



Drug Delivery Stimulation by Alternating Current Using Methylene Blue Dye Absorption by Pig's Skin as a Model

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Abstract

Pig skin has been studied as administration site of drug for its systemic effects, since systemic therapeutic agents can be delivered for long time with a controlled ratio, escaping from the first pass effect by liver through the transdermal delivery, which can decrease the drug dose. The low permeability of drug molecules through stratum corneum (SC) has been the limiting factor for developing transdermal delivery system of therapeutic agents. The aim of the present work is to study the enhancement of drug absorption by the skin using stimulating alternating current (AC). In our study stimulated voltage over range 6.5 V to 200 V had been used to enhancement the absorption of methylene blue (MB) with pig's skin. Our results showed that the highest MB concentration in tissue was (0.239 ± 0.011) mg/g and lowest ohmic resistances ($k\Omega/cm$) (3.15 ± 0.223) for skin were obtained after skin stimulation with 12.5 V; these results had been confirmed using histochemistry study. Dielectric properties of skin in the range of 10 Hz- 100 KHz revealed change in the dielectric dispersion curves immediately after skin stimulation.

Keywords: Alternating current –Drug Delivery - Methylene Blue - Pig skin.

1. Introduction

Topical drug administration has potential advantages over oral, injection, or intravenous drug delivery. These advantages include convenience, noninvasiveness, minimal trauma

induction, and avoidance of "first-pass" degradation or metabolism in the gastrointestinal tract (GIT) or liver. Tightly localized administration is possible, and systemic delivery can be achieved through absorption by the dermal blood supply. The main barrier to cutaneous or transcutaneous drug delivery is the

impermeability of the stratum corneum (SC), which is outermost layer of the skin, constitutes a formidable barrier both to the loss of tissue water and to entry of xenobiotics into the skin. If the integrity of SC is disrupted, the barrier to molecular transit may be greatly reduced and facilitate the transdermal drug delivery (Guy R 1996, Delgado et al. 2001; Singhet al. 2002; Kalia, Yet al. 2004).

Transdermal administration of drug is assuming an important place in modern drug therapy. Transdermal administration can be passive or facilitated. In passive administration, the non-ionized drug transverses the skin through the lipophilic SC (Kassan D et al. 1996), the lipid-protein matrix of SC not only restricts the passive diffusion of lipophilic molecules but also severely limits the transport of hydrophilic compounds across the membrane (Narasimha M, et al. 2007). This matrix makes the skin a semi-permeable membrane, allows only a small amount of any drug molecules, which are small in size and rather lipophilic in nature, to passively penetrate the skin (Kassan D et al. 1996). Ionized drugs do not easily penetrate this barrier and are not suitable for routine transdermal delivery unless an external source of energy is provided to drive the drug across the skin (Green Pet al. 1993). Facilitated diffusion can be utilized by chemical enhancers such as low molecular alcohols (Bhatia K, and Singh J 1999), aliphatic esters, and various vehicle formulations (Williams A, and Barry B 2004; Asbill C et al. 2000). Other methods rely on physical techniques such as iontophoresis (Chien Y, and Banga A 1989), electroporation (Prausnitz M et al. 1993), and phonophoresis (Mitragori S et al. 1995).

Iontophoresis is a method of transdermal local drug delivery using electrical current (Burnette R et al. 1989) iontophoresis increases the penetration of electrically charged drugs into surface tissues. Electrical energy assists the movement of ions across the SC according to the basic electrical principle like charges repel each other and opposite charges attract each other (Honggang S et al. 2002). The drug is applied under an electrode of the same charge as the drug (delivery electrode) and a return electrode opposite in charge to the drug

which is placed at a minimal distance from the other electrode at a neutral site on the body surface (dispersive electrode) (William E et al. 2005).

The applied electric current must be below the level of patient's pain threshold. The two classically considered prerequisites for iontophoretic treatment are that the drug must be charged (or modified to carry a charge) and the distance process must be at or near a body surface (Peter M et al. 2003).

Iontophoresis enhances transdermal drug delivery by three mechanisms: (a) ion-electric field interaction provides an additional force that drives ions through the skin, (b) the flow of electric current increases the permeability of the skin, and (c) electro-osmosis produces bulk motion of solvent that carries ions or neutral species with the solvent stream (Zhu H et al. 2002).

Iontophoresis provides a controlled delivery rate of ionized drugs through variations of current density, pulsed voltage, drug concentration and ionic strength. It eliminates the gastrointestinal incompatibility, erratic absorption, and first pass metabolism. It reduces side effects and avoids the risks of infection, inflammation, and fibrosis associated with continuous injection or infusion. It enhances patient compliance with a convenient and non-invasive therapeutic regimen, and decreased dosing frequency. It improves efficacy by continuous release and decreases the total dose and dosing frequency (Reena R and Srinivas C 2005).

Iontophoresis has been proposed for numerous uses, including delivering local anesthetic before skin puncture or painful dermal procedures, treatment of excessive sweating of the hands and feet, local drug delivery for agents such as nonsteroidal anti-inflammatory drugs or corticosteroids for musculoskeletal inflammatory disorders; and others (Pradas R et al. 2006; Khoo P et al. 2006).

Methylene blue (MB) dye is used in some researches because its color allows visual observation of its location; its absorbance and fluorescence properties permit spectroscopic detection. Its cationic nature can be used as a

model for a number of photosensitizers and chemicals with similar characters (in which the methyl group is replaced by longer chain) (Kirste J et al. 2002) and has similar physicochemical properties to lidocaine (anesthetic drug) (Flucker Cet al. 2000).

Pig skin was selected for the *in vitro* experiments, because it has histological properties comparable to human skin with similarities in epidermal thickness and composition, pelage density, dermal structure, lipid content, and general morphology (Monteiro-Riviere N 1986). The net charge on the skin is zero when the degree of permselectivity (DP) equals unity, corresponding to the IP of the membrane (~ 4.4 for pig skin and ~ 4.8 for human skin). The consistent IPs observed for human and pig demonstrate that porcine skin is an appropriate model for iontophoresis studies (Diego M et al. 2001). Furthermore, in a number of permeation studies, pig skin showed to be a good model for human skin permeability (Chang S, and Riviere J 1991; Woolfson A et al. 1992).

The aim of the present work is to study the effect of stimulating alternating current (AC) on the absorption of MB by pig's skin. This to evaluate how can stimulate voltage accelerate the process of drug delivery.

2. Materials and methods

2.1 Skin sample

Fresh pieces of abdominal skin from domestic pigs were obtained from a local abattoir with dimensions $\approx 50 \times 60 \text{ cm}^2$. Samples, were stored on ice, and used within 48 hours. The skin was shaved by electric clippers to remove all obvious hairs. The skin was then cut into square pieces each one has the area $15 \times 15 \text{ cm}^2$. Before use the skin was washed three times with normal saline then with distilled water. This method was repeated for every piece of skin obtained.

2.2 Electrodes

Two electrodes of diameter $\Phi = 9 \text{ mm}$ made of Ag/AgCl (Leonhard Lang GmbH "UK" FS-521), which they already used in electrocardiogram purposes and their original

function are to transmit the electrical signals across the skin were used and fixed to the skin by adhesive tape.

2.3 Silver-silver chloride ring

Two rings manufactured from Ag/AgCl, its diameter $\Phi = 9 \text{ mm}$ as electrode diameter, and its height is 5 mm. The rings were put between the electrodes and the skin. They acted as vehicle to the drug (wetted cotton piece with MB put in the ring), also the silver ring used to determine the size of punch of skin sample $\Phi = 9 \text{ mm}$.

Seven main pig skin groups used in the work classified as the following:

- G I: 40 samples were stimulated by pulsing half sine shape waves; the pulse height used in this group was 6.5 V, and its duration 5 seconds.
- G II: 40 samples were stimulated by pulsing half sine shape waves; the pulse height used in this group was 12.5 V, and its duration 5 s.
- G III: 40 samples were stimulated by pulsing half sine shapes waves; the pulse height used in this group was 25 V, and its duration 5 s.
- G IV: 40 samples were stimulated by pulsing half sine shape waves; the pulse height used in this group was 50 V, and its duration 5 s.
- GV: 40 samples were stimulated by pulsing half sine shape waves; the pulse height used in this group was 100 V, and its duration 5 s.
- GVI: 40 samples were stimulated by pulsing half sine shape waves; the pulse height used in this group was 200 V, and its duration 5 s.
- G $^{\circ}$: 40 samples weren't stimulated by pulsing half sine waves and were used as control.

2.4 Methylene blue dye preparation

1.122 gm from the MB crystal was taken and dissolved in 100 ml of distilled water to obtain 0.03 M. A specified constant volume of this concentration was used along this study (200 μl) and it was determined in the preliminary

experiments. But the MB which diffused to the skin was determined in basic medium (ammonium hydroxide) (NH₄OH), so the physical and chemical parameters of the MB was determined in basic medium and compared with the characteristics in the aqueous solution.

2.5 Stimulating circuit

Stimulation of the skin was achieved by half-sine waves with different peaks voltages. It was applied to examine the effect of this pulsing stimulus on the absorption of MB. The circuit that shown in figure 1 was attached to the electrodes fitted with Ag/AgCl rings to the skin. The stimulating currents were changed to give (6.5v, 12.5v, 25v, 50v, 100v, and 200v), the duration of this stimulation was 5 seconds. The ohmic resistance was measured within 9 seconds after terminating the stimulus.

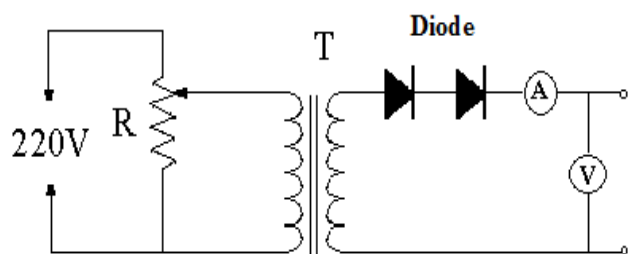


Figure 1. Pulsing sine wave producing circuit

2.6 Experiment set-up

Skin was put dermal side down, on ice covered with cling film. Each skin section was used once. Two Ag/AgCl rings were placed on the skin surface (8 cm apart), then a piece of cotton containing 200 µl of the prepared MB solution was put in cavity of one ring which was used as positive electrodes (anode). The two electrodes were fixed to the skin above the two Ag/AgCl rings by adhesive tape; a slight pressure was applied evenly to the electrodes to ensure a good contact to the skin.

2.7 Absorbance spectra measurements

Punched samples of 9 mm diameter were taken underneath the positive electrode (anode), its WT ≈ (0.3 ± 0.1gm). Each sample was rinsed

in water to remove the superficial dye then it was dissolved overnight in 1 ml NH₄OH. The Solution was centrifuged, and absorbance spectra of the supernatant fractions were recorded spectrophotometrically (λ = 670 nm), to monitor MB level. After measuring the absorbance of MB, the concentration of MB in (mole/liter) was calculated according the following equation:

$$MB \text{ conc.} = \frac{\text{Analyt} - \text{Blank}}{\text{Standard} - \text{Blank}} \times \text{conc. standard} \dots \dots \dots 1$$

2.8 Histological method

Preparation of frozen section: The skin punch was used to prepare a frozen section. It was placed in a holder in a refrigerated chamber (-60 degrees). Then the samples were cut into small sections (10 µm).

Procedure: The section was fixed at a 10% formaldehyde, washed in dis. H₂O. The nuclear fast red was used as a counter stain, then left the slide to dry and mounted by glycerine-gel and covered by cover slip to be observed under light microscope, and then a photograph was taken with digital compact camera.

Image analysis: The image analysis of the depth of invasion (penetration depth) of color was measured by soft imaging system (Pearse A 1972)

2.9 Dielectric properties of the skin

Dielectric properties of the skin (the relative permittivity, the dielectric loss factor, and the conductivity) were determined after applying the stimulating voltage immediately. The measured capacitance "C", and resistance "R" of pig skin were measured over frequency range from 12 Hz to 100 KHz, with LCR meter. Skin tissue which taken under the positive electrodes was placed in glass measuring cell between two circular disc electrodes of silver (figure. 2). The skin was first stimulated by stimulating circuit using (6.5v, 12.5v, 25v, 50v, 100v, and 200v), for 5 s then the dielectric properties of the skin were measured by LCR- Bridge after fixed time (90s). Control was done by measurement the dielectric property directly without any stimulation. This was done at room temperature which was maintained constant at 25°C.

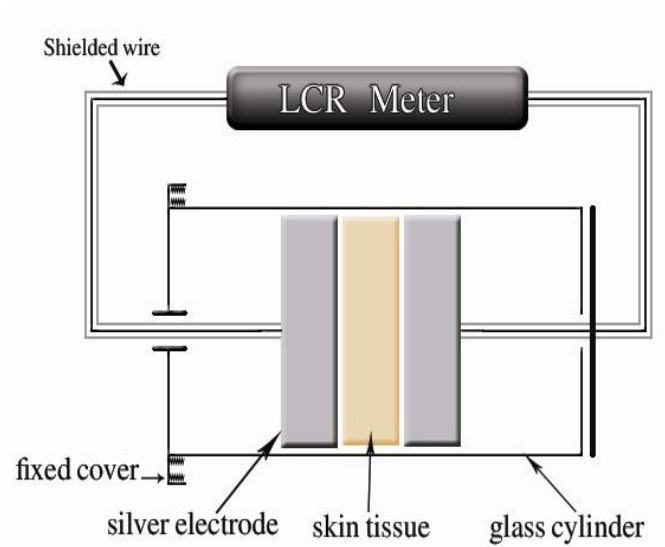


Figure 2.Diagram of the dielectric measuring cell

The relative permittivity (ϵ'), the dielectric loss factor (ϵ''), and the conductivity (σ) were calculated according the following equations.

$$\epsilon' = \frac{\text{Air capacitance}}{\text{Tissue capacitance}} \dots\dots\dots 2$$

$$\text{Air capacitance} = \frac{\epsilon_0 A}{d} \dots\dots\dots 3$$

$$\epsilon'' = \frac{\epsilon'}{2\pi fRC} \dots\dots\dots 4$$

$$\sigma = \frac{d}{RA} \dots\dots\dots 5$$

Where:

ϵ_0 is the vacuum permittivity which equals 8.85×10^{-12} farad/meter.

A is the electrode surface area in meter square.

d is the distance between the two electrodes in meter.

$\pi = 3.14$

f is the frequency in Hz.

R is the resistance in Ω .

C is the capacitance in Farad.

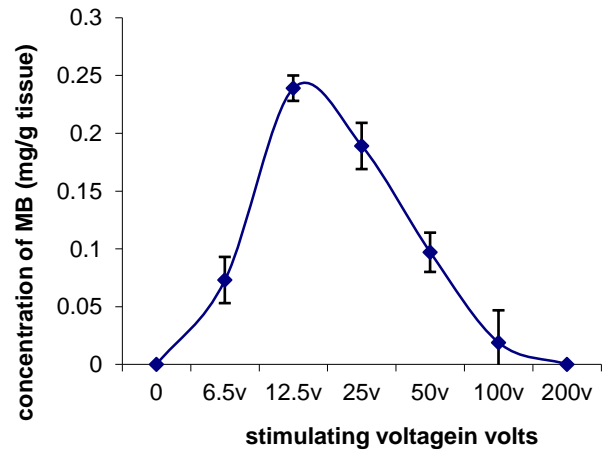
σ in siemen/meter.

2.10 Statistical analysis

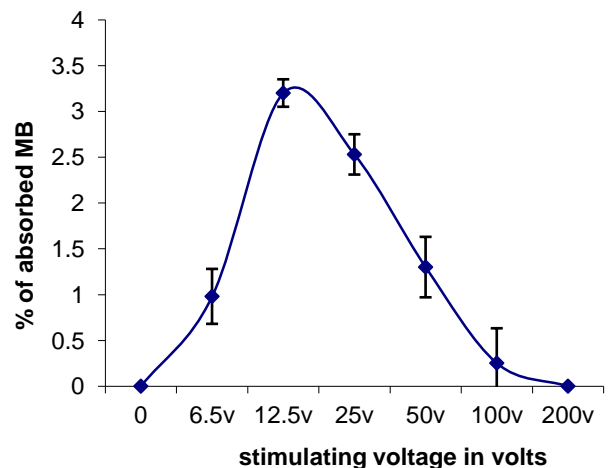
All the parameters measured were represented as the mean \pm Sd. Correlations between different parameters were studied using Excel Microsoft 2007.

3. Results

Electrical studies in the present work showed the effect of stimulating voltage from 0-200 V on the MB concentration (in mg/ g tissue). The concentration percentage reached its highest value at 12.5 volts and began to decrease again (figure 3). Strong correlation between the concentration of MB and stimulating voltage was found ($R^2=0.73$).



a- Dependence of the absorption of MB (mg/g) on the stimulus voltage value



b- The percentage of the absorbed MB as the stimulating voltage varies

Figure 3. Effect of stimulus voltage value on the absorption of MB through pig skin.

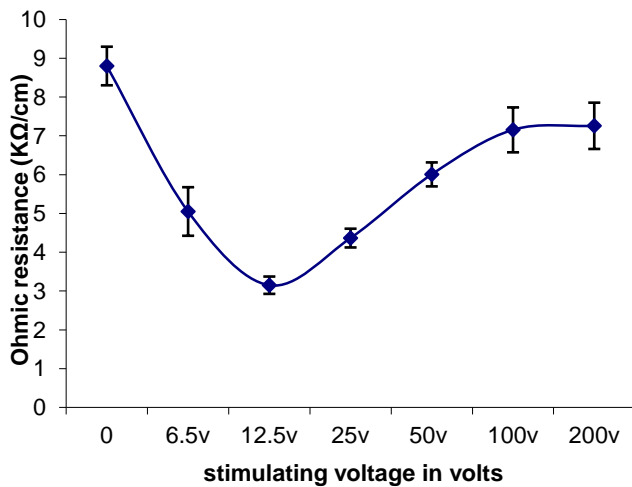


Figure 4. Relationship between the value of the stimulating voltage and the ohmic resistance of the skin in (KΩ/cm).

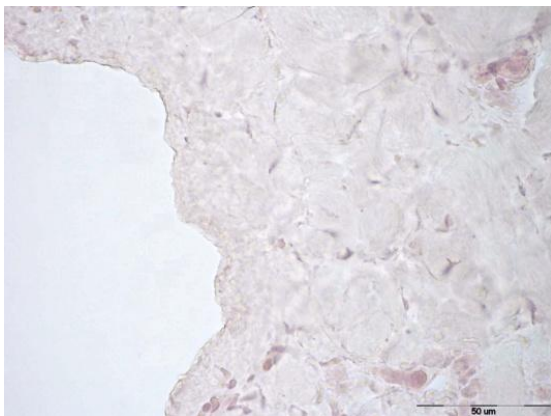


Figure 5. Frozen section micrograph of pig skin stained by nuclear fast red showing the anatomical structure of skin layers (X= 400).

The ohmic resistance of the skin was decreased as the stimulating voltage increased. It reached the lowest values at 12.5 V. From Figure 4. It's clear that the ohmic resistance began to increase for the stimulating voltages higher than 12.5 V.

In comparison between the control skin sample and stimulating samples with AC, histological study showed a clear difference between them in absorption of MB. Figure 6 shows the light micrographs of the correlation of MB penetration depth to the anatomical structure

of the skin after stimulation via AC, the dye enters the SC and its penetration depends on the exposure dose particularly field application time and voltage. The penetration of MB increases following electroperoration and reaches its highest value at exposure dose of 12.5 V and its lower value at 200 V.

Figure.7 shows the relative permittivity of the skin after each stimulating voltage as a function of frequency; the results indicate that the relative permittivity at 100 and 200 V are lower than control and the other stimulating voltages are higher than it and reaches the highest at 12.5 V. Fig.8 illustrates the dielectric loss results and similar results to relative permittivity are obtained. Figure.9 shows the conductivity results which match with relative permittivity and dielectric loss results.

4. Discussion

The major flux enhancing mechanisms of AC transdermal iontophoretic transport include electrophoresis (direct electric field effect), electroosmosis (convective solvent flow), and electric field-induced pore formation (electropermeabilization) (Qingfang X et al., 2009).

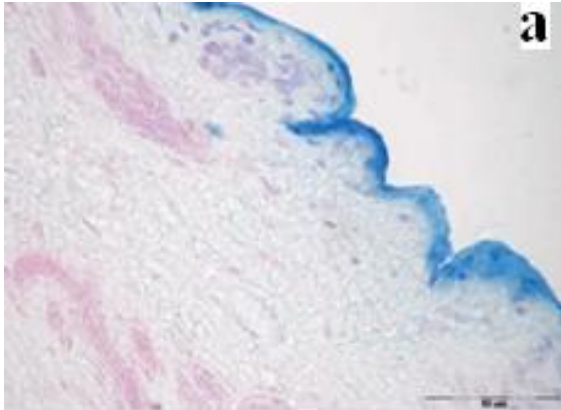
The ability of AC to reduce and control epidermal membrane electrical resistance also makes this a useful method to improve ion-exchange membrane enhanced iontophoresis. A previous study (Xu et al., 2009) has shown that the incorporation of an ion exchange membrane having a charge opposite to that of the drug ion in an iontophoresis system can enhance transdermal iontophoretic transport (i.e., permeant transference number) by approximately 2 to 4 times when the electrical resistance of epidermal membrane was lowered to and maintained at 0.5 kΩ with AC.

AC is more effective in sustaining a constant state of pore induction in epidermal membrane, and constant skin resistance AC iontophoresis has been shown to have less transdermal iontophoretic flux variability for neutral permeants than conventional DC (Zhu et al., 2002). (Shibaji et al., 2001) has concluded that AC enhances membrane transport by

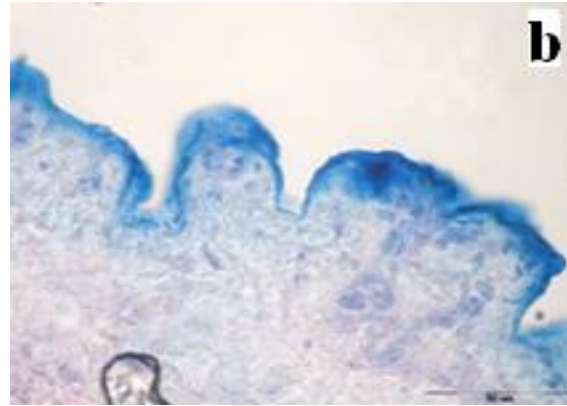
increasing the electromobility of the ions as a result of the decrease in the hydrated ion radii under the AC electric fields.

The increase in MB concentration results from the fact that different aspects of the electroporation, transport phenomenon are being

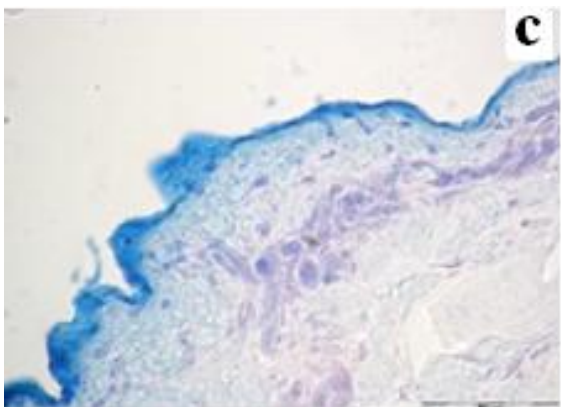
monitored(Patricia G et al.1998). The decrease in MB concentration in the skin with increasing voltage could be contributed to the structure and characteristic differences exist between low and high voltage electroporation(Seiji T et al. 2005).



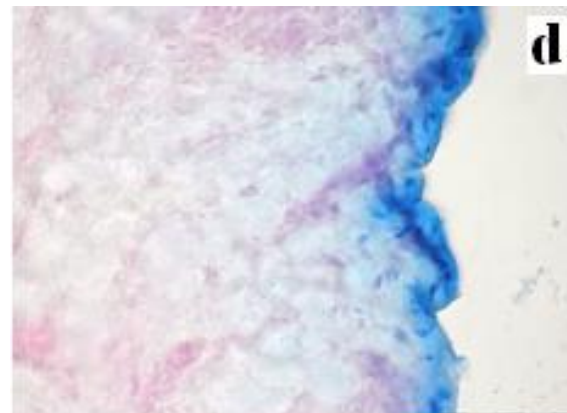
a: pig skin stimulated with 6.5 volts



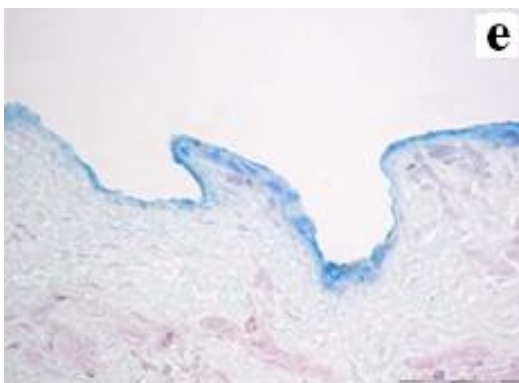
b: pig skin stimulated with 12.5 volts



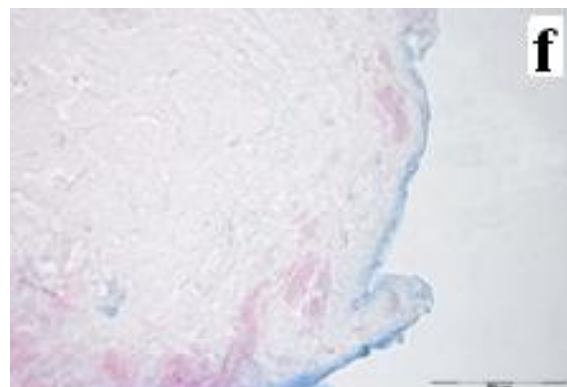
c: pig skin stimulated with 25 volts



d: pig skin stimulated with 50 volts



e: pig skin stimulated with 100 volts



f: pig skin stimulated with 200 volts

Figure 6. Frozen section micrograph of pig skin stimulated with (6.5-200) volts showing the MB depth after stimulation (X= 400).

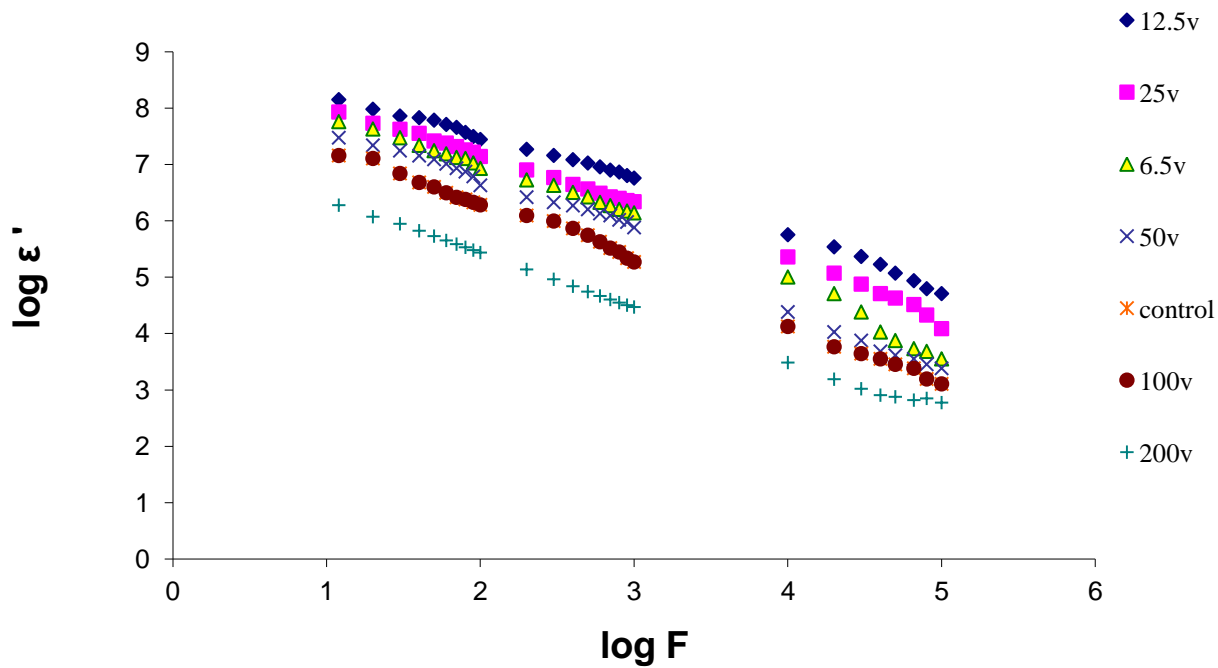


Figure 7. The variation of log permittivity as function of log frequency for skin after stimulation.

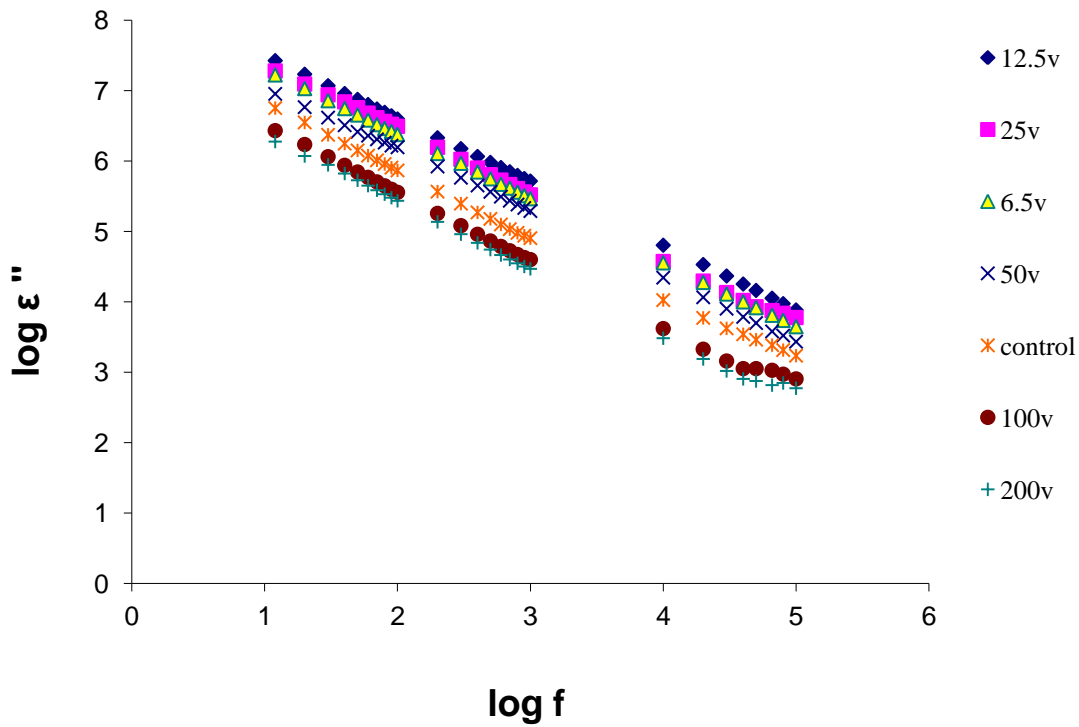


Figure 8. The variation of log dielectric loss as a function of log frequency for normal and stimulating skin.

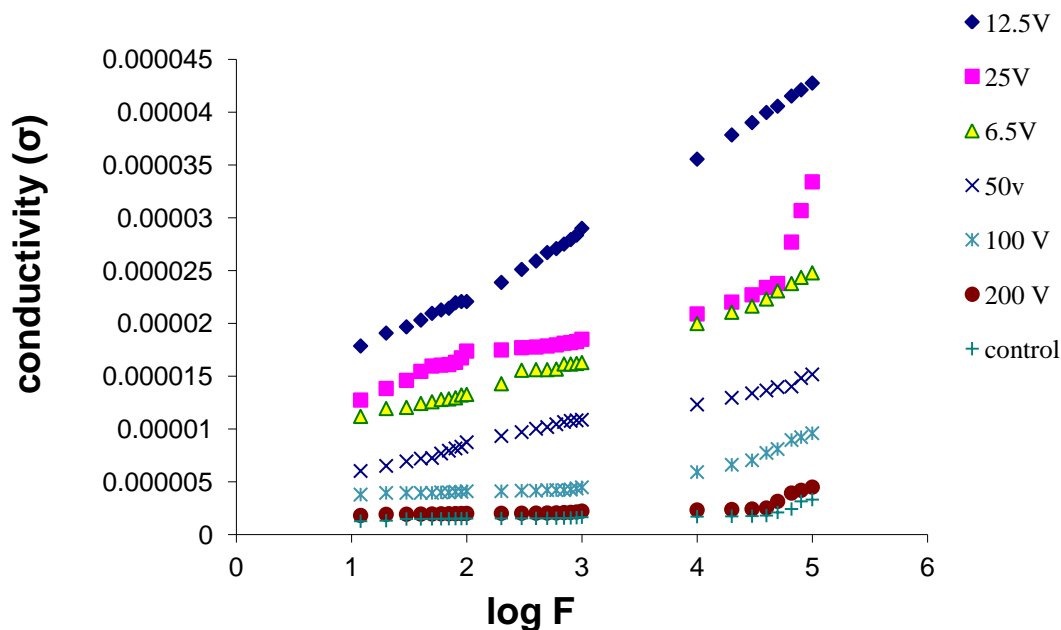


Figure 9. The variation of conductivity as a function of log frequency for skin after stimulation.

There is a high correlation coefficient between resistance and percentage of absorbed MB, ($R^2 = 0.85$). Revere (Riviere J1986), reported that, skin permeability of drug is greatly influenced by skin permselectivity. Since electroporation pretreatment damages the barrier function of the SC but does not strip the layer off.

Voltage, pulse length (pulse duration), number of pulses, and physicochemical properties of chemicals such as MB are among the factors affecting chemical or drug permeation in electroporation. Two different types of electrical pulsing protocols have been used: exponentially decaying pulses (ED) and square wave pulses (SW). A study performed by Vanbever et al. indicated that the intermittent application of short (~1 ms) high-voltage (~100 across skin) ED pulses and a few applications of long (=100 ms) medium-voltage (>30 V across skin) ED pulses had similar alterations and recovery processes of skin electrical resistance, while long pulses of medium-voltage appeared to be more efficient in transporting sulforhodamine (Mwt= 607) across skin. For the same total transported charge, long pulses induced faster and greater molecular

transport across skin than short pulses (Vanbever R et al.1996).

Pulse length could be the second most important parameter next to the voltage determining electroporative drug penetration. In Sharma et al. Study, 20 V pulses were used with lengths of 10, 20, 30, and 40 ms to enhance the in vitro delivery of terazosin hydrochloride through hairless rat skin (Sharma A et al. 2000)

Resistance of skin measurements shown in figure. 4. reflect permeation of SC implying the creation or enlargement of SC opening through which electrolyte flow. The creation and/or the enlargement of aqueous pathways during electroporation has been proposed and observed in many studies. In an X-ray scattering analysis, disordering of lipid lamellar stacking and of the lipid lateral packing was observed after application of long electroporation pulses (Jadoul A et al.1997). Freeze-fracture electron microscopy showed that high voltage pulses induced a general perturbation of the intercellular lipid materials. A light microscopy study found increased detachment in the SC cell layers with increasing electroporation voltages from 100 to

300 V (Jadoul A et al. 1998). As resistance drops, voltage is redistributed across SC and the other layers, reducing the voltage gradient across the SC and reducing the probability that additional poration will occur (Patricia Get al. 1998).

Electroporation produces tiny pores in the skin barrier and this decreases skin resistance (Vanbever R et al. 1997) to confirm these results; we measured the dielectric properties of the pig skin after each stimulation; figures (7-9). The dielectric properties of the skin are largely determined by the SC which has a thickness of 15 μm and consists of dead cells (Gabrial S et al. 1996). By measuring the dielectric properties of pig skin in the frequency range 10 Hz- 100 KHz, it can be noted that skin possesses a relatively weak α -dispersion and this relative lack of significant dispersion is plausibly ascribed to the dead nature and low conductivity of the SC. It was suggested that the origin of this dispersion lay in SC and was associated with the relaxation of ions surrounding the corneal cells (Akimoto M et al. 2006). Since skin tissue after constitutes the interface between the biological and electronic parts of an iontophoretic system, its dielectric properties are of some interest and importance.

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