al.*, 1989;, Theiβ U *et al.*, 1991; Green PG *et al.*, 1993; Gangarosa LP *et al.*, 1995). Another physical means recently introduced in the field of transdermal drug delivery is the use of electroporation (Bommannan DB *et al.*, 1994; Prausnitz MR *et al.*, 1993; 1995a). Both iontophoresis and electroporation involve the use of electrical energy and are separately discussed below.

1.2.1 **Iontophoresis**

Iontophoresis is a non-invasive technique which uses a low electric current, ≤ 0.5 mA/cm^2 (Prausnitz MR, 1996a), to facilitate the transdermal delivery of a variety of drugs. This technique has been in use for a long time and references dating back to the late 19th century can be found.

In principle, iontophoresis is very similar to electrophoresis, in which charged particles migrate through a relatively stationary liquid phase under the influence of an applied electric field provided by immersed electrodes. In iontophoresis, the amount of electricity required to drive the molecules is much smaller than that in electrophoresis. The skin behaves as a drug transport barrier membrane between the two electrodes. The transport of charged species through the skin membrane depends on the magnitude of the applied electromotive force, the physicochemical nature of the species and the medium. Stratum corneum has been shown to be

negatively charged at physiological pH (Burnette RR *et al*, 1987). This property facilitates the transport of cations in anodal delivery and is also coupled with the effect of electro-osmosis. For neutral species, electro-osmosis might affect the transport, in which electrically induced convective solvent flow acts as a driving force (Srinivasan V *et al*, 1989; 1990; Wearley *et al*, 1990). According to the Stokes-Einstein equation,

$$D = kt./6\pi\eta r \qquad \text{(i-1)}$$

k: Boltzmann constant
T: absolute temperature
η: viscosity
r: radius of permeant

diffusivity of molecules, D is inversely proportional to the molecular size of the permeant (Peck KD *et al.*, 1996). For the negatively charged agents with high molecular size, SC is almost a formidable barrier in passive diffusion because of the high lipophilicity of SC itself (Blank HI *et al*, 1969). However, by suitably adjusting the composition of the donor and receiver solutions, electro-osmosis can be increased and also used to deliver the negatively charged species (Santi P *et al*, 1996).

The hydration of skin is reported to enhance iontophoretic delivery. Skin hydration is a reversible process and it is due to disorganization of the lipid layers

of the SC that leads to increased SC permeability (Jadoul A *et al.*, 1996).

Besides enhancing transdermal drug delivery, iontophoretic drug delivery may also be programmed to achieve controlled drug release from a drug reservoir to further improve drug therapy and patient compliance. In comparison with passive transdermal drug delivery, which is usually limited to deliver small, non-polar or lipophilic species, iontophoresis can deliver both ionic and non-ionic molecules including those with high molecular weight (Phipps JB *et al.*, 1989; Singh P et al, 1994). To date, iontophoresis offers a feasible non-invasive alternative for the delivery of some polypeptide and protein drugs (Srinivasan V *et al.*, 1989; Singh P *et al*, 1994; Thyman S *et al.*, 1994; Chen LLH *et al*, 1996; Green PG, 1996; Brand RM *et al*, 1996).

1.2.2 **Electroporation**

In electroporation, an exponential decaying high electric voltage is pulsed for a very short duration of time (micro or milliseconds) to bring in transient changes in cell membranes or lipid bilayers (Pliquett U *et al.*, 1996). The early studies of electroporation dates back to 1960s. It has been widely used in many different fields for different purposes, including gene transfer in mammalian cells, genetic manipulation in plant cells, genetic transformation of bacteria and yeast, electroinjection of exogenous molecules for studying cellular functions and

electroinsertion of protein and DNA into cell membrane (Chang DC *et al*, 1992a). The recent development in its application are in the field of transdermal drug delivery (Prausnitz MR *et al.*, 1993; 1994; 1995a; b; Vanbever R *et al.*, 1994; 1996; Bommannan DB *et al.*, 1994; Edwards DA *et al.*, 1995; Riviere JR *et al.*, 1995; Pliquett U *et al*, 1996) and electrochemotherapy to deliver anticancer drugs to improve solid tumor chemotherapy (Mir LM *et al.*, 1991; 1995; Belehradek J. Jr. S *et al.*, 1991; Belehradek M *et al.*, 1993; Heller R *et al.*, 1995; 1996; Glass LF *et al.*, 1996). The use of electrochemotherapy in human subjects testifies the clinical safety of this technology.

When applied to lipid bilayers, it is hypothesized to destabilize the membrane lipids leading to the formation of transient intercellular pores (Chang DC *et al.*, 1992; Inada H *et al.*, 1993; Orlowski S *et al*, 1993; Bommannan DB *et al.*, 1994; Freeman SA *et al.*, 1994; Edwards DA *et al.*, 1995; Prausnitz MR *et al.*, 1995a). The mechanisms of tissue electroporation are complicated and most of the present hypotheses are scientific speculations. The formation of aqueous pores is believed mainly to be a biophysical process induced by electromagnetic field and can be characterized by a reversible electrical breakdown of the membrane (Powell KT. *et al*, 1986; 1989; Barnett A *et al*, 1991; Weaver JC, 1993, 1995). Under an external electric field, the relatively high potential drop across the membrane is built because of the high electrical impedance of lipid bilayer membrane. Once the

electric potential drop across the membrane is higher than a certain threshold potential, the resting potential, a rapid process of structural rearrangement within the membrane occurs and results in a large increase in transmembrane conductance and molecular transport through temporarily formed hydrophilic porous membrane openings (Orlowski S *et al*, 1993). The number and size of pores can be increased with the increase of pulse length and voltage of the applied pulses (Glaser RW *et al.*, 1988; Weaver JC, 1993). As pores form, the conductivity of the membrane is increased. The induced high conductance state in the membrane can limit the transmembrane voltage and therefore protect it. Similar to lipid bilayer membranes of cells, cell layers, such as SC of the skin can also be temporarily altered by the electroporation (Chizmadzhev *et al.*, 1995). When applied on skin, the electric pulses create transient pores in the SC, which may facilitate drug transport. Pliquett *et al.* studied the effect of voltage and number of electroporation pulses on epidermis. They have reported that highly localized transport regions were formed in the epidermis with the use of high voltage electrical pulses. The increase in the applied voltage increased the number of regions with increased permeability and increase in the number of pulses increased the size of regions (Pliquett UF *et al.*, 1996). The efficiency of transdermal drug transport as well as the extent of changes in the skin are both dependent on the transmembrane voltage and the duration of pulsing. By optimizing the electrical parameters in experiment,

the pores created in SC could temporarily increase its permeability to deliver drug molecules, thereby ensuring that no long term damage is done to the protective property of the skin. Increased pore pathway in the SC could lead to increased skin permeability and prolong the post-pulse diffusion phase. The main driving forces in drug transport process are electrophoresis (or iontophoresis) and electro-osmosis (Dimitrov DS *et al*, 1990; Weaver JC, 1993; 1995; Prausnitz MR *et al.*, 1995a).

1.3 Topical Delivery of Cyclosporin A for the Treatment of Psoriasis

1.3.1 Psoriasis

Psoriasis is a skin disease that is characterized by marked increases in keratinocyte proliferation, abnormal patterns of keratinocyte differentiation and prominent alterations in dermal capillary vasculature and presence of dermal and epidermal T cells, manocytes/macrophages and neutrophils (Das UN *et al.*, 1992). It has a chronic relapsing nature and variable clinical features. The involvement of the skin could be small, isolated and localized region or generalized on the whole body. The morphology of the skin lesions of psoriasis varies considerably and is classified as scaly erythrosquamous that indicates the involvement of both the vasculature (erythema) and epidermis (increased scale formation). Epidermal keratinocytes display marked proliferative activation and differentiation (Fitzpatrick

TB *et al.*, 1993).

The occurrence of psoriasis is universal, ranging from 0.5% to 3% (Krueger GG *et al*, 1994). It diminishes the quality of life for a large number of people by causing not only economic loss but also human sufferings, including disability, discomfort and disfigurement (Krueger GG, 1993).

Although the pathogenesis and etiology of psoriasis are not fully understood, two important mechanisms have been proposed: epidermal proliferation and an inflammatory/repair process (Krueger GG, 1989). It is reported to involve the immune system and has a genetic disposition (Gottlieb AB, 1988; Suarez-Almazor ME *et al*, 1990; Chang EY, 1992; Breathnach SM, 1993). The disequilibrium of certain major histocompatible antigens can be found with the disease expression. Since interleukin-2 (IL-2) therapy for malignancy in patients with a history of psoriasis induces severe psoriatic exacerbation, it proves that T cell-derived cytokines are inducers for psoriatic epidermal changes (Lee RE *et al.*, 1988). Besides the action of IL-2, the overexpression of interleukin-1 (IL-1) and tumor necrosis factor (TNF-α) in psoriatic epidermis, a high level of IL-6 expressed in psoriatic skin and an increase in the level of IL-8 derived from a variety of cells involved in the psoriatic reactions indicate the complexity of the cytokine network in psoriasis (Elder JT *et al.*, 1989; Romero LI *et al.*, 1989; Christophers E *et al.*, 1989; Grossman RM *et al.*, 1989; Das UN *et al.*, 1992). All these facts demonstrate

the participation of the immune system in the etiology and pathogenesis of psoriasis. Moreover, the observation that psoriasis and Reiter's Syndrome (an autoimmune disease) may co-occur and be flared by infection with the human immunodeficiency virus (HIV), which dysregulates and destroys the human immune system, supports the hypothesis that psoriasis is an immunologically mediated disease (Madeleine D, 1990). All these malfunctions of immune system in the psoriatic plaque is believed to occur in the viable epidermis and the junction between epidermis and dermis of the skin (Valdimarsson H *et al.*, 1986; Wong RL *et al.*, 1993).

1.3.2 **Methods Currently Used in the Treatment of Psoriasis**

Various forms of treatment of psoriasis have been developed in the past several decades. The topical corticosteroid creams and ointments remain the most common first-line choice for the management of psoriasis. With the use of potent, fluorinated preparation, such as fluclorolone ointment, the incidence of side effects has dramatically increased (Menter A *et al.*, 1991). The intralesional injection of corticosteroids and other drugs is used to treat small, localized plaques. The total amount of the given drug and the depth of injection must be well controlled to avoid systemic side-effects.

Ultraviolet phototherapy at 311 nm wavelength is useful in treating psoriasis,

particularly in the treatment of superficial types of psoriasis. The photochemo-therapy (PUVA), using systemic psorialens plus ultraviolet light to treat psoriasis, was introduced in 1974 (Parrish JA *et al.*, 1974). Since then, the effectiveness of PUVA in clearing psoriasis has been confirmed by many clinical studies both in United States and in Europe (Melski J *et al.*, 1977; Henseler T *et al.*, 1981). However, the side effects and the consequences of overdosage have limited its usage. The long-term side effects in skin damage are of considerable importance which makes it necessary to restrict PUVA to patients with widespread and severe psoriasis. The PUVA freckles may persist for years and involve the risk for the development of skin cancer. Studies have confirmed that the treatment of psoriasis with PUVA could increase the risk of developing skin cancer (Mali-Gerrite MG *et al.*, 1991; Chuang TY *et al.*, 1992; Studniborg HM *et al*, 1993; Van-Praag MC *et al.*, 1993; Stern RS *et al*, 1994).

In the systemic treatment, use of corticosteroids is still the first-line choice. Improvement of symptoms can be obtained by the use of systemic corticosteroids, especially in the widespread and more acute forms of psoriasis. The mechanism is thought to be "anti-inflammatory" in the broadest sense. The major drawback is that a transient relief is often followed by a severe relapse of the disease and it almost always becomes more severe than before.

Methotrexate (MTX) was used as an antipsoriatic agent for the systemic therapy.

It is known to inhibit DNA synthesis by competing as a substrate for dihydrofolate reductase and is thought to act primarily on the rapidly dividing epidermal cells within psoriatic lesions. It directly affects the proliferation cells, either epidermal keratinocytes or lymphoid tissue cells, by inhibiting cell cycle and by decreasing mitosis resulting in cell death (Weinstein GD *et al* 1990). However, topical use of MTX to psoriatic lesions is not effective (Weinstein GD *et al*, 1976). Systemic use of MTX is often accompanied by severe side effects that may lead to liver damage and cirrhosis. Fibrosis of the liver is seen in approximately 10 percent of patients treated for long period, and cirrhosis is seen in 5 percent (Zachariae H *et al.*, 1980). The cumulative dose of MTX has important impact on the risk of hepatotoxicity. For these reasons, MTX is only used in severe and recalcitrant psoriasis.

Retinoids, derivatives of vitamin A, have been used as a treatment for psoriasis. The response to therapy is variable and depends on the type of psoriasis. The best outcomes have been in the treatment of pustular psoriasis or guttate psoriasis of recent onset (Ellis CN *et al*, 1987). Retinoids are lipophilic and accumulate in the fat tissue after prolonged therapy. Pharmacokinetic studies revealed that after cessation of therapy, retinoids administered for psoriasis could still be detected in the plasma even 4 months after the last dose. Since retinoids have been proved to be teratogenic in several animal studies, they are contraindicated in female patients in their reproductive period of life (Windhorst DB *et al*, 1982).

The combination of several therapies has also been applied to optimize the efficacy and to reduce the toxicity of individual drug treatments (Menter A *et al.*, 1991). The treatment of psoriasis is far from perfect. The quest for a more potent and selective drug and more efficient clinical use of existing drugs are in progress.

1.3.3 The Use of Cyclosporin A in Psoriasis

Treating psoriasis with cyclosporin A (CSA) is thought to be a promising development. It appears to have more potential for treating psoriasis than any other therapy (Mahrle G *et al.*, 1995). Studies have shown over 90% of psoriatic lesion cleared after treating with CSA (Ellis CN *et al.*, 1986; Menter A *et al.*, 1991). In fact, the clinical effectiveness of CSA has been considered a major advancement in the understanding and treatment of psoriasis.

As a novel and effective immunosuppressant, CSA is a main component of metabolites isolated from the fungus, *Tolypocladium inflatum* Gams. It has been used in the prevention and treatment of transplant organ rejection and autoimmune diseases in humans, since its immunosuppressive properties were found in late 1970's (Borel JF *et al.*, 1976a; b; c; Calne RY *et al.*, 1978; Powels RL *et al.*, 1980). Because of its effective inhibition of the antibody and cell-mediated immune responses, CSA is used as an immunosuppressant which has improved the survival of patients receiving a variety of organ allotransplantation (Colonna JO *et al.*, 1988;

Borel JF *et al.*, 1989a; 1991; Yamada S *et al.*, 1994; Thiel G *et al.*, 1994).

Mueller and Hermann (1979) first reported improvement in psoriatic plaques with the use of CSA. Thereafter, many studies have confirmed that CSA is an effective agent in the treatment of psoriasis, including erythodermic, pustular and chronic plaques (Ellis CN *et al.*, 1986; Bos JD, 1988; Talal N, 1988; Heule F *et al.*, 1988; Griffiths CE *et al.*, 1989; 1990; Fradin MS *et al.*, 1990; Meinardi MM *et al* 1990; Farber EM *et al*, 1993; Lewis HN, 1994; Laburte C *et al.*, 1994).

The pharmacology of CSA has been extensively investigated (Borel JF *et al.*, 1989b). Its powerful selective immunosuppressive activity is elucidated as the inhibition of both antibody and cell-mediated immunity, especially of lymphokine production secreted by activated T cells (Borel JF *et al.*, 1989b; 1990). In contrast to classical immunosuppressants, CSA specifically and reversibly acts on the lymphocytes and does not affect the function of phagocytes or hemopoietic stem cells. It affects the early steps immediately following antigen or mitogen recognition, which results in restricting the T-cells to remain in the resting phase of the cell cycle. The restricted T-cells are unable to transform and release lymphokines and thus fail to induce an immune response. CSA can also suppress an ongoing immune reaction or an autoimmune relapse. With reversible action, CSA does not cause lymphocytotoxic or mutagenic changes in the body (Borel JF, 1989b).

In the treatment of psoriasis, CSA has been reported to inhibit several activation pathways related to immune system (Bos JD, 1988). It is also postulated that most of the immunosuppressive effects of CSA are due to the inhibition of gene expression resulting in the inhibition of the production of IL-2 and of other keratinocyte cytokines (Gottlieb AB *et al.*, 1992; Won YH *et al.*, 1994). Specific inhibition of T-cell infiltration and lymphokine secretion, keratinocyte proliferation and cytokine production as well as antigen presentation and cytokine secretion are believed to be involved as well (Valdimarsson H *et al.*, 1986; Shuster S, 1988; Dipadova FE, 1989; Ryffel B, 1989; Griffiths CE *et al*, 1990; Wong RL *et al.*, 1993).

The formulations of CSA are marketed by Sandoz Pharmaceuticals Inc. and are available as intravenous formulations, oral solution (Sandimmune®) and oral soft gelatin capsule (Yee GC, 1991). The oral absorption of CSA from Sandimmune® is slow and incomplete. Peak concentration in patients was reported to occur 1 to 8 hours after oral dosing. Considerable inter-patient variations have been observed in the peak concentration of CSA. Clinical Pharmacokinetic studies revealed that the absorption phase of CSA was the rate-limiting step (Ptachcinski RJ *et al.*, 1986; Fahr A, 1993). In this step, intestinal enzymatic degradation of CSA is substantial (Wu CY *et al.*, 1995).

Many studies have focused on the improvement of systemic absorption and bioavailability of CSA. Attempts were made by taking advantage of techniques of

enteric solid dispersion (Takada T *et al.*, 1989), microemulsion (Tarr BD *et al.*, 1989; Ritschel WA *et al.*, 1990; Mueller EA *et al.*, 1994; Trull AK *et al.*, 1994; Ferea G *et al.*, 1994; Friman S and Bächman, 1996), by means of water-soluble vitamin E (Sokol RJ *et al.*, 1991), oral administration in an olive oil solution (Ueda CT *et al.*, 1983; Takada K *et al.*, 1988), solid micellar solution (Drewe J *et al.*, 1992), liposomal formulation (Vadiei K *et al.*, 1989; Arnoux R *et al.*, 1990; Stuhne-Sekalec L *et al.*, 1986a; 1986b; 1989; 1991; Freise CE *et al.*, 1991) and microsphere technique (Sánchez A *et al.*, 1995). A new oral soft gelatin CSA capsules (Neoral®, Sandimmune, Sandoz) has recently been introduced (Jewkes FME, 1995; Nobel S *et al.*, 1995), which incorporates the drug in microemulsion formulation containing surfactants. The microemulsion formulation dose not depend on the bile salts for solubilization in the GI tract and is claimed to have an improved bioavailability profile compared to Sandimmune®.

CSA improves the skin of patients with psoriasis by a local mechanism of action, in the epidermis and the junction of epidermis and dermis (Ho VC *et al.*, 1990; Griffiths CE *et al*, 1990; Burns MK *et al.*, 1992; Wong RL, 1993). The Inhibition of mitotic activity of CSA in psoriatic lesions to reduce hyper proliferation of epidermal keratinocytes suggests that, systemically administered, CSA could be first taken up preferentially by the lower layers of epidermis and then distributed upward with time. After oral administration, human epidermis contained a high concentration of

CSA. The epidermis of psoriasis patients contained 10 times more CSA than the blood samples after 7-day oral treatment with 14 mg/kg/day of CSA (Fisher GJ *et al.*, 1988). Although no correlation between patient's clinical response and blood level of CSA was observed, a significant difference between the CSA uptake by psoriatic epidermis and non-psoriatic epidermis was found. Furthermore, CSA therapy did not impair normal epidermal functions (Fisher GJ *et al.*, 1988). However, the systemic use of CSA can be accompanied by severe dose-dependent toxicities that include nephrotoxicity, hypertension and hepatotoxicity (Curtis JJ *et al.*, 1988; Mason J *et al.*, 1989; Griffiths CE *et al.*, 1990). The major toxicity of CSA is impairment of renal function, which has a high incidence in recipients with renal transplantation and patients with autoimmune diseases (van Graffenried B *et al*, 1986; Feutren G *et al.*, 1990). Infection and liver malfunction can also occur in these patients. High doses of CSA are toxic to cardiac myocytes in certain strain of mice and rats (Paul LC *et al.*, 1991), and probably in man as well (Oyer DE *et al.*, 1983). CSA-induced neurotoxicity has also been reported (Pace MT *et al.*, 1995). These findings suggest a potential feasibility of using CSA topically in the treatment of psoriasis.

In the treatment of psoriasis, the therapeutic efficiency of CSA with systemic administration is dose-dependent (Borel JF, 1990). The narrow therapeutic window of CSA (Shaw LM *et al.*, 1987; Holt DW *et al.*, 1991; Awni WM, 1992; Kivisto KT.,

1992) combined with the erratic absorption and nonlinearity in the dose versus blood concentration relationship in oral formulation (Reymond JP *et al.*, 1988; Mueller EA *et al.*, 1994) impose a risk of over- or under-dosing that may lead to inconsistencies in the treatment of psoriasis. Another shortcoming in the systemic use of CSA is its low bioavailability. The absolute bioavailability was estimated to be about 30% , ranging from 10% to 60% (Fahr A, 1993). CSA is the most expensive therapy in the treatment of psoriasis due to the relatively higher cost of medication and the laboratory tests to monitor renal status (Sander HM *et al.*, 1993). In contrast, topical use of CSA is expected to require a low dose which would avoid the systemic side effects and would also cost less.

CSA is extensively metabolized by the cytochrome P-450 3A enzyme family, which is a catalyst for the oxygen-dependent metabolisms and the major cyclosporine-metabolizing enzyme family located in both liver and small intestine in humans (Kronbach T *et al.*, 1988; Watkins PB, 1990; Shet MS *et al.*, 1993; Christians U *et al*, 1993). Systemic delivery always leads to first-pass effect in the liver and/or in the gut wall of small intestine (Kolars J *et al.*, 1991; Ducharme MP *et al.*, 1993; Lindberg FA *et al.*, 1994). About 1% unchanged CSA is excreted renally after systemic administration (Shah AK *et al.*, 1995). Cytochrome P450 enzymes are ubiquitously distributed in the whole body. Besides liver, drug metabolic activity involving cytochrome P450 enzymes has been shown in kidney, lung and small

intestine (Gonzalez FJ, 1992). Although human lung and kidney contain P450 3A enzymes, no significant metabolism of CSA has been reported in these organs.

CSA undergoes both phase I and phase II metabolisms. To date there are more than 30 CSA metabolites isolated from bile and urine of different species and from samples obtained after incubation of CSA with liver microsomes (Christians U *et al*, 1993). The sulfate conjugates are formed directly from CSA (Christians U *et al*, 1993). All these metabolites of CSA maintain their cyclic undecapeptide backbone structure intact.

The skin is an active site of metabolic activity. Metabolites generated in cutaneous tissue may diffuse to the bloodstream where they may move to extra-cutaneous tissue and exert effects therein. The epidermis possesses additional subcellular constituents that in general diminish the toxicity of xenobiotics, and one of them is the family of cytochrome P450 enzymes (Mukhtar H *et al*, 1989). Although the epidermal P450 metabolizes a number of endogenous and exogenous compounds (Takahara H *et al.*, 1993; Jugert FK, 1994), CSA was reported not to be metabolized in normal and psoriatic epidermis (Duell E *et al.*, 1991). The probable reason might be attributed to the low content (about 5%) of P450 enzymes in epidermis. These facts suggest that topical delivery of CSA could bypass both hepatic and gut metabolisms. Thus an effective topical formulation of CSA that is able to penetrate SC will exert antipsoriatic therapeutic action at the required site,

without any noticeable systemic side effects (Brown MD *et al.*, 1989; Fradin MS *et al.*, 1990).

Intensive search for an effective topical preparation of CSA for the treatment of psoriasis has been tried by different researchers. Although certain putative penetration enhancers have been used to improve skin permeability and absorption of CSA, such as olive oil, propylene glycol and Azon®, none has been able to deliver sufficient amount of CSA (Gilhar G *et al.*, 1988; Hermann RC *et al.*, 1988; Fradin MS *et al.*, 1990; Bunse T *et al.*, 1990; De-Rie MA *et al.*, 1991; Korstanje MJ, 1992; Mrowietz U, 1992; Duncan JI *et al.*, 1993). Liposomal formulation of CSA is also under *in vitro* evaluation (Egbaria K *et al.*, 1991).

Intralesional injection has also been used in order to deliver CSA (Barker BS *et al.*, 1989; Ho VC *et al.*, 1990; Burns MK *et al.*, 1992). The efficacy of intralesional CSA in the treatment of psoriasis, without any significant systemic absorption, indicated that CSA does not act solely through systemic route and that it has a local mechanism of action. Thus the several unsuccessful attempts to treat psoriasis with topical use of CSA may be due to inadequate penetration in the skin. However, the disadvantages with intralesional injection are that it is extremely painful to patients and that it is only applicable in the case of localized plaques (Ho VC *et al.*, 1990; Burns MK *et al.*, 1992).

A transdermal delivery system that would result in a suitable concentration of

CSA in the skin without the invasive pain and risk of systemic toxicities would be very desirable. The main hurdles in achieving the required topical concentration of CSA are the barrier function of the skin and the physicochemical properties of CSA.

1.3.4 Physicochemical Properties of CSA

CSA is a neutral, hydrophobic cyclic undecapeptide, with a molecular weight 1203 *Da* ($C_{62}H_{111}N_{11}O_{12}$). The molecular structure of CSA is cyclo-[-[4-(*E*)-but-2-enyl-*N*,4-dimethyl-*L*-threonyl]-*L*-homoalanyl-(*N*-methylglycyl)-(*N*-methyl-*L*-leucyl)-*L*-valyl-(*N*-methyl-*L*-leucyl)-*L*-alanyl-*D*-alanyl-(*N*-methyl-L-leucyl)-(*N*-methyl-*L*-leucyl)-(*N*-methyl-*L*-valyl)-] (Figure 3). Ten of the amino acids are known and in position "1" is an amino acid, (4*R*)-4-[(*E*)-2-butenyl]-4-methyl-*L*-threonine (MeBmt), which was never seen before (Rüegger *A et al.*, 1976; Petcher TJ *et al.*, 1976). NMR analysis of the molecule revealed a fairly rigid backbone and fixed combinations for the side chains except the amino acids in the position 1 and 2 (Loosile HR *et al.*, 1985). All amino acids are natural *L*-amino acids with *S*-configuration except for the D-Ala in position 8 with *R*-configuration. Seven of the amino acid residues are *N*-methylated (position 1, 3, 4, 6, 9, 10 and 11). The successful total synthesis of CSA was first reported by Wenger RM (1984).

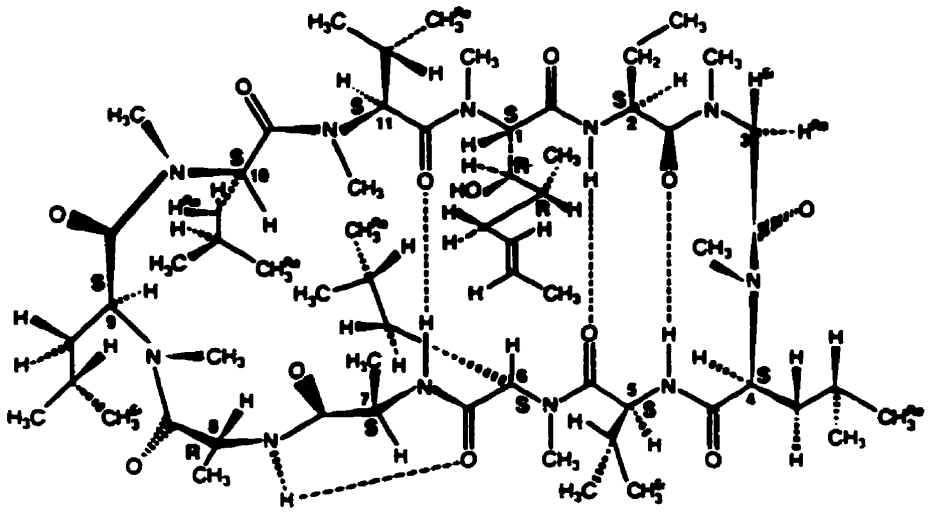


Figure 3 Molecular Structure of CSA

In addition to CSA, the fungus, *Tolypocladium inflatum*, produces a number of metabolites with similar structures. They have been isolated and chemically

characterized (Borel JF *et al.*, 1991). These cyclosporins are all composed of 11 amino acids and with only minor changes in amino acid units. Some of them are biologically inactive. None of the natural cyclosporins or synthetic analogous possess greater pharmacological potency than CSA in either *in vitro* or *in vivo* tests. Only a few of natural cyclosporins, namely cyclosporin A, C, D, G and M, have been found to be effective in *in vivo*. The unusual structure of MeBmt in the CSA cycle structure seems to be involved biological effects of CSA but alone it does not have any immunosuppressive activity.

All metabolites of CSA resulting from the *in vivo* biotransformation found so far have always maintained their cyclic peptide structure intact. The majority of metabolites of CSA have been found to be less effective than CSA itself (Radeke HH *et al.*,1992) and do not contribute to the immunosuppressive effects of CSA (Freed BM *et al.*, 1987; Garraffo R, 1992). Toxicity studies have shown that metabolites of CSA have less nephrotoxic potential and cannot account for the CSA-induced nephrotoxicity (Ryffel B *et al.*, 1988).

The important physicochemical properties of CSA are summarized in Table 2. The behavior of solubility of CSA is unique. It was found to be inversely proportional to temperature (ranging from 5-37°C), and an exothermic heat of solution was indicated. Same solubility behavior had been found in aqueous solution with different pH values (Ismailos G, 1989).

CSA will lose its immunosuppressive activity if its cyclic structure is destroyed. It was reported that acylating the hydroxyl groups of CSA results in significantly reduced activity (Wenger RM, 1989). Thus, changing structure by means of chemical approach to alter its physicochemical properties favorable for drug delivery may not be a useful strategy.

Table 2 The Physicochemical Properties of CSA*

Cyclosporin A	neutral, hydrophobic peptide
Molecular Weight	1202.64
Density	1.042 g
Appearance	white prismatic crystals
Melting range	148-151°C (natural)
	149-150°C (synthetic)
log P_{oct}	2.92**
log P_{hep}	1.40**
Solubility	insoluble in water and n-hexane; very soluble in all other organic solvents, such as methanol, ethanol, ether and chloroform
UV	end absorption at 200 nm

* Hassen MMA *et al.*, 1987
** Tayar NE *et al.*, 1993

II STATEMENT OF RESEARCH PROBLEM

The immunosuppressive peptide drug, CSA is clinically effective in the treatment of psoriasis. **Systemic administration of CSA is fraught with the risk of severe side effects. Treating psoriasis topically with CSA would be desirable but no such delivery system is presently available.**

III RESEARCH OBJECTIVE

SC is the main barrier in the skin for the transdermal drug delivery. Data derived from morphological, histochemical and biochemical studies provide strong evidence that SC can be viewed as a heterogeneous two-compartment system of lipid-laden intercellular domain (Elias PM, 1991). To overcome the barrier function of SC and facilitate the drug delivery through it, electrically enhanced topical and transdermal drug delivery approaches, including iontophoresis and electroporation, have been used. These techniques are currently studied in our laboratory to enhance transdermal delivery of peptide drugs.

The objective of present study is to develop a transdermal delivery system for CSA using iontophoresis and electroporation technologies.

IV RESEARCH HYPOTHESES

In developing the research hypotheses, a brief background information is included as a prelude. The ability of drugs to penetrate human skin depends on several properties: skin structure, lipophilicity and molecular size or configuration of the drug (Yoshida NH *et al.*, 1992). A number of unknown parameters related to these properties make it difficult to predict whether a particular molecule can be delivered transdermally. Several models have been developed to theoretically describe the transdermal transport of drugs (Guy RH *et al.*, 1989; Potts RO *et al.*, 1989; Keister JC *et al.*, 1992; Edwards DA *et al.*, 1994). But they are too complicated to be used in practice. For *in vitro* modeling of percutaneous absorption, Fick's first law of diffusion (ii) is still believed to be applicable in simplifying the analysis (Guy RH *et al.*, 1989; Sugibayashi K *et al.*, 1996).

$$\frac{dQ}{dt} = \frac{DK_pC}{h} \qquad \text{(iv-1)}$$

Equation (iv-1) is valid only in the sink condition, in which the rate of absorption of the drug by the skin (dQ/dt) is proportional to the concentration gradient or thermodynamic activity of the drug (C) across the skin membrane of thickness (h), and the partition coefficient of drug (K_p) between skin and vehicle. D is the diffusion

coefficient. This indicates that the concentration gradient could be as a driving force in passive diffusion. Although more complicated models were proposed (Potts RO *et al.*, 1992; Kontturi K *et al.*, 1996), they give the same prediction in the trend to drug penetration using these parameters. To optimize diffusion, the thermodynamic activity of the drug should be maximized without adversely affecting the partition coefficient of the drug. In order to effectively deliver CSA by iontophoresis and electroporation, the drug solution should not only have sufficient conductivity for the passage of electrical current but also have an optimum thermodynamic activity to facilitate diffusion.

Water is considered as a universal solvent which constitutes 55-65% of human body weight. The state of hydration of SC is one of the most important factors in determining the rate of percutaneous absorption of a given solute. Hydrated SC has higher permeability for drug molecules. In this sense, water is very desirable as a vehicle for drug delivery through the skin. Water molecules exhibit a slight but physiologically important tendency of dissociation to act as either an acid or a base, which allows it to dissolve most physiologically important electrolytes and many drugs as well as to conduct electric current. Due to its ability to conduct electric current, water is a good solvent for iontophoresis and electroporation. However, CSA is poorly soluble in water.

Ethanol can destabilize the lipid phase of SC, leading to increased lipid chain

mobility (or fluidization) and/or lipid extraction (Krill SL *et al.*, 1992b; Ghanem AH *et al.*, 1992). Ethanol has been extensively used in many topical preparations as a penetration enhancer. CSA is quite soluble in ethanol. Aqueous ethanolic solution of CSA has resulted in increased penetration in the skin (Egbaria K *et al.*, 1990; Kim DD *et al.*, 1996). Its systemic toxicology and local tolerability have been well established. Ethanol, being a non-ionizable solvent, is a poor conductor of electric current and may not be a good choice for iontophoresis and electroporation drug delivery. However, a hydroethanolic solution may combine the solvent properties of ethanol and water, and also provide the required conductivity. There is no report of using hydroethanolic solution as a solvent for iontophoresis and electroporation studies.

Thus, the two problems in developing a transdermal delivery system for CSA using iontophoresis and electroporation are of thermodynamic activity of CSA and electrical conductivity of drug solution. This leads us to the following two hypotheses.

Hypothesis 1:

Solid dispersion (such as coevaporate) of CSA prepared with water soluble polymer will have increased aqueous dissolution, which will provide the necessary thermodynamic activity of CSA while maintaining a good electrical

conductivity.

Hypothesis 2:

Hydroethanolic solution will be a good solvent with a balanced solvent property for CSA without adversely affecting the electrical conductivity of the medium.

Hypothesis 3:

Electroporation and iontophoresis can be used as the permeation enhancement techniques to deliver CSA transdermally.

The mechanisms of iontophoresis can be described based on the modified Nernst-Plank flux equation (Keister JC *et al.*, 1992)

$$J_i = -D_i \frac{\partial C_i}{\partial x} - D_i \frac{z_i F C_i}{RT} \frac{\partial \phi}{\partial x} \pm J_v C_i (1 - \sigma_i) \qquad \text{(iv-2)}$$

J_i: *steady-state flux of the permeant i*

D_i: diffusion coefficient of the permeant i

C_i: concentration of the permeant i

z_i: charges of the permeant i

F: Faraday's constant

R: gas constant

T: absolute temperature

ϕ: electric potential at any point *x* in the membrane

J_v: solvent velocity

σ_i: permeant's reflection coefficient
at the membrane-solution boundary.

In this equation the term $\pm J_v C_i(1-\sigma_i)$ is a measure of the transport of permeants resulting from the convective solvent flow or electro-osmosis. In a negatively charged membrane such as the skin, it facilitates the flux of positively charged permeants and impedes that of negatively charged permeants. Therefore, the term $+J_v C_i(1-\sigma_i)$ can be used to account for the effect of convective solvent flow on the flux of positively charged permeants, while the term $-J_v C_i(1-\sigma_i)$ can be used for negatively charged permeants (Srinivasan V *et al.*, 1990).

Based on the modified Nernst-Plank equation (iv-2), drug permeation could take place in three ways: passive diffusion, electro-osmotic flow and electrical migration of ionic drug molecules. For non-ionic species, the electro-osmotic flow acts as an additional driving force complimenting passive diffusion. For lipophilic compounds, transport of relatively small molecules is retarded through the hydrated pores in the skin (Del-Terzo SD *et al.*, 1989). For CSA which is a neutral, lipophilic molecule, iontophoretic delivery would mainly be due to the effect of electro-osmosis.

Electroporation could involve three mechanisms to promote the molecular transport in transdermal delivery, which are electrical drift (electrophoresis, ion migration), electro-osmosis and passive diffusion (Dimitrov DS *et al.*, 1990; Edwards DA *et al.*, 1994; Weaver JC, 1995). Besides electrical drift, the electro-osmosis can enhance the penetration of both water-soluble and water-insoluble as well as neutral molecules. It is speculated to be important in the transport of macromolecules and neutral molecules in iontophoresis (Srinivasan V *et al.*, 1990; Pikal MJ, 1992). In electroporation, increased pore pathways and pore size may facilitate the effect of electro-osmosis and passive diffusional transport for neutral and lipophilic compounds, such as CSA.

V MATERIALS AND METHODS

5.0 Materials

CSA and CSD were obtained from Sandoz Pharma Inc., Switzerland and were used as received. [mebmt-β -^{3}H-labeled] CSA was procured from Amersham Life Sciences, Ontario, Canada (specific activity: 6.00 Ci/mmoL; molecular weight: 1203 at this specific activity; radioactive concentration: 1.0 mCi/mL). Ethanol-1-^{14}C (^{14}C-EtOH) was obtained from Sigma®Chemical Company, St. Louis, MO, USA (molecular weight: 46.07, purity ≥ 98%, specific activity: 8.1 mCi/mmoL, neat density: 0.785) and made up to 50 μCi/mL. Tritium water was obtained from Du Pont, NEN Products, Boston, MA, USA (specific activity: 37.0 mBq/gr or 1.0 mCi/gr, radioactive concentration: 1.00 mCi/mL). The Cyclo-Trac® SP-Whole Blood for the radioimmunoassay of CSA was obtained from Incstar Corporation (Stillwater, Minnesota, USA)

Polyvinyl methyl ether maleic acid copolymer (Gantrez® S-97 BF, PVME/MA) was obtained as a free sample from International Specialty Products Inc., N J, USA and was used as received (molecular formula: -[$C_4H_4O_4$-C_3H_6O]-$_n$; molecular weight: 70,000). The tissue digesting solvent (Tissue and gel solubilizer, Solvable®, 0.5M solution) and scintillation cocktail (high flash-point LSC cocktail Formula-989) were obtained from Du Pont NEN Research Products, MA, USA. All other chemicals and reagents were of analytical or HPLC grade.

The HPLC system comprised a Hewlett-Packard pump system (HP-1050, Waldbroon, FRG), a multiple wavelength UV detector and an autosampler. A C_{18} reverse phase column (Bondclone®, Phenomenex, CA, USA, 150 × 3.9 mm) with a C_{18} guard column (4.60 × 3.9 mm) was used in the analysis of CSA. A liquid scintillation counter (Beckman LS 5000 TD, CA, USA) was used to measure radioactivity of radioisotope-labelled drug solution.

Electrical power supply used for iontophoresis was PS 500 X DC Power Supply, Hoefer Scientific Instruments, San Francisco, LA, USA. A custom made electronic circuit was used to maintain a constant electric current in the *in vitro* experiments. Digital multimeters (Micronta®, Intertan Canada Ltd, Barrier, ON, Canada) were connected in circuit to monitor the voltage and current.

A Cell Porator® (Life Technologies Inc. ON, Canada) and a Gene Pulser® II (Bio-Rad laboratories, CA, USA) units were used respectively, as the source to generate single and multiple exponential electrical pulses in electroporation. The electrodes of Gene Pulser® and BioRad® electroporation units were replaced with custom made Ag/AgCl electrodes. With the Cell Porator® an oscilloscope (Tektronix®, OR, USA) was attached to monitor the pulse waveform and measure pulse voltage and time constant. The Gene Pulser® unit had a built-in panel where the pulse voltage and time constant were displayed.

The Ag/AgCl electrodes used in both iontophoresis and electroporation were

made from silver wire (1.0 mm diameter, purity: 99.99 %, Aldrich® Chemical Co., WIS, USA). A layer of AgCl was deposited on the silver wire by immersing the silver wire in molten AgCl. The linear electrodes were used in iontophoresis with the lengths of 2 cm in donor and of 7 cm in receiver compartments. In electroporation, the silver wire was spiraled to form a circle with diameter of 0.5 cm (area: ~ 0.2 cm^2).

Franz diffusion cells (Figure 4) were used in iontophoresis (Laich®, industries Corp., USA). The iontophoresis electrical circuit is shown in Figure 4, in which a DC constant-current device used in circuit is a slight modification of the device reported by Su MH *et al* (1994). The output current is determined by measuring the voltage drop across a resistor (R = 1000 Ohms) with the Multimeter 1 (Figure 4). The current (mA) is equivalent to the voltage read from the Multimeter 1 divided by R (1000 Ohms). The Multimeter 2 is used to measure the voltage across the electrodes, a fraction of which is transdermal voltage (U_{skin}). A stirring digital dry block heater (model 274, PMC Dataplate®, Crown Bioscientific Inc., NJ, USA) was used to keep the receiver cells at 37°C. The effective diffusional cross area of the skin samples was 0.80 cm^2.

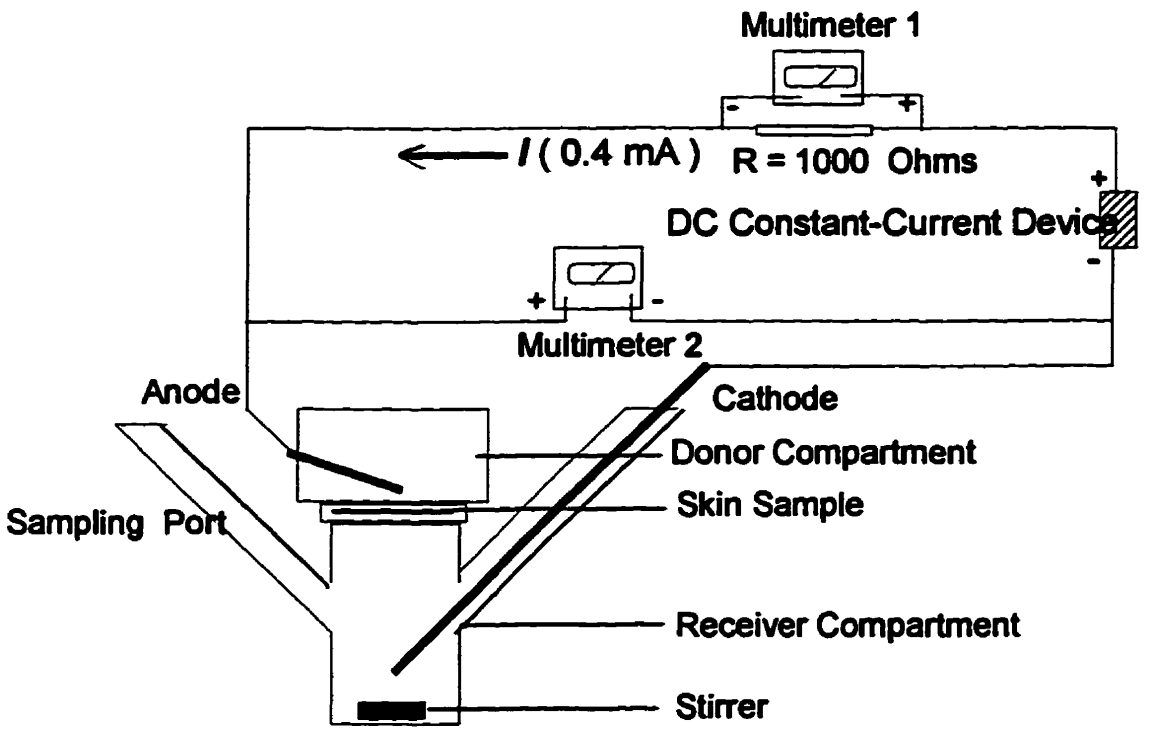
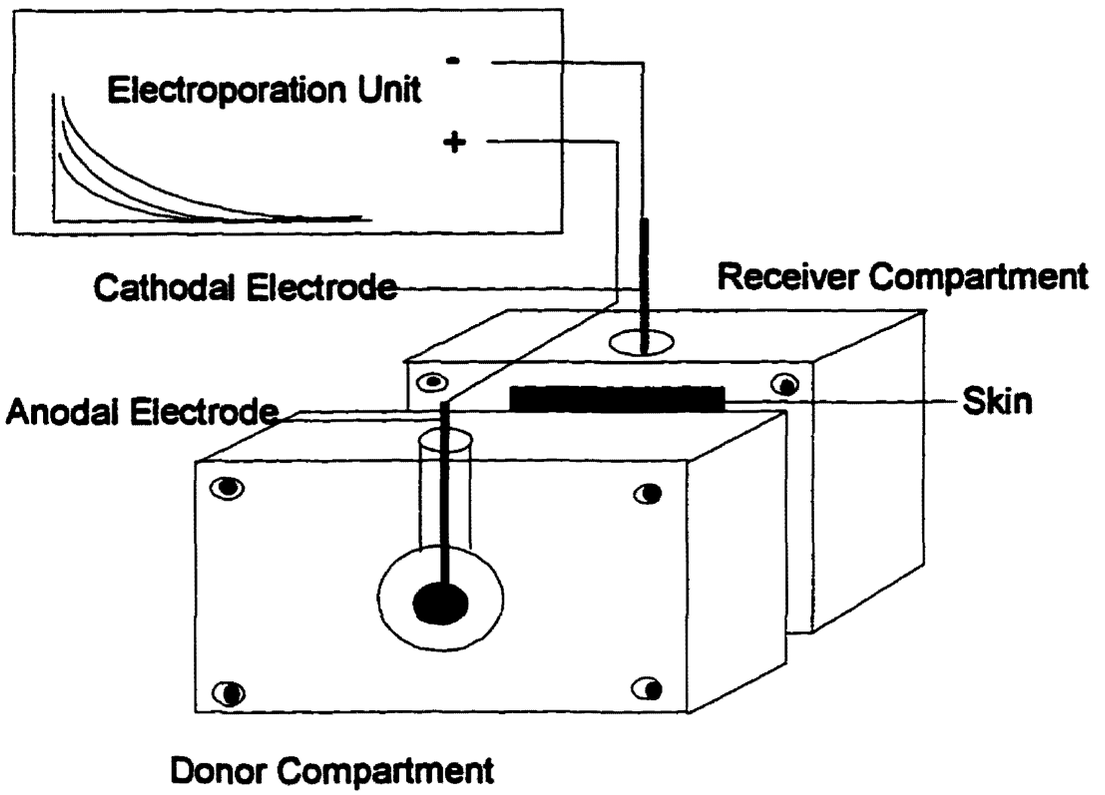


Figure 4 **Schematic Diagram of Franz Cell and Iontophoresis Circuit**

Side-by-side diffusion cells (Figure 5), custom-made of Delrin (polymethanal, Dupont®), were used in electroporation experiments. The effective diffusion cross-area of the cells was 1.3 cm^2. The volume of both donor and receiver compartments were 0.8 mL. The distance between two electrode ports in the two compartments was 1 cm.



igure 5 Schematic Diagram of Side-by-Side Diffusion Cell and Electroporation Unit

Model 925 pH meter (Accumet®, Fisher Scientific. USA) equipped with gel-filled combination pH electrodes (Orion®, Orion Research Incorporated Laboratory Products Group, Boston, MA, USA) was used to measure pH values of all samples.

Hairless rat (Harlan Sprague Dawley, IN, USA, 5-6 weeks old) was selected as

the animal, whose skin was used as model membrane in the transdermal delivery experiment of CSA. Freshly excised skin was used within 48 hours in all the experiments. All fat and muscle tissues were carefully removed with surgical scissors and forceps. The skin was then kept soaked in PBS solution and hydrated for at least 1 hour before use. The integrity of the skin could be ascertained by the multimeter readings and any skin samples showing abnormally low electrical resistance was discarded.

5.1 Analyses

5.1.1 HPLC Analysis

The concentrations of CSA from aqueous test samples were determined using an HPLC procedure described in the U.S.P. XXII [USP XXII, 1990] after modifying it to suit our laboratory conditions. The instrument was controlled and monitored using the ChemStation® software supplied by Hewlett Packard. The column was maintained at 70°C. The mobile phase consisted of 75% acetonitrile in water delivered isocratically at 1.5 mL/min. The UV detector was set at 210 nm to detect CSA in the sample. CSD was used as the internal standard. Standard curves were obtained using standard solutions of CSA, ranging from 0.5 µg/mL to 5 µg/mL. All samples were of 500 µL to which 25 µL of CSD (100 µg/mL in mobile phase) was added. Twenty µL of the solution was injected in duplicates. All measurements

were based on CSA/CSD peak height ratio. Standard curves were prepared with each set of experimental test solutions.

5.1.2 Liquid Scintillation Counting (LSC)

The donor CSA solution was spiked with radiolabelled ^{3}H-CSA to facilitate analysis. CSA concentrations in the skin, receiver and donor compartments before and after iontophoresis and electroporation experiments were determined by measuring the radioactivity of tritium from ^{3}H-CSA using single label LSC program. The measurements were made in DPM (disintegrations per minute). The 2-sigma error was set at $\leq$ 10% (maximum counting time = 20 min). The standard curves were prepared using the DPM of known strengths of CSA solutions which were prepared with each set of experimental test solutions. The background radioactivity was measured in each set of experiment using non-labelled samples and subtracted from samples' DPM values.

In order to study the transport of water and ethanol across the skin, a donor solution containing ^{3}H-H_2O and^{14} C-EtOH was used. For this purpose the scintillation counter was programmed to dual label counting, with 2-sigma error set at $\leq$ 5% (maximum counting time = 10 min). Other procedures used were the same as those in single label LSC.

5.1.3 Radioimmunoassay (RIA)

Analysis of CSA by RIA was attempted to ascertain that the CSA in the skin determined throughout the project using LSC was indeed the intact CSA molecules and not a fragment. Both skin and receiver samples were measured. Ethanolic solution containing 3.00 mg/mL of unlabeled CSA was used as donor solution. The RIA reagents supplied with the RIA kit of CYCLO-Trac® SP-Whole Blood were added to the samples as follows:

(1) Total count tubes: 100 µL of ^{125}I-CYCLO-Trac SP

(2) Nonspecific binding tubes: 50 µL of extracted 0 standard, 100 µL of ^{125}I-CYCLO-Trac SP, 1.0 mL of CYCIO-Trac SP NSB ImmunoSep

(3) CYCLO-Trac SP 0 Standard: 50 µL of extracted CYCLO-Trac SP 0 Standard 100 µL of ^{125}I-CYCLO-Trac SP

(4) CYCLO-Trac SP Standards (A-E): 50 µL of extracted CYCLO-Trac SP Standard, 100 µL of ^{125}I-CYCLO-Trac SP

(5) Control and test samples: 50 µL of extracted samples, 100 µL of ^{125}I-CYCLO-Trac SP

All tubes were incubated at 20-25°C for 1 hour, and then were centrifuged using 1600 × g, where $g = 1118 \times 10^{-8}$ (radius in cm) $(rpm)^2$, for 20 min at 20-25 °C. The supernatant from all the tubes was decanted except the total count tubes and the precipitate of each tube and total count tubes was counted using a gamma scintillation counter (LKB, Wallac OY, Turku, Finland) for 2 min. All determinations

were in duplicate. The standard curve, $\%B/B_0$ (percent of specific binding to nonspecific binding) versus log concentration, was plotted using three cycle semi-log graph paper. The amount of CSA in samples was determined from the standard curve.

5.2 Preparation of CSA-PVME/MA Coevaporate

5.2.1 Preparation

CSA and PVM/MA copolymer in different weight proportions (w/w of CSA to PVMA/MA respectively 1:5, 1:8 and 1:10) were weighed and dissolved separately in distilled ethanol. The two solutions were mixed and solvent was evaporated completely in a flash evaporator, which was set at 100 revolutions per minute (rpm) and 50°C. The coevaporates were further dried in a desiccator under vacuum, pulverized and sifted through a standard sieve (sieve NO. 70., pore size 212 µm).

5.2.2 Equilibrium Solubility of CSA from Coevaporate

The equilibrium solubilities of the coevaporates and untreated CSA were determined in deionized water at room temperature (22 ± 1°C). For this an excess of the test sample in deionized water was shaken using an electrical shaker, until the equilibrium solubility was reached (about 20 hours). The samples were then centrifuged, filtered through 0.45 µm polyvinylidene fluoride filter (Millipore®,

Yonezawa, Japan) and analyzed by HPLC to determine the amount of CSA dissolved in water. The experiments were performed in duplicate or triplicates and the mean values were reported.

5.2.3 Dissolution of CSA from Coevaporate

Dissolution tests were done using a Vanderkamp R 600 [Vankel Industries Inc. NJ. USA] modified USP type II, six-spindle dissolution apparatus. One litre of deionized water at 37°C was used as the dissolution medium. The stirrer was set at 50 rpm. Coevaporate powder sample, equivalent to 2.5 mg of CSA, was placed in a nylon bag and tied to the paddle. This was done to prevent withdrawing any solid particles during the sampling procedure. Aliquots of 1 mL aqueous samples for analysis were withdrawn periodically and centrifuged at 13200 rpm for 5 minutes. To a 500 µL portion of supernatant aqueous samples 25 µL of CSD(100 µg/mL) was added and mixed. Twenty µL of the solution was injected into HPLC column for quantitative analysis. The volume of the dissolution medium was maintained constant by replenishing the aliquot sample with fresh medium equilibrated to 37°C. Cumulative amount of CSA released at each time point was determined by averaging results from four determinations.

Untreated CSA and the physical admixture of CSA and PVM/MA copolymer (1:8) were used as controls. Since one of the factors contributing to poor dissolution

could be inadequate wetting, an experiment was done to determine the role of a wetting agent in improving the aqueous dissolution of CSA, where 5 mg of CSA was mixed with 2 mg of a surfactant mixture of Span-80 and Tween-20 in a w/w ratio 7:3 (HLB 8.0) and the mixture was used as the test sample for a dissolution test in water.

5.3 Coevaporate Solution for Iontophoresis and Electroporation

Based on the above dissolution studies, 1:8 CSA/PVMA/MA copolymer was chosen for further studies. Labelled CSA (^{3}H-CSA) was incorporated during preparation of the coevaporate (0.056 µCi/mg). The donor solution for the transdermal experiments consisted of CSA solution in isotonic phosphate buffered saline (PBS), in which the concentration of CSA was 64 µg/mL (radioactivity of solution: 0.03 µCi/mL, pH 7.0 - 7.5). PBS was made of 138 mM NaCl, 8.1 mM Na_2HPO_4, 2.7 mM KCl, and 1.1 mM KH_2PO_4, and the pH was then adjusted to about 7.4, using NaOH or HCl solution. The donor solution was freshly prepared and used within 48 hours.

5.4 Ethanolic Solution of CSA for Iontophoresis and Electroporation

The hydroethanolic donor solution for the transdermal experiments consisted of 0.3% CSA (3.00 mg/mL) in 40 % ethanol (in PBS, v/v). Dual labelled solution viz.

^{3}H-CSA (0.105 μCi/mL) and C^{14}-EtOH (0.05 μCi/mL) enabled analysis of both the drug and ethanol. The ethanolic donor solution was freshly prepared in each set of experiment.

5.5 Control

The control experiment comprised a passive diffusional transport study of CSA. The composition of donor and receiver solutions and duration of experiments remained the same for both test and control experiment. In the test experiment, iontophoretic current or electroporation pulses were used, while in the control experiment the drug transport was solely due to passive diffusional force.

5.6 Iontophoresis

Iontophoresis was conducted using Franz diffusion cells. The hydrated skin sample was sandwiched between the donor and receiver compartments with SC facing the donor compartment (Figure 4). The Ag/AgCl electrodes were used to conduct DC current passing the diffusion cell during iontophoresis. The anode (positive electrode) was placed in donor compartment while cathode (negative electrode) in receiver compartment (Figure 4). The initial experimental runs resulted in a deposition of AgCl on the SC surface. Therefore, a piece of filter membrane (Millipore®, 1.2 μm, Millipore Corporation, Bedford, MA, USA) was

placed on the surface of SC to prevent occlusion of SC surface from AgCl precipitate. The anodal electrode was inserted into donor compartment while the cathodal electrode was inserted into receiver compartment. A constant DC current was supplied by a DC power supply with an electric current 0.4 mA. After subjecting the skin samples to 8 hours of iontophoresis, the experimental set up was allowed to stand for other 16 hours for passive diffusion. During the iontophoresis experiment, the receiver temperature was maintained at 37°C using a stirring digital dry block heater. The donor samples were taken before and after iontophoresis experiment. The receiver samples were taken before, during and after iontophoresis experiment. After iontophoresis experiment, the skin samples were taken out and the circumference of the skin which didn't contact the donor and receiver solution was trimmed off. The remaining skin (0.80 cm^2) samples were washed with 3 × 1 mL of deionized water, to remove the adhering CSA from the surface of the skin. After air drying for 30-60 min, the skin samples were stripped 10 times using Scotch® adhesive tape (3M, Ontario, Canada). At the end of the stripping process, the skin surface glistened which is an indication of removing all the layers of SC. Then the skin was completely digested in 1 mL of tissue digesting agent (Solvable™) maintained at 50-60°C for 3-5 hours. Thereafter to all the samples, 10 mL of scintillation cocktail (Formula-989) was thoroughly mixed to enable analysis of CSA with the liquid scintillation counter. The amount of CSA

was calculated based on the DPM measured after subtracting the background radioactivity.

5.7 Electroporation

5.7.1 Single and Multiple Pulse Electroporation Experiment

Exponential pulses were used in the electroporation experiment. The experimental parameters that were studied are as follows:

(1) applied electrode voltage ($U_{electrode}$)

(2) decay time constant of applied exponential pulse,
or pulse length (J)

(3) contact duration of experiment

(4) number of pulses - single or multiple exponential pulses

The applied electrode voltage, $U_{electrode}$, is defined as the peak value of voltage of an exponentially decaying electric field applied between electrodes, anode (positive electrode) and cathode (negative electrode), in electroporation. The decay time constant or pulse length, τ, is the time required for the value of $U_{electrode}$ drop to 37 % of original. The contact duration is the period of time for which the donor drug solution is in contact with the skin during the experiment.

Single pulse electroporation experiments were conducted at $U_{electrode}$, ranging from 100 to 370 V. Two pulse lengths, 5 and 10 milliseconds (ms), were tried.

The full-thickness hairless rat skin, including both from ventral and dorsal side, were used in the *in vitro* electroporation experiment. The skin was cut to appropriate size and sandwiched between two custom made side-by-side diffusion cells (Figure 5), with the SC facing the donor compartment.

The donor solution used in this part of experiments was either ^{3}H-CSA labelled CSA/PVMA-MA (1:8) coevaporate aqueous solution or ^{3}H-CSA and ^{14}C-EtOH dual - labelled ethanolic solution of CSA. The concentrations of CSA in both coevaporate aqueous solution and ethanolic solution were lower than their respective equilibrium solubilities of CSA so as to avoid precipitation of CSA. The receiver solution was PBS. The anodal electrode was placed in the donor compartment while the cathodal electrode in the receiver compartment. All the experiments were performed at the room temperature (22±1 °C).

The skin was allowed to equilibrate for one hour with PBS before initiating electroporation experiment. After the electric pulses of known value of $U_{electrode}$ and τ were delivered, the test unit of diffusion cell was left to stand undisturbed for a given contact duration. After experiment, the skin sample was dismantled from diffusion cell and washed with 1 mL of 40% ethanol and 3 × 1 mL deionized water. Thereafter, a skin sample was punched out using a biopsy punch (Area: 0.87 cm^2) to quantify the amount of CSA delivered within the viable layers of the skin. As mentioned earlier under iontophoresis, the SC was stripped using adhesive tape

and the remaining skin was completely digested in tissue digesting agent (Solvable™).

Besides the skin samples, aliquot samples, 50 μL from donor and 250 μL from receiver, were taken before and after the experiments to measure the amount of CSA in the donor and receiver compartments. All digested skin samples, test solution samples and SC tape-stripping samples were analyzed with liquid scintillation counter. The analysis procedure was the same as that described in iontophoresis.

In the multiple pulse mode, totally 25 pulses, at an interval of 1 min between pulses, were delivered. All samples were treated as same as that in single pulse experiment. $U_{electrode}$ used in multiple pulse experiment was chosen as 200 V. Different pulse lengths, τ, from 0.9 to 10 ms, and contact duration, from 2 to 12 hours, were chosen to study the optimum experimental conditions.

To analyze samples with RIA, the skin samples were homogenized after the multiple electroporation experiment at $U_{electrode}$ = 200 V, τ = 10 ms and contact duration = 4 hours. The homogenates were added to 4 mL of methanol to extract CSA for 24 hours. Then, extracts were centrifuged at 3000 rpm for 5 min. Two mL of supernatant of extracts was transferred to a volumetric flask and made up to 10 mL with methanol. Aliquots of 0.4 mL of receiver sample were taken after electroporation, and evaporated with nitrogen flow at 30°C. The residues were

dissolved with 400 µL of methanol. The samples were centrifuged at 3000 rpm for 5 min before the use. The fresh skin was used as a control compared to the skin samples which were subject to the multiple electroporation experiment. The control for receiver samples was done using 40% ethanol (in PBS) as donor solution.

5.7.2 Solvent Transport Induced by Electroporation

In this experiment, single and multiple electroporation pulses were delivered at $U_{electrode}$ = 200 V, τ = 10 ms. The skin sample was allowed to equilibrate for 2 hours to measure transport of solvents due to passive diffusion before subjecting it to electroporation pulses. Aliquots of receiver samples were drawn at periodic intervals up to 24 hours and analyzed. The donor solution was 40% ethanol in PBS, in which both water (0.105 µCi/mL) and ethanol (0.053 µCi/mL) were labelled. The samples were analyzed using scintillation counter to calculate the transport of water and ethanol.

5.8 Electron Microscopy

To observe any microscopic changes in the skin structure after electroporation, electron microscopic study was performed. The samples consisted of freshly excised and hydrated untreated skin and skin after electroporation. Duplicate samples were prepared in each instance. The electroporation experimental set was

the same as that described earlier (section 5.7.1) with multiple pulse mode, $U_{electrode}$ = 200 V and J= 10 ms. Immediately after the electroporation, the skin was fixed in glutaraldehyde fixative (0.1M cacodylate buffer, pH 7.2). The skin samples were left overnight in the same fixative at 4°C and were later washed repeatedly with 0.1M cacodylate buffer and post-fixed in 0.5% buffered ruthenium tetroxide (RuO_4), containing 2% potassium ferrocyanide for 1 hour at 4°C. Then the skin samples were dehydrated in ethanol and embedded in Spurr's low-viscosity resin. Ultrathin sections of the skin samples were stained with uranyl acetate and lead citrate, and viewed under a transmission electron microscope (Jeol, TEM-1200EX, Electron Microscope, Toyota, Japan).

5.9 Statistical Analysis of Data

All experiments have been done as multiple determinations and the $\bar{x}$ (mean) ± SD are reported. Non-parametric statistical methods were used to evaluate data. Analysis of variance and other statistical evaluations were done using Minitab® software (Minitab Inc., PA, USA) with a level of significance: $p = 0.05$.

VI RESULTS AND DISCUSSION

6.0 HPLC Analysis for CSA

To demonstrate that the chemical integrity of CSA in coevaporate was retained, HPLC analysis of untreated CSA and coevaporate were performed. The HPLC chromatograms of the coevaporate were comparable with that of untreated CSA and there were no extra peaks (Figure 6). This indicated that CSA in the coevaporate had maintained its chemical integrity. The chromatogram exhibited well resolved peaks for CSA (retention time ~ 3.50 min) and CSD (retention time ~ 4.30 min). The peak resolution could be measured using:

$$R_S = \frac{(t_2 - t_1)}{0.5(t_{w1} + t_{W2})} \qquad \text{(vi-1)}$$

R_s: resolution of adjacent peaks
t_i: retention time of the peak
t_{wi}: peak width

In our analysis, $R_s > 2.5$.

To measure CSA, standard curves produced on different days using stock solutions with different concentrations of CSA resulted in a statistically well correlated straight line (Figure 7). The regression equation for the standard curve was:

$$\text{Peak height ratio } H_{CSA/CSD} = 0.025 + 0.26\, C_{CSA} \qquad \text{(vi-2)}$$

$R^2 = 0.99$, CV = 4.9 %, n = 5, linear range: 0.5 - 5 µg/mL

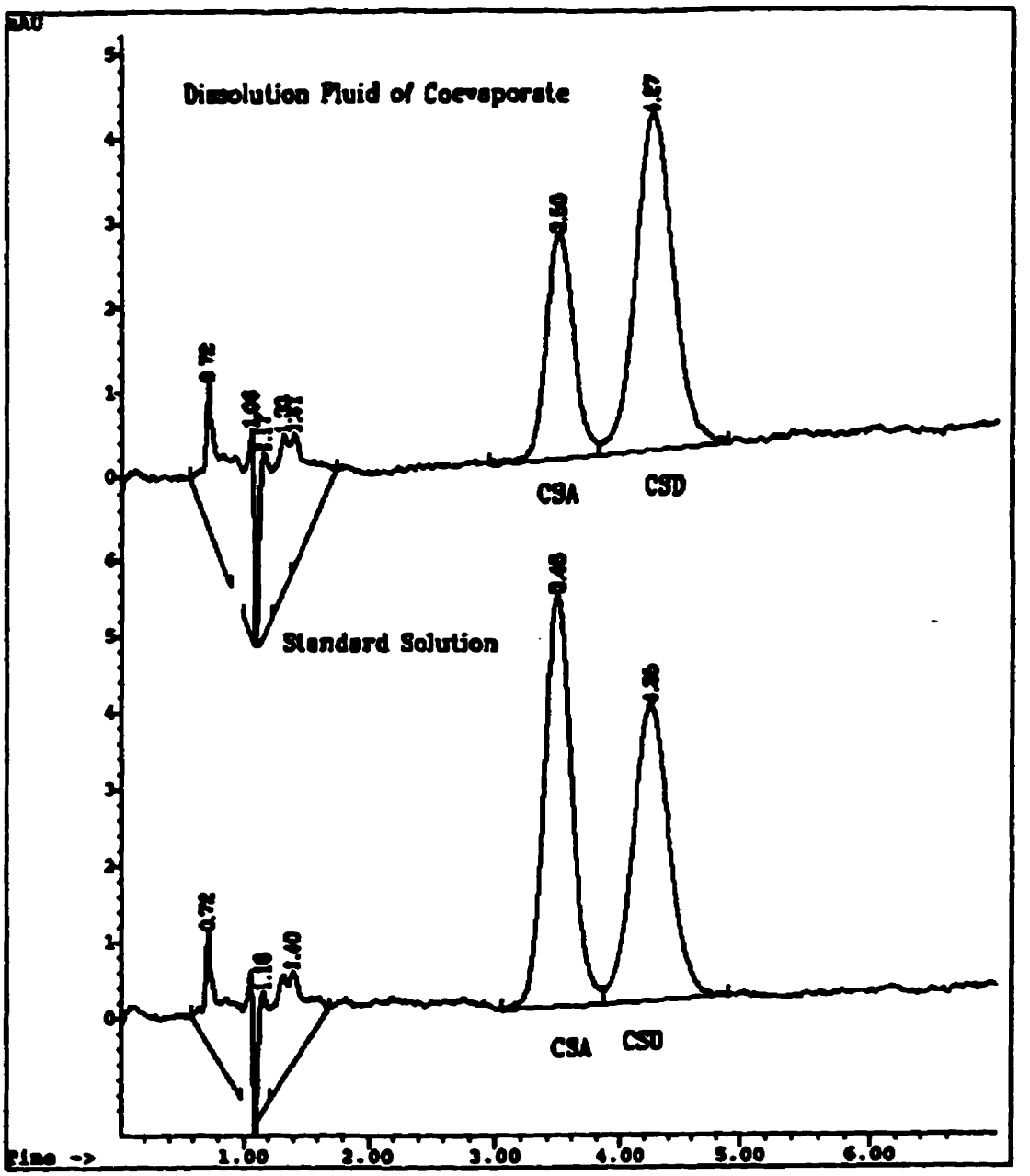


Figure 6 HPLC Chromatograms

Coevaporate VS Standard

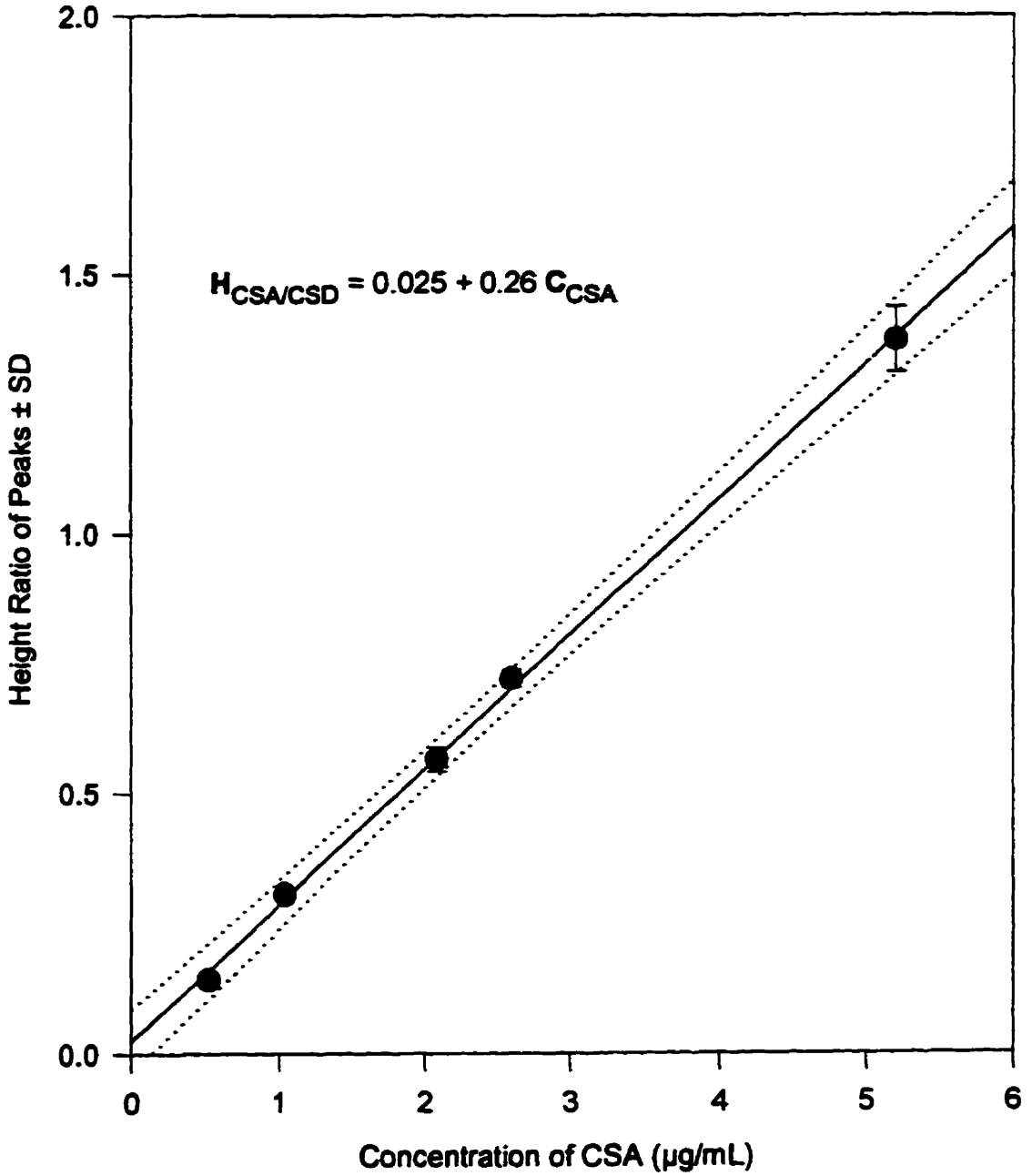


Figure 7 Standard Curve for HPLC Analysis

6.1 Physicochemical Properties of Coevaporate

6.1.1 Equilibrium Solubility of CSA from Coevaporate

The equilibrium solubilities (at 22 ± 1 °C) of CSA from untreated samples and CSA from CSA-PVME/MA copolymer are shown in Table 3. The CSA released from coevaporate had 7 to 9.5 times increased solubility as compared to untreated CSA samples.

Table 3 **Equilibrium Solubility of CSA (22 ± 1°C)**

Samples	Solubility of CSA µg/mL ± SD (n=3)
CSA (untreated)	9.25 ± 0.21
1:5 coevaporate	63.10 ±7.89
1:8 coevaporate	88.43 ± 1.68
1:10 coevaporate	82.17 ± 3.18

6.1.2 Dissolution of CSA from Coevaporate

The CSA released from the coevaporate showed significantly improved aqueous dissolution profiles (Figure 8). The cumulative CSA released from the coevaporates 1:5, 1:8, and 1:10 were, respectively, 82, 95 and 85 %. To elucidate

the kinetics of drug released from the coevaporate, the percentage of CSA remaining to be released from the coevaporate as a function of time was fitted separately to the following equations:

Zero order	$[Q_\infty - Q_t] = Q_\infty - K_0 t$	(vi-3)
First order	$\ln [[Q_\infty -] = \ln Q_\infty - K_1 t$	(vi-4)

In the above equation, parameters Q_∞ and Q represent, respectively, the percentage of CSA released at infinite time and at any time, under the experimental conditions. K_0 and K_1 are, respectively, the zero and first order rate constants. The correlation coefficients (R^2) for the two equations were considered as the indicator for the order of release. For the three formulations 1:5, 1:8 and 1:10, the values of R^2 were, respectively, 0.95, 0.53, and 0.58 for the zero order fitting and 0.96, 0.84, 0.72 for the first order fitting. These results suggest that the release of CSA from the coevaporate could be considered following first order kinetics.

As the percentage of the polymer in the coevaporates was increased from 83.3% (1:5) to 88.9 % (1:8), there was a significant increase ($p < 0.05$) in the cumulative amount of CSA released. However, regardless of further increasing the percentage of polymer in coevaporate to 90.9 % (1:10), the cumulative amount of CSA released did not increase. Since the PVME/MA is a water-soluble polymer, which can form

a viscous gel at a higher concentration, it is envisaged that the reduced cumulative release from 1:10 coevaporate could be due to the increased diffusional barrier at the higher polymer concentration in the 1:10 coevaporate. The same trend was also observed in the equilibrium solubility studies of the coevaporate.

The control experiments using untreated CSA, CSA-PVME/MA admixture and CSA wetted with Span-80 and Tween-20 did not result in any improvement in the dissolution of CSA. The amount of CSA released in all above instances was below the detection sensitivity of our HPLC analysis (0.5 μg/mL). This demonstrated that the hydrophobicity of CSA could not be overcome by simply improving the wetting of CSA particles. The lack of dissolution of the physical admixture of CSA and PVME/MA might suggest that the polymer itself has little or no direct effect in solubilizing CSA in water. It appears that CSA and PVME/MA in the coevaporate are associated in a unique way, which is essential in improving the dissolution of CSA. The formation of molecular dispersion could be a possible mechanism for the improved dissolution of CSA. The polymer, PVME/MA, has a hydrophobic polyvinyl backbone and a maleic acid hydrophilic side chain. Therefore, the polymer can act as an amphipathic molecule and facilitate the dissolution of hydrophobic drugs (Krishnan TR *et al.*, 1991), such as CSA.

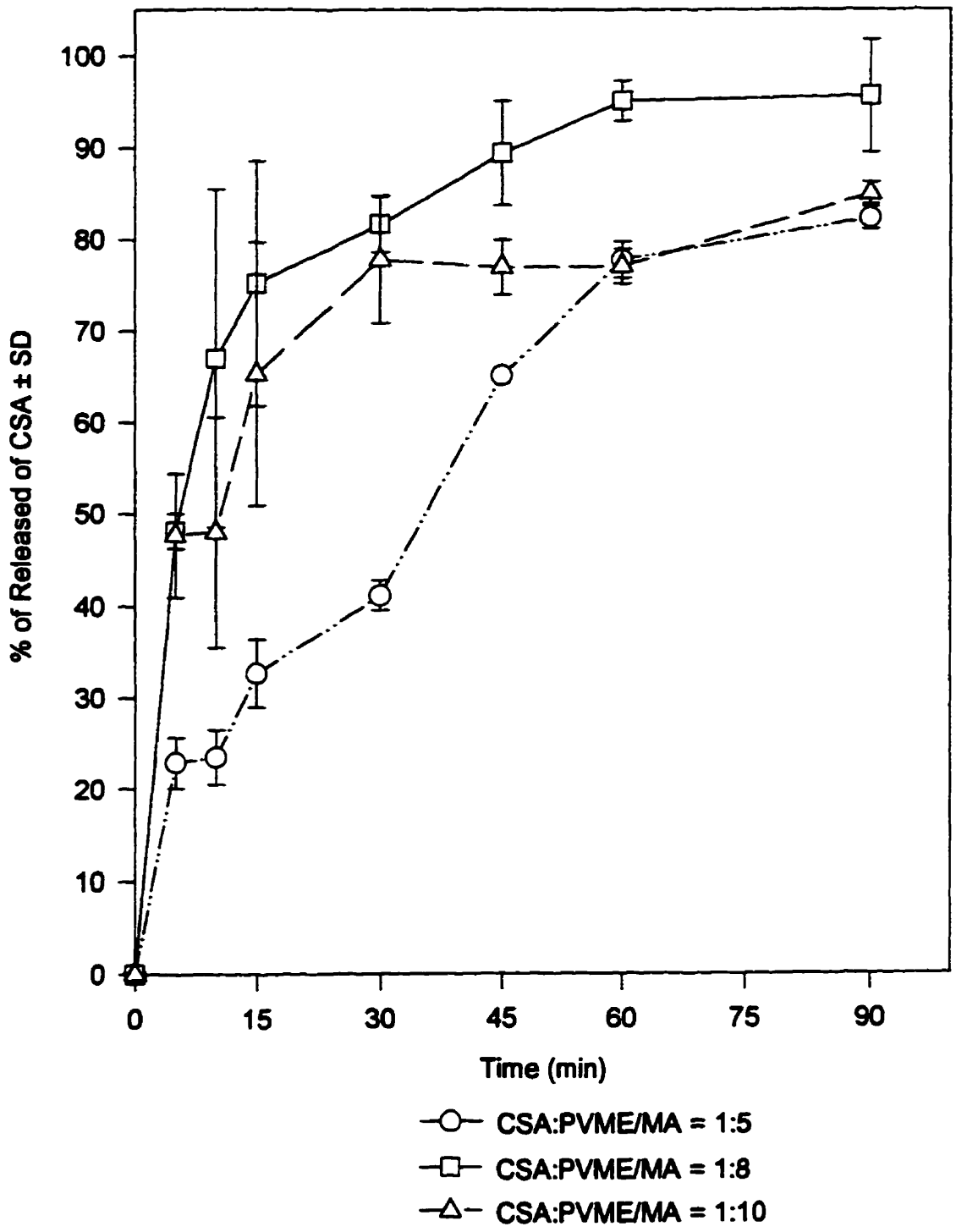


Figure 8 **Dissolution Profiles of Coevaporates (CSA:PVME/MA)**

6.2 Skin Model

In the study of topical and transdermal drug delivery, the challenge of finding a suitable animal model whose skin attributes are similar to the human skin has been pursued by many investigators for several decades. Apparently, human skin is unique. There is no universally acceptable animal skin that could be interchangeably used for transdermal studies. Even for human skin, different skin types have different epidermal permeability barrier function although race and gender do not influence this function (Reed JT *et al.*, 1995). Since the availability of human skin is limited, using an animal model to conduct *in vitro* and *in vivo* experiments is unavoidable. *In vitro* experiments with different animal models have shown that permeability varies from species to species. Studies have also shown that permeability of the pig skin and guinea pig skin seems to be similar to that of man, particularly for the penetration of lipophilic compounds (Priborsky J *et al.*, 1990; Sato K *et al.*, 1991; Dick IP *et al.*, 1992). Hairless rat skin has been extensively used by many investigators. Using hairless skin avoids the need for shaving the skin that could cause accidental cuts leading to deceptive results. It was reported that hairless rat dorsal skin showed similar permeability to human breast and thigh skin (Harada K *et al.*, 1993). However, the different lipid content and water uptake lead to different skin permeability between human SC and that of hairless rat skin (Morimoto Y *et al.*, 1992). It was reported that the permeability of

lipophilic drugs in man is slightly higher than that in hairless rat. In the case of hydrophilic drugs, permeability has been reported much lower in hairless rat than in human (Morimoto Y *et al.*, 1992). Despite all the differences animal models are useful in the development of the most suitable drug delivering conditions that could later be tried in human volunteers.

The heterogeneous skin is composed of a comparatively lipophilic SC and a hydrophilic epidermis and dermis. Based on this fact, a parabolic relationship may be expected between lipophilicity of the drug and skin permeability (Hansch C *et al.*, 1973; Lee CK *et al.*, 1994). The high lipophilicity of CSA could be a disadvantage for its penetration through the skin, because drug molecules may tend to be trapped in the SC rather than penetrate into the relatively hydrophilic deeper skin layers. The successful development of CSA as a transdermal therapeutic agent will clearly be aided by an understanding of the influence of lipophilicity on the absorption of drugs across the skin barrier.

Before conducting iontophoresis or electroporation, we had hydrated the skin for at least one hour. It was found that as the skin was hydrated the electric resistance across the skin declined and approached a stable value, which indicated the extent of hydration of the skin. The fully-hydrated skin is believed to be more permeable. The reason could be that water-hydrated skin has an increased fluidity in the intercellular membranes of SC, which could facilitate the water flux through the skin

(Alonso A *et al.*, 1995; 1996). The structure of fully hydrated SC is reported to be swollen due to the uptake of water (van Hal DA *et al.*, 1996). The existence of localized water domain in the intercellular region of the SC may shorten the diffusional path length through the lipid region of the SC for the hydrophilic molecules. On the other hand, a shorter diffusional path reduces diffusional resistance that may also facilitate the diffusion of lipophilic and amphiphilic molecules.

The passive electrical properties of the skin are reported to exhibit a characteristic change with time after it was separated from the body (Pliquett F *et al.*, 1996). To avoid dramatic changes in the electrical properties in the skin samples, we have used all skin samples within 48 hours after excised from the body.

6.3 Tape-Stripping Technique

The tape-stripping technique has been used for a long time in transdermal research. This technique was initially used to study the rate of epidermal replacement (Pinkus H, 1952; Porter D *et al.*, 1967; Downes AM *et al.*, 1967). By using tape-stripping technique in transdermal drug delivery, we can obtain some insight into drug concentration profile within the SC (Tsai JC *et al.*, 1991a). In the study of iontophoresis, this technique has been used to examine the role of the SC

in defining the electrical behavior of the skin (Ruddy SB *et al.*, 1995). In our experiments, tape-stripping technique was used as a means to remove SC selectively enable us to measure the amount of CSA delivered into the viable skin layers. By removing the SC before determining the amount of drug delivered transdermally, we avoided any artifact effect caused by the drug adsorbed in the superficial layers of the skin. In all the results reported in the this thesis the measurements of transdermally delivered drug were done after removing the SC. With 10 consecutive strippings, the counts of radioactivity in the tapes approached nearly constant, which suggested a complete removal of the SC from the skin. The quality of the adhesive tape was reported to have an influence on the stripping efficiency (Tsai JC *et al.*, 1991b). We used Scotch® (3M, Ontario, Canada) adhesive tape which gave satisfactory results.

6.4 Non-parameter Statistics

Skin samples are known to exhibit a wide variation in its electrical properties (Pliquett U *et al.*, 1995). Since the standard deviations in our results were high a normal distribution of data could not be assumed. Hence it was considered to use nonparametric statistical analysis of variance. Mann-Whitney test and Kruskal-Wallis test were respectively performed for two and several sample populations. Pair-wise multiple comparisons were made using Mann-Whitney test after adjusting

for Type I error. The amount of CSA in the skin (CSA_{skin}), in the receiver ($CSA_{receiver}$) and EtOH delivered into receiver ($EtOH_{receiver}$) and in the skin ($EtOH_{skin}$) are reported.

6.5 *In Vitro* Iontophoresis

In vitro iontophoresis was done using both coevaporate aqueous solution and hydroethanolic solution as donor solutions. The anode electrode was placed in the donor compartment, while the cathode electrode was placed in the receiver compartment to maximize electro-osmosis (Pikal MJ, 1992). It was reported that the maximum painless current for iontophoresis could be estimated using :

$$I = \frac{28.6A}{48.3 + A} \qquad \text{(vi-3)}$$

where I is current (mA) and A is electrical contact area (cm^2) (Prausnitz MR, 1996a). In our experiments, the current we used was 0.4 mA that was lower than 0.47 mA , the calculated value of perception threshold according to the equation vi-3 ($A = 0.8\ cm^2$).

With the use of Ag/AgCl electrodes and chloride-containing donor and receiver solutions, the electrolytic reaction can be represented as:

Cathode $Ag_{(solid)} \rightleftarrows Ag^{+}_{(solution)} + e^{-}_{(metal\ phase)}$ (vi-5)

Anode $Ag^{+}_{(solution)} + Cl^{-}_{(solution)} \rightleftarrows AgCl_{(solid)}$ (vi-6)

Overall reaction: $Ag + Cl^{-} \rightarrow AgCl\downarrow + e^{-}$ (vi-7)

There are two advantages in using Ag/AgCl electrodes. Firstly, the reaction at the electrode does not cause a change in the pH in both donor and receiver solution because the process does not involve dissociation of water. Secondly, the reaction at the electrode does not generate competing ions in the anode because the silver chloride produced during the reaction is virtually an insoluble salt (solubility product: 1.25×10^{-10} at 25°C, Martin A *et al.*, 1983), which precipitates at the electrodes. In most of the instances Ag/AgCl electrodes do not significantly contaminate the medium in which they are used. These two benefits make Ag/AgCl electrodes suitable in iontophoresis (Sage BH Jr. *et al.*, 1992).

The results of iontophoresis were summarized in Table 4. The changes in pH values before and after experiments in both donor and receiver compartments were not large, either with the use of coevaporate donor solution or ethanolic donor solution. This suggested that the electrical property of the skin was not significantly altered, and that anodal delivery and the direction of electro-osmosis were maintained during iontophoresis.

Compared to control, the passive diffusion, the amount of CSA delivered into the

skin (CSA_{skin})and receiver ($CSA_{receiver}$) by iontophoresis was not significant, either using coevaporate aqueous donor solution or ethanolic donor solution. Ethanol delivered to the receiver compartment ($EtOH_{receiver}$) by iontophoresis was not significantly different from that in the control. These results suggested that iontophoresis is not effective in transdermally delivering CSA which is a large, neutral lipophilic species.

Although Ag/AgCl electrodes are approved for use in USA in iontophoretic devices (Sage BH, 1993), recently some concerns have been expressed regarding its safety issue. Migration of ions from donor compartment to receiver compartment across the skin occurs when an electric current is applied. In the relatively long time of delivery, the toxicological aspects of migrating silver ions produced from the electrodes used in iontophoresis must be taken into account because the filter membrane we used may not thoroughly shield the skin from silver ions. Some of the recent findings suggest that movement of silver ions beyond the immediate area of electrode placement is possible (Hollinger MA, 1996). Although silver ions are generally considered to be fixed by tissues due to binding with the protein at the site of application, an iontophoretic device can generate an electrical driving force to deliver silver ions across the tissues (Maloney JM *et al.*, 1992). This makes it possible for the free silver ions to penetrate the skin in depth. The free silver ions can interact with sulfhydryl, amino, imidazole, phosphate, or carboxyl groups in the

skin (Petering HG, 1976). The permeability of the skin may also be altered.

Table 4 The Results of Iontophoresis

	Coevaporate				
	ΔpH_{donor} $\bar{x}$	$\Delta pH_{receiver}$ $\bar{x}$	CSA_{skin} $\bar{x}$(ng)±SD	$CSA_{receiver}$ $\bar{x}$(ng)±SD	n
Control	0.11	0.48	0.615±0.40	0.013±0.00	5
Iontophoresis	0.20	0.51	0.606±0.49	0.024±0.03	5
Mann-Whitney *p*	—	—	1.00 (NS)	0.84 (NS)	

	Hydroethanolic Solution					
	ΔpH_{donor} $\bar{x}$	$\Delta pH_{receiver}$ $\bar{x}$	CSA_{skin} $\bar{x}$(μg)±SD	$CSA_{receiver}$ $\bar{x}$(μg)±SD	$EtOH_{receiver}$ $\bar{x}$(mg)±SD	n
Control	0.30	0.57	0.039±0.04	0.061±0.05	19.616±6.35	5
Iontophoresis	0.78	0.48	0.070±0.04	0.101±0.06	12.835±4.64	5
Mann-Whitney *p*	—	—	0.22 (NS)	0.31 (NS)	0.10 (NS)	

Control: Passive diffusion
NS: Not significantly different
Sig: Significantly different
ΔpH_{donor}: The change in pH in the donor solution before and after experiment
$\Delta pH_{receiver}$: The change in pH in the receiver solution before and after experiment

6.6 *In Vitro* Electroporation

In electroporation experiments, the exponential decaying electric voltage was applied across the skin sample through the side-by-side diffusion cell. The transdermal voltage (U_{skin}) of the skin directly affects the formation of transient pores in the SC. However, it is very difficult to measure it because it depends on a number of factors, such as electrode size, spacing, material and composition of the donor and receiver solutions as well as transdermal resistance (R_{skin}), which is highly time-dependent during electroporation (Pliquett U *et al.*, 1995). The local potential drops across the different media are different after pulsing and are relatively lower for donor and receiver solutions due to their higher conductivities. The skin has high local field strength because of its low electrical conductivity. Compared to other layers of the skin, SC has a significantly high local field strength due to its high lipid content, which leads to very low electrical conductivity. Our electroporation units record only the voltage applied across the electrodes, $U_{electrode}$. It is recognized that only a fraction of the $U_{electrode}$ would appear across the skin (Pliquett U *et al.*, 1995). We did not have the experimental facility and the sophisticated electrical circuit required to determine the corresponding voltage across the skin, U_{skin}, for each $U_{electrode}$. Although not an ideal description, in this thesis reference will be made only to the $U_{electrode}$ which was recorded in all the experiments. The pulse length, τ, is determined by the combination of capacitance

(expressed in farads, F) and resistance (expressed in ohms, Ω) in a circuit. Capacitance depends on the capacitors in the circuit and resistance is largely determined by the sample, which in this case is the skin. Both $U_{electrode}$ and τ together can decide the waveform of applied exponential pulse.

During the electroporation pulse, the transmembrane voltage, U(t), increases and the value depends on the type of membrane. Then, a reversible electrical breakdown (REB) occurs which is accompanied by increased molecular transport across the membrane. Although paradoxical, it seemes that at higher voltage there is a protective mechanism where the pores formed provide large conductance and prevent the U(t) from reaching very high values (Weaver JC, 1995).

6.6.1 Induced Transport of Solvents During Electroporation

The transport of water and ethanol under the effect of electroporation were measured to understand the mechanisms of electroporation. It is expected that transient pore pathways are created during electroporation, which can result in enhanced electro-osmosis and post-pulse diffusion. The results are summarized in Table 5. The results show that electroporation significantly increases the transport of water and ethanol with either single or multiple pulse modes, at $U_{electrode}$= 200 V and τ = 10 ms. The transport of water and ethanol peaked about 2 hours after single pulse electroporation (Figure 9) and after about one hour after

multiple pulse electroporation (Figure 10). The transport profile of ethanol was similar to that of water in both single and multiple pulse electroporation. It was noted that multiple pulse electroporation was more efficient in increasing the transport of the solvents than single pulse mode by a factor of 2 for water and 1.5 for ethanol. Also the transport of water was 3 and 4 times higher than ethanol under single and multiple pulse electroporation, respectively. These observations seem to confirm the theory, in which creation of hydrophilic pores has been attributed as the possible reason for enhancement of aqueous solution transport across the skin under the influence of electroporation (Prausnitz MR *et al.*, 1995a; Pliquett U *et al.*, 1995). The higher transport of water could be attributed to the following reasons: (1) Water being more polar (1.84 Debey units) than ethanol (1.69 Debey units) will be more influenced by the electrophoretic force. (2) Water has a smaller molecular size than ethanol and also has a higher molar concentration in 40% ethanolic donor solution. Hence, water will be better able to compete with ethanol for available pores to go through. It should be noted that despite having a smaller transportation rate, transport of ethanol is very significant since it is valuable for the delivery of compounds, such as CSA, that are insoluble in water but soluble in hydroethanolic solution.

Table 5 **Tansport of Water and Ethanol Induced by Electroporation**

Time (hr)	H_2O $\bar{x} \pm SD$ (mg/1.3cm^2/hr)	Ethanol $\bar{x} \pm SD$ (mg/1.3cm^2/hr)	n = 5
	Single Pulsing		
1	0.38 ± 0.18	0.09 ± 0.02	
2	1.25 ± 0.48	0.32 ± 0.14	
2.5*	5.60 ± 2.56	1.38 ± 0.67	
3.5	5.47 ± 2.00	0.99 ± 0.48	
5	8.17 ± 2.78	1.94 ± 0.64	
7	6.57 ± 1.46	1.55 ± 0.32	
9	5.03 ± 0.43	1.21 ± 0.23	
24	3.71 ± 0.56	0.99 ± 0.16	
	Multiple Pulsing		
1	0.55 ± 0.78	0.51 ± 0.87	
2	1.41 ± 0.72	0.54 ± 0.14	
2.5**	7.16 ± 1.70	1.54 ± 0.52	
3**	13.44 ± 5.27	2.29 ± 1.10	
3.5	14.98 ± 8.82	3.01 ± 1.89	
5	11.56 ± 3.47	2.51 ± 0.96	
7	8.90 ± 1.63	2.19 ± 0.58	
9	6.95 ± 1.19	1.73 ± 0.24	
24	5.09 ± 0.77	1.53 ± 0.20	

* the time of applying electroporation pulse in single pulse mode
** the time of starting and terminating electroporation pulses in multiple pulse mode

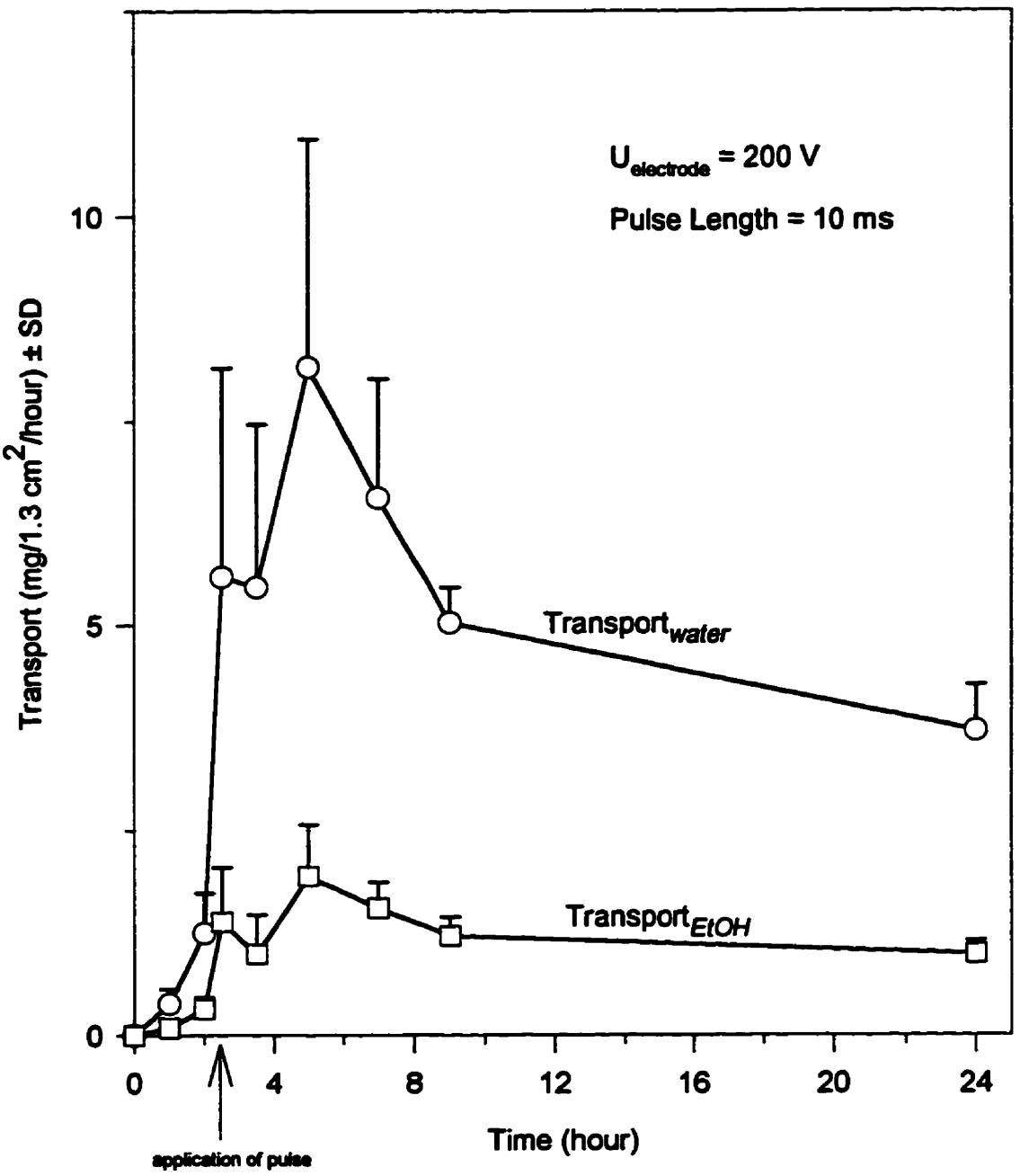


Figure 9 **Transport of Water and EtOH Induced by Single Pulse**

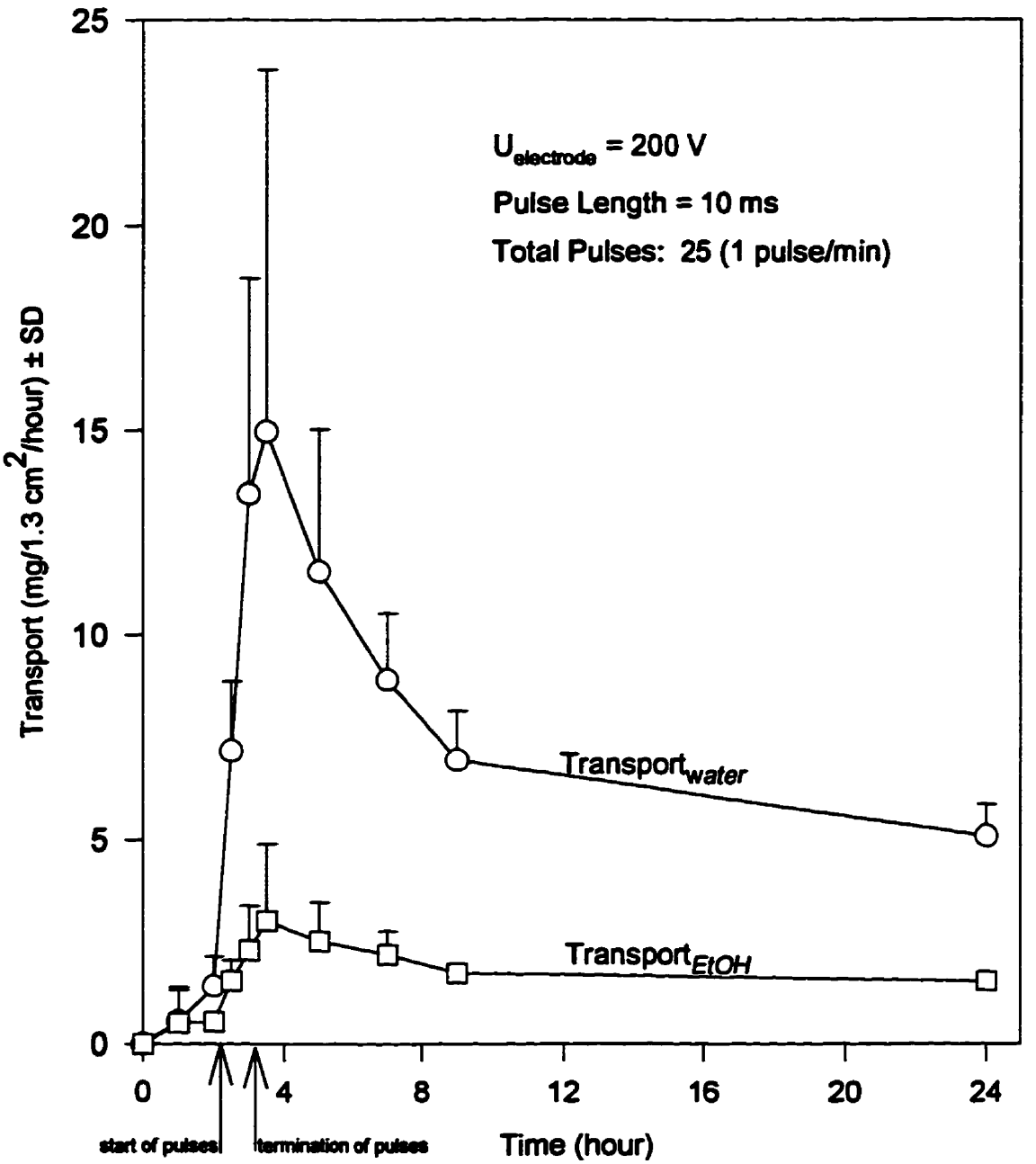


Figure 10 **Transport of Water and EtOH Induced by Multiple Pulses**

6.6.2 Delivery of CSA from Coevaporate Aqueous Solution

CSA is virtually insoluble in water. Previous attempts by other investigators to deliver CSA topically without using physical methods of enhancement had met with only limited success (Gilhar G *et al.*, 1988; Hermann RC *et al.*, 1988; Fradin MS *et al.*, 1990; De-Rie MA *et al.*, 1991; Egbaria K *et al.*, 1991; Duncan JI *et al.*, 1993; Choi HK *et al.*, 1995). The possible reasons for poor topical delivery of CSA could be due to its higher molecular weight, property of neutrality and the lack of a balanced o/w partition coefficient. By using solid dispersion technique, we succeeded in increasing the aqueous solubility of CSA by a factor of 10. The dispersion or coevaporate consisted of CSA and PVMA/MA copolymer in a ratio of 1:8 (w/w). The concentration of CSA released from the coevaporate in donor solution was 64 µg/mL of CSA, which is 7 times higher than its solubility in water. The higher thermodynamic activity of coevaporate aqueous solution of CSA was expected to facilitate the electroporative delivery of CSA.

Experiments were done using both single and multiple pulse electroporation modes. The $U_{electrode}$ used in both single and multiple modes were 150 and 200 V. The τ varied between 5 and 10 ms.

The results of delivery of CSA from coevaporate aqueous solution by electroporation are shown in Table 6. The contact duration in all the experiments was 4 hours. Although the scintillation counter measured the radioactivities (DPM)

for the samples with very low drug content, it was not possible to differentiate between the CSA concentrations below the 10 ng level with enough accuracy. Hence, the CSA concentrations equivalent to or below 10 ng are shown as < 10 ng in Table 6.

Table 6 **Delivery of CSA from Coevaporate Aqueous Solution Single and Multiple Pulse Modes**

$U_{electrode}$ (V)	τ (ms)	Total Pulses	Duration (hr)	CSA_{skin} $\bar{x} \pm SD$ (ng)	$CSA_{receiver}$ $\bar{x} \pm SD$ (ng)	n
150	10	1	4	60 ± 20	< 10	3
150	10	25	4	23 ± 6	17 ± 11	3
200	10	1	4	87 ± 15	< 10	3
200	5	25	4	37 ± 6	40 ± 17	3
200	10	25	4	50 ± 10	27 ± 12	3
Control*			4	<10	< 10	9

* Control: passive diffusion

CSA was found to be confined to the viable epidermis (without SC) and dermis, and only small amount of CSA was transported across the skin into the receiver compartment, which could be perceived as a desirable feature for treating psoriasis topically. This could be attributed to the high lipophilicity of CSA. The amount of drug delivered through the passive diffusion was very low (below 10 ng), both in

the skin and in the receiver compartment. However, with the use of electroporation there was a significant increase (p <0.05, Kruskal-Wallis test) in the amount of CSA delivered into the skin (CSA_{skin}). Compared to the passive diffusion there was a 6 to 8 fold increase in CSA_{skin} with the use of single electroporation pulse at $U_{electrode}$ = 150 and 200 V (τ = 10 ms).

With the use of multiple pulse mode at the two $U_{electrode}$, 150 and 200 V (τ = 10 ms), a smaller accumulation of CSA was found to be in the skin, compared to that in single pulse electroporation. The amount of CSA delivered into the receiver ($CSA_{receiver}$) was lower than the limit of sensitivity of assays (< 10 ng), similar to that in passive diffusion experiments. In the multiple pulse mode at $U_{electrode}$ = 150 and 200 V (τ = 10 ms), detectable quantities of CSA ($CSA_{receive}$) were also transported across the skin to the receiver compartment, significantly different from passive diffusion ($p < 0.05$ Kruskal-Wallis test). However, there was no significant difference in $CSA_{receiver}$ between $U_{electrode}$ = 150 and 200 V (τ = 10 ms) in multiple pulse mode ($p > 0.05$, Mann-Whitney test). Also, increasing the τ from 5 to 10 ms in multiple pulse electroporation at 200 V, $CSA_{receiver}$ did not show any significant difference ($p > 0.05$, Mann-Whitney test).

The total amount of CSA delivered, which is the sum of CSA_{skin} and $CSA_{receiver}$, at 150 and 200 V (τ = 10 ms) with single and multiple pulse mode was not significantly different ($p > 0.05$, Mann-Whitney test). The total amount of CSA

delivered at 200 V using multiple pulse mode with τ = 5 ms and τ = 10 ms was also similar ($p > 0.05$, Mann-Whitney test). This is contrary to the literature reports where an increase in pulse length resulted in increased mass transport of other drug molecules through the cell membrane or skin (Zimmerman U, 1982; 1986; Pliquett U et al., 1996 (a)). It is speculated that the lack of adequate thermodynamic activity of CSA in the donor compartment could be the main reason for not seeing any enhancement in CSA transport with increase in pulse length.

After both single and multiple electroporation, pH did not significantly change in donor and receiver compartments. This indicated that electroporation did not involve any significant amount of ion migration between donor and receiver compartments in our experiments.

The transient pores created in the skin during electroporation lead to increased diffusion of molecules from the donor compartment to the receiver compartment across the skin barrier. Since diffusion is a concentration dependent phenomenon, a seven fold increase in the concentration of CSA in the donor coevaporate solution may not be sufficient to maintain a satisfactorily high thermodynamic activity of CSA during the electroporation experiments.

By analyzing the strippings of SC, it was found that the amount of CSA in SC was 40-50 times higher than that in the viable skin. This, however, may not be of any therapeutic value in treating psoriasis since the CSA in SC is not expected to

penetrate to the deeper part of the skin where it is required (faster desquamation occurs in psoriatic skin than in normal skin). It is recognized that although a 6 to 8 fold increase in topical delivery of CSA compared to passive delivery was obtained with the use of electroporation, it is probably not a therapeutically significant quantity. One way to improve the delivery would be to increase the thermodynamic activity of CSA in the donor solution, which is discussed in the next section.

After a contact duration of 4 hours, donor solutions were analyzed by HPLC. No extra peaks were found in chromatogram after electroporation. It may also be speculated that under the influence of electroporation, some amount of PVME/MA could have been transported from the coevaporate solution in donor site to the skin. No attempt was made to quantify the amount of PVME/MA transported during the electroporation. The amount of PVME/MA contained in donor solution is not expected to cause any toxicity since PVME/MA is considered a safe excipient (Urrtti A, 1985; Palomo F *et al.*, 1989).

6.6.3 Delivery of CSA from Ethanolic Donor Solution

Delivering CSA from ethanolic donor solution by using electroporation as a means of skin permeability enhancement was encouraging. The CSA has a higher thermodynamic activity in hydroethanolic solution (3% of CSA dissolved in 40%

ethanol and 60% PBS), in which the concentration of CSA is 3.00 mg/mL. The increase in solubility was significantly higher compared to 64 μg/mL in coevaporate aqueous solution and about 9 μg/mL in water. The ionic strength provided by PBS also seemed sufficient to provide the required electrical conductivity for the donor solution. It is suitable for as a donor solution in electroporation. The pH values of the donor and receiver solutions were only slightly changed after electroporation experiments in both single and multiple pulsing modes. It ranged from 8.0-7.0 in the donor and 7.4-7.0 in the receiver solution. Maintenance of a neutral pH in the skin would ensure minimal irritation to the skin.

It is reported that external electrical potential leads to deterioration of peptide drugs (Huang YY *et al.*, 1996). We analyzed both donor and receiver solutions after electroporation with HPLC to determine the integrity of CSA. The CSA in receiver compartment was undetectable with HPLC. No extra peak was found in the HPLC chromatogram compared to that obtained with the CSA solution before subjecting it to electroporation pulses.

Skin Variation Both dorsal and ventral parts of the hairless rat skin were used as skin samples. The radioactivity of the strippings on which the SC was lamellarly stripped showed a gradual decrease of radioactivity, which indicated the decrease of amount of CSA as the deeper layers of SC were reached. On comparing the

CSA_{skin}, $CSA_{receiver}$ and $EtOH_{receiver}$ between the dorsal and ventral side skin samples, no significant difference ($p > 0.05$) was seen (Table 7). In passive diffusion where the SC was not under any electrical influence, any regional variation in the skin if present would be expected to result in different amount of EtOH delivered. The data suggest that, in passive diffusion, regional variation in the barrier function of the skin has no influence in the transport of EtOH and CSA.

Deterioration of barrier property of the skin on short term storage of the excised skin was checked by comparing the CSA_{skin} , $CSA_{receiver}$ and $EtOH_{receiver}$ between freshly excised skin and skin taken after 48 hours of storage at 0 - 4°C. The variations in the transport of CSA and EtOH between freshly excised and stored skin were found to be insignificant (Table 8). An important feature of our *in vitro* experiments is the use of full-thickness skin rather than epidermis used by some of the other scientists (Bommannan DB *et al.*, 1994; Pliquett U *et al.*, 1996 (a)). Although it is acknowledged that SC is the main barrier in transdermal delivery, we believe that use of the whole skin would simulate the *in vivo* conditions more completely.

Effect of Contact Duration of Study It was noticed that the contact duration had a varying effect in the efficiency of transdermal delivery, which are summarized in Table 9. The three groups of experiments chosen for comparison were passive

diffusion, single pulse ($U_{electrode}$ = 200 V, τ = 10 ms), and multiple pulse electroporation ($U_{electrode}$ = 200 V, τ = 10 ms, 25 pulses/ 1 pulse per minute). Pair-wise comparisons were made only between adjacent groups such as between 2 and 4 hours, 4 and 6 hours contact duration etc. In the passive diffusion, although Kruskal-Wallis test showed both $CSA_{receiver}$ and $EtOH_{receiver}$ were significantly different ($p < 0.05$), on performing pair-wise Mann-Whitney comparison only $EtOH_{receiver}$ was found to have significantly higher amount delivered when the contact duration was increased from 2 hours to 4 hours. The same observation was made earlier (see Table 7).

Table 7 **Regional Variation of the Skin in Passive Diffusion**

Amount	Ventral Skin $\bar{x} \pm SD$ (n)	Dorsal Skin $\bar{x} \pm SD$ (n)	*p*
	Contact Duration = 2 hours		
CSA_{Skin} (µg)	0.256±0.411 (7)	0.114±0.212 (9)	0.223(NS)
$CSA_{receiver}$ (µg)	0.141±0.148 (7)	0.200±0.235 (9)	0.791(NS)
$EtOH_{receiver}$ (mg)	0.866±0.504 (7)	0.974±0.304 (9)	0.751(NS)
	Contact Duration = 4 hours		
CSA_{Skin} (µg)	0.412±0.175 (6)	0.570±0.394 (5)	0.4286(NS)
$CSA_{receiver}$ (µg)	0.353±0.183 (6)	0.186±0.166 (5)	0.1775(NS)
$EtOH_{receiver}$ (mg)	2.126±0.806 (6)	2.018±0.852 (5)	0.5368(NS)

p value: calculated using Mann-Whitney test, level of significance: 0.05

Table 8 **Effect of Storage of the Skin**

Amount	Freshly Excised Skin $\bar{x} \pm SD$ (n)	48-hour Stored Skin $\bar{x} \pm SD$ (n)	*p*
CSA_{skin} (µg)	1.42±1.55(3)	1.84±2.58(3)	1.00 (NS)
$CSA_{receiver}$ (µg)	0.41±0.31(3)	0.65±0.57(3)	0.70 (NS)
$EtOH_{receiver}$ (mg)	5.84±1.71(3)	7.37±2.41(3)	0.40 (NS)

$U_{electrode}$ = 370 V, τ = 5 ms, contact duration = 2 hours
p value: calculated using Mann-Whitney test

In the passive diffusion study, the amount of CSA delivered into the skin (CSA_{skin}) and receiver ($CSA_{receiver}$) did not significantly increase when the contact duration was ranging from 2 hours to 12 hours (Table 9 and Figure 11). The transport of EtOH ($EtOH_{receiver}$) had a significant increase when contact duration increased from 2 hours to 4 hours.

In single pulse electroporation study, only the amount of CSA_{skin} was significantly increased when contact duration was increased from 2 hours to 4 hours ($p < 0.05$, Mann-Whitney test), while both $CSA_{receiver}$ and $EtOH_{receiver}$ were not significantly different when the contact duration was increased (Table 9 and Figure 12).

In multiple pulse electroporation study, all the values, CSA_{skin}, $CSA_{receiver}$ and $EtOH_{receiver}$, measured at different contact durations, respectively 2, 4, 6 and 12 hour, were significantly different according to Kruskal-Wallis test ($p < 0.05$). However, pair-wise comparison using Mann-Whitney test showed that there were significant difference for CSA_{skin} between 2 hours and 4 hours, for $CSA_{receiver}$ and $EtOH_{receiver}$ between 4 hours and 6 hours ($p = 0.017$, after adjusting for Type I error). No significant increase in the CSA_{skin} were observed when the different contact durations, 4, 6 and 12 hours were applied ($p = 0.3104$, Kruskal-Wallis test, Table 9, Figure 13).

These results suggest the importance of post-pulse diffusion in the electroporative delivery of CSA. Although it may be difficult to make any definite

conclusion from the above observation, it is clear that a study beyond 6-hour contact duration is not of any vital advantage. Keeping in mind the therapeutic benefit of achieving an optimum CSA_{skin} while maintaining the minimum $CSA_{receiver}$ for the topical treatment of psoriasis and the practical problems in conducting studies for prolonged time periods, a 4-hour contact duration was considered as the optimum time period for further studies using multiple electroporative pulse mode.

Table 9 **Effect of Contact Duration of Study on the Delivery of CSA and EtOH**

Duration (hr)	CSA_{skin} $\bar{x}$ (μg) ± SD	$CSA_{receiver}$ $\bar{x}$ (μg) ± SD	$EtOH_{receiver}$ $\bar{x}$ (mg) ± SD	n
		Passive Diffusion		
2	0.25 ● 0.38	0.16 ± 0.17	1.06 ± 0.28	10
4	0.48 ± 0.29	0.28 ± 0.19	2.08 ± 0.79	11
6	0.99 ± 1.19	0.86 ± 0.91	5.16 ± 2.92	5
12	0.93 ± 1.10	1.12 ± 0.70	5.22 ± 2.96	5
$p^{(1)}$	0.073 (NS)	0.042 (Sig)	0 (Sig)	
$p^{(2)}$		NS	0.006 (2-4 hr, Sig)	
		Single Pulse (200 V, 10 ms)		
2	1.04 ● 0.41	0.40 ± 0.23	2.83 ± 1.21	5
4	2.90 ± 1.28	0.24 ± 0.30	3.10 ± 0.98	5
$p^{(2)}$	0.022 (Sig)	0.296 (NS)	0.676 (NS)	
		Multiple Pulse (200 V, 10 ms, 25 pulses)		
2	0.87 ± 0.39	0.87 ± 0.92	4.81 ± 2.29	5
4	29.78 ± 20.14	1.03 ± 0.79	6.74 ± 1.64	5
6	55.42 ± 32.29	3.26 ± 0.72	14.50 ± 2.56	5
12	38.33 ± 15.23	3.45 ± 0.80	13.85 ± 2.65	5
$p^{(1)}$	0.007 (Sig)	0.004 (Sig)	0.002 (Sig)	
$p^{(2)}$	0.012 (Sig 2-4hr)	0.011 (Sig 4-6hr)	0.012 (Sig 4-6hr)	

Sig: significantly different, NS: not significantly different
$p^{(1)}$: p value of Kruskal-Wallis test, $p^{(2)}$: p value of Mann-Whitney test

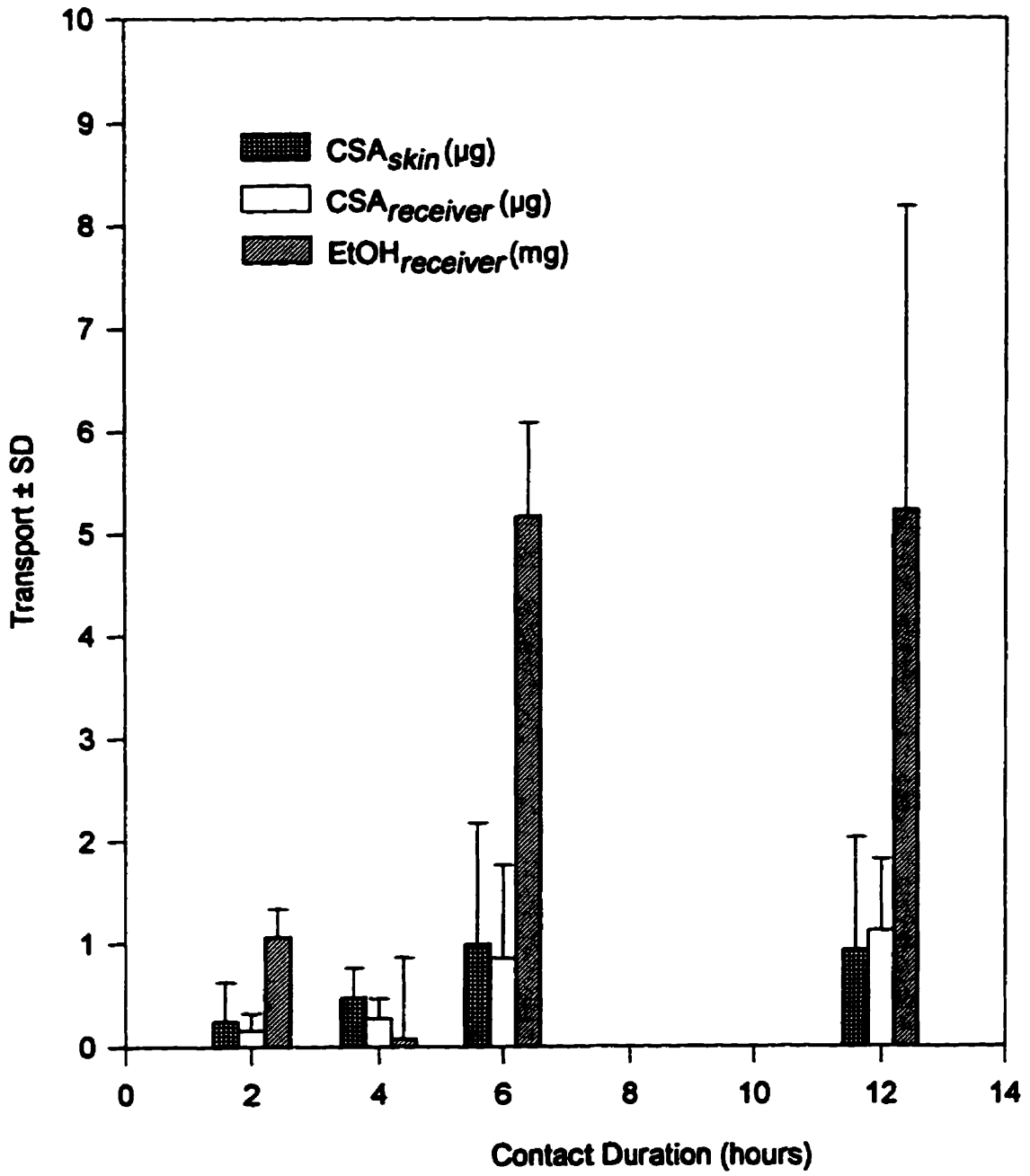


Figure 11 Effect of Contact Duration under Passive Diffusion

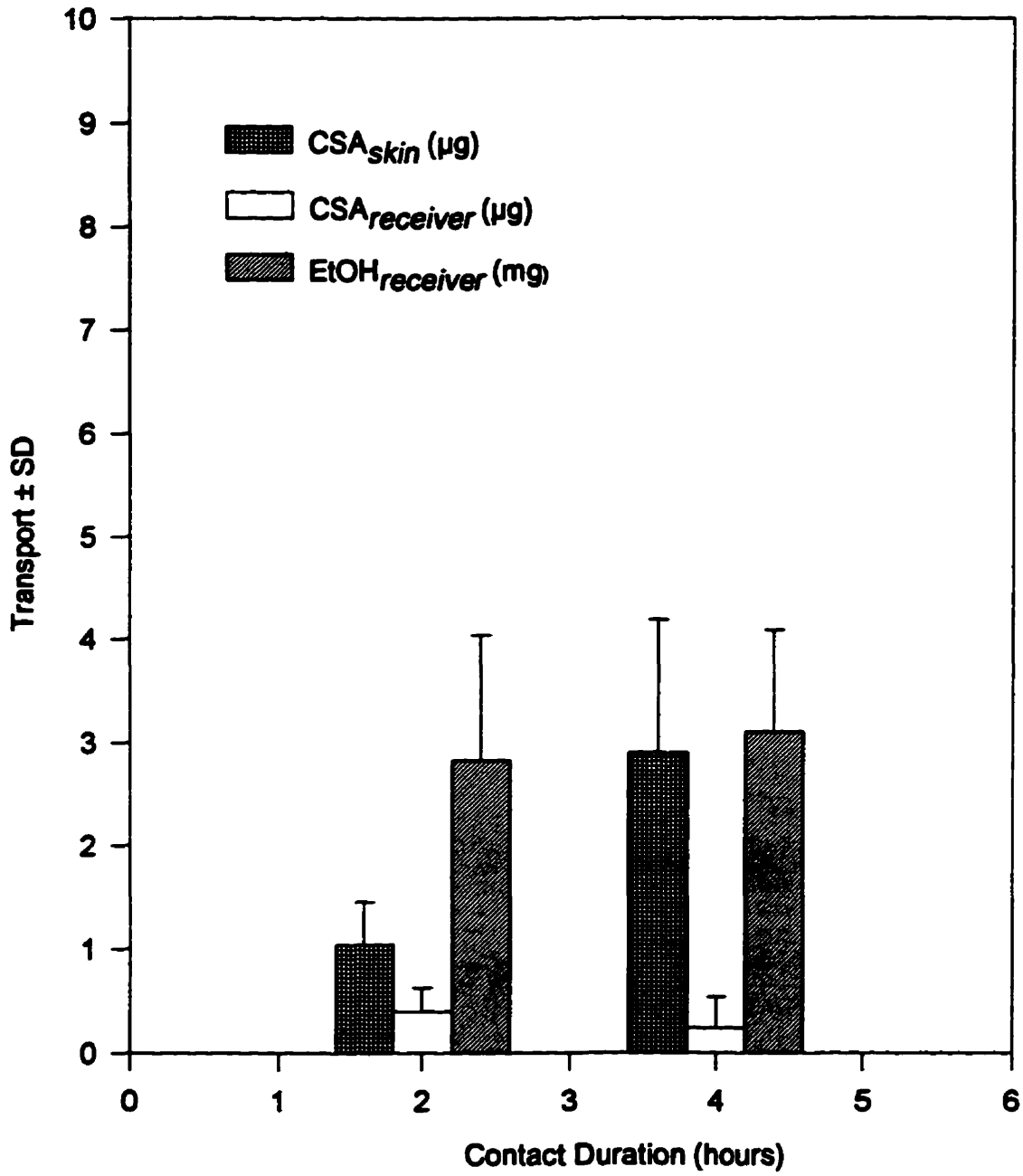


Figure 12 Effect of Contact Duration under Single Pulse Electroporation

($U_{electrode}$ = 200 V, Pulse Length = 10 ms)

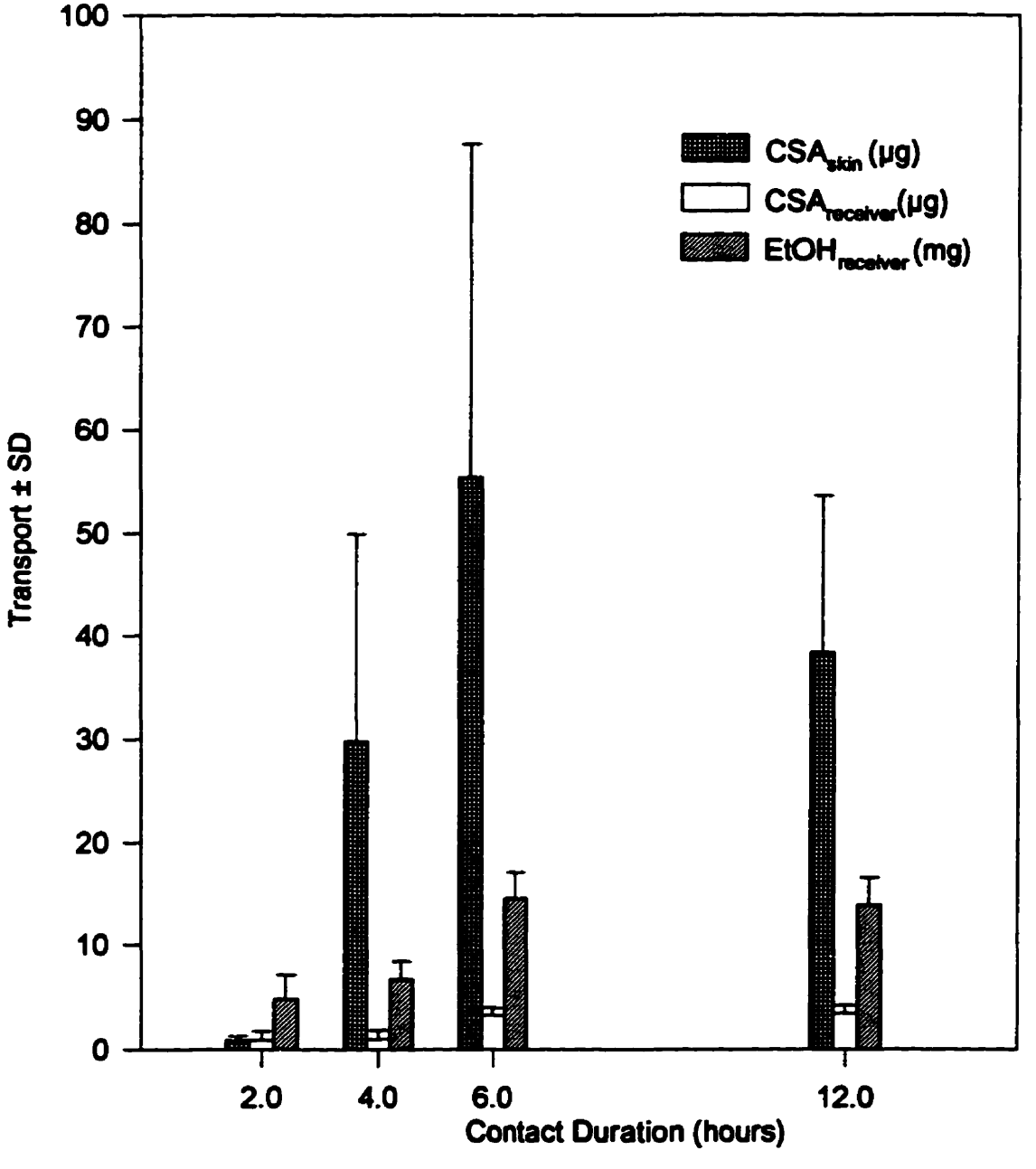


Figure 13 Effect of Contact Duration under Multiple Electroporation

$U_{electrode}$ = 200 V

Pulse Length = 10 ms

Total Pulses: 25 (1 pulse/min)

Single Pulse Electroporative Delivery The results of single pulse electroporation delivery are summarized in Table 10. With our electroporation unit there were limitations to the flexibility and control of electrical parameters. It was not possible to deliver all pulses at 10 ms under all experimental conditions. Experimental variables included $U_{electrode}$, τ, and contact duration. Although regional skin variation was low in passive diffusion, a relatively high fluctuation was found in the transdermal delivery by electroporation. Pulsed electromagnetic field tends to act on lipoidal multilayers of the SC temporarily breaking it down (Weaver JC, 1995). Therefore, the regional variation in the lipid composition in SC has an important influence in drug delivery during electroporation than that in passive diffusion.

The amount of CSA delivered across the skin into the receiver ($CSA_{receiver}$) was erratic and no significant difference as the $U_{electrode}$ was increased (Table 10). In the 2-hour contact duration study (Figure 14), with the increase in the $U_{electrode}$ from 160 to 370 V there was a proportional increase in the amount of $EtOH_{receiver}$ delivered across the skin, except at $U_{electrode}$ = 300 V. It should be noted that due to the lack of flexibility and limitations in the our electroporation apparatus, τ = 10 ms could not be maintained for all the $U_{electrode}$. A regression analysis of the $EtOH_{receiver}$ delivered at 100, 160 and 200 V, τ = 10 ms, yielded a coefficient of determinations, R^2 = 97.8 %. This indicates a linear relationship between $EtOH_{receiver}$ and $U_{electrode}$, when

$U_{electrode}$ < 200 V. As $U_{electrode}$ increased, absence of linearity between $EtOH_{receiver}$ and $U_{electrode}$ could be due to the skin's electrical property which is complicated with the subsequent increase in the voltage. This is consistent with studies reported earlier by other groups (Kasting GB *et al.*, 1990; Pliquett U *et al.*, 1995; Kalia YN *et al.*, 1995).

The mean values of CSA_{skin} delivered by electroporation at the different voltages appeared to be about 3-4 times higher compared to control in both single and multiple pulse electroporation. However, it was not statistically significant with the Mann-Whitney test in pair-wise comparison.

Theoretical estimates suggest that a minimum transdermal voltage, U_{skin}, of about 100 V is necessary for electroporation effect to occur in human SC (Prausnitz MR, 1996a). While there are reports of using $U_{electrode}$ as high as 450 to 1000 V (Vanbever R *et al.*, 1994; Bommannan DB *et al.*, 1994) for delivering drugs transdermally using electroporation, we selected a smaller electrode voltage, viz. $U_{electrode}$ = 200 V for studying multiple pulse mode to minimize any adverse effect on the skin. Although the U_{skin} was not measured, it is expected to be only a fraction of $U_{electrode}$ (Pliquette U *et al.*, 1995).

Compared to the control, using single pulse electroporation with 4-hour contact duration showed significantly higher values for CSA_{skin} and $EtOH_{receiver}$ ($p < 0.05$, Table 10 and Figure 15). However, increasing τ from 5.6 ms to 10 ms did not result

in any significant increase in the $EtOH_{receiver}$ and the CSA_{skin} delivered ($p > 0.05$, Table 10).

Before stripping the SC the skin had to be padded using a filter paper. This process of drying combined with the volatility of ethanol made it difficult to accurately determine $EtOH_{skin}$. However, an attempt was made to determine $EtOH_{skin}$ during the single pulse electroporation experiments by rapidly working to minimize evaporation of ethanol. The results of $EtOH_{skin}$ are given in Table 11. Absence of significant difference between the EtOH uptake of the stripped skin indicates that the skin has a limited capacity for EtOH uptake and after attaining saturation an increase in the field strength does not make any significant difference in $EtOH_{skin}$.

Table 10 **Single Pulse Electroporative Delivery of CSA and EtOH**

$U_{electrode}$ (V)	τ (ms)	Duration (hr)	CSA_{skin} $\bar{x}$ (µg) ± SD	$CSA_{receiver}$ $\bar{x}$ (µg) ± SD	$EtOH_{receiver}$ $\bar{x}$ (mg) ± SD	n
Control*		2	0.25 ± 0.38	0.16 ± 0.17	1.06 ± 0.28	10
100	10	2	0.78 ± 0.55	0.26 ± 0.18	1.01 ± 0.82	5
160	10	2	1.02 ± 1.14	0.11 ± 0.06	1.86 ± 0.49	5
200	10	2	1.04 ± 0.4i	0.40 ± 0.23	2.83 ± 1.21	5
300	7.5	2	0.95 ± 0.87	0.21 ± 0.23	2.25 ± 1.38	5
370	5.0	2	0.99 ± 1.24	0.59 ± 0.45	5.53 ± 1.59	5
Kruskal-Wallis		*p*	0.026 (Sig)	0.053 (NS)	0.001 (Sig)	
Control*		4	0.48 ±0.29	0.28 ± 0.19	2.08 ± 0.79	11
200	5.6	4	2.00 ± 1.40	1.11 ± 0.71	4.03 ± 0.67	3
200	10	4	2.90 ± 1.28	0.24 ± 0.30	3.10 ± 0.98	5
Kruskal-Wallis		*p*	0.002 (Sig)	0.117 (NS)	0.035 (Sig)	

* passive diffusion

Table 11 **The EtOH Uptake of Skin**

$U_{electrode}$ (V)	Contact Duration (hour)	$EtOH_{skin}$ $\bar{x}$ (mg) ± SD
100	2	0.36±0.11
160	2	0.34±0.13
200	2	0.30±0.20
300	2	0.28±0.09
370	2	0.37±0.20
Kruaskal-Wallis *p*		0.746 (NS)

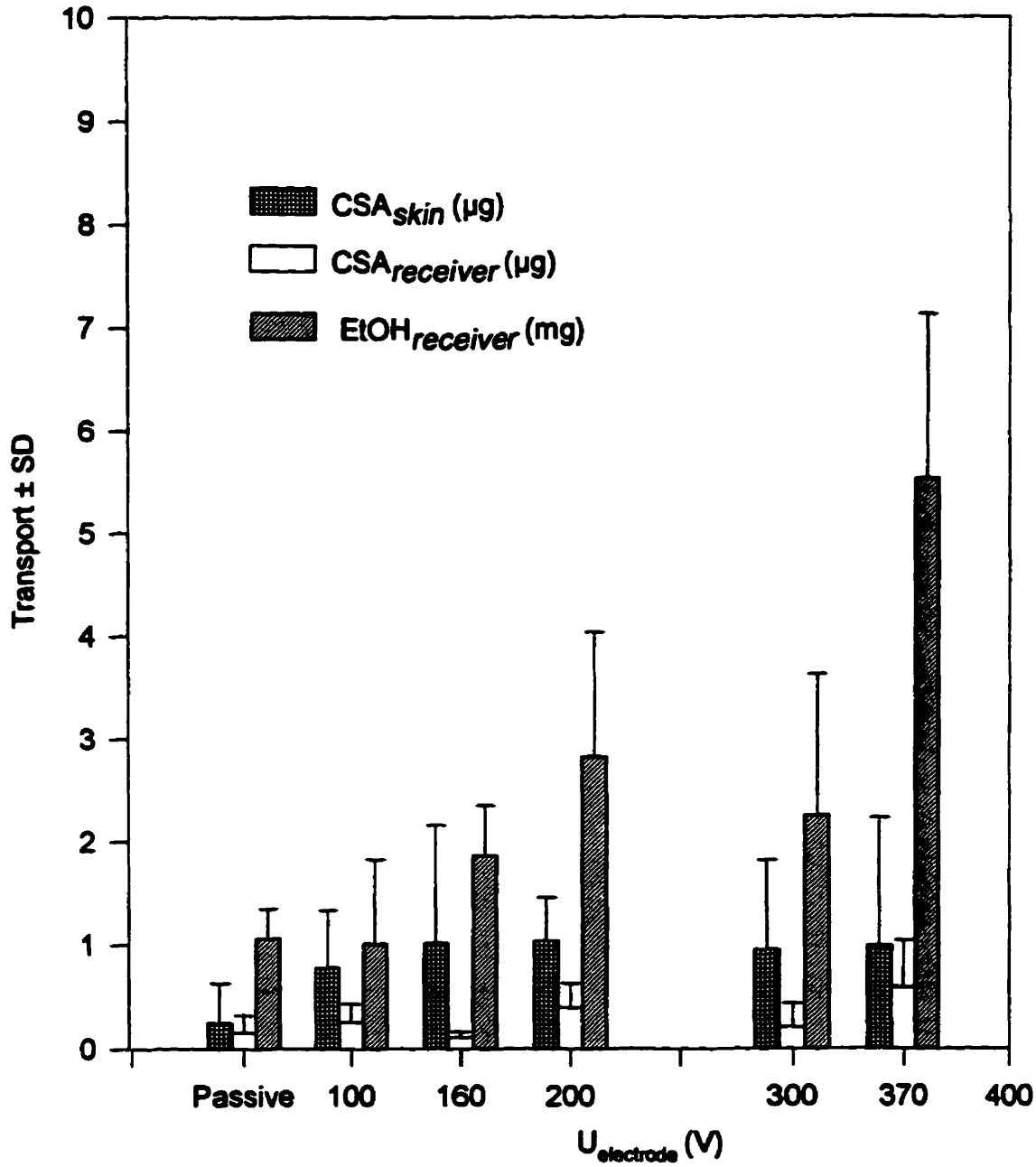


Figure 14 Results of Single Pulse Electroporation

- Effect of $U_{electrode}$

Pulse Length = 10 ms

Contact Duration = 2 hours

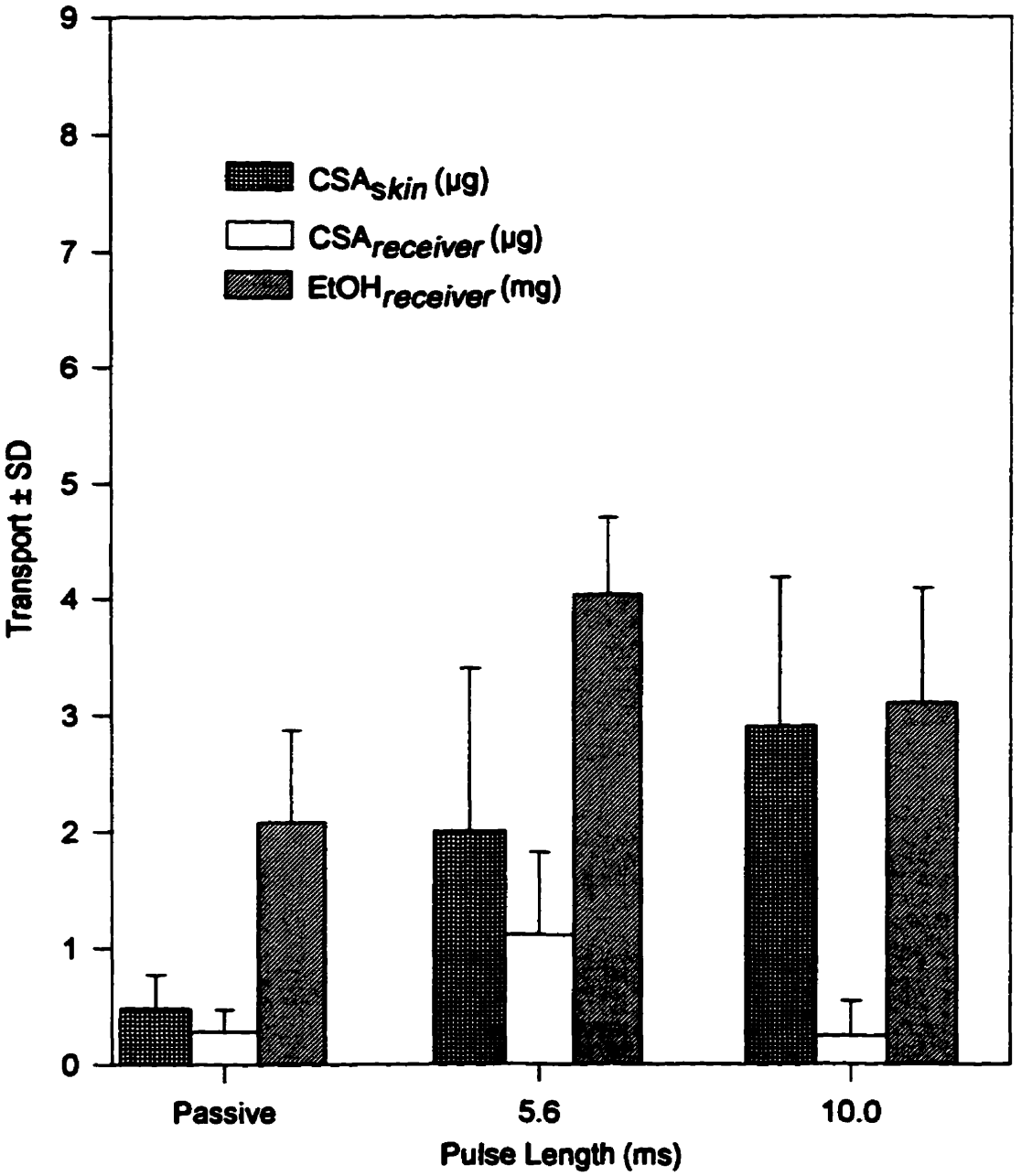


Figure 15 Results of Single Pulse Electroporation - Effect of Pulse Length

$U_{electrode}$ = 200 V

Contact Duration = 4 hours

Multiple Pulse Electroporative Delivery at $U_{electrode}$ = 200 V The results of multiple pulse electroporative delivery are reported in Table 12. As mentioned earlier based on the single pulse electroporation experiments, $U_{electrode}$ = 200 V was selected as the optimum voltage to study multiple pulse electroporation. Compared to control the electroporative delivery with multiple pulse at τ= 0.9 ms resulted in a significant increase ($p < 0.05$) only for the delivery of $EtOH_{receiver}$. Increasing the pulse length from 0.9 to 5.8 ms did not result in any significant increase in the delivery of any of the quantities. However, further increase in the pulse length to 10 ms led to a significant increase ($p = 0.025$, after adjusting for Type I error) in CSA_{skin}, although the other two quantities viz. $CSA_{receiver}$ and $EtOH_{receiver}$ did not show any significant increase (Figure 16). Among the different analyzed quantities $CSA_{receiver}$ was found to be the least in amount. The amount of CSA_{skin} was about 60 fold higher than in the control with multiple pulse at 4 hour contact duration and 10 fold compared to single pulse electroporative delivery with the same $U_{electrode}$, τ and contact duration. With 6 hour and 12 hour contact duration, the increase in CSA_{skin}, compared to the control, was 40 to 55 times higher (Table 9). The amount of $EtOH_{receiver}$ using multiple pulse was only 2-4 times higher than the control. The lack of correlation between $EtOH_{receiver}$ and CSA_{skin} is difficult to explain. It appears that the electroporative force at τ = 0.9 ms is sufficient for the transport of the relatively small and polar ethanol molecules. A further increase in the pulse length

without increasing thermodynamic activity of ethanol does not increase its transport. But for CSA, a large and non-polar molecule, CSA_{skin} and $CSA_{receiver}$ are dependent on the electroporative conditions. Probably due to these differences, there is no correlation between $EtOH_{receiver}$ and CSA_{skin} as expected. Multiple pulse with longer τ and large $U_{electrode}$ provide higher electroporative force for improved CSA_{skin}. A recent finding by the Weaver group (Pliquet *et al.*, 1996 (b)) has reported the dependence of the number of localized transport region and size of the pore, on the transdermal voltage and number of pulses, respectively. The low values of CSA_{skin} with single pulse and a significant increase with multiple pulse is in agreement with these observations. It also appears that there is a threshold value for CSA transport and the pulse length of 5.8 ms does not cross it. High lipophilicity (log P_{oct} = 2.92) and poor aqueous solubility of CSA seem to be two main factors responsible for containing CSA in the skin and minimizing $CSA_{receiver}$. This however could be an advantage in treating psoriasis patients in whom a minimal systemic concentration of CSA would reduce the undesirable side effects. Since there is no reported value for the therapeutically required concentration of CSA to treat psoriasis topically, we can only make a speculation. Topical delivery (*in vitro*) of a 15% CSA in an Azone-containing vehicle in human skin is reported to have resulted in 78 ng/cm^2 in epidermis and 80 ng/cm^2 in the dermis (Biren C *et al.*, 1984). In comparison to that we obtained about a 200 fold higher value in the epidermis

(stripped of SC) and dermis with multiple pulse mode (25 pulses at $U_{electrode}$ = 200 V and τ = 10 ms: CSA_{skin} = 29.78 µg/0.87 cm^2). The amount of CSA_{skin} can be easily reduced, if needed, by adjusting the electroporative conditions. The most desirable testimony for the therapeutic efficacy of the electroporative delivery will be an *in vivo* study which will be done in the near future.

Table 12 **Multiple Pulse Electroporative Delivery of CSA and EtOH**

$U_{electrode}$ = 200 V, Contact Duration: 4 hours, number of Pulses: 25

τ ms	CSA_{Skin} $\bar{x}$ (µg) ± SD	$CSA_{receiver}$ $\bar{x}$ (µg) ± SD	$EtOH_{receiver}$ $\bar{x}$ (mg) ± SD	n
0.9	2.62 ± 2.47	0.23 ± 0.28	4.09 ± 1.47	4
5.8	2.09 ± 1.20	1.33 ± 0.80	5.95 ± 1.34	4
10	29.78 ± 20.14	1.02 ± 0.79	6.74 ± 1.64	5
Kruskal-Wallis				
p	0.014 (Sig)	0.091 (NS)	0.12 (NS)	
Mann-Whitney				
p	0.025 Sig, 5.8-10 ms	—	—	

Sig: significantly different, NS: not significantly different, Level of Sig: 0.05

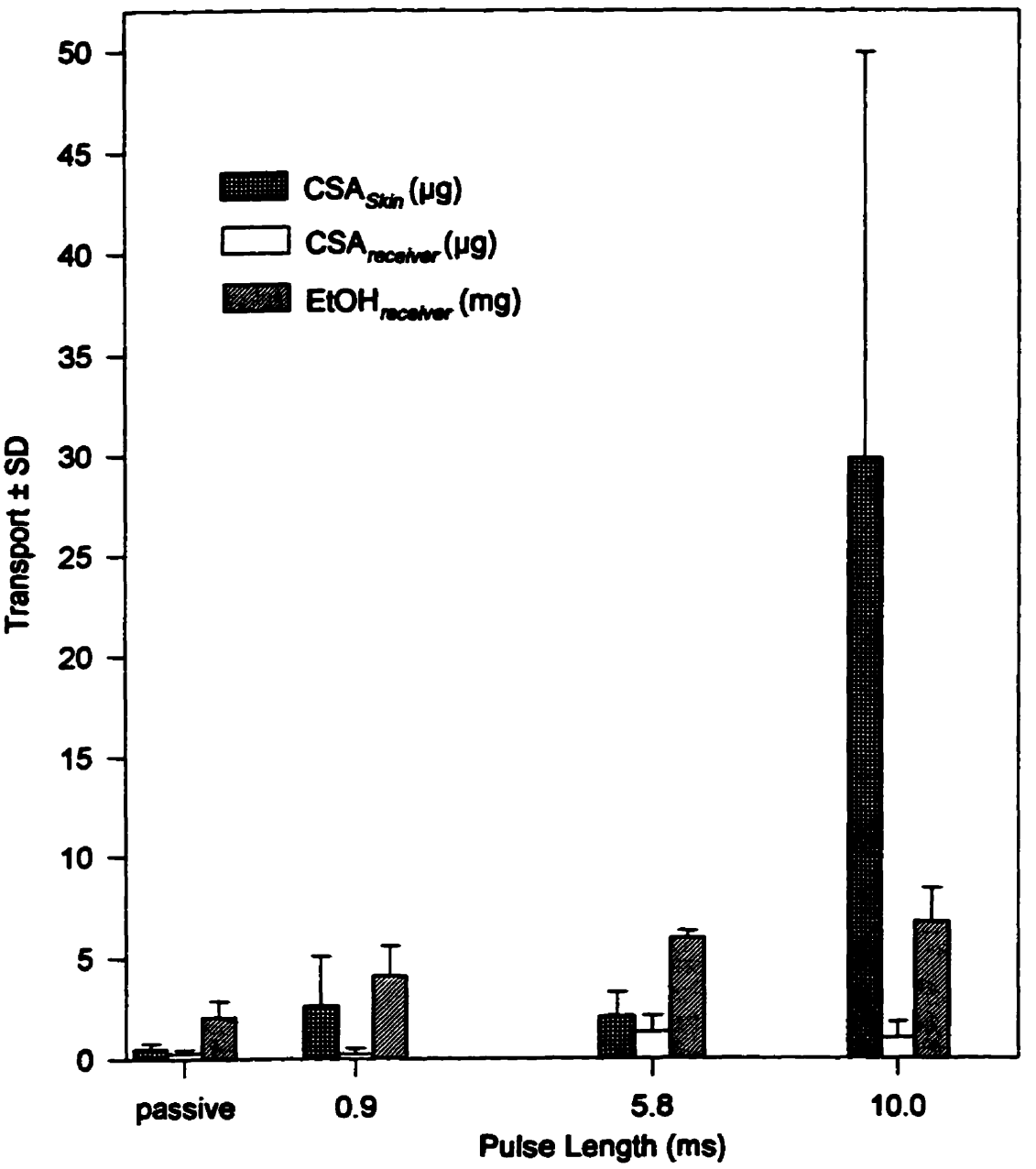


Figure 16 **Results of Multiple Pulse Electroporation**

- Effect of Pulse Length

$U_{electrode}$ = 200 V,

Total Pulses = 25

Contact Duration = 4 hours

Results of RIA The results of RIA are shown in Table 13. All samples are in duplicate and the mean values are reported. The quantitative measuerment of CSA was done using a standard curve, in which $\%B/B_0$ is calculated using following formula:

$$\%B/B_0 = \frac{\text{CPM of standard or samples - CPM of NSB}}{\text{CPM of 0 standard - CPM of NSB}} \times 100$$

The fresh skin seems to interfere with the assay but the measurement of assay is lower than that of passive diffusion. Since % cross-reactivity for CSA metabolites is less than 2 in the RIA kit, our results have confirmed that electroporation can be used to deliver CSA transdermally and the intact CSA exists in the skin samples after electroporation and a 4 hour contact duration. However, the results can be only taken as a qualitative determination because of interferences and possible incomplete extraction of CSA from the skin. The quantity of CSA in the receiver sample were similar for passive diffusion and electroporation. This was the same with LSC analysis.

Table 13 **Results of RIA**

Skin Samples	
	CSA_{skin} (µg/0.87 cm^2)
Passive Diffusion	0.46
Electroporation	2.66
Receiver Samples	
	$CSA_{receiver}$ (µg/0.8 mL)
Passive Diffusion	0.36
Electroporation	0.36

6.7 Effect of Electrical Pulse on the Skin

Electron microscopic photographs show no disruption of the lipid bilayer in the SC interstices after pulsing the skin 25 times at $U_{electrode}$ = 200 V and τ = 10 ms. The outermost layer of SC was noted to have a normal pattern of lamellar sheets, similar to untreated skin used as the control (Figure 17 and 18). It is reported that high voltage pulsing causes a dramatic increase in the transdermal flux for macromolecules with simultaneous change in the skin's structure. But the structural changes in the skin are reversible and also subtle as to be undetectable by electron microscopy (Prausnitz MR, 1996b). It is reported that during high voltage pulsing

the changes of the skin are on sub-microscopic scale and therefore not expected to be detectable by electron microscopy (Pliquette U *et al.*, 1996 (a)). After high-voltage electroporation, lasting effects in the epidermis are reported in which increased transdermal flux generaly persisted for minutes to hours, but the effects are reversible (Prausnitz MR *et al.*, 1994;1995a; Vanbever R *et al.*, 1994; Pliquett U *et al.*, 1996(a)). In our experiment, we observed that, after single or multiple electroporation, transport of water and EtOH across the skin lasted for 24 hours. On the other hand, transport of CSA lasted only about 4-6 hours. This could be due to the large molecular size of CSA, which is much larger than those of the solvents. This result also suggests that after electroporation the skin is expected to undergo a process of recovery.

Figure 17 Electron Microscopic Photograph (I)

Freshly-excised, hydrated skin

Magnification: 3000 × 2

Figure 18 **Electron Microscopic Photograph (II)**

Multiple - pulsed skin

Magnification: 3000 × 2

VII CONCLUSIONS

Transdermal delivery of CSA would be beneficial in the treatment of psoriasis. To deliver CSA transdermally, the electrical means of enhancement seems applicable. Although iontophoresis alone is ineffective in enhancing transdermal delivery of CSA, electroporation enhances the permeation of the skin to deliver CSA. Based on our results, we can make the following conclusions:

1. Hydroethanolic solution (40% ethanol in PBS) can be used as a donor solution in electrically enhanced permeation techniques. It has the solvent property desirable to dissolve hydrophobic drugs while maintaining the electrical conductivity required for electroporation experiment.
2. Preparation of CSA-PVME/MA copolymer helped in enhancing the aqueous solubility of CSA by 10 fold. Electroporative delivery of CSA from coevaporate is 4 - 7 times higher than passive diffusion but the amount delivered is not significant in comparison with the amount delivered from hydroethanolic solution.
3. By using hydroethanolic donor solution, single pulsed electroporative delivery of CSA is significantly higher than the passive diffusion. However, use of multiple pulse electroporation was more effective than single pulse mode in the delivery of CSA into the skin. Multiple pulse electroporative

delivery of CSA using hydroethanolic donor solution is about 60 times higher than passive diffusion. It is also 10 times higher than the single pulse electroporative delivery.

4. The mechanism of enhanced delivery of CSA by electroporation could be attributed to the increase in pore pathway created in SC and electro-osmotic flux of water and ethanol induced by high voltage electrical pulses.

5. The contact duration of donor solution is an important factor in the delivery of CSA. The amount of CSA delivered significantly increased as the contact duration was increased from 2 hours to 4 hours. However, further increase of contact duration from 4 hours to 12 hour did not result in significantly different in the delivery of CSA. The results suggest that post-pulse diffusion is essential to deliver CSA by electroporation.

6. From the single pulse electroporation study, parameters of an exponential pulse, at $U_{electrode}$ = 200 V and τ = 10 ms, were chosen for the multiple pulse electroporation. Further experiments to optimize these conditions for multiple pulse electroporation will need to be conducted.

7. Preliminary electron microscopy study of the skin did not reveal any evidence for gross damage of the skin as a result of electroporation. A more detailed study using techniques, such as TEWL, and in live animal models to evaluate the safety issue of electroporation will have to be performed before

trials in humans.

Now that the *in vitro* parameters are set satisfactorily the first step in this project to achieve a topical delivery of CSA to treat psoriasis has been completed. Further work using an animal model should be carried out.

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