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Effect of Gramicidin on Percutaneous Permeation of a Model Drug

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ABSTRACT This study investigated the enhancement effect of gramicidin, a cationic ionophore, on percutaneous absorption of a model drug, benzoic acid (BA), through rat abdominal skin. The mechanisms by which gramicidin increased skin permeability to BA were also investigated. Degree of hydration measured by the Karl Fisher method, the concentration gradient measured by cryostat analysis, and lipid concentration measured by the Fiske-Subbarow method were evaluated and compared. The results showed that BA permeation profiles through rat abdominal skin followed dose- and volume-dependent patterns. The pretreatment of gramicidin increased the permeation rate of BA through rat abdominal skin compared with the untreated control (18.89 vs. 10.86 μ g/cm^2/hour). Change in skin permeation rate of BA after gramicidin pretreatment was closely correlated with the remaining skin water content. There were no significant differences in the amounts of phospholipid phosphorous between gramicidin pretreated and untreated skin. The enhancing effect of gramicidin on percutaneous absorption of a model drug is mainly attributed to increasing the diffusivity in the hydration domain of the skin and rearranging the lipid bilayer in the stratum corneum.

KEYWORDS: Gramicidin, Permeation, Enhancer, Hydration, Lipid bilayer

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INTRODUCTION

The skin permeability to exogenous compounds is affected by various factors, such as degree of hydration and conformational changes in the membrane [1]. A large number of excipients have been reported to enhance the permeation rate of exogenous compounds by either improving the partitioning coefficient of compounds in the stratum corneum or disrupting the ordered lipid bilayers to reduce their diffusional resistance [2,3]. Compared to enhancers working on a transcellular pathway, those working on a paracellular pathway have been relatively less reported.

Various approaches including ionophore and volatile anaesthetic have been evaluated for their ability to modulate the hydration rate in the membrane [4,5]. Cationic ionophores act as a free mobile carrier to transport calcium and magnesium and to equilibrate endogenous mitochondria divalent cations with external medium [6]. Gramicidin, a linear peptide-type cationic ionophore, has been reported to form specific channels across cell membranes and to enhance the transport of cations. Gramicidin has no charged or hydrophilic chains, and its aqueous solubility is low. Because both the amino and carboxy termini of the molecule are blocked, gramacidin has been found to partition strongly into the hydrophobic region of phospholipid membrane and to maintain the liquid crystalline state [7]. The biological activity of gramicidin depends on the efficacy of its peptide-like interactions with cell membrane. These interactions induce pore formations across cell membrane and subsequent deregulation of cation exchange [8]. By elucidating the changes in the hydration rate and the lipid

bilayer conformation exerted by gramicidin, the role of ionophores in regulation of skin permeability to exogenous compounds can also be clearly elucidated.

The major hypothesis of this work is that pretreatment with gramicidin affects the permeation barrier functions in the skin to exogenous compounds by modulating the hydration rate and disrupting the ordered lipid bilayer in the stratum corneum and the epidermis. Benzoic acid (BA) was chosen as a model drug because it has been extensively used as a model compound for uptake, metabolism, and excretion of carboxylic acids in various studies [9]. The effects of loading doses and loading volumes on the permeation profiles of BA through rat abdominal skin placed in vertical-diffusion cells were evaluated. The permeation profiles of BA through rat abdominal skin pretreated with gramicidin were compared with the untreated control. The degree of hydration measured by the Karl Fisher method [10], the concentration gradient measured by cryostat analysis [11], and the lipid concentration measured by the Fiske-Subbarow method [12] were conducted to evaluate the mechanisms behind the promoting effects of gramicidin.

MATERIALS AND METHODS

Materials and Methods

All materials were obtained from commercial sources and used as received. Gramicidin D, BA, and radioactive [carboxy-^{14}C] BA (specific activity, 250 mCi/mmol) were purchased from Sigma (St Louis, MO). HEPES-buffered (25 mM) Hank's balanced salt solution (HHBSS) was used as a receptor solution. In conducting *in vitro* studies, various concentrations of BA in isopropanol were used as loading doses.

Assay of Benzoic Acid

BA was assayed using HPLC with a reverse-phase Beckman Ultrasphere column and a Waters C_{18} guard column at a wavelength of 230 nm. Solvent elution was carried out at a constant flow rate (1 ml/min), with a mobile phase consisting of 12% MeOH in 0.05 M phosphate buffer (pH 7.0), after degassing by filtration and ultrasonification. Radioactivity of BA was measured by a radiometric 500TR series flow scintillation analyzer (Packard Instrument Co, Meriden, CT).

Evaluation of BA Stability

Experiments were run to evaluate the stability of BA in buffer solutions and the skin extract under various conditions (25° C and 37°C up to 7 days). The stability of BA in the systems can be monitored based on the remaining concentrations of BA. The activation energy of BA is determined using the Arrhenius plots.

Procedure for Percutaneous Permeation Study

Freshly acquired abdominal skin from male rats (Sprague Dawley, 2-3 months old, weighing about 200 g) was used. Due to the difficulty in continuous supply of metabolically intact human skin, the rat was chosen as the animal model. Interspecies differences in both absorption and metabolism will be taken into account when extrapolation to the human situation is needed. The permeation study of BA through rat abdominal skin was performed using a method developed by Kabadi and Chien [13]. Briefly, HHBSS (pH 7.4), as prepared in the material section, was warmed to 37°C. To determine percutaneous permeation profiles of a model drug, the whole abdominal region of the skin was carefully shaved and excised just before the experiment. Skin samples were sandwiched between the half-cells of each Franz permeation cell (PermeGear, Somerville, NJ) with a serosal surface exposed to the receptor half-cell filled with HHBSS as described in the materials section. The donor solution containing varying doses of BA in the same volume or the same dose of BA in different volumes was placed on the skin and permeation profiles of BA across the skin membrane were monitored. Three different loading doses (10, 100, 1000 μ g) of BA (100 μ l), and two different volumes (20 and 100 μ l) with the same loading dose (100 μ g) were used. Loading doses were based on the volume of the skin samples calculated from the surface area and the thickness (cm^2 x mm). Skin integrity was evaluated by monitoring the permeation profile of tritiated water (^{3}H) and skin variability was evaluated by measuring anaerobic glucose utilization throughout the experiment (12 hours).

Evaluation of Gramicidin Effects on Percutaneous Permeation of BA

The effect of gramicidin on skin permeability to exogenous compounds was studied using a concentration of 100 μ g/ml in isopropanol. One hundred μ l of gramicidin solution was added on the donor side of the skin 30 min before BA loading. The procedure in permeation studies section (2.4) was repeated for the evaluation of the enhancement effect of gramicidin. The same volume of isopropanol without any gramicidin was added for the control. To measure the hydration rate and phospholipid phosphorous concentration, the skin samples treated with gramicidin and those treated with the solvent alone (the control) for 30 min were compared. To evaluate drug distribution profiles in the membrane, skin samples were first treated for 30 min with gramicidin or solvent alone (the control), then BA concentrations in each section of the skin samples after 12-hour permeation studies were measured.

Measurement of the Degree of Hydration

A correlation between the degree of skin hydration and skin impedance to exogenous compound penetration was investigated. The change in the degree of hydration upon exposure to enhancers is small, difficult to detect, and subject to large variance. The Karl Fisher titration method was used for the measurement of the hydration rate. The water content of samples was determined by measuring the weight of water per the weight of samples using an Aquar-Star VIB Titrator (EM Science, Gibbstown, NJ) equipped with a water vaporizer.

Evaluation of Drug Distribution in the Membrane

BA distribution in the membrane was measured to evaluate whether each layer makes an equivalent contribution to the overall barrier functions. Following 12-hour permeation studies using radioactive [carboxy-^{14}C] BA (100 μ g), skin samples were carefully rinsed 5 times with 1 ml of water. The skin was placed epidermis side up on a board lined with a parafilm. A chuck was placed on dry ice and a thin layer of optimum cutting temperature (OCT) medium was applied on the chuck. After the OCT medium wasfrozen until it turned white, the skin sample was placed on the frozen medium with the stratum corneum facing a chuck holder, and with OCT medium placed over the skin to cover it completely. Skin was adjusted in such a way that the dermis would be the first part to be reached by the knife.. Skin samples and OCT medium were frozen on dry ice until they were ready for sectioning with microtome. Each sample piece was cut into about 20 μ m thickness using a Carl Zeiss HM 505 microtome (Fisher, PA). Section pieces were collected into five groups in order, with the outmost stratum corneum compartment assigned No. 1 and the dermis compartment assigned No. 5 (used as X-axis in **Figure 3**). Each group was kept frozen on dry ice until they were ready for counting of radioactivity. Any remaining drugs were collected from each skin sample for mass balance counting.

Measurement of the Total Amount of Phospholipid Phosphorous

The amount of phospholipid phosphorous was determined by the modified Fiske-Subbarow method with a Fiske Subbarow reagent obtained from Sigma (St Louis, MO) [12,14]. Briefly, samples were mixed thoroughly with 20% trichloroacetic acid to remove protein and lipid phosphorous. After filtration processes, acid molybdate solution was added to form phosphomolybdate. The Fiske Subbarow reagent was added to reduce phosphomolybdate to form a phosphomolybdenum blue complex and left for 10 min for a color development. The absorbance of the samples was read at 660 nm using the blank as a reference.

Statistical Analysis

The difference in mean flux values determined under various experimental conditions was statistically analyzed by a one-way analysis of variance (ANOVA) with pair-wise multiple comparisons of the Student-Newman-Keuls method.

RESULTS AND DISCUSSION

Permeation Profiles of BA

The permeation profiles of BA through rat abdominal skin, from donor compartments containing varying doses of BA, were typically characterized by a rapid permeation rate with no lag time and reaching a plateau in 10 hours, as shown in **Figure 1A.** This indicated that BA permeation through rat abdominal skin was mainly controlled by partition and diffusion processes. The permeated amount through rat abdominal skin increased steadily as a function of time. As shown in **Table 1**, the drug permeation flux (Q/t) from the donor compartments, calculated from the slope of the linear portion of the Q vs. t profile, increased as a loading dose increased (2.51, 10.86, and 27.0 μ g/cm^2/hour).

Permeation profiles of BA through rat abdominal skin, from donor compartments containing different volumes of BA (20 μ l and 100 μ l) were similar to those that resulted in the presence of different loading doses. The permeation rate of BA through rat abdominal skin using a loading volume of 20 μ l was much higher than that of 100 μ l (25.33 ± 5.04 and 10.86 ± 2.91 μ g/cm^2/hour), as shown in **Figure 1B.** The extent of the BA transport seems to be favored by an aqueous environment, causing the skin barrier function to appear intimately related to the degree of skin hydration. The low loading volume (20 μ l) is physiologically compatible with the amount of water in the skin, facilitating a rapid mixture with the skin components. Skin viability, evaluated by anaerobic glucose utilization, was maintained throughout the experiments. Experiments were also run to ensure the stability of BA in various buffer solutions under the different experimental conditions. The results from the Arrhenius plots using the remaining concentrations of BA in buffer solutions at 25°C and 37°C up to 7 days indicate that BA is quite stable with activation energy of 30.34 kcal/mol for HHBSS buffer and 22.31 kcal/mol for skin extract.

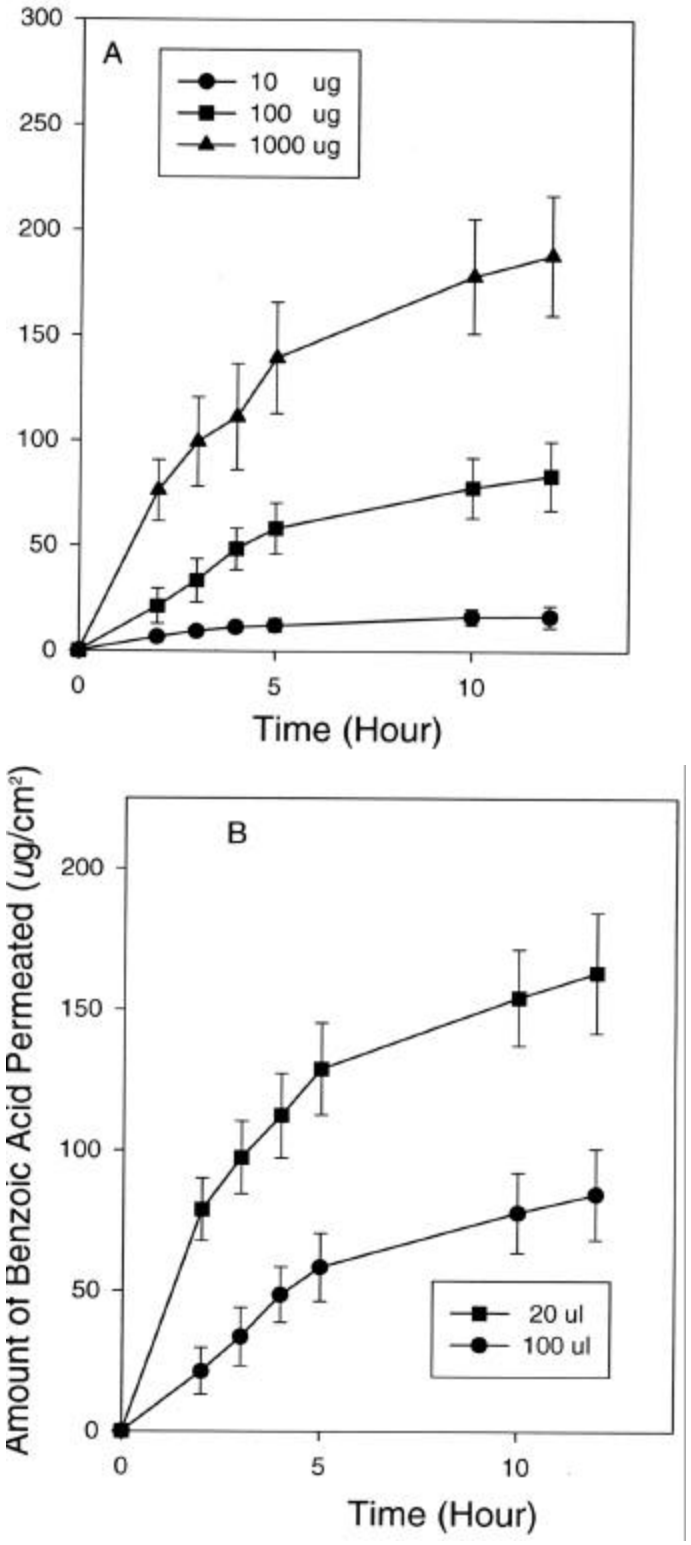

Figure 1. Permeation profile of benzoic acid through rat abdominal skin: Effect of loading dose (A) and loading volume (B) (N=6)

Table 1. Drug permeation flux (Q/t) calculated from the slope of the linear portion of the Q vs. t profile as a function of loading dose or loading volume from a donor compartment

Doses (mg)	Drug Permeation Flux (Average ± SD mg/ cm^2/hr)
10	2.51 (± 0.57)
100	10.86 (± 2.91)
1000	27.01 (± 5.28)
Volume (µl)*	Drug Permeation Flux (Average ± SD µg/ cm^2/hr)
20	25.33 (± 5.04)
100	10.86 (± 2.91)

N=6 for each experiment
*** Loading dose of 100 mg.**

Effects of Gramicidin on BA Permeation through Rat Abdominal Skin

The permeation profiles of BA through rat abdominal skin upon pretreatment of gramicidin were similar to those of BA with treatment by the solvent without gramicidin, but the flux rates of the former were much greater.. When the amount of BA permeated (µg/cm^2) through rat abdominal skin was plotted against time (hour), a linear relationship was established, as shown in **Figure 2** (18.89 ± 3.04 vs. 10.86 ± 2.91 µg/cm^2/hour). On increasing the gramicidin concentration of pretreatment up to 1 mg/mL, the permeation rate of BA through rat abdominal skin also increased, but there was no linear relationship between gramicidin concentration and the permeation rate of BA. The distinctive effect of gramicidin on BA permeation through rat abdominal skin may be due to rearranging a lipid barrier and causing a change in the hydration content in rat abdominal skin. The major mechanism behind gramicidin activity as a permeation enhancer was further studied.

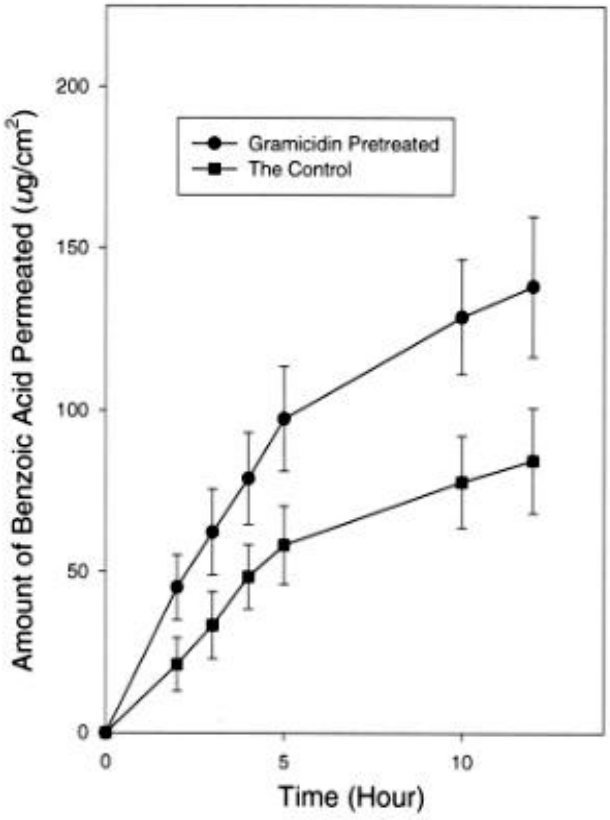

Figure 2. Effect of gramicidin on permeation profiles of benzoic acid through rat abdominal skin (N=6)

Measurement of the Degree of Hydration

To evaluate the effects of gramicidin on the degree of skin hydration and impedance to exogenous substance penetration, the skin's water content was measured by the Karl Fisher titration method and determined by calculating the weight of water per the weight of samples. The degree of skin hydration after pretreatment of gramicidin for 30 minutes was much greater than that of the control, which was treated by the solvent without gramicidin for 30 minutes (5.14% ± 1.12% vs. 3.03% ± 0.83%), as shown in **Table 2.** This further affected the permeation rate of BA through rat abdominal skin. Mechanical measurement—an indirect method of measuring degree of hydration—was used for this study. This method cannot be used to estimate the degree of hydration in a specific skin compartment. Therefore, evaluation of BA concentration gradient in the skin by the cryostat method was used to determine which layer of the skin contributed most to the change in the degree of hydration.

Table 2. The degree of hydration of the skin after treatment with gramicidin

Conditions	Water Content (%) (Average ± SD)
Gramicidin Treated	5.14 ± 1.12 [1)]
Untreated Control [2)]	3.03 ± 0.83 [1)]

N=6

1. Significantly different from each other (p<0.05).

2. Only solvent was treated.

Measurement of Drug Distribution in the Membrane

The results of cryostat sectioning on skin samples after 12-hour permeation processes demonstrated a BA concentration gradient with a high concentration in the stratum corneum (SC) part and a low concentration in the dermis part, as shown in **Figure 3.** As described in the experimental section (2.7), the x-axis in **Figure 3** denotes five sections in the skin, assigning the outermost stratum corneum compartment No. 1 and the dermis compartment No. 5. The difference in BA concentrations between the stratum corneum and the dermis compartment after 12-hour permeation studies is less distinctive when rat abdominal skin has been pretreated with gramicidin. Between the control and the gramicidin-treated samples, there was no significant difference in the BA amount at the stratum corneum, but there was a significant difference toward the dermis compartment. The stratum corneum is known as a hydrophobic barrier, whereas viable dermis is known as a hydrophilic barrier. The different profiles of the remaining amount of BA indicated that gramicidin increased the degree of skin hydration, which was reflected in the increased amount of BA in the dermis compartment. This may imply that each layer in the skin makes a different contribution to overall barrier properties. Some compounds influence skin permeability to exogenous compounds by affecting the degree of hydration of viable epidermis. Gramicidin readily conditions viable epidermis and affects the permeation rate of BA through rat abdominal skin. The results of this work showed a close relationship between degree of hydration and the permeation profile of BA.

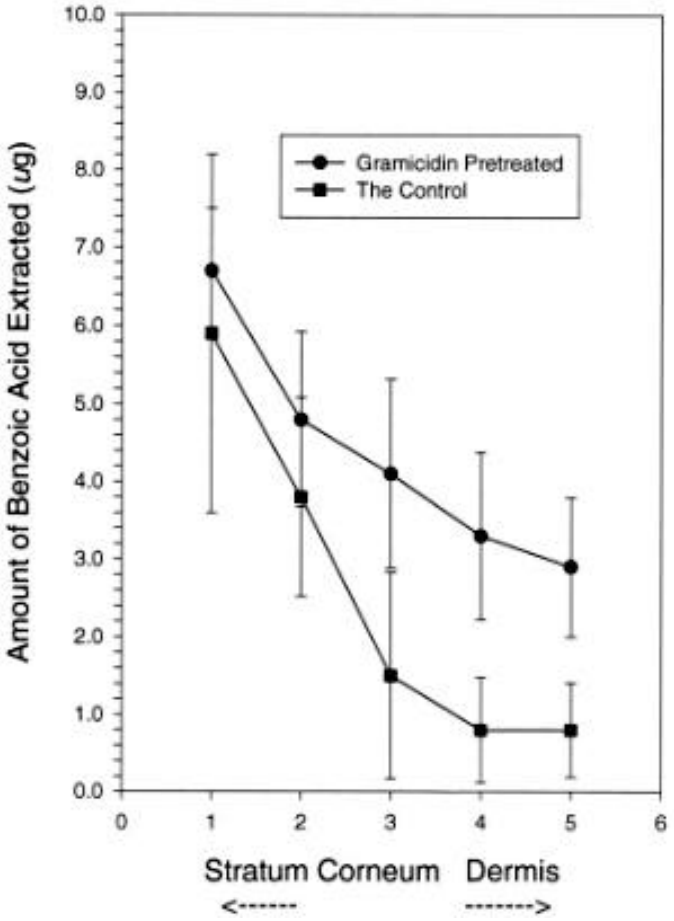

Figure 3. Distribution profile of benzoic acid within rat abdominal skin after 12-hour permeation study (N=6)

Measurement of the Total Amount of Phospholipid Phosphorous

The results of phospholipid extraction study showed that there was no significant difference in the amount of lipid extraction between gramicidin-treated skin samples and the untreated control (8.85 ± 1.08 vs. 9.14 ± 1.11 nmol/mg dry weight of the control), indicating that the lipid amount in rat abdominal skin was not affected by gramicidin pretreatment. Gramicidin did not cause any lipid extraction from the membrane, but seemed to affect the ordered conformation in lipid bilayer. Gramicidin is known to interact with various lipids and maintain the liquid crystalline state [15,16]. An enhancement of disorder in lipid bilayer seems to facilitate the permeation rates of exogenous compounds through the skin membrane.

CONCLUSIONS

The permeation rate of exogenous compounds through the skin membrane can be increased by various strategies, such as an addition of excipients that enhance the skin permeation by altering the solubility of the active ingredient in the formulation, reversibly damaging the membrane conformation or optimizing the ionization state of the drug. Most enhancers increase the permeation rate by regulating lipophilic barriers, by means of improving the partitioning coefficient or disrupting the ordered lipid bilayers, and, consequently, those agents have less significant effect on the permeation rate of hydrophilic compounds than lipophilic compounds.

In this study, the permeation rate is determined by the extent of benzoic acid (BA) transport. BA, which is a hydrophilic compound and is favored by an aqueous environment, is one of the most commonly used preservatives in cosmetics, foods, and drug preparations. The permeation profile of BA through rat abdominal skin followed a dose-dependent pattern. As the loading dose increased, so did the permeation rate of BA. The effects of loading volumes on the permeation rate of BA through rat abdominal skin were also apparent. As the loading volume increased, the permeation rate of BA through rat abdominal skin decreased.

When the skin was pretreated by gramicidin 30 minutes before BA loading, there was a significant increase in the permeation rate of BA through rat abdominal skin, indicating that gramicidin can be used as a permeation enhancer of BA. Most chelating agents, including gramicidin, appear to increase the heterogeneity of the skin and consequently reduce its diffusional resistance by opening new penetration routes and increasing the disorder of the lipid matrix. The gramicidin channel is readily permeable to water and is narrow enough to exclude other non-electrolytes such as urea. The gramicidin channel is regarded as a single-file process for both ions and water.

Most permeation processes of hydrophilic compounds take place in an aqueous phase of the membrane [17]. Water is a small molecule (a diameter of 0.28 nm) and an exceptionally flexible compound, so it can penetrate through microscopic pores between lipid molecules. The major force for organizing biological water is the hydrogen bond, and its organization is influenced by repulsive forces, such as are found near hydrophobic domains of membrane protein and lipid [18]. The most likely targets for water bonding are the oxygen atoms of phosphate and carbonyl groups of gangliosidic sialic acid residue. Phospholipid morphology of the membrane is affected by the presence of water in this residue [19]. A marked increase in water permeability, about 30–100 fold, was found as a bilayer passed through a temperature-dependent phase transition, but water permeability was not strongly influenced by hydrocarbon chain length or unsaturation so long as bilayer was fluid [20].

There was a significant difference in BA content in tissues located toward the epidermis between those that were gramicidin-pretreated and the untreated control. The skin barrier function is intimately related to the degree of the skin hydration, and a low volume is more compatible with the limited amount of water in the skin. The result of the degree of hydration acquired by the Karl Fisher method clearly supported the volume effects on the skin permeability to exogenous compounds. Therefore, the changes in the skin permeability to BA upon gramicidin pretreatment were closely correlated with the content of water remaining in the skin. Gramicidin-induced water transport seems to occur through ion permeable gramicidin channels. There was no significant difference in phospholipid phorous contents in the skin between gramicidin-pretreated and untreated control. The extraction process of lipid, one of the proposed mechanisms previously reported with other enhancers such as ethanol, is not applicable in the case of the skin pretreated by gramicidin. An increase in the heterogeneity of the skin caused by opening new penetration routes and inducing the disorder of the lipoidal matrix appeared to contribute to the effects of gramicidin on the marked enhancement of the permeation rate of BA through rat abdominal skin. The results of this work provided basic information about the role of the lipid bilayer and the hydration rate of the skin, and further elucidated the molecular mechanism that underlies percutaneous absorption.

REFERENCES

1. Yamashita F, Bando H, Koyama Y, Kitagawa S, Takakura, Y, Hashida, M. In vivo and in vitro analysis of skin penetration enhancement based on a two-layer model with polar and non-polar routes in the stratum corneum. Pharm Res. 1994;11:185-191.

2. Phillips CA, Michiniak BB. Transdermal delivery of drugs with differing lipophilicities using azone analogs as dermal penetration enhances. J Pharm Sci. 1995;84:1427-1437.

3. Flynn GL, Yalkowsky SH, Roseman TJ. Mass transport phenomena and models: theoretical concepts. J Pharm Sci. 1974;63:479-510.

4. Ozawa T, Takahashi M. Skin hydration: Recent Advances. Acta Derm Venereol. Suppl. 1994;185:26-28.

5. Iazzo PA, Olsen RA, Seewald MJ, Powis G, Stier A, Van Dyke RA. Transient increases of intracellular Ca++ induced by volatile anesthetics in rat hepatocytes. Cell Calcium. 1990;11:515-524.

6. Reed PW, Lardy HA. A23187: Adivalent Cationionophore. J Biol Chem. 1972;247:6970.

7. Wallace BA. Gramicidin channels and pores. Ann Rev Biophysic Chem. 1990; 19:127

8. Araki M, Inaba H, Kon S, Imai M, Mizuguchi T. Effects of volatile anesthetics on the calcium ionophore A23187-mediated alterations in hepatic flow and metabolism in the perfused liver in fasted rats. Acta Anesthesiol Scan. 1997;41:55-61.

9. Plakas SM, James MO. Bioavailability, metabolism, and renal excretion of benzoic acid in the channel catfish (*Ictalurus punctatus*). Drug Metab Dispos. 1990;18(5):552-556.

10. French DL, Haglund BO, Himmelstein KJ, Mauger JW: Controlled release of substituted benzoic and naphthoic acids using Carbopol gels: measurement of drug concentration profiles and correlation to release rate kinetics. Pharm Res. 1995;12(10):1513-1520.

11. Miller GR, Smith CA, Stauber WT. Determination of fibrosis from cryostat sections using high performance liquid chromatography: skeletal muscle. Histochem J. 1999;31:89-94.

12. Bartlett GR. Phosphorous assay in column chromatography. J Biol Chem. 1959;234:466-468.

13. Kabadi MB, Chien YW. Intravaginal controlled administration of flurogestone acetate IV: *in-vitro* and *in-vivo* correlation for intravaginal drug delivery from rate-control vaginal pessary. Drug Develop Ind Pharm. 1985; 11:1313-1361.

14. Lee CH, Vyavahare N, Zand R, Kruth H, Schoen FJ,Bianco R,. Levy RJ. Inhibition of aortic wall calcification in bioprosthetic heart valves by ethanol pretreatment: Biochemical and biophysical mechanisms. J Biomed Mater Res. 1998;42(1):30-37.

15. Hu W, Cross TA. Tryptophan hydrogen bonding and electric dipole moments: functional roles in the gramicidin channel and implications for membrane proteins. Biochemistry. 1995;34:14,147-14,155.

16. Heitz F, Gavach C. Ca+2 gramicidin A interactions and blocking effects on the ionic channel. Biophysical Chemistry. 1983; 18:153-163.

17. Mitchell DC, Litman BJ. Effect of hydration on receptor conformation: decreased levels of bound water promote metarhodopsin II formation.Biochemistry. 1999; 38: 7617-7623.

18. Kuruc M, Krupey J. Application of an insoluble protein precipitation reagent. Am Biotechnol. 1992;10(3):12-14.

19. Hwang J, Tamm LK, Bohm C, Ramalingam TS, Betzig E, Edidin M. Nanoscale complexity of phospholipid monolayers investigated by near-field scanning optical microscopy. Science. 1995; 270:610-614.

20. Carruthers A, Melchior DL. Human erythrocyte hexose transporter activity is governed by bilayer lipid composition in reconstituted vesicles. Biochemistry. 1984;23(26):6901-6911.