Development of Ethosome Containing Indomethacin for Transdermal Delivery

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ชื่อวิทยานิพนธ์ การพัฒนาอีโธโซมของอินโดเมทาซินเพื่อการนำส่งผ่านผิวหนัง

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บทคัดย่อ

อินโดเมทาซินเป็นยาต้านการอักเสบที่ไม่ใช่สเตียรอยด์ มีประสิทธิภาพในการรักษาอาการปวดและอักเสบในบริเวณที่เกี่ยวข้องกับการเคลื่อนไหวของร่างกาย เช่น โรคข้ออักเสบรูมาตอยด์ เป็นต้น การบริหารยาอินโดเมทาซินโดยการรับประทานอาจก่อให้เกิดอาการข้างเคียงต่อระบบทางเดินอาหารและระบบประสาทส่วนกลาง ซึ่งอาการข้างเคียงเหล่านี้จะมีความสัมพันธ์กับขนาดยาที่ได้รับ ดังนั้นการนำส่งยาอินโดเมทาซินผ่านผิวหนังจึงเป็นอีกทางเลือกหนึ่งในการหลีกเลี่ยงอาการไม่พึงประสงค์ที่อาจเกิดขึ้น แต่อย่างไรก็ตามเนื่องจากอินโดเมทาซินมีความสามารถในการซึมผ่านผิวหนังได้น้อย จึงทำให้การใช้ในรูปแบบยาทามีประสิทธิภาพในการรักษาต่ำ ในการศึกษานี้จึงนำอีโธโซม ซึ่งเป็นระบบนำส่งยาที่สามารถช่วยเพิ่มการซึมผ่านผิวหนังมาประยุกต์ใช้ในการนำส่งยาอินโดเมทาซิน รวมทั้งประเมินประสิทธิภาพในการซึมผ่านผิวหนังของตำรับ

ในการศึกษานี้ทำการวิเคราะห์ปริมาณอินโดเมทาซินโดยใช้วิธี HPLC ซึ่งมีความจำเพาะเจาะจง ถูกต้อง และแม่นยำสูง จากการศึกษาการละลาย พบว่าอินโดเมทาซินละลายได้ดีในสารละลายที่เป็นกลางหรือด่าง และเอธานอลจะสามารถช่วยเพิ่มการละลายของยาได้เช่นกัน ในการพัฒนายาในรูปแบบอีโธโซม พบว่าสูตรตำรับอีโธโซมของอินโดเมทาซินสามารถเตรียมได้โดยวิธี thin film hydration โดยใช้ฟอสเฟตบัฟเฟอร์ pH 7.4 ร่วมกับเอธานอลในความเข้มข้นต่างๆ (10-30% v/v) เป็นตัวกลาง ทั้งนี้ปริมาณของเอธานอล รวมทั้งองค์ประกอบของไขมันในตำรับอีโธโซม จะมีผลต่อลักษณะทางกายภาพ ขนาดอนุภาค และประสิทธิภาพการกักเก็บยา พบว่าสูตรตำรับที่เหมาะสมมีองค์ประกอบของไขมัน คือ 4% w/v SPC:CHOL:DA (อัตราส่วน 6:2:1 โดยโมล) และตัวกลาง คือ 20% v/v เอธานอลในฟอสเฟตบัฟเฟอร์ pH 7.4 ซึ่งจะให้อีโธโซมที่มีขนาดอนุภาค 55.3±7.8 nm (PI=0.207±0.160), ศักย์ซีต้า -39.06±1.53 mV

และประสิทธิภาพการกักเก็บยา 52.51±4.09% อีโธโซมที่เตรียมได้มีความคงตัวดีทั้งทางกายภาพและทางเคมี เมื่อเก็บในภาชนะปิดสนิทที่อุณหภูมิห้องเป็นเวลา 3 เดือน แม้ขนาดอนุภาคของอีโธโซมจะเพิ่มขึ้นอย่างมีนัยสำคัญก็ตาม แต่พบว่าไม่มีการเปลี่ยนแปลงอย่างมีนัยสำคัญของประสิทธิภาพการกักเก็บยาและศักย์ซีต้า นอกจากนี้เมื่อเก็บอีโธโซมในตู้เย็น (4±2°C) เป็นเวลา 2 สัปดาห์ จะมีผลึกรูปเข็มกระจายอยู่ในตำรับ ซึ่งเป็นผลของอุณหภูมิต่อการละลายของอินโดเมทา-ซิน เมื่อศึกษาการซึมผ่านและการสะสมยาในผิวหนังนอกกาย พบว่าตำรับอีโธโซมที่เตรียมได้มีการซึมผ่านผิวหนัง (Q_t ที่ 24 ชั่วโมง, J_{ss} และ K_p) สูงกว่าเมื่อเทียบกับสารละลายอินโดเมทาซินที่จำหน่ายในทางการค้า และสารละลายในเอธานอลซึ่งมีความเข้มข้นของตัวยาเท่ากัน (8 mg/ml) รวมทั้งมีค่า lag time น้อยกว่าอย่างมีนัยสำคัญ นอกจากนี้อีโธโซมยังให้ปริมาณยาสะสมในผิวหนังที่เวลา 24 ชั่วโมง น้อยกว่าสารละลายอินโดเมทาซินที่จำหน่ายในทางการค้า ซึ่งผลจากการทดลองนี้บ่งชี้ถึงความเป็นไปได้ในการนำอีโธโซมมาใช้เป็นตัวนำส่งยาอินโดเมทาซินผ่านผิวหนังและอาจมีศักยภาพในการนำไปพัฒนาต่อในรูปแบบของยาที่บริหารทางผิวหนัง อย่างไรก็ตามควรมีการประเมินประสิทธิภาพทางคลินิกของตำรับในลำดับต่อไป

Thesis Title Development of Ethosome Containing Indomethacin for Transdermal Delivery

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ABSTRACT

Indomethacin is a non-steroidal anti-inflammatory drug which is effective in the management of pain and inflammation within locomotor area such as rheumatoid arthritis. Oral administration of indomethacin can cause gastrointestinal and central nervous system side effects which are related with dose. Therefore, transdermal administration can be considered as an alternative route to avoid its systemic side effects. However, the insufficient of indomethacin skin penetration leads to low clinical efficiency of topically applied indomethacin formulations. The permeation enhancing drug carrier named ethosomes was therefore developed in this study. In addition, the skin permeation of indomethacin from the ethosome formulation was evaluated.

In this study, the analysis of indomethacin was performed using HPLC which gave high specificity, accuracy and precision. From the solubility studies, it has been found that indomethacin was higher soluble in aqueous solutions with neutral or alkaline pH. In addition, ethanol enhanced its solubility. The indomethacin containing ethosomes could be prepared by thin film hydration method using the phosphate buffer pH 7.4 with different concentration of ethanol (10-30% v/v) as dispersion

media. Both ethanol concentration and lipid compositions could affect the physical appearance, vesicular size and drug entrapment efficiency of the ethosomes. It was found that the optimized formulation composed of 4% w/v SPC:CHOL:DA (6:2:1 molar ratio) as lipid component and 20% v/v ethanol in phosphate buffer pH 7.4 as dispersion medium. This formulation gave ethosomes with 55.3±7.8 nm vesicular size (PI=0.207±0.160), -39.06±1.53 mV zeta potential, and the entrapment efficiency of 52.51±4.09%. The ethosome formulation showed good physical and chemical stability after storage in well-closed container at ambient temperature for three months. Although the significant increase in its vesicular size was observed, there was no significant change in the entrapment efficiency and zeta potential. In addition, for the formulation kept in refrigerator (4±2°C) for two weeks, needle-shaped crystals of free drug were observed due to the effect of temperature on the solubility of the drug. The *in vitro* skin permeation and retention study showed that the ethosome formulation provided significantly higher skin permeation of indomethacin (Q_t at 24 h, J_{ss} and K_p) as compared to the commercial solution and ethanolic solution with the same drug concentration (8 mg/ml). In addition, its lag time was significantly reduced. The retained indomethacin in the skin after 24 h application showed that ethosome formulation gave less drug-retained compare to the commercial solution. These results indicated the feasibility of ethosomes as the transdermal drug carrier for indomethacin which consequently could be considered for further development for transdermal applications. However, the clinical efficacy of the ethosome formulation should be evaluated.

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LIST OF ABBREVIATIONS AND SYMBOLS

AUC	area under the curve of drug concentration in plasma-time profile
^{o}C	degree Celsius
CHOL	cholesterol from lanolin
CLSM	confocal laser scanning microscope
cm	centimeter (s)
cm^2	square centimeter (s)
CNS	central nervous system
COX	cyclooxygenase enzyme
DA	deoxycholic acid
DSC	differential scanning calorimetry
e.g.	exempli gratia, for example
EIV	ether injection vesicles
EPC	egg phosphatidylcholine
et al.	et alii, and others
FPV	French press vesicles
g	gram (s)
GI	gastrointestinal
h	hour (s)
HPLC	high performance liquid chromatography
ICH	international conference on harmonization
IS	internal standard
IV	intravenous

LIST OF ABBREVIATIONS AND SYMBOLS (Continued)

J_{ss}	steady state flux
K_p	permeability coefficient
log P	logarithm of the partition coefficient
LUV	large unilamellar vesicles
M	molar (mole per liter)
mA	milli ampere (s)
μg	microgram (s)
mg	milligram (s)
min	minute (s)
μl	microliter (s)
ml	milliliter (s)
MLV	multilamellar vesicles
μm	micrometer (s)
mm	millimeter (s)
MPA	mycophenolic acid
mV	millivolt (s)
nm	nanometer (s)
NMR	nuclear magnetic resonance
NSAID	non-steroidal anti-inflammatory drug
o/w	oil in water
Pa	pascal
%	percent

LIST OF ABBREVIATIONS AND SYMBOLS (Continued)

PBS	phosphate buffer saline solution pH 7.4
PCS	photon correlation spectroscopy
PEG	polyethylene glycol
pH	the negative logarithm of the hydrogen ion concentration
PI	polydispersity index
pKa	the negative logarithm of the dissociation constant
Q_t	cumulative drug permeated
R^2	correlation coefficients
REV	reverse-phase evaporation vesicles
rpm	round (s) per minute
RSD	relative standard deviation
SA	stearylamine
SD	standard deviation
SEM	scanning electron microscope
SMEDDS	self-microemulsifying drug delivery system
SPC	phosphatidylcholine from soybean
SUV	Small unilamellar vesicles
TDD	transdermal delivery device
TEM	transmission electron microscope
THP	trihexyphenidyl hydrochloride
T_m	melting temperature
UD	undetected

LIST OF ABBREVIATIONS AND SYMBOLS (Continued)

ULV	unilamellar vesicles
v/v	volume by volume
vs.	versus
w/v	weight by volume
$\overline{X}$	average value

CHAPTER 1

INTRODUCTION

1.1 Background and Rationale

Indomethacin is a non-steroidal anti-inflammatory drug (NSAID) in indole acetic acid derivative group. It possesses anti-inflammatory, analgesic and antipyretic properties by inhibition of cyclooxygenase enzyme (COX) which catalyzes the biotransformation of arachidonic acid to prostaglandin (McEvoy, 2007). This drug is effective and widely used in the treatment of pain and inflammation in locomotor area such as rheumatoid arthritis, osteoarthritis, ankylosing spondylitis, acute gouty arthritis and acute shoulder pain. Generally, indomethacin is taken orally in the dose of 25-50 mg 3-4 times per day (Klasco, 2008).

Like other NSAIDs, oral indomethacin can cause gastrointestinal (GI) side effects such as nausea, vomiting, dyspepsia. Long-term use of this drug can cause bleeding, ulceration and perforation of GI tract. Moreover, central nervous system (CNS) side effects such as headache, dizziness can be found in most patients. Both GI and CNS adverse reactions of indomethacin are dose related (McEvoy, 2007).

To avoid the systemic side effects from orally taken NSAIDs, the topical formulations such as creams, gels, sprays and transdermal patches are the attractive way to deliver these compounds. The topical formulations of NSAIDs were found to be effective in relieving the pain compared to placebo (Moore *et al.*, 1998)

and provided less plasma drug level compared to oral formulations which could reduce the systemic side effects (Vaile and Davis, 1998; Bannwarth, 2006). However, for indomethacin, insignificant difference in treatment efficacy was observed between the commercial topical formulation and placebo (Moore *et al*., 1998). This result might be explained by the limitation of indomethacin to penetrate the skin due to its unsuitable physicochemical characteristics (Cordero *et al*., 1997; Goosen *et al*., 1998). Since the drug topically applied on the skin should permeate through the skin directly to the inflammation target site (Cevc *et al*., 2008) or to the dermis skin layer before absorption to systemic circulation (Vaile and Davis, 1998), the development of novel indomethacin topical formulations which have higher skin permeation efficiency is still required in pharmaceuticals.

In recent years, the use of permeation enhancing vesicular carriers named ethosomes has evoked an interested in transdermal drug delivery. They are phospholipid bilayer vesicles which contain relatively high concentration of ethanol from 20% up to 45% (Touitou *et al*., 2000). The presence of ethanol in the vesicles which makes them more flexible than the conventional liposomes combined with the permeation enhancing effect of ethanol itself leads to promote more amount and deeper penetration of drug through the skin, as can be clearly evident in many researches (Kirjavainen *et al*., 1999; Dayan and Touitou, 2000; Touitou *et al*., 2000; Lopez-Pinto *et al*., 2005; Elsayed *et al*., 2006). In addition, ethosomes have many suitable characteristics for using as drug delivery carriers such as their smaller size compared to liposomes which comprise the same lipid composition (Dayan and Touitou, 2000; Touitou *et al*., 2000; Lopez-Pinto *et al*., 2005; Dubey *et al*., 2007a; Dubey *et al*., 2007b; Fang *et al*., 2008), high drug entrapment efficiency for both

hydrophilic and lipophilic drugs (Dayan and Touitou, 2000; Touitou *et al*., 2000; Lopez-Pinto *et al*., 2005; Dubey *et al*., 2007b; Fang *et al*., 2008), and good physical stability after storage (Dayan and Touitou, 2000; Touitou *et al*., 2000; Lopez-Pinto *et al*., 2005; Dubey *et al*., 2007a,b; Fang *et al*., 2008).

Indomethacin is hydrolyzed in strong acid (pH<3) and base (pH >8) catalyst (Connors *et al*., 1986), hence the increase in drug solubility of weak acidic indomethacin (pKa=4.5) by increase the pH of the system is limited. However, the decomposition of indomethacin can be minimized in the system composed of ethanol/water and polyethylene glycol (PEG) 400/water (Krasowska, 1974, 1979). In addition, the incorporation of indomethacin in liposomes can reduce the drug degradation from basic hydrolysis (D'Silva and Notari, 1982; Gohel *et al*., 1998; Matos *et al*., 2001). Therefore, the incorporation of indomethacin in ethosome vesicles may be beneficial for chemical stability of the drug since the ethosomal systems compose of ethanol/water and phospholipid vesicles which may helpful for protecting the drug from decomposition.

In this present works, the development of ethosomes containing indomethacin for using as transdermal drug carrier was carried out. The determination of ethosomes characteristics, skin permeation properties as well as physical and chemical stabilities was conducted to determine for the promising indomethacin loaded ethosome formulations which may be efficient for using in clinical treatment.

1.2 Objectives of the study

The objectives of this study were:

1.2.1 To develop and validate a HPLC method for determination of indomethacin from ethosomes.

1.2.2 To formulate and prepare the ethosomes containing indomethacin for using as transdermal drug carriers.

1.2.3 To evaluate the *in vitro* skin permeation and retention efficiency of indomethacin from the ethosomes compared to a commercial solution and ethanolic solution of free drug.

1.2.4 To evaluate the chemical and physical stability of ethosomes containing indomethacin.

CHAPTER 2

REVIEW OF LITERATURE

2.1 Indomethacin

Indomethacin is an indoleacetic acid derivative NSAID. Its chemical structure and physicochemical properties are shown in Figure 2.1 and Table 2.1, respectively.

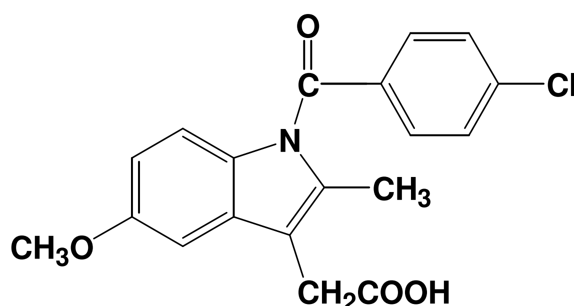

Figure 2.1 Structure of indomethacin

Table 2.1 Physicochemical properties of indomethacin (Florey, 1984)

Generic Name	Indomethacin
Chemical Formula	l-(*p*-chlorobenzoyl)-5-methoxy-2-methylindole-3-acetic acid ($C_{19}H_{16}ClNO_4$)
Molecular Weight	357.79 g/mol
Physical Appearance	Pale yellow to yellow-tan, crystalline powder with a slight odor

Table 2.1 Physicochemical properties of indomethacin (Florey, 1984) (continued)

Melting Point	158 -162 ^{o}C
Dissociation Constant (pKa)	4.5
Partition Coefficient (log P)	3.8 (Goosen *et al*., 1998)
Solubility	1 g dissolves in 30 ml of chloroform, 45 ml of ether and 50 ml of alcohol (Connors *et al*., 1986)

2.1.1 Pharmacological and mechanism of drug action

Similar to other NSAIDs, indomethacin exhibits anti-inflammatory, analgesic and anti-pyretic activity. Although the mechanism of these activities have not been clearly established, but many actions appear to be associated with the inhibition of prostaglandin synthesis in body tissues which is resulted from the inhibition of cyclooxygenase enzyme, the enzyme that catalyzes the formation of prostaglandin from arachidonic acid (McEvoy, 2007).

2.1.2 Clinical application

In clinical treatment, indomethacin has been used in the management of patients with moderate to severe rheumatoid arthritis, ankylosing spondylitis, osteoarthritis, acute painful shoulder (bursitis and/or tendinitis) and acute gouty arthritis. The recommended dosage forms include tablets, capsules, extended release capsules, oral suspensions and suppositories. The suggested dose of indomethacin is

25-50 mg 3-4 times daily, not more than 200 mg/day, for the shortest possible duration in order to reduce the risk of serious adverse effects. Moreover, intravenous (IV) injection of this drug has been used as an alternative to surgical ligation in the treatment of premature neonates with patent ductus arteriosus (Klasco, 2008).

2.1.3 Adverse drug reaction

Adverse effects in patients treated with indomethacin have been estimated to occur 30-60%. Most of the adverse reactions appear to be dose related and mainly involve the CNS and GI tract. The CNS side effect such as headache and dizziness is the most frequently reported (25-50% of patient treated with indomethacin). These side effects are reported to associate with plasma indomethacin concentration. The GI side effect including nausea, vomiting, dyspepsia, diarrhea and gastric mucosal damage, which may result in ulceration and/or bleeding, is less frequent found (3-9% of patients). Although the mechanism of indomethacin inducing GI side effect is not clearly established, the possible mechanism is reported to be involved in the inhibition of prostaglandin E series which exhibits cytoprotective effects on GI mucosa (McEvoy, 2007).

2.1.4 Stability

Indomethacin in powder form is reported to be stable for at least five years at room temperature. The exposure to strong sunlight directly can cause an increase in color of indomethacin powder and aqueous solution with slightly affects to

drug degradation. In aqueous solution, indomethacin can be hydrolyzed at the amide moiety both in acidic and basic pH as shown in Figure 2.2 resulted in two degradation products which are *p*-chlorobenzoate and 2-methyl-5-methoxy-indole-3-acetate (Florey, 1984; Connors *et al.*, 1986).

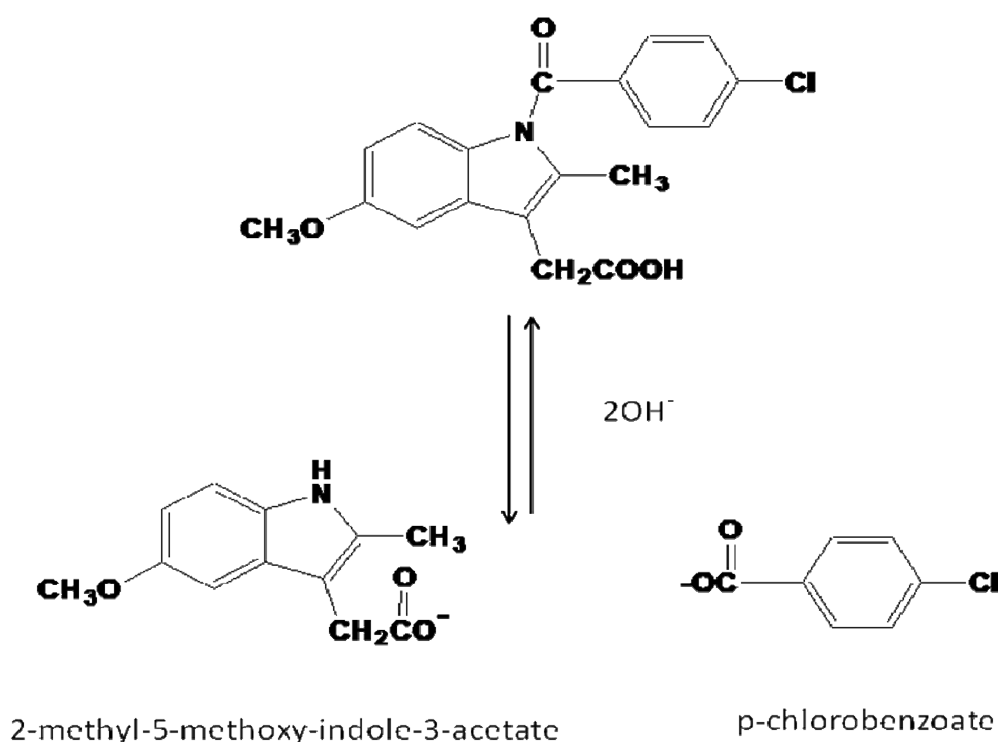

Figure 2.2 Degradation pathway of indomethacin in solutions (Connors *et al.*, 1986)

The pH-rate profile of indomethacin hydrolysis at elevated temperatures is shown in Figure 2.3. This profile includes the region below pH 3 where specific acid catalysis dominates, the broad valley in the region pH 3-5 where water attack is predominant, and the linear region of specific base catalysis above pH 7. At room temperature, indomethacin is maximally stable near pH 3.75, with a calculated shelf life of 8.4 day (Connors *et al.*, 1986).

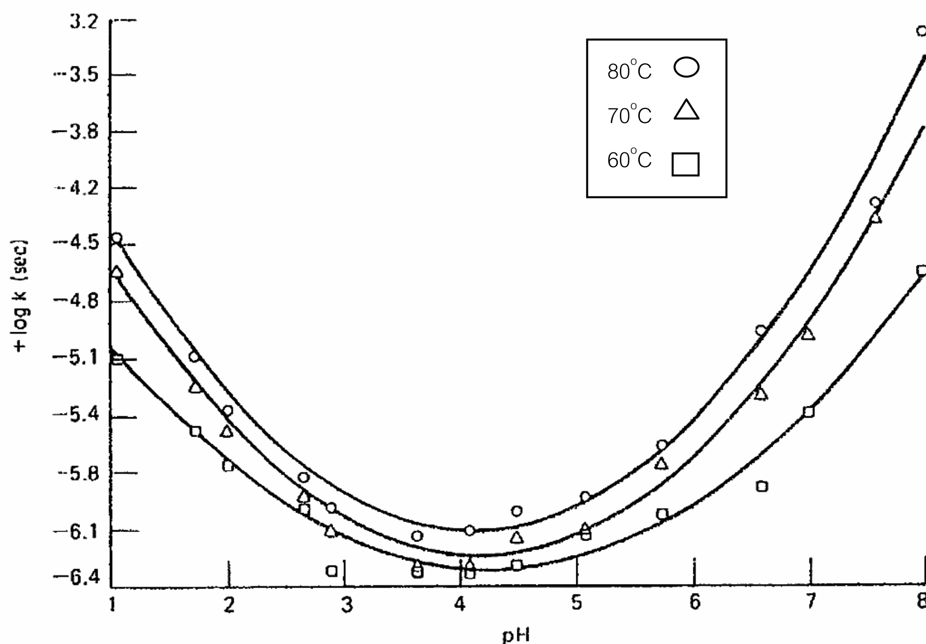

Figure 2.3 pH-rate profile for hydrolysis of indomethacin (Connors *et al.*, 1986).

2.2 Skin structure and route of penetration

Skin is the outermost part of the body which functions as the external barrier to prevent the loss of water and other components of the body to the environment, and protect the body from a variety of environmental insults. The skin also has important immune and sensory functions, helps to regulate body temperature, and synthesizes vitamin D (Wickett and Visscher, 2006). Skin can be divided into two main structural layers, epidermis and dermis. The structure and important of each skin layers for transdermal drug penetration is summarized as follows.

Epidermis

The epidermis is a stratified, squamous, keratinizing epithelium. The keratinocytes comprise the major cellular component (>90%) and are responsible for the evolution of barrier function. The epidermis can be divided into five distinct

strata; stratum basale, stratum spinosum, stratum granulosum, stratum lucidum and stratum corneum; which correspond to the consecutive steps of keratinocyte differentiation (Delgado-Charro and Guy, 2001).

The stratum corneum is known as a main barrier for extraneous substances penetration. Its structure usually thought as a brick wall, the stratum corneum corneocytes (keratin-filled dead cell) with their cornified envelope composed of cross-linked protein. Earlier findings demonstrate that the corneocytes are linked together by the structure called desmosomes which are composed mainly of glycoprotein. The brick wall structure of stratum corneum is embedded in the lipids lamellae which are considered to be the mortar as shown in Figure 2.4. Due to the resistant structure of corneocyte envelope, the main route of drug penetration through skin is intercellular lipid which composes of an approximately equimolar mixture of ceramides, cholesterol and free fatty acid (Bouwstra and Honeywell-Nguyen, 2002; Wickett and Visscher, 2006; Delgado-Charro and Guy, 2001).

Dermis

Dermis layer is the largest skin layer. It comprises primarily connective tissue and provides support to the epidermis. The dermis incorporates blood vessels, lymphatic vessels and nerve endings. The extensive microvasculature network found in the dermis represents the site of resorption for drugs absorbed across the epidermis to the systemic circulation before access to their target site. The hair follicles and sweat glands located in the dermis may also provide shunt pathways for drug penetration, however their low surface area has only little influence on the overall drug flux (Delgado-Charro and Guy, 2001).

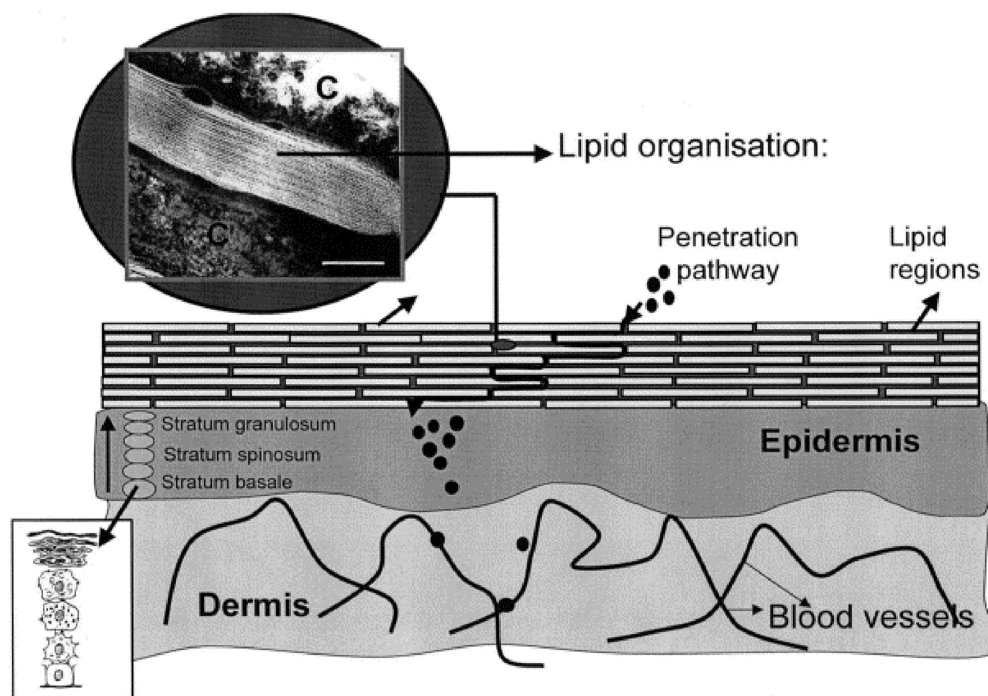

Figure 2.4 Schematic diagram of skin structure showing brick and mortar model of stratum corneum, viable epidermis and dermis (Bouwstra and Honeywell-Nguyen, 2002)

2.3 Transdermal drug delivery

Transdermal drug delivery is the delivery of topically applied therapeutic drugs across the skin aimed for reaching to the systemic circulation. The advantages of this route of administration are;

- Avoidance of significant presystemic metabolism such as the degradation in GI tract or liver which results in reduction of drug daily dose.
- Maintenance of systemic drug levels within the therapeutic window for a long period, hence the duration of drug action following a single administration can be extended and the frequency of dosing can be reduced.

- Improvement in patient compliance and acceptability of the drug therapy.
- Ease of termination of drug input such as by removal of the patch.

2.4 Transdermal delivery of indomethacin

An approach to avoid the systemic side effects of indomethacin from oral therapy is to deliver the drug via transdermal route. Several studies were aimed to develop the efficient means for transdermal delivery of indomethacin in order to increase local soft tissue and joint concentrations after topical application while decrease its systemic distribution so as to limit its harmful side effects (Ricci *et al*., 2005).

The limitation of indomethacin itself is a key role for the development of transdermal formulation since it has low skin penetration efficiency. In the study of Cordero *et.al* (1997), the comparative study of the transdermal penetration of various NSAIDs dissolving in phosphate buffer pH 6.6 was examined *in vitro* using human abdominal skin. In case of indomethacin, the permeation parameters including permeability coefficient (K_p), steady state flux (J_{ss}) and lag time were reported to be $0.7x10^3$ cm/h (at 100% ionization), 0.7 µg/h/cm^2 and 6 h, respectively. These data showed poor skin permeation characteristics of indomethacin. The physicochemical properties of NSAIDs which might affect to skin permeation properties were investigated by Goosen *et al*. (1998). From this study, partition and ionization properties played an important role in skin permeation of indomethacin which was observed from the area under the curve of drug concentration in plasma-time profile (AUC) after topical gel application of various NSAIDs. The log P value of 3.8

indicates the lipophilicity of indomethacin, thus it may retain in the stratum corneum lipid and be more susceptible for enzymatic degradation. At skin pH, indomethacin molecule (pKa 4.5) is minor non-ionized form which is more lipid soluble and may dissolve more rapidly in the skin lipid than ionized form. From this evidence, facilitating transport by passive diffusion of indomethacin is limited in this condition (Goosen *et al*., 1998; Beetge *et al*., 2000).

Many methods are introduced for improving the transdermal penetration of indomethacin. These methods include the use of chemical modification technique such as the formation of ester prodrug containing 1-alkylazacycloalkan-2-one, tertiary N,N-dialkyl functional group, polyoxyethylene, N-acyllactam, terpenoids, N,N-dimethylamino acid and O-acylmenthol (Bonina *et al*., 1991; Jona *et al*., 1995; Bonina *et al*., 1995a.b; Palagiano *et al*., 1997; Novotny *et al*., 2008; Zhao *et al*., 2008). Moreover, the use of various kinds of penetration enhancers such as azone, dimethyl sulfoxide, phospholipids, capsaicin and nonivamide, and essential oils from sweet basil were also investigated to overcome the problems (Chiang *et al*., 1991; Yokomizo and Sagitani, 1996; Fang *et al*., 2001, 2004).

The using of drug delivery system is an alternative choice for enhancing skin penetration of indomethacin. The example of drug delivery approaches aimed for transdermal administration of indomethacin and their skin penetration efficiency investigated are summarized in Table 2.2.

Table 2.2 Transdermal drug delivery systems of indomethacin and their skin permeation efficiency determination

Drug delivery systems	**Criteria under investigation**	**Subjects/species**	**Results**
Silicone polymer matrix-type transdermal delivery device (TDD) (Chien *et al*., 1988)	*In vitro* skin permeation	Hairless mice abdominal skin	The addition of enhancer such as isopropyl myristate, capric acid and octanol in silicone matrix resulted in increase the flux 3.82, 8.75 and 8.99 times more than control.
Lecithin microemulsion gel (Dreher *et al*., 1997)	*In vitro* skin permeation	Human full-thickness skin	The flux of indomethacin dissolved in lecithin microemulsion gel was about 6 fold more than that dissolved in neat isopropyl palmitate (1.2±0.3 vs. 0.21±0.02 $\mu g/cm^2/h$).
o/w nanoemulsion (Shakeel *et al*., 2010)	*In vivo* anti-inflammatory activity	Wistar rats skin	The inhibition of carrageenan-induced paw edema after 12 h application of indomethacin nanoemulsion was higher (83%) compared with marketed Indobene® gel (32.1%)

Table 2.2 Transdermal drug delivery systems of indomethacin and their skin permeation efficiency determination (continued)

Drug delivery system	**Criteria under investigation**	**Subjects/species**	**Results**
Polyamidoamine dendrimer (Chauhan *et al*., 2003)	*In vitro* skin permeation	Male Wistar rats skin	0.2% w/v of G4-NH_2, G4-OH and G-4.5 dendrimer resulted in enhance skin permeation 4.15, 3.25 and 2.81 fold compare to free drug suspension.
	Pharmacokinetics and pharmacodynamics	Male Wistar rats	The AUC of G4-NH_2 and G4-OH formulation were 2.27 and 1.95 times greater than pure drug suspension and the reduction of paw volume in carrageenan-induced edema showed a trend similar to pharmacokinetics data.
Self-microemulsifying drug delivery system (SMEDDS) (Maghraby, 2010)	*In vitro* skin permeation	Rabbit ear skin	The flux of indomethacin from SMEDDS was more than from other formulations including microemulsions, liquid crystalline, coarse emulsion and saturated drug solution in phosphate buffer saline (control). However, the lag time of SMEDDS was longer than others.

2.5 Ethosomes

2.5.1 Introduction

During the past decades, transdermal delivery of drugs by lipid vesicles has evoked a considerable interested. Liposomes are conventional phospholipid bilayer vesicles which have been used as drug carriers in various routes including dermal and transdermal. However, it can be found in most cases that classical liposomes are little or no value as carriers for transdermal drug delivery as they remain confined to upper layers of the stratum corneum and do not deeply penetrate skin (Elsayed *et al*., 2007).

Due to the unsuitability of the classical liposomes in transdermal delivery, the novel permeation enhancing phospholipid carrier namely ethosomes were developed by Touitou *et al*. in 1997. Ethosomes are kind of liposomes which embodying high concentration of ethanol. Although high concentration of ethanol was believed to disturb the bilayer structure of liposomes, however the inventors have found that the vesicles could coexist with up to 45% v/v of ethanol (Touitou *et al*., 1997). The existence of ethanol both inside the core and lamellar of phospholipid bilayer vesicles is believed to affect the vesicular flexibility, thus makes them more malleable and can easily penetrate skin. Furthermore, the permeation enhancement property of ethanol also plays a role in transdermal delivery (Williams and Barry, 2004; Dubey *et al*., 2007b).

2.5.2 Ethosomes composition and methods of preparation

The main compositions of ethosomes are phospholipid, ethanol and water. The other additives such as cholesterol or surfactant can also be added to improve the ethosomes characteristics. Ethosomes can be prepared by two methods. The first preparation method is purposed by Touitou *et al*. (1997) which described briefly; the lipids are dissolved in ethanol and the aqueous components are added slowly in a fine stream with constant mixing in a well-sealed container. The mixing is continued for additional few minutes. The system is kept at 30°C throughout the preparation and then left to cool at room temperature. The second method is thin film hydration method (Dayan and Touitou, 2000; Lopez-Pinto *et al*., 2005; Fang *et al*., 2008). This method is most commonly used in the preparation of lipid vesicles such as conventional liposomes or the others ultraflexible vesicles such as transfersomes and niosomes. Briefly, the lipids are first dissolved in the easily evaporated solvents such as chloroform, and then completely dried in a rotary evaporator above the lipid transition temperature to form a lipid film on the wall of a round-bottomed flask. The film is then hydrated with aqueous ethanolic solution by mixing method such as rotation or sonication at the corresponding temperature.

2.5.3 Physical morphology

The vesicular shape and surface morphology of ethosomes can be visualized by transmission electron microscope (TEM) and scanning electron microscope (SEM), respectively. The ethosomes prepared from 2%

phosphatidylcholine, 30% ethanol and water showed electron micrographs of closed spherical shape with multilamellar (Touitou *et al.*, 2000). This finding was also found in several ethosome preparations, but the lamellar of the vesicle could be varied from unilamellar to multilamellar (Dayan and Touitou, 2000; Godin and Touitou, 2004; Lopez-Pinto *et al.*, 2005; Dubey *et al.*, 2007b).

2.5.4 Physicochemical characteristics

Many researches have investigated the physicochemical properties of the ethosome vesicles. The presence of ethanol in ethosomes affected several physicochemical characteristics of phospholipid vesicles. First, the vesicular size of ethosomes was smaller than the conventional liposomes which contain the same compositions without ethanol. These results were explained to be due to the modification of ethanol on the net charge of ethosome system which conferred some degree of steric stabilization (Dayan and Touitou, 2000; Touitou *et al.*, 2000; Lopez-Pinto *et al.*, 2005; Dubey *et al.*, 2007a,b; Fang *et al.*, 2008).

Moreover, ethosomes could entrap the molecule of various hydrophilicities with higher entrapment efficiency than liposomes. This characteristic could be evident in the study of Touitou *et al.* (2000) by incorporating of three different fluorescent probes which included highly lipophilic rhodamine red, amphiphilic D-289 and hydrophilic calcein in ethosomes and liposomes. The probe entrapment was visualized by confocal laser scanning microscope (CLSM). It was found in ethosomes that all probes filled up the entire volume of vesicles with high fluorescence intensity whereas in liposomes, the lipophilic and amphiphilic probes

were found only in the bilayer and the hydrophilic probe present primarily in the aqueous core of the vesicles as shown in Figure 2.5. The entrapment efficiency of various drug-loaded ethosomes formulations was also found to be higher than in liposomes. These results were indicated to be caused by ethanol in the formulation which could enhance the solubility of the drugs and the multilamellarity of ethosome vesicles as well (Dayan and Touitou, 2000; Touitou *et al*., 2000; Lopez-Pinto *et al*., 2005; Dubey *et al*., 2007b; Fang *et al*., 2008).

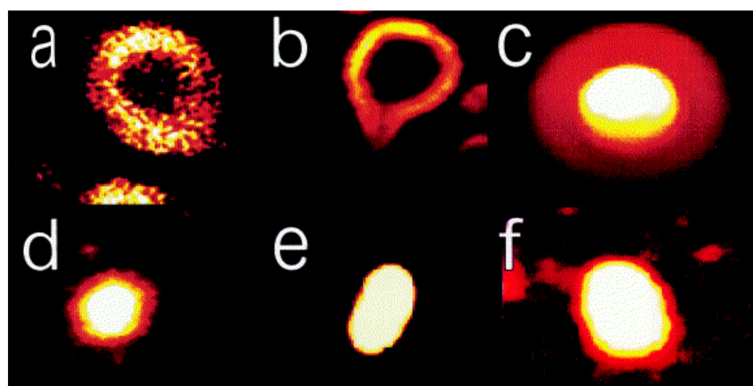

Figure 2.5 CLSM micrographs of liposomes (a-c) or ethosomes (d-f) entrap fluorescent probes rhodamine red (a,d), D-289 (b,e) or calcein (c,f) (Touitou *et al*., 2000).

The flexibility of ethosomes was found to be higher than liposomes. As shown in the study of Jain *et al*. (2007), by conducting the elasticity measurement of ethosome and liposome vesicles using extrusion method, it was found that the elasticity of ethosome vesicle membrane was 7 fold more than liposome membrane. The explanation of the researchers about this finding was the higher concentration of ethanol in ethosomes might reduce the interfacial tension of the vesicle membrane

therefore it could provide the elasticity to the vesicle membrane. Another method to confirm the softness and fluidity of ethosome vesicles is to measure the melting temperatures (T_m) of phospholipid in the ethosomal systems by differential scanning calorimetry (DSC). The lower T_m value of ethosomal system compared to liposomes was found in several studies indicating the fluidizing effect of ethanol on phospholipid bilayer (Dayan and Touitou, 2000; Touitou *et al.*, 2000; Godin and Touitou, 2004; Lodzki *et al.*, 2003; Dubey *et al.*, 2007b).

2.5.5 Skin permeation and deposition properties

Many researches have investigated the skin permeation and deposition properties of ethosomes to ensure that they could be efficiently used as transdermal and dermal drug carriers. The enhancement in skin permeation and deposition properties from ethosomal carrier could be clearly observed in the study of Dayan and Touitou (2000). The incorporations of amphiphilic fluorescence probe D-289 in trihexyphenidyl hydrochloride (THP) loaded ethosomes, liposomes and hydroalcoholic solution were conducted in this study and monitored for probe penetration in the nude mouse skin using CLSM. The results showed that the classic liposomes did not facilitate probe penetration into the skin, rather resulted in only a small reservoir in the upper layers of the skin. For hydroethanolic solution, the relatively deep penetration was observed but with relatively low fluorescence intensity. In case of ethosomal system, the high skin penetration was observed in terms of the depth of penetration and in intensity as shown in Figure 2.6. In the study

of Touitou *et al*. (2000), the same result was also observed when using rhodamine red as the lipophilic fluorescent probe.

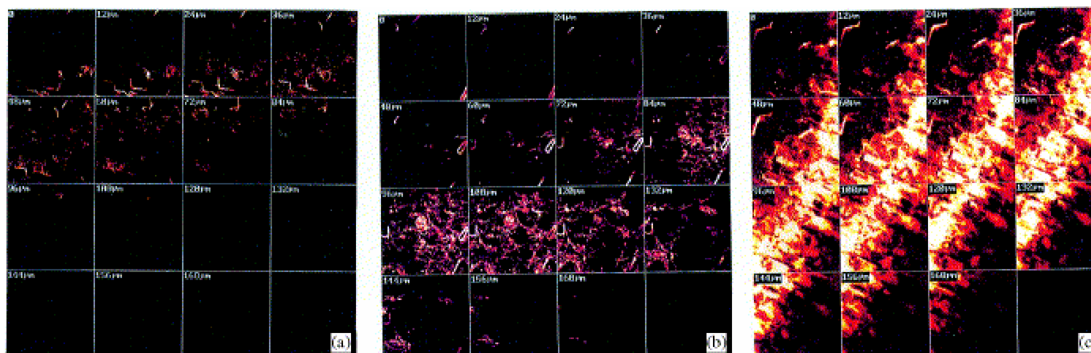

Figure 2.6 CLSM micrographs of mouse skin, after application of the fluorescent probe, D-289 from (a) THP liposomes (b) THP hydroethanolic solution (c) THP ethosomes (Dayan and Touitou, 2000).

Moreover, the skin permeation and deposition properties of ethosomes can be determined *in vitro* by franz diffusion experiments. As demonstrated in Table 2.3 ethosomal system could enhance drug permeation and deposition compared to the drug in other forms. The efficiency of *in vivo* skin delivery of various drugs from ethosomal carriers is also summarized in Table 2.4.

Table 2.3 Summary of ethosomes *in vitro* skin permeation/disposition studies (Elsayed *et al*., 2007)

Drug	Enhancement ratio		References
	Permeation[a]	Disposition	
Sotalol	7.1[c]	Not determined	Kirjavainen *et al*., 1999
Sodium salicylate	3.8[c]	Not determined	Kirjavainen *et al*., 1999
Propranolol	1.4[c]	Not determined	Kirjavainen *et al*., 1999
Trihexyphenidyl HCl	51[b], 4.5[c], 87[e]	4.6[b], 1.4[c], 1.4[e]	Dayan and Touitou, 2000
Minoxidil	45[c], 35[d], 10[f]	7[c], 5[d], 2[f]	Touitou *et al*., 2000
Minoxidil	1.2[e]	Not determined	Lopez-Pinto *et al*., 2005
Testosterone	30[g]	7[g]	Touitou *et al*., 2000
Ketotifen	1.2[b], 1.4[c], 1.2[e]	1.2[b], 1.4[c], 1.2[e]	Elsayed *et al*., 2006

[a] Estimated based on cumulative amounts permeated at the end of the experiment or on flux data.

[b] Relative to aqueous solution.

[c] Relative to hydroethanolic solution.

[d] Relative to absolute ethanol.

[e] Relative to traditional liposomes.

[f] Relative to lipid ethanolic solution.

[g] Relative to commercial patch.

Table 2.4 Summary of *in vivo* efficiency studies of ethosomes as carriers for skin drugs delivery (Elsayed *et al*., 2007)

Drug	Criteria under investigation	Subjects/species	Results
Acyclovir (Horwitz *et al*., 1999)	Clinical efficacy in treatment of recurrent herpes labialis	Humans	Time to crusting of lesions and time to loss of crust were shorter with the ethosomal acyclovir than with the commercial cream (Zovirax® cream).
Testosterone (Touitou *et al*., 2000)	Pharmacokinetics	Rabbits	After application for 5 days (new patch applied daily), AUC was 125% greater with ethosomal patch than with commercially available patch.
Cannabidiol (Lodzki *et al*., 2003)	Suppression of carageenan-induced aseptic paw edema (anti-inflammatory action)	Male mice	Development of edema was prevented entirely only in pretreated (ethosomal patch) group of mice. Delta in paw thickness of pretreated mice was statistically different from that of the non-pretreated mice starting from 1 h post-carrageenan injection and lasting until the end of the inflammation course.

Table 2.4 Summary of *in vivo* efficiency studies of ethosomes as carriers for skin drugs delivery (Elsayed *et al*., 2007) (continued)

Drug	Criteria under investigation	Subjects/species	Results
Testosterone (Ainbinder and Touitou, 2005)	Pharmacokinetics	Male Sprague-Dawley rats	AUC was about 64% greater with ethosomes than with commercial gel.
Erythromycin (Godin and Touitou, 2005)	*In vitro* antibacterial efficiency	*S. aureus* inoculated mice skin	Ethosomal erythromycin resulted in complete inhibition of infection while hydroethanolic erythromycin solution caused deep dermal and subcutaneous abscesses within 5 days after challenge.
Ammonium glycyrrhizinate (Paolino *et al*., 2005)	Suppression of chemically induced erythema (anti-inflammatory action)	Human volunteers	Ethosomes reduced the erythema more rapidly with respect to drug solutions. Ethosomes also showed sustained effect.

The model of how ethosomes may enhance penetration of drugs through the skin was purposed by Touitou *et al.* (2000) as illustrated in Figure 2.7. Firstly, the structure of the stratum corneum lipid bilayer was disturbed by ethanol and therefore enhanced its lipid fluidity. This process provided the penetration of the flexible ethosome vesicles through the skin by virtue of their particulate nature. The release of drug in the deeper skin layers and transdermal drug absorption could be due to the fusion of ethosomes with skin lipids and the release of drug at various points along the penetration pathway.

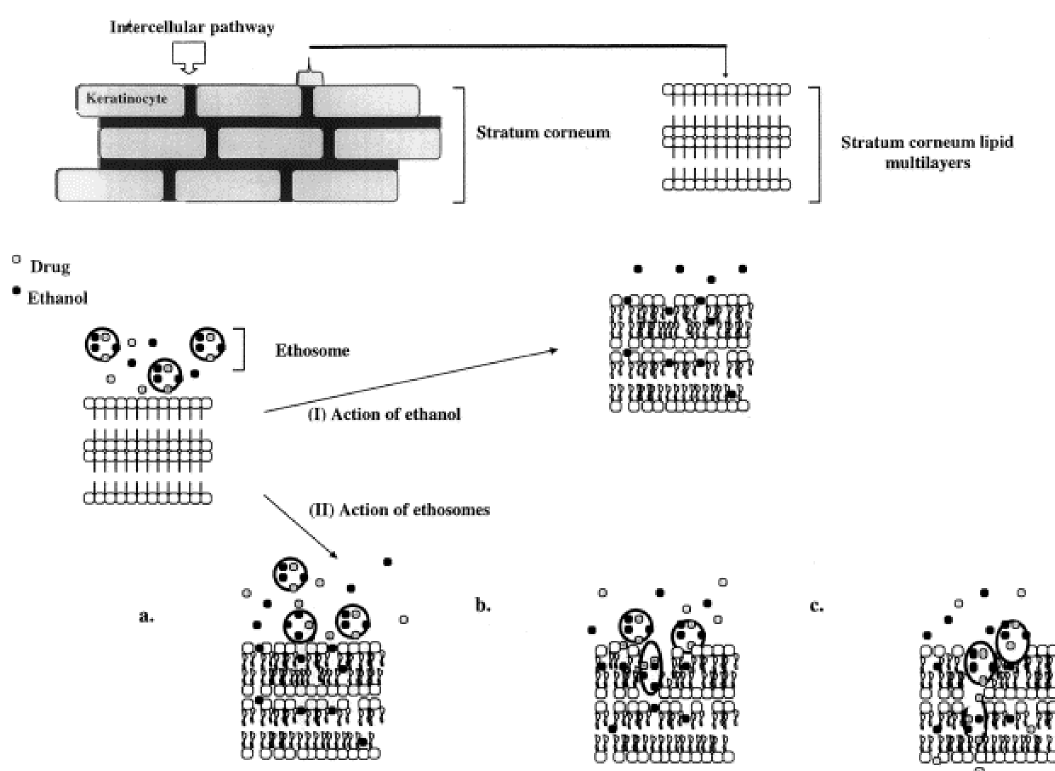

Figure 2.7 Proposed model for skin delivery from ethosomal systems (Touitou *et al.*, 2000)

2.5.6 Skin irritancy and tolerability

Since potential transdermal drug delivery systems need to be safe for using *in vivo* especially for the skin which is the part of the body that directly contact to the drug formulations, the *in vivo* skin irritancy and tolerability is the important parameter to evaluate before utilizing in human. In the study of Paolino *et al*. (2005), the reflectant spectrophotometry was used to determine the erythema index in human volunteer after applying empty ethosomes (2% w/v Phospholipon 90® and 45% v/v ethanol), hydroethanolic solution (45% v/v ethanol) and using saline solution as a control. After 48 h of treatment, no significant difference in erythema index was observed between the group applied ethosomes and saline solution, but remarkably different in the group applied hydroethanolic solution. The similar result was observed in the study of Dubey *et al*. (2007b). The safety of the formulations was evaluated in term of erythema score and conducted in hairless albino rabbit skin. These results indicated that high concentration of ethanol in ethosomes was not act as a skin erythema inducing agent.

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Drug

Indomethacin BP 2002 (P.C. Drug Center Co.,Ltd., Bangkok, Thailand)

Indomethacin Reference Standard (Fluka®, Italy)

3.1.2 Chemicals and reagents

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

Acetic acid (BHD AnalaR®, Poole, United Kingdom)

Acetonitrile, HPLC grade (Lab-Scan®, Bangkok, Thailand)

Cholesterol from lanolin (Fluka®, Japan)

Deoxycholic acid (Fluka®, Italy)

Hexane, AR grade (Lab-Scan®, Bangkok, Thailand)

Hydrochloric acid (Merck®, Darmstadt, Germany)

Methanol, AR grade (Lab-Scan®, Bangkok, Thailand)

Mycophenolic acid (sponsored by the Molecular Pharmaceutical Research Center, Songkhla, Thailand)

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

Sodium acetate (Univar®, New South Wales, Australia)

Sodium chloride (Carlo Erba®, Italy)

Sodium dihydrogen orthophosphate (Univar®, New South Wales, Australia)

di-Sodium hydrogen orthophosphate anhydrous (Univar®, New South Wales, Australia)

Sodium hydroxide (Merck®, Darmstadt, Germany)

Stearylamine (Aldrich®, United States)

Triton®X-100 (Baker analyzed®, New Jersey, United States)

3.2 Instruments

Table 3.1 Experimental instruments

Instrument	**Model**	**Company**
Centrifuge	Z323K	Hermle Labortechnik GmbH, Germany
Chromatographic column	Pinnacle II C_{18} column, 5 µm particle size, 250 × 4.6 mm	Restek Corporation, United States
High performance liquid chromatography	SpectraSystem P1000 pump SpectraSystem UV1000 detector ChromQuest software program SN 4000	Thermo Electron Corporation, United States
Homogenizer	PT 1200 E	Polytron, Switzerland

Table 3.1 Experimental instruments (continued)

Instrument	Model	Company
Modified Franz diffusion apparatus	57-6M	Hanson Research Corporation, United States
Optical microscope	Olympus CK2	International Co.,Ltd., Japan
Rotary evaporator	Eyela N-1000 series	Tokyo Rikakikai Co.,Ltd., Japan
Scanning electron microscope (SEM)	JSM-5800LV	JEOL Ltd., Japan
Sonicator	HT Crest	S.V. Medico Co.,Ltd., Thailand
Ultracentrifuge	Optima L-100XP, equipped with SW 60 Ti rotor	Beckman Coulter, United States
Vortex mixer	G-560E,	Scientific Industries Inc., United States
Zeta potential analyzer	ZetaPALS,	Brookhaven Instruments Corporation, United States

3.3 Methods

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

3.3.1.1 Instruments and chromatographic conditions

The HPLC method for quantitative determination of indomethacin used throughout this study was modified from the method described by Chauhan *et al.* (2003). The analysis was performed at room temperature on reversed phase Restek® Pinnacle II C_{18} column (5 µm particle size, 250 × 4.6 mm). A mixture of acetonitrile and 0.1 M acetic acid (60:40 v/v) was used as mobile phase with the flow rate of 1.0 ml/min. The mobile phase was filtered through 0.45 µm nylon membrane filter and degassed by sonication prior to use. The 20 µl sample solution was injected and the absorbance was detected at 254 nm.

3.3.1.2 The validation procedures

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

Specificity

The specificity of the HPLC method for determination of indomethacin in ethosome formulations, receptor fluid of skin permeation study, and in the skin model was tested. The experiments were performed by spiking the drug in the tested matrices. For ethosome samples, the total drug content was determined using the procedure described in section 3.3.4.3. In addition, the drug spiked phosphate buffer saline solution pH 7.4 (PBS) which was a receptor fluid for permeation study as well as the drug spiked pig skin were determined as described in section 3.3.6.2 and 3.3.6.3, respectively. Mycophenolic acid (MPA) solution was added as an internal standard (IS) at the final concentration of 15 µg/ml in all samples. The peak of any excipients in ethosome formulation, in breaking procedures, in PBS, as well as in pig skin and extraction procedures must not interfere with the drug and IS peaks.

Linearity

The standard solutions containing 5, 7, 10, 15 and 20 µg/ml indomethacin were prepared by diluting the aliquot of 100 µg/ml indomethacin stock solution in methanol with appropriate volume of the mobile phase. MPA solution was added at the final concentration of 15 µg/ml. These solutions were freshly prepared and analyzed in five replicates. The peak area ratios of indomethacin and MPA were plotted versus indomethacin concentrations, and the linearity was analyzed by least square regression analysis.

Accuracy and precision

Accuracy and precision for analytical of indomethacin content in ethosome formulations were determined by spiking the indomethacin standard solution in drug-free ethosomes to obtain the final concentration of indomethacin 6, 8

and 10 mg/ml. These three concentrations represent to 75%, 100% and 125% of indomethacin concentration in the prepared indomethacin-loaded ethosomes. The obtained samples were then lysed with 20%v/v Triton®X-100 in the same volume and diluted with mobile phase. MPA solution was added at the final concentration of 15 µg/ml in all samples prior to analyze with HPLC. These samples were prepared and analyzed in triplicate for each indomethacin concentration in the single assay day to determine the intra-day accuracy and precision. The same procedure was also carried on for three consecutive days to determine the inter-day accuracy and precision.

For accuracy, the concentrations of indomethacin calculated from the linear regression equation obtained from linearity test were compared to true value and expressed as percentage of recovery which could be calculated by the following equation:

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

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

C_{actual} : actual concentration of indomethacin

For precision, the closeness of indomethacin concentrations detected by HPLC was expressed as percentage of relative standard deviation (%RSD) which could be calculated by the following equation:

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

Where; *SD*: standard deviation of indomethacin concentration

$\overline{X}$: average concentration of indomethacin

The acceptable value of %recovery and %RSD is supposed to be in the range of 80-110% and not more than 7.3%, respectively (AOAC, 1993).

3.3.2 Solubility studies of indomethacin

The effects of pH and ethanol content on indomethacin solubility were evaluated to find out the suitable dispersion medium for the preparations. The study was conducted by adding an excess amount of indomethacin (2 g) in 5 ml of the desired media which include the buffer solutions pH 3.0, 4.5, 5.5, 7.0 and 7.4 and the hydroethanolic solution with 0-50% v/v ethanol. The samples were agitated at room temperature for 48 h until equilibrium and the excess drug was filtered through 0.45 µm syringe filter membranes. The solubility of indomethacin in the samples was determined by HPLC. All determinations were performed in triplicate.

3.3.3 Formulation and preparation of ethosomes containing indomethacin

The ethosomes containing indomethacin were formulated using indomethacin at the concentration of 8 mg/ml. The compositions of ethosomes including phospholipid, hydroethanolic mixture, as well as the additives such as

cholesterol (CHOL) and surface charge agent were varied in term of type, concentration and ratio added as follows;

Phospholipid:

2%, 4% and 6% w/v of phosphatidylcholine from soybean (SPC)

Hydroethanolic mixture:

Aqueous phase: Distilled water, acetate buffer pH 5.5, phosphate buffer pH 7.0 and 7.4

Ethanol: 10%, 20%, 30% and 40% v/v ethanol

Additive:

Cholesterol in the molar ratio of SPC:CHOL = 4:1 and 3:1

Surface charge agents:

Cationic: Stearylamine (SA) in the molar ratio of SPC:CHOL:SA = 6:2:1

Anionic: Deoxycholic acid (DA) in the molar ratio of SPC:CHOL:DA = 6:2:1

The ethosomes containing indomethacin were prepared by thin film hydration method. Firstly, indomethacin, phospholipid and other additives (if need) were dissolved in the mixture of chloroform and methanol (2:1 v/v) in round-bottomed flask followed by evaporation at 60°C using rotary evaporator to make the thin lipid film on the flask wall. The flask was continued on rotary evaporator for an hour to ensure that the organic solvents were completely evaporated. This thin lipid film was then hydrated with hydroethanolic mixture by sonication at 60°C for 30 min in the well-sealed flask.

For comparison, the corresponding liposomes containing indomethacin were also prepared using the same materials and procedure described for preparing ethosomes but without ethanol. All of these formulations were prepared in triplicate.

3.3.4 Characterization of ethosomes containing indomethacin

3.3.4.1 Physical appearance examination

The physical appearances of all prepared ethosomes and liposomes were visually observed for colloidal appearance, color, phase separation and precipitation. In addition, the optical microscope was utilized to examine for crystal of drug precipitation in the formulations.

3.3.4.2 Particle size and size distribution measurement

The size and size distribution of ethosomes and liposomes containing indomethacin were determined using photon correlation spectroscopy (PCS) at 25°C after dilution of 200 µl ethosome suspension with 3 ml Milli-Q water. All determinations were performed in triplicate.

3.3.4.3 Determination of drug content and entrapment efficiency

The entrapment efficiency of ethosomes and liposomes containing indomethacin was evaluated by ultracentrifuge technique. The obtained ethosome and

liposome formulations were centrifuged at 60,000 rpm, 4°C for 2 h using ultracentrifuge. The supernatant was collected to determine the amount of non-entrapped drug by HPLC. The ethosomes or liposomes were also lysed with the same volume of 20% v/v Triton®X-100 and diluted with mobile phase to determine total drug amount in the formulation. The entrapment efficiency can be calculated from the equation;

$$\text{Entrapment efficiency (\%)} = \frac{T - F}{T} \times 100 \qquad (3)$$

Where; *T*: total drug amount in the formulation

F: non-entrapped drug amount.

3.3.4.4 Surface morphology

Surface morphology of ethosomes containing indomethacin was examined using SEM. Firstly, 200 µl of ethosome suspension was diluted with 3 ml MilliQ water. A drop of diluted ethosomes was allowed to dry on the cover slip and then stained with crystal violet solution for 1 min. An excess dye was rinsed out with water followed by fixing with Gram's iodine solution for 1 min. This staining method was based on Gram's Method where the positive charge of crystal violet interacted with negative charge of the phospholipid in ethosomes and later formed complex with I^- or 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 40,000X magnification.

3.3.5 Stability study of ethosomes containing indomethacin

The optimized ethosomes containing indomethacin formulation was selected for stability and skin permeation studies based on optimal particle size and entrapment efficiency. Chemical and physical stabilities of the selected ethosome formulation were evaluated after storage of the formulation in well-closed containers at ambient temperature and in refrigerator (4^oC) for three months. At the initial time and after storage of 1, 2, 4, 8 and 12 weeks, the aliquot of the formulation was collected to estimate the physical appearance, particle size and size distribution, total indomethacin content and entrapment efficiency as described in section 3.3.4. In addition, the zeta potential of ethosomes was analyzed using zeta potential analyzer at 0, 4, 8 and 12 weeks of storage.

3.3.6 *In vitro* skin permeation experiments

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3.3.6.1 Skin preparation

The pig skins used in permeation study were obtained from 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 fatty tissue was removed from the skin using a surgical scissors. The skin surface was then cleaned with PBS and allowed to dry. The skin was packed in aluminum foil and stored in freezer (-20^oC) until used. Before permeation

experiment was performed, the full-thickness skin prepared in the aforementioned way was soaked overnight in PBS.

3.3.6.2 *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. The diffusion cells were maintained at 37°C with stirring at 500 rpm throughout the experiment. The skin permeation of the indomethacin-loaded ethosomes was determined compared to the commercial drug solution and the ethanolic solution containing the same amount of indomethacin (8 mg/ml). The 1 ml of each sample was applied on the skin surface in the donor part of the Franz diffusion cells. The 1 ml sample of receiver medium was withdrawn at predetermined time intervals (0.5, 1, 2, 4, 6, 8, 12 and 24 h) and an equivalent volume of fresh PBS was then replaced in. All withdrawn samples were filtered through a 0.45 µm syringe filter membranes and analyzed for the amount of indomethacin permeated by HPLC. All determinations were performed at least in triplicate.

The cumulative amount of indomethacin permeated (Q_t, µg/cm^2) was calculated according to the following equation;

$$Q_t = \frac{P_n V_o + \sum_{i=1}^{n-1} P_i V}{A} \quad (4)$$

Where; P_n and P_i: the drug concentration determined at No.n and No.i (µg/ml)

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

A: effective diffusion surface area

The cumulative amount was then plotted as a function of time and the flux of indomethacin at steady state (J_{ss}, µg/cm^2/h) through the pig skin was calculated from the slope of linear portion of the plot. The lag time or the time required for drug to permeate through skin layer before reaching the receptor fluid was calculated from the X-intercept of the plot. In addition, the permeability coefficient (K_p, cm/h) was calculated from the equation;

$$K_p = \frac{J_{ss}}{C_0} \quad (5)$$

Where; C_0: the initial concentration of drug in the donor compartment.

3.3.6.3 *In vitro* skin retention studies

At the end of the *in vitro* skin permeation experiments (24 h), the formulations were removed from the skin by wiping with cotton balls soaked with PBS. The skin was then cut-off in small pieces and homogenized in 5 ml of suitable extracting solvent at 24,000 rpm for 5 min. The resultant mixture was filtered through

filter cloth and centrifuged at 12,000 rpm 4°C for 30 min to separate the skin lipid. The clear supernatant was collected, filtered through to 0.45 μm syringe filter membrane and determined for the amount of indomethacin retained in the skin by HPLC.

The suitable solvent for extraction of the drug from pig skin was validated in this experiment. Various solvents including PBS, methanol, acetonitrile, chloroform and hexane were examined for their efficiency for indomethacin extraction. The 1.77 cm^2 pig skin was first cut-off in small pieces and then spiked with 1 ml of indomethacin standard solution (100 μg/ml). The 5 ml of test solvent was then added and the extraction procedure was further conducted as previously described. The extraction recovery was determined by computing the ratio of the amount of the drug extracted from spiked skin to the amount of the drug added.

3.3.7 Statistical analysis

All experimental data were presented as means±standard deviation (SD). The significance of the differences between parameters was tested using the Student's independent *t-test*. The differences were considered statistically significant when $p < 0.05$.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Quantitative analysis of indomethacin using HPLC

The HPLC method for quantitative determination of indomethacin in our study was based on the method of Chauhan *et al*. (2003) with minor modifications. A typical chromatogram of a standard solution of indomethacin is shown in Figure 4.1. The retention time of MPA and indomethacin were about 4.3 and 7.6 min, respectively.

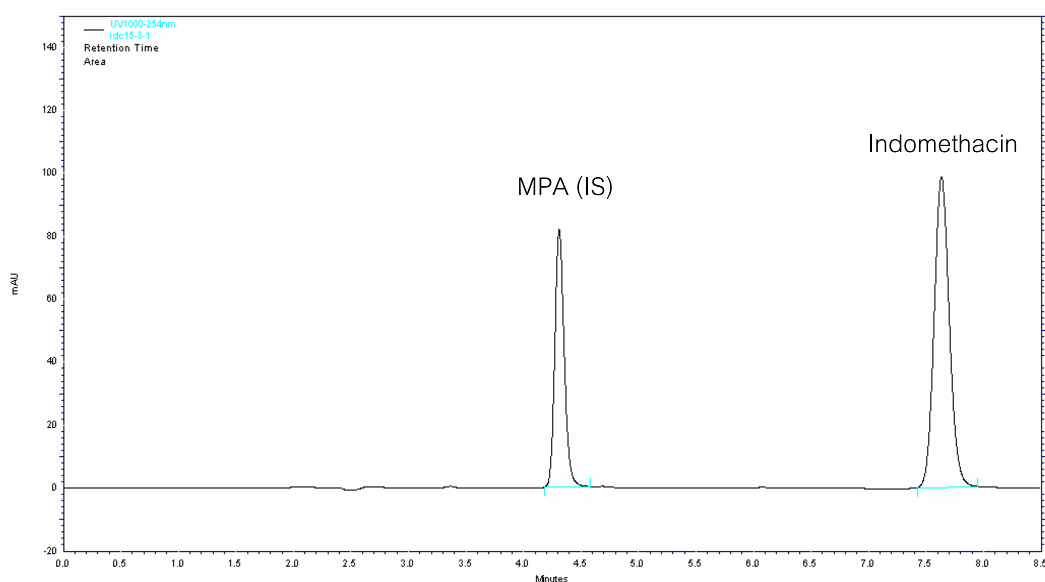

Figure 4.1 Typical chromatogram of standard solution of indomethacin and IS

Specificity

In our study, the analytical method was intended for the determination of indomethacin in the formulations and skin permeation studies, therefore it must be able to discriminate the peak of indomethacin from those of impurities and/or other components in the formulations, in the receptor fluid, and in the skin model of skin permeation study. Figure 4.2, 4.3 and 4.4 demonstrates the specificity of the chromatogram obtained from the analysis of indomethacin in ethosomes, PBS and the pig skin, respectively. As could be seen in all figures, no interfering peak was observed at the retention times of indomethacin, indicating the good specificity of this indomethacin assay.

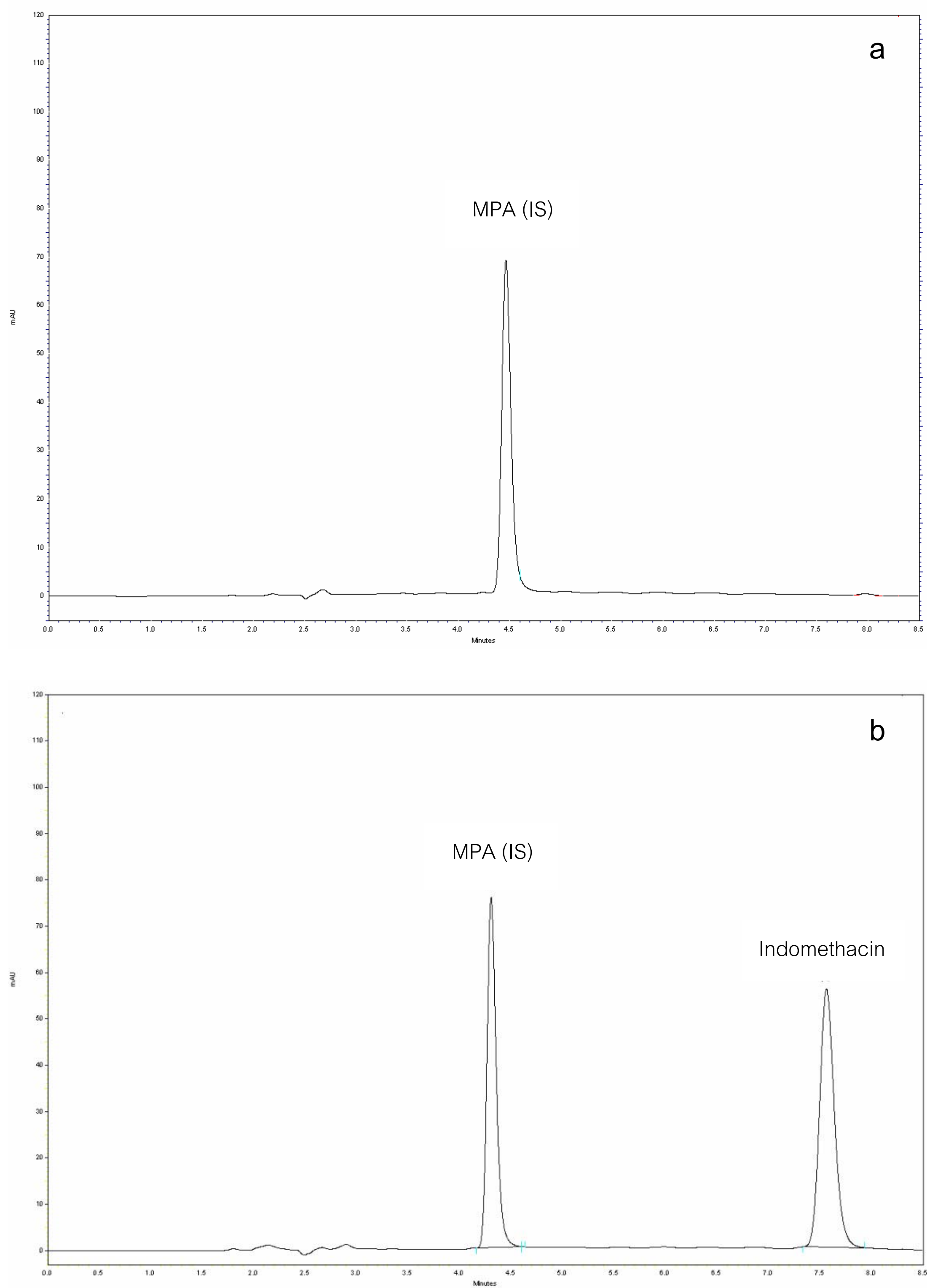

Figure 4.2 Chromatograms obtained from the analysis of blank ethosomes (a) and indomethacin spiked ethosomes (b)

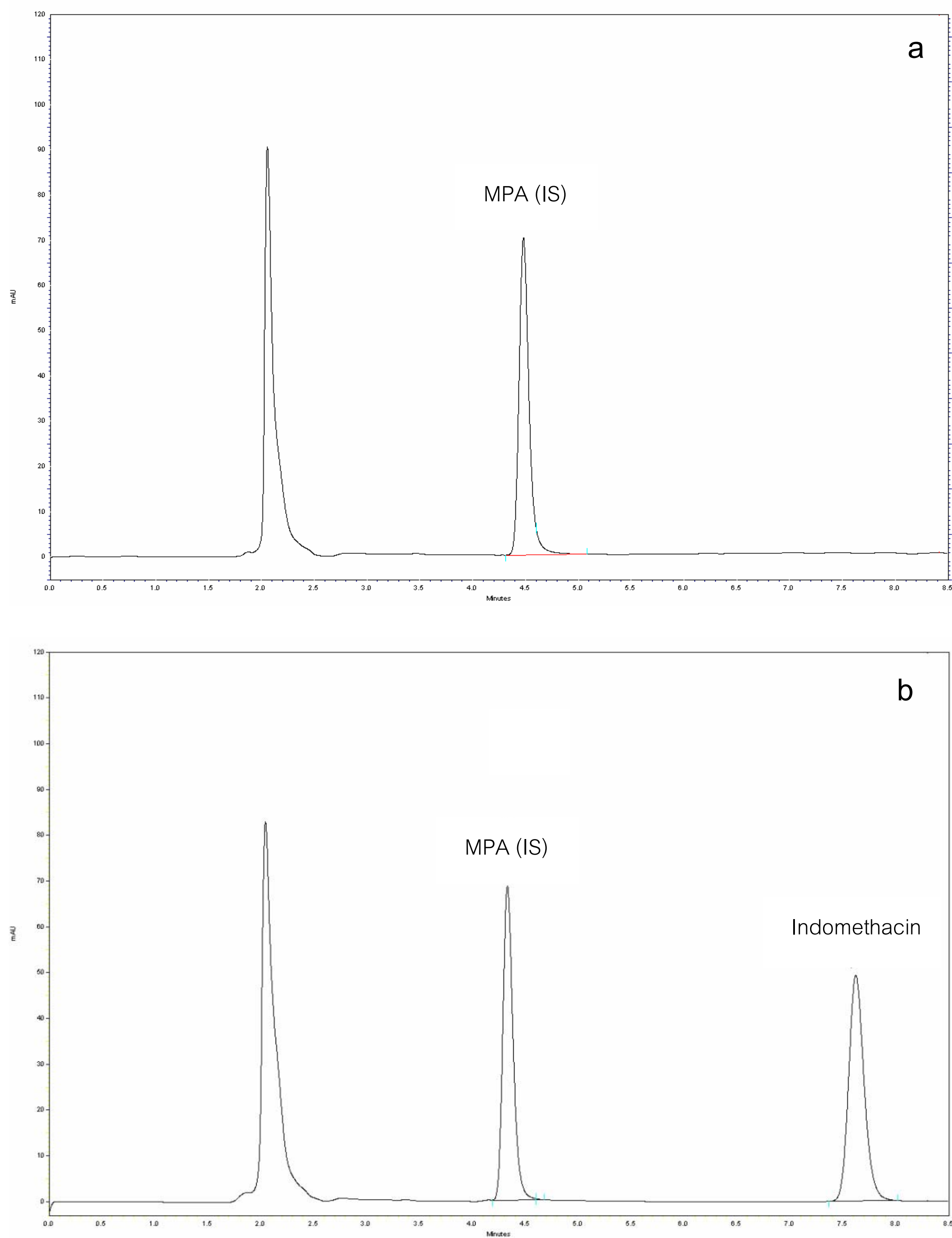

Figure 4.3 Chromatograms obtained from the analysis of blank PBS (a) and indomethacin spiked PBS (b)

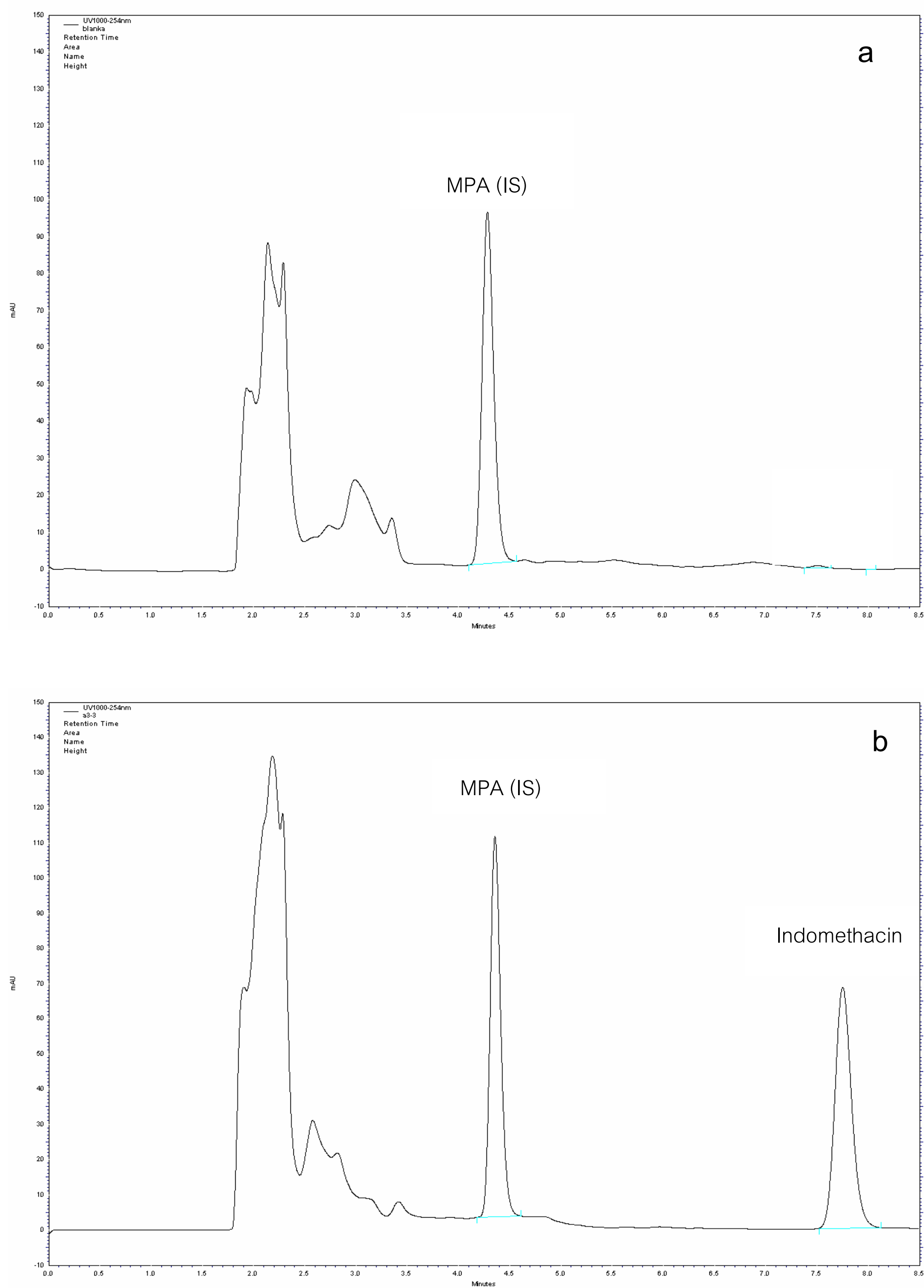

Figure 4.4 Chromatograms obtained from the analysis of blank skin (a) and drug spiked skin after extraction with acetonitrile (b)

Linearity

The linearity of the assay was determined by plotting the peak area ratio of standard indomethacin and MPA (IS) over the concentration range of 5-20 μg/ml. The linear relationship was obtained with correlation coefficients (R^2) of more than 0.999 [y = 0.1322 (±0.0034)x − 0.0107 (±0.0452), n= 5]. Figure 4.5 shows a standard calibration curve of indomethacin.

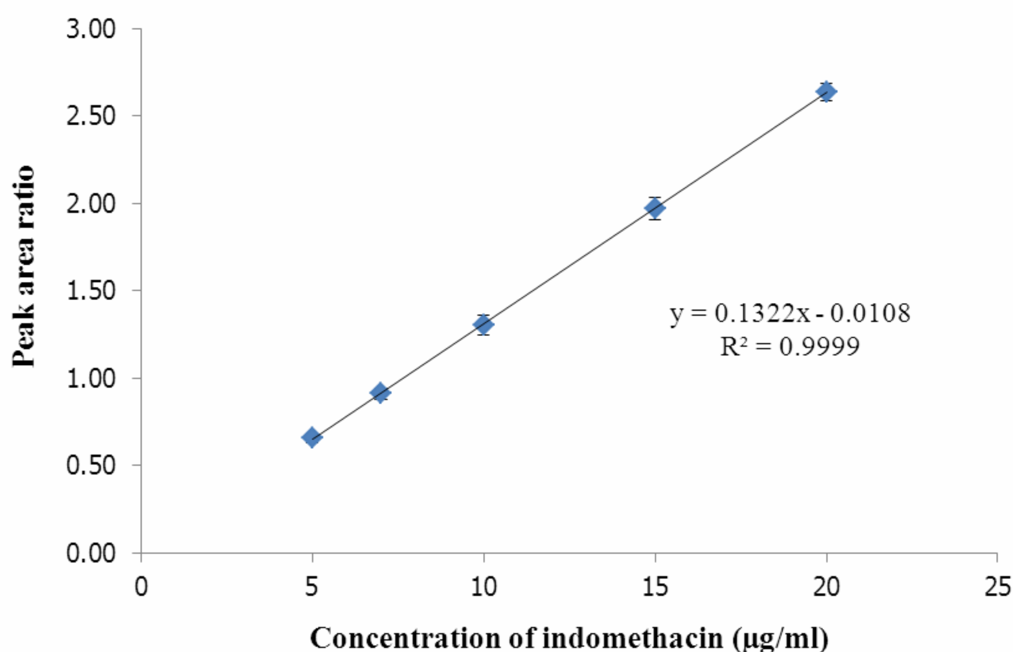

Figure 4.5 A standard calibration curve of indomethacin. The plotted data are mean±SD (n=5). Solid line is a linear regression fit to the data.

Accuracy and precision

The analytical accuracy and precision were evaluated from intra- and inter-day variability of the assay. Table 4.1 shows the intra-day reproducibility of the indomethacin assay. At each concentration, the intra-day RSD varied between 1.20 and

4.41%. The accuracy of the average measured concentration for each day, reported in terms of %recovery was between 94.31-101.75%. The inter-day reproducibility of the method, given in Table 4.2, showed that the intra-day RSD varied between 3.45 to 4.69% and the recovery was in the range of 96.94-98.55%. These values were within acceptable limits of AOAC (1993) for both recovery (80-110%) and RSD (less than 7.3%). These results indicated that the assay was accurate and reproducible enough for its application.

Table 4.1 Intra-day variability of indomethacin analysis

Spiked conc. (mg/ml)	**Day**	***Mean of measured conc. (mg/ml)**	**Average recovery (%)**	**%RSD**
5.99	1	5.65±0.22	94.31	3.82
	2	6.09±0.21	101.75	3.52
	3	5.97±0.20	99.60	3.31
7.99	1	7.61±0.27	95.22	3.52
	2	7.93±0.18	99.26	2.24
	3	7.70±0.26	96.34	3.35
10.00	1	9.93±0.12	99.31	1.20
	2	9.52±0.26	95.24	2.76
	3	9.86±0.44	98.65	4.41

*n=3

Table 4.2 Inter-day variability of indomethacin analysis

Spiked conc. (mg/ml)	*Mean of measured conc. (mg/ml)	Average recovery (%)	%RSD
5.99	5.90±0.28	98.55	4.69
7.99	7.75±0.27	96.94	3.45
10.00	9.77±0.34	97.73	3.50

*n=9

4.2 Effect of pH and ethanol on solubility of indomethacin

Indomethacin is a lipophilic drug (log P=3.8). It is very slightly soluble in water (5.2 μg/ml at 25°C) (Florey, 1984). In order to prepare a high dose (8 mg/ml) formulation of indomethacin ethosomes, we need to find an appropriate dispersion medium that is able to avoid drug precipitation in the formulation. In this case, the effects of pH and ethanol on the aqueous solubility of indomethacin were investigated. Figure 4.6 shows its pH-solubility profile.

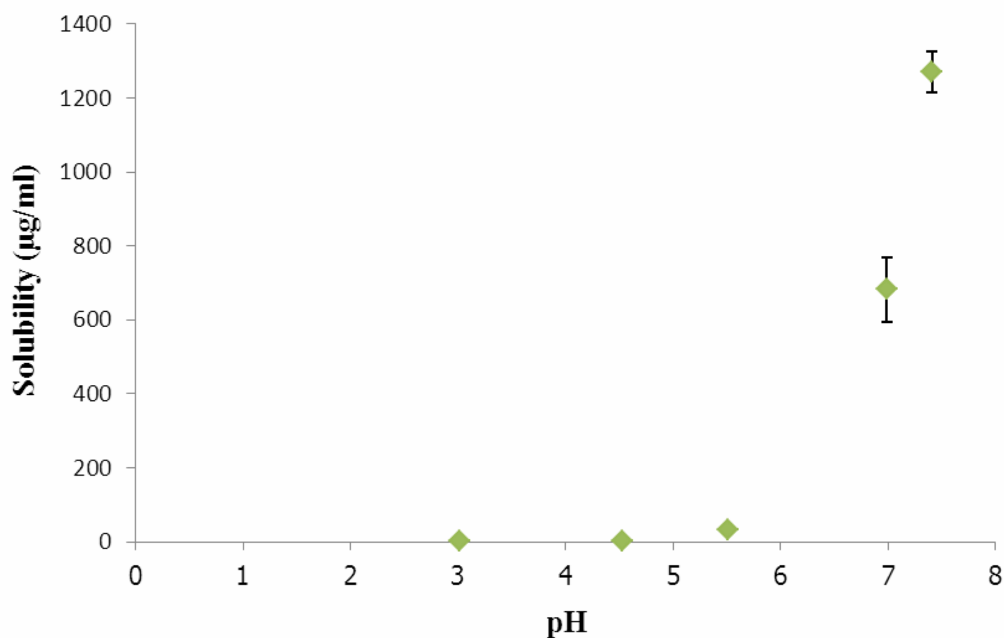

Figure 4.6 pH-solubility profile of indomethacin. The plotted data are mean±SD (n=3).

Since indomethacin is a weak acidic compound (pKa=4.5), its solubility increases as pH increases. At pH 7.0 and 7.4, the solubility of the drug increased to about 0.7 and 1.2 mg/ml, respectively. Although indomethacin is increasingly soluble in the alkaline aqueous solutions, it is not stable in those conditions (pH>8) due to base catalyzed hydrolysis (Connors *et al*., 1986). Therefore, to minimize this problem, the aqueous medium with neutral or slightly alkaline pH (pH<8) was considered. In addition to the pH adjustment, ethanol could be applied as a co-solvent to enhance the aqueous solubility of the drug. The solubility profile of indomethacin in different concentrations of ethanol is illustrated in Figure 4.7.

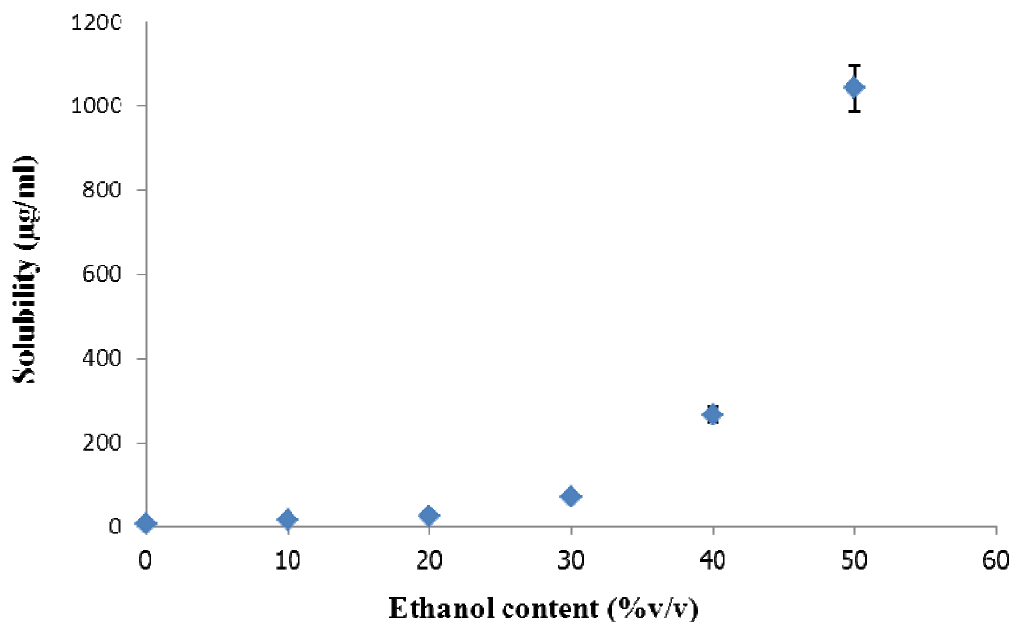

Figure 4.7 Solubility profile of indomethacin in aqueous solutions with various ethanol contents. The plotted data are mean±SD (n=3).

Notice that, the solubility of indomethacin also increased as ethanol content increased which could be clearly seen when ethanolic content was higher than 30% v/v. However, the high concentration of ethanol (>45% v/v ethanol) could disturb the vesicles structure of ethosomes and cause skin irritation (Touitou *et al.*, 2000; Darnpanid, 2004). Therefore, in our study, 10-40% v/v ethanol was added in dispersion media of ethosome formulations since ethanol might affect both indomethacin solubility and ethosomal characteristics.

4.3 Formulation and preparation of ethosomes containing indomethacin

Since the aqueous solubility of indomethacin (5.2 µg/ml) is lower than its dose in ethosome formulations (8 mg/ml), the suitable disperse medium was required to prevent the precipitation of the active drug. At first, the indomethacin ethosomes were prepared using 2% w/v SPC and hydroethanolic mixture containing 10-40% v/v ethanol in water, acetate buffer pH 5.5 and phosphate buffer pH 7.0 as dispersion media. It was found in all formulations that the colloidal characteristic of ethosomes could not form and the white needle-shaped crystals of drug were visualized under optical microscope. Figure 4.8 shows the physical appearance and optical image of indomethacin ethosomes where 30% v/v ethanol in water was used as dispersion medium. This result was expected to be due to the crystallization of un-entrapped drug in the formulation.

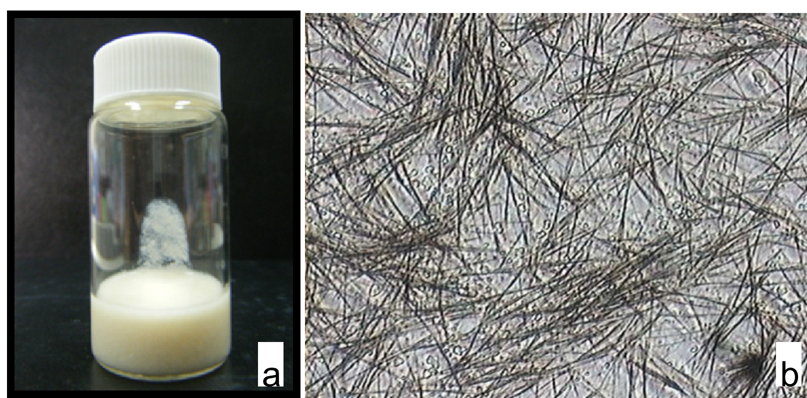

Figure 4.8 The physical appearance of indomethacin ethosomes prepared using 30%v/v ethanol in water as dispersion medium (a) and optical image showing needle-liked crystals of indomethacin dispersed in the medium (x400 magnification) (b)

In order to increase the solubility of the free drug in the formulation, 10-40% v/v ethanol in phosphate buffer pH 7.4 was used as dispersion media. In the formulation containing 10-30% v/v ethanol, the yellowish colloidal appearance with no drug crystallization was observed as seen in Figure 4.9. This result confirmed the need of the slightly alkaline medium to maintain solubility of the un-entrapped drug in the formulations.

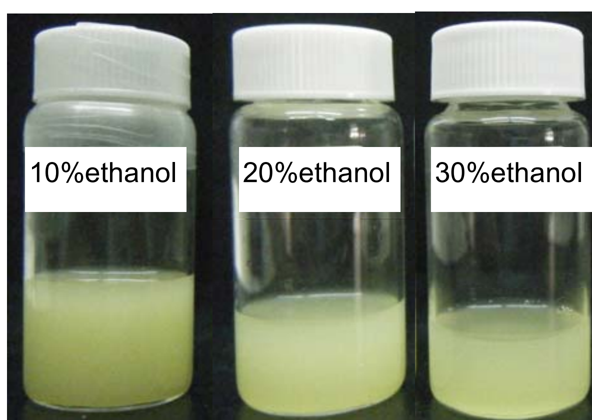

Figure 4.9 Physical appearance of the ethosome formulations prepared from 2% w/v SPC using 10-30% v/v ethanol in phosphate buffer pH 7.4 as dispersion media

Notice that the effect of pH (7.4) and ethanol content (10-30% v/v) should not provide the solubility of indomethacin in the formulation (8 mg/ml). However, no precipitation of free drug was observed. This result might be due to the synergist effect of pH, co-solvent system, and micellar solubilization due to the presence of phospholipid.

However, as increasing ethanol content to 40% v/v in phosphate buffer pH 7.4, it was found that the indomethacin-loaded ethosomes could not be performed due to phase separation as shown in Figure 4.10. These similar results were also

reported by Darnpanid (2004) where ketoprofen-loaded ethosomes were prepared using 10% phospholipid and 40% ethanol in water. This might be due to the effect of high concentration of ethanol on disruption of lipid vesicles (Touitou *et al*, 2000). However, it was reported that in several drug-loaded ethosomes, more than 40% up to 60% ethanol was able to add without breaking of the lipid vesicles (Lodzki *et al*., 2003; Lopez-pinto *et al*., 2005; Paolino *et al*., 2005; Dubey *et al*., 2007a; Jain *et al*., 2007). The effect of ethanol content on bilayer structure of ethosome vesicles has been investigated by Touitou *et al*. (2000) using the phosphorus Nuclear Magnetic Resonance (NMR) technique. They reported that the ethosomal lipids prepared from 5% phospholipids in 20-45% ethanol dispersion were mostly organized in phospholipid bilayers, whereas those in 50% ethanol were formed as fast-tumbling micelles. In addition, it has been stated that the drug might also have influence on the formation of the lipid vesicle. From these evidences, the effect of ethanol concentration on the phospholipid vesicles might depend on various factors such as concentration of phospholipid used, type of drug incorporated, as well as the method of preparation.

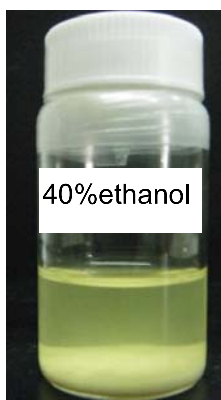

Figure 4.10 Physical appearance of the ethosome formulation prepared from 2% w/v SPC using 40% v/v ethanol in phosphate buffer pH 7.4 as dispersion medium

As shown in our previous studies, to obtain the ethosome formulation with good physical appearance, the dispersion media containing 10-30% v/v ethanol in phosphate buffer pH 7.4 were required. Table 4.3 lists the indomethacin ethosome formulations using 2-6% w/v SPC as lipid vesicular structure and phosphate buffer pH 7.4 containing 0-30% v/v ethanol as dispersion media, along with their vesicular size, polydispersity index (PI) and entrapment efficiency.

Table 4.3 Compositions and physical characteristics of ethosome and liposome formulations

No.	Composition		Vesicle size (nm)	PI	Entrapment efficiency (%)
	SPC (%w/v)	Ethanol (%v/v)			
1	2	0	320.5±55.8	0.397±0.096	77.57±4.03
2		10	291.0±17.2	0.313±0.060	56.38±4.58
3		20	49.3±9.5	0.527±0.212	20.46±5.37
4		30	220.6±48.8	0.388±0.087	32.01±7.34
5	4	0	445.9±32.6	0.471±0.036	81.47±2.45
6		10	400.0±116.3	0.470±0.172	63.15±2.22
7		20	72.0±23.0	0.465±0.137	37.03±9.84
8		30	164.9 ± 72.0	0.335 ± 0.099	31.64 ± 7.31
9	6	0	449.9 ± 13.4	0.352 ± 0.103	87.25 ± 1.18
10		10	414.8 ± 15.2	0.372 ± 0.078	79.24 ± 3.76
11		20	452.7 ± 28.4	0.305 ± 0.147	56.19 ± 1.59
12		30	247.4 ± 71.8	0.331 ± 0.118	37.67 ± 7.14

In formulation no.1, 5 and 9 with no ethanolic content (or liposomes), the high entrapment efficiency were initially found, however, the aggregation of the lipid components was observed within one week. This might be caused by the unsuitability of the formulation which contained only SPC as the main lipid structure of liposome wall. Without any stabilizing agents such as CHOL and/or surface charge agents, the aggregation of lipid vesicles might occur due to electrostatic and hydrogen

bonding interaction between choline head group of SPC (Mura *et al*., 2007). Srinath *et al.* (2000) has reported that indomethacin liposomes could be prepared using egg phosphatidylcholine (EPC), CHOL, and SA with the molar ratio of 1:0.5:0.1. However, a significant variation in encapsulation of the drug in liposomes might be observed when prepared by different methods. In this study, the indomethacin-loaded liposomes were not future developed, since we wanted to focus on the development of the ethosome formulations.

As compared to the liposomes (0% ethanol) with the same lipid concentration, the vesicular size examined in all ethosome formulations was likely decrease. This could be explained due to the effect of ethanol on their net surface charge. It has been described by Touitou *et al*. (2000) that ethanol provided a surface negative net charge to the liposomes. From their report, the zeta potential investigated from ethosomes (2% phospholipid, 30% ethanol) and liposomes (2% phospholipid 0% ethanol) was found to be -4.3±0.2 mV and 4.6±0.2 mV, respectively. The decrease in the zeta potential might provide some degree of steric stabilization and consequently lead to reduce the vesicular size (Dayan and Touitou, 2000; Touitou *et al*., 2000; Lopez-Pinto *et al*., 2005; Dubey *et al*., 2007a,b; Fang *et al*., 2008). Moreover, the addition of ethanol was stated to decrease in membrane thickness of ethosomal vesicles since ethanol might form a phase with interpenetrating hydrocarbon chains (Lopez-pinto *et al*., 2005, Dubey *et al*., 2007a).

The effect of ethanol content on size and entrapment efficiency of ethosomes was reported in many studies (Dayan and Touitou, 2000; Touitou *et al.*, 2000; Lopez-Pinto *et al*., 2005; Paolino *et al*., 2005; Dubey *et al*., 2007a,b; Jain *et al*., 2007; Fang *et al*., 2008). With increasing ethanol concentration, the size of ethosome

vesicles was reported to decrease whereas the entrapment efficiency was increased (Touitou *et al.*, 2000; Paolino *et al*., 2005; Dubey *et al*., 2007a; Jain *et al*., 2007). The increment of entrapment efficiency in the presence of ethanol could be explained by the increasing solubility of drug in both core and lamellar of ethosome vesicles, since ethanol distributed throughout the vesicles (Dayan and Touitou, 2000; Touitou *et al*., 2000; Lopez-Pinto *et al*., 2005; Dubey *et al*., 2007b; Fang *et al*., 2008). However, the results from our study showed that as the ethanol content increased, the entrapment efficiency of indomethacin-loaded ethosomes was likely decrease. This consequence might be due to the fact that the effect of ethanol on indomethacin solubility had less influence than that on the ethosomal size. Since indomethacin is a lipophilic drug, it is more likely exist in the phospholipid bilayers than in the aqueous core of the liposome/ethosome vesicles. When the vesicular size of ethosomes was reduced, their volume of both core and bilayer membrane was also decreased, resulting in lower drug entrapment efficiency. In addition, the effect of phospholipid concentration on size and entrapment efficiency of ethosomes was investigated in this study. It was shown that by increasing the phospholipid concentration, both size and entrapment efficiency of ethosomes was increased. The results are in agreement with previous reports of ethosomes (Touitou *et al*., 2000; Paolino *et al*., 2005). These could be clarified by the increase of phospholipid molecules in bilayers of the vesicles where indomethacin resided.

It has been reported that particle size of liposome played the key factor for skin penetration of substances. Verma *et al*. (2003) reported that the small size of liposomes (average diameter of 71 and 116 nm) could enhance the delivery of both lipophilic and hydrophilic fluorescence probes into the deeper skin layers and through

the human skin compared to the larger ones (average diameter of 272 and 586 nm). Therefore, in our study, the ethosome formulation no.7 composing of 4% w/v SPC and 20% v/v ethanol which had the size of 72.0±23.0 nm was selected for further development. The next formulation development was performed to improve its entrapment efficiency by adding some additives such as CHOL, SA and DA. The formulations are listed in Table 4.4 along with their vesicular size, PI and entrapment efficiency.

Table 4.4 Composition and physical characteristics of ethosome formulations in the presence of additives

No.	**Additives**	**Vesicle size (nm)**	**PI**	**Entrapment efficiency (%)**
7	-	72.0±23.0	0.465±0.137	37.03±9.84
13	SPC:CHOL = 4:1	160.0±84.4	0.307±0.122	39.12±5.85
14	SPC:CHOL = 3:1	322.9±13.1	0.340±0.119	43.85±4.34
15	SPC:CHOL:SA = 6:2:1	384.1±23.1	0.360±0.071	56.83±4.71
16	SPC:CHOL:DA = 6:2:1	55.3±7.8	0.207±0.160	52.51±4.09

As shown in Table 4.4, adding CHOL in the plain ethosomes (formulation no.13 and 14) could increase both size and entrapment efficiency. As described elsewhere, CHOL could increase the rigidity of bilayers of lipid vesicles. Its molecule was inserted into liposomal bilayers with the hydroxyl group oriented towards the aqueous phase and the aliphatic chain parallel to the acyl chains of

phosphatidylcholine. The existence of CHOL prevented partial dilution and reduced the membrane permeability of liposomes leading to higher vesicular size and entrapment efficiency (Fang *et al*., 2006; Lopez-Pinto *et al*., 2005; Mura *et al*., 2007).

The effect of positive and negative surface charge agent, stearylamine (SA) and deoxycholic acid (DA), on ethosome characteristics were also evaluated. The addition of SA in formulation no.15 resulted in an increase of drug entrapment efficiency compared to formulation no.14. This might be due to the electrostatic interaction between acid moiety of indomethacin and amine moiety of SA (Srinath *et al*., 2000). However, SA leaded to increase in vesicle size (about 20%). On the other hand, by adding DA in the formulation no.16, although the comparable increase in entrapment efficiency was observed, there was significantly decrease in the lipid vesicular size as compared to formulation 14 and 15.

The appropriate indomethacin-loaded ethosome formulation was selected for stability and skin permeation studies based on the size and entrapment efficiency. In this study, the formulation no.16 which using 4% w/v SPC:CHOL:DA (6:2:1 molar ratio) as lipid component and 20% v/v ethanol in phosphate buffer pH 7.4 as dispersion medium was selected. It gave the vesicular size of 55.3±7.8 nm (PI=0.207±0.160) and the entrapment efficiency of 52.51±4.09%. The zeta potential of this ethosomal formulation was -39.06±1.53 mV. The morphology of the indomethacin-loaded ethosomes was evaluated using SEM as shown in Figure 4.11. The micrograph could prove the existence of nanosize closed-vesicles in the colloidal ethosomal suspension.

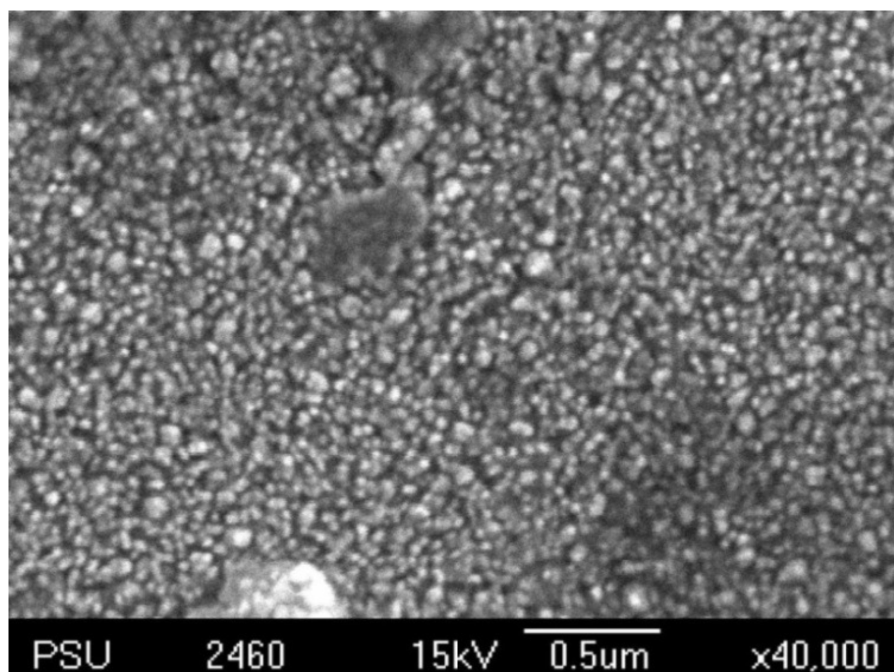

Figure 4.11 Scanning electron micrograph showing ethosome vesicles (x40,000 magnification)

Liposomes and other vesicular carriers can be classified into several types due to their size, the number of bilayers present in the vesicles, and the method of preparation as illustrated in Table 4.5. In the aspect of the lipid vesicular size, the indomethacin-loaded ethosomes (55.3±7.8 nm) developed in this study might be characterized as the small unilamellar vesicles (SUV).

Table 4.5 Classification and approximate sizes of various vesicles (Verumi and Rhodes, 1995)

Vesicles	Classification	Approximation size (µm)
By size	Small unilamellar vesicles (SUV)	0.025-0.05
	Large unilamellar vesicles (LUV)	0.1
By lamellarity	Multilamellar vesicles (MLV)	0.05-10
	Unilamellar vesicles (ULV)	0.025-0.1
By method	Reverse-phase evaporation vesicles (REV)	0.5
	French press vesicles (FPV)	0.05
	Ether injection vesicles (EIV)	0.02

4.4 Stability study of ethosomes containing indomethacin

At the beginning of stability study, ethosomes containing indomethacin appeared as a yellowish colloidal dispersion. After storage for two weeks in refrigerator, the white precipitations were observed at the bottom of the containers as shown in Figure 4.12a. The precipitation was collected to examine for its microstructure by optical microscope. As illustrated in Figure 4.12b, the clusters of needle-liked crystals were found indicating the presence of the free drug precipitation in this system. However, the physical appearance of the formulation kept in ambient temperature did not change throughout the study period as shown in Figure 4.13a,b. This could be explained by the decrease in indomethacin solubility due to low

temperature and therefore resulting in the precipitation of the free drug in the dispersion medium.

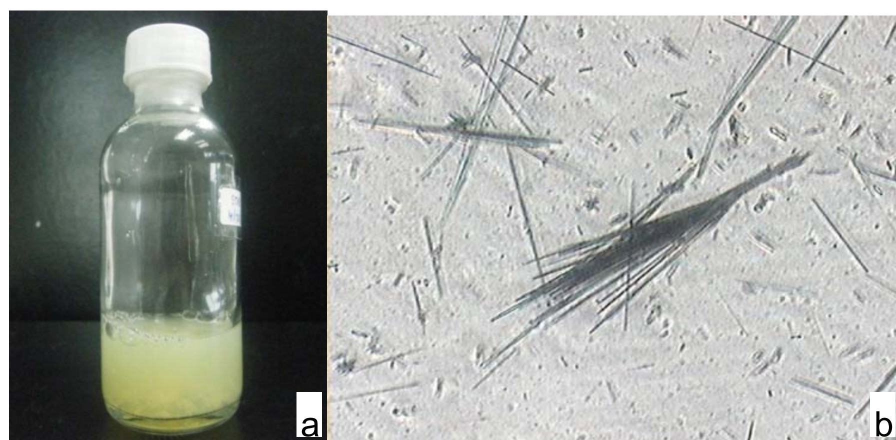

Figure 4.12 Physical appearance of indomethacin-loaded ethosomes kept at 4°C for two weeks (a) and its optical micrograph image (x400 magnification) (b)

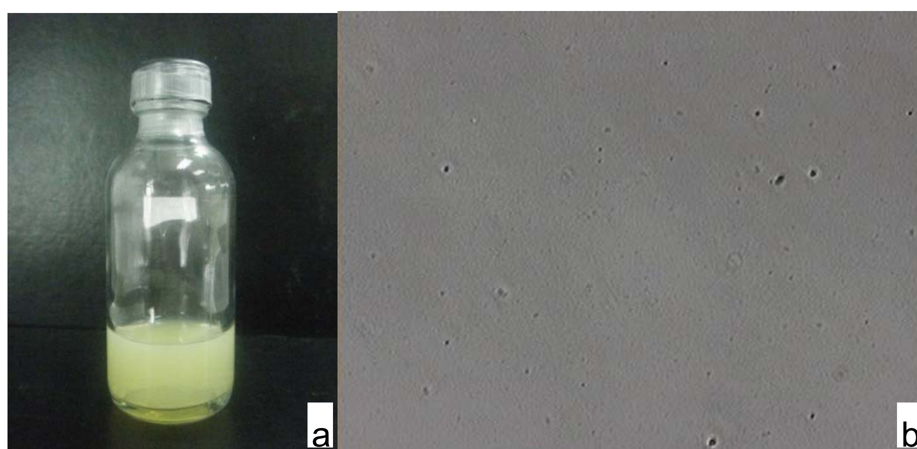

Figure 4.13 Physical appearance of indomethacin-loaded ethosomes kept at ambient temperature for three months (a) and its optical micrograph image (x400 magnification) (b)

Since the formulation kept in refrigerator showed precipitation within two weeks, only data from the formulation kept in ambient temperature was expressed

in the stability profile. Figure 4.14 illustrates the stability profile of the ethosome formulation in term of indomethacin content calculated as percentage of drug remaining after storage. It was clearly seen that, indomethacin content was not significantly change over the storage period of three months.

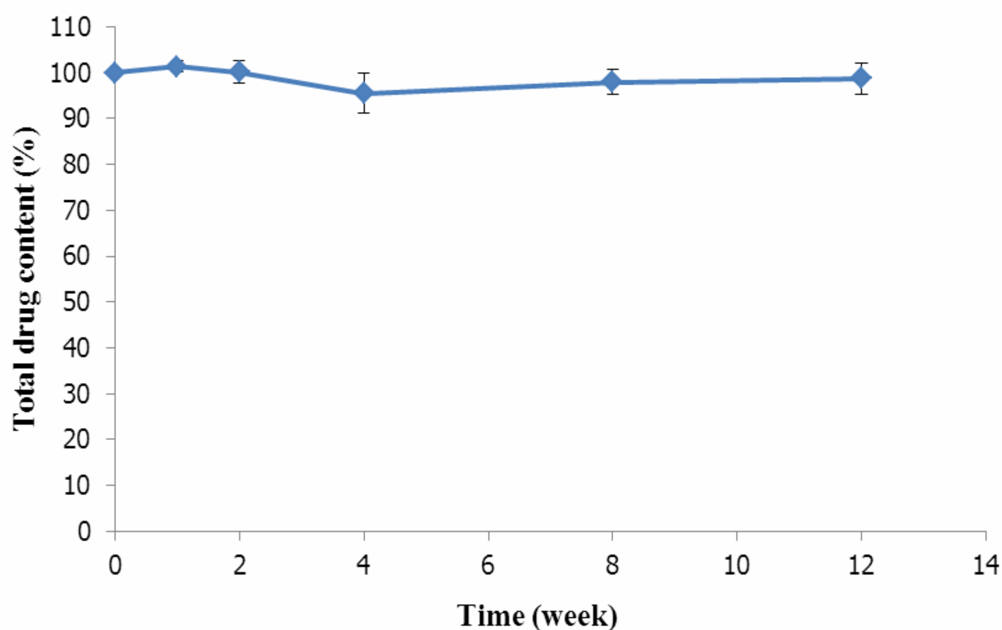

Figure 4.14 Stability profile of indomethacin-loaded ethosomes in term of total drug content (%) after storage at ambient temperature

The stability profiles of indomethacin-loaded ethosomes in terms of vesicular size and zeta potential are shown in Figure 4.15. The results showed that the size of ethosomes did not change after storage for four weeks. However, significant increase in ethosomes size was observed thereafter. Although, the size of ethosomes was reported to be stable due to electrostatic repulsion between negative surface

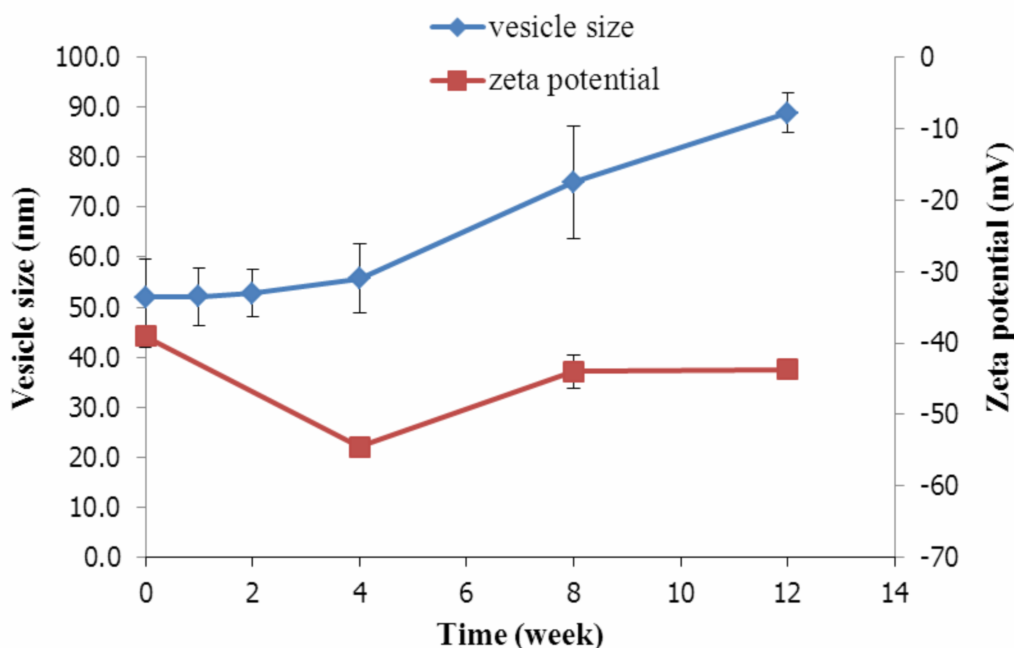

Figure 4.15 Stability profiles of indomethacin-loaded ethosomes kept at ambient temperature in terms of vesicular size (left axis) and zeta potential (right axis)

charged-ethosomes which could avoid vesicles aggregation (Dayan and Touitou *et al.*, 2000; Lopez-Pinto *et al.*, 2005; Dubey *et al.*, 2007a; Dubey *et al.*, 2007b; Fang *et al.*, 2008). However, the growing in ethosomal size found in our study might be caused by the aggregation and/or fusion of the SUV ethosome vesicles. The mechanism might due to the high surface free energy of the nano-vesicular system originating from the distortion of phospholipid molecular packing in a bilayer (Saez *et al.*, 1985; Lentz *et al.*, 1987). Moreover, in the ethosome formulations, ethanol might displace to the water molecules which hydrated the phospholipid head group and served a repulsive force (hydration force) between adjacent bilayers (Komutsu and Okada, 1995). When the hydration force became weak, the aggregation of the vesicles was then initiated. As the vesicles aggregated, the head groups of phospholipids might

come into contact and form adhesive, interdigitated, joining and fission between the two apposed membranes as illustrated in Figure 4.16 (Komutsu and Okada, 1995).

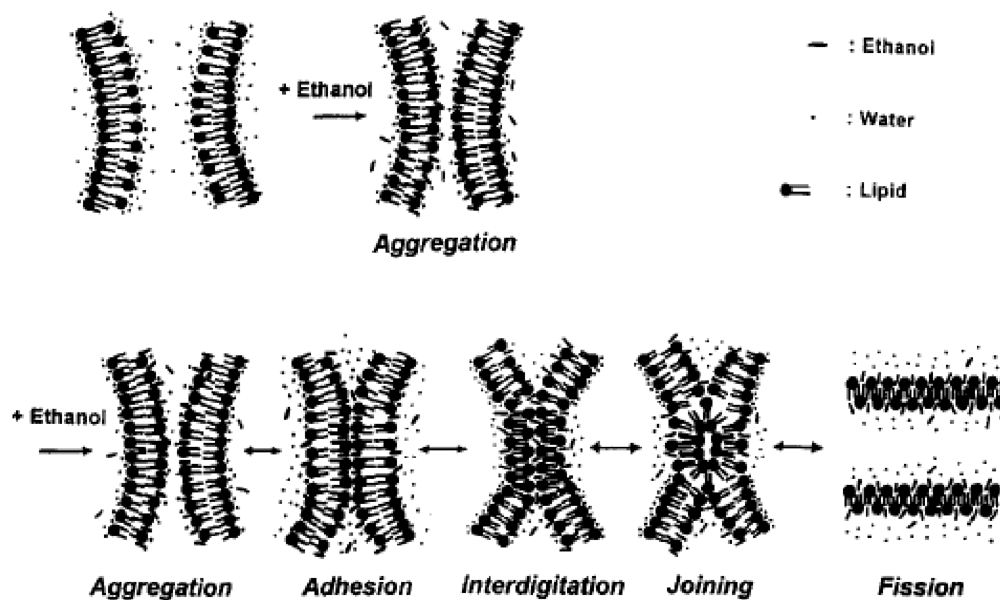

Figure 4.16 Mechanism of the ethanol-induced SUV-SUV aggregation and fusion

Notice that, there was no significant change in the zeta potential of the ethosome formulation (-39.06±1.53 mV at 0 week, and -43.67±1.33 mV after three months of storage) as shown in Figure 4.15. In addition, only minor changes in entrapment efficiency of the ethosome vesicles were observed (from 51.70±3.00% at 0 week to 59.66±5.48% at 12 weeks) indicating the non-significant leakage of the drug from ethosome vesicles as shown in Figure 4.17.

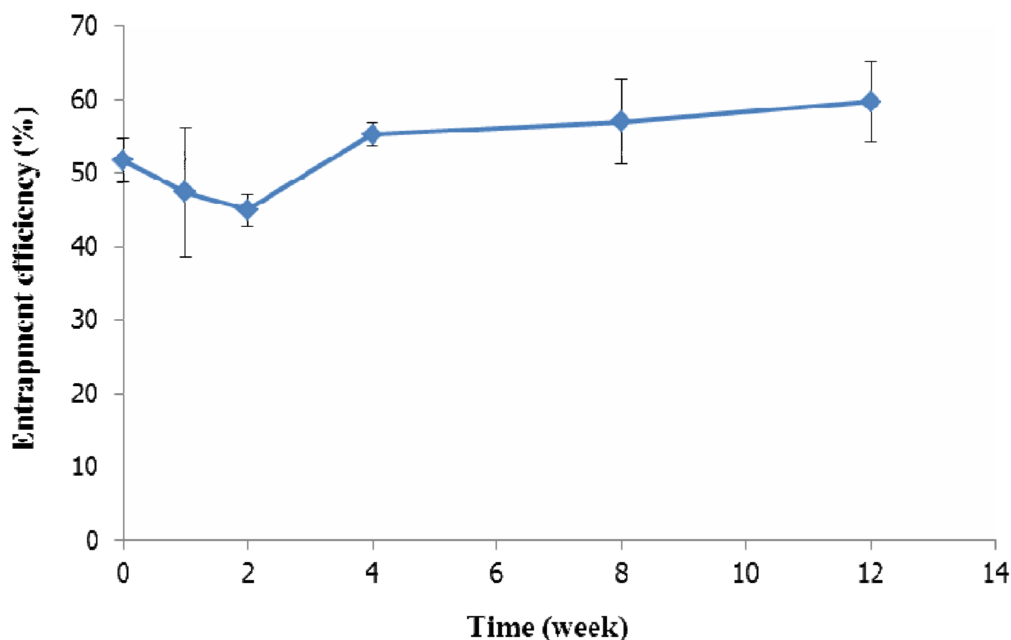

Figure 4.17 Stability profile of indomethacin-loaded ethosomes kept at ambient temperature in term of entrapment efficiency

4.5 *In vitro* skin permeation and skin retention studies

In order to examine for the feasibility of the ethosomes as the transdermal delivery system, the *in vitro* skin permeation and retention studies were then carried out using modified Franz diffusion cells. The newborn pig skin was used as a skin model in these studies due to its similar stratum corneum as human skin in terms of lipid composition and thickness (Cilurzo *et al*., 2007). The skin permeation profiles and the permeation parameters e.g. J_{ss}, K_p and lag time of the investigated indomethacin formulations are shown in Figure 4.18 and Table 4.6, respectively. It could obviously seen in permeation profile that throughout the study period (24 h), the ethosomal system gave about 3 fold higher amount of indomethacin permeated

through skin comparing to commercial product (194.92±24.97 $\mu g/cm^2$ vs. 68.52±17.04 $\mu g/cm^2$; $p<0.05$). The transdermal flux calculated from the slope of linear portion of the skin permeation profiles were 8.81±0.90 $\mu g/cm^2/h$ for the ethosome preparation which was about 2 fold higher than commercial product (4.10±1.25 $\mu g/cm^2/h$). In addition, the lag time for ethosomes calculated from the X-intercept of the profiles was significant shorter than commercial product (1.97±0.87 h vs. 7.10±0.23 h; $p<0.05$). For ethanolic solution of indomethacin, the drug could not be detected in receptor fluid throughout the experiment. This evidence confirmed the lipophilic nature of indomethacin which might form reservoirs in stratum corneum lipid and not deeply penetrate the skin (Goosen *et al.*, 1998). From the skin permeation study, it could be indicated that the ethosomes could provide higher amount, rate and faster time for delivery of indomethacin through the skin than other formulations.

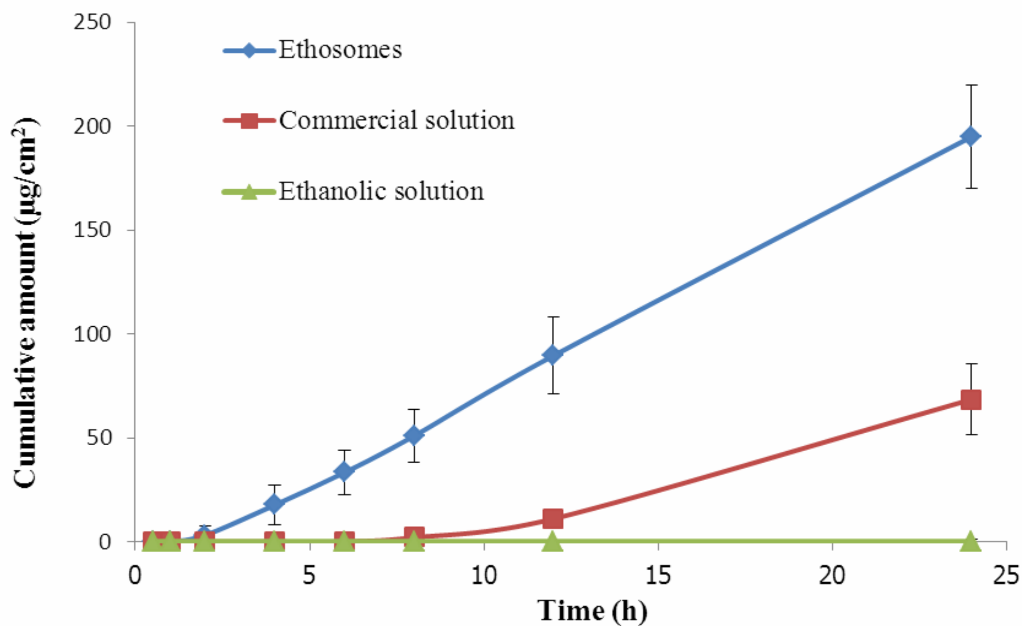

Figure 4.18 *In vitro* cumulative amount-time profiles of indomethacin permeated across pig skin from the ethosome formulation, commercial solution and ethanolic solution. Each point represents the mean±SD (n = 4)

Table 4.6 *In vitro* skin permeation parameters of indomethacin from the ethosome formulation and commercial solution

Formulations	**J_{ss} (µg/cm^2/h)**	**K_p (x10^{-4} cm/h)**	**Lag time (h)**
Ethosomes	8.81±0.90	11.01±1.12	1.97±0.87
Commercial solution	4.10±1.25	5.12±1.56	7.10±0.23

For the skin retention study, the suitable solvents used for extracting the drug from the pig skin were evaluated. It was found that acetonitrile provided the highest recovery (83.68±6.07%) of indomethacin after extraction as shown in Table 4.7. In addition, the extraction procedure gave no interfering peak at the retention time of the drug as previously reported in section 4.1.

Table 4.7 Percentage of recovery for the extraction of indomethacin in the pig skin using various solvents

Solvent	*Recovery (%)
Phosphate buffer pH 7.4	48.62±4.02
Methanol	65.21±15.20
Acetonitrile	83.68±6.07
Chloroform	42.74±10.90
Hexane	3.10±1.97

*n=3

The illustration of *in vitro* amount of indomethacin accumulated in the newborn pig skin and in receptor chamber after 24 h permeation study was expressed in Figure 4.19. The ethosome formulation and ethanolic solution gave comparable amount of indomethacin accumulated in the skin (169.07±101.61 μg vs. 210.51±90.19 μg) but significantly less than from commercial solution (472.58±185.05 μg). These results indicated that ethosomal carrier could provide a mode for transdermal delivery of indomethacin and also reduce the amount of drug deposited within skin layers compared to other formulations.

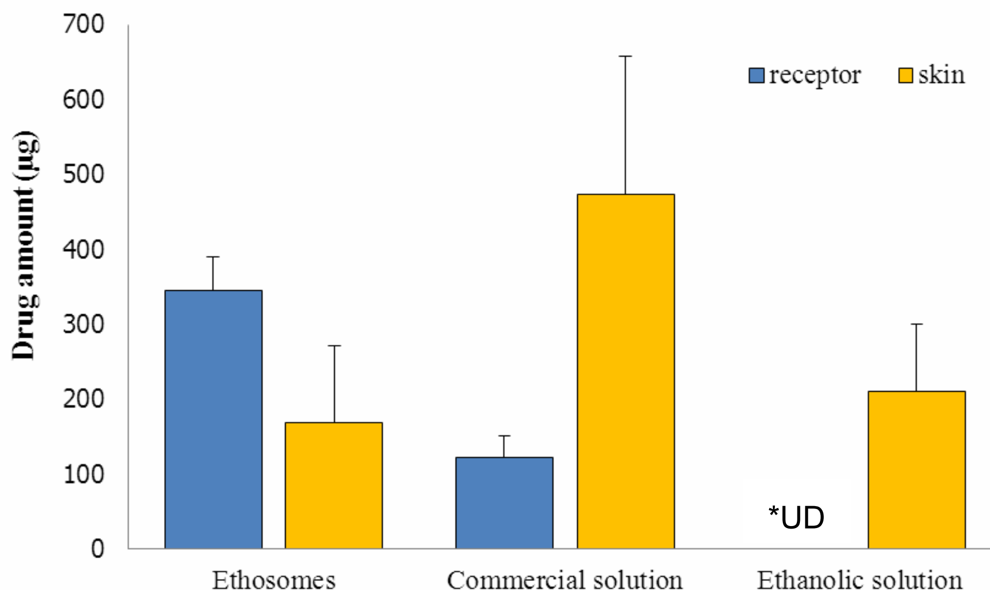

Figure 4.19 *In vitro* amount of indomethacin accumulated in receptor chamber of diffusion cells and in pig skins from various formulations. Each bar represents the mean±SD (n = 4). *UD = undetected

Indomethacin is a drug with poor skin permeation due to two main factors. First, its lipophilic property makes it more likely form the reservoir in the stratum corneum lipid. Second, its weak acidic nature makes it ionize at the skin pH leading to limit transdermal permeation (Goosen *et al.*, 1998). In this study, ethosomes was introduced to overcome the poor skin permeation problems of indomethacin. As expect, the significant improvement in the permeation parameters was observed compared to commercial solution and ethanolic solution which comprised the same drug concentration. This finding is in good agreement with previous researches comparing skin permeation efficiency of ethosomes with other formulations such as aqueous solution, hydroethanolic solution, ethanolic solution,

lipid ethanolic solution, liposomes and commercial formulation (Kirjavainen *et al.*, 1999; Dayan and Touitou, 2000; Touitou *et al.*, 2000; Lopez-Pinto *et al.*, 2005; Elsayed *et al.*, 2006; Fang *et al.*, 2006).

Ethanol has long been used as a skin penetration enhancer in transdermal formulations. The mechanisms reported involve in increasing solubility of poorly soluble drug, alteration of stratum corneum's barrier properties, increasing in thermodynamic activity due to evaporation of ethanol, and permeation of ethanol with drug molecule across the skin (Williams and Barry, 2004; Dubey *et al.*, 2007b). Phospholipid vesicular system such as liposomes is also used as dermal and transdermal drug delivery system. A variety of its skin penetration enhancing mechanisms were stated including intact vesicular skin penetration, the penetration enhancing effect, the adsorption effect, and the penetration of liposomes through the transappendageal route (Elsayed *et al.*, 2007). However, the skin penetration enhancement effect of ethosomes was greater than the combination effects of each ethanol and liposomal vesicles. Touitou *et al.* (2000) suggested the model of ethosomes for enhance the penetration of drugs through the stratum corneum lipids. It was reported to be due to the synergist effects between ethanol, phospholipid vesicles and skin intercellular lipid. When applying ethosomes on the skin, the organization of the stratum corneum lipid bilayer was disturbed by the effect ethanol and therefore enhanced its lipid fluidity. This provided the penetration of the flexible ethosome vesicles through the skin by virtue of their particulate nature. The release of drug in the deeper skin layers and transdermal drug absorption could be due to the fusion of ethosomes with skin lipids and the release of drug at various points along the penetration pathway.

CHAPTER 5

CONCLUSION

Indomethacin is a NSAID which is effectively used in the treatment of locomotor associated pain and inflammation such as rheumatoid arthritis. Oral administration of indomethacin can cause gastrointestinal as well as central nervous system side effects in dose related manner therefore limit its use in clinical management. Although topically applied indomethacin formulations can reduce the plasma drug concentration which then can reduce the side effects, the clinical efficiency of these formulations is not different from the placebo. This effect may be due to the insufficient of indomethacin skin penetration. Therefore, to improve the skin permeation efficiency of indomethacin, the permeation enhancing drug carrier named ethosomes was introduced in this study.

The HPLC quantitative analysis of indomethacin used in this study was developed from the method of Chauhan *et al*. (2003) with some modifications. This HPLC analysis allowed the specificity of indomethacin detection both in ethosome formulations and in skin permeation studies. In addition, this method was accurate and reproducible in both intra-day and inter-day validation with its linear correlation coefficient.

Indomethacin is a very low water soluble drug (5.2 µg/ml at 25°C). Since it is an acidic compound, its solubility could be increased mainly due to the effect of alkaline pH. In addition, ethanol could promote its solubility. However, it

has been reported that it is not stable in basic pH (pH > 8). The indomethacin-loaded ethosomes could be prepared by thin film hydration method using phosphate buffer pH 7.4 with various ethanol contents (10-30% v/v) as dispersion media to avoid the precipitation of the free drug in the formulation. The optimized formulation composed of 4% w/v SPC:CHOL:DA (6:2:1 molar ratio) as lipid component and 20% v/v ethanol in phosphate buffer pH 7.4 as dispersion medium gave 55.3±7.8 nm vesicular size (PI=0.207±0.160), -39.06±1.53 mV for zeta potential and the entrapment efficiency of 52.51±4.09%. This ethosome formulation showed acceptable stability in terms of physical appearance, total drug content after storage in well-closed container at ambient temperature for three months. However, the size of ethosomal vesicles was significantly increased after four weeks of storage, whereas the insignificant changes of their zeta potential and entrapment efficiency were observed. For the formulation kept in refrigerator (4±2°C), the needle-shaped crystals of free drug were observed under optical microscope after two weeks of storage due to the decrease in indomethacin solubility at low temperature. The *in vitro* skin permeation and retention study showed that the ethosome formulation provided significantly higher skin penetration parameters such as Q_t at 24 h (194.92±24.97 μg/cm^2), J_{ss} (8.81±0.90 μg/cm^2/h) and K_p (11.01±1.12 cm/h), compared to a commercial solution and ethanolic solution. In addition, it could significantly reduce the lag time (1.97±0.87 h), which might resulted in reducing the onset of drug action. In the skin retention study, it was found that after 24 h application, the ethosome formulation gave less drug-retained compared to the commercial solution. These results indicated that the ethosome could enhance the skin permeation of indomethacin and also reduce the drug retention in the skin. In consequence, these evidences showed the feasibility of

ethosomes as the transdermal drug carrier for indomethacin. However, the *in vivo* and clinical evaluation should be performed to ensure the clinical efficiency and safety of this drug carrier.

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