Skin Permeation Study of Liposomes Containing Nicotinamide for Cosmeceutical Application

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ชื่อวิทยานิพนธ์ การศึกษาการซึมผ่านผิวหนังของนิโคตินามายด์ในรูปแบบลิโปโซมเพื่อการประยุกต์ใช้ทางเวชสำอาง

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บทคัดย่อ

นิโคตินามายด์มีฤทธิ์ในการยับยั้งอาการอักเสบของสิวที่เกิดจากความผิดปกติของต่อมไขมันในรูขุมขน อย่างไรก็ตามการใช้นิโคตินามายด์มีปัญหาคือ การซึมผ่านผิวหนังได้ยาก เนื่องจากนิโคตินามายด์ละลายน้ำได้ดี ทำให้ยากต่อการซึมผ่านชั้นสตราตัม คอร์เนียม ซึ่งประกอบด้วยโครงสร้างของไขมันที่เรียงตัวซ้อนกันสองชั้น เทคโนโลยีลิโปโซมเป็นวิธีการที่ชัดเจนว่าอาจจะเอาชนะปัญหานี้ได้ ด้วยโครงสร้างของไขมันที่เรียงซ้อนกันของลิโปโซมเหมือนกับโครงสร้างของชั้นสตราตัม คอร์เนียมในผิวหนัง การศึกษาในครั้งนี้ ลิโปโซมที่บรรจุนิโคตินามายด์ได้ถูกพัฒนาเพื่อช่วยเพิ่มการซึมผ่านผิวหนังของนิโคตินามายด์ ลิโปโซมที่ประสิทธิภาพในการกักเก็บสารสูงสุดถูกเลือกเพื่อศึกษาความคงตัวต่อไป รวมทั้งศึกษาการซึมผ่านผิวหนังนอกกาย และ การเตรียมตำรับรูปแบบครีม ตำรับนิโคตินามายด์ลิโปโซมทั้งหมดมีประสิทธิภาพในการกักเก็บสารอยู่ในช่วงร้อยละ 12.04-73.86 และมีขนาดอนุภาคอยู่ในช่วง 110.20-430.59 นาโนเมตร จากการศึกษาพบว่าตำรับที่ดีที่สุดได้แสดงผลของคุณสมบัติทางกายภาพและทางเคมีดีที่สุด ได้แก่ ประสิทธิภาพในการกักเก็บสาร (ร้อยละ 73.86±0.09) ขนาดอนุภาค (185±1.25 นาโนเมตร) ศักย์ซีตาร์ (-32±1.87 มิลลิโวลต์) และค่าพีเอช (6.17±0.07) ตำรับนี้ประกอบด้วย ฟอสฟาทิดิลโคลีนจากถั่วเหลือง คอเลสเตอรอล ทวีน$_{80}$ อัตราส่วนโดยโมล 4:1:1 ปริมาณไขมันทั้งหมด 80 ไมโครโมลต่อมิลลิลิตร และปริมาณนิโคตินามายด์ในตำรับลิโปโซมคิดเป็นร้อยละ 5 ของตำรับ (โดยน้ำหนักต่อปริมาตร) ตำรับนี้ถูกพบว่ามีความคงตัวดีภายใต้การทดสอบความคงตัวที่อุณหภูมิ 4 องศาเซลเซียส เป็นเวลา 2 เดือน การศึกษาการซึมผ่านผิวหนังนอกกายด้วยการใช้เทคนิค โมดิฟายด์ ฟรานซ์ ดิฟฟิวชัน เซลล์ แสดงปริมาณในการซึมผ่านผิวหนังของนิโคตินามายด์ลิโปโซมร้อยละ 1.02±0.02 ในขณะที่สารละลายนิโคตินามายด์คือร้อยละ 0.70±0.03 ยิ่งไปกว่านั้นปริมาณของนิโคตินามายด์จากลิโปโซมที่ถูกสะสมในผิวหนังก็สูงกว่าสารละลายนิโคตินามายด์ (ร้อยละ 1.02±0.03 และร้อยละ 0.80±0.01 ตามลำดับ) การศึกษาในครั้งนี้นิโคตินามายด์ลิโปโซมในรูปแบบตำรับครีมได้ถูกเตรียมขึ้น และการศึกษาการซึมผ่านผิวหนังนอกกายของตำรับถูกเปรียบเทียบกับ นิโคตินามายด์

ครีม และผลิตภัณฑ์จากท้องตลาด ปริมาณในการซึมผ่านผิวหนังของนิโคตินามายด์จากลิโปโซม ครีมคือร้อยละ 7.25±0.05 ซึ่งสูงกว่านิโคตินามายด์ครีม และผลิตภัณฑ์จากท้องตลาด (ร้อยละ 1.03±0.01 และร้อยละ 1.47±0.05 ตามลำดับ) นอกจากนี้ปริมาณการสะสมของนิโคตินามายด์ในผิวหนังจากนิโคตินามายด์ลิโปโซมครีมคือร้อยละ 1.47±0.05 ซึ่งมากกว่าปริมาณการสะสมของนิโคตินามายด์ครีม และผลิตภัณฑ์จากท้องตลาด จากผลการทดลองดังกล่าวนี้ได้บ่งบอกว่าตำรับลิโปโซมสามารถเพิ่มการซึมผ่านผิวหนังและเพิ่มการสะสมของนิโคตินามายด์ในผิวหนัง ข้อสังเกตดังกล่าวนี้ได้แสดงศักยภาพของลิโปโซมในบทบาทการนำส่งยาทางผิวหนัง อย่างไรก็ตาม ประสิทธิภาพทางคลินิกของตำรับลิโปโซมควรได้รับการประเมินผล

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ABSTRACT

Nicotinamide has anti-inflammatory activities of acne vulgaris which caused from disorders of sebaceous glands in hair follicles. However, the problem of using nicotinamide is difficult to skin permeation because its hydrofilicity, difficult to permeate into stratum corneum which composed of lipid bilayer structure. Liposomes technology is an obvious approach that might overcome this problem with lipid bilayer structure of liposomes as same as stratum corneum layer in skin structures. In this study, the liposomes containing nicotinamide were developed to enhance skin permeation of nicotinamide. The liposome with the highest entrapment efficiency was selected for further stability studies including *in vitro* skin permeation, and formulation of cream dosage form. All nicotinamide liposomal formulations have percent entrapment efficiency in range of 12.04-73.86% and particle size in range of 110.20-430.59 nm. It was found that, the best formulation exhibited the best result in physicochemical properties such as entrapment efficiency (73.86±0.09%), particle size (185±1.25 nm), Zeta potential (-32±1.87 mV), and pH (6.17±0.07). This formulation was composed of soybean phosphatidylcholine (SPC): Cholesterol (CHO): Tween80; 4:1:1 molar ratio, total lipid of 80 μmol/ml, and 5% nicotinamide

(weight/volume). This formulation was found to be stable under stability test at 4 °C for 2 months. The *in vitro* skin permeation studies, using modified Franz diffusion cells technique, showed that the percent cumulative amount of nicotinamide liposomes was 1.02±0.02% while that of the nicotinamide solution was 0.70±0.03%. Moreover, the amount of nicotinamide from liposomes accumulated in the skin was higher than nicotinamide solution (1.02±0.03% and 0.80±0.01%, respectively). In this study, nicotinamide liposome cream was prepared and its *in vitro* skin permeation study were performed comparing with nicotinamide cream and the commercial product. The cumulative amount of nicotinamide from liposome cream was 7.25±0.05% which is higher than the values of nicotinamide cream and the commercial product (1.03±0.01% and 0.99±0.02%, respectively). In addition, the accumulation of nicotinamide in the skin, from nicotinamide liposome cream was 1.47±0.05% which is more than those of nicotinamide creams and the commercial product. From these results, it is indicated that the liposomal formulation could increase skin permeation and accumulation of nicotinamide in the skin. This investigation showed potential of liposome as drug skin delivery. However, the clinical efficacy of the liposomal formulations should be evaluated.

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LIST OF ABBREVIATIONS AND SYMBOLS

AFM	Atomic Force Microscopy
BKK	Bangkok
bw	body weight
^{o}C	degree celsius
DMPC	Dimyristoylphosphatidylcholine
DOPE	1, 2-Dioleoyl-*sn*-glycero-3-phosphoethanolamine
DSPC	1, 2-Distearoyl-*sn*-glycero phosphocholine
CHO	Cholesterol
CLSM	Confocal Laser Scanning Microscopy
cm	centimeter (s)
cm^2	square centimeter (s)
EDTA	Ethylene diamine tetra-acetic acetate
EPC	Egg phosphocholine
ESEM	Environment Scanning Electron Microscopy
et al.	and others
FT	Freeze thaw cycle
g	gram (s)
h	hour (s)
HLB	Hydrophilic-Lypophilic Balance
HPLC	High Performance Liquid Chromatography
J_{ss}	steady state flux

LIST OF ABBREVIATIONS AND SYMBOLS (Continued)

K_p	permeability coefficient
Log P	Logarithm of the partition coefficient
LUV	Large Unilamellar Vesicles
mg	milligram (s)
µg	microgram (s)
µmol	micromole (s)
µm	micrometer (s)
min	minute (s)
ml	milliliter (s)
MLV	Multilamellar Vesicles
MW	Molecular Weight
nm	nanometer (s)
NA	Nicotinic Acid
NAD	Nicotinamide Adenine Dinucleotide
NADP	Nicotinamide Adenine Dinucleotide Phosphate
NCM	Nicotinamide / Niacinamide
NMR	Nuclear Magnetic Resonance
o/w	oil in water
p.	page
Pa	Pascal
PBS	Phosphate Buffer Saline

LIST OF ABBREVIATIONS AND SYMBOLS (Continued)

pH	The negative logarithm of the hydrogen ion concentration
PI	Polydispersity Index
pKa	the negative logarithm of the dissociation constant
Q_{cum}	Cumulative amount of drug permeated
R^2	coefficients of determination
REV	Reverse-phase evaporation
rpm	round (s) per minute
RSD	Relative Standard Deviation
RT	Room temperature
SEM	Scanning electron microscope
SPC	Soybean Phosphatidylcholine
SUV	Small Unilamellar Vesicles
TEM	Transmission Electron Microscopy
TEWL	Transepidermal water loss
ULV	Unilamellar vesicles
USA	United Stated of America
v/v	volume by volume
vs.	versus
w/o	water in oil
w/v	weigh by volume
%	percent

CHAPTER 1

INTRODUCTION

1.1 Background and Rationale

Nicotinamide is one of the newest vitamin-based components of cosmeceutical products. Most of the available studies have focused on its anti-inflammatory and anti-acne action (Griffiths, 1995). Nicotinamide is also believed that its anti-inflammatory effect may improve skin appearance by reducing leucocytes peroxidase systems that may lead to localize tissue damage as well as by ameliorating the cutaneous barrier (Berson *et al.*, 2003). In a comparative study, the anti-inflammatory effect of 4% nicotinamide gel in the management of acne vulgaris was as good as the benefits of 1% clindamycin gel (Shalita *et al*., 1995). This anti-inflammatory effect is also useful to reduce cutaneous erythema in various disorders (Bisset and Oblong, 2005).

Acne vulgaris is a multifactorial disease involving excessive sebum production by the sebaceous glands related to an increase in the androgen levels in the onset of puberty, ductal hyperconification of the follicles, and proliferation of *Propionibacterium acnes* and other bacterial that activate the toll like receptors, resulting in attraction of lymphocytes, neutrophils, and macrophages (Monica and Edileia, 2009). Although, there are many options available to treat the disease, topical antibiotic therapy is preferred option for the treatment of mild to moderate acne

because topical use of antibiotic (such as clindamycin phosphate and benzoyl peroxide) is generally well tolerated but these drugs may cause skin irritation despite this therapeutic effectiveness (Srinivasan *et al*., 2009). Nicotinamide is nonirritating to facial skin, easily formulated, chemically stable, and compatible with other formulation components, it has been considered an ideal cosmeceutical agent; nevertheless, it is from one-third to one-fifth as effective as topical 0.025% tretinoin (Bisset *et al*., 2003). So nicotinamide was replaced antibiotic in anti-inflammation of acnes for reducing skin irritation problem. However, topical use of nicotinamide as gel or cream is not enough effective to its difficulty in permeating the stratum corneum layer of the skin (Sara *et al*., 2008). Skin permeation development and topical formulation of nicotinamide were interested.

Recently, numerous drug delivery systems have been explored to achieve optimal drug transport into the skin, and one such promising approach is entrapment of drug into lipid constructs, commonly, known as liposomes. Because of physicochemical characteristic and construct diversity, liposomes proved to be an efficient drug delivery system for topical administration (Kumar *et al*., 2007). However, the skin permeation of liposome vesicles significantly varies depending on lipid composition and particle size (El Maghraby *et al*., 2006; Choi and Maibach, 2005). Different lipid compositions that constitute the liposomes interact differently with the skin layer. The most of liposomal formulations are designed for reduction toxicity, inhibition of rapid clearance of liposome, controlling size, charge, and surface hydration. Enhanced safety heightened efficacy has been achieved for a wide range of drug classes (Tianshun and Rodney, 2001).

Because the barrier properties of the skin is stratum corneum and the hydrophilic make nicotinamide difficult to permeate through the skin and reach to its site of action. Since preparing liposome containing nicotinamide for skin permeation improved was investigated because the lipid bilayer properties of liposomes which that likely the layer of stratum corneum in the skin (mostly composed of lipid) (Tianshun and Rodney, 2001). Though, the delivery of nicotinamide with the aid of liposome technology has a potential for the prevention inflammation of acnes, until now a very few work have been performed. Beside, no report is available on the characterization, formulation, and evaluation of skin permeation behavior of liposomal vesicles containing nicotinamide.

In this present study, preparation and physicochemical study of liposomes containing nicotinamide was determined for skin delivery. Nicotinamide liposome characteristics, skin permeation properties, and stability studies for conducted suitable liposomes were evaluated. The suitable liposomes were prepared to cream formulations for future.

1.2 Objective of the study

The objectives of this study were to:

1.2.1 Formulate the liposomes containing nicotinamide

1.2.2 Evaluate the physicochemical property and stability of liposomes containing nicotinamide

1.2.3 Evaluate the *in vitro* skin penetration retention efficiency of nicotinamide from the liposomes compared to a nicotinamide solutions

1.2.4 Prepare o/w cream formulations containing nicotinamide liposomes

1.2.5 Evaluate the physicochemical property and stability of o/w cream formulations containing nicotinamide liposomes

1.2.6 Evaluate the *in vitro* skin penetration retention efficiency of o/w cream formulation containing nicotinamide liposomes compared to a nicotinamide creams and commercial product

CHAPTER 2

REVIEWS OF LITERATURE

2.1 Nicotinamide

Nicotinamide is a water-soluble amide of nicotinic acid. It is one of two principal forms of B-complex vitamin, B_3, active form that acts as constituent of the enzyme cofactors NAD and NADP. These functions were performed in cell metabolism of carbohydrates, fatty acid, and amino acids. Structure of nicotinamide and its derivatives showed as in **Table 2.1** and the **Table 2.2** shows physicochemical properties of nicotinamide.

2.1.1 Nicotinamide in cosmeceutical applications

The likely usefulness of topical nicotinamide for the improvement of skin appearance may be related to its action in the synthesis of sphingolipid, free fatty acid, cholesterol, and ceramide, thus, decreasing transepidermal water loss (Berson *et al*., 2003; Bisset *et al*., 2003; Tanno *et al*., 2000). Additionally, nicotinamide increases collagen production in fibroblast culture and this effect may be responsible for the improvement of skin elasticity and reduction of fine wrinkles (Bisset *et al*., 2003), improvement of facial dyspigmentation is also likely mediated by the suppression of melanosome transfer from melanocyte to keratinocytes (Hakozaki *et al*., 2002). All of these effects may help to reverse some of the aging skin signs. Concentration

ranging of nicotinamide has been used in cosmetic products from 2-5%, so it has sufficient (Bisset and Oblong, 2005).

Table 2.1 Shows structure of nicotinamide and its derivatives (Ping and Antony, 2010)

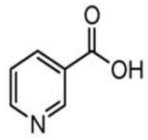
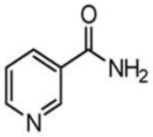
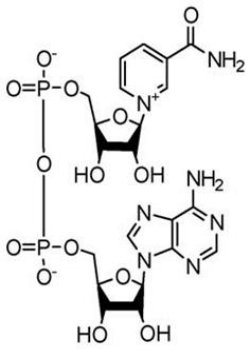
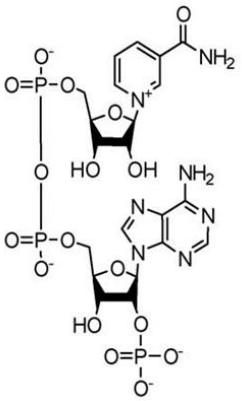
Structure formula	Structure formula names
	Nicotinic Acid (NA)
	Nicotinamide / Niacinamide (NCM)
	Nicotinamide adenine dinucleotide (NAD)
	Nicotinamide adenine dinucleotide Phosphate (NADP)

Table 2.2 Physicochemical properties of nicotinamide

Physicochemical properties	**Forms of nicotinamide**
Molecular Formula	$C_6H_6N_2O$
Chemical Name	3-pyridine carboxamide
Molecular Weight (MW)	122.1
Appearance	White crystalline powder or colorless crystals
Solubility (water solubility)	1g/ml
Partition coefficient n-octanol/water (log value)	-0.38 (22°C) (Georg, 2002)
%Use in cosmetic	2-5%
LD_{50} in rat by injection	1.7 g/kg
Pharmacological dose	500 mg-2 g/day (non toxic), ≥ 3 g/day (toxic)
pKa	0.5 and 3.35 (http:www.drugs.com)

(http://www.scribd.com/doc/30137426/Niacin-Nicotinamide-And-nicotinicotinicacid)

2.1.2 Nicotinamide for skin penetration

Sara *et al.* determined the nicotinamide accumulation in the dermal and epidermal layers of human *in vitro* skin after application of topical gels. It was found the accumulation in the skin *in vitro* after 20 min application. Most of drug amounts were accumulated in the epidermis and adsorbed to deeper layer in few amount because the hydrophilic properties is barrier in skin penetration (Sara (1) *et al*., no date).

Sara *et al*. also study the enhancement effect of nicotinamide in methyl paraben solutions by Franz diffusion cells with rabbit ear skin. Amount of methyl paraben with nicotinamide solution (20% w/v) could be permeated to the skin higher methyl paraben with water for 8 hours. Beneficial of it is enhanced permeation of substance for permeation skin improving (Sara (2) *et al*., no date).

Imokawa *et al*. presented for nicotinamide improves epidermal ceramide synthesis with concurrent epidermal barrier benefits. Sphingolipids and other stratum corneum lipids, particularly ceramides, are known to play central roles in the structural and functional integrity of the epidermal permeability barrier, and decreased in aged and a topic skin (Imokawa *et al*., 1997).

Tanno and colleagues shows nicotinamide inducing up to a 5-fold up-regulation in ceramide synthesis, in a dose dependent fusion, in cultured human epidermal-keratinocytes. The *in vivo* clinical significance of these findings was demonstrated (Tanno *et al*., 1997). And topically 2% nicotinamide was recovered ceramide in stratum corneum and free fatty acid lipid fraction. This reduced transepidermal water loss (TEWL) in the skin human (Tanno *et al*., 2000).

2.1.3 Toxicological data of nicotinamide

Nicotinamide is very low acute toxicity to mammals, both orally and dermally. For oral, the acute toxicity of nicotinamide after oral exposure was LD_{50} 3-7 g/kg bw in rodents. Its toxicity after dermal application was very low with a dermal LD_{50} >2000 mg/kg bw in rabbits. Skin irritation studies indicated that nicotinamide shown no potential to irritate the skin. In additionally, nicotinamide irritated to the eyes, application of 0.1 g nicotinamide to the eyes of 3 rabbits induced irritation in two animals, which was reversible within 7 days (Keri *et al*., 2005).

2.2 Inflammatory of acne vulgaris

Acne vulgaris is a disorder of the pilosebaceous unit, characterized by comedones, inflammatory lesion and scars on the face and trunk. The processes in the pathogenesis of acne were the increase of sebum production in first step; perifollicular hyperkeratinization and follicular obstruction; colonization with *Propionibacterium acnes*; and released of enzymes which induce humoral and cell mediated inflammations which presented as in **Figure 2.1** (Monica and Edileia, 2009; Guy and Webster, 1995), and inflammation skin appearances of acnes versus normal skin as shown in **Figure 2.2**. Nicotinamide was used for anti-inflammatory of acnes by reducing sebum production. It was reported about anti-inflammatory action affecting neutrophil chemotaxis by inhibited histamine released and to suppress the lymphocyte transformation (Yesim and Meltem, 2008).

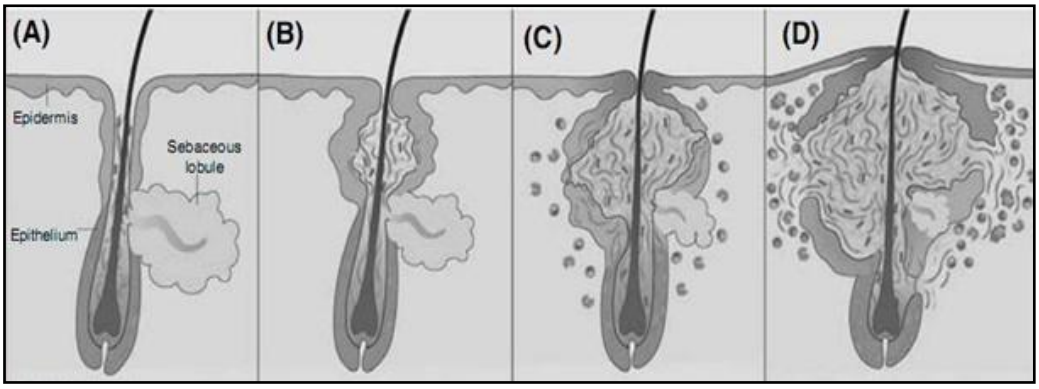


Figure 2.1 Acto pathogenesis, the steps of acne phathogenesis: (**A**) normal skin, (**B**) increased sebum production, (**C**) perifollicular hyperkeratinization and follicular obstruction and colonization with *Propionibacterium acnes* (**D**) released of enzymes which induce humoral and cell mediated inflammations (Zoe and Lauren, 2008)

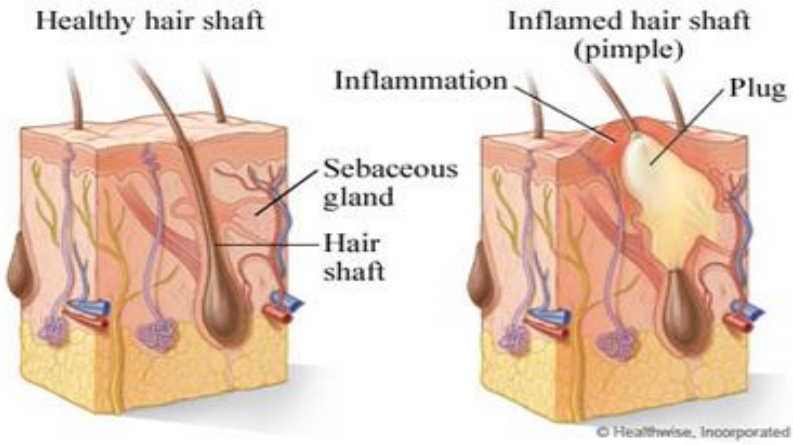


Figure 2.2 Normal skin and skin inflammatory acnes (http://www.healthchrome.com)

2.3 Skin structure

The skin has the primary function to provide a barrier against environmental influences and protect the body against the loss of endogeneous substances. Macroscopically, two distinct skin layers are apparent, an unvascularized outer layer (epidermis) and an inner vascularized layer (dermis and hypodermis) as

shown in **Figure 2.3**, an overview about the dimensions and the stratified appearance of the skin (Heiko *et al*., 2006). The several layers of the skin were complained as following.

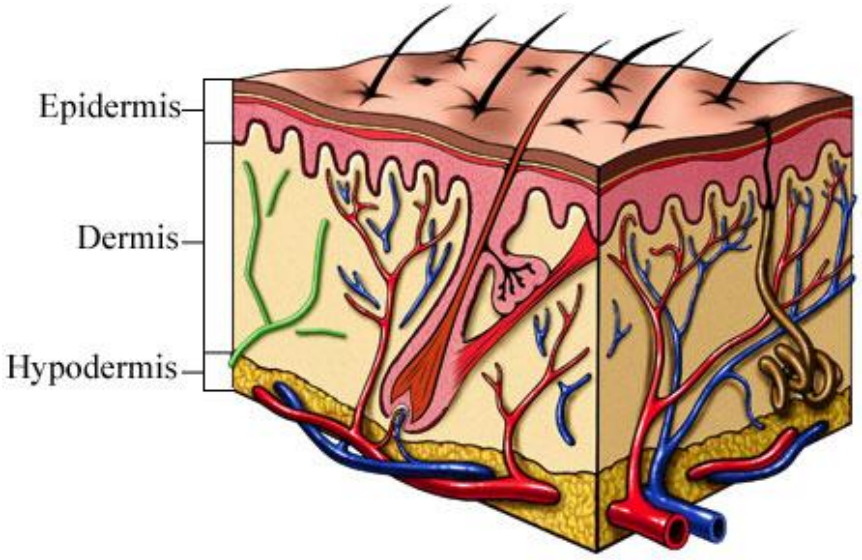


Figure 2.3 The different layers of the skin (http://www.1wipe1week.com)

Epidermis

The viable epidermis is divisible into five distinct layers, namely, from inside to outside: stratum basale, stratum spinosum, stratum granulosum, stratum lucidum, and stratum corneum. The cells of these layers undergo continuous differentiation to produce the outermost layer of the skin. The stratum basale is a single layer of epidermal stem cells which are anchor to a basement membrane that separates the epidermal tissue from the underlying dermis. Above, these differentiating cells are forming the stratum spinosum. The cells are now of spiny appearance, due to abundance of desmosome (Heiko *et al*., 2006). The stratum corneum constitutes the outermost layer of the epidermis and represents the main barrier function, although it is the thinnest and smallest compartment of the skin. It is about 10 to 20 µm thick and consists of several layers of dead, keratin filled

corneocytes that represent the final state of epidermal differentiation. These cells are embedded in a matrix of lipid lamellas, which gives the stratum corneum a brick and mortar organization (Menon, 2002). Another specialty of the stratum corneun is the lipid composition which differs significantly from those of the cells of the lower epidermal layers. The extracellular lipid matrix of the stratum corneum is finally free of phospholipids and consists of ceramides, cholesterol, and free fatty acids in roughly equimolar ratio. The ceramides are crucial for the lipid organization of the stratum corneum barrier, while the cholesterol is promoting the intermixing of different lipid species (Heiko *et al*., 2006).

Dermis and hypodermis

The dermis is connected by the basement membrane to the stratum basale. Main components of the dermis are collagen and elastin fibers that form a vast network of filamentous and amorphous connective tissue that prevents strength and flexibility to the skin (Schaefer and Redelmaier, 1996). Furthermore, the dermis accommodates cellular residents such as fibroblasts, endothelial cells, mast cells, and under conditions of inflammation, macrophages, lymphocytes, and leucocytes.

Underneath the dermis is the hypodermis situated that contains, in contrast to the dermis, loose connective tissue and adipocytes as the main cellular exponent which represent energy source for the body (Heiko *et al*., 2006).

2.4 Liposomes

Liposomes are self assemble colloidal particles that occur naturally and can be prepared artificially, as shown by Bangham and his students in the mid-1960. At first, they were used to study biological membrane. Several practical applications, most not ably in drug delivery, emerged in the 1970 (Dan, 1998). The structural of it

is spherical self-closed, composes of curved lipid bilayers, which enclose part of the surrounding solvent into their interior as shown in **Figure 2.4**. Their size ranges from some 20 nm up to several µm and liposomes layer may be composed of one or several concentric membranes, each with a thickness of about 4 nm. Liposomes are possessing unique properties owing to the amphiphilic character of the lipids, which make liposomes suitable for drug delivery (Nill, 2003).

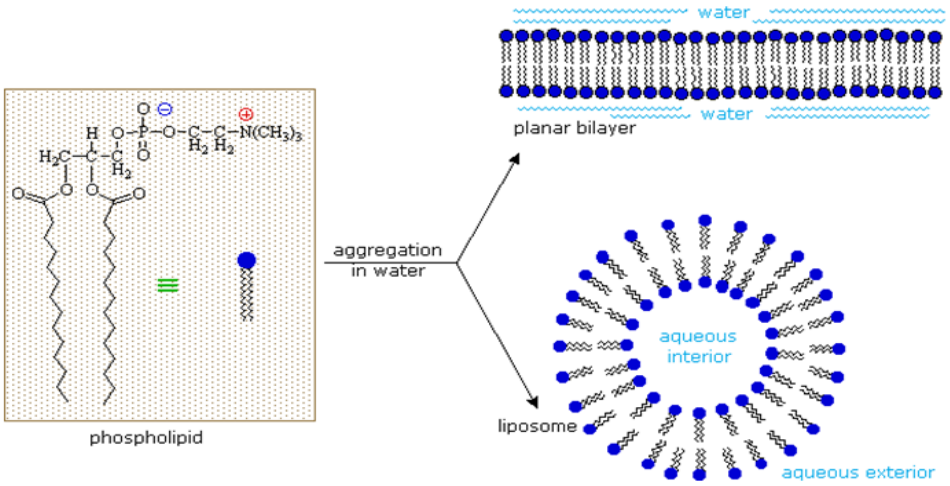


Figure 2.4 A schematic bilayer membrane of liposomes (http://www.2chemistry.msu.edu/faculty/reusch/VirtTxtJml/lipids.htm)

2.4.1 The benefit of liposomes

(i) Improved solubility of lipophilic and amphiphilic drugs. (Lasic, 1992);

(ii) Passive targeting to the cells of the immune system, especially mononuclear phagocytic cells

(iii) Sustained release system of systermically or locally administered liposomes.

(iv) Site-avoidance mechanism: liposomes do not dispose in certain organ, such as heart, kidneys, brain, and nervous system, and this reduce cardio-, nephro-, and neuro-toxicity.

(v) Improved transfer of hydrophilic, changed molecules such as chelators, antibiotics, plasmids, and genes into cells

(vi) Improved penetration into tissue, especially in the case of dermally applied liposomal dosage forms.

In general, liposome encapsulation is considered when drugs are very potent, toxic, and very short life times in the blood circulation or at the sites of local (subcutaneous, intramuscular, or intrapulmonary) administration.

2.4.2 Classification of liposomes

The liposome size can range from very small (0.025 μm) to large (2.5 μm) vesicles. Furthermore, it may have single or multiple bilayers membranes. The vesicle size is a critical parameter in determining circulation half-life of liposomes, and size and number of bilayers influence the extent of drug encapsulation in the liposomes. On the basis of their size and number of bilayers, liposomes can also be classified into one of three categories: multilamellar vesicles (MLV); large unilamellar vesicles (LUV); and small unilamellar vesicles (SUV) as shown in the **Figure 2.5**. The size and characteristics of these types of liposomes are listed in **Table 2.3** (Amarnath and Uma, 1997).

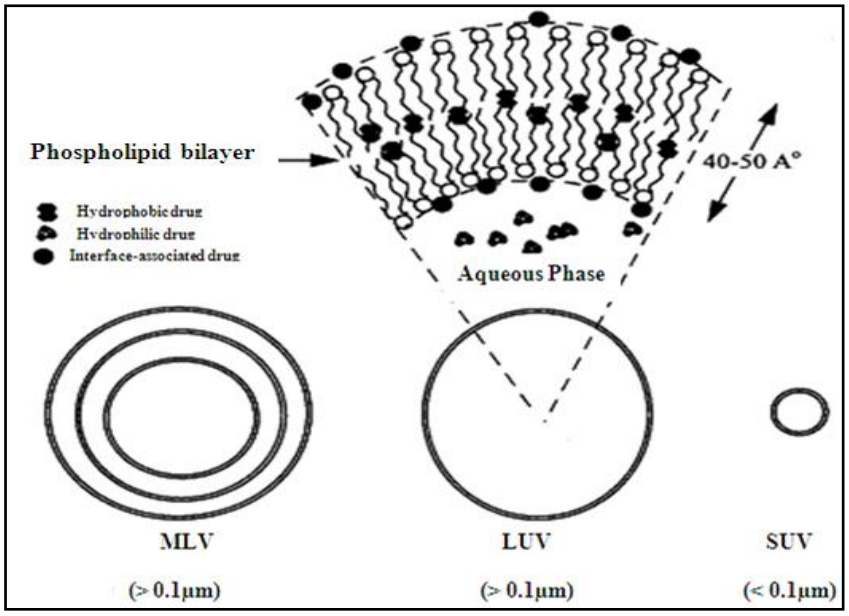


Figure 2.5 Type of liposomes depending on size and number of lamellae (Amarnath and Uma, 1997)

Table 2.3 Liposome classifications by size and number of lamellae (Amarnath and Uma, 1997)

Type	Usual size	Characteristics
MLV	> 0.1µm	More than one bilayer; greater encapsulation of lipophilic drugs; prepared by thin film hydration method or hydration of lipids in presence of an organic solvent
LUV	>0.1µm	Single bilayer; useful for hydrophilic drug; high capture of macromolecules; prepared by ether injection, reverse-phase evaporation or active loading methods

Table 2.3 Liposome classifications by size and number of lamellae (continued), (Amarnath and Uma, 1997)

Type	Usual size	Characteristics
SUV	≤0.1μm	Single bilayer; homogenous in size; thermodynamically unstable; susceptible to aggregation and fusion at low or no charge; limited capture of macromolecules; prepared by reducing the size of MLV or LUV using solvent injection techniques

2.4.3 Compositions of liposome formulations

The main components of liposomes are phospholipid and cholesterol. A phospholipid has two acyl chains linked to a head group by means of a glycerol-backbone. The **Figure 2.6** shows the structural formula of a phospholipid, where R1 and R2 are saturated or unsaturated acyl chains and R3 is the polar head group.

Phosphatidylcholines or PC-lipids are the most widely used lipids in liposome work. PC-lipids are zwitterionic at all relevant pH, therefore, form lamella structure independently of the pH in the solution. EPC and DSPC are showed structures in the **Figure 2.6.** DSPC is a synthetic lipid with only saturated chains, while EPC is a natural PC-lipid with both saturated and unsaturated fatty acids. Phosphatidylethanolamines (PE) have pH dependent phase behavior. At physiological pH, the PE-lipids have zwitterionic head group, they are not capable of forming lamellar structures (Nill, 2003).

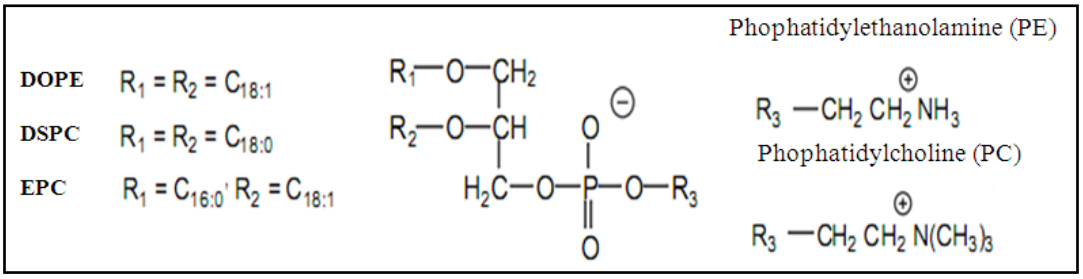


Figure 2.6 The general structure of a phospholipid and the structure of DOPE, DSPC, and EPC (Nill, 2003)

Additionally, also cholesterol is component in liposomal formulation. Cholesterol incorporated in liposome bilayer brings about major changes in the preparation of these membranes. It can be incorporated into phospholipid membranes in very high concentration up to 1:1 or even 2:1 molar ratios of cholesterol to PC. Cholesterol inserts into the membrane with its hydroxyl groups oriented towards the aqueous surface and aliphatic chain aligned parallel to the acyl chains in the center of the bilayer. However, the over concentration of cholesterol was used for condensation fluidity and increase rigidity of liposomes bilayer membrane (Emnet, 2010; http://www.pharmaxchange.info).

2.4.4 Liposomes preparation methods

Liposomes with different sizes and characteristics usually require different methods of preparation. The most simple and widely used method for preparation of MLV is the thin-film hydration procedure in which a thin film of lipids is hydrated with an aqueous buffer. The drug to be encapsulated is included either in the aqueous hydration buffer (for hydrophilic drugs) or in the lipid film (for lipophilic drugs). Thin-film hydration method produces a heterogeneous population of MLV (1-

5 μm diameters) which can be sonicated or extruded through polycarbonate filters to produce small (up to 0.025 μm) and more uniformly size population of SUV. One of the major disadvantages of this method is relative poor encapsulation efficiency (5-15%) of hydrophilic drugs. Reduction of liposome size further decreases the amount of encapsulated drug. MLV with high entrapment efficiency (up to 40%) can be prepared by freeze-drying preformed SUV dispersion in an aqueous solution of the drug to be encapsulated (Ohsawa *et al*., 1984).

Additionally, several methods have been developed for the preparation of LUV, including solvent injection of ether and ethanol, detergent dialysis, calcium induced fusion, and REV techniques. SUV can be prepared from MLV or LUV by sonication using probe sonicator or extrusion passage through a small orifice under high pressure (Amarnath and Uma, 1997; Mohammad, 1996).

2.4.5 Physical morphology and physicochemical characteristics

The vesicular shape and surface morphology of liposomes can be visualized by atomic force microscopy (AFM), environment scanning electron microscopy (ESEM), transmission electron microscopy (TEM), and confocal laser scanning microscopy (CLSM; labeling using a fluorochrome marker). Barbara *et al*. reported about the improved evaluation of physicochemical and technological properties of drug delivery by liposome systems with several equipments. Aim in research was to determine the details of the morphology and structure of conventional liposomal formulations (Barbara *et al*., 2011).

The **Figure 2.7** shows images morphology of liposome by many techniques. In summary, microscopic studies improve the characterization of

nanoscale structures of liposomes and provide information about shape and morphology (AFM, TEM), dimensions (AFM, ESEM, TEM, and CLSM), surface properties (AFM), and internal structure (CLSM).

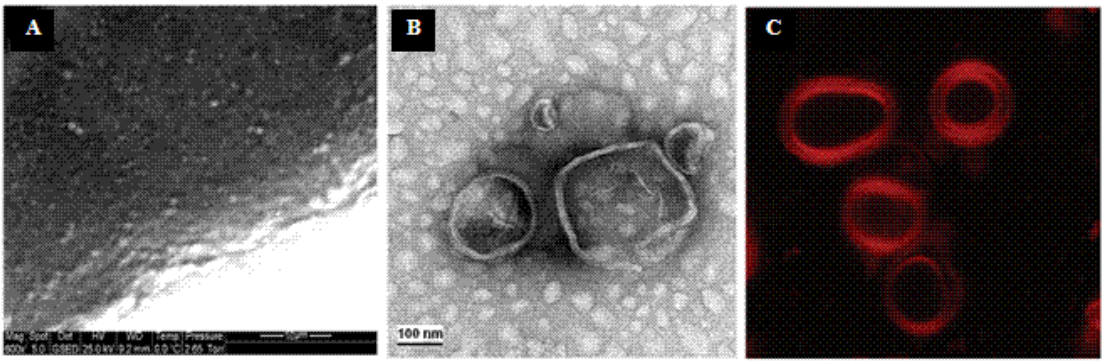


Figure 2.7 **(A)** Environment scanning electron microscopy (ESEM) micrographs liposomes 9.0 ºC, 4.32 Torr; (**B**) Negative staining transmission electron microscopy (NS-TEM) images; and **(C**) Confocal laser scanning microscopy (CLSM) images illustrating the architecture of liposomes (Barbara *et al*., 2011).

The behaviors of liposomes in both physical and biological systems are depended on factors such as physical size, membrane permeability, percentage of entrapped solutes, and chemical composition.

2.4.6 Mechanisms of liposomes as skin drug delivery systems and liposomes limitation

Mechanisms of action of liposomes as skin drug delivery systems

The skin barrier is situate in the stratum corneum and consists of the keratinocytes, proteins, and lipids. Thus, the skin barrier contains hydrophilic and lipophilic compartments, which act as buffer to retard both water loss and absorption of water (Imokawa *et al*., 1989). Alternative mechanisms have been suggested for liposomes acting as skin drug delivery systems as shown in the **Figure 2.8**. The drug

is permeates the skin independently after exiting from the vesicles as presented at position **A** (**Figure 2.8**). Kato *et al*. concluded that lecithin enhances skin delivery by lowering the permeability barrier of the skin. Changes in the ultrastructures of the intercellular lipids were seen after application of the vesicle suggesting a penetration enhancing effect (Kato *et al*., 1987). In another study, Zellmer *et al*. treated human stratum corneum with dimyristoylphosphatidylcholine (DMPC) liposomes, follow by differential scanning calorimetric investigations. DMPC vesicles did not penetrate into stratum corneum but the lipid can penetrate and change the enthalpy of the lipid-related transitions of the stratum corneum as shown in position **B** (**Figure 2.8**) (Zellmer *et al*., 1995). From the literature reports can be attributed penetration enhancing mechanism of liposome through using different lipid components in the vesicles and non-rigid lipids tending to produce the greatest enhancing effects. The vesicles may adsorb to the stratum corneum surface with subsequent transfer of drug directly from vesicles to skin, or vesicles may fuse and mix with the stratum corneum lipid matrix, increasing drug partitioning into the skin as seen in position **C** (**Figure 2.8**). Ultradeformable liposomes have been reported to invade the skin intact and go deep enough to be absorbed by the systemic circulation. The transdermal hydration gradient is produce force sufficient to drive ultradeformable vesicles through the intact stratum corneum and into the epidermis as presented in at position **D** (**Figure 2.8**). Another one pathway for liposomes to the skin is tranappedageal penetration as shown at position **E**, but, this route not necessarily for liposomes transdermal.

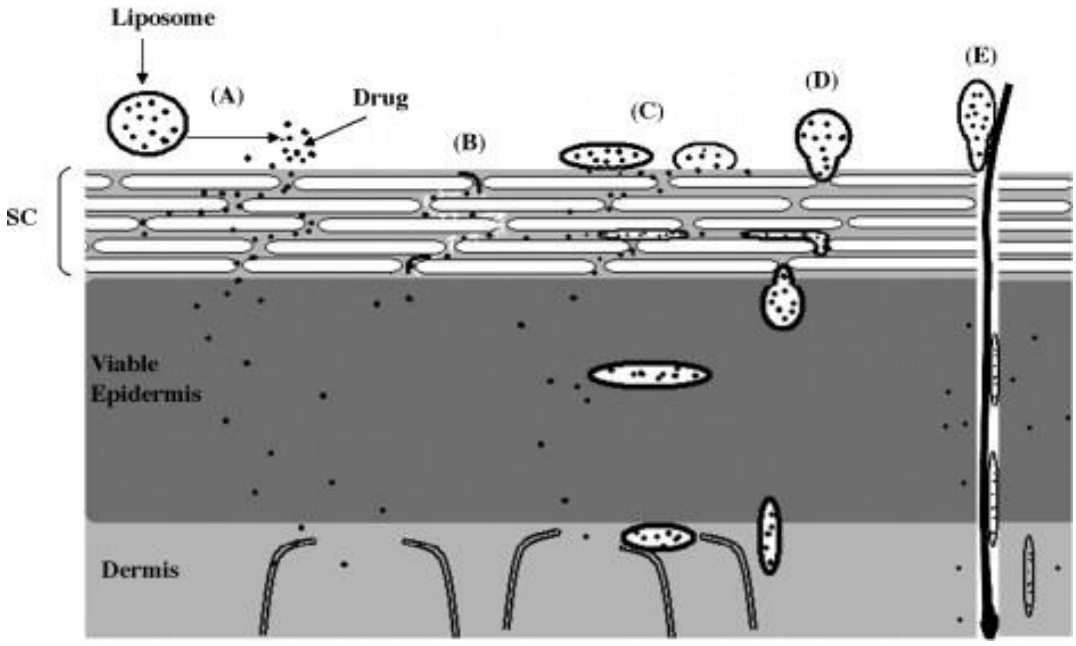


Figure 2.8 Possible mechanisms of action of liposomes as skin drug delivery systems. (**A**) is free drug mechanism, (**B**) is the penetration enhancing process of liposome components, (**C**) indicates vesicle adsorption to and/or fusion with the stratum corneum, (**D**) illustrates intact vesicle penetration into and through the intact skin, and (**E**) transappendageal penetration (El Maghraby *et al*., 2008)

Limitation

The liposomal formulation have various limitations will be presented for its important as following;

Stability: Stability problem of liposomes was limited both physical and chemical. Chemical instability might be caused by hydrolysis of ester bond and/or oxidation of unsaturated acyl chains of lipids. Physical instability might be caused by drug leakage from the vesicle and/or aggregation of fusion of vesicles to form larger

particles. Both of these processes, drug leakage and change in liposome size were effected the *vivo* performance of the drug formulation.

Encapsulation efficiency: Encapsulation efficiency was depended amount of lipid. Since lipid in high dose might be toxic and also cause non-linear (saturable) pharmacokinetics of liposomal drug formulations. Additionally, other limitations of liposome are not mention such as sterilization method, particle size control, and short circulation half life (Amarnath and Uma, 1997).

2.5 Emulsion cream formulations

Emulsions are heterogeneous systems containing two immiscible phases; a hydrophilic liquid phase and a lipophilic or oil phase. For the mixture consists of hydrophilic droplet dispersed in oil, they refer to it as water in oil (w/o) emulsion, even when the hydrophilic liquid is not water, they refer to it as oil in water (o/w) emulsion. It has non polar liquid droplet dispersed in the aqueous phase. Additionally, multiple emulsions are composed of droplets of one liquid dispersed in larger droplets of a second liquid, which is dispersed in a final continuous phase. Also systems may be w/o/w emulsions where the internal and external phases are hydrophilic; or o/w/o, which have the reverse composition. **Figure 2.9** gives an overview about these emulsion types (Heiko *et al*., 2006).

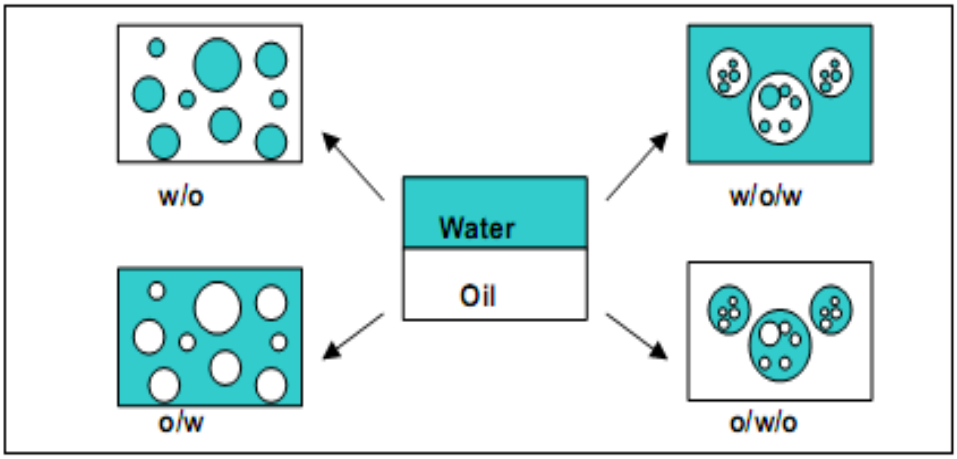


Figure 2.9 Overview about different emulsion types (Heiko *et al*., 2006)

Physicochemical properties and preparation of creams

An emulsifier as the additive was added in emulsions for reducing the interfacial area. It may be divided into four groups as follows: adsorbed non surfactant ionic materials, colloidal solids, and polymers, which represent the most common stabilization mechanism. Type of emulsion that is produced with given hydrophilic and lipophilic compounds, o/w or w/o, depends primarily on the properties of the surfactant. This characteristic is referred to as hydrophilic-lipophilic balance (HLB) that is the polar-non polar nature of the emulsifiers (Heiko *et al*., 2006).

In principal, two common methods are used to prepare cream formulations. The continental method which the emulsifier, the lipophilic phase, and parts of the hydrophilic phase forms is primary w/o emulsion. The final o/w emulsion is obtained due to phase inversion following addition of more hydrophilic solution. The second method was by dissolving the emulsifier in the hydrophilic phase and the preparation step involves slowly titration with the oil. This method is referred to as English method. Each methods, however, requires that energy put into the system in

some forms. The energy may supply in a variety of ways, such as triturating, heat, agitation, or homogenization (Heiko *et al*., 2006).

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Active ingredient

Nicotinamide (P.C. Drug center Co., Ltd., BKK, Thailand)

Nicotinamide Standard (Fluka, switzerland)

3.1.2 Chemicals and reagents

Absolute ethanol, AR grade (Merk®, Darmstadt, Germany)

Absolute methanol, AR grade (Merk®,Darmstadt, Germany)

Cetyl alcohol (Vidhyasom, BKK, Thailand)

Cholesterol from lanolin (Fluka®, Buchs, Japan)

Cremophor A 6 (BASF, Ludwigshafen, USA)

Cremophor A 25 (BASF, Ludwigshafen, USA)

Crystal violet (Sponsored by Faculty of Sciences, Department of Microbiology, Prince of Songkla University, Songkla, Thailand)

Ethanol 95%, HPLC grade (Merk®,Darmstadt, Germany)

Glycerin (P.C. Drug Center Co., Ltd., BKK, Thailand)

Glyceryl monostearate (P.C. Drug Center Co., Ltd., BKK, Thailand)

Gram's iodine (Sponsored by Faculty of Sciences, Department of Microbiology, Prince of Songkla University, Songkla, Thailand)

Isopropyl myristate (P.C. Drug Center Co., Ltd., BKK, Thailand)

L-∂-Phosphatidylcholine from soybean, Type IV-S, ≥30% (TLC) (Sigma-Aldrich®, USA)

Mineral oil (P.C. Drug Center Co., Ltd., Bkk, Thailand)

Orthophosphoric acid 85% (Merk®, Darmstadt, Germany)

Polyoxyethylene (20) sorbitan monooleate, Tween80® (Srichand Co., Ltd., BKK, Thailand)

Potassium dihydrogen phosphate (KH_2PO_4) (Merk®,Darmstadt, Germany)

3-sn-Phosphatidylcholine from soybean (Fluka®, Buchs, United States)

Sodium metabisulfite, AR grade (Namsaing international Co., Ltd., BKK, Thailand)

Stearic acid (Srichand Co., Ltd., BKK, Thailand)

3.2 Instruments

Table 3.1 Experimental instruments

Instruments	Model	Company
Centrifuge	Z323K	HermleLabortechnik GmbH, Germany
Chromatographic column	Reverse phase BDS HYPERSIL C_{18} column, 150×4.6 mm particle size, 5µm	Restek Corporation, USA
High performance liquid chromatography	SpectraSystem P1000 pump SpectraSystem UV1000 detectorChromQuest software program SN 4000	Thermo Electron Corporation, USA
Hot air oven	DIN 12880-KI	Polytron, Switzerland
Magnetic stirrer	MR 3000D	Heidolph, Germany
Sonicator	HT Crest	S.V. Medico Co.,Ltd., Thailand
Syringe filter	-	Vertical chromatography Co., Ltd., Thailand
Ultracentrifuge	Optima L-100XP, equipped with SW 60 Ti rotor	Beckman Coulter, USA
Viscometer	RVT	Brook fielded dial reading, USA
Zeta potential analyzer	Zeta PALS	Brookhaven Instrument Corporation, USA

3.3 Methods

3.3.1 Quantitative analysis of nicotinamide using High-Performance Liquid Chromatography (HPLC)

3.3.1.1 Instruments and chromatography conditions

The HPLC method for quantitative determination of nicotinamide was modified from the method described by Junaid *et al*., (2008). The analysis was performed at room temperature on reverse phase BDS HYPERSIL C_{18} column (150 × 4.6 mm, particles size, 5µm). A mixture of methanol and buffer of 0.05 M potassium-dihydrogen phosphate having pH 3.6±0.1, adjusted with orthophosphoric acid (75:925 ml) was used as mobile phase with the flow rate of 0.6 ml/min. The mobile phase was filtered through 0.45 µm nylon membrane filter and degassed by sonication before using. The 20 µl sample solution was injected and absorbance was detected at 254 nm. The concentrations of nicotinamide were quantified from the standard curve by plotting the peak area of nicotinamide against the nicotinamide concentration.

3.3.1.2 Preparation of nicotinamide standard solution

A 0.10 gram of nicotinamide was accurately weighted, dissolved, and adjusted volume with distilled water to 100 ml in order to reach the concentration of 1000 µg/ml to form a stock solution. It was diluted with mobile phase which their concentrations of 1, 5, 10, 20, 30 and 40 µg/ml (Tsang and Zeng, 2007).

3.3.1.3 The validation procedures

The HPLC method used for analysis of nicotinamide was validated in terms of linearity, accuracy, precision, specificity, and limit of detection and quantification according to International Conference on Harmonization (ICH) guideline (ICH, 1996) as following;

Linearity

Three sets of nicotinamide standard solution and nicotinamide spiked in pig skin were analyzed in triplicate. Linear regression analysis of means peak area versus their concentrations was performed. The coefficient of determination (R^2) $\geq$ 0.99 is the acceptable criteria (ICH, 1996).

Accuracy

Nicotinamide stock solution was spiked in blank liposomes to the concentration of 30, 50, and 70 mg/ml. These three concentrations represent to 60%, 100%, and 140% of nicotinamide concentration in the prepared nicotinamide-loaded liposomes. Three sets of sample were analyzed in triplicate. Additionally, also the accuracy for skin permeation studies was determined. Nicotinamide stock solution was spiked in blank skin to the concentration 20, 30, and 40 µg/ml. To prove the accuracy of nicotinamide amount determination in the skin permeation study procedure by Franz diffusion cells. The concentrations of nicotinamide were calculated from the linear regression equation obtained from linearity test compared with true value and expressed as percentage of recovery which could be calculated by the following equation:

$$\%\text{Recovery} = \frac{\mathbf{C}\, measured}{\mathbf{C}\, actual} \times 100 \quad (1)$$

Where; $C_{measured}$: concentration of nicotinamide detected by HPLC

C_{actual}: actual concentration of nicotinamide

The percent recovery should be in the range of 98 to 102% (ICH, 1996).

Precision

Precision evaluation was divided to the intra-and inter-day precision.

The intra-day precision

Three concentrations of the nicotinamide standard solution (1, 10 and 30 µg/ml) were analyzed by HPLC in the same day. Each set of sample was analyzed in triplicate.

The inter-day precision

Three concentrations of nicotinamide standard solution (1, 10, and 30 µg/ml) were analyzed by HPLC in 3 continuously days. Each set of sample was analyzed in triplicate.

The evaluation of both intra- and inter-day precision expressed as percentage of relative standard deviation (%RSD) of peak area in every concentration, both intra-day and inter-day precisions which their values should not be over 2.0% (ICH, 1996) was calculated by the following equation:

$$\%\text{RSD} = \frac{SD}{\bar{X}} \times 100 \quad (2)$$

Where; *SD*: Standard deviation of nicotinamide concentration

$\bar{X}$: Average concentration of nicotinamide

Specificity

The specificity of the HPLC method was determined for nicotinamide in liposome formulations, receptor fluid of skin permeation study, and the pig skin model. The experiments were performed by spiking nicotinamide in the tested matrices. The peak of any excipients in liposome formulations, PBS, as well as pig skin must not interfere with the nicotinamide peak.

Limit of detection (LOD) and quantification (LOQ)

Stock solution of nicotinamide at a concentration of 1 µg/ml was prepared by dissolving the drug in mobile phase. Through successive dilutions, the stock solution was diluted to the lowest concentration of 0.1 µg/ml. The LOD and LOQ were assessed as lowest concentration which provided response of linearity and precision (RSD < 2%). The LOD and LOQ were calculated based on standard deviation of response obtained from solution diluted and the slope value of calibration curve. The solution was injected in three replicates (Thais *et al*., 2008). The equations of detection and quantification limits are, thus:

$$\mathrm{LOD} = 3.3 \times \frac{S}{b} \quad (3)$$

$$\mathrm{LOQ} = 10 \times \frac{S}{b} \quad (4)$$

Where; S: standard deviation of response

b: the slope of the calibration curve

3.3.2 Formulations and preparation of nicotinamide liposomes

The liposomes containing nicotinamide were formulated using nicotinamide at the concentration of 50 mg/ml (5% in formulations) and prepared by modified ethanol injection method. The formulations were optimized in terms of type, ratio and concentration of lipids and additives as shown in **Table 3.2**. Firstly, the oil phase was prepared by dissolving soybean phosphatidylcholine (SPC) and cholesterol (CHO) in 10 ml ethanol. The water phase was prepared by dissolving nicotinamide and Tween80 in 10 ml distilled water. Both phases were sonicated at 30 ºC until homogeneity was obtained. Then, they were repeatedly heated at 60 ºC by swirl continuously for 1 minute in round bottom flask. Subsequently, the water phase was poured into the oil phase and ethanol was evaporated by Rotary evaporator at 60 ºC to obtain the liposome suspensions.

Table 3.2 Compositions of nicotinamide liposome formulations

Composition	**Composition ratio of lipid (*Weight ratio, **Molar ratio)**	**Total lipid (µmole/ml)**
1. SPC:CHO		20
2. SPC:CHO	4:1**	40
3. SPC:CHO		60
4. SPC:CHO		80
5. SPC:Tween80		20
6. SPC:Tween80	84:16*	40
7. SPC:Tween80		60
8. SPC:Tween80		80
9. SPC:Tween80:CHO		20
10. SPC:Tween80:CHO	4:1:1**	40
11. SPC:Tween80:CHO		60
12. SPC:Tween80:CHO		80
13. SPC		20
14. SPC	-	40
15. SPC		60
16. SPC		80

3.3.3 Characterizations of nicotinamide liposomes

3.3.3.1 Physical appearances

The physical appearances of all liposomes were visually observed for colloidal appearance, color, phase separation, and precipitation.

3.3.3.2 Particle size and zeta potential

The size and zeta potential of liposomes containing nicotinamide were determined using zeta potential analyzer at 25 ºC. Before determination, the liposomes containing nicotinamide suspension was diluted about 15 folds with distilled water.

3.3.3.3 Entrapment efficiency

The entrapment efficiency of liposomes containing nicotinamide was evaluated by ultracentrifugation technique. The liposome formulations were centrifuged at 60,000 rpm, 4 ºC for 2 h using ultracentrifuge. The supernatant was collected to determine the amount of non-entrapped nicotinamide by HPLC. The liposomes formulation (not centrifuged) was busted with the 20% v/v Triton®X-100 and diluted with mobile phase to determine the total amount of nicotinamide in liposomes formulation. The entrapment was calculated from the equation;

$$\text{Entrapment efficiency (\%)} = \frac{T-F}{T} \times 100 \quad (5)$$

Where; T: total amount of nicotinamide in formulation

F: free nicotinamide (non-entrapped amount)

3.3.3.4 Surface morphology

Surface morphology of liposomes containing nicotinamide was examined using scanning electron microscopy (SEM). The amount of liposome formulations 200 µl was diluted with 3 ml MilliQ-water. A drop of diluted liposomes was allowed to dry on the cover slip and then stained with crystal violet solution for 1

minute. An excess dye was rinsed out with water followed by fixing with Gram's iodine solution for 1 minute. This staining method was based on Gram's Method where the positive charge of crystal violet interacted with negative charge of the phospholipids in liposomes and then formed complex with I^- and I_3^-(Davies *et al*., 1983). The sample was then coated with gold in a sputter coater under an argon atmosphere (50 Pa) at 50 mA for 50 seconds and investigated under SEM at 50,000X magnification.

3.3.3.5 pH

pH of liposome formulations was evaluated by pH meter. All determinations were performed in triplicate.

3.3.4 Stability study of liposomes containing nicotinamide

The best liposomes containing nicotinamide were selected for stability testing based on physical appearance (color, precipitation, and separation) as well as the optimal particle size, and percent entrapment efficiency. Liposome formulations were storage at 4±2 ºC and 25±2 °C for 2 months compared to the freshly prepared liposome formulations (Pinsuwan *et al*., 2010).

3.3.5 *In vitro* skin permeation study

3.3.5.1 Skin preparation

The experiments were performed by using the skin of naturally died newborn pig which the age was not more than three days. The pig skins were cleaned and removed hair off with clipper without any damage on the skin surface. The

subcutaneous fat was carefully removed by surgical scissors. The skin samples were packed in aluminum foil and stored at -20 ºC until used. Two hours before the beginning of the experiments, the skin was pre-equilibrated in phosphate buffer solution (PBS) pH 7.4 at 25 ºC (Pinsuwan *et al*., 2010).

3.3.5.2 *In vitro* skin permeation procedures

The skin samples were mounted carefully on Franz diffusion cells which the effective diffusion area was 1.77 cm^2. The stratum corneum of pig skin was set side up for contacted with sample. The receptor compartment was filled with 11 ml of PBS. Temperature and magnetic stirrer were controlled of 37 ºC and 200 rpm, respectively. The skin permeation of nicotinamide-loaded liposomes was determined compared with nicotinamide solution containing the same amount of nicotinamide (50 mg/ml). Moreover, nicotinamide liposome creams was determined compared with nicotinamide cream and commercial product. The 1 ml of liposome sample and a 1 gram of cream sample were applied on the skin surface in the donor part of the Franz diffusion cells. The sample of receiver medium in receptor fluid was sampled of 1 ml at period times of 0.5, 1, 2, 4, 6, 8, 12 and 24 hours and replaced with the same amount of PBS every sampling (Pinsuwan *et al*., 2010). All withdrawn samples were filtered through a 0.45 µm syring filter membrane and analyzed of amount of nicotinamide permeated by HPLC. The data were plotted between amounts of nicotinamide permeation skin versus times. All determinations were performed in triplicate. The cumulative amount was calculated according to the following equation;

$$Qcum = \frac{PnVo + \sum_{i=1}^{n-1} PiV}{A} \quad (6)$$

Where; *Pn* and *Pi*: the nicotinamide concentration determined at No. n and No. i (µg/ml)

Vo and *V*: the volume of a single Franz cell and withdrawn sample (ml)

A: effective diffusion surface area (cm^2)

The cumulative amounts were then plotted as a function of time. Additionally, the flux of nicotinamide at steady state (J_{ss}, µg/cm^2/h) through the pig skin was calculated from the slope of linear portion of the plot and the permeability coefficient (K_p, cm/h) was calculated from the equation;

$$Kp = \frac{Jss}{Co} \quad (7)$$

Where; *Co*: the initial concentration of nicotinamide in the donor compartment

3.3.5.3 Determinations of nicotinamide content in pig skin

At the end of Franz diffusion cell experiment (24 hours), abundant nicotinamide was removed from the skin by wiping with cotton balls soaked with PBS pH 7.4. The part of skin was cut into the small pieces and homogenized in 5 ml of methanol at 24,000 rpm for 2 minutes. The mixture was centrifuged at 12,000 rpm 4 ºC for 30 minutes to separate supernatant from the skin lipid. The supernatant was filtered through to 0.45 µm membrane and determined for nicotinamide content by HPLC. The determination was performed in triplicate (Padula *et al*., 2008).

3.3.5.4 Calibration curve of nicotinamide in pig skin

Blank skin was cut to small pieces. Then, the nicotinamide standard solution was spiked in the six of blank skin samples to obtain nicotinamide concentration in blank skin of 1, 5, 10, 20, 30, and 40 µg/ml. After 12 hours, the skin was managed in the same method **3.3.5.3**. The calibration curve of nicotinamide in pig skin was plotted peak area of nicotinamide versus as its concentration. The determination was performed in triplicate.

3.3.6 Preformulation study of cream containing nicotinamide liposomes

3.3.6.1 Formulation of cream base

Preparation of o/w emulsion creams was conducted by beaker method. All chemicals used in this experiment were showed in **Table 3.3**. These chemicals were divided into 2 phase; oil phase, and water phase. The both oil and water phases were heated to about 75 ºC using water bath. The water phase was then poured into the oil phase and continuously stirred until congealed. The formulation was kept overnight at room temperature.

3.3.6.2 Formulations of nicotinamide cream and nicotinamide liposome creams

Nicotinamide cream and nicotinamide liposome cream were prepared in the same method. A 2% nicotinamide (20 mg/g) was added in creams formulation. Firstly, 40 grams of nicotinamide liposomes or nicotinamide solution were added into the 60 grams of cream base after its temperature decreased to about 45 ºC. The mixture was stirred until congealed at room temperature.

Table 3.3 Ingredients of the o/w emulsion cream bases (Sukhapat, 2005)

Ingredients	Content (g)
Oil phase	
Mineral oil	0.8
Cremophor A6	1.8
Cremophor A25	1.8
Glyceryl monostearate	3.8
Isopropyl myristate	2
Stearic acid	2
Water phase	
Glycerin	10
Disodium EDTA	0.1
Potassium sorbate	0.2
Sodium metabisulfile	0.1
Purifier water to 100 g	

3.3.6.3 Quantitative determinations of nicotinamide liposome creams and nicotinamide cream

Each 1 gram of the nicotinamide liposome cream or nicotinamide creams was dissolved in 10 ml of methanol. The mixture was then sonicated for 30

minutes, centrifuged at 4 ºC, 4,500 rpm for 10 minutes (Jansuk, 2007). The supernatant was filtered using 0.45 µm membrane. Quantitative analysis of nicotinamide was determined by HPLC method. The determination was performed in triplicate**.**

3.3.6.4 Physical evaluations of cream formulation

3.3.6.4.1 Physical appearances

The physical appearances of each formulation were investigated such as color, phase separation, and smoothness or roughness of creams.

3.3.6.4.2 pH measurement

The pH value of each formulation was determined using pH meter. The measurement was performed in triplicate.

3.3.6.4.3 Viscosity measurement

The viscosity of each formulation was determined by Brookfield DV-III Ultra Rheometer (Brookfield Rheocale operating software version 3.1-1) controlled the Rheometer. The measurement was performed at 25 ºC. All measurements were performed in triplicate.

3.3.6.5 Stability study of cream formulations

The stabitlity of cream base, nicotinamide creams, and nicotinamide liposome creams were tested by Freeze thaw cycle method for 6 cycles (1 cycle; 4±2 ºC for 24 hours and 45±2 ºC 24 hours). pH, viscosity, and physical appearances

(phase separation, smoothness, or roughness) were observed before and after the Freeze thaw testing. Each sample was considered for triplicate.

3.3.6.6 *In vitro* skin permeation study of cream formulation

The *in vitro* skin permeation studies of nicotinamide liposome creams were performed comparing to nicotinamide cream and the commercial product using modified Franz diffusion cell, as described in section **3.3.5.** The experiment was performed in triplicate.

3.3.7 Statistical analysis

All experiment data were presented as mean±standard deviation (SD). Analysis of variance (ANOVA) was used to test the statistical significance of difference among groups. The significance of the difference of the mean was tested using the Student's *t-test*. The differences were considered statically significant when $p < 0.05$.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Quantitative analysis of nicotinamide using HPLC

Figure 4.1 shows a typical chromatogram of a nicotinamide standard solution. The retention time of nicotinamide was about 6 minutes.

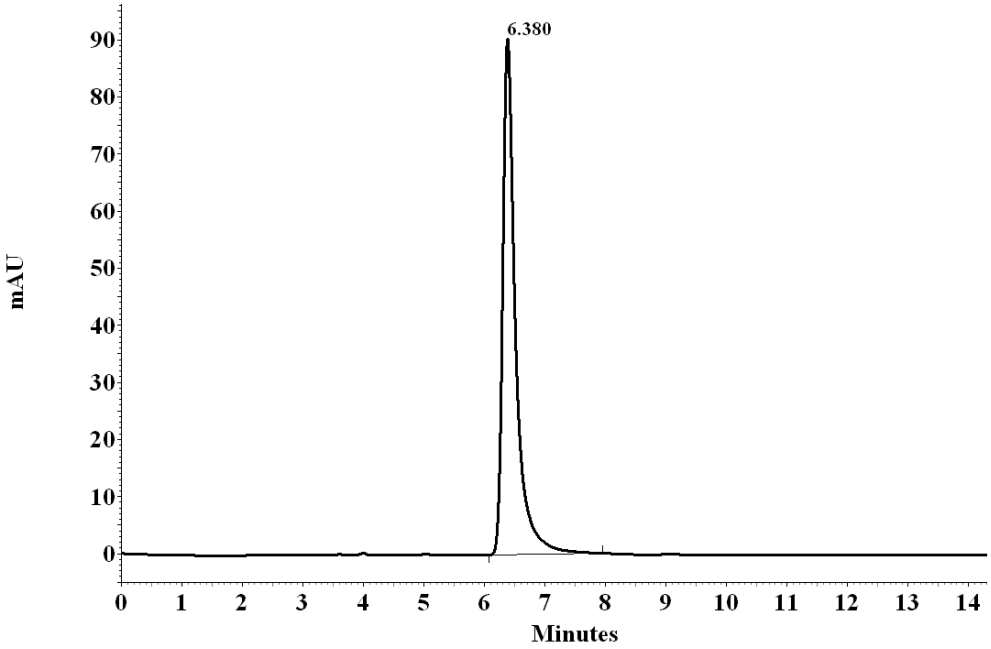


Figure 4.1 Typical chromatogram of nicotinamide standard solution (10 µg/ml)

Linearity

The standard curves of nicotinamide standard solution and nicotinamide spiked in pig skin were constructed as shown in **Figure 4.2** and **4.3**,

respectively. In both cases, the linear relationships were obtained with coefficients of determination (R^2) of more than 0.99.

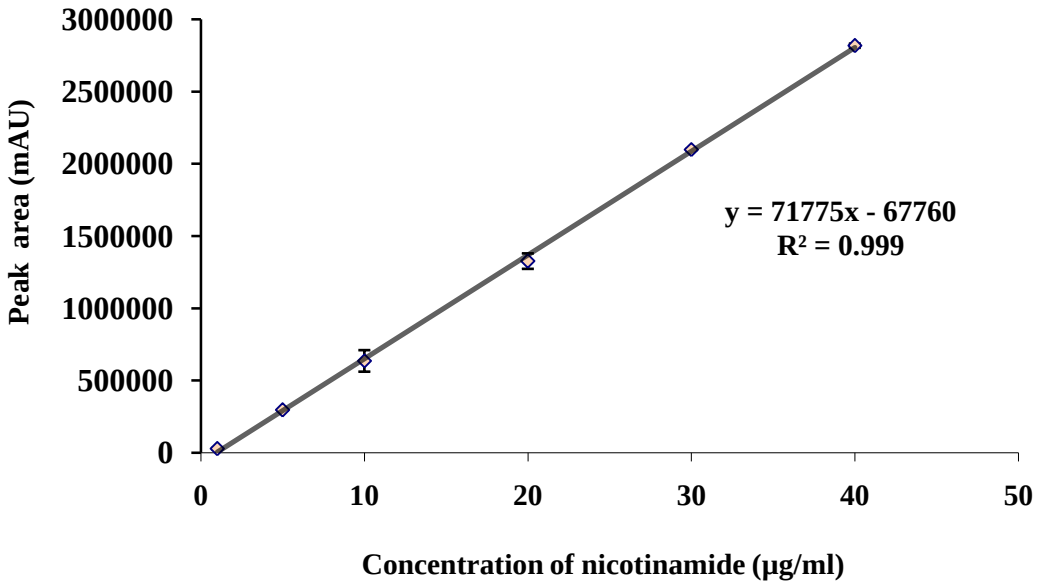


Figure 4.2 A calibration curve of nicotinamide standard solution

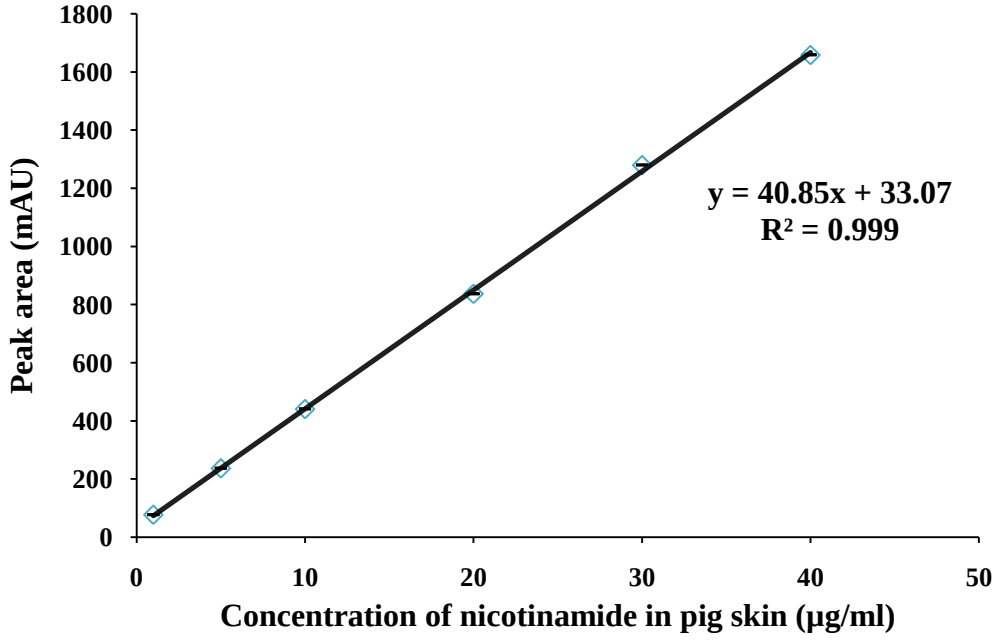


Figure 4.3 A calibration curve of nicotinamide spiked in blank skin

Accuracy

As shown in **Table 4.1**, the average recoveries from nicotinamide analysis varied between 99.90-100.29%, with RSD in range of 0.17-0.76%. These values were within acceptable limits of ICH guideline (1996) for both recovery (98-102%) and RSD (less than 2.0 %).

Table 4.1 Accuracy of nicotinamide determination (n=3)

Spiked conc. (mg/ml)	**Mean of measured Conc. (mg/ml)**	**Average recovery (%)**	**% RSD**
30.05	30.02±0.05	99.90	0.17
50.06	50.04±0.09	100.29	0.43
70.10	70.05±0.04	99.46	0.76

To investigate the possibilities of the liposomes as skin drug delivery system, the *in vitro* skin permeation study was carried out using modified Franz diffusion cells. The average recoveries of nicotinamide were founded in range of 98.30-101.83% as shown in **Table 4.2**. These values were within acceptable limits of ICH guideline (1996).

Table 4.2 Accuracy of nicotinamide spiked in blank skin determination (n=3)

Actual concentration of nicotinamide (µg/ml)	Measured concentration of nicotinamide (µg/ml)	% Recovery
20	19.66±0.26	98.30
30	30.55±0.12	101.83
40	39.86±0.21	99.65

Precision

Table 4.3 and **4.4** are showing the intra- and inter-day precision of nicotinamide analysis, respectively. The %RSD was in range of 0.12-1.98% for intra-day precision and 0.08-1.08% for inter-day precision. Additionally, the average recoveries of both analyses were between 98-102%. All of these data were acceptable for the ICH guideline (1996).

Table 4.3 Intra-day precision of nicotinamide determination (n=3)

Spiked conc. (µg/ml)	Mean of measured Conc. (µg/ml)	Average recovery (%)	% RSD
0.99	0.97±0.03	101.28	0.12
10.10	9.99±1.04	100.24	1.98
30.00	29.61±0.29	98.69	0.98

Table 4.4 Inter-day precision of nicotinamide determination (n=3)

Spiked conc. (µg/ml)	**Day**	**Mean of measured Conc. (µg/ml)**	**Average recovery (%)**	**%RSD**
0.99	1	0.95±0.03	99.68	0.22
	2	1.00±0.01	101.50	0.50
	3	0.97±0.01	101.13	0.16
10.10	1	10.08±0.02	100.24	0.08
	2	9.88±1.02	98.39	1.08
	3	10.04±0.51	98.79	0.17
30.00	1	29.61±0.29	98.69	0.98
	2	29.96±0.34	99.90	0.79
	3	29.80±0.20	99.76	0.89

Specificity

Since this analytical method was applied for the determination of nicotinamide in formulations and skin permeation studies, therefore it should be able to identify the peak of nicotinamide from those of impurity or other components in formulations, as well as the receptor fluid, and the skin model which used in skin permeation study. The results were illustrated through the specificity of the chromatograms obtained from the analysis of nicotinamide in liposomes, PBS pH 7.4, and the pig skin as given in **Figure 4.4**, **4.5**, and **4.6**, respectively. No interfering peak was observed at the retention times of nicotinamide, indicating the good specificity of this nicotinamide assay.

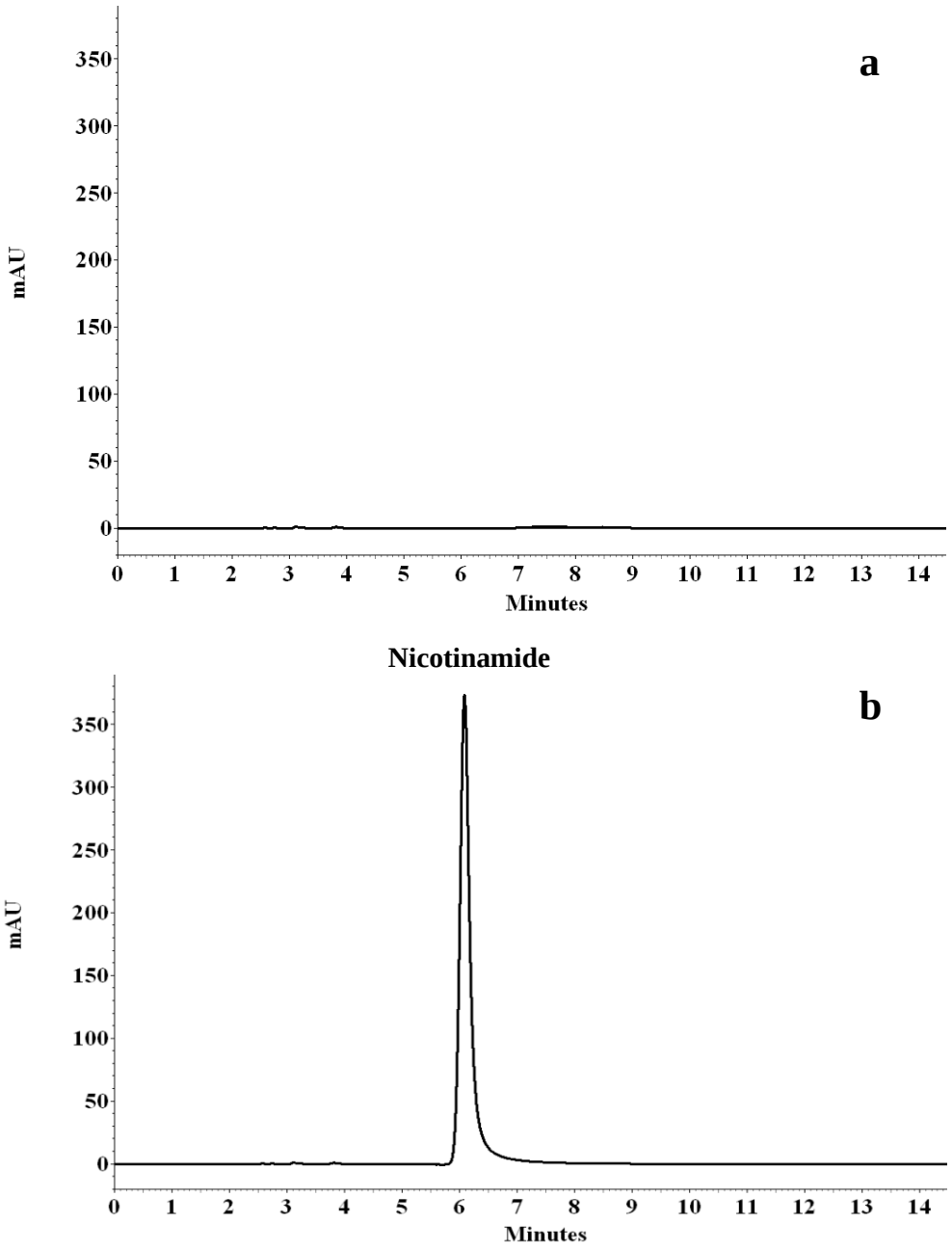


Figure 4.4 Chromatograms of (**a**) blank liposomes and (**b**) nicotinamide spiked in liposomes (30 µg/ml)

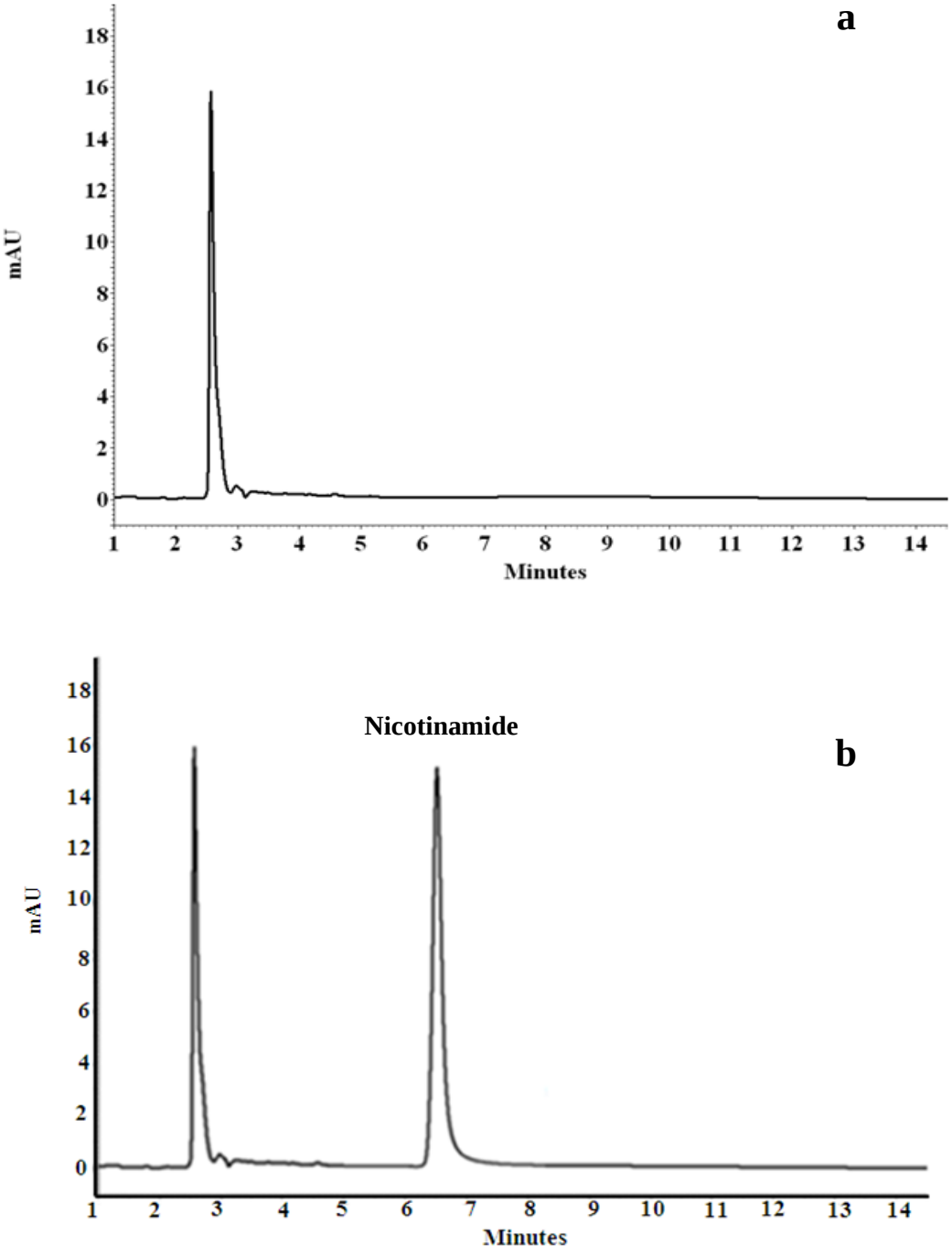


Figure 4.5 Chromatograms of (**a**) blank PBS pH 7.4 and (**b**) nicotinamide spiked in PBS pH 7.4 (10 µg/ml)

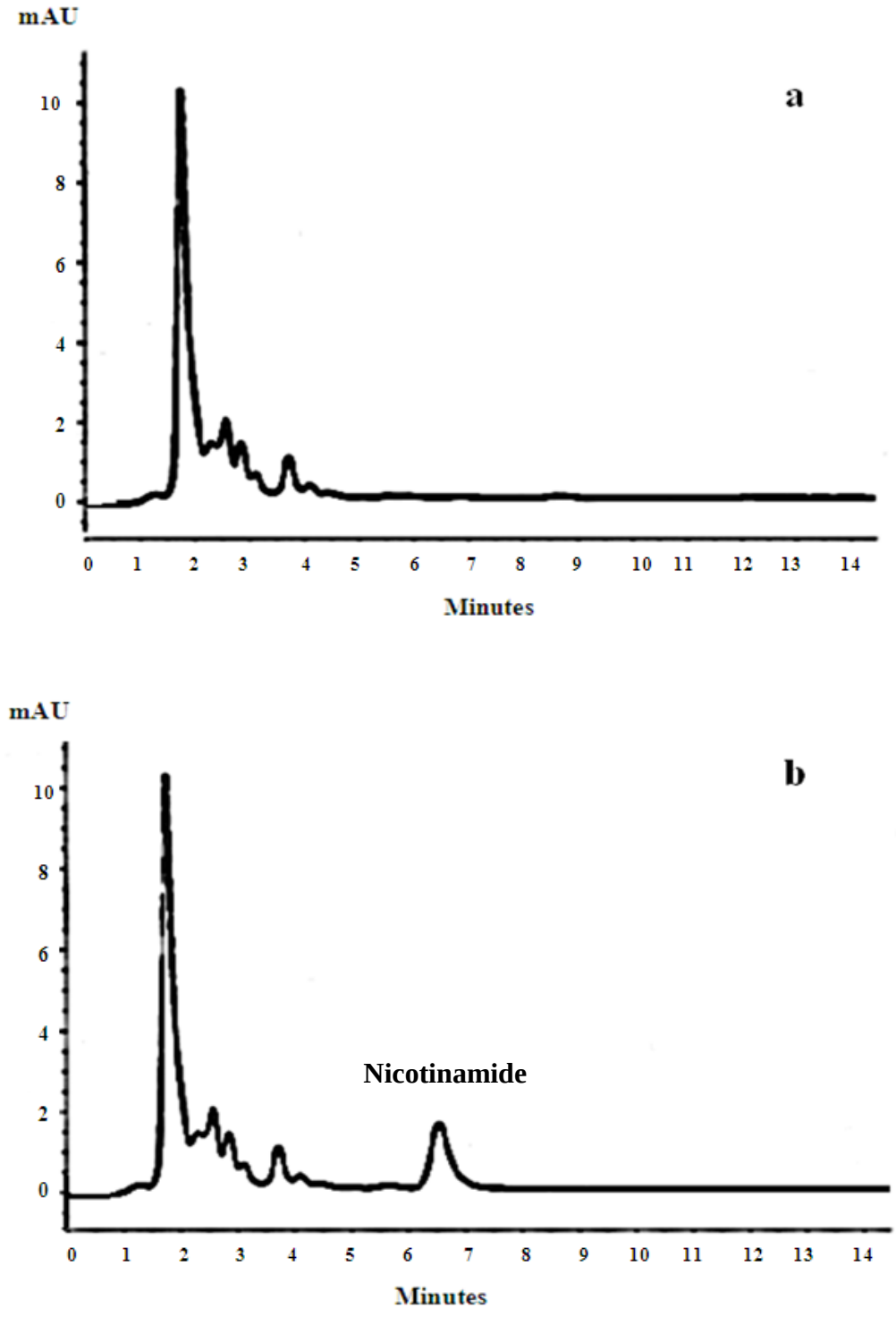


Figure 4.6 Chromatograms of (**a**) blank skin and (**b**) nicotinamide spiked in blank skin (20 µg/ml) after extraction with methanol

LOD and LOQ

The obtained detection and quantification limits of nicotinamide analysis were 0.050±0.04 µg/ml and 0.136±0.03 µg/ml, respectively.

4.2 Formulations and preparation of liposomes containing nicotinamide

The nicotinamide liposomes were prepared by modified ethanol injection method. The advantage of this method is the simplicity and the absence of many potentially harmful chemical and physical treatments (Minghui *et al*., 2008). In this experiment, 5% nicotinamide (w/v) was cooperated in the liposome formulations with various lipid composition and amount. The physical appearances of all liposome formulations were shown in the **Figure 4.7**. All formulations gave the milky to yellowish colloidal appearance depending on the amount of total lipid content in formulations. The liposomes with lowest total lipid content exhibited the milky colloidal appearances as shown in formulations no. 1, 5, 9, and 13, whereas the formulations with highest total lipid content showed the yellowish colloidal appearances as shown in formulations no. 4 and 8. In addition, no precipitation and phase separation were observed in all formulations.

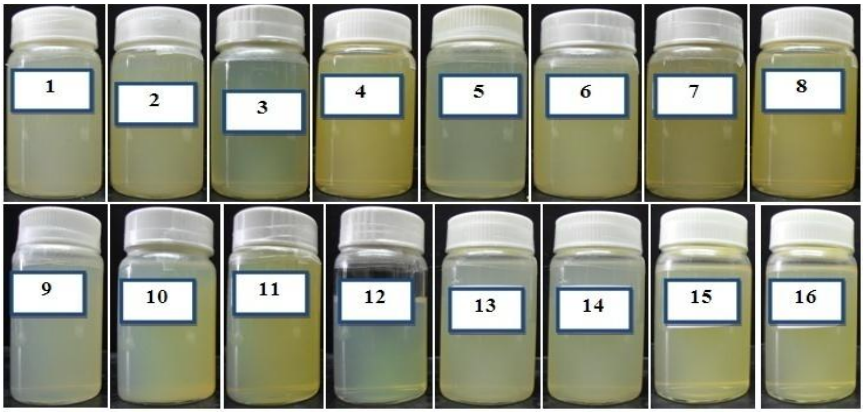


Figure 4.7 Physical appearances of nicotinamide liposome formulations

Nicotinamide liposomes consisting of SPC and CHO were formulated in formulations 1-4, as listed in **Table 4.5**. It was found that, the total lipid content affected the degree of entrapment efficiency. The 40 μmol/ml of total lipid content (formulation 2) gave the highest entrapment efficiency. However, as total lipid increase led to 60 and 80 μmol/ml, the percent entrapment efficiency decreased from 65% to 60% and 48% (formulations 2-4). CHO could increase the rigidity of liposomes and affect on their entrapment efficiency (Minghui *et al*., 2008). It was inserted into the liposome membrane with its hydroxyl groups oriented toward the aqueous surface and aliphatic chain aligned parallel to the acyl chains in the center of bilayer (El Maghraby *et al*., 2004; Emnet, 2010). In the other hand, CHO regarded as a crystal-breaker of the gel phase and an inducer of chain-ordering in the fluid phase without rigidification of the overall phase (Lasic, 1996). But, the CHO level beyond a certain level might start disrupting the bilayer structure leading to loss of solute molecules encapsulated (Minghui *et al*., 2008). Then, it could be predicted that CHO strongly enhanced the liposomes bilayer but at its certain, the breaking of liposomal vesicle and the decreasing of percent entrapment efficiency were observed. Therefore, the appropriate proportions between CHO and total lipid amounts were important to the percent entrapment efficiency (Minghui *et al*., 2008). In addition, increasing amount of the total lipid affected to particle size of nicotinamide liposomes formulation. The particle size of nicotinamide liposome formulations 1-4 increased from 172 nm to 240 nm. These might be due to the larger volume of the aqueous central core in liposome vesicles. This investigation was in agreement with the report of Prasanth *et al*. (2012).

Table 4.5 Percent entrapment efficiency and particle size of liposome formulations

No.	Composition	Total lipid (µmol/ml)	Ratio		Entrapment efficiency (%) (n=3)	Particle size(nm) (n=3)
			weight	molar		
1	**SPC:CHO**	20		4:1	50.06±0.02	172.96±1.79
2		40			65.61±1.15	206.28±2.95
3		60			60.21±0.18	212.89±3.04
4		80			48.59±0.01	240.30±3.50
5	**SPC:Tween80**	20	84:16		58.59±0.10	331.20±3.18
6		40			64.85±0.58	430.59±6.86
7		60			69.33±0.03	380.29±1.27
8		80			66.43±0.09	355.10±9.90
9	**SPC:Tween80:CHO**	20	-	4:1:1	12.04±0.12	110.20±6.41
10		40			35.85±1.47	163.60±2.20
11		60			48.43±2.21	181.20±1.62
12		80			73.86±0.09	185.40±1.25
13	**SPC**	20	-	-	34.45±0.25	200.90±2.10
14		40			38.53±0.45	216.20±1.31
15		60			43.08±0.39	232.16±2.26
16		80			72.38±0.53	261.35±4.16

In our study, the surfactant added into liposomal formulation is Tween80. It was added in nicotinamide liposome formulations 5-8 as shown as well in **Table 4.5**. The percent entrapment efficiency of liposome was increased from 58% to 69% as total lipid was increased from 20 to 60 µmol/ml. However, the total lipid increasing to 80 µmol/ml, percent entrapment efficiency of nicotinamide liposome was decreased. The resulting of Tween80 in formulations might to weaken the bilayer membrane of liposomes, easy bilayer leakage. Moreover, Tween80 had influenced on amount affected particle size of nicotinamide liposomes. As the increasing amount of the total lipid from 20 to 40 µmol/ml, particle size was increased as shown in formulations 5-6. However, the increasing total lipid to 60 and 80 µmol/ml, particle size was decreased as shown in formulations 7 and 8. Since Tween80 is a nonionic surfactant with a large head group (El Maghraby *et al*., 2004). El Maghraby *et al*. (2004) reported that Tween80 was living between bilayer by the head group of Tween80 linked to the head group of the phospholipids and the oleate residue (lipophilic part) parallel to the acyl chains of phospholipid. As Tween80 appearing in lipophilic region of liposome, the bilayer of liposome vesicle has thicken, lipophilic region area increased but the aqueous central core volume of liposomes decreased, whereas, if the Tween80 exposing aqueous central core, the bilayer of liposomes was thin, and aqueous central core volume of liposome vesicle increased (Minghui *et al*., 2008). The high aqueous central core volume, nicotinamide amount was highly loaded in the liposome. These investigations have influence on the change of percent entrapment efficiency and particle size of liposome. However, this experiment could not verify about the position of Tween80 leaved in liposome vesicle, but the change amount of total lipid might be describe the physicochemical properties of liposome

which it composed of SPC and Tween8o in formulation. Our experiment showed that Tween80 many increase or decrease the particle size of liposome. The effect of Tween80 on particle size depended on its position inserted in liposome vesicle. Although Tween80 in liposomal formulation could enhance the flexibility of bilayer, but its unsuitable quantities could also effect to physicochemical properties of liposome (El Maghraby *et al*., 2004).

Formulation 9-12 was prepared with adding both Tween80 and CHO in formulation. Adding of CHO and Tween80 in formulation affected the percent entrapment efficiency and particle size as shown in **Table 4.5**. The increase total lipid to 80 µmol/ml, formulation 12 showed the highest percent entrapment efficiency. So, the suitable amount and ratio of Tween80 to CHO could reduce broken or leakage of liposomes vesicle, leading to increase percent entrapment efficiency. Additionally, as the total lipid content increased, the particle size of liposome formulations 9-12 were increased. In this experiment, adding of CHO and Tween80 in liposomal formulations showed good formulation (high percent entrapment efficiency and small particle size) when compared to the formulations contained only either CHO or Tween80.

To compare with liposome formulation without any additives, formulations containing only SPC were prepared as shown in **Table 4.5** (formulation 13-16). Increasing the total lipid content, percent entrapment efficiency and particle size was increased. Theoretically, entrapment efficiency should increase with increasing lipid concentration both in the lipophilic and hydrophilic drug (Brisaert *et al*., 2001). The ultimate lipid concentration in liposomal dispersion depended on hydration volume used during the preparation, hydration volume higher than lipid

concentration, the low liposomal dispersion (Minghui *et al*., 2008). At the low total lipid content, lowering nicotinamide was encapsulated in liposomes vesicle, percent entrapment efficiency was decreased as shown in formulation 13 in **Table 4.5**. Also, the enough lipids concentrations, the higher nicotinamide were encapsulated, and the higher percent entrapment efficiency was exhibited as in formulation 16. Additionally, the percent entrapment efficiency and particle size of liposomes was depended on composition of formulations, ratio, and total lipid content (Pinsuwan *et al*., 2010). The particle size of all nicotinamide liposomes in this preparation (110-430 nm) might be characterized as the LUV or MLV types.

4.3 Stability study of nicotinamide liposomes

The formulations with percent entrapment efficiency more than 50% were selected to stability study. Nine formulations were selected including formulation 1, 2, 3, 5, 6, 7, 8, 12, and 16. Before testing, all formulations were freshly prepared. It was found that no changed of color, and no precipitation and phase separation was observed in freshly prepared formulations.

After stability study at 4 °C and 25 °C for 2 months, no precipitation and phase separation was investigated for both temperatures as shown in **Figure 4.8**. However, the physicochemical properties of all formulations were evaluated before and after stability study. Percent entrapment efficiency of all formulations decreased after storage of 4 °C and 25 °C for 2 months as shown in Table 4.6. Notice that, all formulations kept at 4°C had more decreases of entrapment efficiency comparing to these at 25 °C.

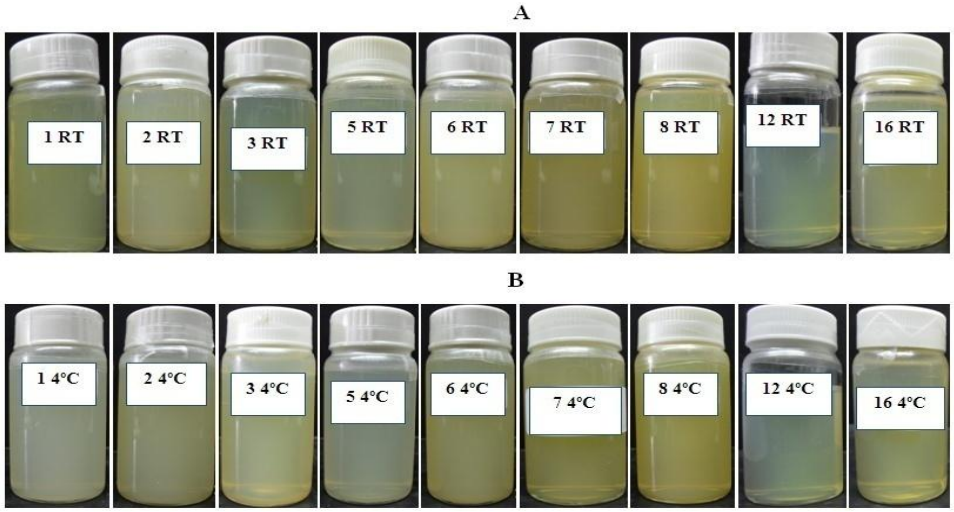


Figure 4.8 Physical appearances of various nicotinamide liposome for after storage at (**A**) 25±2 ºC (**RT**) and (**B**) 4±2 ºC for 2 months

Table 4.6 Percent entrapment efficiency of nicotinamide liposome for stability study before and after 2 months (n=3)

No.	**Entrapment efficiency (%)**		
	Before stability test	**After stability test**	
		4±2 °C	**25±2 °C**
1	50.06±0.02	4.93±0.12	3.53±0.10
2	65.61±1.15	46.66±0.04	39.88±0.25
3	60.21±0.18	39.73±0.07	30.77±0.18
5	58.59±0.10	30.09±0.28	25.15±0.18
6	64.85±0.58	42.25±0.29	30.89±0.21
7	69.33±0.03	37.88±0.09	33.70±0.27
8	66.43±0.90	43.96±0.69	37.39±0.14
12	73.86±0.09	57.16±0.15	33.35±0.42
16	72.38±0.53	38.00±0.15	37.89±0.24

Omar (2002) reported that liposome was exposure to higher oxidation at 25 °C than 4 °C, increased leakage or peeling of liposome bilayer and decreased percent entrapment efficiency. His report was accorded to the result of our experiment. Additionally, a water soluble drug was incorporated into the aqueous compartments by associated with the polar head groups of bilayer via electrostatic interaction and entrapment within the aqueous compartments depend on the aqueous volume. Then, the aqueous volume was depended on the ratio, total lipid, and composition in formulation (Kulkarni *et al*., 1995).

The particle size evaluation was also investigated. The **Table 4.7** shows the change of particle size of liposomes kept in both 4 °C and 25 °C for 2 months. Phospholipids are susceptible to oxidation and hydrolysis reactions, effective lipid bilayer degradation, particle aggregation, fusion and/or vesicle disruption tendencies, effective increasing or decreasing of particle size (Sriram and Rhodes, 1995; Mitkari *et al*., 2010). Increasing particle sizes might be occurred by aggregation or fusions of particles, and decreasing particle size might be occurred with liposome bilayer degradation. Additionally, a PI value exhibited narrow as shown in **Table 4.7**. A PI value has very narrow, liposome particle showed a better uniform in diameter (Minghui *et al*., 2008). After 2 months, PI value increased in both 4 °C and 25 °C. The highest PI of liposomes was 0.38±0.05 for 4 °C and 0.30±0.12 for 25 °C. The changing of PI values was affected of disrupting the bilayer structure (Minghui *et al*., 2008).

Table 4.7 Particle size and polydispersity index (PI) of nicotinamide liposome for stability study before and after 2 months

No.	Particle size (nm)			PI		
	Before stability	After stability test		Before stability test	After stability test	
		4±2 °C	25±2 °C		4±2 °C	25±2 °C
1	172.96±1.79	170.20±0.90	167.30±1.70	0.12±0.02	0.27±0.03	0.16±0.02
2	206.28±2.95	263.20±4.00	203.70±1.40	0.15±0.01	0.33±0.03	0.23±0.04
3	212.89±3.04	189.30±1.00	196.90±1.10	0.13±0.09	0.38±0.05	0.16±0.05
5	331.20±3.81	308.20±6.70	289.00±5.20	0.23±0.02	0.32±0.02	0.30±0.12
6	430.59±6.86	406.40±3.40	383.50±7.20	0.16±0.01	0.24±0.08	0.26±0.04
7	380.29±1.27	401.40±0.43	304.20±3.40	0.13±0.02	0.22±0.06	0.06±0.03
8	355.10±9.90	342.10±3.12	310.30±3.30	0.05±0.02	0.29±0.08	0.09±0.16
12	185.40±1.25	197.80±1.62	196.00±1.60	0.08±0.02	0.10±0.07	0.20±0.05
16	261.35±4.16	317.20±3.72	311.70±1.30	0.22±0.06	0.12±0.02	0.14±0.03

In addition, zeta potentials of liposomes were investigated. Zeta potential is an important and useful indicator of particle surface charge, which can use to predict and control the stability of colloidal suspensions or emulsion (Minghui *et al*., 2008). All formulations in this study showed negative charges of zeta potentials, because of zwitterionic nature of phosphatidylcholine monomers and negative charge of nicotinamide (**Table 4.8**). It was regarded that the absolute value of zeta potential under ±30 mV is required for full electrostatic stabilization; zeta potential between ±5 and ±15 mV is in the region of limited flocculation (Minghui *et al*., 2008). Then, zeta potential values more and less than 30 were indicated stable and unstable of formulation, respectively. This rule was in agreement of our results as shown in **Table 4.8**. It was found that the changes of zeta potential were not significant after stability test. Additionally, zeta potential values presented increasing trend at 4 °C more than those at 25 °C. Since, the bilayer of liposomes was damaged by oxidation or hydrolysis at 25 °C more than at 4 °C, the electrostatic force of particle decreased, leading to decrease of zeta potential (Omar, 2002).

Table 4.8 Zeta potential of nicotinamide liposome before and after keeping the liposome at 4 °C and 25 °C for 2 months

No.	Zeta potential (mV)		
	Before stability test	After stability test	
		4±2 °C	25±2 °C
1	-16.47±4.65	-20.63±2.96	-11.43±2.47
2	-19.57±5.44	-41.92±1.77	-31.78±2.45
3	-21.56±4.71	-39.80±1.60	-33.83±1.49
5	-31.75±4.94	-30.59±2.88	-34.86±1.50
6	-30.22±2.50	-37.04±3.24	-35.38±1.42
7	-27.90±2.45	-37.78±0.37	-30.73±1.14
8	-41.30±1.40	-41.15±0.42	-36.59±0.02
12	-24.20±1.87	-38.95±0.30	-30.95±0.50
16	-23.49±1.93	-34.38±4.32	-34.30±2.00

The change pH values of formulations were subsequently in the stability test. It was found that the pH values of formulation were initially in the range of 5.93±0.03- 6.35±0.04. After stability test, no significant change of pH values was observed in both pHs as shown in **Table 4.9**. Grasso (1972) reported, the pH of solution influences percutaneous absorption principally by determining the state of ionization of a particular compound. A unionized substance penetrated to skin lipid layer greater than ionized substances. Weak acid or alkaline has ionized less than strongly acid or alkaline, then, the penetration of weak acid or alkaline more than

strongly acid or alkaline. If the substance is strongly acid or alkaline a destructive effect of skin and skin irritancy occurs which will be decrease skin permeability. Then, the nicotinamide solution and nicotinamide liposome formulation have pH value in range of 6.0-7.5 and 5-6, respectively. They are weak acid, which are suitable for applying to the skin and should not make skin irritation.

Table 4.9 pH of nicotinamide liposome before and after keeping at 4 °C and 25 °C for 2 months

No.	**pH value**		
	Before stability test	**After stability test**	
		4±2 °C	**25±2 °C**
1	6.07±0.28	6.08±0.22	6.07±0.06
2	6.35±0.04	6.03±0.33	5.88±0.03
3	6.21±0.08	6.00±0.02	6.01±0.02
5	6.32±0.03	5.94±0.05	5.69±0.01
6	6.27±0.03	5.98±0.02	5.89±0.09
7	6.15±0.03	5.86±0.01	5.82±0.05
8	6.10±0.06	5.91±0.04	5.65±0.05
12	6.17±0.07	5.86±0.02	5.55±0.10
16	5.93±0.03	5.75±0.02	5.63±0.06

The temperature affected on stability of liposomes was reported by Tiwari Sandip *et al*. (2000). Liposomes stability is thought to be affected by lipid composition and type of liposomes. The assessment of leakage degree of drug from the vesicles and drug decomposition were used as the parameters to ascertain the stability of the vesicles at various temperatures. In this study, a physicochemical

property of nicotinamide liposomes has changed at different temperatures. At the low temperature (4 °C), nicotinamide liposomal formulations were more stable than those at high temperature (25 °C). Therefore, the changes in their physicochemical properties were depended on the ratio, and total amount of composition, as well as drug behavior in formulation, these results were according to the study of Tiwari Sandip *et al*. (2000).

After stability test for 2 months, the appropriate nicotinamide liposomal formulation was selected for skin permeation studies based on their physicochemical properties. In this study, the formulation 12 which composed of SPC: Tween80: CHOL (4:1:1 molar ratio), total lipid content of 80 μmol/ml was selected. The morphology of nicotinamide liposome formulation 12 was evaluated using SEM as shown in **Figure 4.9**. The micrograph could prove the existence of nanosize closed-vesicles in the colloidal liposomal suspension.

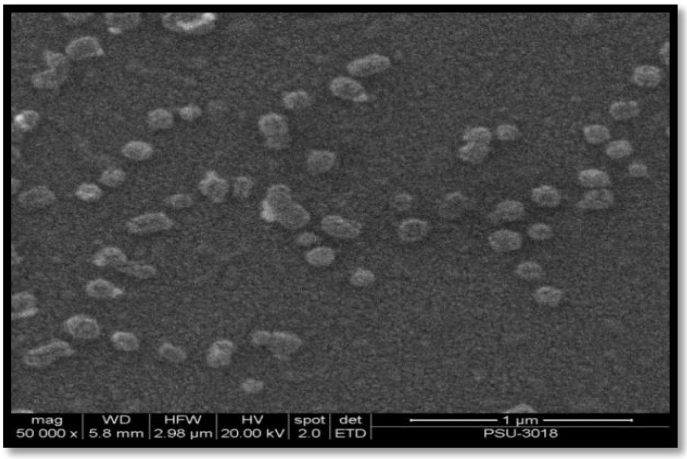


Figure 4.9 SEM of nicotinamide liposomes 12 (×50,000 magnification).

4.4 *In vitro* skin permeation study

The **Figure 4.10** shows the skin permeation of nicotinamide from liposomes and solution. After 24 hours, skin permeation of nicotinamide liposomes was significantly higher when comparing with nicotinamide solution (0.59±0.02% vs. 0.31±0.06%). Flux values of the nicotinamide liposome and solution were 12.03 and 8.18 μg/cm^2/h, respectively as shown in **Table 4.10**. The K_p values was 2.41 $\times 10^{-4}$ and 1.64 $\times 10^{-4}$ cm/h for nicotinamide liposomes and solutions, respectively. The enhanced skin permeation of nicotinamide liposomes might be the increased fluidity of the skin barrier, caused by the interaction of phospholipid molecules of the liposome structure (Valenta *et al*., 2000; Yarosh, 2001; Sigh *et al*., 2005; Yu and Liao, 1996). Likewise, increasing skin permeation affected to increasing flux and K_p values. These values of nicotinamide liposomes were higher than those values of nicotinamide solution. A different flux value between nicotinamide liposomes and nicotinamide solutions was due to the repulsion between charge of liposomes or solutions and skin membrane. Low repulsion, resulting in high flux value was presented as shown in **Table 4.10**. Additionally, **Figure 4.10** exhibits sink condition. A sink condition occurs when the amount of drug that can be dissolving in the dissolution medium is three times greater than the amount of drug to be dissolved. The rate of drug dissolution will be showed by the limited solubility of the drug in that medium (Tejal *et al*., 2008). The account of nicotinamide dissolved in receptor fluid (PBS 7.4) was shown three times greater than the amount of nicotinamide to dissolved. After 12 hours, the nicotinamide solution was constant permeated to skin, while the nicotinamide liposomes showed higher skin permeation.

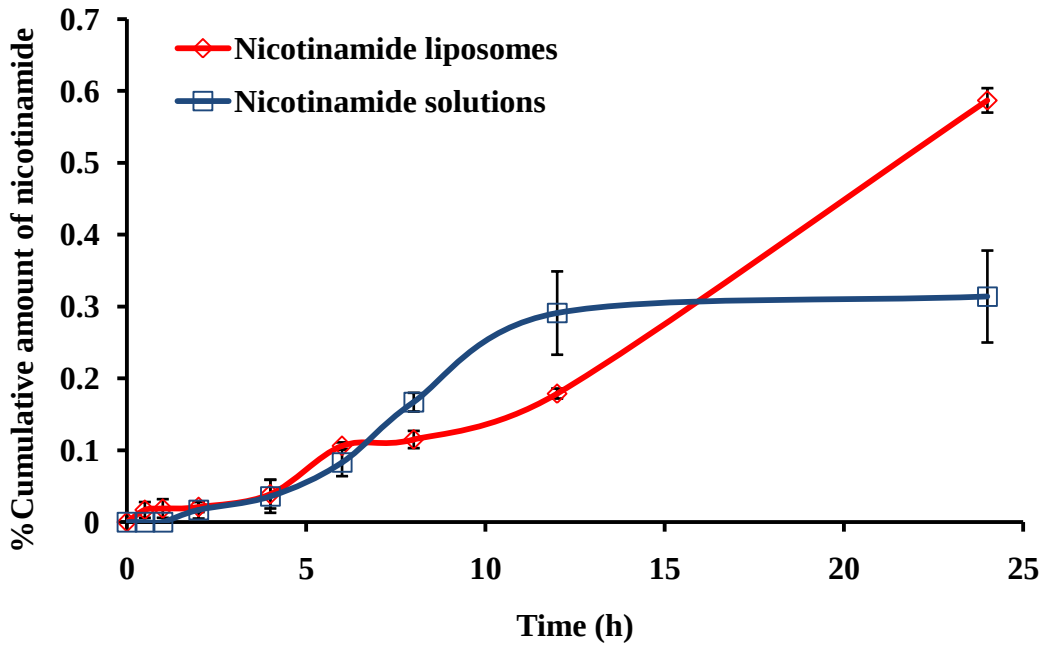


Figure 4.10 *In vitro* %cumulative amount-time profiles of nicotinamide permeated across pig skin from the liposome (formulation 12) and solution at same concentration (50 mg/ml), for 24 hours ($p < 0.05$)

Table 4.10 *In vitro* skin permeation parameters of nicotinamide from the liposome formulation and solution after 24 hours (n=3)

Formulations	**Q_{cum} (%)**	**J_{ss} (µg/cm^2/h)**	**K_p (×10^{-4} cm/h)**
Nicotinamide liposomes 12	0.59±0.02	12.03±0.35	2.41±0.21
Nicotinamide solutions	0.31±0.06	8.18±0.54	1.64±0.42

Moreover, the amount of nicotinamide accumulated in the pig skin and receptor were investigated as shown in **Figure 4.11**. After 24 hours, nicotinamide liposomal formulation was higher accumulated in the skin than that of solution (1.02±0.03**%** and 0.80±0.01%, respectively). Similarly in receptor fluid; nicotinamide

from liposome was found higher in the receptor fluid than that from solution (1.02±0.02% and 0.70±0.03%, respectively). These results showed that liposome formulation could be suitable for skin delivery and enhance the amount of nicotinamide penetrated to the skin comparing to nicotinamide. This investigation could be described that the stratum corneum is the primary barrier for the delivery of nicotinamide into the dermal layer. Liposome structure is phospholipids as skin structure, and then the nicotinamide liposome could easily penetrate into the skin better than nicotinamide solution. Additionally, virtues of structural characteristics of liposome are proven to deliver and accumulate nicotinamide in the skin by overcoming the barrier effect of stratum corneum (Srinivasan *et al*., 2009).

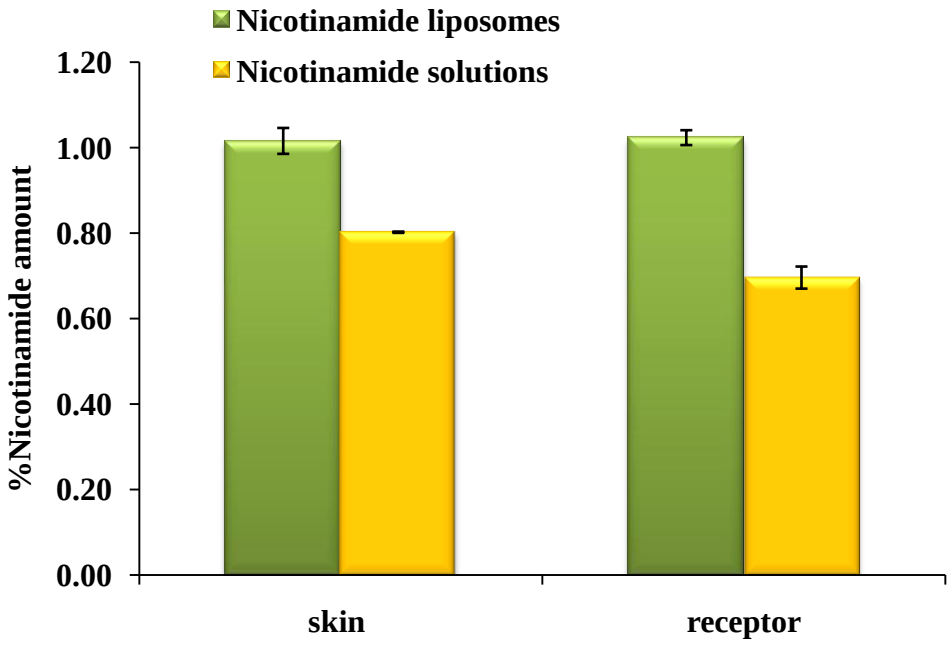


Figure 4.11 *In vitro* amount of nicotinamide (%) accumulated in pig skins and receptor chamber of diffusion cells from liposome (formulation 12) and solution

4.5 Formulations and stability study of nicotinamide cream formulations

The nicotinamide liposome (formulation 12) was selected to formulate into dosage form. The characteristics of cream base, nicotinamide cream, and nicotinamide liposome cream were shown in **Figure 4.12** for **A1**, **B1**, and **C1,** respectively. Each formulation was tested for its stability using Freeze-thaw cycles method. The appearances of each cream formulation after stability test were illustrated in **Figure 4.12** for **A2**, **B2**, and **C2**. No significance change of the physical appearances (color, smelt, smoothness, and separation) the freshly prepared was observed before and after stability test. In addition, the freshly prepared cream formulation was evaluated for pH and viscosity. All freshly prepared formulations gave pH values of approximately 5 as shown in **Table 4.11,** which is similar to the human skin pH (4-6). After stability test, no change in pH values was observed in all formulations.

The viscosity of cream formulation was determined before and after stability testing. Cream base had showed the highest viscosity following by nicotinamide cream, and liposome cream. The last two formulations had similar viscosity. No significant change of the viscosity was observed before and after stability test for all formulations as shown in **Table 4.11**. The viscosity was affected by temperature using in Freeze thaw method. Additionally, changes of temperature affected to inter facial tension changes, viscosity, as well as the vapor pressure of the liquid phases, and the thermal agitation of the molecules. Thus, an emulsion cream was very sensitive to temperature changes. Emulsions were more stable when the

temperature is near the point of minimum solubility of emulsifying agents. Emulsion stability was decreased when temperature increased (Abdurahman and Rosli, 2006).

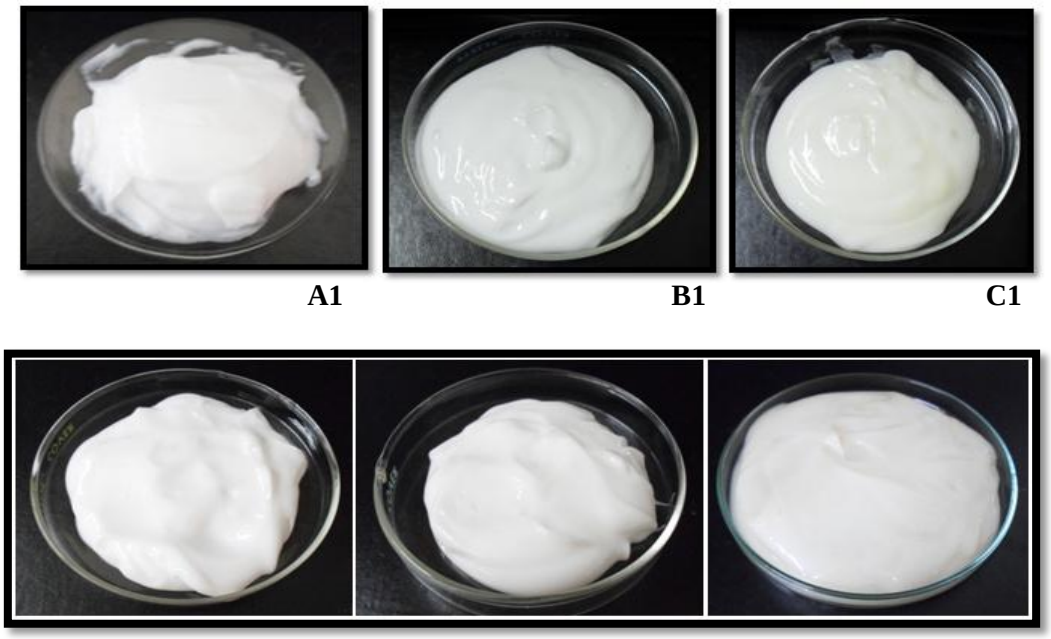


Figure 4.12 Characteristic appearances of cream base, nicotinamide cream, and nicotinamide liposome cream before (**A1**, **B1**, **C1**) and after (**A2**, **B2**, **C2**) stability test under Freeze thaw cycles

Table 4.11 pH and viscosity of cream formulations before and after stability test by Freeze thaw cycles

Formulation	pH		Viscosity (Pa)	
	before FT	**after FT**	**before FT**	**after FT**
Nicotinamide cream	5.65±0.03	5.55±0.02	10706±18.37	9088±119.98
Nicotinamide liposome cream	5.51±0.02	5.45±0.01	10364±14.84	9148±49.99
Commercial product	5.47±0.02	5.40±0.01	Not detected	Not detected
Cream base	5.11±0.03	5.20±0.02	16257±419.91	14632±64.29

4.6 Quantitative determination of nicotinamide liposome cream formulations

The amount of nicotinamide content in cream formulations was determined as shown in **Table 4.12**. The mean recovery assay for each formulation was in range of 70-130% which is acceptable according to ICH guidline (1996).

Table 4.12 Nicotinamide amount in cream formulations (n=3)

Formulations (1 g)	**Actual concentration (mg/ml)**	**Measured concentration (mg/ml)**	**% Recovery**
Nicotinamide creams	20	17.55±52.77	87.74±0.26
Nicotinamide liposome creams	20	18.01±56.08	90.07±1.28

4.7 *In vitro* skin permeation study of cream formulations

After 24 hours for *in vitro* skin permeation study, the cumulative amount of nicotinamide form cream formulations was illustrated in **Figure 4.13**. Maximum cumulative amount of nicotinamide liposomes cream was 7.25±0.05%, whereas those form nicotinamide cream and commercial product were 1.03±0.01% and 0.99±0.02%, respectively. Nicotinamide liposome cream could significantly penetrate into the skin better than nicotinamide cream and commercial product for about 7 folds. Cream formulations could also affect the skin permeation of nicotinamide. Cream is emulsion of oleaginous substances and water. For this experiment, type of cream was o/w emulsion where water is incorporated into the continuous phase of the emulsion. Using this type of cream for skin delivery will

enhance made to the accumulation of large moisture between the skin and the ointment layer that caused hydration of the stratum corneum and increasing humidify of the stratum corneum. Hydration of stratum corneum was allowed 'opening up' of intra- and inter- cellular channels and pathways for easier passage of drug molecules. A drug molecule is able to easily enter the stratum corneum (Ratna, 2004). As shown in **Figure 4.13**, the cumulative amount after 24 hours from nicotinamide liposome cream was found higher than those from nicotinamide cream and commercial product. That may due to the synergist effect of phospholipids bilayer of liposome and the emulsion. From this result, nicotinamide liposomes cream seemed to be suitable as carriers for skin delivering when compared with other formulations.

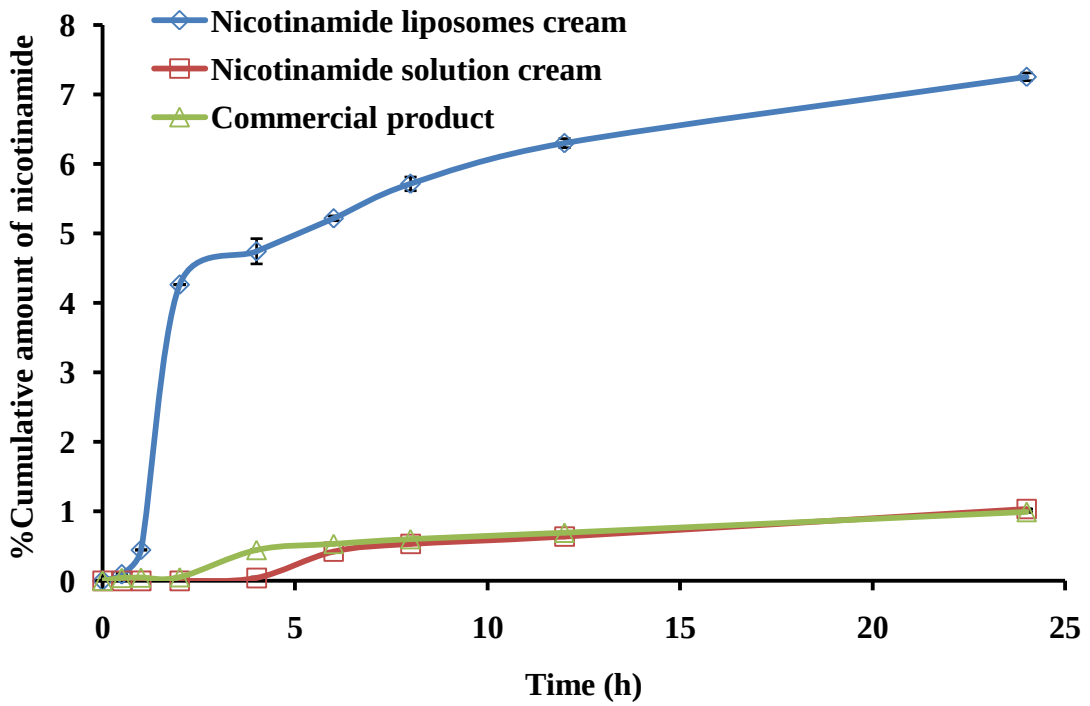


Figure 4.13 *In vitro* %cumulative amount-time profiles of nicotinamide permeated across pig skin from nicotinamide cream, nicotinamide liposome cream, and the commercial product for 24 hours (n=3) ($p < 0.05$)

Additionally, the accumulation of nicotinamide in the skin and receptor fluid was determined. **Figure 4.14** shows that in all cream formulations, nicotinamide passed into the receptor more than accumulated in the skin. The nicotinamide liposome cream shows maximum accumulation of nicotinamide in the skin as 1.47±0.05 %, whereas nicotinamide cream and commercial product gave 0.79±0.27% and 0.40±0.03%, respectively. In receptor fluids, the accumulated amount of nicotinamide amount from nicotinamide liposome cream, nicotinamide cream, and commercial product were found to be 11.98±0.13%, 1.38±0.50%, and 0.70±0.03%, respectively. The liposome cream shows the highest amount of accumulated nicotinamide in receptor fluids. Cream formulations could enhance skin permeation of nicotinamide because of the skin permeation enhancer in creams. Isopropyl myristate could increase skin permeation of nicotinamide by generating disordered bilayers in the corneocyte-bound lipids (Nava, 2005). Other compositions in creams formulation could also increase skin permeability. Nicotinamide liposomes cream gave the highest accumulation of nicotinamide in receptor because phospholipids composition of liposomes could be mixed with the stratum corneum lipid or penetrated though the skin by inter cellular pathway mostly when nicotinamide liposomes was contained in creams formulation. In addition, cell of stratum corneum layer was disrupted, leading to reversible deformation in the bilayers structure that allowed the creation of various types of “opening” the bilayers.

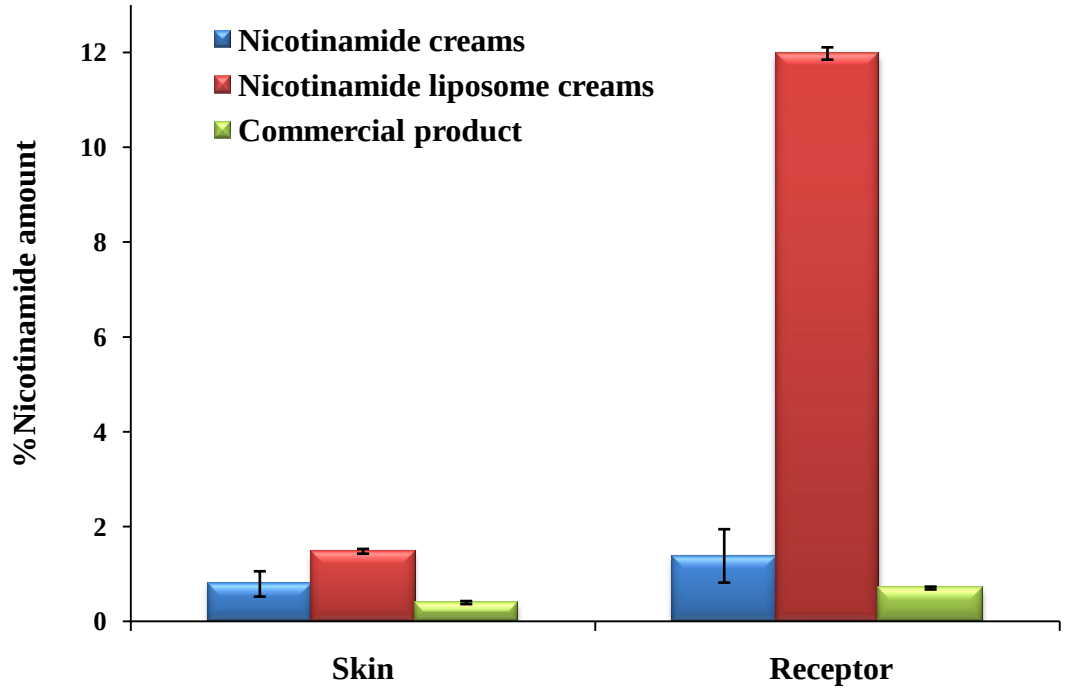


Figure 4.14 *In vitro* amount of nicotinamide (%) accumulated in pig skins and receptor chamber of diffusion cells from nicotinamide cream, nicotinamide liposome cream, and commercial product (n=3)

Moreover, flux of nicotinamide liposome cream was shown in **Table 4.13**, the maximum flux and permeation coefficient were 60.45±0.46 µg/cm^2/h and 30.22±2.31×10^{-4}cm/h, respectively. Flux of nicotinamide creams and commercial product were 8.62±0.02 and 8.26±0.01 µg/cm^2/h. The flux of nicotinamide liposome cream was increased for about 52% comparing to nicotinamide cream and commercial product. Nicotinamide liposomes creams exhibited highest permeability and steady state flux. Liposomes are potential drug carriers for a variety drugs that includes the traditional small molecular weight drugs, hydrophilic or hydrophobic drugs, and hydrophilic and lypophilic drugs. The liposomes vesicle consists of simple lipid bilayers that resemble biological membranes, in the form of a spherical shell can be enhances skin permeation of drug (Sriram, and Rhodes, 1995). Nicotinamide have

Table 4.13 *In vitro* skin permeation parameters of nicotinamide from cream, liposome cream, and commercial product after 24 hours (n=3)

Formulations	**Q_{cum} (%)**	**J_{ss} (μg/cm^2/h)**	**K_p (×10^{-4} cm/h)**
Nicotinamide creams	0.79±0.27	8.62±0.02	4.31±2.08
Commercial product	0.40±0.03	8.26±0.01	9.20±0.01
Nicotinamide liposome creams	1.47±0.05	60.45±0.46	30.22±2.31

hydrophilicity leading to difficult for skin permeation. Thus, the development of nicotinamide in liposomal formulations could increase its skin permeation. The nicotinamide was loaded into vesicle of liposomes, topically applied liposome vesicles could be mixed with the stratum corneum lipid matrix and penetrate to skin. So, liposomes proposed the skin penetration and increased substance transport across the skin (Bouwstra and Honeywell-Nguyen, 2002). Moreover, the objective of this experiment is to deliver nicotinamide liposome for anti-inflammatory of acne vulgaris into the dermis layer of skin. Although, the amount of nicotinamide founded in the receptor fluid was more than that in the skin but this amount has no effect to the systemic absorption and no toxic because it is not over its pharmacological dose (≥ 3 g/day). Thus, this research indicates that the nicotinamide liposomal preparations could improve the skin permeability. The nicotinamide liposome cream was value added products for nicotinamide. However, this research should be evaluated for *in vivo* study for justifying the efficacy of nicotinamide liposome cream.

CHAPTER 5

CONCLUSION

To improve the skin permeation efficiency of nicotinamide, the permeation enhancing substance carrier named liposomes was explained in this research. For this experiment, sixteen different formulations of nicotinamide liposomes were prepared with the composition such as SPC, CHO, and Tween80. The different formulations of nicotinamide liposome were formulated and evaluated in physicochemical property such as the appearance of formulation (non sedimentation, non separation), entrapment efficiency, and particle size. This study had purposed to select the suitable physicochemical properties of formulations for stability study. Thus, nine suitable formulations were selected such as formulations 1, 2, 3, 5, 6, 7, 8, 12, and 16. These formulations showed the range of entrapment efficiency of 50-74 %, and particle size of 172-430 nm. After stability test for 2 months, nicotinamide liposomal formulation 12 was stable after storage at 4 ºC with the entrapment efficiency of 73.86±0.09%, particle size of 185.40±1.25 nm, PI of 0.08±0.02, zeta potential -32.20±1.87 mV and pH of 6.17±0.07. Thus, nicotinamide liposomal formulation 12 was selected for cream preparation and skin permeation study comparing to nicotinamide solution. It was found that, compositions in formulations affected the physicochemical property of liposomes. In addition, appropriate ratio caused satisfying entrapment efficiency of the formulation. The best formulation was then formulated in to cream and evaluated for *in vitro* skin permeation comparing

with nicotinamide cream and commercial product. The *in vitro* skin permeation of nicotinamide liposome was found significantly potential when compared with nicotinamide solution. The skin permeation parameters of nicotinamide liposomes such as Q_{cum}, J_{ss}, and K_p were 0.59±0.02%, 12.03±0.35 μg/cm^2/h, and 2.41±0.21 (×10^{-4}cm/h), respectively, these all values were higher than those of nicotinamide solution. Additionally, the *in vitro* skin permeation of nicotinamide liposome cream gave significantly amount when compared with nicotinamide cream and commercial product. The nicotinamide liposome creams shows higher skin permeation parameter than nicotinamide creams and commercial product, Q_{cum}, J_{ss}, and K_p values of nicotinamide liposomes cream were 7.25±0.05%, 60.45±0.46 μg/cm^2/h, and 30.22±2.31 (×10^{-4} cm/h), respectively. The accumulation of nicotinamide in the skin and receptor fluid, from nicotinamide liposomes formulation were 1.02±0.03% and 1.02±0.02%, respectively. Thus, nicotinamide liposomes gave significantly higher nicotinamide accumulated in the skin and receptor fluid than that of nicotinamide solution. The nicotinamide liposome creams showed that nicotinamide accumulated in skin and receptor were 1.47±0.05% and 11.98±0.13%, respectively providing significantly higher compared with nicotinamide cream and the commercial product. It is indicated that the liposomes could be enhanced the skin penetration of nicotinamide, and therefore its concentration could be reduced in cream formulations. Skin permeation efficacy of nicotinamide liposomes and nicotinamide liposome cream was higher than those of other formulations. In conclusion, these studies showed the potential of liposomes as the skin carrier for nicotinamide. However, the *in vivo* and clinical evaluation should be achieved to confirm the clinical efficiency.

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