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Transdermal absorption enhancement through rat skin of gallidermin loaded in niosomes

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ABSTRACT

Gallidermin (Gdm) loaded in anionic niosomes composed of Tween 61/CHL/DP (1:1:0.05 molar ratio) gave the highest entrapment efficiency (45.06%). This formulation gave antibacterial activity against *Propionibacterium acnes* and *Staphylococcus aureus* with the MIC and MBC of 3.75 and 7.5; 7.5 and 15 μg/μl, respectively. Gdm loaded in niosomes was more chemically stable than Gdm in aqueous solution of about 1.5 times. Gdm loaded and unloaded in niosomes were not found in the receiver solution investigated by vertical Franz diffusion cells at 37 °C for 6 h. Gdm loaded in niosomes showed higher cumulative amounts in viable epidermis and dermis (VED) of rat skin of about 2 times more than unloaded Gdm. Gdm loaded in niosomes and incorporated in gel exhibited the highest cumulative amounts ($82.42 \pm 9.28\ \mu g\ cm^{-2}$) and fluxes ($13.74 \pm 1.55\ \mu g\ cm^{-2}\ h^{-1}$) in stratum corneum (SC) and comparative cumulative amounts ($183.16 \pm 30.32\ \mu g\ cm^{-2}$) and fluxes ($25.74 \pm 5.05\ \mu g\ cm^{-2}\ h^{-1}$) in VED to the unloaded Gdm incorporated in gel. This study has suggested that Gdm loaded in anionic niosomes and incorporated in gel is the superior topical antibacterial formulation because of the high accumulation in the skin with no risk of systemic effect.

1. Introduction

Topical application of drugs promises many advantages over oral or intravenous administration. It offers many advantages over conventional administration such as avoidance of first pass metabolism and elimination of gastrointestinal irritation resulting in the improvement of patient convenience and compliance (Verma and Chandak, 2009). However, the major barrier of the skin is the stratum corneum (SC), the top layer of the epidermis. Low molecular weight (≤500 Da), lipophilicity, and effectiveness at low dosage are the ideal characteristics of the drugs for transdermal delivery. However, many drugs do not possess ideal physicochemical properties. Thus, manipulation of the drug or vehicle to enhance diffusion through skin becomes necessary.

Gallidermin (Gdm), a result of post-translational enzymatic modifications, contains some unusual amino acid residues such as lanthionine, β-methyllanthionine or α,β-didehydroamino acids. These amino acids building intramolecular thioether bridges are used for the chemical characterisation of a group of peptide antibiotics called lantibiotics (Kellner et al., 1988). Lantibiotics are generally divided into two classes on the basis of their structures: type-A lantibiotics are elongated, amphiphilic peptides, while type-B lantibiotics are compact and globulars. The principle biological activity of most type-B lantibiotics appears to be directed at the inhibition of enzyme functions and type-A lantibiotics kill bacterial cells by forming pores in the cytoplasmic membrane (Hans-Georg et al., 1995). Gdm is a 22 amino acid type-A lantibiotic secreted by *Staphylococcus gallinarum* TÜ 3928 (Kempf et al., 1999) which occurs as part of the natural flora of chicken crests. Gdm is a smallest so-far-reported type-A lantibiotic with tetracyclic structure, 4 thioether bridges and net charge +3 (Kellner et al., 1988). Gdm is soluble in water, ethanol and methanol (Rollema et al., 1995). The three-dimensional structure analysis of several type-A lantibiotics in solution state indicated that they can form amphiphilic structure with the hydrophobic and hydrophilic residues aligning on opposite faces of the cylinder, the central region of which contains a 'hinge' with increase flexibility (Freund et al., 1991). Gdm has a potential advantage for the treatment of endocarditis, abscesses and skin infections. This drug is as effective as the renowned antibiotics in current clinical practice like erythromycin or fusidin (Kellner et al., 1988). Furthermore, Gdm is highly active

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against *Propionibacterium acnes*, the cause of human acne disease. In clinical investigation, 25 patients with bacteria-induced skin diseases were treated with Gdm in preliminary clinical tests. Improvement of the condition or complete healing was observed over 70% of the cases (Actapharm, 2002). In human medicines, Gdm are particularly promising for the topical pharmaceutical therapy and cosmetic treatment of acne. Gdm may be useful for the treatment of multidrug resistant *Staphylococcus aureus* strains, which is an increasing problem world-wide especially in the hospitals (Kempf et al., 1999). The bacterial strains that produce Gdm are isolated from human skin and are most probably normal flora of the skin, and provide natural protection (Cotter et al., 2005). Gdm is stable at the skin pH (5.4) and has quite narrow spectrum of inhibition, thereby reducing possible side effects. Gdm has no major toxicological side effects or skin problems. Series of clinical tests have supported. No negative effects were observed when Gdm was added to lymphocyte cultures. Only reversible eye irritations were observed in eye tests using rabbits which were probably caused by the alcoholic formulation. Toxicological tests in mice gave similar results as with other highly active antibiotics (Actapharm, 2002). Maher and McClean (2006) have investigated the cytotoxicity of natural magainin peptides, nisin A, Gdm, and melittin on two robust intestinal epithelial cells (HT29 and Caco-2) with the MTT conversion assay when compared with vancomycin. Gdm was the least cytotoxic antimicrobial peptides followed by nisin A. Gdm is a most promising therapeutic agent, with relatively low cytotoxicity and potent antimicrobial activities (Maher and McClean, 2006). However, long-term stability is required for Gdm especially in the case of products with a required long shelf-life. Because of its large molecular structure, Gdm has limited absorption through skin. It also has problem of chemical stability because of its peptide nature. Niosomes are bilayer vesicular carriers composed of nonionic surfactants such as Tween and Span. The encapsulation of drugs such as peptide drugs in these vesicular systems offers several advantages, such as decreasing of toxicity, increasing of stability, circulation time and absorption of the drug (Gude et al., 2002; Manosroi et al., 2005). There is no work has been recently done on Gdm entrapped in nanovesicles and its absorption profile through rat skin. The objective of this study was to investigate transdermal absorption enhancement through rat skin of Gdm loaded in niosomes in order to evaluate its potential topical use for pharmaceuticals and cosmeceuticals.

2. Materials and methods

2.1. Materials

Gallidermin (Gdm) was from Boehringer Ingelheim International GmbH, Ingelheim am Rhein, Germany. The male Sprague–Dawley rats were from National Laboratory Animal Centre, Mahidol University, Salaya, Nakhon Pathom in Thailand, weighing between 150 and 200 g. Cholesterol (CHL, 99.6%), polyoxyethylene sorbitan monostearate (Tween 61), Dimethyldioctadecylammonium bromide (DDAB) and dicetylphosphate (DP) were from Sigma Chemical Co. (St. Louis, MO, USA). Chloroform and methanol were analytical grade from Lab-Scan. Sodium hydrogen phosphate anhydrous (Na_2HPO_4) was from Merck Company in Germany. Sodium dihydrogen orthophosphate (NaH_2PO_4) was from VWR International Ltd. in England. All other reagents were analytical grade and used without further purification.

2.2. Experimentals

2.2.1. Preparation of drug-loaded niosomes

Empty niosomes were prepared by freeze-dried empty liposomes (FDEL) method (Kikuchi et al., 1999). The neutral niosomes were composed of Tween 61/CHL in the molar ratio of 1:1, whereas cationic and anionic niosomes were prepared from Tween 61/CHL/DDAB and/or DP in the molar ratio of 1:1:0.05. Briefly, the surfactants together with cholesterol and anionic (DP) or cationic lipid (DDAB) were dissolved in chloroform. The solvent was removed by a rotary evaporator (R-124 Buchi, Switzerland) to get a thin film. The film was dried by evacuation in a desiccator under reduced pressure for over 12 h. Distilled water was added to the film to obtain a dispersion, which was further swelled by swirling in a water bath at 45 °C for 30 min. The resulting dispersion was sonicated by a microtip probe sonicator (Vibra CellTM, Sonics & Materials, Inc., Newtown, CT, USA) at pulse on 3.0 and pulse off 2.0, 25% amplitude, for 5 min. The dispersion was then lyophilized by a freeze-dryer (Alpha 1-2 LD model Christ, Germany), and kept at 4 °C until use. Gdm was entrapped in the dried powder empty niosomes by reconstitution. Briefly, Gdm (1 mg/ml) in phosphate buffer (20 mM, pH 5.4) was used to disperse the niosome powder. The dispersion was gently mixed by hands for 10 min at room temperature (30 ± 2 °C). The resulting dispersion was in white dispersion with no precipitation.

2.2.2. Physical characteristics of Gdm loaded in niosomes

2.2.2.1. The morphology of Gdm loaded in niosomes. The lamellarity of Gdm entrapped in niosomes was observed by a TEM (80 kV, TEM1200SJEOL, JEOL Ltd., Tokyo, Japan) using negative staining technique employing 2% (w/v) of ammonium molybdate solution. A drop of the dispersion was applied on a 300-mesh formvar copper grid on paraffin and allow the sample to adhere on the formvar for 10 min. The remaining dispersion was removed and a drop of 2% aqueous solution of ammonium molybdate was applied for 5 min. The remaining solution was then removed. The sample was air dried and examined with a TEM. The morphology and lamellarity of the bilayer vesicles were observed.

2.2.2.2. The vesicular sizes and ζ (zeta) potential determination. The vesicular sizes of the bilayer vesicles were determined by dynamic light scattering (Zetasizer Nano Series Nano-S, Malven instrument). The diameter of the vesicles was measured in three individual runs at 25 °C using the non-negative constrained least squares (NNLS) algorithm mode by the particle size distribution (PSD) analysis on a dynamic light scattering (DLS) technique. The ζ (zeta) potential of all samples was obtained by the phase analysis light scattering (PALS) software for data analysis in five individual runs. The time-dependent correlation function on the scattered light intensity was measured at a scattering angle of 90°.

2.2.2.3. Entrapment efficiency determination of Gdm in niosomes. The entrapped Gdm in niosomes was separated from the free peptide by gel filtration using Sepharose CL 6 B (Fluka Chemicals, Gillingham, Dorset, UK) as a packing material and purified water as an eluent. Eluates were collected in fractions using a fractional collector (Foxy JR, Isco Inc., Lincoln, USA) at the flow rate of 10 ml/min. The fractions containing the peptide-loaded vesicles which were detected at 470 nm were pooled, collected and dried using a freeze-dryer (Alpha 1-2 LD model Christ, Germany). The remaining residues were dissolved in absolute methanol and assayed for the Gdm contents by HPLC (AS 1000, Thermo Finigan, USA) using a Luna C 18 HPLC column (250 mm × 40 mm, 10 μm) and acetonitrile/water (6:4, v/v) mixed with 0.1% TFA as the mobile phase, isocratically at ambient temperature with the flow rate of 1 ml/min at 267 nm. Peak areas were calculated and the concentrations of Gdm were determined from the standard curves. The percentage of Gdm entrapment in niosomes was calculated from the ratio of the peptide entrapped in the vesicles to the total initial peptide contents multiplied by 100.

2.2.3. Physicochemical stability of the loaded and unloaded Gdm in niosomes

An aliquot of the freshly prepared Gdm solution and Gdm loaded in niosomes were filled in transparent vials. The vials were kept at 4 ± 2 °C, room temperature (30 ± 2 °C) and 45 ± 2 °C for 4 months. The physical stability of the dispersions was observed visually. The morphology of the niosomes was observed under TEM. At 0, 1, 2, 3 and 4 months, the samples were withdrawn and the Gdm contents in the samples were analyzed by HPLC. A Luna C 18 (250 mm × 40 mm, 10 μm) HPLC column was used. The mobile phase was a mixture of acetonitrile/water (6:4, v/v) mixed with 0.1% TFA with UV detection at 267 nm. HPLC was performed isocratically at ambient temperature with the flow rate of 1 ml/min. The peak areas were calculated and the concentrations of Gdm were determined from the standard curve.

2.2.4. In vitro antibacterial assay of Gdm loaded in niosomes

2.2.4.1. Agar diffusion method. Antibacterial activity was evaluated by agar diffusion method (James, 1990). BHI (Brainheart infusion broth) was dissolved in distilled water. The solution was then made up to 1000 ml with distilled water and the agar was added before sterilization. *P. acnes* and *S. aureus* (clinically isolated from Maharaj Nakorn Chiang Mai Hospital, Chiang Mai, Thailand) were streaked on BHI agar and incubated in anaerobic and aerobic condition (37 ± 1 °C, 72 h), respectively. Four to five colonies were inoculated into BHI broth (10 ml) and incubated in anaerobic or aerobic condition (37 ± 1 °C, 24 h). Then, the suspension of the bacteria was adjusted to have the density equivalent to McFarland 0.5. The suspension was spread on the BHI agar plate. When surface inoculation was used, the samples were applied to the plate in the form of impregnated paper discs (Oxoid, Basingstoke, UK). Plates were incubated at 37 ± 1 °C. Inhibition zones were measured after 24-h incubation.

2.2.4.2. Macrodilution method. Dilution susceptibility testing method (James, 1990) was used to determine the minimal concentration of the sample required to inhibit or kill the microorganisms. To prepare the basal medium composed of BHI and Tween 80, BHI was dissolved in distilled water. The solution was then made up to 1000 ml with distilled water and added with 0.5% Tween 80 before sterilization. The samples were diluted two-fold from tube nos. 1–9, then mixed with 0.005% TTC and 20 μl of *P. acnes* or *S. aureus*. The basal medium was added to each tube to adjust the volume to 1 ml. The positive and negative controls were composed of 5 μl of 0.005% TTC solution mixed with or without 20 μl of *P. acnes* or *S. aureus* and the basal medium was added to adjust the volume to 1 ml. All samples were incubated in anaerobic condition for *P. acnes* and in aerobic condition for *S. aureus* at 37 ± 1 °C for 36–48 h. The lowest concentration of the sample that completely inhibited the visible growth of the organism as detected by the unaided eye (MIC) was determined. The solution from the tube used to determine MIC and the tube with clear solution (the next tube) were inoculated in the tube with the new broth and incubated in anaerobic or aerobic condition at 37 °C for 48 h. The lowest concentration of the sample that completely inhibited the visible growth of the organism as detected by the unaided eye (MBC) is determined.

2.2.5. Transdermal absorption through rat skin by vertical Franz diffusion cells

2.2.5.1. Preparation of rat skin. Full-thickness abdominal skin was obtained from the male Sprague–Dawley rats (National Laboratory Animal Centre, Mahidol University, Salaya, Nakhon Pathom, Thailand), weighing between 150 and 200 g. Hair on abdominal area was shaved off and left overnight. The rats were sacrificed and the abdominal skin was removed. The subcutaneous fat was trimmed off and the prepared skin was freshly used. The investigational protocol for all procedures has adhered to the "Principles of Laboratory Animal Care".

2.2.5.2. Transdermal absorption experiment. Samples used for the permeability study through the excised rat skin were Gdm loaded in anionic niosomes, Gdm incorporated in gel, Gdm loaded in anionic niosomes incorporated in gel and Gdm in phosphate buffer pH 5.4. All formulations used in this study contained 1 mg/ml of Gdm.

The rat skin was mounted on the receptor compartment of the vertical Franz diffusion cells with the stratum corneum (SC) side facing upwards to the donor compartment. The donor compartment was filled with 1 ml of the samples. An amount of 12 ml of phosphate buffer saline (PBS), pH 7.4 was put in the receiver chamber. The available diffusion area (rat skin) was 2.46 cm^2. The receiver compartment was maintained at 32 ± 2 °C and stirred by a magnetic bar. All experiments were done in triplicate and carried out with non-occluded donor compartments. At 1, 3 and 6 h, the diffusion cells were withdrawn. Gdm contents in the SC, whole skin and the receiver chamber were extracted and analyzed by HPLC.

2.2.5.3. Extraction of Gdm from skin strips and whole skin. After the experiment, the rat skin was washed twice with 2 ml of distilled water. The skin was removed from the cell and swung twice in 100 ml of distilled water, and the rinsed water was discarded. The amount of Gdm in SC was collected by stripping the skin with a 3M Scotch Magic™ tape (1 cm × 1 cm) (Plessis et al., 1992). Nine tapes were used for each skin sample and were pooled in a 5 ml vial, containing 5 ml of methanol. The vial was vortexed for 5 min, allowed to stand for 10 min, and vortexed again for 5 min, prior to the analysis. The amount of Gdm in the viable epidermis and dermis (VED) was determined by cutting the stripped skin into small pieces, and pooled in a 5 ml vial, containing 5 ml of methanol, and the subsequent steps followed similar procedures as described for the extraction of Gdm content from SC.

2.2.5.4. Quantitative analysis of Gdm by high performance liquid chromatography (HPLC) with UV detection. Gdm contents were analyzed by HPLC according to the procedure previously described in Section 2.2.3.

3. Results and discussion

3.1. Characteristics of niosomes loaded with Gdm

3.1.1. The morphology, vesicular size and zeta potential of niosomes loaded with Gdm

The advantage of the preparation of the vesicles by the freeze-dried method with reconstitution is that the drug will not expose to high temperature of solvent evaporation and lyophilization, thereby reducing the risk of drug degradation especially for Gdm. Stability studies of Gdm have demonstrated that it is stable in aqueous or ethanolic solution or in propylene glycol at 37 °C of up to 1 month. Moreover, it appears to be most stable at acidic pH in the pH range of 3.0–5.0 of phosphate and acetate buffers (Rollema et al., 1995). Thus, a freeze-dried method with reconstitution at 45 °C in phosphate buffer at pH 5.4 for 30 min was selected to use in this study. This procedure has also been applied to our previous studies for entrapping tyrosinase-encoding plasmid (Manosroi et al., 2008). After lyophilization, all empty niosomes (neutral, cationic and anionic) were in white powder. When reconstituted in phosphate buffer (20 mM, pH 5.4), all empty niosomal dispersion showed best physical stability with no sedimentation for 3 months at 4 ± 2, 45 ± 2 °C and room temperature (30 ± 2 °C). The morphology, vesicular size and zeta potential of all niosomes loaded with Gdm determined by TEM and DLS technique were

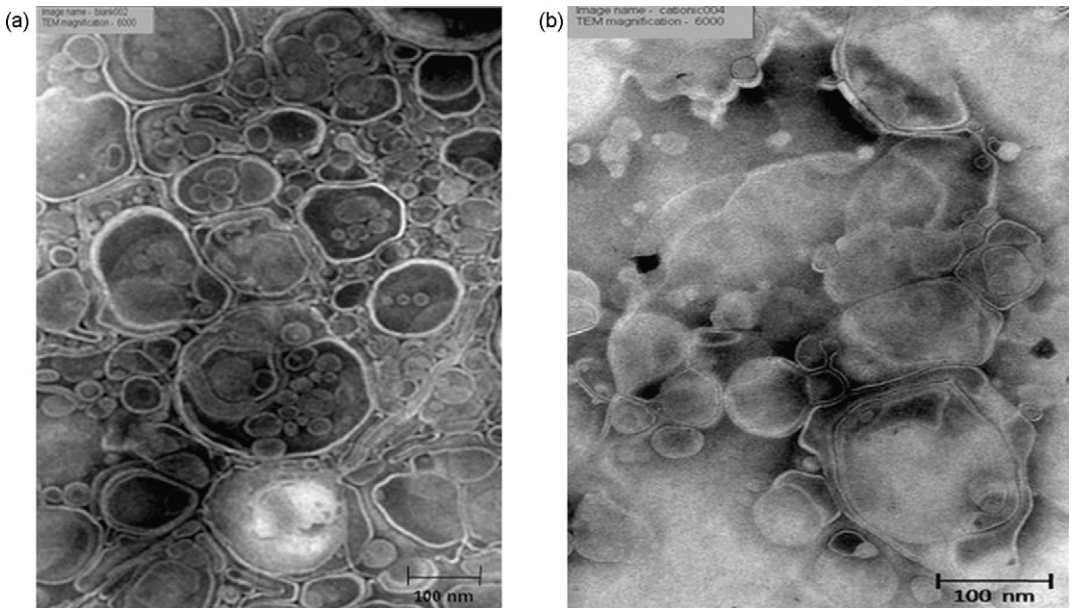

Fig. 1. The negative staining TEM images of (a) blank anionic niosomes (Tween 61/CHL/DP at 1:1:0.05 molar ratio) and (b) Gdm loaded anionic niosomes (Tween 61/CHL/DP at 1:1:0.05 molar ratio).

in oligolamellar structure and in nanosize ranges of 145–275 nm, respectively. Fig. 1 shows the negative staining TEM images of the anionic niosomes loaded and not loaded with Gdm. The vesicular sizes of neutral and cationic niosomes loaded with Gdm were 273.67 ± 2.34 and 275.78 ± 3.15 nm, respectively which were larger than the anionic niosomes loaded with Gdm (148.56 ± 1.19 nm). Due to the structure of the bilayer vesicles in an aqueous solution that usually obtained by the arrangement of the amphiphilic molecules for example Tween 61, the hydrophilic and hydrophobic chains at these molecules can be assembled by arranging with the hydrophobic heads facing each other as the bilayer membrane and the polar or hydrophilic heads expose to water. The aqueous phases (layers) are between the vesicular bilayers. The charges of the vesicles are usually located on the bilayers and the outer membrane surface (Kikuchi et al., 1999). The adsorbed or entrapped Gdm (with the positive charges) can neutralize the anionic charges of the anionic (negatively charged) niosomes and reduce the repulsion force between the bilayers resulting in the decrease of particle size, while the cationic or positively charged niosomes have larger size from the repulsion between the cationic niosomes and Gdm. The zeta potentials were positive for Gdm solution (+45.7 ± 0.66 mV), blank cationic niosomes (+3.63 ± 0.16 mV) and cationic niosomes loaded with 1 mg/ml of Gdm (+41.6 ± 0.21 mV). The zeta potentials were negative for blank anionic niosomes (−42.5 ± 0.39 mV), blank neutral niosomes (−24.5 ± 0.41 mV), neutral niosomes loaded with 1 mg/ml of Gdm (−18.7 ± 0.47 mV) and anionic niosomes loaded with 1 mg/ml of Gdm (−49.8 ± 0.32 mV). The negative zeta potential value of blank neutral niosomes was conformed with our previous study (Manosroi et al., in press) that the blank neutral niosomes (cholesterol/Tween 61 at 1:1 molar ratio) both dispersed in distilled water and phosphate buffer system indicated negative zeta potential values. Moreover, the vesicles prepared from only cholesterol and dispersed in distilled water and phosphate buffer at pH 5.4 also showed negative zeta potential value of (−)36.8 ± 5.27 and (−)41.2 ± 2.11, respectively (data not shown). This may be due to the hydroxyl group in the cholesterol molecule which has an uneven distribution of polarity. The partial negative property from the oxygen molecule and the partial positive property from the hydrogen molecule in the hydroxyl group have contributed to the positive and negative poles of the cholesterol molecule (Masterjohn, 2005). In a buffer system, the vesicles such as niosomes are surrounded by the double layers of dense layers (counter ions) which have opposite charges to the electrical charges of niosomes (McLaughlin et al., 1971). The binding of the charged ions in the buffer system on the niosomal membrane can affect the zeta potential values. The partial positive charge distribution of the cholesterol molecules composed in niosomes may be binded by the phosphate ions in the phosphate buffer resulting in the negative zeta potential values detected by PALS. The negative zeta potential value of anionic niosomes even loaded with Gdm was still observed. This can be described by the previous study of Kotyńska and Figaszewski (2005) that have evaluated the effect of the adsorption of ions in the solution to the electric charge of the phospholipid membrane. In acidic solution, the adsorption of negative charge ions in solution to the phospholipid membrane was increased resulting in the decrease of the positive charge of the system.

3.1.2. *Entrapment efficiency of Gdm loaded in niosomes*

The percentages of entrapment efficiency of Gdm in neutral, cationic and anionic niosomes determined by gel filtration and HPLC were 30.51, 10.57 and 45.06%, respectively. Anionic niosomes gave the highest entrapment efficiency of Gdm at 45.06%. Many literatures have reported that the water soluble substances can be entrapped in bilayer vesicles of not more than 10–20% (Hauser, 1982; Peltonen et al., 2002; Ferreira et al., 2004). In this study, the entrapment efficiency of Gdm in anionic niosomes was much higher than this amount owing to the charge interaction between the anionic niosomes and the positive charge of Gdm (p*I* 9.8) in the system of phosphate buffer (pH 5.4) and the adsorption of Gdm on the niosomal membrane. Moreover, the neutral niosomes also showed higher entrapment efficiency than the theoretical value due to the negative property of neutral niosomal membrane that gallidermin can not only entrapped in the aqueous layer between the bilayers, but also adsorbed on the niosomal membrane. The zeta potential value of the blank neutral niosomes is (−)24.5 ± 0.41 mV. This has agreed with the previous report that the cationic type-A lantibiotics can accumulate at the membrane and also on artificial phopholipid bilayers through ionic interaction (Kordel et al., 1989; Sahl et al., 1991). The total entrapment efficiency was calculated

Table 1
Quantitative analysis of Gdm remaining in various systems by HPLC when kept at 4 ± 2 °C, room temperature (30 ± 2 °C) and 45 ± 2 °C for 4 months.

System	Percentages remaining of Gdm					
	At initial			4 months		
	4 ± 2 °C	RT (30 ± 2 °C)	45 ± 2 °C	4 ± 2 °C	RT (30 ± 2 °C)	45 ± 2 °C
Gdm in phosphate buffer, pH 5.4 (1 mg/ml)	100%	100%	100%	75.0%	68.6%	60.7%
Gdm (1 mg/ml) in neutral niosomes (Tween 61/CHL 1:1)	100%	100%	100%	94.0%	92.9%	90%
Gdm (1 mg/ml) in cationic niosomes (Tween 61/CHL/DDAB 1:1:0.05)	100%	100%	100%	95.6%	94.8%	91.0%
Gdm (1 mg/ml) in anionic niosomes (Tween 61/CHL/DP 1:1:0.05)	100%	100%	100%	94.0%	93.5%	92%

Table 2
MIC and MBC values against *P. acnes* and *S. aureus* of the unloaded and loaded Gdm in anionic niosomes.

Systems	*P. acnes*		*S. aureus*	
	MIC (μg/μl)	MBC (μg/μl)	MIC (μg/μl)	MBC (μg/μl)
Gdm (15 μg/μl) in phosphate buffer, pH 5.4	0.468 (1/32 dilution)	0.937 (1/16 dilution)	0.937 (1/16 dilution)	1.875 (1/8 dilution)
Gdm (15 μg/μl) loaded in anionic niosomes	3.750 (1/4 dilution)	7.5 (1/2 dilution)	7.5 (1/2 dilution)	15 (1/1 dilution)

Note: MBC: minimum bactericidal concentration; MIC: minimum inhibitory concentration.

from both adsorbed and encapsulated Gdm. The sustained release profile from the first rapid release of the adsorbed drug and followed by the slow release of the entrapped Gdm was expected. This anionic niosomal formulation was selected to investigate for antibacterial and transdermal absorption through rat skin.

3.2. Chemical stability of Gdm loaded in niosomes

Gdm solution and Gdm loaded in niosomes (at 1 mg/ml) were kept in transparent glass vials and stored at 4 ± 2 °C, room temperature (30 ± 2 °C) and 45 ± 2 °C for 4 months. The pH of Gdm solution and Gdm loaded in niosomes was in the range of 5.50–5.55 and 5.45–5.55, respectively. The amount of Gdm was determined by HPLC (λ = 267 nm) at different time intervals (0, 1, 2, 3 and 4 months). The percentages of Gdm remaining in the niosomal formulations were calculated from the Gdm content at each time intervals in comparing to the initial amount. At all temperatures, the physical changes of all niosomes were not observed in 4 months. The initial pH values were 5.45, 5.43 and 5.44 for neutral, cationic and anionic Tween 61 niosomes, respectively. The pH values of all formulations slightly increased in 4 months. This may be from the niosomal formulations containing ether linkage between the polar head group and the acyl in Tween 61 molecule chains which may reduce the hydrolysis of the acyl chain. At optimal pH (normally between 5 and 6), only 5–10% of the chains were hydrolyzed in 1 year (Torchilin and Weissig, 2003). Table 1 shows the effect of temperature on the degradation of Gdm especially when in an aqueous solution. After 4 months, the remaining percentages of Gdm in solution at 4 ± 2 °C, room temperature (30 ± 2 °C) and 45 ± 2 °C were 75.0, 68.6 and 60.7%, respectively. The remaining percentages of Gdm loaded in neutral niosomes, cationic niosomes and anionic niosomes after 4 months at 4 ± 2 °C, room temperature (30 ± 2 °C) and 45 ± 2 °C were 94.0, 92.9 and 90.0%; 95.6, 94.8 and 91.0%; 94.0, 93.5 and 92.0%, respectively. Gdm loaded in all niosomes exhibited more chemically stable during 4 months than in an aqueous solution. The vesicles appeared to protect Gdm from vigorous conditions during the entrapment process and the high storage temperature. This has agreed with our previous study which has demonstrated that stability of the entrapped plasmid in the vesicles was due to the vesicular protection of the plasmid against high temperature (Manosroi et al., 2008).

3.3. In vitro antibacterial assays of Gdm loaded in niosomes

3.3.1. Agar diffusion assay

Gdm (30 μg) in distilled water, the positive control (15 μg of erythromycin), blank anionic niosomes and Gdm (26.7 μg) loaded in anionic niosomes gave the inhibition zone diameters of 19, 7.5, 0 and 7 mm against *P. acnes* by disc diffusion method and 21, 0, 0 and 10 mm by surface inoculation technique (data not shown). It is known that Gdm is as effective as the renowned antibiotics in current clinical practice like erythromycin with the MIC value against *P. acnes* of 0.125 μg/μl (Kempf et al., 1999). At the same concentration of 15 μg, Gdm indicated same inhibition zone in comparing to erythromycin. However, when Gdm loaded in anionic niosomes, it gave half the inhibition zone of erythromycin. The loaded Gdm may be not released and only the unentrapped or the adsorbed Gdm on the niosomal membrane gave this effect. In fact, the entrapment of Gdm in this niosomal system was about 50%. Thus, the remaining half of the total Gdm which was unloaded in niosomes should have the same inhibition effect as erythromycin or Gdm at 15 μg. This may be due to the masking effect from niosomes which may interfere with the diffusion of Gdm in the agar plate. Although Gdm loaded in anionic niosomes gave lower inhibition zone than erythromycin, it is advantageous not only for the nonbacterial resistant property of Gdm (Kellner et al., 1988), but also the enhancement

Table 3
The cumulative amounts (μg cm^{-2}) and fluxes (μg cm^{-2} h^{-1}) of Gdm from various systems in stratum corneum (SC), viable epidermis and dermis (VED) and receiver compartment solution following transdermal absorption across excised rat skin at 6 h by vertical Franz diffusion cells.

Systems	Cumulative amounts (μg cm^{-2}) ± S.D.			Fluxes (μg cm^{-2} h^{-1}) ± S.D.		
	SC	VED	Receiver compartment	SC	VED	Receiver compartment
Gdm solution (phosphite buffer, pH 5.4)	40.08 ± 2.81	36.10 ± 0.36	0	6.68 ± 0.47	6.02 ± 0.06	0
Gdm loaded in niosomes (Tween 61/CHL/DP 1:1:0.05)	22.62 ± 1.16	66.19 ± 14.19	0	3.77 ± 0.19	11.38 ± 2.36	0
Gdm incorporated in gel	54.09 ± 3.50	211.54 ± 9.51	0	8.61 ± 0.58	35.25 ± 1.59	0
Gdm loaded in niosomes and incorporated in gel	82.42 ± 9.28	183.16 ± 30.32	0	13.74 ± 1.55	25.74 ± 5.05	0

Note: Each value represented the mean ± S.D. (n = 3).

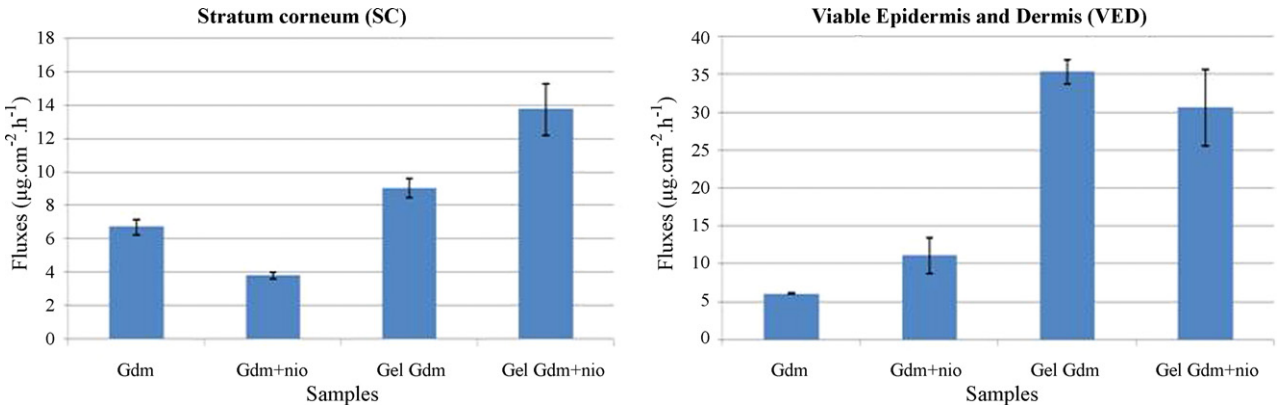

Fig. 2. The fluxes ($\mu g\,cm^{-2}\,h^{-1}$) of Gdm from various systems in stratum corneum (SC) and viable epidermis and dermis (VED), at 6 h by vertical Franz diffusion cells (each value represents mean ± S.D., $n = 3$). Gdm: Gdm in phosphate buffer (20 mM, pH 5.4); Gdm + nio: Gdm loaded in niosomes (Tween 61/CHL/DP at 1:1:0.05 molar ratio); Gel Gdm: Gdm incorporated in gel; Gel Gdm + nio: Gdm loaded in niosomes and incorporated in gel.

of chemical stability of Gdm when loaded in niosomes. The similar anti-*S. aureus* effects of Gdm loaded in niosomes to *P. acnes* were also observed. Gdm (30 μg) in aqueous solution, the positive controls (15 μg of erythromycin), blank anionic niosomes and Gdm (26.7 μg) loaded in anionic niosomes gave the inhibition zone diameters of 19, 7.5, 0 and 7 mm against *S. aureus* by disc diffusion method and 27, 12.5, 0 and 17 mm by surface inoculation technique, respectively. Gdm loaded in anionic niosomes gave lower inhibition zone than Gdm in aqueous solution owing to the stability and the sustained release property of Gdm in niosomes.

3.3.2. Macrodilution assay

Table 2 shows the MIC and MBC values of all systems against *P. acnes* and *S. aureus*. The MIC and MBC of Gdm solution against *P. acnes* were at 1/32 and 1/16 dilutions while those of *S. aureus* were at 1/16 and 1/8 dilutions. Gdm loaded in anionic niosomes showed MIC and MBC against *P. acnes* at 1/4 and 1/2 dilutions and against *S. aureus* at 1/2 and 1/1 dilutions. Gdm loaded in anionic niosomes gave higher MIC and MBC indicating lower activity than Gdm solution. Similar to the agar diffusion assay, when Gdm entrapped in anionic niosomes, the antimicrobial property of Gdm was from the Gdm which was not loaded but adsorbed on the surface of the niosomal membrane. The unloaded and the adsorbed Gdm appeared to have the initial antimicrobial activity and the loaded Gdm will be further slowly released to give the sustained antimicrobial effect.

3.4. Transdermal absorption through rat skin

The cumulative amounts of Gdm in all systems were increased with times during the period of 6 h (data not shown). The cumulative amounts of Gdm from various systems in stratum corneum (SC), viable epidermis and dermis (VED) and the receiver solution following transdermal absorption across the excised rat skin were shown in Table 3. No Gdm from all systems was found in the receiver compartment. This may be due to the large molecular structure of Gdm that limits the absorption through skin (Amsden and Goosen, 2004) as well as the large niosomal structure. Thus, no risk of systemic side effects even Gdm was loaded in niosomes. However, Gdm loaded in niosomes showed more Gdm accumulation in VED of about 2 times than the unloaded Gdm. This has demonstrated the transdermal enhancement of Gdm when entrapped in niosomes. Nevertheless, when the unloaded Gdm incorporated in gel formulation, the accumulation of Gdm in VED was increased of about 6 and 3 times in comparing to the Gdm in solution and Gdm loaded in niosomes, respectively. As known, the gel formulation can also give the occlusion effect thereby increasing the absorption of Gdm. The retarding effect of the loaded Gdm when incorporated in gel was obviously observed since the highest cumulative amount of Gdm was observed in SC.

Table 3 and Fig. 2 show the fluxes at 6 h of Gdm from various systems. Gdm loaded in niosomes incorporated in gel exhibited the highest flux in SC at $13.74 \pm 1.55\ \mu g\,cm^{-2}\,h^{-1}$, while Gdm solution, Gdm loaded in niosomes and Gdm loaded in niosomes incorporated in gel gave the fluxes at 6.68 ± 0.47, 3.77 ± 0.19 and $8.61 \pm 0.58\ \mu g\,cm^{-2}\,h^{-1}$, respectively. Similar to cumulative amounts, fluxes of Gdm in VED increased when loaded in niosomes and incorporated in gel. The synergistic effects on fluxes in SC but not in VED were observed when Gdm loaded in niosomes and incorporated in gel. Thus, Gdm loaded in niosomes and incorporated in gel can provide the reservoir of Gdm in SC for the sustained release effect. This system also gave high accumulation of Gdm in the dermis and epidermis (VED) almost the same as the unloaded Gdm incorporated in gel. Moreover, Gdm loaded in niosomes did not only demonstrate more chemical stability than the unloaded Gdm but also superior and similar cumulative amounts and fluxes to the unloaded Gdm incorporated in gel in SC and VED, respectively. Entrapped drugs in niosomes protected from the degradation by proteolytic enzyme including the stratum corneum chymotryptic enzyme (SCCE) has been reported (Sondell et al., 1994; Biju et al., 2006). As known, the gel structure can promote the penetration of Gdm across the SC owing to the occlusion effects from the gel formulation which can enhance skin hydration and consequently increase the absorption and penetration of Gdm across the rat skin (Mak et al., 1991; Cevc et al., 2008). However, Gdm loaded in niosomes and incorporated in gel appeared to have less risk of systemic effects owing to the lesser cumulative amounts in VED than the unloaded Gdm incorporated in gel.

4. Conclusion

The anionic niosomes composed of Tween 61/CHL/DP (at the molar ratio of 1:1:0.05) loaded with 1 mg/ml of Gdm gave the nanosize range of about 149 nm, the zeta potential of about −50 mV and the highest entrapment efficiency of Gdm at 45.06% which were higher than the theoretical value due to the effect of charge interaction between the anionic niosomes and cationic charge of Gdm at pH 5.4. The antibacterial activity assay of Gdm loaded in niosomes gave lower antibacterial activity against *P. acnes* and *S. aureus* than the unloaded Gdm owing to the masking and sustained release effect of niosomes. Gdm loaded in niosomes was more chemically stable than Gdm in an aqueous solution when exposed to high temperatures. Gdm loaded or unloaded in niosomes was not found in the receiver compartment solution investigated by vertical Franz diffusion cells. In gel formulation, Gdm loaded in niosomes gave

superior and similar fluxes to Gdm not loaded in niosomes in SC and VED, respectively. This study has demonstrated that when Gdm was loaded in anionic niosomes, its chemical degradation against high temperatures was not only protected, but also its antibacterial activity was also existing with more sustained release effects. Gdm loaded in anionic niosomes and incorporated in gel was the best system with the superior cumulative amounts and fluxes in the skin but no risk of systemic effects. The results from this study can be used for the further development of Gdm for topical antibacterial treatment.

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