EFFECTS OF HIGH VOLTAGE PULSED CURRENT

ON VITAMIN C PERMEATION THROUGH THE PORCINE SKIN MODEL

A THESIS

BY

SOAWALUK POLDECH

Presented in Partial Fulfillment of the Requirements for the

Master of Science in Physical Therapy

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Advisor Committee: Asst. Prof. Dr. Nopporn Jongkamonwiwat,
Asst. Prof. Dr. WarinKrityakiarana and Dr. ChittimaManagit.

Transdermal drug delivery is consider the route of choice for drug administration, which has many advantages. The administered drug in transdermal application will not be subjected to degrade along the first pass effect by the liver stomach and intestine, specific dose for constant application or time-varying control delivery, reduce transport lag time. Iontophoresis is the conventional technique for transdermal drug delivery in Physical Therapy by using galvanic current (GC). Recently, the newer approach for transdermal drug delivery has been established. This theory refer to the capability of high voltage pulsed current (HVPC) which generated electrical current up to 100 volt for short duration within microsecond range. There is still a lack of evidence support in using HVPC for transdermal drug application in physical therapy practice. Therefore, the aim of our study was to compare the effect of magnesium ascorbyl phosphate (MAP) permeability across the porcine skin model among iontophoresis, electroporation and passive diffusion at 5, 10 and 20 minutes time points. MAP use in this study, which the effects on inhibiting of melanogenesis, promotion of collagen and prevention of free radical. MAP is the derivative form of vitamin C, which more stable than other vitamin C. Franz diffusion cell and high performance liquid chromatography (HPLC) techniques were used in this study. HVPC was shown to enhance MAP permeability greater than passive diffusion and GC conditions. Moreover, the results showed that HVPC 400 V significantly enhanced MAP permeability when compared to other conditions in every time point. Therefore, this study provided the evidence using electroporation in transdermal application by using therapeutic electrical stimulator with MAP which is one step closer to the clinical application in the near future.

ผลของการใช้กระแสไฟตรงศักย์สูงในการผลักดันวิตามินซีผ่านแบบจำลองผิวหนังหมู

บทคัดย่อ

โดย

เสาวลักษณ์ พลเดช

เสนอต่อบัณฑิตวิทยาลัย มหาวิทยาลัยศรีนครินทรวิโรฒ เพื่อเป็นส่วนหนึ่งของการศึกษาตาม

หลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต สาขากายภาพบำบัด

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การนำส่งยาผ่านทางผิวหนัง เป็นทางเลือกหนึ่งสำหรับการนำส่งยาเนื่องจากการนำส่งยาด้วยวิธีนี้ ยาไม่ผ่านกระบวนการดูดซึมที่ตับและระบบย่อยอาหาร ยาจึงสามารถซึมผ่านไปยังอวัยวะเป้าหมายได้โดยตรงในปริมาณยาที่แน่นอน ลดความเสี่ยงการใช้ยาปริมาณที่สูงหรือต่ำเกินไปได้ เห็นผลของการรักษาได้รวดเร็วสามารถหยุดการรักษาได้ตามต้องการ ทำให้ผู้ป่วยยินยอมเข้ารับการรักษาเพราะเป็นวิธีที่ง่าย สะดวก ปลอดภัยและลดความเสี่ยงในการติดเชื้อ วิธีการรักษาด้วยการผลักดันยาผ่านผิวหนังที่ได้รับความนิยมในทางกายภาพบำบัดประกอบด้วย วิธีไอออนโตโฟเรซิส (Iontophoresis) เป็นวิธีการนำส่งยาที่ใช้รักษาทางกายภาพบำบัดโดยใช้ไฟฟ้ากระแสตรง (galvanic current; GC) ซึ่งเป็นกระแสไฟฟ้าที่มีการออกของคลื่นอย่างต่อเนื่อง ในปัจจุบันได้มีการศึกษาวิจัยถึงวิธีการผลักดันยาอีกรูปแบบหนึ่งคือ อิเล็กโตรโพเรชั่น (Electroporation) ซึ่งรูปแบบของกระแสไฟฟ้าที่ใช้คือกระแสตรงศักย์สูง (high voltage pulse current; HVPC) โดยกระบวนการนี้อาศัยการใช้กระแสไฟตรงศักย์สูงมากกว่า 100 โวลต์ ในระยะเวลาสั้นๆในช่วงไมโครวินาที ทั้งนี้ยังไม่มีการศึกษาก่อนหน้าที่ใช้เครื่องกระตุ้นไฟฟ้าในรูปแบบ HVPC ทางกายภาพบำบัดในการผลักดันยาผ่านผิวหนังงานวิจัยนี้จึงต้องการเปรียบเทียบความสามารถในการผลักดันยาผ่านแบบจำลองผิวหนังระหว่างวิธีไอออนโตโฟเรซิส วิธีอิเล็กโตรโพเรชั่นและไม่เปิดกระแสไฟ (passive diffusion) ตัวยาที่ใช้ในการศึกษาคือ magnesium ascorbyl phosphate (MAP)ซึ่งเป็นยาที่ใช้ยับยั้งการเกิดกระบวนการสร้างเม็ดสี เพิ่มการสร้างคลอลาเจน และป้องการการเกิดอนุมูลอิสระ ซึ่งเป็นอนุพันธ์ของวิตามินซีที่มีความเสถียรมากกว่าและมีผลข้างเคียงน้อยกว่าวิตามินซีตัวอื่นๆ ทำการทดลองโดยใช้ Franz diffusion cell และเปรียบเทียบความสามารถในการผลักดันยาที่เวลา 5 10 และ 20 นาที โดยวัดความเข้มข้นของ MAP ด้วยวิธี High Performance Liquid Chromatography (HPLC) เพื่อเปรียบเทียบค่าความเข้มข้นของยา ผลของการศึกษาพบว่า HVPC มีแนวโน้มเร่งการผลักดันยาผ่านแบบจำลองผิวหนังได้ดีกว่า GC และpassive diffusionนอกจากนั้นยังพบว่าในการใช้กระแสไฟรูปแบบ HVPC ที่ 400 V มีค่าในการผลักดันยาเพิ่มขึ้นอย่างมีนัยสำคัญทางสถิติเมื่อเทียบกับการทดลองกลุ่มอื่นในทุกช่วงเวลา ดังนั้นการใช้กระ แสไฟตรงศักย์สูง (HVPC) สามารถเพิ่มการผลักดันยาผ่านแบบจำลองผิวหนังได้

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Soawaluk Poldech

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CHAPTER 1

INTRODUCTION

Background

The application of drugs for treatment can be delivered through various forms and routes. The administrative routes of drugs into the body can be applied via the enteral application where the drugs are taken orally or sublingually. The parenteral applications of the drugs are injected through intravascular, intramuscular and subcutaneous routes or through the inhalation and topical application can apply the drugs into the skin or mucosal membranes. Each route has specific purposes based on its aims and the ultimate effectiveness of treatment by drugs. Some application methods may also have more advantages or disadvantages than the others, considering the factors affecting the delivery of drugs (1).

Currently, the research studies on topical application especially via transdermal route have become one of the most successful and innovative studies focusing on the drug delivery. The technology of transdermal delivery of drugs has been collectively developed by several researchers and it also has been proven by the record from Food and Drug Administration (FDA) since the first approval in 1979 (2).

An increasing number of recommended drugs for administration via transdermal delivery has been continuously supported by several experimental studies demonstrating real therapeutic benefits to patients (3). Transdermal drug delivery offers a number of potential advantages over conventional methods, because drugs do not subject to degrade along the digestive tract; in stomach, intestine, or first pass metabolite by the liver. It has demonstrated that the transdermal drug delivery has improved patients' compliance

because of user-friendly method of applications. Moreover, this application has shown potential benefits for constant application or time-varying control delivery (4). It emphasized the importance of this delivery route as having high potential for major impact on the delivery of medicine. A number of approaches have been employed to increase the efficiency of transdermal transport (4). The most common approach enhancing the pharmacodynamic of drug is the addition of chemical enhancers, which is believed to increase the concentration of drug when passing through the skin. Another approach involves the chemical modification of drugs into the form of “pro-drug” which potentiates the penetration through the skin and it is converted by epidermal enzymes into the original pharmacologically which actives drug formulation. Applying external forces to enhance efficiency of drug permeation has been widely used especially in the physical therapy profession. Iontophoresis and electroporation have been used to promote transdermal flux and to reduce transport lag times (5). These approaches have utilized the movement of drugs across the skin by an electric field, which is believed to act primarily by moving charged molecules across the skin via an electrical force (4). The barrier properties of skin are attributed primarily to the stratum corneum, the skin’s outermost layer. The stratum corneum is a dead tissue composing of flattened cells filled with cross-linked keratin and extracellular matrix made up of lipids which arranged largely in bilayers (4). This part is different from the unilamellar phospholipid bi-layers of cell membranes according to its containing multilamellar layers, no phospholipids and primarily composing with ceramides, cholesterol, and fatty acids (4). The overall hydrophobic character and net negative charge of the stratum corneum have proved to be major obstacle challenging the transdermal delivery of negatively charged hydrophilic drugs especially (6).

Iontophoresis is a physical method which uses an electrode of the same polarity to the drug charge and subsequently drive into the body by using galvanic current (GC) current to stimulate (6). The use of iontophoresis to treat at local conditions of the skin is

well known (3). Iontophoresis is also useful for targeting deeper underlying tissue such as muscle in the conditions of osteoarthritis, musculoskeletal spasms and other local inflammations associated with sport injuries or accidents (3). Iontophoresis has been restricted to low molecular mass hydrophilic drugs (7). In cosmetic purposes, iontophoresis has been used in various conditions, especially in treating melasma conditions. Vitamin C is one of the drug of choices in treating melasma, and it is well known as expressing physiological effects on inhibiting of melanogenesis, promoting of collagen and preventing of a free radical (8). However, the major obstacles of using vitamin C treatment by iontophoresis are the high molecular weight and quickly oxidize (9). There is evidence in using iontophoresis to enhance transdermal delivery of vitamin C through the skin. The use of vitamin C alone does not encourage owing to its poor penetrating ability, which is presented a limited factor in transdermal of drug delivery (10). Magnesium ascorbyl phosphate (MAP) is the derivative form of vitamin C, which is more stable than vitamin C. It has been proved that MAP molecular structure showed less oxidation and better dissolution in hydrophilic solvent (11). Therefore, MAP is commonly used in cosmetic treatment when being compared to vitamin C. Recently, a novel transdermal of drug delivery approach has emerged, which involves in skin membrane especially the level of stratum corneum (12). The approach is electroporation which is generated by high voltage pulses up to 100 volt for short duration (from 100 μs to less than 1s) which is known as the high voltage pulsed current (HVPC) (13-15). It has been shown that HVPC increases drug transport across the skin by creating new aqueous pores in lipid bi-layers as a result from the application of the short (microseconds to milliseconds) electrical pulse involving in the transient structural changes (4). This theoretical model supports the hypothesis of “pores” or aqueous pathway formation (16, 17). These data suggest that electroporation may express more effectiveness in delivering compounds through cutaneous cells than iontophoresis.

Therefore, in this study used the therapeutic electrical stimulator with MAP which generated a stronger evidence and more implacable for clinically application in the near future. The researcher examined the hypothesis that electroporation is more effective than iontophoresis on MAP permeability through porcine skin model in the laboratory setting.

Research question

Does physical therapy electrotherapeutics modalities by high voltage pulsed current can generate electroporation and be used in enhancing permeability of MAP across the porcine skin model?

Objectives

General objectives

The study was conducted to compare the permeability of MAP among porcine skin models using different types of electrical stimulation.

Specific objectives

1. To compare the permeated MAP concentration among three types of experimental settings.
    - Passive diffusion (control)
    - Galvanic current stimulation (GC)
    - High voltage pulse current (HVPC)
2. To compare the permeated MAP concentration in different time points at 5, and 10 minutes.
3. To compare the permeated MAP concentration in different HVPC parameter settings at the different intensities at 100 V, 200 V and 400 V.

4. To compare the permeated MAP concentration between GC and HVPC at the different intensities at 100 V, 200 V and 400 V.

5. To compare the types of electrical current on reversibility of skin (electrical breakdown)

Hypothesis

High voltage pulsed current (HPVC) can improve the permeability of MAP through skin models when being compared to the galvanic current (GC) stimulation and passive diffusion control respectively.

Expected benefits

The results of this study could be used for further clinical application study to improve the transdermal application of drugs in physical therapy treatment. The experiment is important to test *ex vivo* delivery of the drug before applying to clinical practice in the future.

Conceptual framework

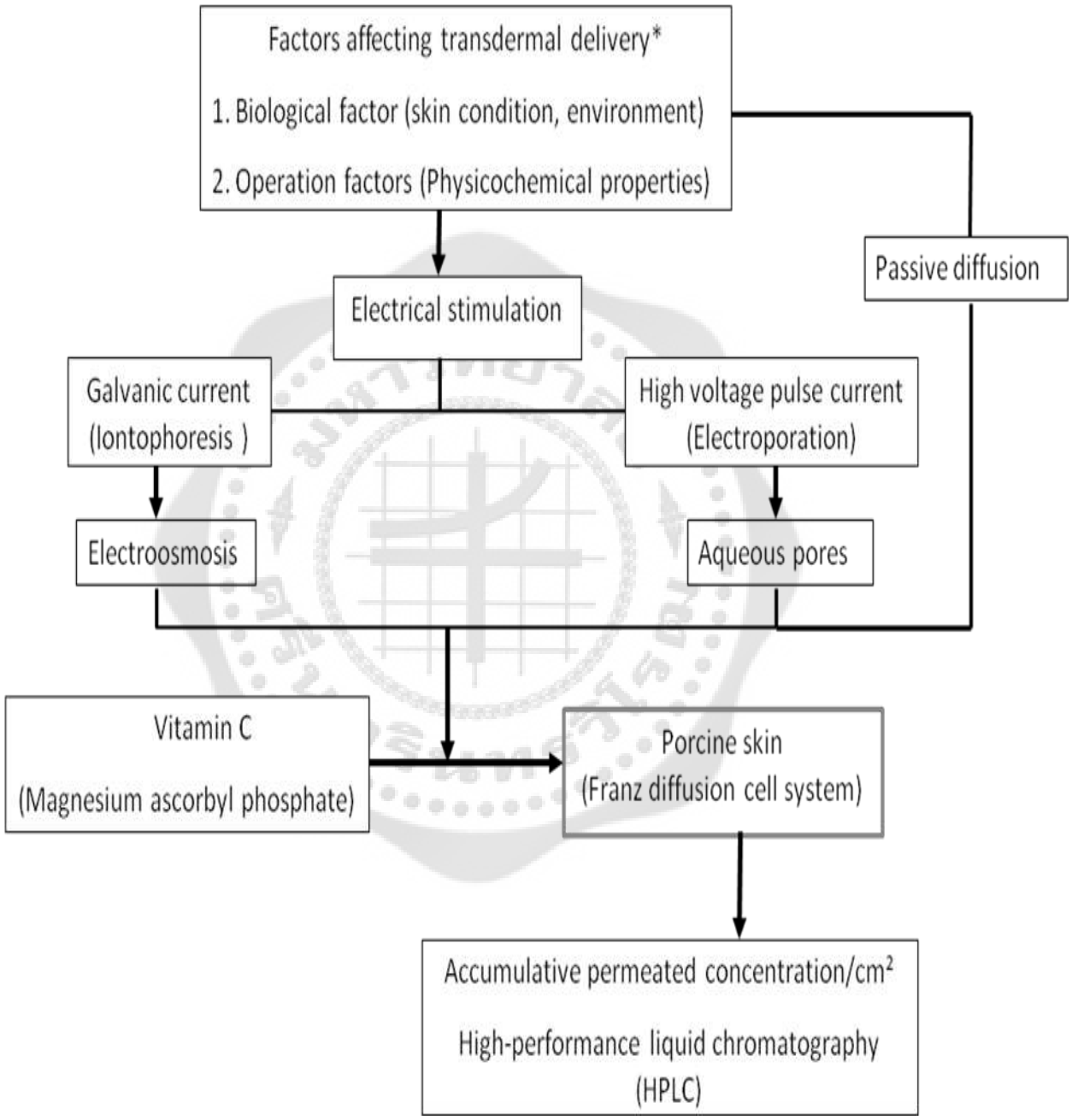

Figure 1 Conceptual framework of this study

CHAPTER 2

LITERATURE REVIEW

For several centuries, humankind has been employed substances onto the skin for some therapeutic effects. In the modern time, numerous practices have been investigated to study the mechanism of its effectiveness. The technique of transcutaneous drug delivery into systemic circulation has received much attention (5). A number of research studies in transdermal drug delivery have shown important contributions to clinical practice due to its non-invasive approach and safety issues. However, the application of transdermal drug delivery needs to be further developed in order to be improved to achieve its potential as an alternative to oral and intravenous deliveries of drugs (2, 18).

In the modern medicinal practice, transdermal delivery is represents an alternative to the application of drugs. This is because this approach offers safety and effective manner on an administration to patients (2). A variety of topical formulations has been developed to provide a local indication of treatments especially a low-cost and easy to approach and design for the possibility of self-administration (2, 5, 18). Apparently, there are immediate key benefits of transdermal application such as a significant difference because of an incomplete absorption of drugs from the gastrointestinal tract as well as a subjection to first-pass metabolism. The results showed high levels of metabolites formed on their first passage through the liver (1, 2, 18-20). The oral application needs to take drugs frequently (2-4 times a day) due to the short half-life of drugs and to maintain optimal treatment effects. The bitter taste of the oral solution is another disadvantage, adding to bias oral bioavailability and avoidance of frequent administration (18).On the contrary, a number of evidences showed that transdermal delivery of drugs also had some major advantages. Transdermal routes could provide the administration in a longer period of time in which drug

levels could also be maintained within a range of effective therapeutic dose (1, 2, 18-20). Furthermore, the low concentration of the administered drugs in the blood circulation system avoided its potential toxicity (1). Transdermal delivery has benefits over an intravenous delivery. It could decrease the risk of infections and pain from an invasive approach (2, 18).Moreover, transdermal delivery of the drugs is safe, effective and easy to use as it is designed for self-administration by patients at the target rates of therapeutic delivery.

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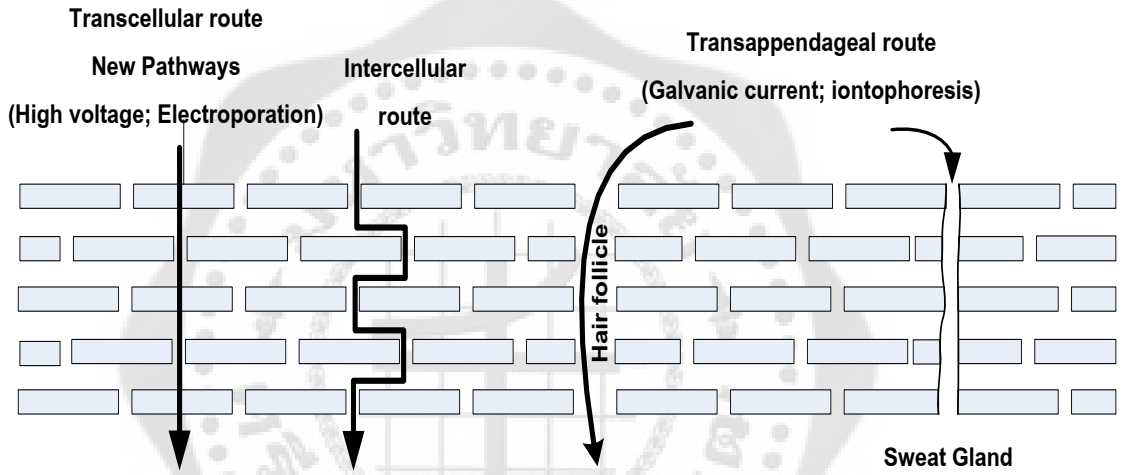

Figure 2 The comparison of drug delivery route between iontophoresis and electroporation (21).

The routes of transdermal delivery can be concluded in 2 major pathways; transappendages (shunt); and transepidermal pathway (Figure 2) (21). Transappendages pathway; these skin appendages are about 0.1% of the total human skin surface. The skin appendages comprise with hair follicles, sweat glands, and sebaceous glands. , which form shunt pathways through the intact epidermis (22). Generally, sweat ducts, pilosebaceous unit and sebaceous glands usually form the transepidermal pathway around $\geq$ 50 μm, 5-70 μm and 5-15 μm respectively. These pathways contribute the largest width and lowest resistant of the skin (23). Another pathway is transepidermal pathway. It composes of the

intercellular pathway and transcellular pathway. Intercellular lipid route is the latter channel for transepidermal diffusion. This component is in the stratum corneum comprising of the crystalline lipid lamellae and fluid lipid in the intercellular region. These structures are crucially important for transepidermal diffusion. The lipophilic molecules are to be predominantly diffusible rather than hydrophilic molecules, because of its composition in its region (24). The weight of molecular is more considered in the transepidermal diffusion rather than molecular size in this area. The molecular weighting more than 400-500 Da is too heavy and insufficient to pass through this intercellular lipidic matrix (22).Transcellular diffusion is the most practical diminishing pathway for transepidermal permeation (25). This transcellular route contemplates the crossing through the membrane of corneocytes and the intervening lipid (28). The narrow transcellular pores have been observed by using confocal laser scanning microscopy in the intact skin (23). This region mostly coincides within wrinkles on the skin surface, and it is also the lowest skin resistance in transport of hydrophilic entities (22).

Transdermal drug delivery has 3 generations. The first generation is transdermal drug delivery patch in which its technology has been continuously increased in the clinical. This system has been widely used for a delivering of a small molecule, lipophilic and low-dose drugs in clinical purposes and settings. Therefore, the first generation is the limited outermost performing as a barrier layer (stratum corneum) of skins. The second generation of delivery system involves a chemical enhancer and also physical agent such as iontophoresis. The third generation physical enhancement system has set a target to the skin’s barrier layer of stratum corneum using the cavitational ultrasound and electroporation (2, 26). The second and third generation enhancement strategies of transdermal delivery are significantly increasing impact on medicine (2). Although several experiments have confirmed that transdermal delivery had variety of advantages compared to oral application. The investigation still relies on the delivery system that achieves a better drug administration in order to improve the clinical impact especially when comparing between iontophoresis and electroporation systems (2).

1. Mechanism of transdermal transport of drug by iontophoresis and electroporation

1.1 Iontophoresis

In physical therapy, iontophoresis is described as the use of a continuous and direct electrical current to deliver therapeutically charged ions through skins into the blood circulation. The ions are driven through the target if a polarity matches between the charged ions and the placed electrode (Figure 3). Therefore, the drugs must be charged molecules and placed under one or both electrodes. In order words, negatively charged ions must be placed under the negatively charged electrode. Electrorepulsive forces (or repelling force) will drive the drug molecules away from the electrodes and pass it into the skin via pathways of molecular transport. The drug permeation may enter through two main pathways;

Figure 3 Direct current passes in the same direction constantly (27).

1. Intercellular (paracellular) pathway between the corneocytes.

2. Transappendages (shunt) pathway through hair follicles, sweat ducts, secretory glands (Figure 2) (28, 29).

According to Barry (2002), there are three principle processes in provoking a transmission of drugs: (i) the driving electrode repels ions; (ii) the flow of the current which may decrease the resistance of the skin so as to increase is permeability; and (iii)

electroosmosis which may affect unchanged molecules and large polar peptides (30). In clinical setting of physical therapy, iontophoresis has been tested by different drug concentrations and a variety of electrical stimulation typesin order to combine its effects with other means of the treatment using the electric current and physical therapeutic methods to enhance a patient recovery (5). Following the clinical protocol, the repelling force has been set between the current and drug charges having similar polarity is driving force in the background of iontophoresis phenomenon. Penetration is the key factor which is crucial for iontophoresis treatment. In this way, the penetration rate of drug charges relatively through the impermeability of stratum corneum is greatly enhanced when compare to passive diffusion technique (2, 18, 28, 29, 31-36).

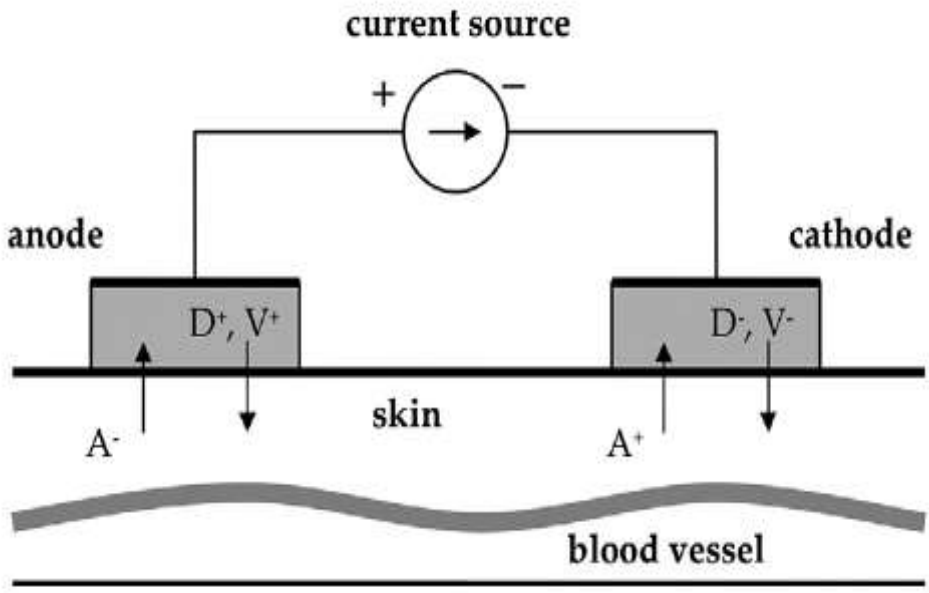

Figure 4 Schematic representation of transdermal iontophoresis D+ (positively) and D— (negatively) are charged drug ions. V+ (positively) and V— (negatively) are charged co-ions in the drug solution. A+ and A— are counter-ions traveling from the skin towards the electrode to maintain electroneutrality (37).

Moreover, it has been reported that the rate of penetration of the drugs is proportional to the amplitude of GC (38). Dosimeters is another factor that physical therapist

must concern. It associated with 4 parameters; (1) drug ions; (2) polarity; (3) the chemical concentration; and (4) the dose used (Figure 4). The dose (D) is referred to the amount of drug which is delivered into tissues. It is proportional to the current magnitude (A) and the total application time (T). Dose is calculated by the following equation.

$$D\ (mA.min) = A\ (mA) \times T\ (min)$$

However, there is limitation of applied current amplitude within the optimum dose range since the electrical stimulation can cause the skin irritation and burn under the electrode (15, 39, 40). The amplitude limit is based on the electrode current density (CD). The CD is calculated by the amount of current magnitude (A) divided by a total surface of treatment area (S) (41). Current density is calculated as equation.

$$CD\ (mA/cm^2) = A\ (mA) / S\ (cm^2)$$

The maximum of iontophoresis current (CD) which is considered as a safe therapeutic range has been suggested that the electrode polarity should be 0.5mA/cm² in using cathode and 1mA/cm² for anode (2, 41, 42). The dose of weak current at 0.06 mA/min iontophoresis has been used for non-steroidal anti-inflammatory drug. The results showed that the iontophoresis improve the drug penetrating into the hypodermis, dermis and epidermis, more deeply than the condition of passive diffusion (43). Transdermal iontophoresis therapeutic systems have been employed in pediatric, ranitidine (gastric acid reduction). It offered a significant enhancement of drug penetrating when being compared to the passive diffusion (18, 21). The number of evidences has been proved that the iontophoresis can enhance the therapeutic efficacy for the treatment of hypertrophic scar that iontophoresis can delivered medicaments across the barrier resistance in scar epidermis (35, 44).

The mechanism of the drug penetration by using the iontophoresis is caused by altering the stratum corneum (SC) structure. The construction of intercellular lipids becomes disorder and allows an increasing of the drug penetration into the skin when the iontophoresis is being employed. Furthermore, the capability of skin permeability is expanded by iontophoresis (43). In addition, the supplementary transport pathways in the bilayers lipid regions in the intercellular spaces might be developed by these Iontophoresis(44). The evidence employed iontophoresis (DC voltage 250 - 4000 mV), and low voltage < 750 mV) with the duration at 10 second, 1 minute, 10 minute and 60 minute. As a result, a complete recovery of skin was found at low voltages (<750 mV) within 60 minute while there was partial recovery (40%) within 480 minute after higher voltages (2000 mV), 60 minute (45).

1.2 Electroporation

In determining the transdermal delivery of drugs, an attention should be given to the recently employed technique which is known as *"Electroporation"*. The development of this challenging topic during recent years has captured much interest among researchers in the electrotherapeutic field. The electroporation has been firstly used for delivering DNA into culture cells or bacteria to manipulate the gene and protein expression which named as "DNA transfection" (46). The focus of those researches have been placed on how electroporation can be used as an *in vivo* model for studying physiologic mechanisms to overcome the skin barrier and/or provide a driven force on the drug penetrating into the cells and tissues. This method provides the effective transmission of drug delivery compared to other passive methods which are based on the formulation and chemical approaches (5, 47, 48).

The evidences of electroporation technique have generally used high voltage pulses up to 100 V(14, 15) for short duration (from 100 μs to less than 1s) (13) (Figure 5). When applying into the skin, the electrical pulse transiently created aqueous pores in phospholipid bilayers membranes in order to increase the permeability of drugs through the skin (2, 5,

47, 48). This phenomenon can drive the drug molecules via three main pathways; (1) Intercellular (paracellular) pathway between the corneocytes; (2) transappendages (shunt) pathway through hair follicles, sweat ducts, secretory glands; and (3)transcellular (intracellular) pathway directly through cells (Figure 3) (28, 29).The effect of high voltage was shown to develop the “large” regions of an altered SC. This is named as local transport regions (LTR) which was produced by “defects” on the stratum corneum that were enlarged by the resistive (Joule) heating (47, 49, 50). These pathways could enlarge the scale of defect up to hundreds of micrometer (47, 49, 50).

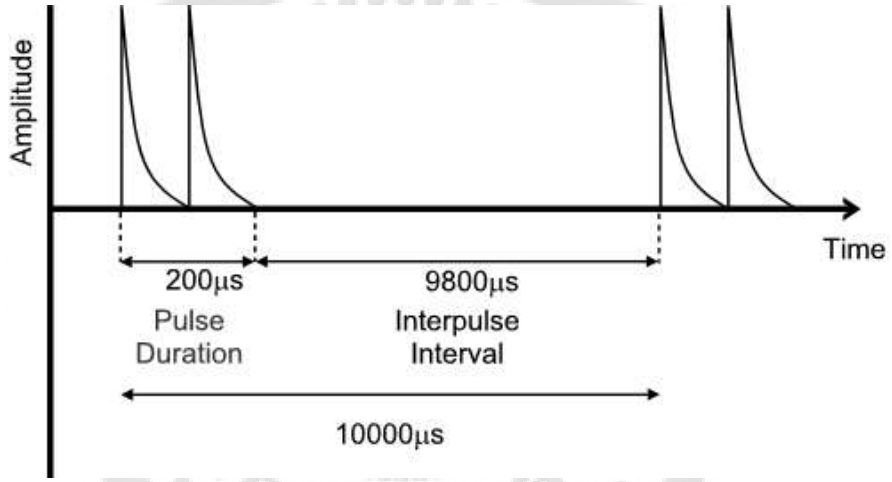

Figure 5 Parameters showed twin spike monophasic pulse waveform which are major characteristic of HVPC (51).

It is believed that the development of the LTR depends on Joule heating. Such the heating was tested under certain experimental pulse conditions. It is found that this type of heating could increase the temperature of some parts of the LTR to 60 ˚C (47, 49, 50).New aqueous pathways are produced by the stratum corneum via electroporation of its lipid bilayers. The changeable enlargement in transmembrane transport and the fundamental changes in membrane barriers that high voltage pulse current (electroporation) application associates with the production of “pores” or aqueous pathways (<10 mm , 0.1% of surface

area) (52). They are approximately 0.02% and 25% of the total current applied skin surface. The LTR are encompassed by localized dissipation regions (LDR). The LDR offers a low resistivity where transport of only small icons can take place at local transport regions in an human stratum corneum due to short and long high-voltage pulses (Figure 6) (52).

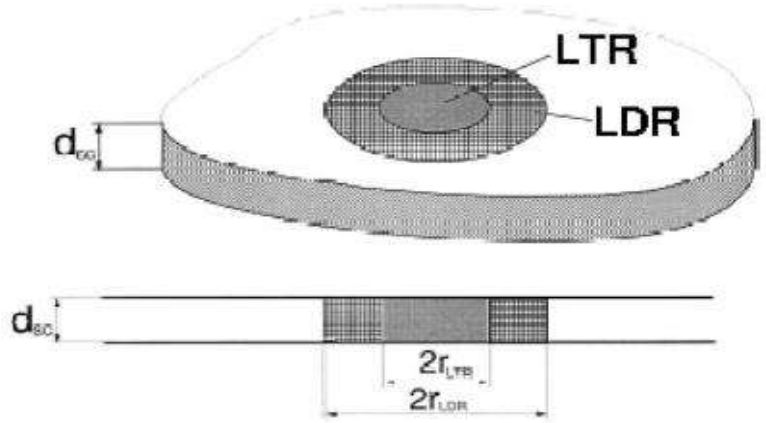

Figure 6 Structure of the local transport region (LTR) and surround LTR is local dissipation region (LDR) (52).

By using a high voltage pulse within a short duration times, it was found that the number of LTR has been increased. The obtained LTR number was between 2 and 10 per 0.1 cm^2 (49). The higher temperature could induce the sphingolipids of the stratum corneum, resulting in an increase of the permeability through the skin. It is also discovered that the LTR could be advanced by the Joule heating and energy (47, 50).

The transdermal transport routes employed during different electrical exposures are used by a fluorescence microscopy as if it is the process of appearance changes in the skin microstructure generated by high-voltage pulses showing that the skin electroporation is able to strongly promote the transdermal delivery of drugs (14, 52). The fluorescence micrograph of skin emerges in different high voltage and numbers of pulses in the presence of calcein transport through the cadaver's skin. This experiment use the electroporation of range 75-160 V, 0.1 ms. It was discovered that places where the brilliant staining located

are similar to places where drugs were delivered through the skin. The results found a large number LTR and sizes when increasing the voltage current and number of pulses (53). The areas of drug transport are different from iontophoresis. They did not involve hair follicles or sweat ducts during high-voltage exposures. Also, they were routes that moved directly through the bulk of stratum corneum. Moreover, they are totally different from the passive diffusion (53). Based on the characteristics of electroporation which increases fluxs through the skin reviewed by a classic single bilayer, the molecule is large and reversible. It changes the skin microstructure (54). The charged compounds are forced to move across the skin by electroporation that electroosmosis are not necessary in this process. Moreover, the use of high voltage electrical field pulses has been found as advancing a delivery in the skin of hydrophilic molecules, and also enhancing the delivery of neutral or highly charged compounds, and of macromolecules with a molecular weight up to 40 kDa (55). The creation of new transport pathways could describe the increases of a large flux generated by high-voltage pulses (54).

The results from several studies showed that electroporation was the issue that is free of harm. This was because it could give a very little damage to the skin (7, 16).The evidence of the electroporation in the transdermal delivery of fentanyl in vivo in the rat by using 15 pulse of 250V-200 ms showed a significant effect on the stronger fentanyl plasma concentrations than the 15 pulses of 100V-500 ms, and 500V-1.3 ms. As a result, a drug deliver could be affected by electric parameters which included a voltage, duration and number of pulses (56).It was also discovered that the use of medium voltage-medium duration pulses (400V-1, 10 and 20 ms) provided better results of the transmit ability of Timolol® (non-selective beta-adrenergic receptor antagonist indicated for treating hypertension, arrhythmias, angina pectoris and myocardial infarction) by having electroporation with square wave pulses across human skin than the use of low voltage-long duration pulses (250 V - 100 and 200 ms). In addition, the long pulse of medium voltage (400 V, 20 ms) had more capability of transporting compared 400 V, 1 ms and 400 V, 10 ms. This made Denet (2003) conclude that using of medium voltage-medium duration

pulses (400V) was more effective than using low voltage-long duration pulses (250V-100 and 200 ms) regarding its transport and energy required for achieving such therapeutic fluxes of Timolol®(13).

Chen (2006) and Weaver (2003) have explained the process of electroporation as follows (57, 58).

- The application of electrical pulse on a microsecond to millisecond time scale which are sufficient to produce a transient, elevated, trans-membrane potential of typically 200 mV – 1V
- Charging of the membrane due to an ion flow.
- Rapid, localized, rearrangement of the molecular structure of the membrane
- Formation of pores which perforates the membrane and are filled by water molecules (the so-called aqueous, or hydrophilic, pores)
- An increase, by several orders of magnitude, in ionic and molecular transport through the cell membrane.
- In some circumstances, and following removal of the external field, membrane recovery.

There is evidence showing the difference of high voltage current amplitudes (55V, 90V, 165V and 300V) in an electroporation. This study evaluated the calcein (623 DA, negative charge hydrophilic molecules) transmission through the skin before pulsing (1hr), during pulses (1hr) and after pulsing (18-24hr) with two conditions of electrical stimulation, forward pulsing (negative electrode on the stratum corneum) and reverse pulsing (positive electrode on stratum corneum). The experiment employed in epidermis of cadaver skin which the results was revealed the effect of electroporation could arise in the stratum corneum even if there were no living cells in this skin layer. There was powerful increase of the permeation flux correlating well with the increase of voltage current. The increase of transdermal fluxes was found under the condition of reversing the pulsing in which finally affected changes of skin deterioration at 165 V and 300 V over 18-24 hours. The skin electroporation may be completely reversible at below 100 V (4).

There are evidences focusing on an electronically failure transmission connected with a sharp increase of transdermal voltages at 30-100 V (100-1500 V applied voltages). Clearly, this range of voltage coincides the voltage used for electroporation in cell 0.2-1.0 V per bilayer (usually SC consisted with 100 bilayer membranes) (47, 48, 54, 57, 59). Moreover, the previous study in high voltage application showed that 1.0 kV/cm² to 10 kV/cm² could lead to a changeable electrical breakdown in an epidermis membrane. The phenomenon was similar to what has been occurred with cell membranes and lipid bilayer membranes (60, 61).

2. Structure of Skin

2.1 The human skin

The integument or skin is the biggest part of the human's body. It covers a surface area of 1.8 m² of the whole body. The integument is up to 16 % weight of the whole body weight (62). Human skin consists of three main layers: the epidermis, dermis, and hypodermis (63, 64). The stratum corneum (SC) is the uppermost of the five layers of the epidermis. Even if it thickness is around 15-20 μm (63, 64), the SC provide the majority of the barrier function of the skin. It is comprised of densely packed, dead corneocytes filled with keratin, surrounded by a lipid matrix. Intercellular lipids within this layer are organized into bilayer structures. This lipid matrix is primarily composed of ceramide (50%), cholesterol (25%) and other free fatty acids. A percentage of composition varies according to the depth of skin layers which is approximately 100 μm thick, and it is composed of keratinocytes below the SC (Figure 7) (63). Epidermis acts as a protective membrane preventing water loss from the skin and limiting the access of chemicals from the environment. There are basically four layers of the skin epidermis as showing below (Figure 8).

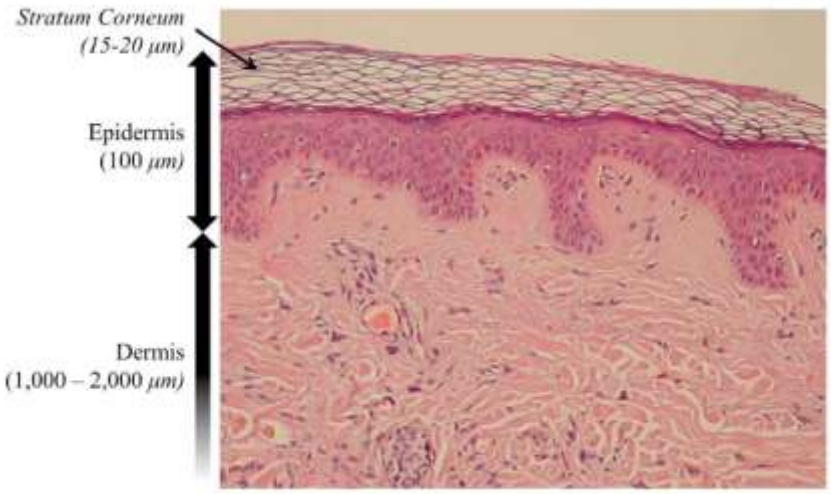

Figure 7 The composition of the skin in the outermost skin is the epidermis and deep skin is dermis (64).

2.1.1 Stratum corneum

The stratum corneum is the first top of skin epidermis. Generally, it is 10 – 12 μm thick and consisted of 15 – 25 layers of flattened, stacked, hexagonal, dead keratinocytes and cornfield cells lying in an intercellular matrix of lipids (22).This layer functions as the skin protection. Furthermore, Lippens (2009) noted that a friction would cast off keratinocytes in the stratum corneum. They would be then substituted by another cells produced by the inner layers of the epidermis. There are epidermis lipids (ceramides, fatty acids, and lipids) among the kerationcytes in the stratum corneum. These epidermis lipids are a cement (or mortar) between the skin cells (bricks)(Figure 9).The association between keratinocytes and interspersed epidermal lipids (brick and mortar) produced an obstacle that is watertight and resisted to moist (65). The stratum corneum is cells without their nuclei and other organelles. Proteins are cross-linked inside the cytoplasmic membrane. The dead cells associated with corneodesmosomes lipids are forced out to produce a water-repelling envelope. They assure an adequate impermeability of the mammalian epidermis (62).

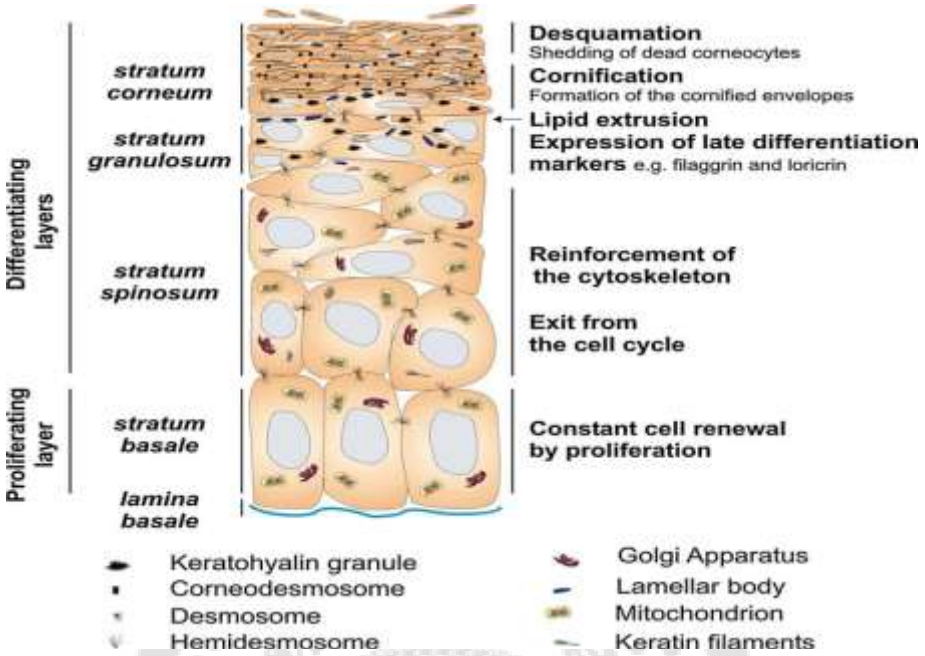

Figure 8 Basic four layers of the skin epidermis comprising with stratum corneum, statum granulosum, statum spinosum and stratum basale (66).

2.1.2 Stratum granulosum or granular layer

There are about 3-5 layers of flattened keratin and fibrous protein with no nuclei and their cytoplasm within a stratumgranulosum or granular layer. Cells within this layers are died because they are too far to absorb any nutrient from the diffusion (62, 66).

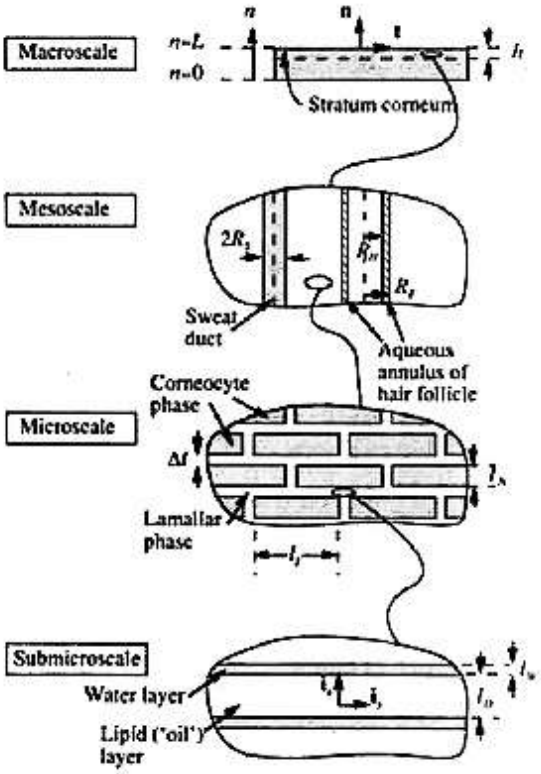

Figure 9 Brick and mortar. These epidermis lipids are a cement (or mortar) between the skin cells (bricks) (65).

2.1.3 Stratum spinosum or prickle-cell layer

The stratum spinosum consists of 8-10 layers of cells. These cells strengthen their cytoskeletal keratin filament network. Each cell interacts one another through desmosomes called ‘prickles’ at a microscopic level as well as connects the cells together (66). Langerhans cells, the cell that is originated by the bone marrow (62), are dendritic immunologically active cells. Basal cells are repeatable and grown-up cells. In other words, they could migrate around the outer layer of the skin and shape the stratum spinosum(62).

2.1.4 Stratum basale

Stratum basale is located at the top of the dermis (66). It is the deepest layer of the epidermis that consists of melanocytes, Langerhans cells, Merkel cells, and two major keratinic cell types. The first type of keratinic cells is stem cells. These stem cells are able to separate cells as well as to create new cells. The other type of keratinic cells connects the epidermis to the basement membrane (lamina basalis) by hemidesmosomes. This layer is 50–70 nm thick (22). Melanocytes are in the stratum basale. It produces melanin or skin pigment. Also, it delivers this melanin close to keratinocytes which finally moves to the surface of the skin (62).Melanin is the skin pigment that could prevent the skin from an ultraviolet radiation (sun exposure). Regularly exposing to the sunlight could multiply the ratio of melanocytes to keratinocytes. Rather than on the lower back and the outer arm, this phenomenon more occurred on facial skin. Even though the number of melanocytes in the equivalent body among white and black skin is similar, their ability to develop melanin is different in terms of their distribution and rate of melanin production. Intrinsic ageing diminishes the melanocyte population (67). The deepest layer of the skin is the dermis which is approximately 1,000-2,000 μm in thickness (63). It is made of connective tissues including collagen and elastic fibers, and it also contains of macrophages, fibroblasts and adipocytes (64, 68). There is a big vascular network inside the dermis. This network functions as developing nutrition for the skin. It restores and provides immune responses for the rest of the body, heat exchange, immune response, and thermal regulation (22). The dermis contains approximately 70% of collagen fibers. This made them strong and firm. Elastin stays at regular resilience whereas proteoglycans offers thickness and hydration (62). The third layer is hypodermis which includes the major composition of free fatty acids and minority collagen (64). The hypodermis is the fat and thin tissue that locates under the dermis. This tissue protects the body from a cold temperature. It also offers a shock absorption and energy storage region. The stratum corneum is thick only about 10-20 μm. It is too thin when comparing to the total thickness of skin which is 2-3 mm (22).The removal of the viable epidermis and SC results in an order-of magnitude increase in a delivery of substance over the SC alone. This is an important

discovery because the drugs must reach the capillaries found in the dermis for systemic delivery (64).

2.2 The porcine skin

The human and porcine skins posses the comparable permselective properties when a current is applied to allow the passage of the drug transport into the skin. The net charge on the skin is zero when the degree of permselective equals, and corresponds to the isoelectric point (pI) of the membrane (~4.4 for pig skin and ~ 4.8 for human skin) (69).The consistent isoelectric point and similar pH-dependent permselectivities which can be observed in human and porcine skins demonstrated that porcine skin is the appropriate model for iontophoresis and electrophoration studies. The porcine skin is commonly accepted as a decent resemblance of the human counterpart (70, 71) because it has a similar composition to human skin structure (69, 71) especially the structure of epidermal thickness and lipid composition (70).

3. Cosmetic purpose of using vitamin C in melasma

According to the market volume of cosmetics industry amounting to multi-billion dollars. The worldwide cosmetic industries continuously develop their skin care services and products. Referring to the physical therapy with skincare science, an application of transdermal therapeutic system in beauty and esthetic field of skin cosmetics has been the major topic of studies in the modern era (35, 44, 72). Skin care products are behind the general category of cosmetics. These are care substances which use to enhance the appearance of human skin and improve the health of skin such as the use of acetic acid(35, 73), and vitamin C in skin cosmetics (10). The absorption of acids through the skin is accelerated when being compared to an oral delivery of vitamin C which has a half-life after ingesting about 12-20 days (74). Vitamin C has become very popular as one of the efficient non-operative cure in melasma(10). Melasma is the example of hyperpigmentation. This condition is characterized by tan or brown patches most commonly found on the face. Generally, melasma is classified into 3 histological types; epidermal, dermal, and

mixed, as they were identified by the depth of melanin pigmentation (75). A clinical examination of melasma have 3 patterns, these are centrofacial, malar, and mandibular patterns. The centrofacial is the most common pattern of melasma with brown patches appearing on the cheeks, forehead, upper lip, nose, and chin. Second, the malar pattern is lesions which are merely found on cheeks and nose. Finally, the mandibular pattern is lesions usually found the ramus of the mandible. In some case, these three patterns of melisma may appear together on the same part of the skin care if that area exposes with a lot of sunlight for instances forearms an neck (75). Sun exposure triggers melasma and the condition usually develops slowly and symmetrically. It can last for many years. It becomes worse in the summer and reimprovement its self during the winter (75).The observant results from pregnancy or changes in uterine and ovarian hormones revealed melasma. It is sometimes called “chloasma” or the mask of pregnancy when it occurs in pregnant women. It is considered to be the stimulation of cells in the layers of skin which produces a pigment called melanin by the estrogen and progesterone in order to generate more melanin pigments when the skin is exposed to the sunlight(75). The most common contributing factors include genetic predisposition, pregnancy, use of oral contraceptives, endocrine dysfunction, or hormonal treatments. External factors can also be the causes increase melanin such as lifestyle (outdoor sports, smoking), and environmental stresses such as UV radiation, and chemical pollutants (75, 76).Melanin is produced in melanocytes or cells in layers of the skin and stored in melanosomes within the keratinocytes. The color of a person’s skin is determined by the number, melanin content, and location of these melanized cells along with oxygenated and deoxygenated hemoglobin. Melanosomes contain tyrosinase which is a copper-containing enzyme that catalyzes the conversion of L-tyrosinase to L-dopa and L-quinone in melanin synthesis. (9, 10, 77, 78). Causes of melasma remain unclear. It probably occurs when the color-making cells in the skin (melanocytes) produce the excessive pigment.In other words, it may cause from dysfunction of pigmentary system resulting in brown or grayish-brown facial hypermelanosis (9, 10, 77, 78). Vitamin C (ascorbic acid) is known to effect both on inhibiting melanin formation by reducing DOPA-quinone formation and synthesized is for reducing oxidized melanin (9-11,

77, 78). Vitamin C has been shown to be effective as a skin whitening agent in cosmetics (77). The only obstacle in using vitamin C is its molecule that quickly oxidizes and decomposes in aqueous solutions(9). Researchers have an attempt to make the vitamin C stable, effective and easy to be controlled (76). Vitamin C derivatives have been tested by adding minerals or metabolites into its the formulation that is Magnesium – L – Ascorbyl – 2 – Phosphate, (MAP) (9, 10, 76). MAP is stable in an aqueous solution especially in a neutral or alkaline solution containing the boric acid or its salt (9, 10). A report revealed that MAP was found to be safe for cosmetic usage. Magnesium ascorbyl phosphate functioned as an antioxidant in cosmetics, and it has been used in 37 formulations over a wider concentration range (0.001% to 3%) (79). The experiments demonstrated that the MAP cream (10%) used for percutaneous absorption in the dermatome human skin, yielded effective results in reducing skin hyperpigmentation within 3 months (9). However, MAP did not easily penetrate into the skin. Despite the benefits of vitamin C, there are some complicate factors in usage such as it can be severely depleted after UV irradiation, and only a very small portion of ingested vitamin C can give an effect into the skin. Continuous large oral doses of vitamin C can cause gastrointestinal discomfort and diarrhea (76). Moreover, the molecular weight of vitamin C leaded to the poor penetrability into the highly resistant barrier of the skin (10).

4. Evidence of using electrical stimulation in enhancing vitamin C permeation.

There is an interesting research finding which compared the use of iontophoresis to enhance penetration of nanosome vitamin C and glycolic acid in melasma conditions. The results indicated that pigmented area was improved, and the nanosome vitamin C offered a better result than 70% glycolic acid. This investigation indicated that the effect of vitamin C can achieve to melasma treatment. It was also the safe, easy and painless application (53). However, vitamin C was not a stable compound resulting in a low absorption when being compared with the magnesium ascorbyl phosphate (MAP). The MAP synthesized vitamin C derivative which aimed to improving its stability and produce more effective in treatment

(Zhang, 1999). MAP exhibited significantly lower cytotoxicity to fibroblast in all age groups of subject (Zhang, 1999). Iontophoresis has been used to enhance MAP penetration through the skin, and it revealed significant results when being compared to control (Huh, 2003).

However, the determination of an active MAP concentration and the improvement of delivering of this large and weight molecule are still required. There is an interesting evidence of using electroporation-mediated topical delivery (EMTD) at 60 or 100 V, pulse lengths of 2.7 - 30 ms in the application of vitamin C in L-ascorbic acid (Asc) form. This study showed that cream form offered a better result than a suspension form (51). However, this study was conducted to examine the electrical stimulation by using the DNA electroporation stimulation in an uncontrolled environmental condition. Moreover, using a high oxidized form of vitamin C was rarely found in clinical experiments. Therefore, to support the use of this therapeutic electrical stimulator with less oxidized form of Vitamin C (MAP) in the near future, more evidences related to this area need to be more conducted. The summary of previous studies concerning on the applying of vitamin C through the skin by the electroporation and ionphoresis is presented in table 1.

5. Factors Affecting Transdermal Delivery of Drugs

The delivery of a drug through the skin using the techniques of iontophoresis and electroporation is influenced by several factors. It can be broadly classified into 3 categories; biological factors, operational factors (drug delivery system factors), and environmental factors.

5.1 The biological factors

The biological factors directly involve with the skin. Apparently, human skin is not all the same. There are numerous differences among patient groups as well as between various regions of the body. A variation in the drug delivery is also related to skin ages, skin conditions and differences between species (5, 26). The conditions of the skin can affect the penetrating properties of drugs. The previous studies *in vivo* passive diffusion of methyl

Table 1 The evidences of transdermal vitamin C delivery by electroporation and iontophoresis.

Study	Methods	Results
Sobhi et al., 2011 (iontophoresis + ascorbic acid nanosome compared glycolic acid 70%)	- 14 patients of melasma - Applying glyocolic acid 70% on the right side(1-3 min) - Applying nanosome vitamin C by iontophoresis on the left side (iontophoresis ; 5mA x 10 min, Ascorbic acid nanosomes ; size 117.6 nm (±11.9nm)) - Measuring by the melasma area and severity index score after 6 sessions	The side treated with more iontophoresis with nanosome vitamin C than glycolic acid 70%.
Huh et al., 2002 (iontophoresis + MAP)	- Iontophoresis ; 0.5 and 0.3 mA, time 6-8 min, twice a week, for 12 weeks, MAP 3.75% - Measuring by the luminance (pigmentation parameter)	Iontophoresis of the treated site showed a significant compared to control.
Zhang et al., 1999 (Electroporation + Asc)	- Cadaver skin 100 V, 20 ms, pulses, 15 s time interval for cream - Fresh skin (neck skin) 60 V, 30 ms, 6 pulses for cream and 60 V, 2.7 ms, 6 pulses for suspension (25 s time interval) - 20% Asc (cream), 33% Asc (crystal suspension) -Measuring by HPLC	Electroporation with cream resulted in a greater enhancement of vitamin C penetration than with Suspension

salicylate using skin from different areas of human body showed the permeability range as abdomen, forearm, instep, heel, plantar (5, 80, 81). Both *in vivo* and *in vitro* experiments have carried out to study differences among flux rate following transdermal iontophoresis which were applied to male and female patients. The results obtained from experiments revealed the similar fashion when being compared between hairy and hairless skin conditions (82). Number of evidence in passive diffusion study showed that passive diffusion of hydrocortisone occurred maximally from the area with numerous hair follicle while it was less than in an area with thickest stratum corneum(81, 83). Species variation means the wide differences in physical characteristic such as appendages per unit area. The thickness of skin and structural changes between human and laboratory rodent display a variation in penetration of drugs. The previous studies investigating this factor showed the average penetration of drugs ranging from rabbit, rat, guineas, pig and human respectively (24).

5.2 The operational factors

The second factor affecting transdermal drug delivery by iontophoresis and electroporation is an operational factor (drug delivery system factors). In the operation process, there are various physicochemical properties such as molecular size, molecular weight, charge, polarity, lipophilicity and solubility of drugs (5, 24, 49, 81). The various compositions of formular for instance drug'sconcentration, drug's pH, ionic strength and viscosity and other experimental conditions such as current intensity, current profile, duration of treatment, electrode material and parameter settings.

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5.2.1 Physicochemical properties

Importantly, the molecular size and molecular weight must also need to be considered. The molecular size of drug is the major factor governing its feasibility for transdermal delivery and hence the amount transported. Smaller and more hydrophilic ions are transported at a faster rate than the larger ions (81, 82). There was one investigating study has investigated the correlation between flux and molecular weight of drugs. The results revealed that the transport of compounds decreased with the molecular weight

increased (81). Charges are involved when considering at the pH of the drugs and the pH of the delivered solution. There are essential parameters influencing the electric charges of the molecule to deliver the drug. However, there are not simply determinations because an increasing charge is required pH to be decreased. As a result, it directly decreased the electroosmosis and electrotransport process (81). The increasing charge of drugs consequence the permanent, enhanced its transport, and influenced the efficiency of the drug delivery (24, 26, 49, 81). At the normal physiological conditions, the skin was negatively charged, which finally presented a better permselectivity to the cations (49). This suggested that divalent ions may had a better interaction with charged sites in the skin than monovalent ions resulting in a slower migration (24). Effective delivery of drugs through the skin relies on a number of factors relating to the components of transdermal delivery techniques set-up and employed in particular cases. It needs to be taken into account in the aspect of polarity and lipophilicity of the compounds which traverse through the membrane of skin. Generally, the hydrophilic compounds are considered as an ideal for the optimum flux (82). Polarity causes an increase of flux, but as the lipophilicity of the compound decreases.

Next factors to be take into consideration are the various compositions of formula such as a drug's concentration, drug's pH, ionic' strength and viscosity. The concentration of drugs is one of the most important factors affecting its transdermal delivery. The effects of drugs concentration have been therefore studied in a number of drugs. The increasing of drug concentration directly relates to influx of drugs (81). Therefore, the increasing concentrations of the drug will result in greater drug delivery. The higher concentration, it is possible that transport becomes independently due to saturation of the boundary layer referring to the donor solution. However, if the concentration of ions in a solution is too great, it causes a bottleneck effect as a large number of ions attempts to pass through the available pores. Drug pH influences the charge on protein. This means that the polarity of electrodes is the important factor to be taken into account during drug delivery. The pH condition can exert its effects to transdermal delivery of drugs in two ways as (1) the pH of the donor solution influences and (2) pH of the skin. The donor solution pH affects the

ionization of the drug itself. Generally, basic weak drugs are ionized at the higher pH than pKa, and they could not be permeated by electromigration in the presence of iontophoresis. The drugs are then more dependent from electroosmosis. It could travel across the skin (1, 3). Controlling of pH offers the advantage of polarization effects on skin and enhances the permselectivity of skin during iontophoretic delivery (1, 3). It appears that pH is the important factor governing the iontophoretic delivery of drugs. Moreover, it also influences the chemical stability of the drugs. Ionic strength serves the main aim that the drug ion should carry a maximum charge across the membrane. If buffer ions are included in the solution of drugs, they compete with the drug to delivery across the membrane and resulting in decrease the quantity of drugs deliver. Especially since buffer ions are generally smaller and more mobile than the larger active drugs. Thus, an increase in ionic strength could decrease the drug delivery as the consequence (24, 81, 82). The viscosity relates to the migration of drugs in an inverse fashion. The more viscosity of drug solution the lesser drug can transport across membrane.

5.2.2 Experimental conditions

There are various operational factors involving in a parameter setting in the transdermal delivery of drugs for instances experimental conditions like a current intensity, current profile, duration of treatment, electrode material and waveform. The Iontophoresis, most of the current profile which has been performed in the animal studies *(in-vitro)* is kept constantly and applied at a very low voltage of less than 100 V is applied. The technique of iontophoresis employs continuous current or direct current is applied in a continuous manner to the stratum corneum to facilitate the transport of charge molecules. Iontophoresis causes an electro-chemical polarization in the skin (81). This causes a reduction of transport of drug molecules and efficiency of iontophoretic drug delivery system (49). Dose of current intensity used in iontophoresis ranges between 10 mA to 40 mA. The current ranging from 5 to 10 mA has been found to cause painless to patients (24). In general, 0.5 mA/cm^2 is often stated to be the maximum iontophoretic current which is suitable for use (81). The suitable duration of treatment and pulses duration cause the significant increase

of in the transdermal drug transport and transdermal flux. The increased strength of current dose also depends on the sensitivity of patients. It is designed to increase power slowly and to remain at a predetermined level as long as the treatment required (56). Care must be taken for long period of time while this could possibly cause an irritation and damage of skin (53). Electrode material mostly affects the localized spots within the superficial layer of the skin. The electrode is subjected to change in pH which are generally seen during the use of platinum, Zinc chloride or silver/silver chloride electrodes. The efficacy of drug transport and tolerance varies depended on electrode material, because the distribution and intensity of the electrical field in the skin are variably affected (3).

The technique of electroporation, electrical pulses, is characterized by their parameters for instance patterns of waveform (exponential decay or square wave), voltage (50-1500 V), duration (μs to ms) and interval between pulses (sec to min) (49). While using continuous and pulse current in electroporation, two kinds of pulse generators are usually employed to transport molecules by electroporation, such as a exponential decay, or square wave pulses. Both of these techniques are used for different applications. Advantages of exponentially decaying pulses are maintenance or expanding of the high permeability state of the skin and the electroporation system (electrodes, conducting medium). In contrast, voltage and duration of square wave pulses remain constant whatever the skin or drug reservoir. Recently, the square wave pulses have been used for electrochemotherapy and DNA electrotransfer, whereas exponentially decaying pulses have been restricted to the transdermal drug delivery to take advantage of the long voltage tail (49). The electroporation technique is high voltage pulses > 100 V short duration (1-2 ms) (12). The effects of pulse current have been verified and shown to have beneficial to the continuous used of the direct current (DC) which is proportional to time, and reducible the iontophoretic flux because of its polarizing effects on the skin (3). Various factors affected the results of electroporation. The following factors have to be considered (Table 2) because they may improve the delivery of the device and drug released kinetics.

Table 2 Parameters affecting drug transport by skin electroporation (49, 84).

Parameters	Increase in	Effect
Electrical Parameters	Pulses voltage	+
	Pulse number	+
	Pulse length	+
Physicochemical Properties of drugs	Charge	+
	Molecular weight	-
	Conformation	?
	Lipophilicity	-
Formulation of drug reservoir	Competitive ions	-
	Ionization (pH)	+
	Viscosity	-

+ = positive effect; - = negative effect, ? = unclear

5.2.3 Environmental factors

Finally, the environmental factors influencing the transdermal efficiency are affected by the skin hydration and effects of temperature. The effects of skin hydration can expand and become soften the stratum corneum of the skin resulting in a rapid increase of drug permeation across of the skin layers. Normally, there is 5-15% of water contained in the skin layers. The water covered the area of skin with thin plastic film about 4-5 fold increase in transportation of drugs (26). Other means to increase skin hydration is applying ointment substances that it will form a thin film covering the area of skin to create the same effect. These are lipid compounds such as lanolin, petrolatum, fat, oil, and isopropyl myristate (26). Temperature can also influence the transport of drugs through the skin as it increases the dynamic energy to faster move molecules of drugs and affects the physiologic condition of the skin. Normally, the temperature of the stratum corneum layer is more or less equivalent to the body temperature. The skin layer temperature can be decreased down to 20°C or increase to as high as 42 °C, at every 10 °C of change will cause skin permeability to change approximately 2 folds. Moreover, a higher temperature can increase a blood circulation. Such the environment should take the drugs away from the specific

area. However the effects of temperature are less when they were compared to the effects of skin hydration (26).

Summary of the factors affecting electrically assisted drug delivery of iontophoresis and electroporation are summarized in Table 3.

Table 3 Factors affecting electrically assisted drug delivery (36).

Factor	Iontophoresis	Electroporation
Electric input	Constant current (low voltage); Current density (<0.5 mA/cm²)	High voltage pulses current (HVPC); Pulse voltage (≥100V); pulse duration (ms-μs); number of pulses given (one-many); spacing between pulses given (all at once or in curtain interval).
Electrode material	The electrode must avoid electrolysis of water (e.g. Silver-silver chloride).	The electrode must avoid electrolysis of water and must withstand high instantaneous current
Physicochemical properties of drugs	Charge of drugs (high charge density better); sizes of drugs (smaller ions are better); structure of drugs (compact structure); lipophilicity (drugs must be watered-soluble); molecular weight of the drugs (maximum about 12,000).	Charge of drugs (not as critical as in iontophoresis); molecular weight of drugs (upper limit not known).
Formula factors	Concentration of drugs in solution pH (adjust to keep drug charged); ionic strength (just adequate buffer capacity).	Concentration of drugs
Driving mechanism	Electric forced on the drugs. No new pathway (transappendageal route).	Electric forced on the drugs and the permeabilization of skin. New created pathways
Reversibility of skin	Reversibility at the low current density.	Reversibility at the low pulse voltage
Electroosmosis	Significant electroosmotic flow accompanies with iontophoresis	Non-significant

6. Technique of drug permeation study (Franz diffusion cell)

Drug permeation study can be done both in vivo and in vitro. In vivo method, it is performed in animal or human. Drugs are administered into the body, and their pharmacological effects such as blood pressure, heart rate, and urine sugar are measured. In vitro method, it is applicable by using Franz diffusion cell which is equipment that can simulate the permeation of drugs through the skin. Franz diffusion cell (Figure 10) is widely employed in pharmaceutical studies to investigate the permeation of drug (85). Franz diffusion (side-by-side) composes of two identical clean glasses which place side-by-side and sandwich between by membranes. The drug solution and phosphate buffer saline (PBS, pH 7.4) are placed into donor and receptor chambers respectively, at 37°C and stir throughout the study (14, 85). The solution from the receptor chamber is taken out at predetermined time in order to measure the amount of drug (63).

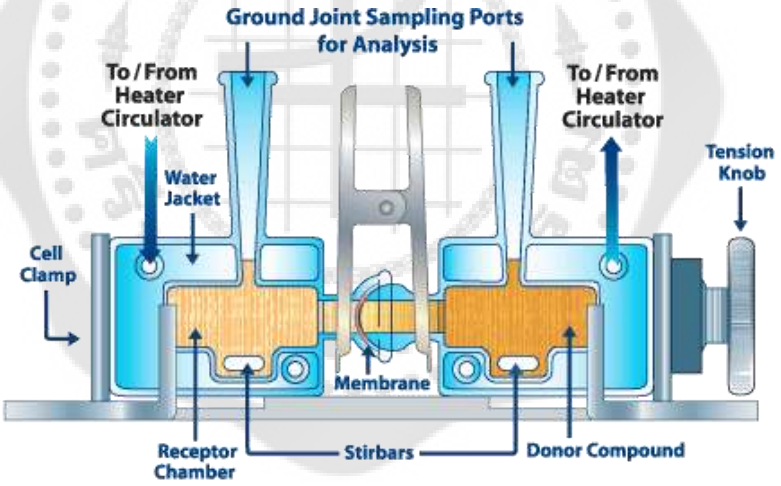

Figure 10 Franz diffusion cell system (86).

7. High-performance liquid chromatography (HPLC)

High-Performance Liquid Chromatography (HPLC) is an analytical technique used to separate, identify and quality each chemical in the mixture (21, 68). HPLC composes of three main parts; HPLC column; pump; and UV detector. The sample mixture and pressurized liquid solvent (mobile phase) a solid adsorbent material (stationary phase) by pumping which leads to

the separation of each chemical as they flow out the column. Each chemical has a specific retention time (RT) which relates to its polarity to the stationary phase and mobile phase. The chemicals which have the similar polarity to the mobile phase will rapidly elute out off the column (87-89). The chemicals which have the similar polarity to the stationary phase will slowly elute out off the column. The UV detector measures the absorbance of each chemical and present as the area under a peak of a chromatogram which in turn can calculate to the amount of each chemical (Figure 11) (87-89).

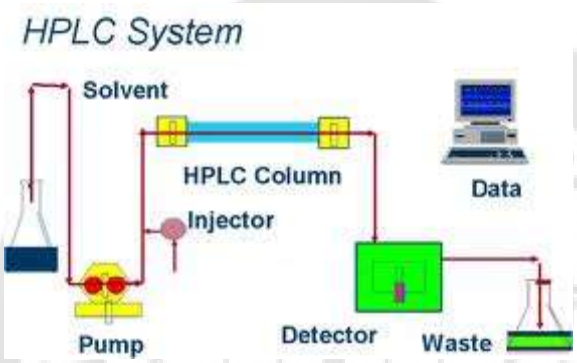

Figure 11 High Performance Liquid Chromatography System (90).

CHAPTER 3

METHODOLOGY

1. Drug

Magnesium – L – ascorby1 – 2 – phosphate (MAP; $C_6H_6O_9P.3/2Mg.5H_2O$) was used as drug model in this study because it has stability in neutral and alkaline solution (10, 79). Magnesium – L – ascorby1 – 2 – phosphate purchased earlier had high molecular weight around 438.98 Da from Nikkol chemical, CO., LTD (Tokyo, Japan).The standard solution of MAP was prepared by dissolving MAP in a phosphate buffer solution (pH 7.4) in order to produce the standard concentration at 0.1 - 100 µg/ml. Sample solution of MAP was prepared at the 3% W/V of concentration by dissolving MAP in phosphate buffer solution (pH 7.4). According to previous experiment was used MAP concentration ranging from 0.1 – 3.75 % w/v (10, 79). The 3 % MAP concentration was chosen in this study caused from the distinctive detection of permeated MAP across the skin by HPLC.

2. Skin model preparation

The porcine abdominal skins were used in this study. The specimens used the aborted pig fetus obtained from local farms, washed them with distilled water, soaked in phosphate buffer solution (pH 7.4), and exfoliated connective tissue until there was 300 - 400 micron in its thickness left. The porcine abdominal skin was cut into a small piece of 3x3 cm^2 using a surgical blade, and kept at - 20°C until being used. The porcine abdominal skin samples were thawed by soaking in the phosphate buffer solution (pH 7.4) for 30 minutes before the experiment (18).

3. Permeation study using Franz diffusion cell

In order to compare effects of electroporation and iontophoresis on the permeation of MAP in porcine skins, Franz diffusion cell was used to carry out the experiment. Franz diffusion

cell is equipment that can simulate the permeation of drug through various skin models including porcine skins, shred snake skins and other artificial membranes (60). The side-by-side type of Franz diffusion cell was used among used with active transport area at 1.77 cm^2 showing in Figure 12 (Permagear Inc., Hellertown, PA (USA)) (85).

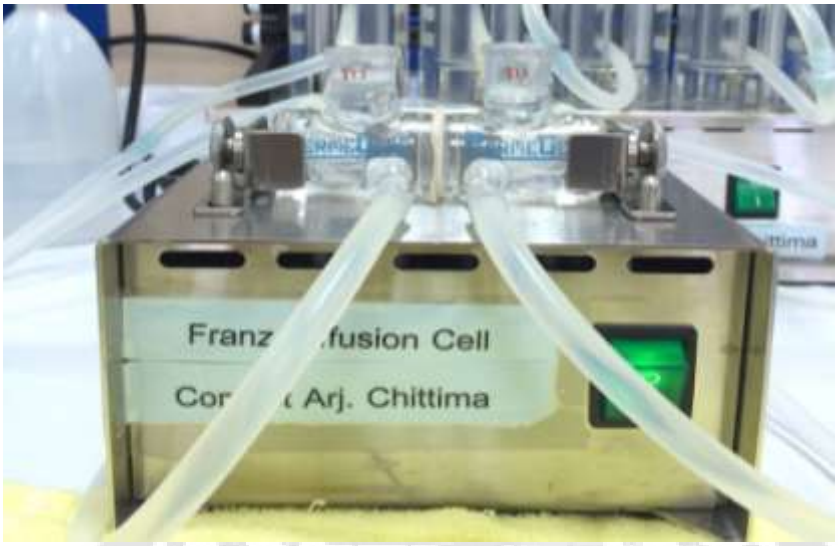

Figure 12 Experimental setting chambers of the Franz diffusion cell system.

Franz diffusion cell composes of two glass chambers (capable for 9 mL each); donor chamber and receptor chamber which place side by side, and sandwiched between membranes. MAP was dissolved in PBS and added into the donor chamber (9 ml). PBS at some volumes was added into the receptor chamber. The temperature was controlled at 37°C throughout the experiments. The donor and receptor glass chambers were covered with a piece of foil to protect the MAP from a light along the study. The solution from the receptor chamber was taken out around 0.5 ml at 5, 10 and 20 minutes for the measurement of MAP by HPLC, and the fresh of PBS (pH 7.4) was placed back into the receptor chamber each time (43, 91). The MAP of each sample was calculated to discover its flux by the high performance liquid chromatography (HPLC).

4. Electrical modalities for permeation study

The HVPC parameter configuration was set at 100 V, 200 V and 400 V, and stimulus frequency was 200 Hz and pulse duration was used as determine by stimulator. The intensity of galvanic current was determined by an equal I_{rms} (root mean square of current) to HVPC at each specific intensity to control an effects from electrical pulse charge. The Agilent 3458A multimeter (Agilent Technologies, USA) was used to analyze a comparable average current from HVPC in apparently experimental condition setting with system solution and skin. An intensity setting for galvanic current was then determined at 4.8 mA, 8.6 mA and 15.8 mA in according to HVPC at 100 V, 200 V and 400 V, respectively. An overall of experimental conditions in this study were presented as follow.

4.1 Passive diffusion study (control); no electrical stimuli, time 5, 10 and 20 minutes (N = 6) in time duration.

4.2 Iontophoresis (Galvanic) set-up; iontophoresis was set to the current intensity at 4.8, 8.6 and 15.8 mA with 5, 10 and 20 minutes (N = 6) in time duration.

4.3 Electroporation (HVPC) set-up; electroporation was set to the current intensity at 100, 200 and 400 V, frequency 200 Hz and pulse duration 200 μs with 5, 10 and 20 minutes (N = 6) in time duration.

4.4 The electrical transmission (iontophoresis and electroporation) was employed during the experiments. The results of each experiment were measured by the concentration of MAP, before pulsing at 10 minutes (passive diffusion), during pulsing at 5 and 10 minutes, after pulsing at 1 hour (passive diffusion) and during pulsing at 5 and 10 minutes respectively (Figure13) (N = 3).

Electroporation was carried out among different short and high-voltage square wave pulses, in the range between 100 V, 200 V and 400 V over 5, 10 and 20 minutes in time. The current study employed electrical stimulation using Endomed 482 (ENRAF-NONIUS, Rotterdam, Netherlands) with silver chloride electrode (AgCl) as the stimulation probe.

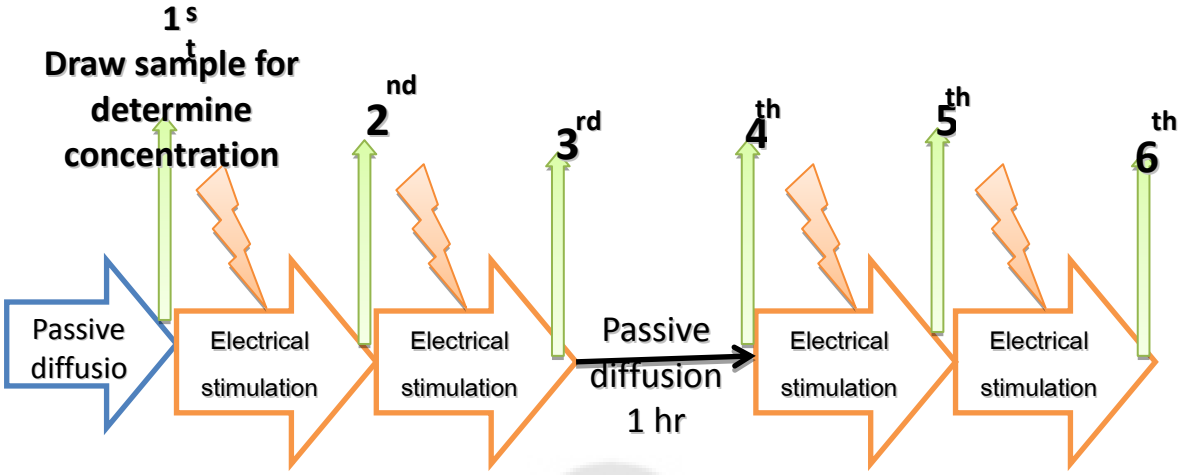

Figure 13 Experimental time plan for evaluation of drug permeation.

5. Evaluation of skin model resistance and its solution (skin model and solution system)

The resistance among skin models during the experiment was determined by electrical multimeter (agilent 3458A multimeter) to verify the resistance solution system and skin model which are involved in electrical application both iontophoresis and electroporation. The data obtained set the optimal averaged intensity current root mean square (Irms) among the GC and HVPC to generate the iontophoresis and electroporation respectively.

6. Drug quantification by HPLC

The concentration of MAP was measured by using high performance liquid chromatography (HPLC) (Agilent Technologies, USA). HPLC column was luna C18 (5µm, 250 mm x 4.6 mm) (Phenomenex Inc, USA) and the security guard cartridge was C18 (4mm x 3mm) (P/N AJ0-4287) (Phenomenex Inc, USA). The mobile phase was acetonitrile - 0.3 M phosphate buffer (pH 4) (volume ratio 40:60 v/v). The flow rate was 1 ml/min (8, 91) and the injection volume was 100 µl. The absorbance of MAP was measured at wavelength at 255 nm (8). Chromatography was performed at room temperature and the retention time of magnesium ascorbyl phosphate at 4.391 minutes (8, 91).

7. Data analysis

K-Independent samples and related samples were used for non-distribution data. In order to compare the mean effect of the different stimulation parameters and times, a difference with $p<0.05$ was considered to be statistically significant. All statistical data were analyzed by SPSS version 11.0.

8. Framework of this study

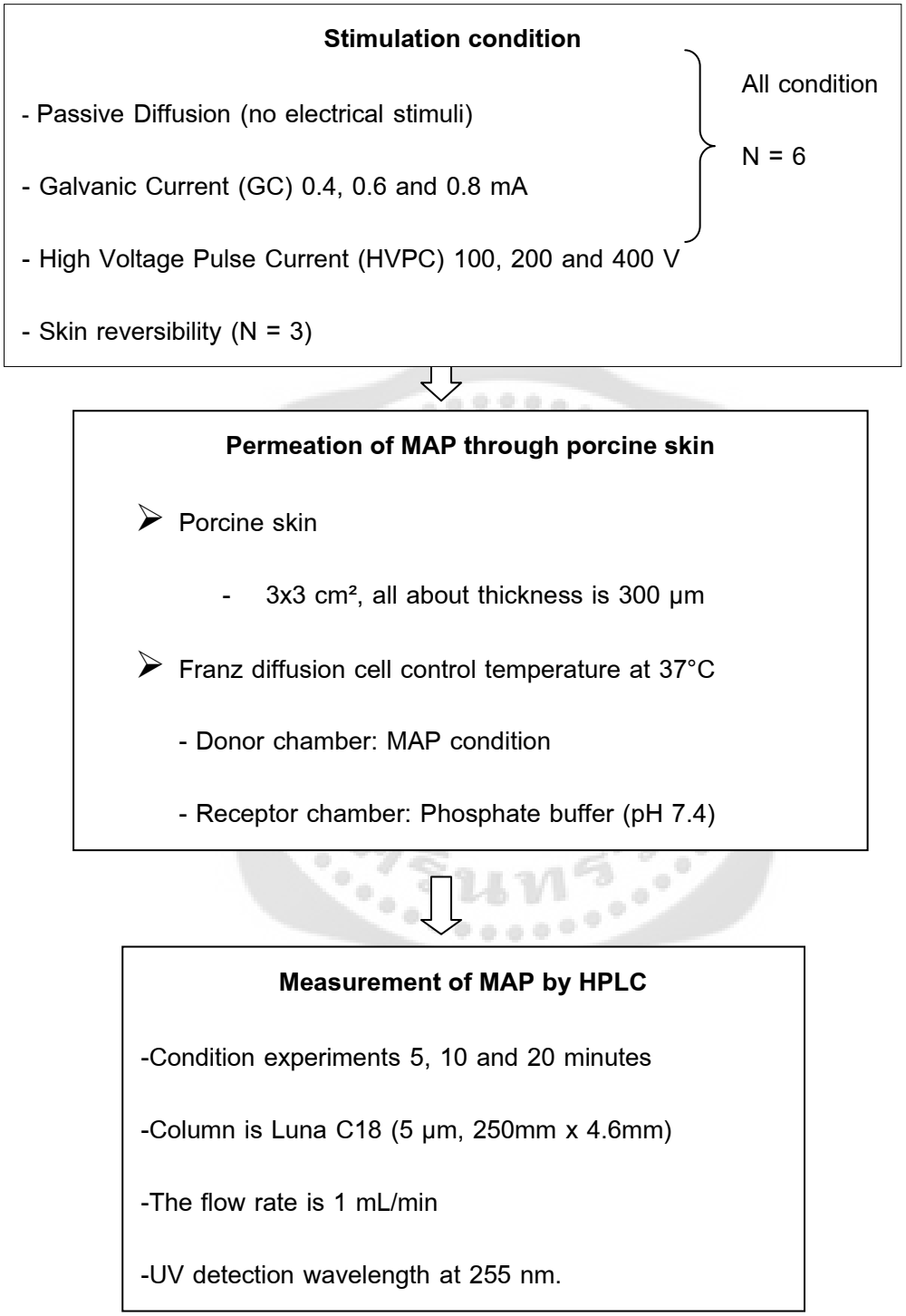

Figure 14 Framework of this study

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CHAPTER 4

RESULTS

This study aimed to investigate the effects of high voltage pulsed current on vitamin C permeation through the porcine skin model. The results of this study are presented as follows. The porcine abdominal skin was obtained from aborted porcine fetuses. The hair, fascia and subcutaneous fat approximately thickness at 300 μm and 3x3 cm^2 area were then carefully removed from the skin. Porcine skin samples from 6 females were randomly assigned to each condition, which were HVPC 100 V, HVPC 200 V, HVPC 400 V, GC 4.8 mA, GC 8.6 mA, GC 15.8 mA and passive diffusion control. Franz diffusion cell system was used to evaluate drug permeation through the skin samples. Finally, the penetrated MAP concentration was determined by high performance liquid chromatography (HPLC).

1. The Characteristics of Porcine Skin

Table 4 shows the characteristics and thickness of the skin samples which were collected from 6 female pigs. These skin samples were randomly separated into 7 conditions. The thickness of each skin sample was measured by a digital venier caliper (MitutoyoCo.Ltd, Kawasaki, Japan). It was summarized in the Appendix A.

Table 4 Thickness of the skin.

Condition	Mean ± SD (mm)
Passive diffusion	0.311±0.021
GC 4.8 mA	0.306±0.023
GC 8.6 mA	0.300±0.019
GC 15.8 mA	0.312±0.015
HVPC 100 V	0.307±0.020
HVPC 200 V	0.312±0.020
HVPC 400 V	0.305±0.019

Data of each condition are reported by mean ± standard deviation (SD). There was no difference among each experimental condition ($p = 0.971$). *p – *value* of one-way ANOVA analysis (*P-value* statistically significant at $p<0.05$) (N = 6)

Table 5 shown characteristics and thickness of skin samples which were collected from 3 female pigs and were randomly separated into mixed conditions. The thickness of each skin sample was measured by digital venier caliper (MitutoyoCo.Ltd, Kawasaki, Japan). The data were summarized in Appendix A.

Table 5 Thickness in skin reversibility study

Mix condition	Mean ± SD
Passive	0.32±0.026
GC 4.8 mA	0.32±0.015
GC 8.6 mA	0.32±0.010
GC 15.8 mA	0.32±0.015
HVPC 100 V	0.32±0.035
HVPC 200 V	0.32±0.032
HVPC 400 V	0.32±0.035

Data of each condition are reported by mean ± standard deviation (SD). There was no difference among each experimental condition (p = 0.971). **p – value* of one-way ANOVA analysis (*P-value* statistically significant at $p<0.05$) (N = 3)

2. The stability of drug

The stabilities of 3% w/v MAP in standard solution phosphate buffer saline (pH 7.4) were stored at room temperature and4 °C in the dark for 1,2,3,4,5,6, 12 and 24 hours. The results showed a minimal decline of MAP concentration to (%) after storage in the room temperature and 4 °C (Figure 15). The results revealed no significant difference between the different time points and 2 types of temperature (room temperature and 4 °C). It was found that the MAP had the greatest stability within 24 hours of storage. It would be the perfect concentration for a derivation of vitamin C.

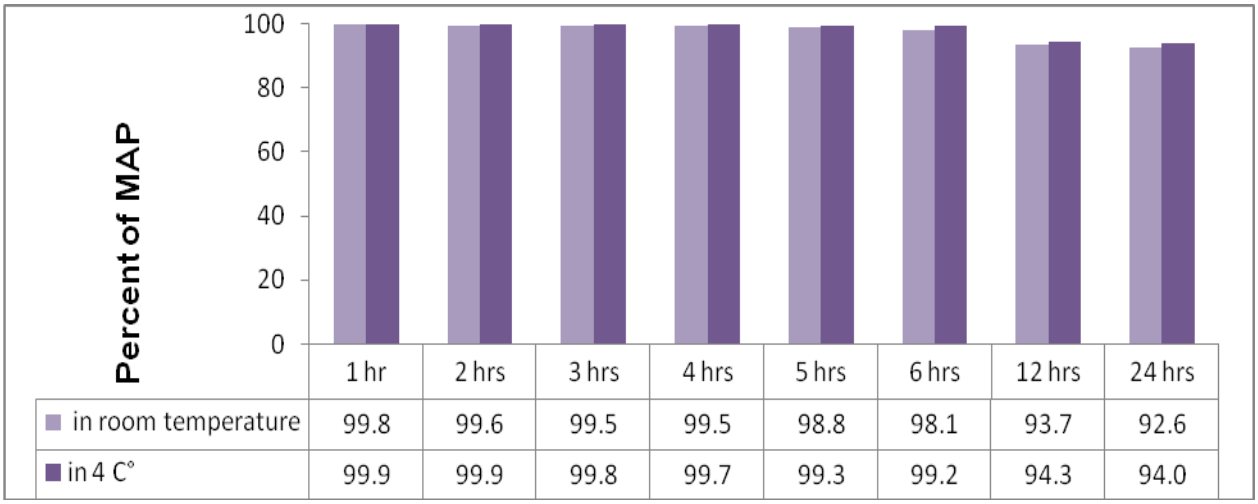

Figure 15 Effect of temperatures on the stability of MAP (N = 3).

3. Resistance of skin model and solution system

The sample of skin determined the skin resistance and the resistance of Franz diffusion cell's system (included the drug and PBS solution) by using Agilent 3458A multimeter. Data were presented in Table 6.

The resistant system of Franz with skin model yielded 44.7 KΩ (without electrical stimulation), and also resulted 27.13 KΩ, 24.75 KΩ and 15.76 KΩ in electrical intensity at 100V, 200V and 400V respectively. The outcome measurement also revealed mean current which could be obtained to determine the intensity setting in galvanic current comparable HVPC intensity (Table 6).

Table 6 The average intensity current and skin electrical resistance.

Condition	Average Resistance (kΩ)	Mean Irms, AC (mA)	I (mA), CV Electrical stimulation	Average Irms (mA)/surface area Franz 1.77 cm² (mA/cm²)
No Electrical stimulation	44.7	-	-	-
HVPC 100 V	27.13	0.171	4.8	0.096
HVPC 200 V	24.75	0.412	8.6	0.232
HVPC 400 V	15.76	0.880	15.8	0.497

Data shown the values of skin electrical resistance and mean intensity current root mean square (Irms) of various electrical modalities, HVPC and GC measured from Agilent 3458A multimeter (N = 3).

4. Validation of the method

Preparation of standard solution

Magnesium ascorbyl phosphate was accurately weighed and dissolved in 100 mL phosphate saline volumetric flasks of these stock diluents to be used as the reference substance. This solution was further appropriately diluted into the range of concentration cover 0.1– 100 µg/mL, which was then analyzed in each point. The obtained results were used to develop the calibration curve in triplicate value of each concentration. The results were analyzed with the linear regression analysis for calculating the calibration curve, which was constructed by plotting the HPLC peak areas of each versus at various points of known concentration and plotted the slope. The correlate efficiency was then calculated. The linear regression was observed over the concentration described in Figure 16. High regression

coefficient (r^2) values were at $r^2 = 0.997$ from the linear regression equation $y = 132.8X + 245.1$ (where, x is concentration (µg/ml), y is the peak absolute area).

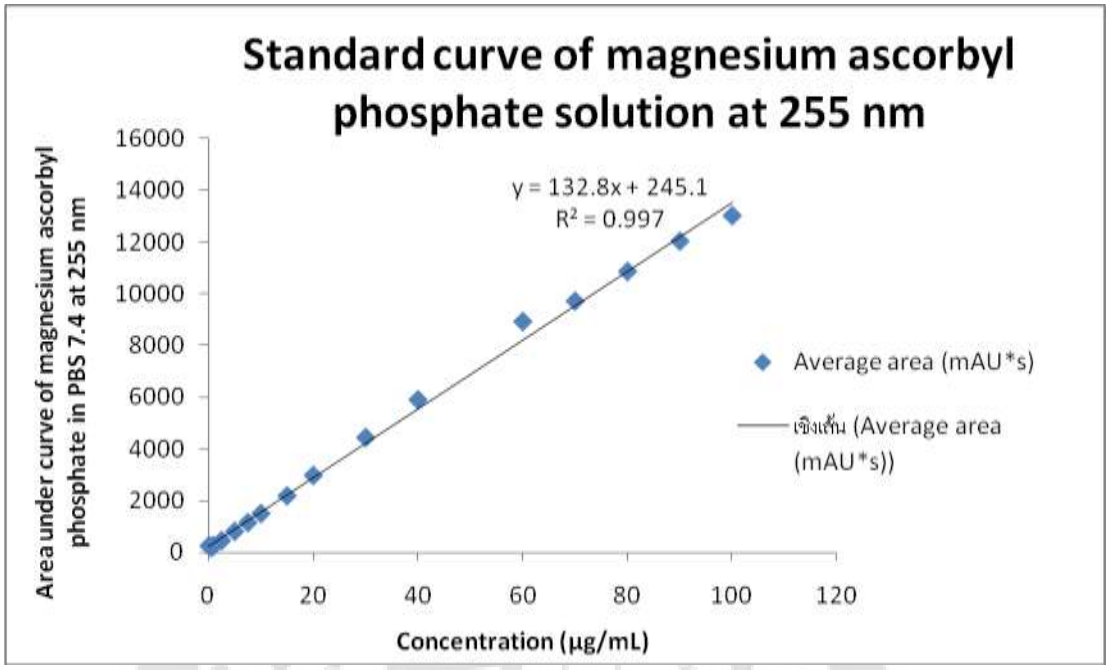

Figure 16 Validation data from standard curve of MAP. (Y, peak absolute area; X, concentration) (N = 6)

5. Stability of MAP molecule

The stability of MAP in PBS was analyzed in terms of relative standard deviations (RSD) within a time series of measurements. The intra-day precision was evaluated by six samples (n=6) of each concentration using the HPLC method. Results were recorded in table 7. The precision of the method was evaluated as the repeatability of an intermediate precision, and it was reported as RSD%. The RSD% results were 0.145 – 0.845%. The precision data obtained for the presented RSD values were 0.145 – 0.845 %, assuring of the good precision.

Table 7 Method validation analysis for MAP by high performance liquid chromatography

Sample	Concentration (μg/mL)	Mean ± SD	RSD	RSD%[a]
Magnesium ascorbyl phosphate solution in PBS 7.4	1	259.96±2.2	0.00845	0.845
Magnesium ascorbyl phosphate solution in PBS 7.4	7.5	1155.74±2.72	0.00235	0.235
Magnesium ascorbyl phosphate solution in PBS 7.4	15	2189.19±3.17	0.00145	0.145

[a] Percentage relative standard deviation (N = 6)

6. The results of this study

This study evaluated MAP concentration of each condition by using HPLC, which were calculated from the standard curve of MAP. The results had shown the total cumulative amount of MAP per area. It was significantly different when comparing with a passive diffusion and HVPC 400 V at every experimental condition in the total time showing in Figure 17. In addition, there was no significant difference in a total time of MAP in other conditions.

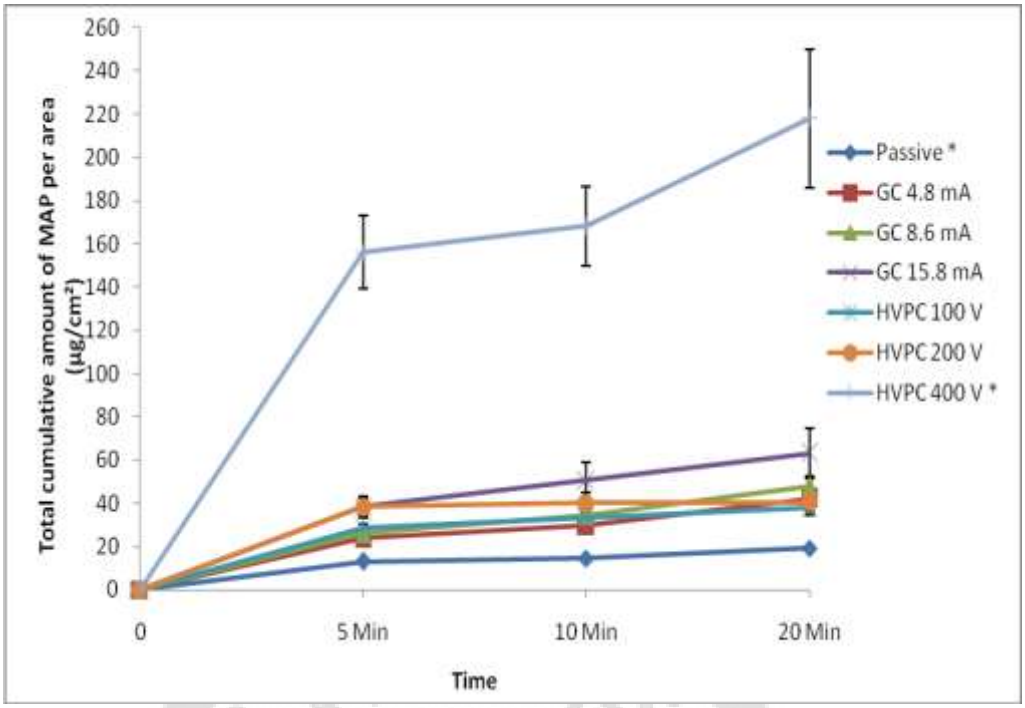

Figure 17 Comparison of the total times of 7 conditions (N = 6).

Data shown mean ± standard error of mean (SEM)

Note: GC = Galvanic, HVPC = high voltage pulse current

*Statically significant difference in every condition (*p-value* from K-independent samples)

The Figure 18 represented the significant difference in the GC 8.6, HVPC 100, 200 and 400 V with passive diffusion and HVPC 400 V with all conditions at 5, 10 and 20 minutes and GC 4.8 mA with passive diffusion at 5 minutes, whereas the other conditions showed no significant difference in groups at 5, 10 and 20 minutes. Moreover, an increasing of flux has been found in every condition after the time increased.

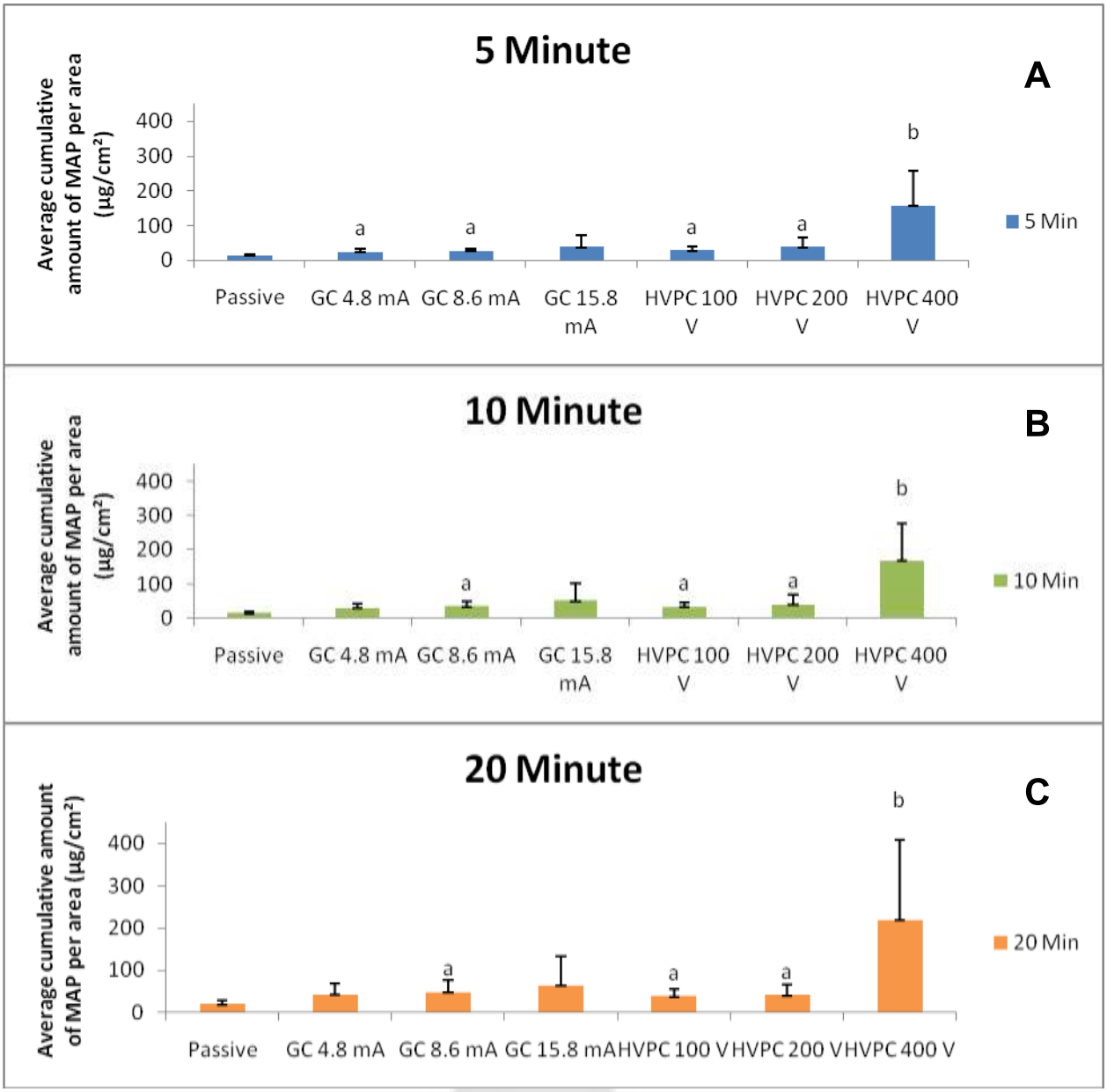

Figure 18 shown the comparison of time points used in each condition of the average cumulative amount of MAP per area. (A; at 5 minutes, B; at 10 minutes, C; at 20 minutes). Data shown their mean ±SD (N = 6)

Note: GC = Galvanic, HVPC = high voltage pulse current, a = significant difference with passive diffusion control, b = significant difference with all condition *(p-value* from K-independent samples)

This study employed an electrical stimulator in physical therapy practice. The study also employed two different waveform patterns with comparable stimulation intensities based on the theory of iontophoresis and electroporation, respectively. According to mean of Irms, this study classified the group of both GC and HVPC current by the mean intensity of electrical stimulation for control intensity in each level into 3 subgroups that were; (1) low intensity (GC 4.8 mA, HVPC100V); (2) medium intensity (GC 8.6 mA, HVPC 200V); and (3) high intensity (GC 15.8 mA, HVPC100V). Figure 19 (A) shown a significant difference in the low intensity between HVPC 100 volt with the passive diffusion at 5 and 10 minutes and GC 4.8 mA with passive diffusion at 5 minutes. The medium intensity revealed the significant difference in HVPC 200 volt with passive diffusion and GC 8.6 mA with passive diffusion at every time point, as shown in Figure 19 (B). The reports of high intensity showed significant difference between HVPC 400 volt with passive diffusion and HVPC 400 volt with GC 15.8 mA at every time point, it was shown in Figure 19 (C). While the other conditions yielded no significant difference in low intensity, medium intensity and high intensity, in 5, 10 and 20 minutes respectively.

7. The results of experiment in Mix condition

To determine whether the physiological conditions of an electrical stimulation under the porcine skin had been changed, quantitative measurements of permeated MAP (438.98 Da, negative charge) before, during and after pulsing at GC 4.8 mA, GC 8.6 mA, GC 15.8 mA, HVPC 100 V, HVPC 200 V, HVPC 400 V and passive diffusion were employed.

Flux was hardly increased in GC 15.8 mA after 1 hour of passive diffusion in time during pulsing (90 minute). There was significantly statistic increase in flux in GC 15.8 mA of time point at 90 minutes in three conditions, GC 15.8 mA with passive diffusion, GC 15.8 mA with HVPC 100 V and GC 15.8 mA with HVPC 200 V. While the other condition, namely GC 15.8 mA with GC 4.8 mA, GC 15.8 mA with GC 8.6 mA and GC 15.8 mA with HVPC 400 V showed no significant statistical difference in a mixed condition as showing in Figure 19. Each time point represented the average permeation of MAP in six skin samples from six different subjects.

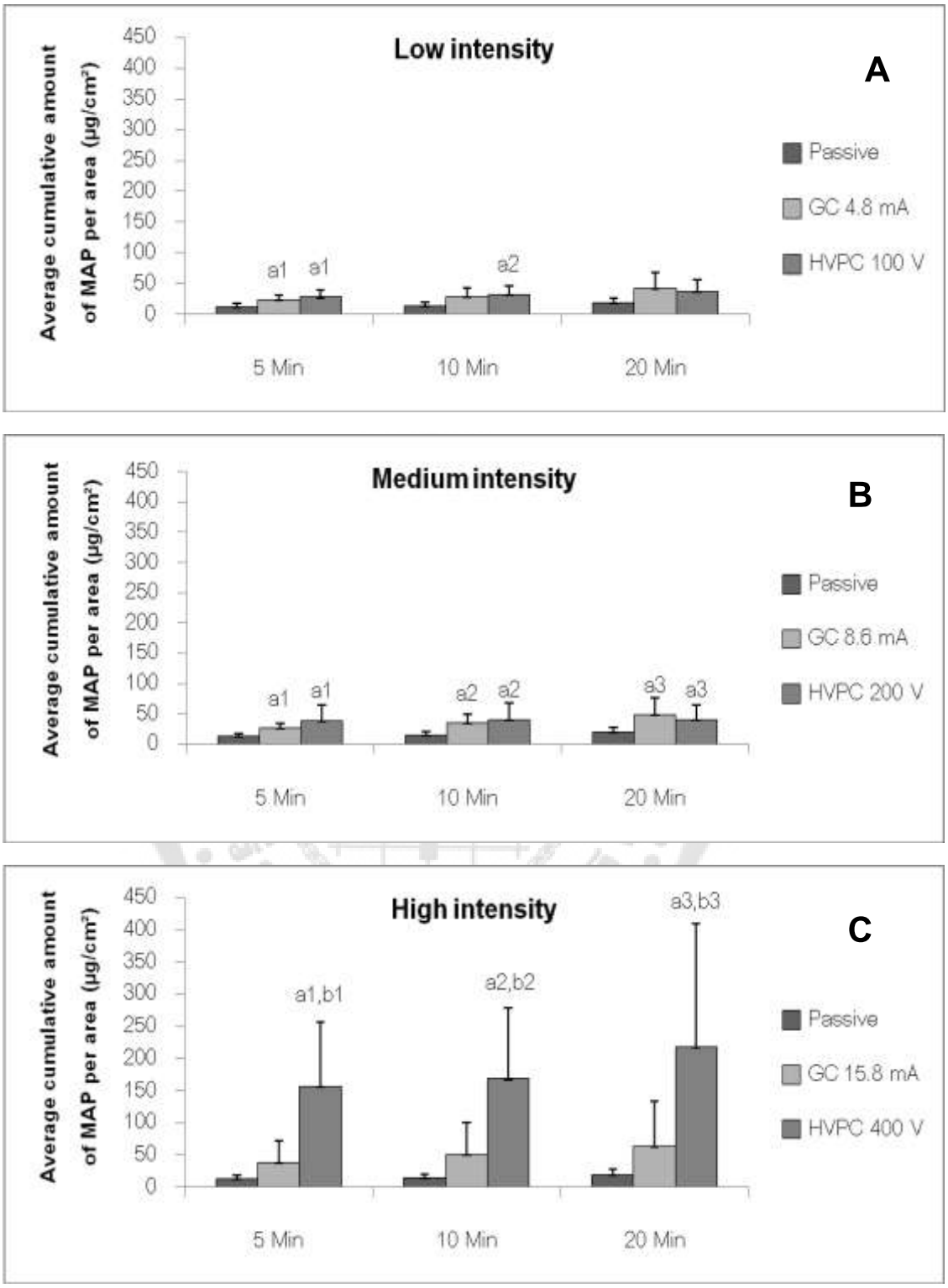

Figure 19 Representation of average cumulative amount of MAP per area comparing in each level of specific intensity. Data shown mean ± SD (N = 6) A; low intensity B; medium intensity C; high intensity, GC = Galvanic, HVPC = high voltage pulse current, a1 = significant difference with passive diffusion control at 5 minutes, a2 = significant difference

with passive diffusion control at 10 minutes, a3 = significant difference with passive diffusion control at 20 minutes, b1 = significant difference with GC at 5 minutes, b2 = significant difference with GC at 10 minutes and b3 = significant difference with GC at 20 minutes *(p-value* from K-independent samples)

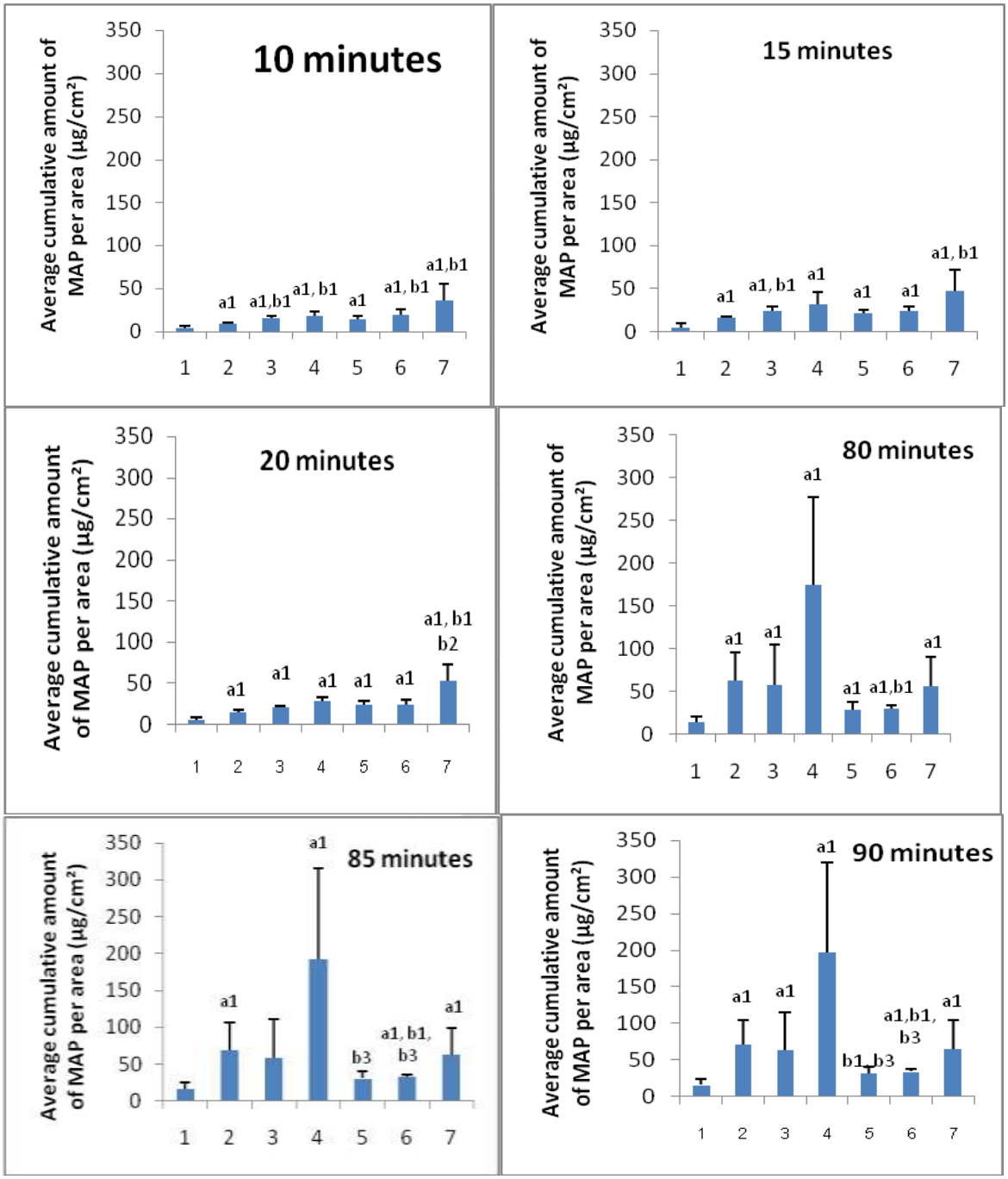

Figure 20 Representation of average cumulative amount of MAP per area comparing with each condition at specific time points. Data are mean ± SD (N = 3). GC = Galvanic, HVPC = high

voltage pulse current, 1 = Passive diffusion control, 2 = GC 4.8 mA, 3 = GC 8.6 mA, 4 = GC 15.8 mA, 5 = HVPC 100 V, 6 = HVPC 200 V and 7 = HVPC 400 V, 10 minutes = before pulsing by passive diffusion, 15 and 20 minutes = pulsing follows in each condition, 80 minutes = 1 hour after passive diffusion, 85 and 90 minutes = pulsing follows in each condition, a1 = significant difference with passive diffusion control, b1 = significant difference with GC 4.8 mA, b2 = significant difference with GC 8.6 mA and b3 = significant difference with GC 15.8 mA. *(P-value* from K-independent samples)

This study revealed no significant difference in the mixed condition between 20 minutes and 80 minutes in all conditions. The data were shown in Figure 20.

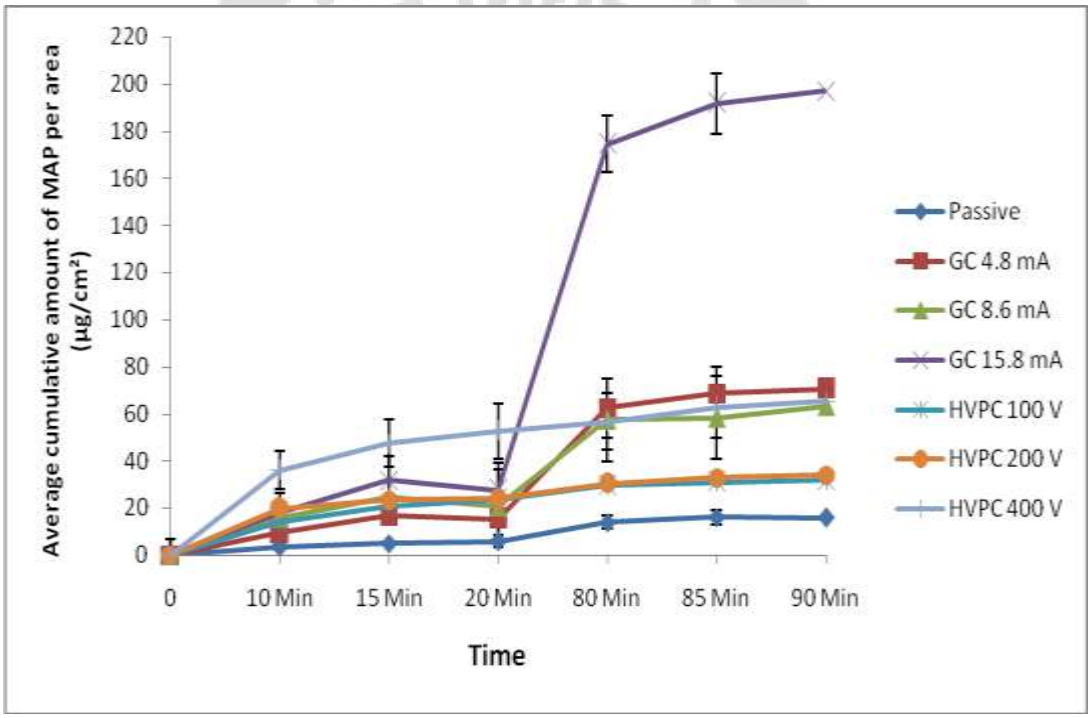

Figure 21 Overall results of the experiment shown average transdermal fluxes of MAP before, during and after pulsing through an electrical stimulation. Data are presented by mean ± standard error of mean (N = 3), GC = Galvanic, HVPC = high voltage pulse current, 10 minutes = before pulsing by passive diffusion, 15 and 20 minutes = pulsing follows in each condition, 80 minutes = 1 hour after passive diffusion, 85 and 90 minutes = pulsing follows

in each condition *Statically significant difference at p<0.05 (*p-value* from K-independent sample

CHAPTER 5

DISCUSSION AND CONCLUSION

The effects of high voltage pulses current on vitamin C permeation through the skin model were evaluated in this study. The concentration of MAP was comparison to various forms and doses of physical therapy electrotherapeutic stimulation which were deviled into7 conditions (1 = passive diffusion, 2 = GC 4.8 mA, 3 = GC 8.6 mA, 4 = GC 15.8 mA, 5 = HVPC 100 V, 6 = HVPC 200 V and 7 = HVPC 400 V) at 5, 10 and 20 minutes. The MAP concentration was then determined by HPLC. This experiment was employed franz diffusion cell system which was known as one of the most important methods of transdermal drug administration research area (92). Vitamin C is one of the drugs of choice in treating. Melasma, according to its physiological effects on inhibiting of melamogenesis. Vitamin C also promoted collagen, prevented free radical and protected inflammatory skin disorders (93, 94). However, vitamin C is quickly oxidized and large molecular weight drug ((9, 10, 91). Therefore, in this work was used MAP, the derivatives form of vitamin C, to reduce the defect of vitamin C (93). MAP has better stable derivative of vitamin C than ascorbyl acid and ascorbic palmitate (91). There was evidence proving that MAP which dissolved in various form of solutions and pH had no significant concentration loss after storage at room temperature for 5 months When comparing to vitamin C, the MAP seem to be the better choice of derivatives from of vitamin C used to improve skin conditions in cosmetic purpose. This experiment was found the similar outcome to previous studies in greater stability of MAP. The stability of MAP revealed good correlation coefficient (r^2 = 0.997) in giving concentration ranging from 0.1 to 100 µg/ml. The good precision of relative standard deviations (%RSD) of the experiment was < 2% of RSD (95-97). By the same token, the results of this study yielded 0.145 – 0.845 % of RSD values.

The full thickness of skin samples used in this study shown no significant difference among group of experimental conditions. The information of porcine skin properties

revealed relevant results to human skin profiles (25). Electrical resistant measured by the porcine skin was found as 20 – 23 kΩ approximately (98, 99). In this study, the resistances of porcine skin samples were decreased by the higher intensity of electrical stimulation ranging from 15.76, 24.75 and 27.13 kΩ according to 400, 200 and 100 V of HVPC stimulation respectively. The increment in voltages was yielded the decrease of skin resistance following to Ohm's law. The stratum corneum is most important barrier in transdermal application which has the highest electrical resistance when comparing to deeper skin layers (100). The previous evidence reported that skin resistance was rapidly decreased due to an electroporation (54). Slow resistance of changes was observed in iontophoresis which altered by the duration and magnitude of the electrical stimulation and upon the ionic strength of the solution (16). Hence, the drop of skin resistance might be due to increasing concentration in aqueous pathways which may be affected by the increasing of the conductance in cellular membrane (101). The changes in lipid structure to stratum corneum allowed transport of drugs to be easily permitted (54).

1. The effects of different time.

Cumulative amount of permeated drug per area (μg/cm²) after iontophoresis, electroporation and passive diffusion up to 20 minutes of electrical stimulation were evaluated. The permeation of MAP was found to correspond well with experimental time factors, with the increasing of permeated drug over time points in all conditions. In this study, at 20 minutes of HVPC 400 V stimulation was shown significantly different ($p=0.000$) when comparing to others stimulus conditions. The results of this study confirmed that HVPC 400 V is achieved the highest permeated MAP after 20 minutes of electrical stimulation. There evidence used to explained the administrator of trysin through abdominal human skin 200 μm thickness and applied high voltage current ranging from 100 and 300 V, pulse duration from 60 and 120 ms, 5 and 20 Hz for 5 and 20 minutes. The results shown an increasing permeability after 5 minutes of high voltage stimulation, which may be explained by the generation of aqueous pores in the stratum corneum lipid structure. However, the application at 100 V was found as the weak enhancement in the drug

transport which did not lead to large perturbation in lipid organization (16). The application of high voltage current at 400 V intensity and pulse duration at 10, 20 ms of timolol across human abdominal skin was found to be more efficient when comparing to passive diffusion for permeation and energy required to enhance drug delivery. Moreover, the application at 400 V was more efficient than low voltage at 250 V at 100 and 200 ms for promoting the transdermal of timolol by electroporation using square wave form as follow for 6 hours (13). Furthermore, the study of calcein permeation by using electroporation revealed was shown the amount of permeated calcein transport across the skin by high voltage stimulation at 270 V, 1 Hz for 1 hour. The rate of calcein transport increased significantly at 5 minute (102). The results of this study similarly found that the pulse parameters at 500 pulses, 40 ns duration, 1.3 MV/m^2 and 2, 5, 10 and 30 Hz was rapidly increased the permeation of drug at 5 minutes after electroporation in mammalian cells. The electroporation onset is related to increasing pore density which considerably enhanced the membrane conductivity of cell resulting in a faster transportation of drug across the skin (103). These results were in agreement with previous literature showing very fast and high of pore density area on the order of 5 – 10 minutes due to high voltage pulsed current such as in mammalian cells (103). and human skin of luteinizing hormone releasing hormone (104). Thus, molecular transport due to high voltage pulses is rapidly response which an onset times for transport and lag times to reach to steady-state of drug permeation ranging only in minutes (7). The generation of pore density was related to several factors such as electrical stimulation parameters, characteristics of the biological samples, properties of drugs etc.

In contrast, the mechanism of iontophoresis is correlates merely with the persisted shunt pathways of hair follicles and sweat ducts which bypass the tissue transport (54). Whereas, the mechanism of passive diffusion could be described following the Flick's first law of the gradient of concentration that is believed to occur largely in intercellular lipid bilayers across stratum corneum (105).

2. The effects of type of the electrical stimulation

This study discovered an increasing of the field strength by electrical intensity, which resulting in the increasing delivery efficiency of across the tissue in every time point. However, the permeation of MAP by the electroporation at 400 V revealed the highest permeation when comparing to other conditions. It showed the significant difference at every time point. Our data corresponded well with previous evidences using the HVPC (150 – 350 V, 1.9 ms, 12 Hz), iontophoresis (0.1 – 1 mA/cm²) and passive diffusion enhanced the transport of heparin across human skin *in vitro*. This showed the greater enhancement of the transport of drug in larger electric fields. Moreover, high voltage pulse allowed more efficiency transports of heparin through the skin than iontophoresis (106). Conversely, there was also the evidence showing no significant difference between the control group of high molecular weight heparin (12.9 kDa) in delivery of drug through the living rat skin via the pulsed current iontophoresis (5 mA, pulse 2,200 Hz, burst time 10 ms, burst 50 Hz and 10 minutes). Nonetheless, pulsed current iontophoresis significantly increased the transdermal transport of low molecular weight heparin (4.5 kDa) (107). The evidence used iontophoresis at 0.4 mA/cm² to investigate cyclosporine-A transdermal administration which was found as having no significant difference between iontophoresis and passive diffusion control. Whereas, the significant difference was found in the condition of electroporation at 160V-10ms, 200V-10ms and 370V-5ms when comparing to the passive diffusion. Furthermore, increasing an electrical field strength result to an increase in the enhancement of skin permeation (108). The electroporation can lead to the increase of the electrical field strength, extend the pore size, as well as increase the number of local transport regions (LTR) in the highly permeabilized membrane (47, 109, 110). Therefore, high voltage pulse supported the delivery of large molecule. As previously reported, the macromolecules size at least 40 kDa could be delivered transdermally by electroporation. The experiment, that electroporation parameters were set at 150 V, 150 ms, 10 pulses for 30 s in delivering of FITC-dextran (FD) molecular weight at 4.4, 12 and 38 kDa across the hairless rat skin in vitro. Electroporation enhanced the transport of the FD as being compared to passive diffusion. The FD showed a significant transport of FD 4.4, 12 and 38 kDa by the creation

of new pathways. The new pathways or pores are the new opening route in keratinocyte cell layers which drive molecules through membrane (55). In contrast, the use of iontophoresis has mainly been restricted to low molecular mass hydrophilic drugs. The data suggested that electroporation might be more efficient for delivering inside cutaneous cells than iontophoresis (7). Nevertheless, the previous study used iontophoresis to increase the transport, comparing to passive transdermal transport of luteinizing hormone releasing hormone (LHRH) through human skin *in vitro*. The results revealed that iontophoresis significantly increased the flux of LHRH at current densities more than 1.25 mA/cm² for 30 minutes, but the therapeutically acceptable limit of current for iontophoresis delivery was about 0.5 mA/cm² in cathode (104). Likewise, this experiment found a significant difference in iontophoresis condition lower than 2.71 mA/cm² (4.8 mA/1.77 cm^2 surface area) when comparing to passive diffusion control, whilst significantly lower than electroporation at 400 V condition.

3. The effects of different intensity

This study used the clinical electrical stimulation in physical therapy practice. There are two difference waveform patterns with comparable mean intensity in electroporation and iontophoresis in three different levels, which are low intensity (HVPC 100 V, GC 4.8 mA) medium intensity (HVPC 200 V, GC 8.6 mA) and high intensity (HVPC 400 V, GC 15.8 mA). Electroporation by HVPC seems to more improve MAP permeation than the iontophoresis in every time point except in the low intensity and medium intensity at 20 minutes. When compared to iontophoresis, electroporation was shown minor improvement of MAP permeability of but show no significant results. The effectiveness of iontophoresis in transdermal enhancement correlated with the influence of tissue in which the staggered corneocytes rearranging in a lipid condition and improved diffusion fluxes of drug ions transported into the SC (38, 111-113). The shunt pathway is believed to be a major iontophoresis transport route via the hair follicles, sweat ducts and sebaceous glands (114). Furthermore, the SC structure can be loosen and increased the intercellular gaps due to the electrical current and allowed the drug to pass through (43). This experiment

may shown the affects from electrical breakdown in iontophoresis conditions. Iontophoresis enhanced the transdermal drug delivery by three main mechanisms, the first step involved current flow that drove ions of drug through the SC, the second step involved the penetration in which the flow of electrical current may decrease the resistance of the tissue yet increases the permeability of the skin and finally, the electroosmosis may be an effective mechanism of drug delivery (30, 38, 115). In many cases, iontophoresis was shown an increasing permeation of the drug molecule, but the pathways may not be suitable for a delivery of high molecular drugs (30, 43). Hence, the other evidences used iontophoresis with various chemical enhancers that increased the transdermal delivery of drugs and could decreased side effects of treatment such as skin irritation, erythema and burns in the same time (111, 116). The stimulation intensity per transmitted surface area in iontophoresis conditions research area was determined at GC 4.8, 8.6 and 15.8 mA/cm² which approximate current intensity per area at 2.71, 4.85 and 8.92 mA/cm², respectively. Nonetheless, this current intensity in the clinical setting in using cathode for treatment is determined at 0.5 mA/cm² (18). The effect of high intensity revealed tremendous higher efficiency in enhancing MAP through the model of porcine skin. Similar results confirm the correlation between the current strength and duration of treatment. The experiment of their study showed an increase of transdermal iontophoresis for the delivery of ranitidine hydrochloride across porcine skin *in vitro.* Three current intensities via iontophoresis were examined. They were 0.13, 0.26 and 0.39 mA/cm^2 at 6 hours respectively. The transference number of drug increased the delivery of rantidine when the time of treatment and current intensity was increased (18). The application of drugs via electroporation into living cells *in vivo* with the current intensity at 1,000, 800 and 400 V/cm^2 (high intensity range) can improve the permeability of drug, respectively (117).

Electroporation required changes in the skin microstructure that was believed to be involved in the creation of new transient aqueous pathways (pores) in lipid bilayers, where aqueous pathways are less than 10 – 60 nm, sparse about 0.1 % of the total skin surface area allowing the transport of drugs via transcellular, intercellular and shut pathways due to

the electrical field (16, 76, 108, 118). In the principle, the pores in lipid bilayers and through keratinocyter were known as a transcellular pathway (54). Molecular dynamic stimulation of lipid bilayers proposed the following outcomes; 1) application of very short lifetime; 2) molecular dynamics stimulations charging of lipid bilayer membranes; 3) the rapid of rearrange structure formed by the appearance of water fingers that enter from the lipid headgroup/water layers in the lipid core; 4) transitions to water-filled membrane structure perforated the membrane (aqueous pathways or pores or hydrophilic pores) by the appearing local transport regions (LTR) and the surrounding local dissipation regions (LDR); 5) the provision of a local driving force ionic and molecular transport across the membrane, and finally the membrane was reversible (58, 60, 76, 119-121). However, the event of pores and complete recovery of cells depends on several applicable factors by electroporation in the treatment (58, 120).

4 Skin electrical breakdown

The results of skin reversibility test also showed transdermal fluxes of MAP before, during and after pulsing at GC 4.8 mA, GC 8.6 mA, GC 15.8 mA, HVPC 100 V, HVPC 200 V and HVPC 400 V as well as the passive diffusion control. The experiment was conducted by employing the stimulation as well as the second set of stimulation after being stopped intermittent for 1 hour. The massive flux of permeated MAP after 1 hour intermittent may indicate the electrical breakdown at the skin structure. These data showed higher of MAP permeation after 1 hour intermittent in the iontophoresis conditions at GC 15.8, 8.6 and 4.8 mA than in other conditions, respectively. The iontophoresis studies used GC in the electric field strengths at 250 – 4,000 mV through human's epidermal membrane by measuring the electrical resistance before and after voltage termination. The results were represented at 2,500 mV, 10 min and 1,000 mV, 60 min that incomplete recovery within 480 min after voltage drops. Thus, the data showed that the effects of duration and voltage have changed human epidermal membrane and the period of the recovery (45). This study may be partially recovered in 1 hour by iontophoresis that found slowly of the recovery. It took a

longer period of time to be completely recovered. Electroporation pulsing allowed the changes in skin permeability. It may be fully reversible at HVPC 100, 200 and 400 V. The previous experiment demonstrated that the electroporation at below 100 V, 5 S for 1 hour, was completely reversed through human cadaver skin within 24 hours (4). Consequently, the electroporation could be reversible or irreversible, due to several factors such as strength, duration and waveform of electric field parameters (122). Interestingly, there was evidence in applying the high voltage current into skin cancer cells which aimed to destroy the cells. The strength of the electric fields in irreversible electroporation was set at 2,200 V, 90 μs, 90 pulses and frequency of 1 Hz in the 10 patients. Irreversible electroporation could change the skin structure as well as damage tissues (123). The evidence used electrical measurements to determine electrical changes in the SC. The results represented the voltage at higher than 130 V, which caused the recovery of approximately 50 % (59). In addition, the field strengths at 1,000 – 10,000 V/cm² for electroporation which are typical for inducing electrical breakdown increased the molecular transport across membranes (61). In the present study, the highest intensity of HVPC at 400 V showed reversible properties of the skin, and it could increase the flux of MAP for the second set of the stimulation. Several investigations have presented the summarized the theory of the reversibility of the skin after applying electrical breakdown in the lipid bilayer membranes. Their results showed that the electrical measurement could determine electrical changes of the SC in three unique characteristics of electroporation; 1) the rate of increase in molecular flux of ionic conductance depended on the applied electroporation parameter; 2) the reversible electroporation where new aqueous pathways are created and the resistance of the skin are recovery in seconds to minutes; 3) the irreversible electroporation caused by changes in barrier membrane structure and aqueous pathway recovery is incomplete of greater magnitude and times for attaining significant electrical breakdown (4, 45, 49, 59).

5. Application of electroporation in clinical setting

According to the intensity setting in this experimented was ranged from 100 V to 400 V that covered voltage intensity in the clinical setting in using high voltage pulse current

via electroporation for treatment is determined at 50 – 500 V (124). In clinical setting for reduce facial skin pigmentation was determined in 0.3 – 0.5 mA/cm^2 in iontophoresis application (10). Therefore, our experiment was used the over ranged intensity than the clinical setting approximately 6 times. However, the skin reversibility in this experiment was shown unlikely affects from electrical breakdown in all HVPC conditions. Therefore, using electroporation by HVPC in facial skin treatment may improve the drug permeation and may lead to increasing the effectiveness in treatment even though the intensity was higher than the determined range in iontophoresis study. Furthermore, the advice effects from HVPC would be seriously concerned in accordance to the unwanted muscle contraction when applying the current over the fleshy area of muscle. An alteration of electrode to be suitable with transdermal drug application and reduce unwanted muscle contraction may needed to improve the real clinical application of electroporation. Summary of cutaneous effects of iontophoresis and electroporation are listed as follows; 1) electroporation at pulse between 10 μs to 1 ms shown significantly reduce sensation therefore HVPC may cause less irritation than iontophoresis; 2) high voltage pulses shown greater effect on drug transport through new pathways than high current densities associated with skin appendages which could irritate by causing pH changes of the skin (118).

Application of electroporation through the skin did not cause adversely or unpleasant side effects such as tissue injury and muscle contraction when being applied on the skin for transdermal application purposes. The previous studies suggested that electroporation was safe and had no long-term side effects inpatients (125, 126). Referring to the damage of tissue caused by the skin electroporation, there is literature presented that electroporation can decrease skin resistance within a few minutes after applying electrical stimulation. This indicated that the skin was damaged by HVPC (122, 127). Conversely, iontophoresis required a positive or negative charge on the molecule to deliver drug, its relatively long time for GC application. This possibly caused skin irritation (125). *In vivo* experiments in hairless rat, the skin biophysical parameters measured factors associated with delayed inflammation of the underlying tissue by HVPC application (100 – 500 V, 1.3

ms, 15 – 60 pulses) versus iontophoresis (0.5 mA/cm²) treatment. The changes in skin capacitance had no significance that demonstrating the safety of using HVPC for transdermal delivery (56). While the limitation for iontophoresis was 0.5 mA/cm² approximately (18). The possibility of decreasing the skin irritation caused by an acid and base from chemical reactions in clinical electrotherapy are as follows; 1) clean the skin before and after the treatment; 2) decrease the average current per square inch of pad, increase the size of the pads and secure the electrode to the skin; 3) use a unit with symmetrical biphasic waveform (128). Clinical studies of electroporation applied HVPC at 1,300 V/cm^2, 100 µs, 8 pulses and frequency 1 Hz for electrochemotherapy treatment in 14 patients. The patients reported less sensation because adipose tissue prevented electric field from distributing deeper into the underlying tissue. Therefore, less muscle contractions were observed. However, the patients did not feel painful after having the pulses of HVPC (129). The electric field in the skin caused minor muscle contractions during pulsing of HVPC and transient erythema. These possibly happened due to the sensation of skin. The increase in the application of electrical stimulation such as pulse rate, time duration, and voltage tends to enhance levels of sensation which causes a direct excitation of underlying nerves and muscles. The skin biophysical parameters were associated with damage of underlying tissue as follows; 1) the increase of tissue hydration; 2) the disorganization of the SC lipid bilayer; 3) the increase transepidermal water loss; and 4) the increase in the blood flow (16, 49). This study demonstrated that HVPC appeared to make significant changes in the skin permeability. It is believed that a mechanism associating with the HVPC is the most important mechanism showing advantages of this approach which are the dramatic increase of flux, short lag times, rapid steady-state flux and having no harmful effects of tissue. Together, these results suggested that electroporation may be useful tools to enhance transdermal drug delivery. This allowed the clinical benefits in using physical therapy electrotherapeutic modality to improve drug permeation to achieve the target dose and may reduce the unpleasant side effects and discomfort of an electrical field to the SC in the near future. Consequently, the delivery of a drug by using HVPC should require a smaller averaged current than iontophoresis. This caused less irritating and more safety.

Iontophoresis needs the prolong application by the polarizing effects of a continuous or pulsed direct current that may cause changes of skin pH. The chemical reaction significantly affected an irritation especially under the active and dispersive electrodes during treatment (128).

Conclusions

The enhancing of MAP permeation across the membrane using HVPC depends on various parameters such as time duration and intensity of application. Abdominal porcine skin can be used to determine the effects of permeable penetration enhancement of drugs via physical therapy therapeutic electrical stimulator. These informative results showed advantages of investigating the effects of electroporation as pioneer data by using electrical stimulation. High voltage pulsed current exhibited a better permeation of MAP than galvanic current and passive diffusion control on porcine skin models. Moreover, the result HVPC 400 V showed to enhance MAP permeability significant difference as compared to other condition. In summary, this study suggested that HVPC application could increase transdermal permeation of drug and it could be reversible stimulate in the tissue. Therefore, this study provided the evidence using electroporation in a transdermal application by using therapeutic electrical stimulator with MAP which is one step closer to the clinical application in the near future.

Strengths of the Study

This study used electrical stimulation in physical therapy practice for real clinical setting that the evidence was never been constructed before. The experimental design has obviously point out the concentration of permeated drug with reliable quantification method. The porcine skin samples were randomly used in each experimental condition which yields the strongest design in a laboratory experiment. This method assured the good reliability and validity in precision of analytical samples by HPLC.

Limitations of the Study

The experiment cannot control the hairless follicle density in each skin sample. The evidence did not show the structural changes of tissue at before, during and after pulsing that were caused by electrical stimulation.

Suggestions for Further Studies

Further studies are required to evaluate in cell structures the compliance of this electrical stimulation in physical therapy to obtain thoroughly understanding and setting up application parameter to the real clinical application, which had the safety and efficiency in protocols for skin electroporation.

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APPENDIX

APPENDIX A

Table was shown the thickness of each sample. These skin samples were randomly separated into 7 conditions from 6 female pigs. The thickness of each skin sample was measured by a digital venier caliper (Mitutoyo Co.Ltd, Kawasaki, Japan). Full thickness skin samples were evaluated at 5 repeated in each number such as right-upper, right-down, centre, left-upper and left-down of surface area in 3x3 cm^2.

N	Passive diffusion	GC 4.8 mA	GC 8.6 mA	GC 15.8 mA	HVPC 100 V	HVPC 200 V	HVPC 400 V
N1	0.302	0.294	0.328	0.316	0.342	0.318	0.302
N2	0.296	0.334	0.312	0.306	0.312	0.31	0.298
N3	0.290	0.280	0.280	0.300	0.290	0.310	0.300
N4	0.304	0.288	0.280	0.328	0.288	0.346	0.322
N5	0.342	0.334	0.302	0.294	0.310	0.300	0.278
N6	0.330	0.308	0.296	0.33	0.300	0.288	0.330
Average	0.311	0.306	0.300	0.312	0.307	0.312	0.305
SD	0.021	0.023	0.019	0.015	0.020	0.020	0.019

Table was shown the thickness of each sample. These skin samples were randomly separated into mixed conditions from 3 female pigs. The thickness of each skin sample was measured by a digital venier caliper (Mitutoyo Co.Ltd, Kawasaki, Japan). Full thickness skin samples were evaluated at 5 repeated in each number such as right-upper, right-down, centre, left-upper and left-down of surface area in 3x3 cm^2.

N	Passive diffusion	GC 4.8 mA	GC 8.6 mA	GC 15.8 mA	HVPC 100 V	HVPC 200 V	HVPC 400 V
N1	0.29	0.31	0.3	0.29	0.28	0.27	0.27
N2	0.34	0.33	0.29	0.28	0.34	0.33	0.3
N3	0.3	0.3	0.28	0.31	0.28	0.28	0.34
Average	0.32	0.32	0.32	0.32	0.32	0.32	0.32
SD	0.026	0.015	0.010	0.015	0.035	0.032	0.035

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