Development of Ethosome Containing Mycophenolic Acid and *In Vitro* Skin Permeation Study

Tunyaluk Limsuwan

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Author Miss Tunyaluk Limsuwan

Major Program Pharmaceutical Sciences

Major Advisor :

(Asst. Prof. Dr. Thanaporn Amnuaikit)

Examining Committee

---Chairperson

(Asst. Prof. Dr. Chalermkiat Songkram)

(Asst. Prof. Dr. Thanaporn Amnuaikit)

(Asst. Prof. Dr. Sarunyoo Songkro)

(Dr. Wanchai Chongcharoen)

The Graduate School, Prince of Songkla University, has approved this thesis as partial fulfillment of the requirements for the Master of Pharmacy Degree in Pharmaceutical Sciences

---

(Prof. Dr. Amornrat Phongdara)

Dean of Graduate School

This is to certify that the work here submitted is the result of the candidate's own investigations. Due acknowledgement has been made of any assistance received.

.....Signature

(Asst. Prof. Dr. Thanaporn Amnuaikit)

Major Advisor

.....Signature

(Miss Tunyaluk Limsuwan)

Candidate

I hereby certify that this work has not already been accepted in substance for any degree, and is not being concurrently submitted in candidature for any degree.

----------Signature

(Miss Tunyaluk Limsuwan)

Candidate

ชื่อวิทยานิพนธ์ การพัฒนาเอทโธโซมของยาไมโครฟีโนลิก เอซิดและการศึกษาการซึมผ่านผิวหนังนอกกาย

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สาขาวิชา เภสัชศาสตร์

ปีการศึกษา 2554

บทคัดย่อ

ไมโครฟีโนลิก เอซิด หรือ MPA เป็นตัวยับยั้งแบบไม่แข่งขันและเลือกยับยั้ง Inosine monophosphate dehydrogenase type II หรือ IMPDH ซึ่งทำให้เกิดการยับยั้งการแบ่งตัวของเซลล์ลิมโฟไซต์ทั้งชนิดทีเซลล์และบีเซลล์ พบว่า MPA ถูกใช้เป็นสารกดภูมิคุ้มกันในกรณีการปลูกถ่ายอวัยวะและในโรคแพ้ภูมิตนเอง การบริหารยา MPA โดยการรับประทานมีชีวปริมาณออกฤทธิ์ต่ำ ผ่านการเมตาบอไลท์โดยตับและสามารถก่อให้เกิดผลข้างเคียงต่อระบบทางเดินอาหารซึ่งส่งผลให้ผู้ป่วยต้องการเปลี่ยนแปลงขนาดของยาหรือต้องการหยุดการรักษา ดังนั้น การให้ยาทางผิวหนังเฉพาะที่สามารถพิจารณาให้เป็นวิถีทางเลือกหนึ่งของการบริหารยาที่หลีกเลี่ยงผลข้างเคียงดังกล่าว อย่างไรก็ตาม เนื่องจาก MPA สามารถซึมผ่านผิวหนังได้ในปริมาณที่ไม่เพียงพอจึงทำให้มีประสิทธิภาพทางคลินิกต่ำ ระบบนำส่งยาแบบถุงเอทโธโซมเป็นที่ทราบกันดีว่ามีศักยภาพทั้งในแง่การนำส่งทางผิวหนังแบบเฉพาะที่และแบบผ่านผิวหนัง ดังนั้น ในการศึกษานี้จึงพัฒนาและประเมินเอทโธโซมที่บรรจุ MPA เพื่อให้ได้มาซึ่งสูตรตำรับที่เหมาะสมสำหรับการนำส่งทางผิวหนัง

ในการศึกษานี้ การวิเคราะห์ปริมาณ MPA จะใช้วิธี HPLC อิทธิพลของพีเอชที่มีต่อค่าการละลายของ MPA แสดงให้เห็นว่า MPA สามารถละลายในพีเอชช่วงด่างได้สูงกว่าช่วงพีเอชที่เป็นกรด การละลายจะเพิ่มขึ้นตามการเพิ่มพีเอช ดังนั้น สารละลายบัฟเฟอร์พีเอช 7.4 จึงถูกเลือกเพื่อเตรียมเอทโธโซม เตรียมเอทโธโซมที่บรรจุ MPA 10 มิลลิกรัมต่อมิลลิลิตรโดยวิธีธินฟิล์มไฮเดรชั่น สูตรตำรับเอทโธโซมที่เหมาะสม (สูตรตำรับ Etho-25) ประกอบด้วย ส่วนของไขมันร้อยละ 4โดยน้ำหนักต่อปริมาตรของ SPC:CHOL:Tween 80:DA (ในอัตราส่วนเท่ากับ 6:2:1:1 โดย

โมล) และมีเอทธานอลในปริมาตรร้อยละ 30 ร่วมกับฟอสเฟตบัฟเฟอร์พีเอช 7.4 เป็นตัวกลางในการกระจาย เอทโธโซมตำรับนี้ให้ขนาดอนุภาคของถุงเอทโธโซมเท่ากับ 370.90±7.91 นาโนเมตร (ค่าดัชนีการกระจายตัวเท่ากับ 0.270±0.02) ค่าศักย์ซีต้าเท่ากับ -45.58±4.50 มิลลิโวลต์และมีประสิทธิภาพการกักเก็บตัวยาเท่ากับร้อยละ 56.01±1.10 เอทโธโซมที่เตรียมได้มีความคงตัวดีทั้งลักษณะทางกายภาพ ปริมาณตัวยาทั้งหมดและประสิทธิภาพการกักเก็บตัวยาเมื่อเก็บที่อุณหภูมิห้องเป็นเวลา 90 วัน แม้ว่าขนาดอนุภาคเฉลี่ยของเอทโธโซมที่เตรียมได้จะเพิ่มขึ้นอย่างมีนัยสำคัญ (p<0.05) แต่กลับพบว่าศักย์ซีต้าไม่ได้เปลี่ยนแปลงอย่างมีนัยสำคัญไปด้วย เป็นการบ่งชี้ว่าเอทโธโซมนี้มีความคงตัว ในขณะที่เอทโธโซมที่เก็บไว้ที่ 4±1 องศาเซลเซียส เป็นเวลา 21 วัน พบว่ามีการเกิดตะกอนของทั้งตัวยาและหรือองค์ประกอบอื่นๆซึ่งแสดงถึงความไม่คงตัวของตำรับ

ตำรับเอทโธโซมที่เหมาะสมมีปริมาณยาที่ซึมผ่านผิวหนังที่ 24 ชั่วโมงเท่ากับ 307.29±24.93 ไมโครกรัมต่อพื้นที่ผิว 1 ตารางเซนติเมตร มี Steady-state flux เท่ากับ 13.20±0.91 ไมโครกรัมต่อพื้นที่ผิว 1 ตารางเซนติเมตรต่อชั่วโมง มีค่าสัมประสิทธิ์การซึมผ่านของตัวยาเท่ากับ 1.32±0.09 $\times 10^{-3}$ เซนติเมตรต่อชั่วโมง ซึ่งสูงกว่าเมื่อเทียบกับตำรับอื่นๆอย่างมีนัยสำคัญ (p<0.05) ยิ่งไปกว่านั้น ตำรับเอทโธโซมยังให้ระยะเวลาในการเริ่มซึมผ่านสั้นกว่าซึ่งนำไปสู่โอกาสการลด onset ของยาลง ถึงแม้ว่าตำรับเอทโธโซมจะมีปริมาณยาที่สะสมในผิวหนังไม่แตกต่างอย่างมีนัยสำคัญกับตำรับสารละลาย hydroethanolic (45.46±6.80 เทียบกับ 49.73±3.71 ไมโครกรัมต่อพื้นที่ผิว 1 ตารางเซนติเมตร) แต่ตำรับเอทโธโซมยังคงมีปริมาณยาที่สะสมในผิวหนังมากกว่าเมื่อเทียบกับตำรับอื่นๆอย่างมีนัยสำคัญ (p<0.05) ผลลัพธ์เหล่านี้แสดงให้เห็นว่า เอทโธโซมสามารถเพิ่มประสิทธิภาพการซึมผ่านและการสะสมของ MPAในผิวหนัง ผลที่ได้นี้คาดว่าระบบนำส่งยาแบบถุงเอทโธโซมสามารถนำส่ง MPAไปยังตำแหน่งออกฤทธิ์ที่ส่งผลให้มีประสิทธิภาพมากขึ้นในการรักษาโรคสะเก็ดเงิน อย่างไรก็ตามควรมีการประเมินการซึมผ่านผิวหนังในกายและประสิทธิภาพทางคลินิกของตำรับเอทโธโซมที่เหมาะสมต่อไป

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ABSTRACT

Mycophenolic acid (MPA) is a non-competitive and selective inhibitor of inosine monophosphate dehydrogenase (IMPDH) type II which leads to the inhibition of T and B cell proliferation. MPA was found to be used as immunosuppressive agent in organ transplantation and in autoimmune diseases. The MPA oral administration has low bioavailability, has underwent extensive first-pass metabolism by the liver and can cause gastrointestinal (GI) side effects which lead to most of patients requiring dose changes or discontinuation of treatment. Thus, using topical administration can be considered as an alternative route to avoid these side effects. However, the insufficiency of skin permeation of MPA formulations leads to low clinical efficiency of topical administration. Ethosome is known as being potential both in topical and transdermal drug delivery. Therefore, the ethosome containing MPA was developed and evaluated to obtain the optimized formulation which was for application as skin delivery system.

In this study, the analysis of MPA was carried out by using HPLC method. The effects of pH on MPA solubility showed that its solubility in the alkaline range was higher than that in the acidic range. The solubility has increased with

increasing pH. Accordingly, the buffer solution pH 7.4 was selected for preparing ethosomal formulation. The ethosome containing MPA was prepared according to the thin-film hydration method by using the concentration of MPA 10 mg/ml.

The optimized ethosome formulation (Etho-25) was composed of 4%w/v SPC:CHOL:Tween 80:DA (6:2:1:1 molar ratio) as lipid component and 30% v/v ethanol in phosphate buffer pH 7.4 as dispersion medium. This ethosome gave the vesicles size of 370.90±7.91 nm (*PI*=0.27±0.02), the zeta potential of -45.58±4.50 mV and the entrapment efficiency of 56.01±1.10%. It showed good physical appearance, total drug content and entrapment efficiency after storage at room temperature for 90 days. Although the average sizes of ethosomes significantly increased ($p<0.05$), its zeta potential did not significantly change. It indicated that the ethosomal formulation remained stable. Whereas, the same formulation storage at 4±1˚C for 21 days showed that that the precipitation of the drug and/or other components, which represented unstable formulation. The appropriate ethosome provided significantly higher skin permeation parameter such as the cumulative amount at 24 hours Q_{24} (307.29±24.93 μg/cm^2), the steady state flux J_{ss} (13.20±0.91 μg/cm^2/h) and skin permeability coefficient K_p (1.32±0.09×10^{-3}cm/h) compared with other formulations ($p<0.05$). Moreover, it could significantly shorter the T_{lag} (0.53±0.26 hours), which might result in reducing the onset of the drug. Although the ethosome system provide the MPA accumulated in the skin did not different with hydroethanolic solution (45.46±6.80 μg/cm^2 vs. 49.73±3.71 μg/cm^2), it provide the MPA accumulated significantly higher than other formulations ($p<0.05$). These results demonstrated that the ethosome could enhance the skin permeation and

retention of MPA. This result was expected that the ethosome carrier could deliver MPA to the site of action which resulting more effective in psoriasis treatment. However, the *in vivo* skin permeation and clinical efficiency of the appropriate ethosome should be evaluated further.

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LIST OF ABBREVIATIONS AND SYMBOLS

AcMPAG	acyl glucuronide
ALA	5-aminolevulinic acid
BH	Buspirone hydrochloride
ºC	degree Celcius
CHOL	cholesterol from lanonlin
CLSM	confocal laser scanning microscopy
cm^2	square centimeter (s)
conc.	concentration
DA	Deoxycholic acid
DLS	dynamic light scattering
DNA	Deoxyribonucleic acid
DSC	differential scanning calorimetry
e.g.	example grati, for example
EPC	phosphatidylcholine from egg yolk
et al.	et alli, and others
g	gram (s)
FITC-Bac	fluorescently labeled bacitracin
GI	gastrointestinal
GPx	glutathione peroxidase
GTP	guanosine triphosphate
h	hour (s)

LIST OF ABBREVIATIONS AND SYMBOLS (Continued)

HPLC	high performance liquid chromatography
IMP	inosine monophosphate
IMPDH	inosine monophosphate dehydrogenase
J_{ss}	permeation rate at steady state
K_p	permeability coefficient
kg	kilogram (s)
LOD	limit of detection
LOQ	limit of quantitation
M	Molar (mole per liter)
µg	microgram (s)
µl	microliter (s)
µm	micrometer (s)
µmol	micromole (s)
mg	milligram (s)
ml	milliliter (s)
mm	millimeter (s)
mV	milli volt (s)
min	minute (s)
MW	molecular weight
MMF	mycophenolate mofetil
MPA	mycophenolic acid

LIST OF ABBREVIATIONS AND SYMBOLS (Continued)

MPAG	phenyl mycophenolic acid glucuronide
nm	nanometer (s)
no.	number
%	percent
PBS	phosphate buffer saline pH 7.4
pH	the negative logarithm of the hydrogen ion concentration
PCS	photon correlation spectroscopy
PCS	photon correlation spectroscopy
PE	Phosphatidylethanolamine
PI	polydispersity index
Q_t	cumulative drug permeated
R^2	correlation of determination
PEG	polyethylene glycol
RNA	ribonucleic acid
rpm	round (s) per minute
RSD	relative standard deviation
SA	stearylamine
SD	standard deviation
SEM	scanning electron microscopy
SPC	phosphatidylcholine from soybean
TEM	transmission electron microscopy

LIST OF ABBREVIATIONS AND SYMBOLS (Continued)

T_m	transition temperature
Tween 80	polyoxyethylene sorbitan monooleate
UD	undetected
UGTs	UDP-glucuronsyl-transferase enzymes
v/v	volume by volume
w/v	weight by volume
w/w	weight by weight
XMP	xanthine monophosphate

CHAPTER 1

INTRODUCTION

1.1 Background and Rationale

Mycophenolic acid (MPA) isolated from the fungus *Penicillium stoloniferum* was firstly discovered by Gosio in 1896 (Kitchin *et al*., 1997). MPA is a non-competitive, selective and reversible inhibitor of inosine monophosphate dehydrogenase (IMPDH) type II. IMPDH is a key enzyme that controls the rate of guanine nucleotides synthesis in the *de novo* pathway which is used for the proliferation of activated T and B lymphocytes. Therefore, IMPDH inhibitions through the MPA administration lead to inhibition of cell proliferation (Chaigne-Delalande *et al*., 2008). MPA is administered as an ester prodrug Mycophenolate mofetil (MMF), it was found to be used as an immunosuppressant in organ transplantation and in a variety of autoimmune diseases such as scleroderma, systemic lupus erythematosus and psoriasis (Elbarbry *et al*., 2007). Jones *et al.* (1975) demonstrated that oral administration of MPA was effective and safe in the treatment of psoriasis. However, its oral administration has disadvantages because it has underwent extensive first-pass metabolism by the liver and its tolerability was limited by gastrointestinal (GI) upset; it caused nausea, soft stools, abdominal cramps, anorexia, diarrhea and frequent stools leading to the most patients wanted to change the dose or discontinuation of treatment (Kitchin *et al*., 1997; Arns, 2007; Orvis *et al*., 2009).

Using the topical route eliminates these side effects, increase patient compliance, target of the active ingredient for a local effect and avoid first-pass metabolism. However, the insufficiency of skin permeation of MPA formulations leads to low clinical efficiency of topical administration (Geilen and Mrowietz, 2000). Thus, an improved MPA formulation with a high degree of permeation could be useful for the treatment of psoriasis.

Ethosomes are soft vesicles carrier, described by Touitou *et al.,* (2000). This carrier was modified form liposome which mainly composes of phospholipids, ethanol in relatively high concentration and water. It is an interesting system which has important features related to ability to increase skin permeability. The size of ethosomes ranges from tens of nanometers to microns which have a small size related to liposome (Touitou *et al.,* 2000). The ethosomal formulations possess greater entrapment capability than liposome. It possesses ability for various entrapment molecules, like hydrophilic, lipophilic and amphiphilic molecules. It could be explained by the high degree of lamellarity and the presence of ethanol in its vesicles, which allows better solubility of many drugs (Touitou *et al.,* 2000). It was found that the physicochemical characteristics of ethosome could assist in the delivery of various active agents through the stratum corneum into the skin deeper layers or to the systemic circulation. One of the most important characteristics of ethosome is the elasticity and deformability property tailored for enhancing delivery of active agents (Jain *et al.,* 2007). The better permeability of ethosome carriers may be due to the synergistic effect of combination of phospholipids vesicles, ethanol at high concentration and skin lipids. Ethanol interacts with skin lipid molecules in the polar head group region that the result of fluidity increased and may finally lead to increase

membrane permeability. In addition, ethanol may provide the lipid vesicles with soft, flexible and malleable characteristics which are easy to penetrate into deeper layers of the skin. The release of drug could be the result of fusion of ethosome with skin lipids and drug release at various points along the penetration pathway (Touitou *et al.,* 2000). Many studies used ethosome as a carrier system of drug delivery for the transdermal or topical administration. It was reported to improve various drug deliveries both *in vivo* and *in vitro* skin, such as acyclovir, testosterone, cannabidiol, buspirone hydrochloride, erythromycin, ammonium glycyrrhizinate, lamivudine, minoxidil and 5-aminolevulinic acid (Elsayed *et al.,* 2007; Jain *et al*., 2007; Fang *et al*., 2008; Shumilov *et al*., 2010).

In this study, the ethosome containing MPA was developed and evaluated in terms of physicochemical characteristics, physicochemical stability, *In vitro* skin permeation and skin retention properties in order to obtain the optimized formulation which suits for application as skin delivery system and efficiency in treatment.

1.2 Objectives of the study

The objectives of the present study were:

1.2.1 To prepare and evaluate physicochemical characteristics of the ethosome containing MPA for using as skin delivery system.

1.2.2 To evaluate physicochemical stability of ethosome containing MPA.

1.2.3 To study the skin permeation and retention properties of MPA from using ethosome system compared with MPA from aqueous suspension, hydroethanolic solution at the same percentage of ethanol (30%v/v), liposome at the same composition and concentration of lipid component without ethanol in the formulation and liposome developed by Rattanat, 2008.

CHAPTER 2

REVIEW OF LITERATURE

2.1 Mycophenolic acid

Mycophenolic acid (MPA) is a weak organic acid and isolated from the fungus Penicillium stoloniferum. It was firstly discovered by Gosio in 1896 (Kitchin *et al.,* 1997) and was named by Alsberg and Black in 1913 (Jones *et al*., 1975). The complete structure of MPA was reported by Birkinskaw *et al*. in 1952 as shown in Figure 2.1 (Jones *et al*., 1975; Kitchin *et al*., 1997; Orvis *et al*., 2009).

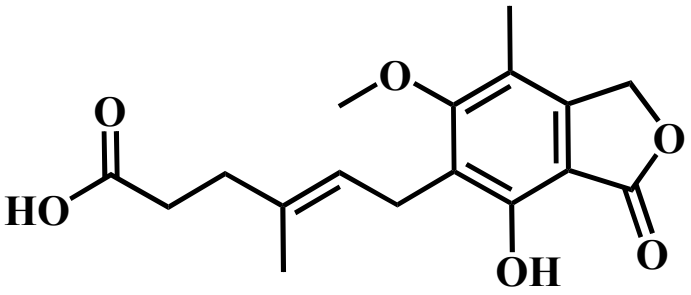

Figure 2.1 Structure of mycophenolic acid

2.1.1 Derivative of MPA

MPA is clinically administered either by using the 2-morpholino ethy ester prodrug, mycophenolate mofetil (Cellcept®, Roche, Basel, Switzerland) or the sodium salt of MPA (Myfortic®, Novatis Pharma, Basel, Switzerland) as shown in Figure 2.2 and 2.3, respectively.

Mycophenolate mofetil (MMF) is the morpholinoethyl ester prodrug from MPA. It was proved to have higher bioavailability and efficacy. In 1995, MMF was approved by the US Food and Drug Administration (FDA) for the prevention of both renal and cardiac allograft rejection, and later for the prevention of hepatic allograft rejection. Soon thereafter, MMF showed promise for several immune mediated dermatologic conditions (Zhou *et al*., 2003; Orvis *et al.*, 2009). However, the administration of MMF is associated with adverse gastrointestinal events, which can lead to withdrawal of therapy. In an effort to reduce the gastrointestinal effects of MMF, an enteric-coated formulation of the drug was developed, based on the sodium salt of MPA (Johnston *et al*., 2006).

Mycophenolate sodium (MPS) is an enteric coated, delayed release monosodium salt of MPA. It was developed for the potential to reduce the adverse GI events of MPA (in particular nausea, vomiting, dys-pepsia and abdominal pain), while allowing more sustained absorption of the MPA delivered to the small intestine (Bjarnason *et al.*, 2001).

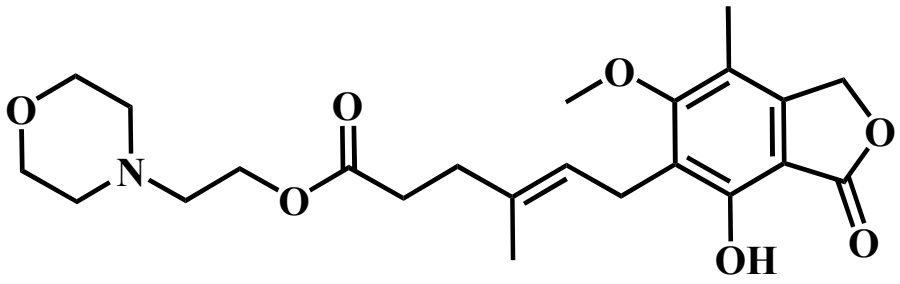

Figure 2.2 Structure of mycophenolate mofetil

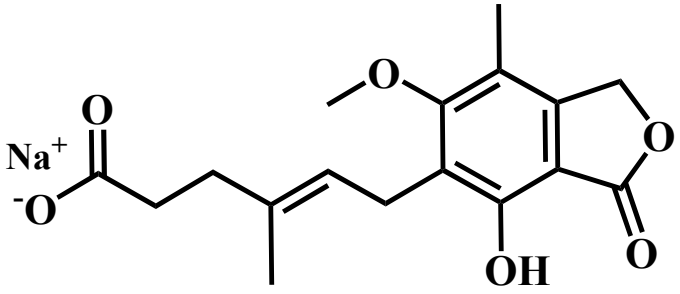

Figure 2.3 Structure of mycophenolate sodium

2.1.2 Physicochemical properties of MPA

MPA or IUPAC name is (*E*)-6-(4-hydroxy-6-methoxy-7-methoxy-3-oxophthalanyl)-4-methyl-4-hexenic acid, its physicochemical properties are listed in Table 2.1.

Table 2.1 Physicochemical properties of MPA (Wishart *et al.*, 2006)

Generic Name	Mycophenolic acid (MPA)
Chemical Name	(*E*)-6-(4-Hydroxy-6-methoxy-7-methyl-3-oxo-5-phthalanyl)-4-methyl-4-hexenoic acid
Chemical Formula	$C_{17}H_{20}O_6$
Molecular Weight	320.337 g/mol
Percent Composition	C 63.74%, H 6.29%, O 29.97%
Appearance	Solid (White to Off-White crystalline powder)
Solubility	Ethanol (30 mg/ml), Methanol (50 mg/ml), Dimethylsulfoxide (200 mg/ml), Dichloromethane Very slightly soluble in water (13 µg/ml at 25°C) (Lee *et al*., 1990)
Melting Point	141°C (285.8°F)
Log P	1.66 (pH 7.4) (Lee *et al*., 1990)
pKa	4.5

2.1.3 Pharmacokinetics of MPA and metabolites

2.1.3.1 Absorption

MPA is administered as an ester prodrug MMF that is completely absorbed and readily hydrolyzed by esterase to MPA (Elbarbry and Shoker, 2007).

2.1.3.2 Distribution

MPA is an acidic compound which is more than 97% bound to albumin at clinically relevant to concentrations (Elbarbry and Shoker, 2007)

2.1.3.3 Metabolism and elimination

MPA is administered as an ester prodrug MMF, a morpholinoethyl ester of MPA. It is extensively metabolized by UDP-glucuronsyl-transferase enzymes (UGTs) in the liver, gut and kidney to its inactive metabolite, phenyl mycophenolic acid glucuronide (MPAG). This metabolite exists in plasma in up to 100-fold greater concentration than MPA. MPAG is extensively bound to serum albumin, from which it can displace MPA, and is excreted in the urine and bile. Accordingly, two MPA peaks in plasma are usually seen: one following the first absorption and occurs 0.5-1 hours after oral administration; and the second and smaller peak represents absorption from the distal bowel following enterohepatic recirculation occurs 4-6 hours after drug administration. A second and less abundant metabolite is the acyl glucuronide (AcMPAG). Unlike MPAG, AcMPAG is pharmacologically active and cross-reacts with antibody used for MPA quantification (Elbarbry and Shoker, 2007).

Pathways of Purine Biosynthesis

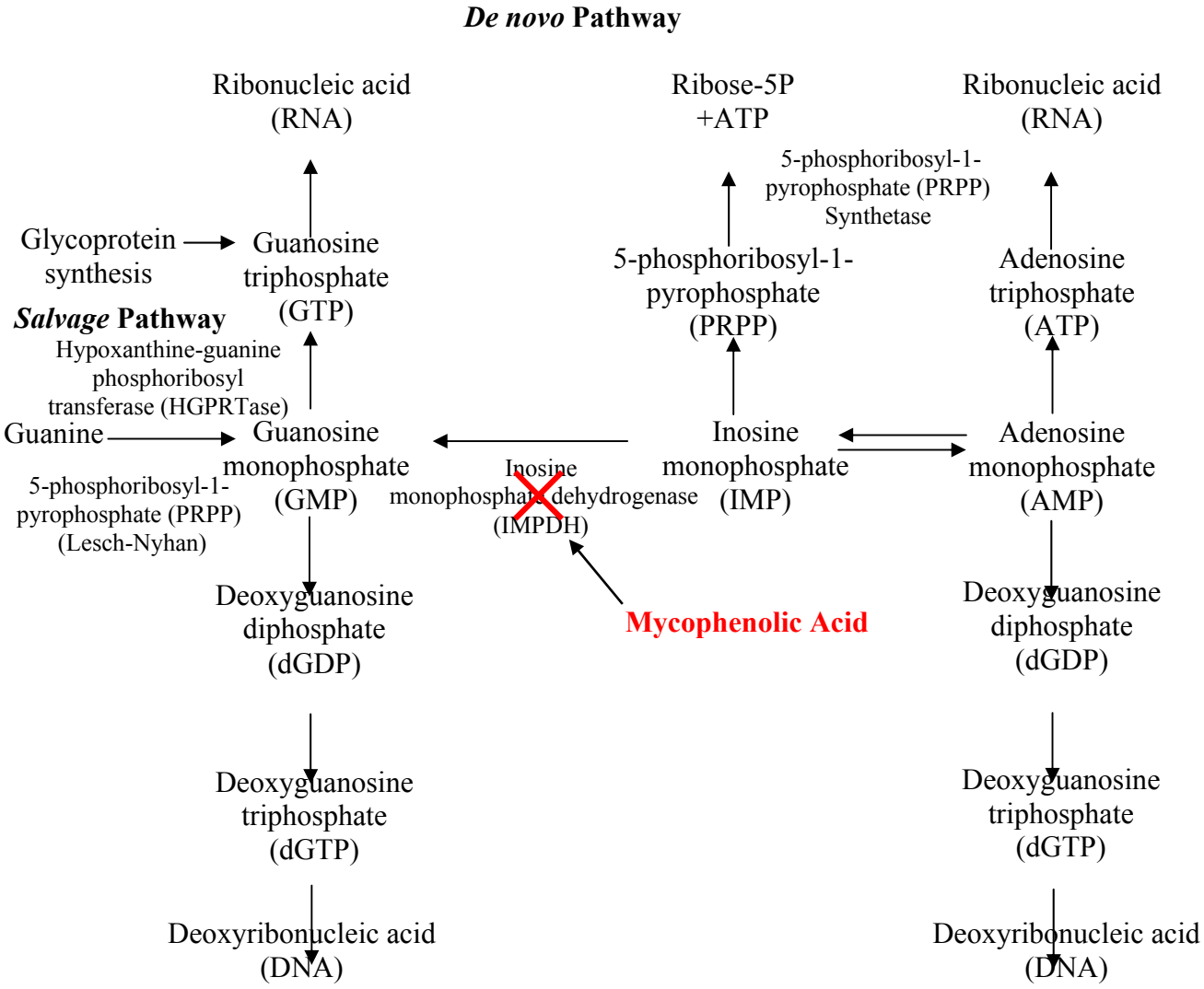

Figure 2.4 Pathway of purine biosynthesis, showing the central position of IMPDH. MPA inhibits IMPDH, therebe depleting GMP, GTP and dGTP. (Allison *et al*., 1993; Alllison and Eugui, 2000)

2.1.4 Pharmacological and mechanism of MPA action

MPA possesses antibacterial, antifungal, antiviral, antitumor and immunosuppressive properties (Westley *et al*., 2005). It has been widely used as an immunosuppressive agent in organ transplantation and in a variety of autoimmune diseases such as scleroderma, systemic lupus erythematosus and psoriasis (Elbarbry *et al*., 2007).

MPA's immunosuppressive activity is mediated through potent, non-competitive, selective and reversible inhibitor of the enzymes inosine monophosphate dehydrogenase (IMPDH) in the *de novo* purine synthesis pathway. This enzyme converts inosine monophosphate (IMP) to xanthine monophosphate (XMP). Xanthine monophosphate is an intermediate metabolite that becomes the purine nucleotide, guanosine triphosphate (GTP). GTP is needed for RNA, DNA, and protein synthesis as shown in Figure 2.4 (Allison *et al*., 1993; Mydlarski *et al*., 2005; Chaigne-Delalande *et al*., 2008).

The purine bases, adenosine and quanosine, might be synthesized through two pathways: the *de novo* purine synthesis pathway, and the *hypoxanthinequanine phosphoribosyl transferase salvage* pathway. As lymphocytes lack the salvage pathway, MPA selectively inhibits lymphocyte proliferation and antibody production (Chaigne-Delalande *et al*., 2008). Moreover, MPA could also induce of T-lymphocytes apoptosis. Furthermore, MPA preferentially blocks the type II isoform of IMPDH predominantly located on lymphocytes. Thus, it also holds potent cytostatic effects on T and B cells (Allison *et al*., 1993). MPA also prevented the glycosylation of lymphocyte and monocyte glycoprotein that were involved in adhesion to endothelial cells. It might be further inhibit the recruitment of leukocytes

to sites of information and impaired antigen presentation. While it does not inhibit early events in the activation of human peripherall blood mononuclear cells (i.e., IL-1 and IL-2 production), MPA blocks the coupling of these events to DNA synthesis and proliferation (Allison *et al*., 1993; Mydlarski *et al*., 2005).

2.1.5 Literature review of use of MPA in psoriasis

Due to the mechanism of action of MPA which specific to T-lymphocyte cells which cause inflammation in psoriasis. Therefore, MPA was used in studies to treat patients with psoriasis. A previous study of Jones *et al.* in 1975, they evaluated the therapeutic and side effects of MPA in treatment of psoriasis lesions. This study has used a mean dose of 3600 mg/day (range 1600 mg to 9600 mg/day) in 29 patients. They reported that the oral MPA therapy has effectiveness in reducing or clearing psoriatic skin lesions of 28 patients with a mean severity score decreased by 68% in 12 weeks. However, its oral administration has disadvantages that this drug has underwent extensive first-pass metabolism by the liver and its tolerability was limited by gastrointestinal (GI) side effect; it caused nausea, weakness, diarrhea, abdominal cramping, soft bowel movements, decreased appetite, perianal tenderness, and abdominal distention which lead to most patients requiring dose changes or discontinuation of treatment (Kitchin *et al*., 1997; Arns, 2007; Orvis *et al*., 2009).

Therefore, using the topical route could eliminate these side effects, increased patient compliance, target of the active ingredient for a local effect and avoid first-pass metabolism. Shoji *et al.* (1994) reported that the examination of topical MPA efficiency in treat to allergic contact dermatitis in guinea pigs which was induced by dinitrofluorobenzene. They found that the topical MPA therapy has

effectiveness in treat for up to 3 days. Moreover, they speculated that topical MPA may be useful for the treatment of psoriasis, but this has not been investigated (Shoji *et al.*, 1994; Kitchin *et al.*, 1997).

These results led to the study of Geilen and Mrowietz in 2000, they investigated efficacy and tolerability of MPA in 7 patients with psoriasis vulgaris. In randomized, placebo-controlled, 1% MPA ointment was compared with the corresponding ointment base after 3 weeks of treatment. Their results indicated that MPA is ineffective for topical treatment of plaque-type psoriasis using the psoriasis plague test and no report about side effects during the time of study. They explained that the low clinical efficiency of MPA topical administration due to the insufficiency of skin permeation of MPA formulations. Thus, an improved MPA formulation with a high degree of permeation could be useful for the treatment of psoriasis.

2.2 Ethosome

The skin as a route for drug delivery can offer many advantages, including avoidance of first-pass metabolism, lower fluctuations in plasma drug levels, targeting of the active ingredient for a local effect and good patient compliance. However, the natural barrier of the skin makes it more difficult for penetration and permeation of drugs (Elsayed *et al*., 2007).

During the past decades, the vesicles carriers have been widely interested in their important characteristic to increase drug transportation into or through the skin. Liposome has the potential of overcoming the skin barrier, as these are conventional vesicles carriers which consist of phospholipids and cholesterol. However, it was found in the most researches showing that classic liposomes are of little or no value as carriers for drug delivery. The researchers explained that it does not deepen penetrate through skin but rather remain in upper layers of the stratum corneum.

One of the advances in vesicles carrier research was the discovery of a vesicles derivative, known as the ethosomes. Ethosomes are soft vesicles carrier, described by Touitou *et al.* (2000). This carrier was modified form liposome which is mainly composed of phospholipids, ethanol in relatively high concentration and water as shown in Figure 2.5. Although the presence of ethanol in the formulation was commonly believed that the ethanol might disrupt to the bilayer structure of liposome from the interdigitation effect between ethanol and the lipid vesicle, but Touitou *et al.* (1997) was found that the vesicles carrier could coexist with ethanol up to 45% v/v.

The existence of ethanol both inside the core and lamellar of phospholipids bilayer vesicles (Figure 2.5) is believed to cause the vesicles having soft flexible characteristics which can easily penetrate into stratum corneum. Moreover, it interacts with lipid molecules in the polar head-group region, lead to an increase in fluidity and may finally lead to increased membrane permeability of drug.

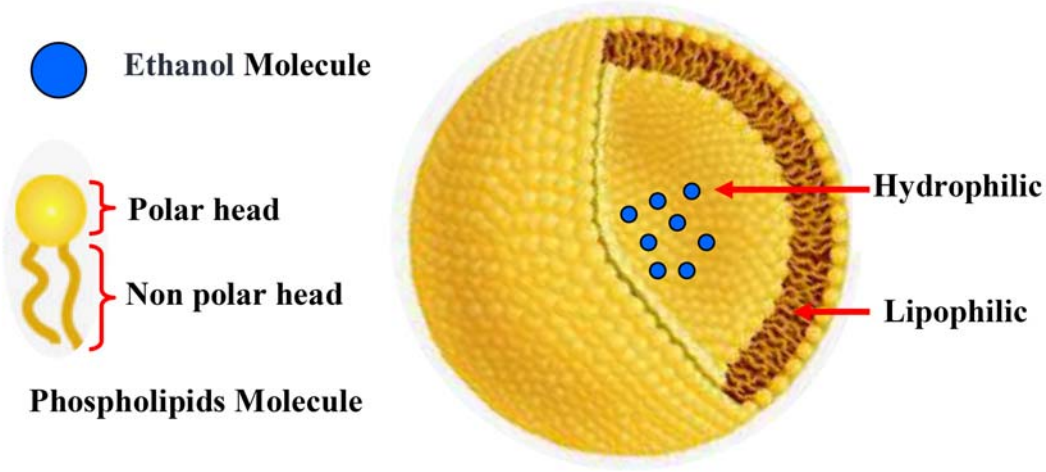

Figure 2.5 The structure of ethosome vesicles which is mainly composed of phospholipids, ethanol in relatively high concentration and water. The blue circles represent the existence of an ethanol molecule in vesicles

2.2.1 Ethosome Composition

Ethosome vesicle was modified form of liposome which is mainly composes of drug or active substance, phospholipids, ethanol in relatively high concentration and water. Table 2.2 shows the different additives employed in formulation of ethosome. Typically, the ethosome might contain phospholipids with various chemical structures like phosphatidylcholine (PC), hydrogenated PC, phosphatidic acid (PA), phosphatidylserine (PS), phosphatidyl ethanolamine (PE), phosphatidylglycerol (PPG), phosphatidylinositol (PI) and hydrogenated PC. Some preferred phospholipids are soya phospholipids, such as Phospholipon 90 (PL-90). It is usually employed in a range of 0.5 to 10% w/w. Examples of alcohols which can be used including ethanol and isopropyl alcohol. The concentration of alcohols in the final product might range from 20 to 50% (Jain *et at*., 2011; Chandel *et at*., 2012; Gupta *et at*., 2012)

In addition, the other additives such as cholesterol or surfactant can be added to improve the ethosome characteristics. Cholesterol at concentrations ranging from 0.1 to 1.5% can also be added for preparation. Among of glycols, propylene glycol and Transcutol are generally used. Moreover, 0.1 to 1% of non-ionic surfactants (PEG-alkyl ethers) can be combined with the phospholipids for these preparations. Cationic lipids such as cocamide, POE alkyl amines, dodecylamine, cetrimide. could be added in formulation. (Jain *et at*., 2011; Gupta *et at*., 2012)

Table 2.2 Different additives employed in formulation of ethosome (Jain *et at*., 2011; Gupta *et at*., 2012)

Material	Examples	Uses
Phospholipid	Soya phosphatidyl choline Egg phosphatidyl choline Dipalmityl phosphatidyl choline Distearyl phosphatidyl choline	Vesicles forming component
Polyglycol	Propylene glycol Transcutol RTM	As a skin penetration enhancer
Alcohol	Ethanol Isopropyl alcohol	For providing the softness for vesicle membrane As a penetration enhancer
Cholesterol	Cholesterol	For providing the stability to vesicle membrane
Dye	Rhodamine-123 Rhodamine red Fluorescen Isothiocynate (FITC) 6- Carboxy fluorescence	For characterization study
Vehicle	Carbopol 934	As a gel former

2.2.2 Method of ethosome preparation

Ethosome could be prepared by two methods as described below. Both method are convenient, do not require any sophisticated equipment and easy to scale up at an industrial scale.

1. The method described by Touitou *et al.* (2000)

This is the most common method utilized for the preparation of ethosomal formulation. In this method, the lipid was dissolved in ethanol. The aqueous component was slowly added in a fine stream at constant rate in a well-sealed container and constant mixing.

2. The thin film hydration method

In this method, the lipids were dissolved in the organic solvent such as chloroform and then evaporated in a rotary evaporator above transition temperature of the phospholipids used to form a thin lipid film on the inner wall of the flask. The thin film is then hydrated with hydroethanolic solution at corresponding temperature.

2.2.3 Characterization of ethosome (Jain *et al*., 2011; Chandel *et al*., 2012)

1. Vesicle shape: The ethosome could be visualized by using either transmission electron microscopy (TEM) or scanning electron microscopy (SEM).

2. Size and zeta potential: Particle size of the ethosome could be determined by either dynamic light scattering (DLS) or photon correlation spectroscopy (PCS). Zeta potential of the formulation could be measured by Zeta meter.

3. Transition temperature: The transition temperature of the vesicular lipid systems could be determined by using differential scanning calorimetry (DSC).

4. Drug entrapment: The entrapment efficiency of ethosomes could be measured by the ultracentrifugation technique.

5. Drug content: Drug content of the ethosomes could be determined using validated method of determination. This could also be quantified by a modified high performance liquid chromatographic method.

6. Surface tension measurement: The surface tension activity of drug in aqueous solution could be measured by the ring method in a Du Nouy ring tensiometer.

7. Stability studies: The stability of vesicles could be determined by assessing the size and structure of the vesicles over time. Mean size is measured by DLS while the structure changes are observed by TEM.

8. Skin permeation studies: The ability of the ethosomal preparation to penetrate into the skin layers could be determined by using confocal laser scanning microscopy (CLSM).

2.2.4 Physicochemical characteristic of ethosome

2.2.4.1 The vesicle morphology

The morphology investigation of the ethosome has been reported as a closed spherical (Figure 2.6A) shape when visualized by scanning electron microscope (SEM). In addition, it also has different characteristics of lamellarity ranging from unilamellar to multilamellar when visualized by transmission electron microscope (TEM) as shown in Figure 2.6B and C, respectively.

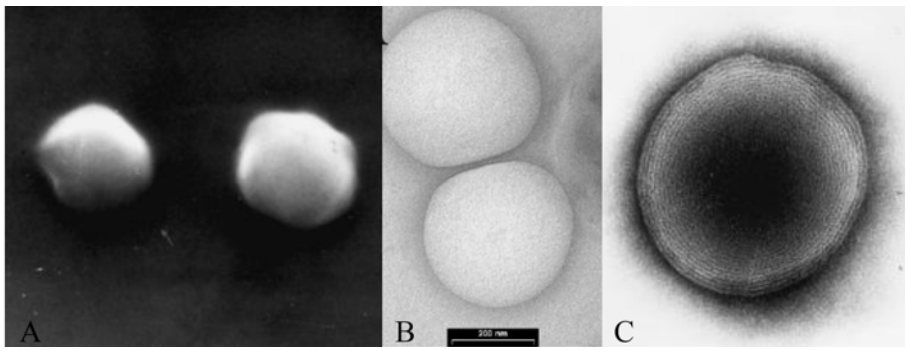

Figure 2.6 Visualization of ethosomal vesicles. (A) A closed spherical shape, (B) unilamellar and (C) multilamellar

2.2.4.2 The vesicle size and size distribution

The size of ethosome ranges from tens of nanometers to a micron which is influenced by the composition of the formulation. The ethosome has narrow size distributions (Figure 2.7) and has a smaller size than liposome when both systems were obtained from the same method of preparation and without involving of size reduction steps (Dayan and Touitou, 2000). In addition, the effect of phospholipids and ethanol concentration on the size distribution of ethosome vesicles was investigated in many studies. For the ethanol concentration ranging from 20 to 45%, the size of the vesicles decreased with increasing ethanol concentration as shown in Figure 2.8A. The ethosome was prepared from 2% phosphatidylcholine of soybean, 30% ethanol and water showed that the largest vesicles in preparations containing 20% ethanol (193±8 nm) and the smallest vesicles size in preparations containing 45% ethanol (103±9 nm).

This reduction in vesicle size could be explained as a result of incorporation of high ethanol concentration. Ethanol confers a surface negative net

charge in the liposome which causes the size of vesicles to decrease (Touitou *et al*., 2000; Lopez-Pinto *et al.,* 2005; Bendas and Tadros, 2007; Jain *et al*., 2007). Moreover, the addition of ethanol results in a decrease in the membrane thickness of the vesicles which is probably due to ethanol and might form a phase with interpenetrating hydrocarbon chain (Barry *et al*., 1994; Bendas and Tadros, 2007). An increase in phospholipids concentration from 0.5 to 4% resulted in increasing size of ethosome as shown in Figure 2.8B. It could be clarified by the increase of phospholipids molecules in bilayers of the vesicles where MPA reside.

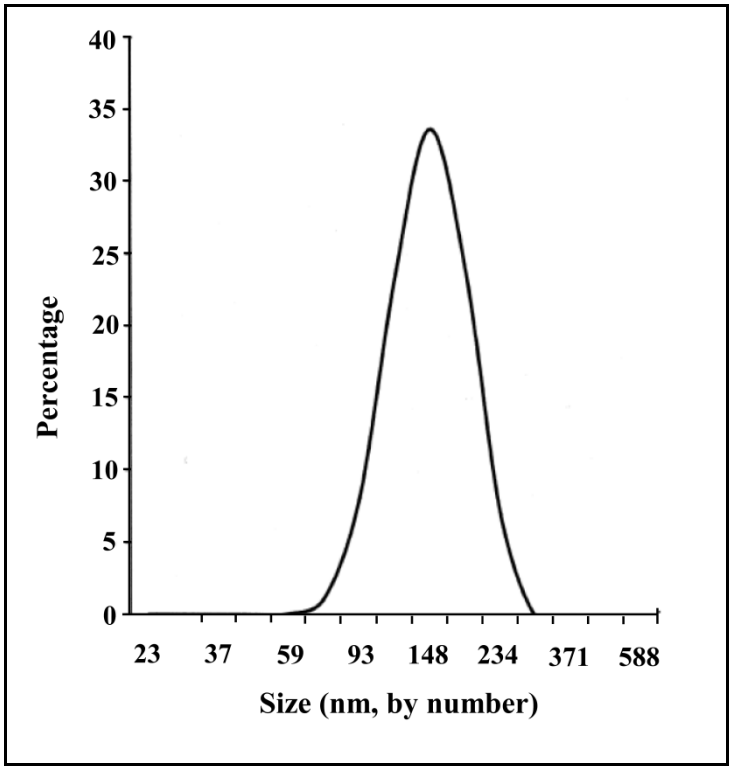

Figure 2.7 Size distributions of ethosome vesicles composed of 2% phosphatidylcholine of soybean, 30% ethanol and water as determined by DLS (Touitou *et al*., 2000)

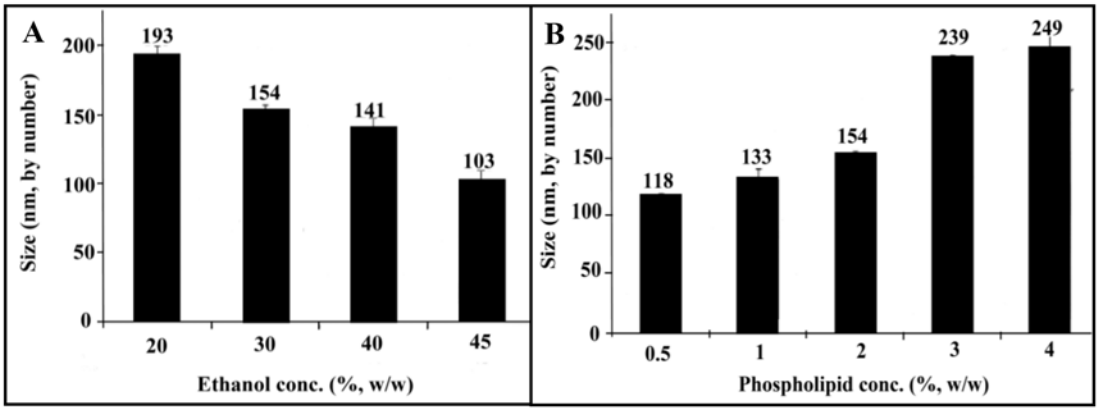

Figure 2.8 Size of ethosome vesicles composed of 2% phosphatidylcholine of soybean, 30% ethanol and water as determined by DLS. (A) Effect of ethanol concentration on the vesicles size. (B) Effect of phospholipids concentration on the vesicles size (Touitou *et al*., 2000)

2.2.4.3 Drug entrapment efficiency by ethosome vesicles

Ethosome has exhibited high entrapment efficiency for a wide range of molecules including hydrophobic, hydrophilic and amphiphilic molecules. This characteristic was evaluated in the study of Touitou *et al.* (2000) by incorporating three different fluorescence probes which included hydrophobic rhodamine red, amphiphilic D289 and hydrophilic calceine molecules. The probes entrapment efficiency was visualized by confocal laser scanning microscope (CLSM).

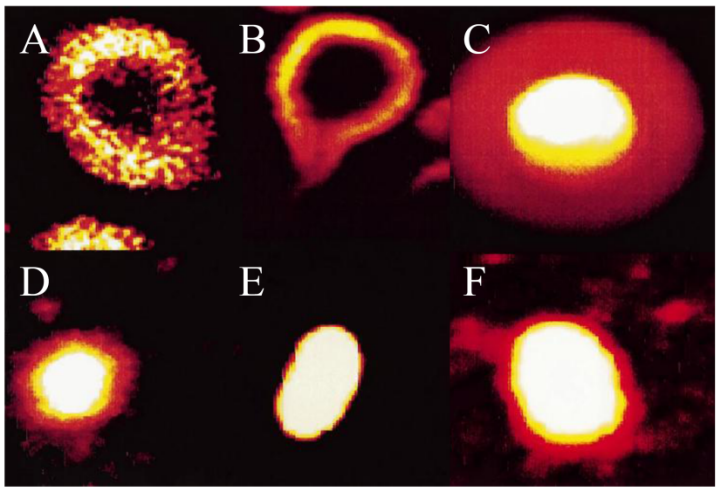

Figure 2.9 CSLM micrographs of liposomes (A-C) or ethosomes (D-F) entrap probes rhodamine red (A, D), D-289 (B, E) or calceine (C, F). White represents the highest concentration of probe, followed by yellow, with red being the lowest probe concentration (Touitou *et al*., 2000)

Their results showed that the ethosomes could entrap all probes filled up the entire volume of vesicles which high fluorescence intensity. Whereas in liposome, all probe were found only in the bilayer and did not enter the aqueous core.

The hydrophobic and amphiphilic probes were entrapped only in the bilayer and the hydrophilic probe presented primarily in the aqueous core of the vesicles as shown in Figure 2.9. In addition, ethosomal formulation possesses greater entrapment capability than liposomes system. Dayan and Touitou (2000) showed that entrapment efficiency of trihexyphenidyl hydrochloride increased from 36% for liposomes to 75% for ethosomes. This could be explained by multilamellarity of ethosome vesicles as well as by the presence of ethanol in ethosomes, which allows for better solubility of many drugs (Touitou *et al*., 2000; Elsayed *et al*., 2007).

2.2.4.4 Elasticity properties of ethosome vesicles

The elasticity of ethosomal vesicles membrane was reported in the study of Jain *et al*. (2007). The ethosomes prepared from 2% phospholipid, 15% ethanol and water showed the elasticity of 38.6±2.7. The liposomes prepared from 2% phospholipid, 0.15% cholesterol showed the elasticity of 4.96±0.8. It was found that the elasticity of ethosomal vesicles was 7 fold more than liposomes. This characteristic could be explained by higher concentration of ethanol present in ethosome that might provide elasticity to vesicle membrane by reducing the interfacial tension of the vesicle membrane. In addition, the incorporation of cholesterol in liposome formulation also significantly decreased the elasticity of vesicles membrane (Jain *et al*., 2007).

The fluidizing effect of ethanol on ethosomal vesicle membrane was already confirmed by differential scanning calorimetry and fluorescent anisotropy studies. SEM photomicrograph in Figure 2.10 illustrated the membrane surface after treatment with ethosome and liposome formulation. The ethosome could penetrate 50 nm pores of filter membrane (Figure 2.10A). However, after treatment with conventional liposomes, a large amount of liposome was retained on the filter surface (Figure 2.10B). These observations could be explained the difference in elasticity of these vesicles (Jain *et al*., 2007). Therefore, these results indicate that ethosome possess a flexible membrane and can penetrate pores much smaller than their diameter.

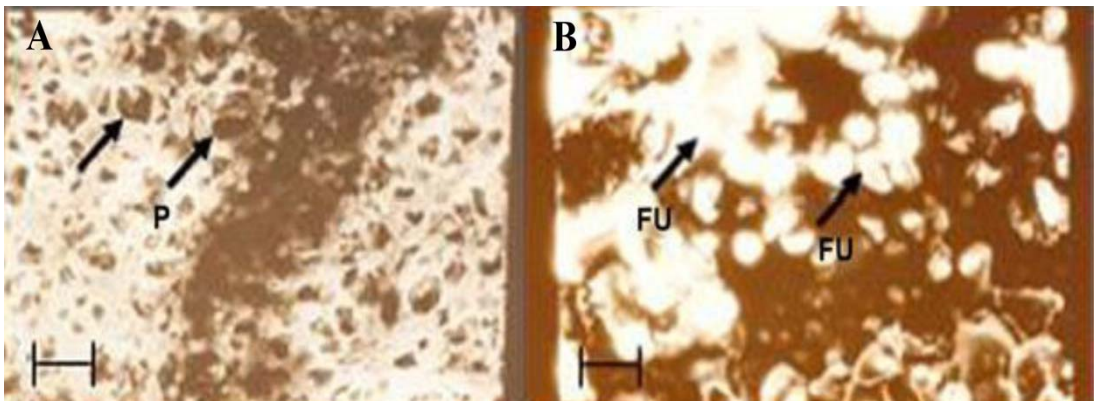

Figure 2.10 SEM photomicrograph (×400 magnifications) of membrane surface after treated with (A) ethosomal and (B) liposomal formulation. P, filter membrane pore; FU, fusion of vesicles on membrane surface. Scale bar = 100 nm (Jain *et al*., 2007)

2.2.4.5 Zeta potential of ethosome vesicles

Zeta potential is an important and useful indicator of particle surface charge, which could be used to predict the stability. In general, particles could be dispersed stable when the absolute value of zeta potential is above 30 mV. Moreover, the zeta potential below 20 mV is of limited stability and that below 5 mV show rapid aggregation.

However, several studies have reported that the zeta potentials of ethosome formulations ranged between -10 to -20 mV. The measured zeta potential was not sufficiently high to vesicle stability. So, that is the limited stability and should be improved in the future.

2.2.5 Stability of ethosome

The stability of ethosome was investigated in many studies. It was reported that the ethosomal formulation were stability after being stored over time. In the study of Jain *et al.* (2007), lamivudine loaded ethosome and liposome formulation were prepared and evaluated in term of vesicle size, zeta potential, and entrapment efficiency of the vesicles after 180 days. Their data indicate that there was no significant change in vesicle size, zeta potential, and the entrapment efficiency of ethosomal formulation. In addition, the ethosome possess reasonably good stability in comparison to liposome because of the presence of ethanol. Ethanol provides a net negative surface charge, which avoids aggregation of vesicles due to electrostatic repulsion (Dayan and Touitou, 2000; Lopez-Pinto *et al.*, 2005; Jain *et al*., 2007; Fang *et al*., 2008).

2.2.6 Skin permeation and deposition of ethosome

In vitro and *in vivo* skin permeation studies have demonstrated the ability of ethosomal formulation to enhance permeation of many molecules as compared to conventional liposomes. Ethosome was reported that the effectiveness for delivering molecules to and through the skin to the systemic circulation (Dayan and Touitou, 2000; Godin and Touitou, 2004; Lopez-Pinto *et al.*, 2005; Jain *et al*., 2007)

2.2.6.1 *In vitro* skin permeation and deposition of ethosome

Several researches have investigated the skin permeation and deposition of ethosome. The enhanced delivery of molecules from the ethosomal carrier was evaluated in permeation experiments with fluorescent probes. The amphiphilic fluorescent probe D-289 in trihexyphenidyl HCl loaded ethosomes, liposomes and hydroethanolic solution were used to study the skin permeation and deposition of nude mouse skin, after non-occlusive application (Dayan and Touitou, 2000). Confocal laser scanning micrographs (CLSM) showed the penetration of rhodamine red from ethosomes, liposomes and hydroalcoholic solution into nude mouse skin (Figure 2.11). The classic liposomes did not facilitate probe penetration into the skin. Rather, it resulted in only a small reservoir in upper layers of skin (Figure 2.11B). CLSM of hydroethanolic solution appeared to have a deep penetration, but with a relatively low fluorescence intensity (Figure 2.11C). In the case of ethosomal, it was found to be high both in terms of the deep of penetration and the fluorescence intensity (Figure 2.11A).

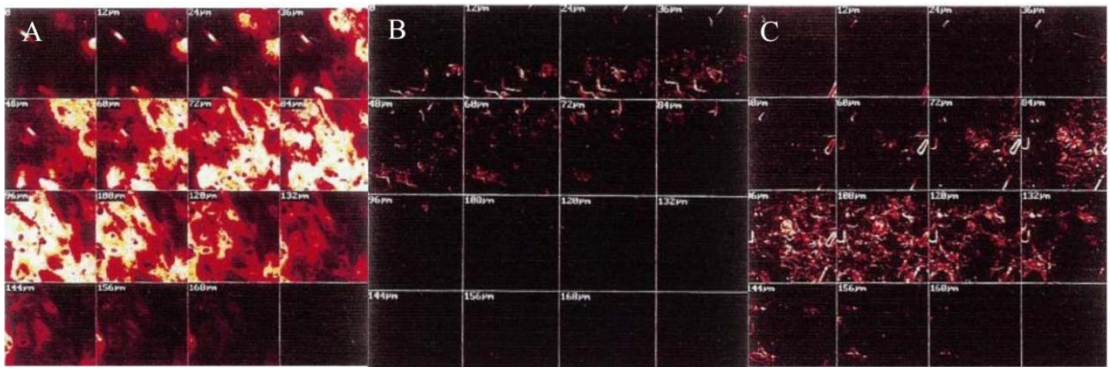

Figure 2.11 CLS photomicrographs presented the penetration of rhodamine red from ethosomes (A), liposomes (B) and hydroalcoholic solution (C) after applying nonocclusively into the back skin of 8-week old male nude mice (Dayan and Touitou, 2000)

Similarly, the study of Dubey *et al.* (2007) reported that the use of the lipophilic rhodamine red fluorescent (RR) loaded ethosomal system lead to an increase both in the depth of penetration and fluorescence intensity as compared with liposomes and hydroethanolic solution as shown in Figure 2.12.

A depth of RR penetration in the rat skin was 160, 80 and 40 mm from ethosome, hydroethanolic solution and liposomes, respectively. The maximum fluorescence intensity measured for RR delivered from ethosome, hydroethanolic solution and liposomes were 240, 160 and 80 μm, respectively. These results could confirm the effectiveness for increasing the permeability of many molecules by using the ethosome system (Dubey *et al*., 2007).

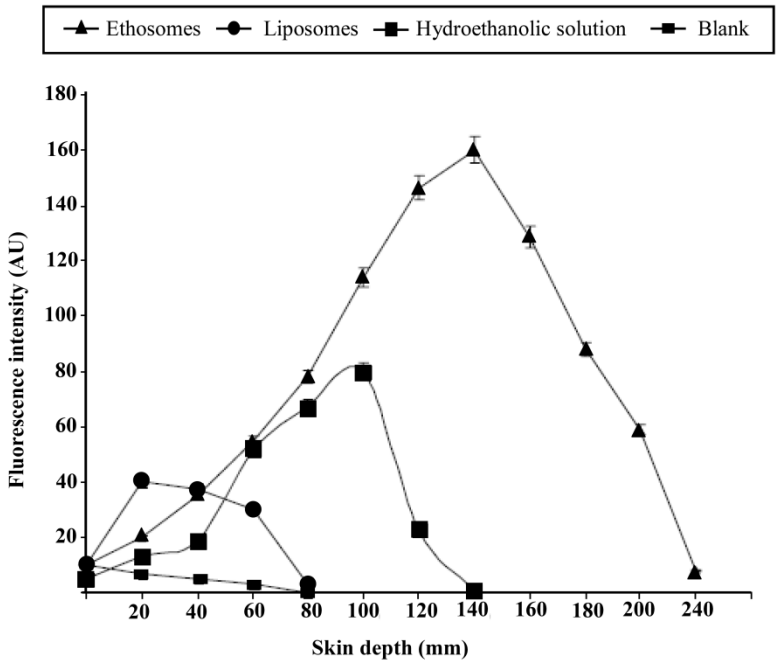

Figure 2.12 Fluorescence intensity (AU) vs. skin depth (mm) studies revealing comparative skin penetration profile of ethosomes, liposomes, hydroethanolic solution and blank. AU, arbitrary units. Values represent means±SD (n = 3) (Dubey *et al*., 2007)

2.2.6.2 *In vivo* skin permeation and deposition of ethosome

The skin permeation and skin deposition efficiency of ethosome were examined in many researches. The ability of ethosomal systems to deliver fluorescently labeled bacitracin (FITC-Bac) into the rat skin was investigated by *in vivo* technique. After 8 hours, the skin samples were cryo-sliced and visualized under CLSM to estimate the skin depth penetration of bacitracin from ethosome, liposome and hydroethanolic solution as shown in Figure 2.13.

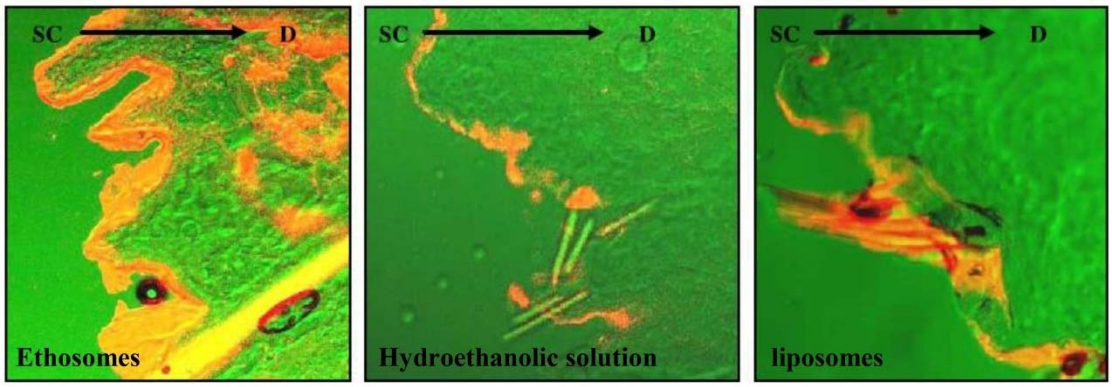

Figure 2.13 Skin cryo-slices following application of various systems containing 0.1% FITC-Bac for 8 hours *in vivo* in rat skin. Depths of FITC-Bac penetration as observed by CLSM (SC-stratum corneum; D-dermis) (Godin and Touitou, 2004)

They found that the application of bacitracin from ethosome was fairly evenly distributed throughout the stratum corneum, viable epidermis and dermis with high fluorescence intensity. However, the application of antibiotic from a hydroethanolic solution caused weak staining of upper skin layers. When FITC-Bac was administered by liposomes, the highest fluorescent intensity was associated with hairs and upper skin layers. *In vivo* skin delivery results demonstrated the ability of

ethosomal formulation to penetrate the skin and to enhance compound delivery to deep skin stata or systemically. It was also effective in terms of penetration depth and fluorescent intensity than other system (Godin and Touitou, 2004).

In addition, Ethosomes were reported to improve *in vitro* and *in vivo* skin delivery of various drugs as shown in Table 2.3 and 2.4, respectively. It was shown to improve skin delivery of drugs both under occlusive (Dayan and Touitou, 2000; Ainbinder and Touitou, 2005; Lopez-Pinto *et al*., 2005; Paolino *et al*., 2005) and non-occlusive conditions (Dayan and Touitou, 2000; Elsayed *et al*., 2007).

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

Drug	Tissue used	Enhancement ratio		Remarks
		Permeation[a]	Deposition	
Sotalol	Heat separated human epidermis	7.1[c]	ND	Franz diffusion cells
Sodium salicylate	Heat separated human epidermis	3.8[c]	ND	Franz diffusion cells
Propranolol	Heat separated human epidermis	1.4[c]	ND	Franz diffusion cells
Trihexyphenidyl HCl	Male nude mouse dorsal skin	51[b], 4.5[c], 87[e]	4.6b, 1.4c, 1.4e	Side-by-side diffusion cells
Minoxidil	Male nude mouse abdominal skin	45[c], 35[d], 10[f]	7c, 5d, 2f	Side-by-side diffusion cells
Minoxidil	Rat abdominal skin	1.2[e]	ND	Franz diffusion cells
Testosterone	Rabbit pinna skin	30	7	Franz diffusion cells
Testosterone	Dermatomed cadaver human skin	6.4	ND	Franz diffusion cells
Zidovudine	Rat skin	15.1[c], 10.9[d], 12.9[e], 7.7[f]	ND	Keshary-Chien diffusion cell
Ketotifen	Rabbit pinna skin	1.2[b], 1.4[c], 1.2[e]	3.3[b], 6.2[c], 1.7[e]	Franz diffusion cells

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

Drug	Tissue used	Enhancement ratio		Remarks
		Permeation[a]	Deposition	
Azelaic acid	Synthetic membranes	Release rate was higher from ethosomes than from liposomes. Ethosomes having the highest ethanol concentration released the drug more rapidly	–	Diffusion through synthetic membranes
Ammonium glycyrrhizinate	Human epidermis	Ethosomes improved cumulative drug permeated after 24 h and reduced lag time relative to aqueous solution, hydroethanolic solution, and mixture of empty ethosomes/hydroethanolic drug solution	ND	Franz diffusion cells

ND: Not determined.

[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.

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

Drug	Criteria under investigation	Subjects/species	Results
Acyclovir	Clinical efficacy in treatment of recurrent herpes labialis	Humans	Time to crust of lesions and time to loss of crust were shorter with the ethosomal acyclovir than with the commercial cream (Zovirax cream).
Testosterone	Pharmacokinetics	Rabbits	After application for 5 days (new patch applied daily), AUC was 125% greater with ethosomal patch than with commercially available patch.
Cannabidiol	Suppression of carrageenan-induced aseptic paw edema (anti-inflammatory action)	Male mice	Development of edema was entirely prevented 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 some reports *in vivo* studies investigating efficiency and applications of ethosomes as carriers for skin delivery of drugs (Elsayed *et al*., 2007) (continued)

Drug	Criteria under investigation	Subjects/species	Results
Erythromycin	In vivo antibacterial efficiency	*S. aurues* 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.
Testosterone	Pharmacokinetics	Male Sprague-Dawley rats	AUC was about 64% greater with ethosomes than with commercial gel.
Ammonium glycyrrhizinate	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.

2.2.7 Mechanism of drug penetration

Although the mechanism of skin penetration of ethosomes is unclear, a possible mechanism for this interaction has been proposed. Touitou *et al*. (2000) have purposed a hypothetical model of how ethosomes might enhance penetration of drugs through stratum corneum lipids as shown in Figure 2.14. They suggested that the great penetration efficiency of ethosome was generated of combination in processes lead to the enhance effect.

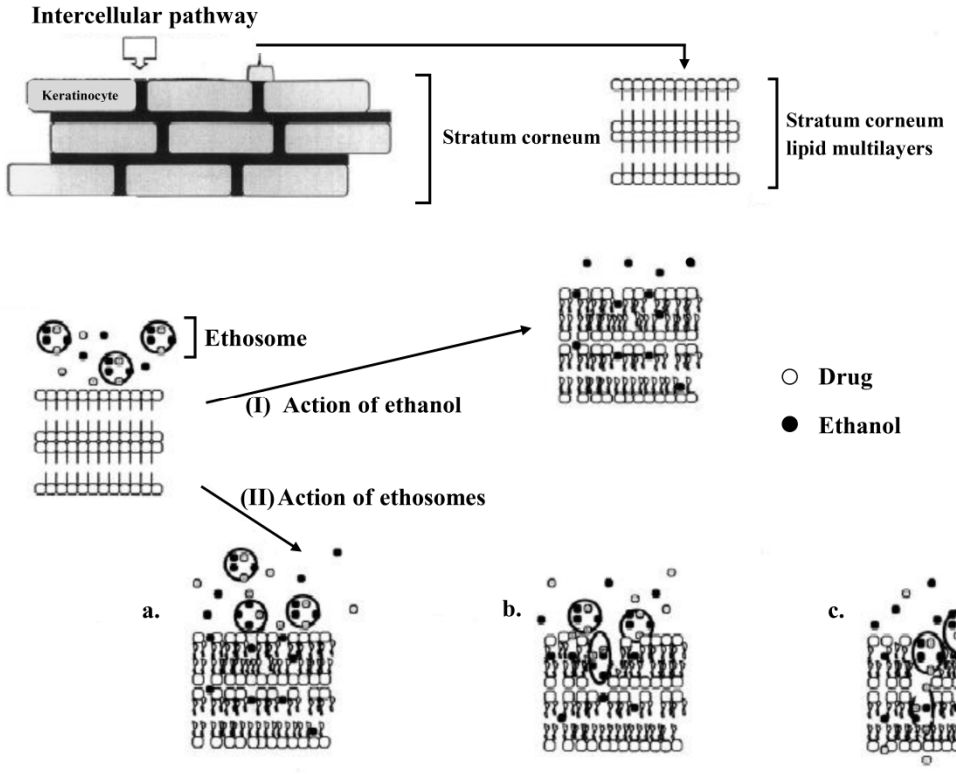

Figure 2.14 Proposed model for skin delivery from ethosomal systems (Touitou *et al*., 2000)

It is thought that the first part of the mechanism is due to the 'ethanol effect' whereby intercalation of the ethanol into intercellular lipids increasing lipid fluidity and decreasing the density of the lipid multilayer. This is followed by the 'ethosome effect', which includes inter lipid penetration and permeation by the opening of new pathways due to the malleability and fusion of ethosomes with skin lipids, resulting in the release of the drug in deep layers of the skin (Jain *et al*., 2011). The drug permeation probably occurs in following two phases:

Action of ethanol: The stratum corneum lipid multilayers, at physiological temperature, are densely packed and highly conformationally ordered. Ethanol interacts with lipid molecules in the polar head group region, resulting in a reduction in the T_m of the stratum corneum lipids, increasing their fluidity and decreasing the density of lipid multilayer of cell membrane. The intercalation of ethanol into the polar head group environment can result in an increase in the membrane permeability.

Action of ethosome: Ethanol might also provide the ethosome vesicles with soft flexible characteristics which could allow them to be more easily penetrate into deeper layers of the skin. The flexible ethosome vesicles could then penetrate the disturbed stratum corneum bilayers and even forge a pathway through the skin by virtue of their particulate nature.

The release of drug in the deep layers of the skin and its transdermal absorption could then be the result of fusion of ethosomes with skin lipids and drug release at various points along the penetration pathway (Dayan and Touitou, 2000; Jain *et al*., 2011).

2.2.8 Skin irritancy and tolerability of ethosome

Safety for topical and transdermal drug delivery systems is one of the important factors needed to be considered to be safe for using *in vivo* especially for the skin which is a part of the body contacting to drug formulation. The *in vivo* skin irritancy and tolerability is the parameter to evaluate before utilizing in human. Several studies have investigated the skin irritancy and tolerability after application of ethosome as a drug delivery system. The ethosome reported that it has the efficiency and safe both in topical and transdermal drug delivery.

The safety of ethosome was confirmed by examining the cell viability of a skin fibroblast cell line. The cell viability of fibroblasts following treatment with 5-aminolevulinic acid (ALA) loaded ethosome (0.13 mM Phosphatidylethanolamine (PE), 15% v/v ethanol) and a hydroalcoholic solution (15% v/v ethanol) was compared with the blank. The results showed that cell viability (%) of fibroblasts treated by ethosome was not different from that treated by the blank as shown in Figure 2.15. There are various types of skin damage which occurs of change in the skin structure. One type of the damage occurs in intercellular lipid areas that it might create gaps which increase the amount of drug. They suggested that the composition of PE might play a role in protecting skin. In additions, the skin damage could be repaired by phospholipids in the formulation (Fang *et al.*, 2009).

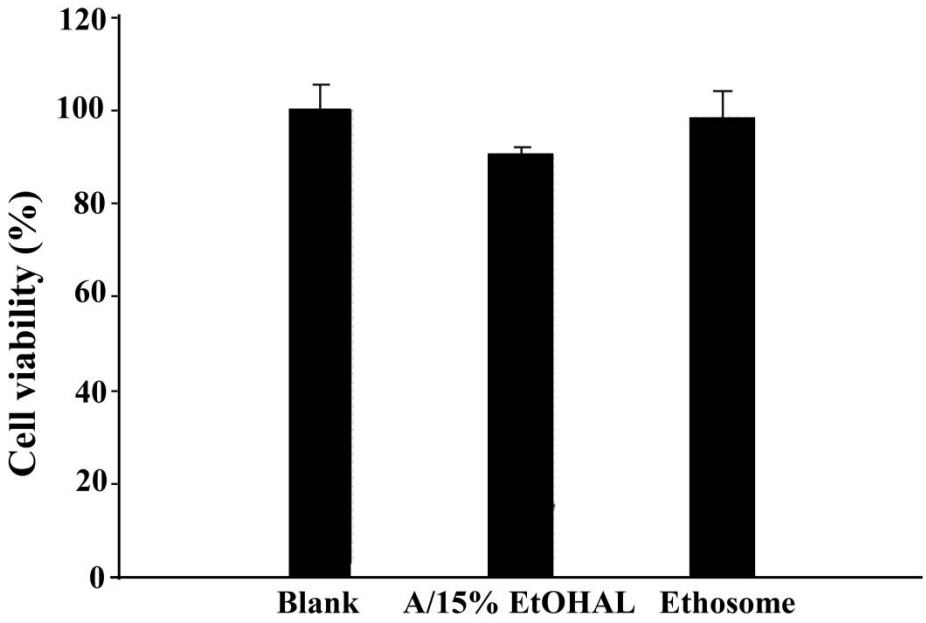

Figure 2.15 The cell viability of fibroblasts following treatment with a hydroalcoholic solution or ethosome with ALA. Data represent the mean ±SD (n = 6) (Fang *et al*., 2009)

The similar result was obtained on observed in the study of Shumilov *et al.* (2010). Histological examinations of the skin at the site of treatment showed that the application of Buspirone (BH) ethosomes for 24 hours did not induce changes in the skin structure and in the thickness of the horny layer. There was no infiltration of inflammatory cells to the skin. The results indicate that the BH ethosomal transdermal system is safe and does not irritate the skin.

2.2.9 Advantages of ethosome (Pratima and Shailee, 2012)

1. Enhanced permeation of drug molecules to and through the skin to the systemic circulation.
2. Contrary to deformation liposomes, ethosomes improve skin delivery of drugs both under occlusive and non-occlusive conditions.
3. Since composition and components of ethosomes are safe, they have various applications in pharmaceutical, veterinary and cosmetic field.
4. Better patient compliance.
5. Better solubility of many drugs as compared with conventional vesicles.
6. Relatively smaller size as compared with conventional vesicles.

2.2.10 Limitation of ethosome (Pratima and Shailee, 2012)

1. Poor yield.
2. less stability
3. In case if shell locking is ineffective then the ethosomes may coalescence and fall apart on transfer into water.
4. Loss of product during transfer form organic to water media.

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Drug

MPA is an immunosuppresive drug which was interested in the Molecular Pharmaceutical Research Center, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat Yai, Songkhla, Thailand. It was developed to enhance the skin penetration, lead to an effectiveness in psoriasis treatment. It was kindly supplied by the Molecular Pharmaceutical Research Center. It was prepared by hydrolysis of mycophenolate mofetil (MMF), which was obtained from the extraction of Cellcept®capsule (Mycophenolate mofetil 250 mg/capsule) and identified by using chromatographic and spectroscopic techniques (MS and H-NMR) compared to the authentic MPA (purity more than 99%).

3.1.2 Chemicals and reagents

1. Absolute ethanol (Merck, Darmstadt, Germany)
2. Acetonitrile (Labscan Asia Co., Ltd, Bangkok, Thailand)
3. Cholesterol from lanolin (Fluka, Buchs, Switzerland)
4. Deoxycholic acid (Fluka, Milan, Italy)

5. Disodium hydrogen orthophosphate anhydrous (Univar®, New South Wales, Australia)
6. Ethanol 95% (Merck, Darmstadt, Germany)
7. Methanol (Labscan Asia Co.,Ltd, Bangkok, Thailand)
8. Methanol (Labscan Asia Co.,Ltd, Bangkok, Thailand)
9. Octylphenoxypolyethoxyethanol, Triton®X-100 (J.T. Baker Chemical Co., New Jersey, United States)
10. Orthophosphoric acid (Merck, Darmstadt, Germany)
11. L-α-Phosphatidylcholine from soybean , Type IV-S, ≥ 30% (TLC) (Sigma-Aldrich, St Louis, MO, United States)
12. Phosphoric acid (Merck, Darmstadt, Germany)
13. Polyethylene glycol (Drug Center Co., Ltd., Bangkok, Thailand)
14. Polyoxyethylene (80) sorbitan monooleate, Tween 80® (Srichand United Dispensary Co., Ltd, Bangkok, Thailand)
15. Potassium dihydrogen orthophosphate (VWR International Ltd., Poole, UK)
16. Sodium chloride (Carlo Erba, Milan, Italy)
17. Sodium dihydrogen orthophosphate (Univar®, New South Wales, Australia)
18. Sodium hydroxide (Merck, Darmstadt, Germany)
19. Stearylamine (Sigma-Aldrich Chemie GmbH, Steinheim, Germany)
20. Triethylamine (Fluka, Buchs, Switzerland)

3.2 Instrumentations

1. Centrifuge (Z323K, Hermle Labortechnik GmbH, Wehingen, Germany)
2. Electrical balance (AB 135-S, Mettler-Toledo Ltd., Bangkok, Thailand)
3. High performance liquid chromatography (Thermo Electron Corporation, SpectraSystem P1000 pump, SpectraSystem UV1000 detector, ChomQuest software program SN 4000, Massachusetts, United States)
4. High performance liquid chromatography (Agilent Technologies Deutschland GmbH, Agilent 1100 series pumping system, Waldbronn, Germany)
5. Homogenizer (Polytron® system PT 1200 E, Kinematica AG, Littau-Luzern, Switzerland)
6. Hot air oven (DIN 12880-KI, Memmert GmbH, Schwabach, Germany)
7. Magnetic stirrer (MR 3000D, Heidolph, Schwabach, Germany)
8. Modified Franz diffusion apparatus (57-6M, Hanson Research Corporation, California, United States)
9. Optical microscope (Olympus CK2, International Co., Ltd., Tokyo, Japan)
10. pH meter (Mettler-Toledo, Greifensee, Switzerland)
11. Rotary evaporator (Eyela® N-1000, Tokyo Rikakikai Co., Ltd., Tokyo, Japan)
12. Scanning electron microscope (Quanta 400, FEI, Brno, Czech Republic)

13. Sonicator (HT Crest, S.V. Medico Co., Ltd., New York, United States)
14. Ultracentrifuge (Optima^{TM} L-100XP, equipped with SW60 Ti Rotor, Beckman Coulter, California, United States)
15. Zeta Potential Analyzer (ZetaPALS, Brookhaven Instruments Corporation, New York, United States)

3.3 Methods

3.3.1 Solubility studies of MPA (Limsuwan and Amnuaikit, 2012)

The effects of pH on MPA solubility were evaluated to find out the suitable dispersion medium for the preparations of ethosome containing MPA. The study was conducted by adding an excess amount of MPA (0.1 g) into glass vials containing 10 ml of hydrochloric acid buffer pH 1.5 and 2.0, acetate buffer pH 4.5 and 5.5, and phosphate buffer pH 6.5, 7.0, 7.4 and 8.0 and prepared according to USP 30. Samples were sonicated for 30 minutes and observed at room temperature (37±2ºC) for 48 and 72 hours to ensure that the solubility of MPA reached equilibrium. Saturated solutions were subsequently filtered through a 0.45 µm syringe filter membrane. They were diluted with appropriate volume of mobile phase before determination with validated method.

3.3.2 Formulation and preparation of ethosome containing MPA (Limsuwan and Amnuaikit, 2012)

The formulation and preparation of ethosome containing MPA were prepared according to the thin-film hydration method. This method is convenient and does not require sophisticated equipment. It also takes less time to prepare and is easy to scale up. The main composition of ethosome included of MPA 10 mg/ml, phospholipids and hydroethanolic solution which is a mixture of phosphate buffer pH 7.4 with ethanol. All the components of the main composition were varied in terms of concentration and ratio as follows;

Phospholipids:

- 2%, 4% and 6% w/v of L-α-Phosphatidylcholine from soybean (SPC)

Hydroethanolic solution:

- Phosphate buffer pH 7.4 which was selected by the pH-solubility profile in section 3.3.1.
- 10%, 20%, 30%, 40% and 50% v/v ethanol

The main composition of ethosome was prepared and evaluated in terms of physical appearances, particle size, size distribution, zeta potential and entrapment efficiency to obtain the optimized composition.

The optimized composition was developed by adding various additives to improve the properties of ethosome. All the components of ethosome were varied in terms of types, concentration and ratios as follows;

Additives:

Cholesterol (CHOL):	0.1-1.5 % w/v
Polyoxyethylene sorbitan monooleate (Tween 80):	0.1-1 % w/v
Polyethylene glycol (PEG):	0.1-1 % w/v
Surface charge agent:	0.1-1 % w/v
Cationic: Stearylamine (SA)	
Anionic: Deoxycholic acid (DA)	

These formulations were prepared and evaluated in terms of physical appearances, particle size, size distribution, zeta potential and entrapment efficiency to obtain the optimized ethosomal formulation. The optimized MPA ethosome was selected from the particle size less than 500 nm, a narrow size distribution, Zeta potential more than 30 mV and entrapment efficiency more than 50% to assess the stability and skin permeation studies.

The preparation process of ethosome was described follows; firstly, MPA, SPC and other additives were dissolved in absolute ethanol and sonicate at 60˚C for 30 minutes until all components were dissolved. Then, these components were added in a 500-ml round-bottomed flask followed by evaporation at 60˚C by using rotary evaporator to form the thin lipid film on the flask wall. The flask was continued on rotary evaporator for 20 minutes to ensure that the absolute ethanol was completely evaporated. Secondly, the thin lipid film was hydrated with hydroethanolic solution followed by shaking for 5 minutes. The flask was tightly sealed to prevent the evaporation of the ethanol in the formulation. Finally, these

formulations were sonicated at 60˚C for 30 minutes to achieve complete ethosome formulations. All formulations were prepared three times.

3.3.3 Characterization of ethosome containing MPA (Limsuwan and Amnuaikit, 2012)

3.3.3.1 Physical appearances

The physical appearances of all prepared ethosome were visually observed in terms of color, phase separation and precipitation. In addition, the optical microscope was utilized to examine the precipitation of drug or other component in the formulation.

3.3.3.2 Particle size, particle size distribution and zeta potential analyzer

The particle size, size distribution and zeta potential of ethosome formulation were determined by Zeta Potential Analyzer (Model ZetaPALS, New York, United States) at 25˚C with a scattering angle (θ) of 90 degrees after diluting of 10 μl ethosome with 4 ml Milli-Q water. All determinations were performed in triplicate.

3.3.3.3 Determination of MPA content and entrapment efficiency

The MPA entrapment efficiency of ethosome formulation was measured by the ultracentrifugation method. This formulation was centrifuged in a Ultracentrifuge (Optima^{TM}L-100XP, Beckman, USA) equipped with SW60 Ti Rotor

at 60,000 rpm, 4°C for 2 hours. The supernatant was collected for determination of free or non-entrapped drug. For the total drug or drug content, the vesicles were also lysed with 30% v/v Triton®X-100 and diluted with mobile phase to determine the total drug amount. The MPA entrapment efficiency was calculated from the following equation (Garg *et al*., 2010; Madsen *et al*., 2010):

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

Where; T : the total drug amount in the formulation

F : the non-entrapped drug amount in the formulation

3.3.3.4 Visualization of ethosome using scanning electron microscopy

Vesicles morphology were visualized by using Scanning Electron Microscope (SEM) (Quanta 400, FEI, Czech Republic) at an accelerating voltage of 20 kV. Prior to visualize, 100 µl of ethosome was diluted with 3 ml MiliQ water. The diluted ethosome was dropped on the cover slip after cleaning. This sample was dried and stained with crystal violet solution for 1 minute. A drop of Gram's iodine solution was added on to the dry sample and the excess of the solution was drained off with a filter paper. This staining method was based on Gram's Method where the positive charge of crystal violet interacted with negative charge of the phospholipids in ethosome and later formed complex with I^- and I_3^-. The sample was coated with gold in a sputter coater under an argon atmosphere (50 Pa) at 50 mA for 70 seconds and viewed under SEM at 30,000X magnification.

3.3.4 Stability study of ethosome containing MPA

The optimized ethosome formulation was stored in sealed vials at 4±1^{o}C and at room temperature for 1, 30, 60 and 90 days. The stability of these formulations at different temperatures for different period's time were assessed to study in terms of the physical appearance, particle size and particle size distribution, zeta potential analyzer, total MPA content and entrapment efficiency as described in section 3.3.3. All evaluations were performed in triplicate. In addition, the vesicle morphology of the selected ethosome storage at room temperature for 90 days was visualized under SEM at 30,000X magnification.

3.3.5 *In vitro* skin permeation study (Limsuwan *et al*., 2012)

3.3.5.1 Preparation of skin model

In vitro skin permeation studies, full-thickness flank skins of newborn pigs which were obtained from a local pig farm in Songkhla Province where is regulated by the Department of Livestock Development, Thailand was used as an animal model. The newborn pigs weighed 1.4 to 1.8 kg, naturally died after birth and were cleaned on the skin surface. The epidermal hair at the flank area was clipped with an electric hair clipper without any damages on the skin surface and excised with a scalpel. The subcutaneous fat and underlying tissues were carefully removed from the dermal surface by using a surgical scissors (Songkro *et al*., 2003). The obtained membrane was cleaned with isotonic phosphate buffer pH 7.4 (PBS), allowed to dry and wrapped with aluminum foil and stored at -20˚C until used in the permeation

studies. Before beginning the experiments, the stored membrane was hydrated by placing in PBS at room temperature overnight.

3.3.5.2 *In vitro* skin permeation experiments

The *in vitro* skin permeation of the optimized ethosome formulation and control formulations were measured through newborn pig skin using a modified Franz-diffusion cell (Model Hanson 57-6M, California, United States). The optimized ethosome system was compared with the control systems to evaluate skin permeation property. The control systems used were as follows: (a) MPA liposomes; (b) 30% hydroethanolic solution of MPA; (c) aqueous suspension of MPA; and (d) MPA liposome that was developed by Rattanat (2008). The effective diffusion area of the diffusion cell was 1.77 cm^2. The receptor compartment was filled with 11 ml of PBS. The 1 ml of each sample was applied on the skin surface in the donor compartment. The newborn pig skin was placed between the donor and the receptor compartment with the stratum corneum side facing upward into the donor compartment. The modified Franz- diffusion cells were maintained at 37˚C with stirring at 500 rpm throughout the experiment. The 1 ml sample of receiver medium was withdrawn through the sampling port of the diffusion cell at 0.5, 1, 2, 4, 6, 8, 12 and 24-hours time intervals. The receptor medium was immediately replenished with equal volume of fresh PBS to maintain the sink conditions throughout the experiment. The receptor medium was immediately replenished with equal volume of fresh medium to maintain the sink conditions throughout the experiment.

The experiments were repeated for three times, and all samples were analyzed in triplicate.

3.3.5.3 Experiments to determine the amount of MPA remaining in the donor compartments

At the end of the study (after 24 hours), the formulation was collected. The skin surface was cleaned with cotton ball soaked in 10 ml methanol for ten times. Then, it was sonicated at room temperature for 30 minutes. After that, the samples were filtered through the filter paper, lysed with 30% v/v Triton®X-100 and analyzed to determine the amount of residual MPA in donor compartment after the end of the skin permeation studies by HPLC. The experiments were repeated for three times, and all samples were analyzed in triplicate.

3.3.5.4 *In vitro* skin retention experiments

At the end of the *in vitro* experiments (after 24 hours), the formulation was collected from skin surfaces according to the processes described in section 3.3.5.3. Then, the experimented skin was removed from the diffusion cells and cut into small pieces with a pair of surgical scissors. This membrane was homogenized in 5 ml of methanol to extract the skin at 24,000 rpm, and room temperature for 5 minutes. Subsequently, the sample was sonicated at room temperature for 30 minutes and centrifuged by using a Hermle Z323K Centrifuge (Wehingen, Germany) at 6,000 rpm, 4˚C for 30 minutes to separate the skin lipid. The clear supernatant was collected

and filtered through at 0.45 μm nylon membrane to determine the amount of MPA retained in the skin by using HPLC at 254 nm. The experiments were repeated for three times, and all samples were analyzed in triplicate.

3.3.5.5 Data analysis

For permeation data analysis, the cumulative amount of MPA permeated per unit area of skin (Q_t, μg/cm^2) was calculated from the following equation (Thakker and Chwern, 2003);

$$Q_t = \frac{C_n V + \sum_{i=1}^{n-1} C_i S}{A} \quad (6)$$

Where; Q_t : cumulative amount of MPA permeated per unit area of skin (μg/cm^2)

C_n : concentration of MPA determined at No.n sampling interval (μg/ml)

C_i : concentration of MPA determined at No.i sampling interval (μg/ml)

V : volume of individual Franz diffusion cell (ml)

S : volume of sampling aliquot, 1 ml

A : effective diffusion surface area, 1.77 cm^2

The cumulative amount of MPA permeated per unit area of skin was then plotted as a function of time and the steady state flux (J_{ss}, μg/cm^2/h) of the MPA through the pig skin was calculated from the slope of linear portion of the permeation profile. The lag time (T_{lag}, h) for MPA to permeate through pig skin before reaching the receptor fluid was calculated from the X-intercept of the plot. In addition, the

permeability coefficient (K_p, cm/h) of the MPA through pig skin was calculated by dividing steady state flux with initial concentration of MPA in donor compartment using the following equation (Errabelli *et al*., 2011);

$$K_p \text{ (cm/h)} = \frac{J_{ss}}{C_0} \quad (7)$$

Where; C_0 : the initial concentration of MPA in the donor compartment

3.3.6 Statistical analysis

For statistical analysis, all the experimental data were presented as mean±standard deviation (SD). All results were statistically evaluated using one-way analysis of variance followed by post hoc analysis for significant at $p<0.05$.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 pH-Solubility Profile (Limsuwan and Amnuaikit, 2012)

MPA is very slightly soluble in water (13 μg/ml at 25°C) because it is a lipophilic drug (Lee *et al*., 1990). However, the solubility properties influenced on formulation and preparation of ethosome containing MPA. We need to find an appropriate dispersion medium that is able to prevent the drug precipitation in the formulation. In this case, the effects of pH on MPA solubility were evaluated. Figure 4.1 shows its pH-solubility profile. Since MPA is known as a weak organic acid with pKa value of 4.5. Accordingly, the solubility of MPA in the alkaline range was higher than in the acidic range and the solubility increased as the pH increased. At pH 7.0, 7.4 and 8.0, the solubility of MPA increased to 4.1, 5.7 and 7.3 mg/ml, respectively. However, MPA was not stable in buffer solutions pH 8 because it was hydrolyzed by base-catalyzed (Connors *et al.*, 1986). Therefore, the buffer solution pH 7.4 was selected for using to prepare ethosome formulation. Moreover, this result showed that there were no significant differences in the solubility of MPA in various pH between 48 and 72 hours which demonstrated that the solubility of MPA reached equilibrium.

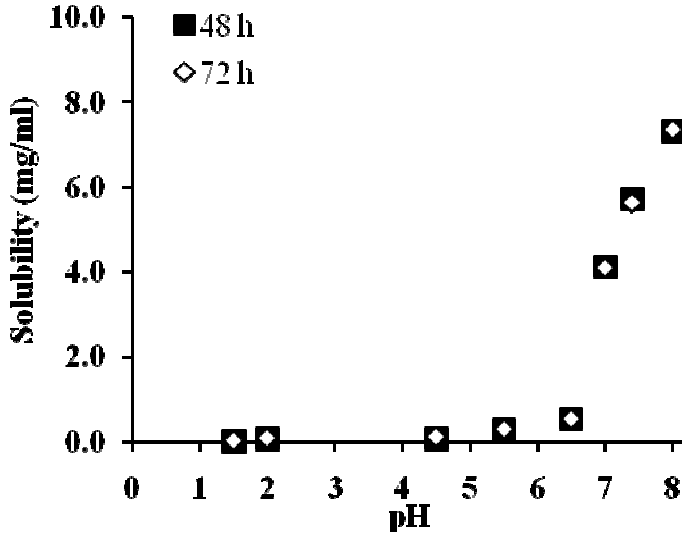

Figure 4.1 pH-solubility profile of MPA (at 37±2 ºC). The plotted data are mean ± SD (n=5) **(**Limsuwan and Amnuaikit, 2012)

4.2 Formulation and preparation of ethosome containing MPA (Limsuwan and Amnuaikit, 2012)

The effect of ethanol and phospholipids at different concentrations on the ethosome formulations were evaluated in terms of the physical appearances. Figure 4.2 shows the physical appearances of the ethosome formulations prepared from 2, 4 and 6% w/v SPC by using 10-50% v/v ethanol in phosphate buffer pH 7.4 as dispersion media. It was found that the formulations containing 10-30% v/v ethanol and 2-6% w/v SPC (F.1-9) had a yellowish colloidal appearance with no precipitation of free drug. This phenomenon may be due to a synergistic effect between buffer and ethanol as a co-solvent system with lipid vesicles of phospholipids, which can increase the solubility of MPA in the formulation (Ran *et al*., 2001; Dong *et al.*, 2008; Seedher and Kanojia, 2009).

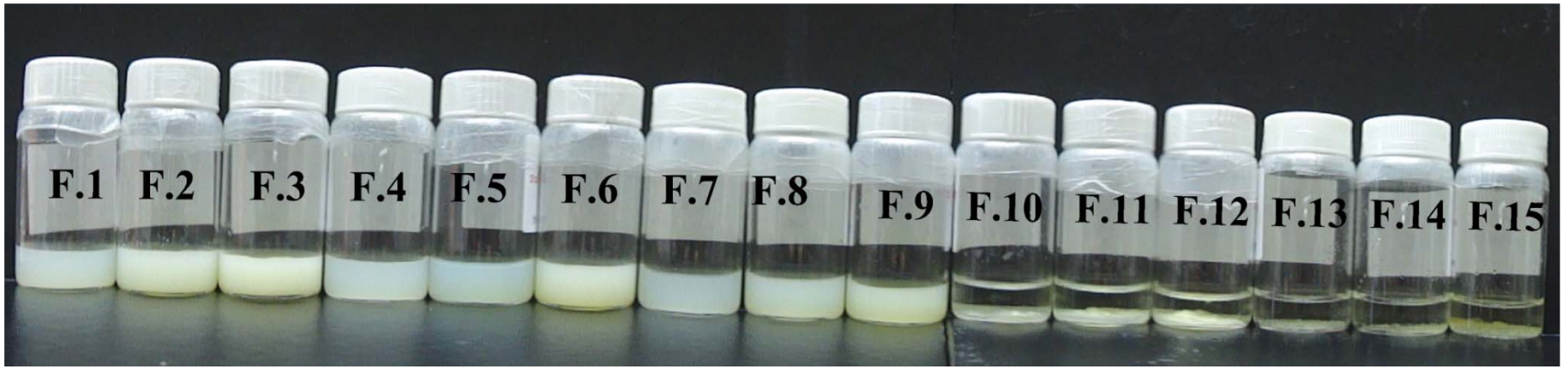

Figure 4.2 Physical appearances of the ethosome prepared from 2, 4 and 6% w/v SPC by using 10-50% v/v ethanol in phosphate buffer pH 7.4 as dispersion media; F.1,2,3 was 2, 4 and 6% w/v SPC by using 10% v/v ethanol; F.4,5,6 was 2, 4 and 6% w/v SPC by using 20% v/v ethanol; F.7,8,9 was 2, 4 and 6% w/v SPC by using 30% v/v ethanol; F.10,11,12 was 2, 4 and 6% w/v SPC by using 40% v/v ethanol; F. 13,14,15 was 2, 4 and 6% w/v SPC by using 50% v/v ethanol, respectively **(**Limsuwan and Amnuaikit, 2012)

However, when increasing concentrations of ethanol up to 40 and 50% v/v in phosphate buffer pH 7.4, it shown that the formulation has precipitation of the drug and/or other components in the formulation (F. 10-15). This could be explained by the phase separation of ethosome formulation which results from the effects of high ethanol concentration on disruption of lipid vesicle. When ethanol concentration increased probably made the vesicle membrane more leak. Lead to decreasing in drug entrapment efficiency, precipitation of the drug and/or other components in the formulation (Touitou *et al.,* 2000; Jain *et al.*, 2007). Our previous study showed that the formulation containing 10-30% v/v ethanol in phosphate buffer pH 7.4 as dispersion media was provided the stable physical characteristics. Therefore, these formulations were selected for further experiments.

Table 4.1 shows the properties of the MPA ethosome and liposome formulations by using 2-6% w/v SPC as lipid vesicle structure and hydroethanolic solution which is a mixture of phosphate buffer pH 7.4 with 10-30% v/v ethanol. Formulation Etho-1, -5 and -9 without ethanol content as a liposome showed a high entrapment efficiency. However, the aggregation and precipitation of liposome were appeared within one week (37±2ºC). This phenomenon reflected the instability of the liposome, which contained only SPC as the main lipid vesicles without other additives. The aggregation of lipid vesicle may occur due to electrostatic and hydrogen bonding interaction between choline head group of SPC in the formulation (Mura *et al*., 2007). In addition, ethanol might provide a surface negative net charge leading to avoidance and delayed the vesicle aggregation, due to the electrostatic repulsions (Bendas and Tadros, 2007). Therefore, the formulations without ethanol

showed greater aggregation than those of ethanol contained formulation (Lopez-Pinto *et al*., 2005; Dubey *et al*., 2007).

The effect of ethanol concentration on the vesicle size and entrapment efficiency was investigated in many studies (Dayan and Touitou, 2000; Touito *et al*., 2000; Lopez-Pinto *et al*., 2005; Paolino *et al*., 2005; Dubey *et al*., 2007; Jain *et al.*, 2007; Fang *et al*., 2008). Increasing ethanol concentration in the range of 20–45%, and the vesicle size were reported to decrease while the entrapment efficiency was increased (Touitou *et al*., 2000; Paolino *et al*., 2005; Dubey *et al*., 2007; Jain *et al.*, 2007). In our experiment, it was found that the ethosome vesicles decreased with increasing ethanol concentration in the range of 20–30%. The low polydispersity index of MPA loaded ethosomal formulation indicates that the ethosomal formulation has narrow size distributions (Table 4.1). These results are consistent with the findings of other researchers who conclude that the decrease in vesicles size is due to the presence of ethanol in the vesicles (Touitou *et al*., 2000; Paolino *et al*., 2005; Dubey *et al*., 2007; Ehab and Mina, 2007; Jain *et al.*, 2007). Ethanol confers a surface negative net charge in the system which causes the size of vesicles to decrease (Touitou *et al*., 2000; Lopez-Pinto *et al.,* 2005; Bendas and Tadros, 2007; Jain *et al*., 2007). Moreover, the addition of ethanol results in a decrease in the membrane thickness of the vesicles which is probably due to the fact that ethanol might form a phase with interpenetrating hydrocarbon chain (Barry *et al*., 1994; Bendas and Tadros, 2007). The entrapment efficiency of ethosomal vesicles was reported to increase with increasing ethanol concentration in the range of 20–45%. This finding could be explained by the presence of ethanol in vesicles membrane. It increases the solubility of the drug in both core and lamellar ethosome due to the fact that the

ethanol distributed throughout the vesicles. Further increasing in the ethanol concentration (>45% v/v) probably made the vesicle membrane leakier, thus leading a decrease in the entrapment efficiency (Touitou *et al*., 2000). However, our study demonstrated that the entrapment efficiency of ethosome was decreased with the increasing of ethanol concentration. Our findings are consistent with the results obtained by Kumar *et al.* (2010). They pointed out that the decreasing of the entrapment efficiency due to the increasing of fluidity and presence of the thinner membrane with higher ethanol concentration in the formulation. Since MPA is a lipophilic drug, it is more likely to exist in the phospholipid membrane than the aqueous core of the vesicles. When the thickness of the membrane in ethosome was decreased, it resulted in lowering drug entrapment efficiency. In addition, the effect of phospholipid concentration on the vesicle size and entrapment efficiency was investigated in several studies (Touitou *et al*., 2000; Paolino *et al*., 2005). Our results showed that increasing the phospholipid concentration, both size and entrapment efficiency of ethosome were increased. These could be clarified by the increase of phospholipid molecules in bilayers of the vesicles where MPA resided. The zeta potential is an important indicator of the surface charge of particles, which can be used to predict stability of formulation. Liu and Hu (2007) reported that the zeta potential exceeding 60 mV indicated good stability, 40-60 mV considerable stability, 30-40 mV moderate stability, and 10-30 mV instability of liposomes. The measured zeta potential of MPA-loaded ethosome formulations ranged from -47.61±2.78 mV to -82.53±4.54 mV which indicated high stability of ethosome. However, the absolute value of zeta potential in liposome is below 30 mV, and represents as instability of liposome.

Table 4.1 The main compositions and physical characteristics of ethosome and liposome formulations **(**Limsuwan and Amnuaikit, 2012)

No.	Composition		Vesicle size	Polydispersity	Zeta potential	Entrapment efficiency
	SPC(%w/v)	Ethanol(%v/v)	(nm)	Index	(mV)	(%)
Etho-1	2	0	415.14 ± 7.85	0.269 ± 0.02	-28.54 ± 2.75	57.59 ± 0.60
Etho-2	2	10	319.63 ± 8.20	0.320 ± 0.02	-47.61 ± 2.78	23.42 ± 0.22
Etho-3	2	20	331.68 ± 11.63	0.357 ± 0.01	-66.30 ± 8.41	22.82 ± 1.39
Etho-4	2	30	158.05 ± 3.11	0.290 ± 0.01	-63.15 ± 4.09	12.96 ± 0.25
Etho-5	4	0	440.28 ± 8.06	0.338 ± 0.01	-28.15 ± 6.15	78.22 ± 0.39
Etho-6	4	10	349.13 ± 6.43	0.346 ± 0.02	-66.75 ± 8.61	40.91 ± 0.22
Etho-7	4	20	621.83 ± 3.78	0.360 ± 0.06	-71.43 ± 2.90	23.60 ± 1.71
Etho-8	**4**	**30**	**153.60 ± 1.93**	**0.291 ± 0.01**	**-79.65 ± 6.67**	**16.52 ± 0.08**
Etho-9	6	0	452.53 ± 4.06	0.337 ± 0.02	-24.50 ± 7.43	82.46 ± 0.20
Etho-10	6	10	361.20 ± 4.23	0.332 ± 0.01	-75.10 ± 3.65	41.10 ± 0.92
Etho-11	6	20	464.20 ± 6.81	0.343 ± 0.02	-82.53 ± 4.54	25.02 ± 0.24
Etho-12	6	30	261.15 ± 3.80	0.295 ± 0.02	-82.20 ± 8.37	17.27 ± 0.66

*All values were mean ± S.D. (n = 3)

It is well known that particle sizes and ethanol concentrations influence the permeability of drug into/through the skin. The small particle size can penetrate through the skin over the larger particle size and the permeation of drug increased with the increase of ethanol volume of concentration (Verma *et al*., 2003; Dragicevic-Curic *et al*., 2009).

The main component of ethosome formulation will focus on the MPA ethosome that has the smallest particle size and appropriate ethanol. Thereby, the formulation Etho-8 containing 4% w/v SPC and 30% v/v ethanol with the smallest particle size (153.60±1.93 nm) was selected as the main component of MPA ethosome for further development. However, the drug entrapment efficiency of the formulation Etho-8 has relatively low (16.52±0.08 nm). Therefore, the main composition of MPA ethosome was developed to improve its entrapment efficiency by adding an addition, such as CHOL, Tween 80, PEG and surface charge agent, such as SA and DA and it was evaluated to select the optimized MPA ethosome as shown in Table 4.2.

Ethosome properties were probably improved due to the addition of various additives. Cholesterol (CHOL) is a stabilizing agent which prevents leakage and reduces the membrane permeability of the vesicles (Bendas and Tadros, 2007), leading to an increase in both size and efficient entrapment **(**Xia and Xu, 2005; Fang *et al*., 2006; Yang *et al.*, 2007). Its molecule was inserted into lipid bilayer whereas the hydroxyl group was connected to the aqueous phase. The aliphatic chain paralleled to the acyl chains of SPC that resulted to the rigidity in the lipid vesicles increased from intravesicle interaction (Lopez-Pinto *et al*., 2005). The addition of CHOL in formulations no.Etho-13, -14, -15 and -16 resulted in an increase of drug

entrapment efficiency compared to non-additive ethosome (Etho-8). Similarly, Tween 80 could enhance both the entrapment efficiency and vesicle size from its solubilizing effect (Yang *et al*., 2007). The addition of polyoxyethylene sorbitan monooleate (Tween 80) in the formulation no.Etho-8 leads to an increase in the entrapment efficiency and particle size as shown in formulation no.Etho-17 and -28. Moreover, it could enhance stability of vesicles due to its hydrocarbon tail ability to penetrate into the lipid bilayer, leave the polyethylene oxide groups on the surface of the vesicle thereby introduced a steric barrier on the surface. It decreased in fusion of the vesicles and the exchange between lipid and drug when the vesicles particles collide (Xia and Xu, 2005; Yang *et al*., 2007). The effects of positive and negative charge agent, stearylamine (SA) and deoxycholic acid (DA), on ethosome characteristics were evaluated. The formulations with added SA greatly increased the particle size but the entrapment efficiency decreased (Etho-18). However, the aggregation of lipid components was observed within one week. This may be due to the incompatibility between the positive charge of SA and the negative charges of SPC and MPA.

In contrast, the negative charge agent of DA could increase the entrapment efficiency of ethosome (Etho-19 and -20) when compared to formulations no.Etho-16 and -13, respectively, because of high interbilayer distance by electrostatic repulsive forces (Pinsuwan *et al*., 2010). The increase of negative surface charge of ethosome can also help to prevent vesicular aggregation (Fang *et al*., 2006; Pinsuwan *et al*., 2010). Besides, the effect of PEG on the ethosome formation in formulations no.Etho-26 and -27 showed an increase of vesicle size compared to formulation no.Etho-16 and -13, respectively.

As a result of polyethylene glycol (PEG) chains covalently bonded to a surface of phospholipids (Xu *et al*., 2008). However, The appropriate formulation, is dependent not only on the additives but also on other factors such as influence of ethanol and phospholipids, the concentration ratio, type of incorporated drug, the method and condition of preparation.

The optimized MPA ethosome was considered from the vesicle size less than 500 nm, a narrow size distribution, Zeta potential more than 30 mV and entrapment efficiency more than 50%. The result of our studies was found that both formulations no.Etho-20 and Etho-25 formulations were matched with the criteria set. Although, the entrapment efficiency of the two formulations has similarity but the formulation no.Etho-25 has a smaller particle size and narrower size distribution than the formulation no.Etho-20. This may be the result of the solubilizing effect of Tween 80 leading to a reduction of the vesicle size. Therefore, the formulation no.Etho-25 composing of 4% w/v SPC:CHOL: Tween80:DA (6:2:1:1 molar ratio) as a lipid component and 30% v/v ethanol in phosphate buffer pH 7.4 as dispersion medium was selected as the optimized MPA ethosome. This formulation gave ethosome with 370.90±7.91 nm vesicular size (*PI* = 0.27±0.02), the zeta potential of -45.58±4.50 mV and the efficient entrapment of 56.01±1.10% as shown in Table 4.2. The physical appearance of the optimized ethosome was yellowish colloidal appearance as shown in Figure 4.3A. The examination in the optimized formulations by optical microscope and SEM revealed the close spherical vesicles (Figure 4.3B and C).

Table 4.2 The main compositions (4% w/v SPC, 30% v/v ethanol) with the adding of additives and physical characteristics of ethosome **(**Limsuwan and Amnuaikit, 2012)

No.	Additives composition	Ratio		Vesicle size (nm)	Polydispersity Index	Zeta potential (mV)	Entrapment efficiency (%)
		Molar	Weight				
Etho-8	-	-		153.60±1.93	0.29 ± 0.01	-79.65±6.67	16.52±0.08
Etho-13	SPC:CHOL	3:1		161.98±1.72	0.22 ± 0.01	-34.48±2.70	18.71±1.10
Etho-14	SPC:CHOL	4:1		473.87±20.20	0.34 ± 0.02	-63.65±1.86	26.26±0.40
Etho-15	SPC:CHOL	3:2		706.72±19.86	0.35 ± 0.02	-57.24±3.79	61.64±1.32
Etho-16	SPC:CHOL	2:1		257.72±4.78	0.28 ± 0.04	-58.32±3.79	19.45±0.14
Etho-17	SPC:Tween80	4:1		189.80±0.71	0.17 ± 0.03	-48.13±2.01	30.12±0.59
Etho-18	SPC:CHOL: SA	2:1:1		1898.50±41.91	0.39 ± 0.04	31.34±1.15	5.42±2.09
Etho-19	SPC:CHOL:DA	2:1:1		546.45±32.55	0.35 ± 0.03	-52.96±6.50	34.37±1.14
Etho-20	SPC:CHOL:DA	6:2:1		419.63±1.60	0.32 ± 0.03	-53.37±8.28	58.31±1.60
Etho-21	SPC:CHOL:Tween80	2:1:1		190.25±1.46	0.13 ± 0.01	-43.32±3.01	20.61±0.35
Etho-22	SPC:CHOL:Tween80	3:2:1		198.20±1.56	0.09 ± 0.02	-28.44±1.66	28.82±0.50
Etho-23	SPC:CHOL:Tween80	6:2:1		173.25±3.00	0.12 ± 0.05	-30.66±4.82	12.32±0.50
Etho-24	SPC:CHOL:Tween80:DA	2:1:1:1		130.65±17.18	0.27 ± 0.03	-39.10±3.56	34.80±0.13
Etho-25	**SPC:CHOL:Tween80:DA**	**6:2:1:1**		**370.90±7.91**	**0.27± 0.02**	**-45.58±4.50**	**56.01±1.10**
Etho-26	SPC:CHOL: PEG4000	2:1:1		525.48±8.15	0.31 ± 0.07	-34.48±2.70	8.98±1.36
Etho-27	SPC:CHOL: PEG4000	6:2:1		304.95±4.35	0.21 ± 0.06	-63.65±1.86	32.36±0.60
Etho-28	SPC : Tween 80		84:16	259.35±7.47	0.25 ± 0.03	-50.18± 1.81	32.94±1.63
Etho-29	SPC : Tween 80 : DA		84:16:25	461.525±7.29	0.22 ± 0.05	-35.38± 3.96	15.69 ±1.05

Each data represents the mean±S.D. (n = 3). SPC, L-α-phosphatidylcholine from soybean; CHOL, Cholesterol from lanolin; DA, deoxycholic acid; SA, stearylamine. Tween 80, polyoxyethylene sorbitan monooleate

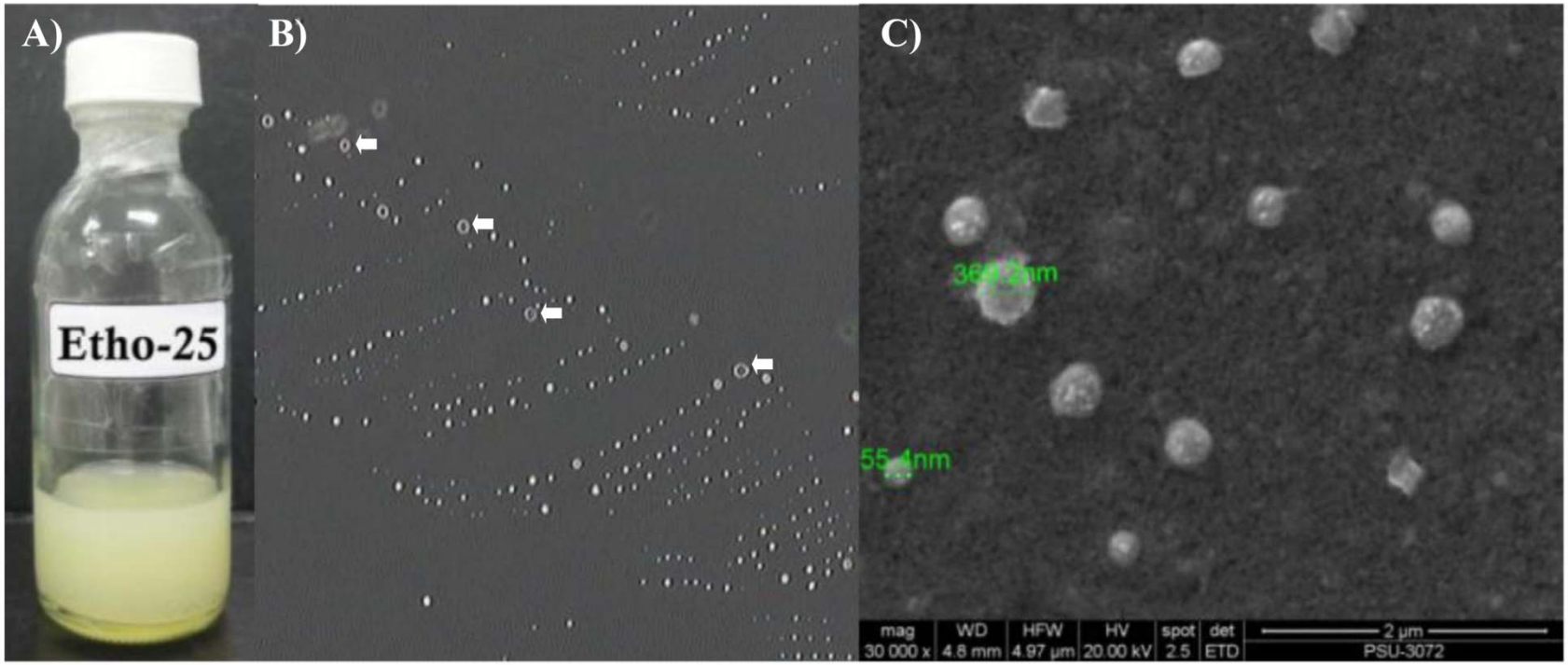

Figure 4.3 The physical appearance (A), optical micrograph image (×400 magnifications) (B) and Scanning Electron microscope or SEM (×30,000 magnifications) (C) of the optimized formulation (Etho-25). Arrows represent to ethosome vesicles in the formulation

4.3 Stability study of ethosome containing MPA

The stability of the optimized ethosome was carried out for three months at 4±1 ºC and at room temperature (37±2 ºC) for 0, 30, 60 and 90 days. In this study, no precipitation was found in all freshly prepared ethosome samples. After storage at 4±1˚C for 21 days, the formulation displayed white precipitation as shown in Figure 4.4A. The precipitation was collected to examine for its structure by optical microscope. Figure 4.4B showed the precipitation of the drug and/or other components in the formulation, which represented unstable formulation. However, no sedimentation was found in the ethosome formulation after storage at room temperature for 90 days as shown in Figure 4.5A and B, respectively. This could be explained that the changes in MPA solubility at low temperature.

Figure 4.6A showed the stability profile of the ethosome formulation in term of MPA content calculated as percentage of drug remaining after storage. It was clearly seen that MPA content did not significantly change over the storage period of three months. Nevertheless, only insignificantly changes in entrapment efficiency of the ethosome vesicles were observed (from 54.40±4.35% at 0 day to 59.63±4.28% at 90 days) as shown in Figure 4.6B. This might be a result of the increased in vesicle size causes an increase in the volume of drug entrapment, resulting in increased the drug entrapment efficiency.

Although, the physical characteristics of the ethosome formulation that stored at room temperature for 90 days did not change, but the average size had been significantly increased (362.86±11.69 nm at 0 day and 616.73±40.19 nm after 90 day of storage; $p<0.05$) as shown in Figure 4.6C. Moreover, the SEM micrograph also supported the increment of vesicle size when stored for 90 days (Figure 4.8A).

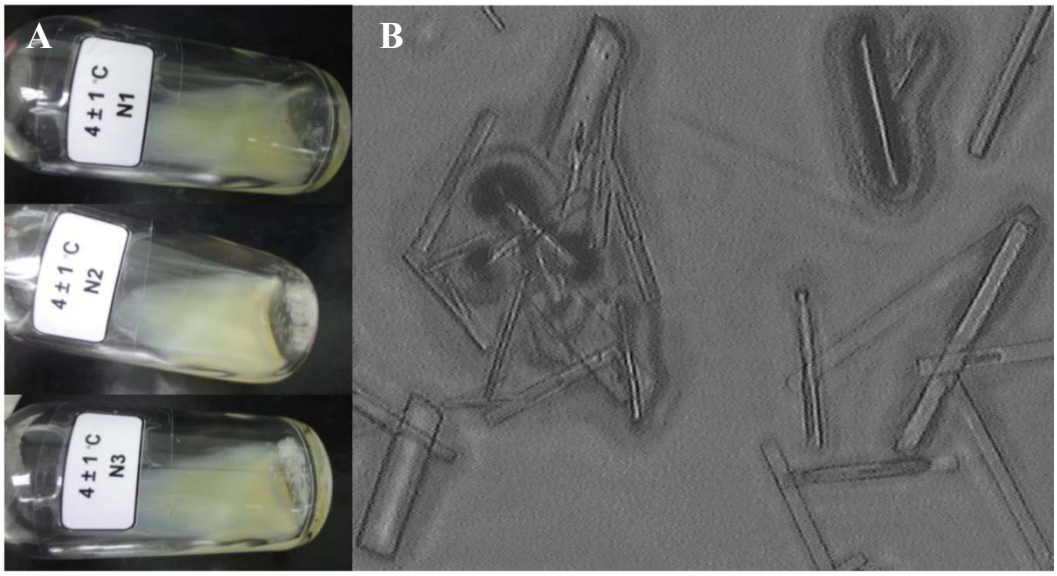

Figure 4.4 The physical appearance of MPA-loaded ethosome storage at 4±1°C for 21 days (A) and its optical micrograph image (×1000 magnifications) (B)

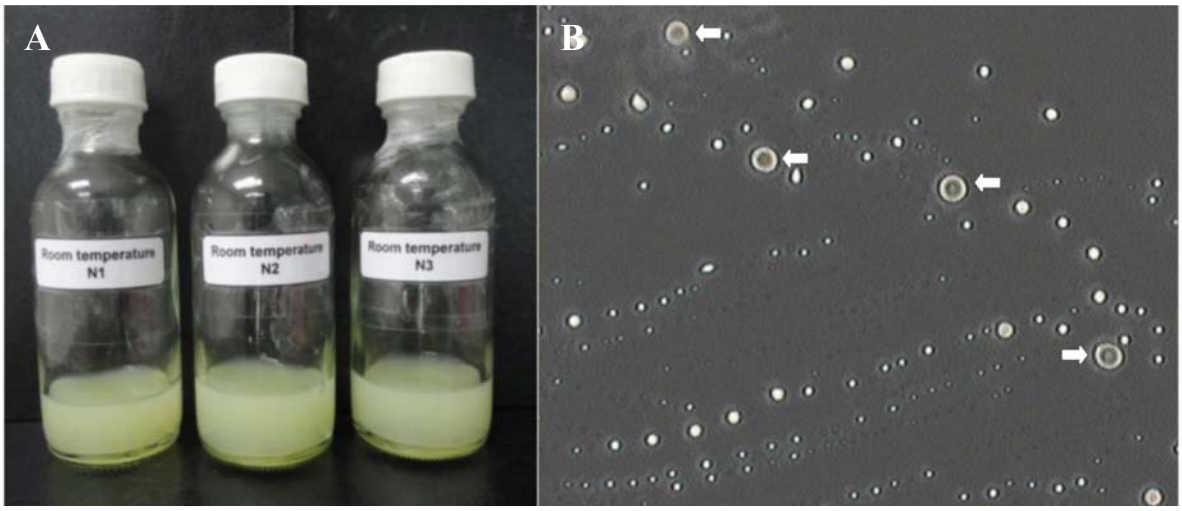

Figure 4.5 Physical appearance of MPA-loaded ethosome storage at room temperature for 90 days (A) and its optical micrograph image (×1000 magnifications) (B). Arrows represent to ethosome vesicles in the formulation

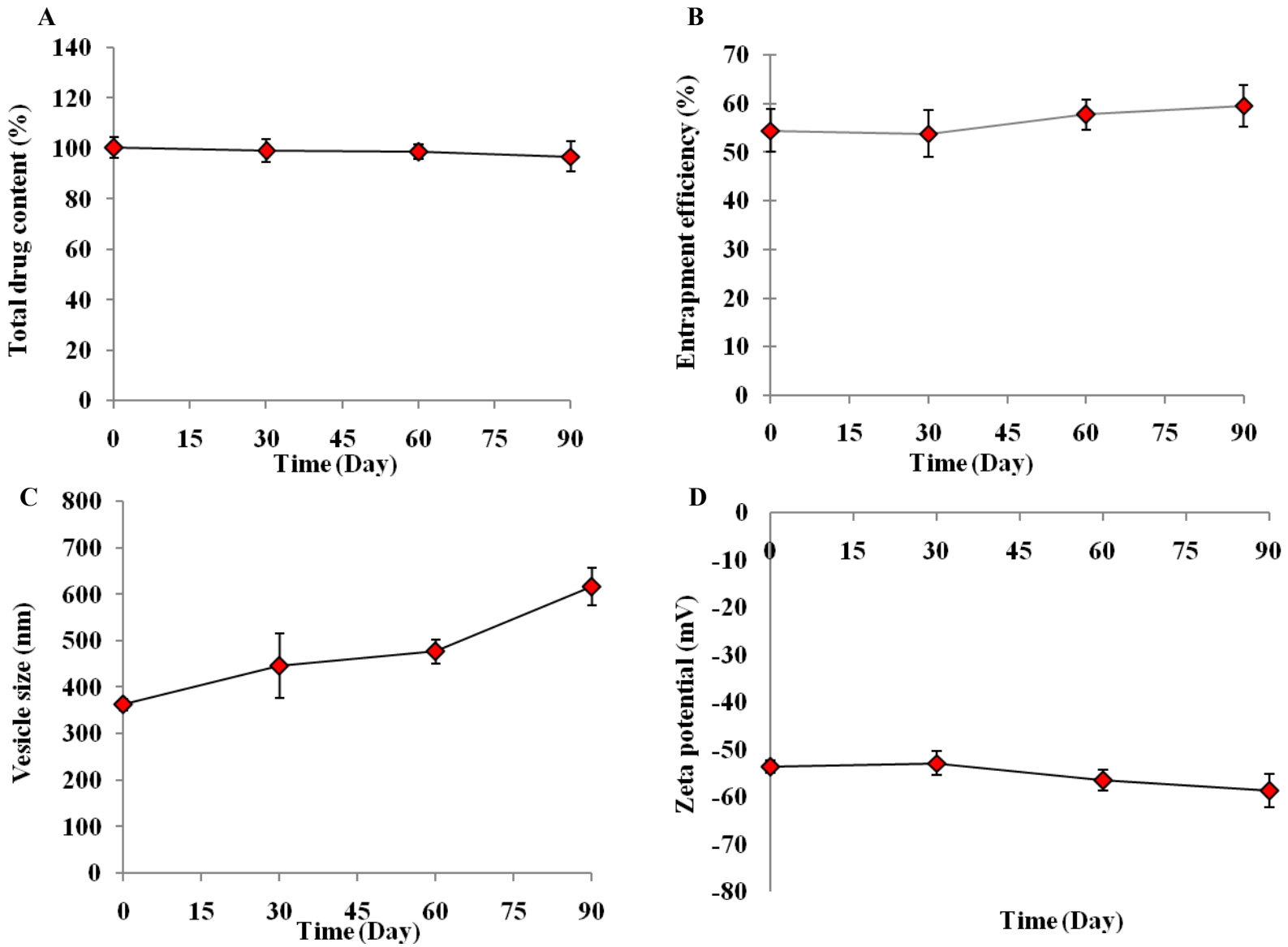

Figure 4.6 Stability profile of MPA-loaded ethosome in term of %total drug content (A), % entrapment efficiency (B), vesicle size (C) and Zeta potential (D) after strong at room temperature

The growth in ethosome size might be caused by the aggregation and/or fusion of the ethosome itself. Since the role of lipid membranes seems to be the most important factor affecting the vesicles fusion process (Ekerdt *et al.*, 1981; Ohki and Ohshima, 2000; Kasson and Pande, 2007).

Figure 4.7 shows the basic steps of fusion of lipid vesicle (Mondal and Sarkar, 2011). The mechanism for fusion of lipid vesicles was explained as follows; (1) fusion occurring through a hemifused intermediate state of a nonbilayer structure formed between two interacting membranes; (2) membrane fusion through the stalk structure formed by the fusion of the outer monolayer of two interacting membranes which is caused by undulation or local interaction of the membranes. Then, through such a structure, the total membrane fusion proceeds; (3) fusion of two membranes through a phase defect in membranes which is created by the mixture of different membrane molecules or impurities in the membrane; (4) an increase in membrane surface tension or dehydration which results in a strong adhesion of two interacting membranes causing the deformation of the membranes (Ohki and Ohshima, 2000). Figure 4.8B shows the SEM morphology confirmed that the fusion of developed ethosome vesicles has been occurred after storage for 90 days, which led to the growth of vesicle size in the formulations.

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. When the hydration force became weak, the vesicles would initiate aggregation (Komutsu and Okada, 1995). However, there was no significant change in zeta potential of the ethosomal formulation (-53.57± 1.23 mV at 0 day, and -58.64±3.43 mV at 90 days) as shown in Figure 4.6C, this indicate that the formulation is stable and not likely to aggregate after storage for 90 days.

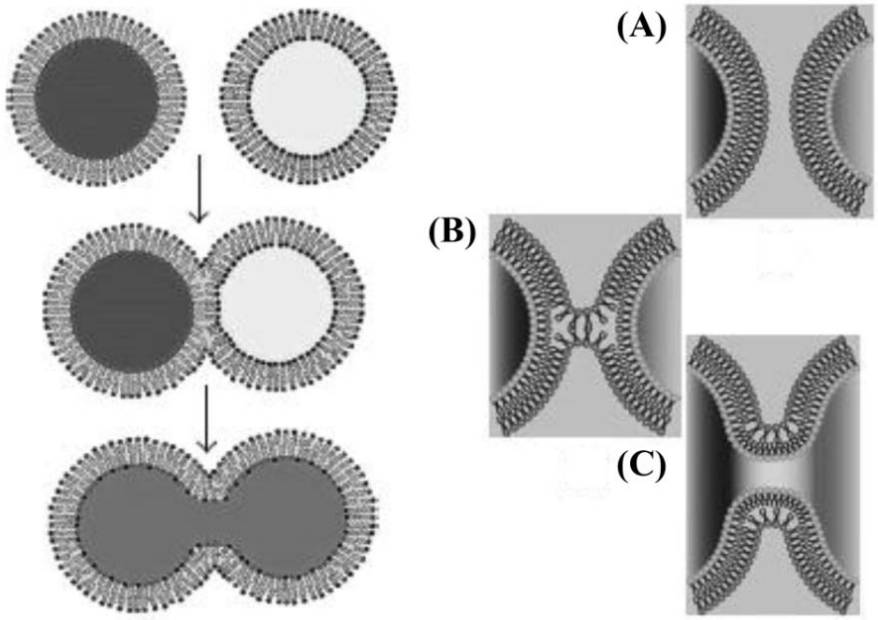

Figure 4.7 Basic Steps of membrane fusion. Membrane contact (A), outer leaflet lipid mixing to form the hemifused state (B), and inner leaflet lipid mixing, a pore formation and content mixing (C) (Mondal and Sarkar, 2011)

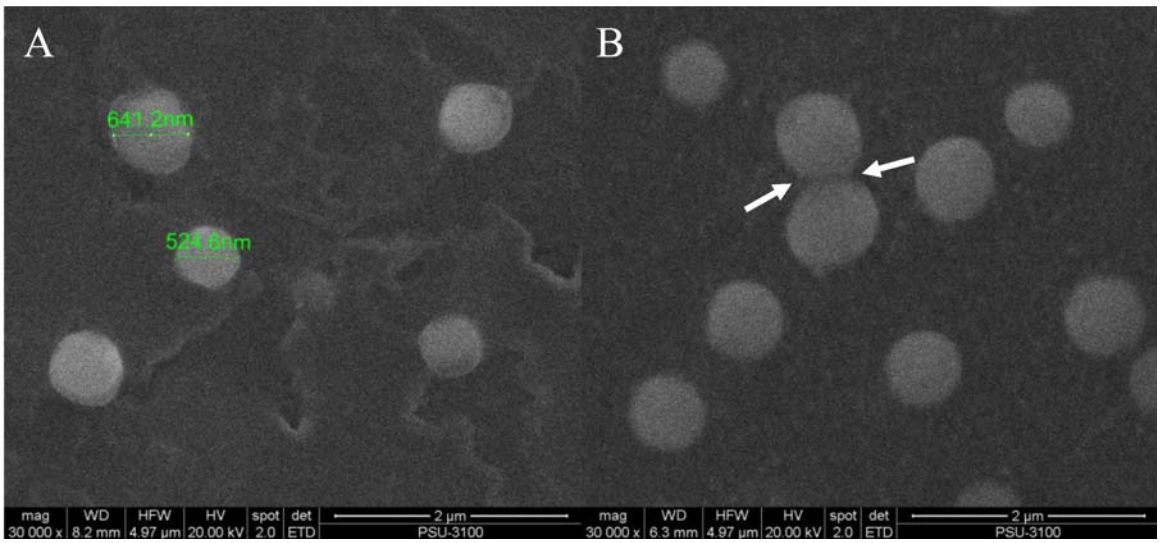

Figure 4.8 Scanning Electron microscope or SEM showing ethosome vesicles after storage at room temperature for 90 days (A) and its fusion (B). Arrows represent to vesicle fusion either via a hemifused intermediate or a stalk-like (×30,000 magnifications)

4.4 *In vitro* skin permeation and skin retention studies (Limsuwan *et al*., 2012)

The ability of ethosomal carrier to deliver MPA through membrane pig skin was evaluated for study the possibility of the ethosome as the skin delivery system through human skin. The newborn pig skin was used because of its similar stratum corneum as human skin in term of lipid composition and thickness (Magnusson *et al*., 2001).

The relations between the MPA in the three compartments (donor and pig skin) were analyzed after the end of experiment at 24 hours. Table 4.3 shows the amount recovery (% of the applied dose) of MPA from various formulations. The amount recovery of MPA per the total recovery of MPA in various formulations (% of the applied dose) was calculated as shown in Table 4.4.

Table 4.3 The amount recovery of MPA from various formulations (Limsuwan *et al*., 2012)

Formulations	Amount recovery (% of the applied dose)			
	Donor compartment	Newborn Pig skin	Receptor compartment	Total
Aqueous suspension	85.40±9.38	0.14±0.07	*UD	85.56±9.39
30% Hydroethanolic solution	93.64±4.58	0.88±0.07	1.39±0.26	94.92±4.80
Liposomes	93.92±11.91	0.52±0.16	2.98±0.32	97.43±12.11
Liposomes (Rattanat, 2008)	88.67±5.61	0.43±0.12	2.81±0.24	91.91±5.51
Ethosome	**84.15±5.46**	**0.80±0.13**	**4.80±0.44**	**89.76±5.41**

Data are expressed as % of total MPA in the applied dose (mean±SD, *n* = 5). *UD = undetected

Table 4.4 The ratio of the amount recovery of MPA in various formulations.

Formulations	Ratio of the amount recovery of MPA			
	Donor compartment	Newborn Pig skin	Receptor compartment	Total
Aqueous suspension	0.998±0.001	0.002±0.001	*UD	
30% Hydroethanolic solution	0.976±0.002	0.009±0.001	0.014±0.002	
Liposomes	0.964±0.003	0.005±0.001	0.031±0.003	1.000
Liposomes (Rattanat, 2008)	0.965±0.005	0.005±0.001	0.031±0.004	
Ethosome	**0.937±0.006**	**0.009±0.001**	**0.054±0.006**	

Data are expressed as the ratio of the amount recovery of MPA (% of the applied dose) per the total recovery of MPA in various formulations (% of the applied dose). (mean±SD, *n* = 5). *UD = undetected

The total MPA recovery from the three compartments for ethosome, aqueous suspension, hydroethanolic solution, liposome and liposome that was developed by Rattanat (2008) was calculated to evaluate the performance and reliability of the method (Table 4.3). These recovery values were within acceptable limit of AOAC (1993). It indicates that this method has performance and reliability.

When MPA was administered to skin, most of the MPA from all formulation remained in the donor compartment (Table 4.3 and 4.4). The low permeability of the MPA might be due to the influence of the structure of the outermost skin layer, the stratum corneum. It consists of protein-rich corneocytes embedded in a matrix of lipids. This domains form is the rate-limiting step for the penetration of drug (Long *et al.*, 1985). In addition, the octanol/water partition coefficient (log P) of MPA at pH 7.4 was 1.66 (Lee *et al.,* 1990) which exhibit intermediate partition coefficients (log P value between 1 and 3). It could soluble in the lipid domains of the stratum corneum and penetrate through the skin via intercellular pathway (Forster et al., 2009) which leads to low permeability of the skin. However, it is seen from Table 4.4 that the amount of MPA from the ethosome remained in the donor compartment significantly lower than other formulations ($p<0.05$). This result shows that the delivery of MPA into/through the skin by using the ethosome system efficient than other systems.

In the skin retention study, the amount of the MPA accumulated in the full-thickness newborn pig skins was analyzed after the end of the skin permeation at 24 hours (Figure 4.9, Table 4.3 and 4.4). The ethosome system and hydroethanolic solution gave comparable amount of MPA accumulated in the full-thickness newborn pig skin. However, the two systems displayed significantly higher amount of MPA

accumulative than the aqueous suspension, liposome and liposome developed by Rattanat (2008) (p<0.05).

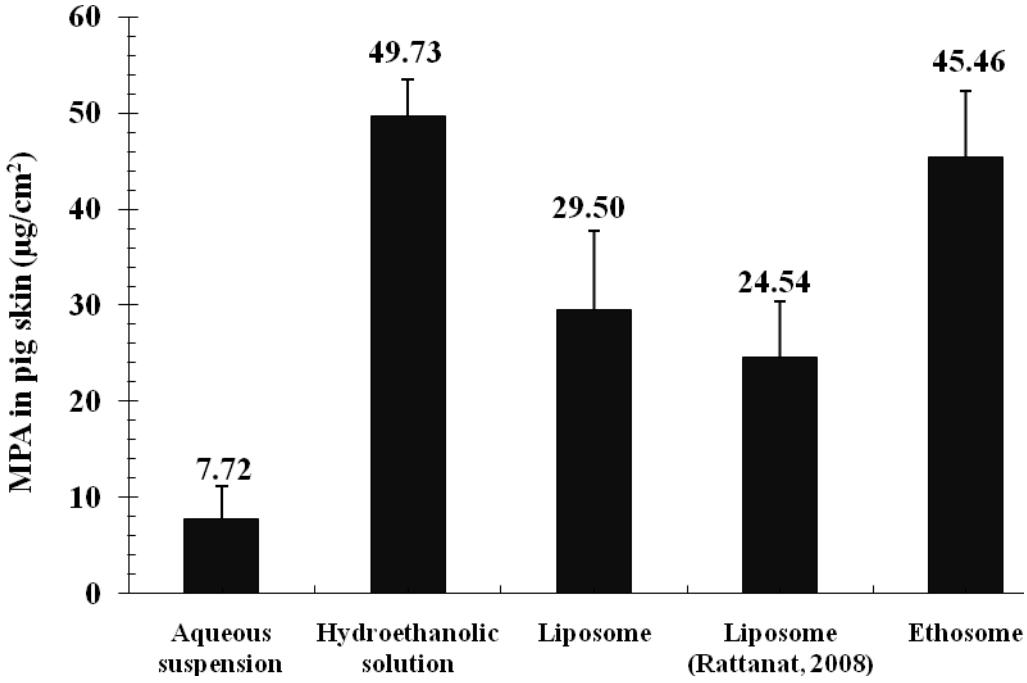

Figure 4.9 *In vitro* accumulated amount of MPA in full-thickness newborn pig skins after 24 hour. Each bar represents the mean±SD (n = 5) (Limsuwan *et al*., 2012)

This result might be due to the presence of ethanol at the same concentration (30% v/v ethanol) in formulations. Ethanol could increase MPA solubility in the formulation, change the stratum corneum's barrier property and increase the thermodynamic activity due to evaporation of ethanol (Williams and Barry, 2004; Dubey *et al*., 2007). It leads to enhance the skin permeation of MPA.

However, the effect of ethanol alone is not sufficient to deliver the MPA through the skin, but might depend on the phospholipids vesicle and skin lipids.

This could be observed from the amount of MPA from hydroethanolic solution in the receptor is low.

In the skin permeation study, it is seen from table 4.3 and 4.4 that the amount of MPA from the ethosome accumulated in the receptor compartment significantly higher than other formulations (p<0.05). In addition, the skin permeation profiles and the permeation parameters e.g. steady state flux J_{ss}, permeation coefficient K_p and lag time T_{lag} of this study were shown in Figure 4.10 and Table 4.5, respectively. It could be clearly seen in the permeability profile that throughout the assay period of 24 hours, the cumulative amount at 24 hours (Q_{24}) of MPA ethosome formulation permeated through pig skin was 307.29±24.93 μg/cm^2 (Figure 4.10). The Q_{24} from ethosome was 3.58-fold higher than that of the hydroethanolic solution (85.91±14.73 μg/cm^2; p<0.05), 1.66-fold higher than that from the liposome (185.32±18.27 μg/cm^2) and 1.72-fold higher than that from the liposome developed by Rattanat, 2008 (178.88±13.31 μg/cm^2). For MPA from aqueous suspension, the drug could not be detected in receptor fluid throughout the experiment. This incident could be confirmed that the MPA is a lipophilic drug. The octanol/water partition coefficient of MPA at pH 7.4 was 1.66 (Lee *et al.,* 1990). It is also slightly soluble in water (13 μg/ml at 25ºC), which might still remain on stratum corneum lipid and not penetrate into the skin (Goosen et al., 1998).

Further, the steady state flux J_{ss} (13.20±0.91 μg/cm^2/h) and the skin permeability coefficient K_p (1.32±0.09 ×10^{-3}cm/h) from ethosome were significantly higher than other formulations (p<0.05). Furthermore, the lag time of various formulations was observed, representing the time required for the drug to permeate through the skin layer before reaching the receptor chamber. Lag time of ethosome

(0.53±0.26 hours) was significantly shorter than other formulations (p<0.05). This result indicated that the ethosome could provide faster skin delivery of MPA than other formulations. These results indicated that ethosome was effective in delivering MPA into/through the skin to the systemic circulation.

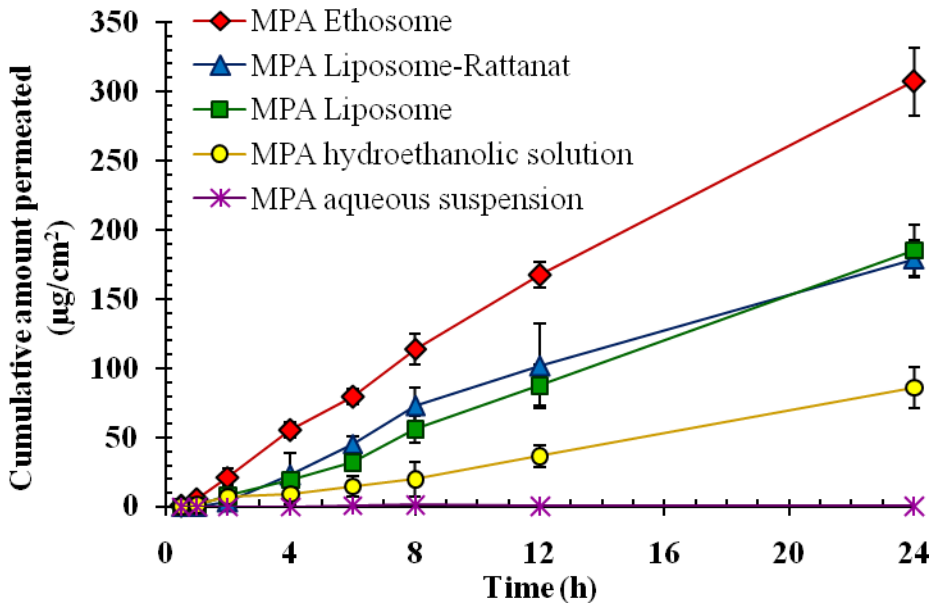

Figure 4.10 *In vitro* cumulative permeation profiles of MPA across membrane pig skin from the aqueous suspension, 30% hydroethanolic solution, liposomes, liposomes developed by Rattanat (2008) and ethosome. Each point represents the mean±SD (n=5) (Limsuwan *et al*., 2012)

Table 4.5 *In vitro* skin permeation parameters of MPA from the aqueous suspension, 30% hydroethanolic solution, liposomes, liposomes developed by Rattanat (2008) and ethosome (Limsuwan *et al*., 2012)

Formulations	J_{ss} (μg/cm^2/h)	K_p (×10^{-3} cm/h)	T_{lag} (h)
Aqueous suspension	*UD	*UD	*UD
30% Hydroethanolic solution	3.55±0.58	0.36±0.06	3.26±0.15
Liposomes	8.02±0.60	0.80±0.06	2.08±0.54
Liposomes (Rattanat, 2008).	8.01±0.36	0.80±0.04	2.50±1.08
Ethosome	13.20±0.91	1.32±0.09	0.53±0.26

Each data represents the mean±SD (n = 5). *UD, undetected; J_{ss}, steady state flux; K_p, permeability coefficient. T_{lag}, Lag time

However, the use of liposome delivery systems composed of lipid phase which same as the ethosome but without ethanol in the formulation, and the use of hydroethanolic solution at the same ethanol concentration with ethosome deliver MPA into/through the skin. These results showed that the effect of ethanol or phospholipid vesicle alone is not sufficient to deliver the MPA into/through the skin. Thus, it appears that the low amount of MPA in the skin and the receptor compartment. While the ethosome including phospholipid vesicle and ethanol in the formulation. It shows that the ethasome has the potential to deliver MPA into/through the skin than the hydroethanolic solusion and liposome. These results demonstrate the synergistic effect between high concentrations of ethanol and phospholipids vesicles in the ethosome formulation, and skin lipids (Table 4.3 and 4.4).

Touitou *et al.,* (2000) suggested a hypothetical model of ethosome for enhance the penetration of drug through the stratum corneum lipid. It was explained

that the better skin permeation of ethosome might be due to the synergistic mechanism among high concentration of ethanol, phospholipids vesicles and skin lipids. The stratum corneum lipid at physiological temperature are densely packed and highly conformationally ordered. Ethanol interacts with lipid molecules in the polar head group region, resulting that the result in increasing fluidity may finally lead to increase membrane permeability. In addition, it may provide the vesicles with soft flexible characteristics which are easy to penetrate into deeper layers of the skin. The malleable ethosome vesicle can forge paths in the disordered stratum corneum. The release of drug could be the result of fusion of ethosome with skin lipids and drug release at various points along the penetration pathway.

It is known that the enzyme inosine monophosphate dehydrogenase (IMPDH) catalyzes the rate-limiting step in the *de novo* biosynthesis of guanine nucleotides. Inhibition of IMPDH leads to immunosuppression by decreasing guanine nucleotides that it is required for the proliferation of lymphocytes. IMPDH activity is mediated by two highly conserved isoforms type I and type II which are the targets of mycophenolic acid (MPA) (Jain *et al*., 2004). The result of the skin permeation and retention showed that the ethosome system could deliver MPA into the skin. This result leads to inhibition of type II IMPDH, which is expressed in activated lymphocytes. It could also deliver MPA through the skin into receptor compartment which representing the blood system. This result leads to inhibition of type I IMPDH, which is expressed in most cell and resting lymphocyte. These results were expected that the ethosome carrier could deliver MPA to the site of action which resulting more effective in psoriasis treatment.

CHAPTER 5

CONCLUSION

In this contribution, we developed and evaluated the ethosome containing MPA to obtain the optimized formulation which suit for application as skin delivery system. The MPA solubility properties influenced formulation and preparation of the ethosome containing MPA. Since MPA is very slightly soluble in water (13 µg/ml at 25˚C) and a weak organic acid whose pKa value is 4.5. Its solubility in the alkaline range is higher than in the acidic range and increase as pH increase. Accordingly, the buffer solution pH 7.4 was selected to prepare ethosomal formulation. It was expected that it could help to avoid the precipitation of the free drug in the formulation. The ethosome containing MPA was prepared according to the thin-film hydration method by using the concentration of MPA 10 mg/ml. The suitable ethosomal formulation (Etho-25) was composed of 4% w/v SPC with CHOL, Tween80 and DA used as additives with a molar ratio of SPC:CHOL:Tween80:DA was 6:2:1:1. The dispersion medium was 30% v/v ethanol in phosphate buffer pH 7.4. This formulation gave ethosome with 370.90±7.91 nm vesicular size (*PI* = 0.27 ± 0.02), the zeta potential of -45.58±4.50 mV and the entrapment efficiency of 56.01±1.10%. This ethosomal formulation showed acceptable stability in term of physical appearance, total drug content and entrapment efficiency after storage in well-closed container at room temperature for 90 days. Although the average size of

ethosome which was stored at room temperature for 90 days significantly increased (p<0.05) but its zeta potential has not significantly changed. This indicates that the ethosomal formulation remains stable and is not aggregate after storage at room temperature for 90 days. For the formulation storage in refrigerator (4±1˚C), the precipitation of the free drug and phospholipids were observed under optical microscope after storage for 21 days. This result demonstrated unstable formulation under this condition. *In vitro* skin permeation and skin retention of the optimized ethosome formulation were evaluated by using modified Franz-type diffusion cell. The total MPA recovery from the three compartments for aqueous suspension, hydroethanolic solution, liposome and liposome that was developed by Rattanat (2008) was calculated to evaluate the performance and reliability of the method. These recovery values were within acceptable limit of AOAC (1993). This indicates that this method has performance and reliability. In addition, the optimized ethosome formulation provided significantly higher skin permeation parameters such as Q_{24} (307.29±24.93 µg/cm^2), J_{ss} (13.20±0.91 µg/cm^2/h) and K_p (1.32±0.09 ×10^{-3}cm/h), compared with aqueous suspension, hydroethanolic solution, liposome and liposome developed by Rattanat (2008) (p<0.05). Moreover, it could significantly reduce the T_{lag} (0.53±0.26 hours), which may result in reducing the onset of the drug. Although the ethosome system gave the MPA accumulated in the pigs skin was not different from hydroethanolic solution (45.46±6.80 µg/cm^2 vs. 49.73±3.71 µg/cm^2), but it gave the MPA accumulated more significantly higher than the other formulations (p<0.05). These results demonstrated that the ethosome could enhance the skin permeation and retention of MPA. This result was expected that the ethosome carrier could deliver

MPA to the site of action which resulting more effective in psoriasis treatment. However, the *in vivo* skin permeation and clinical evaluation study should be conducted to ensure that this drug carrier was good clinical efficacy and safe.

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APPENDIX

1. Quantitative analysis of MPA used high-performance liquid chromatography (HPLC)

1.1 Materials and methods

1.1.1 Standard solution

The stock solution was prepared by dissolving the 12.5 mg of MPA in 50 ml of methanol to obtain the final concentration of 250 µg /mL. The stock solution was stored at 4˚C until used. Working standard solutions were freshly prepared before using by diluting the stock solution with methanol.

1.1.2 Instrument and chromatographic conditions

The HPLC method for quantitative determination of MPA used throughout this study was modified from the method described by Gopalakrishnan *et al*., 2010. The chromatographic analysis was carried out with a reverse phase BDS HYPERSIL C_{18} column (250x4.6 mm, 5 µm) at room temperature. The mobile phase was a mixture of methanol/acetonitrile/phosphate buffer pH 6 (10:30:60 v/v) that was filtered through 0.45 µm nylon membrane filter and degassed by sonication prior to use. The flow rate was maintained at 1.0 ml/min. The 20 µl sample solution was injected and the absorbance was detected at 254 nm (Limsuwan and Amnuaikit, 2012).

The analysis of MPA in ethosome formulations and in *in vitro* skin permeation study was performed by using HPLC Thermo LC (Massachusetts, United States) and HPLC Agilent 1100 series (Waldbronn, Germany), respectively.

1.1.3 The validation procedures

The HPLC method used for analysis of MPA in ethosome formulations and *in vitro* skin permeation study were validated through the analysis of specificity, linearity, limit of detection (LOD), limit of quantitation (LOQ), accuracy and precision according to the Association of Official Analytical Chemists (1993) as follows:

Specificity

Specificity is the ability of a method to discriminate between the analyte of interest and other components that are presented in the sample matrix. For HPLC method, the specificity is demonstrated by the separation of the peak of MPA from other components in the samples, such as impurities, degradants and excipients.

The specificity of the HPLC method for determination of MPA in ethosome formulations and in *in vitro* skin permeation study was tested by spiking the drug in the tested matrices. In ethosome formulations, the total drug content was analyzed by using the procedures described in section 3.3.3.3. In *in vitro* skin permeation study, the specificity of MPA remaining in the donor compartments was determined as described in section 3.3.5.3. For the receptor compartments, the drug spiking isotonic phosphate buffer pH 7.4 (PBS) which was a receptor fluid was analyzed as described in section 3.3.5.2. In addition, the MPA spiking membrane pig as the skin model was performed as described in section 3.3.5.4. The peak of all

excipients in ethosome formulations, PBS and membrane pig after extraction must not interfered with the MPA peak.

Linearity

Working standard solutions were prepared by diluting the stock solution with methanol at least five concentrations. The standard solutions were analyzed at five replicates in the range of 3-40 μg/ml for the analysis of MPA in ethosome formulations, 1-40 μg/ml for the analysis of MPA in the donor compartments, 0.2-20 μg/ml for the analysis of MPA in membrane pig skin and 0.2-30 μg/ml for the analysis of MPA in receptor compartments. The calibration curves were evaluated by plotting the average of peak area (y) against the concentration range of MPA (x). The correlation coefficient (R^2) was calculated and had to be equal to or greater than 0.999 to demonstrate an acceptable linearity of analytical method.

Limit of detection and Limit of quantification

The limit of detection (LOD) is the lowest quantity of analyte in a sample which can be detected but not necessarily quantified with accuracy. The limit of quantitation (LOQ) is the lowest concentration of analyte in sample that can be quantitatively determined with precision and accuracy. The LOD and LOQ were calculated from the standard deviation of the response and the slope of the calibration curve by using equations (1) and (2), respectively (Uma and Sanjeeva, 2010).

$$LOD = 3.3\sigma/S \quad (1)$$

$$LOQ = 10\sigma/S \quad (2)$$

Where; σ: the standard deviation of the response

S: the slope of the calibration curve

Accuracy and precision

Accuracy and precision for the analytical of MPA content in both ethosome formulations and skin permeation study were assessed by using the concentration of MPA at 7.50, 10.00 and 12.5 μg/ml. These three concentrations represent 75%, 100% and 125% of the MPA concentration in the prepared ethosome.

The analytical of MPA content in the ethosome formulations was performed by spiking the three concentrations of MPA into the drug-free ethosome. The obtained samples were lysed with 30% v/v Triton® X-100, diluted with mobile phase and analyzed. Skin permeation study was consisted of the analysis of MPA content which remained in the donor compartment, retained in the membrane pig and permeated through pig skin into the receptor compartment. The process of the analysis of MPA content at the remaining in the donor was the same as the analysis of MPA in the ethosome formulation.

For the process of the analysis of MPA content at retention in the membrane pig was described as follows. The 1 ml of three concentrations of MPA was spiked on the skin surface (1.77 cm^2) and incubated for 24 hours. After 24 hours, the MPA was extracted from the skin according to the process as described in section 3.3.5.4. For the process of the analysis of MPA content at permeation through pig skin

into the receptor compartment, the membrane pig were incubated in 11 ml of PBS which was a receptor fluid for 24 hours. The three concentrations of MPA were spiked in the PBS at the equal volume and analyzed. All procedures were performed in three times and analyzed at least triplicate on the same day to evaluate the intra-day accuracy and precision. The same procedure was also carried on for three consecutive days for determine the inter-day accuracy and precision.

The accuracy of an analysis, and the concentrations of MPA calculated from the linear regression equation obtained from linearity test were compared to actual concentration and expressed as percentage of recovery (% recovery) which can be calculated by the following equation:

$$\% \text{ Recovery} = \frac{C_{measured}}{C_{actual}} \times 100 \qquad (3)$$

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

C_{actual} : actual concentration of MPA

For precision of an analysis, the system precision was expressed as percent relative standard deviation (% RSD) which can be calculated by the following equation:

$$\% \text{ RSD} = \frac{SD}{\overline{X}} \times 100 \qquad (4)$$

Where; SD : standard deviation of MPA concentration

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

1.2 Results and discussion

1.2.1 Quantitative determination of MPA using HPLC

The typical chromatogram of standard solution of MPA was analyzed using an HPLC Thermo LC (Massachusetts, United States) and HPLC Agilent 1100 series (Waldbronn, Germany) which showed the retention time of MPA were approximately 5.455 and 5.460 minutes as shown in Figure 1(A) and (B), respectively.

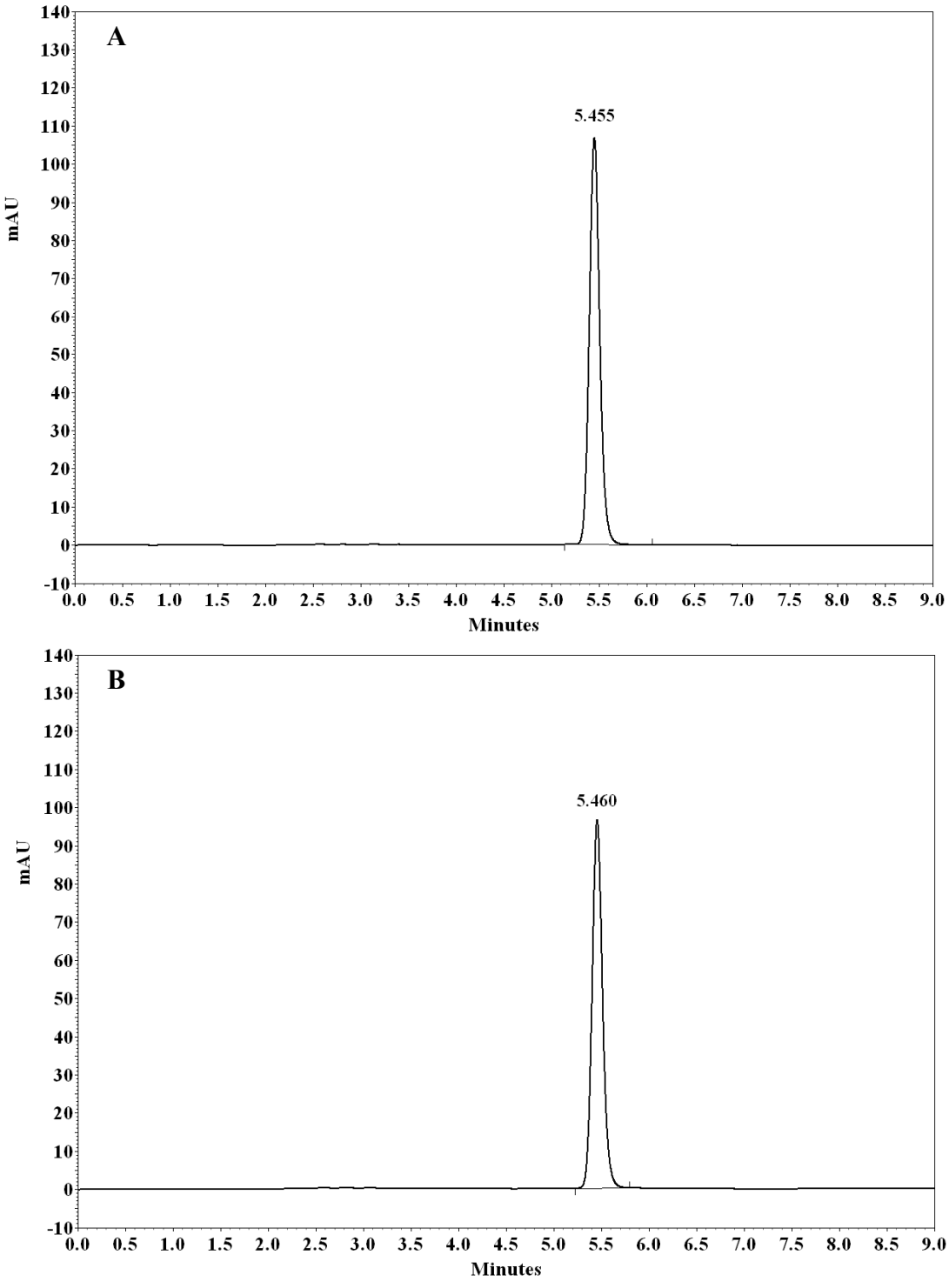

Figure 1 (A) Typical chromatogram of standard solution of MPA was analyzed by using a HPLC Thermo LC (Massachusetts, United States) and (B) HPLC Agilent 1100 series (Waldbronn, Germany)

1.2.2 Validation of analytical method

The validation of HPLC method for analysis of MPA in ethosome formulations and *in vitro* skin permeation study were examined in terms of specificity, linearity, limit of detection (LOD), limit of quantitation (LOQ), accuracy and precision.

Specificity

In this study, the specificity of the analytical method was validated for the determination of MPA in the formulations and skin permeation study. The specificity of the analysis of MPA in ethosome formulations was evaluated by comparing the chromatogram of blank ethosome and chromatogram of MPA spiked ethosome as shown in Figure 2 (A) and (B), respectively. The specificity of the analysis of MPA in skin permeation study, the chromatograms of the analysis of MPA in the donor compartments, membrane pig and receptor compartments were examined as shown in Figure 3, 4 and 5, respectively. All the figures showed that the peak of the other components in the samples did not interfere with the peak of the MPA. These results indicate that the methods of analysis of MPA have an acceptable specificity.

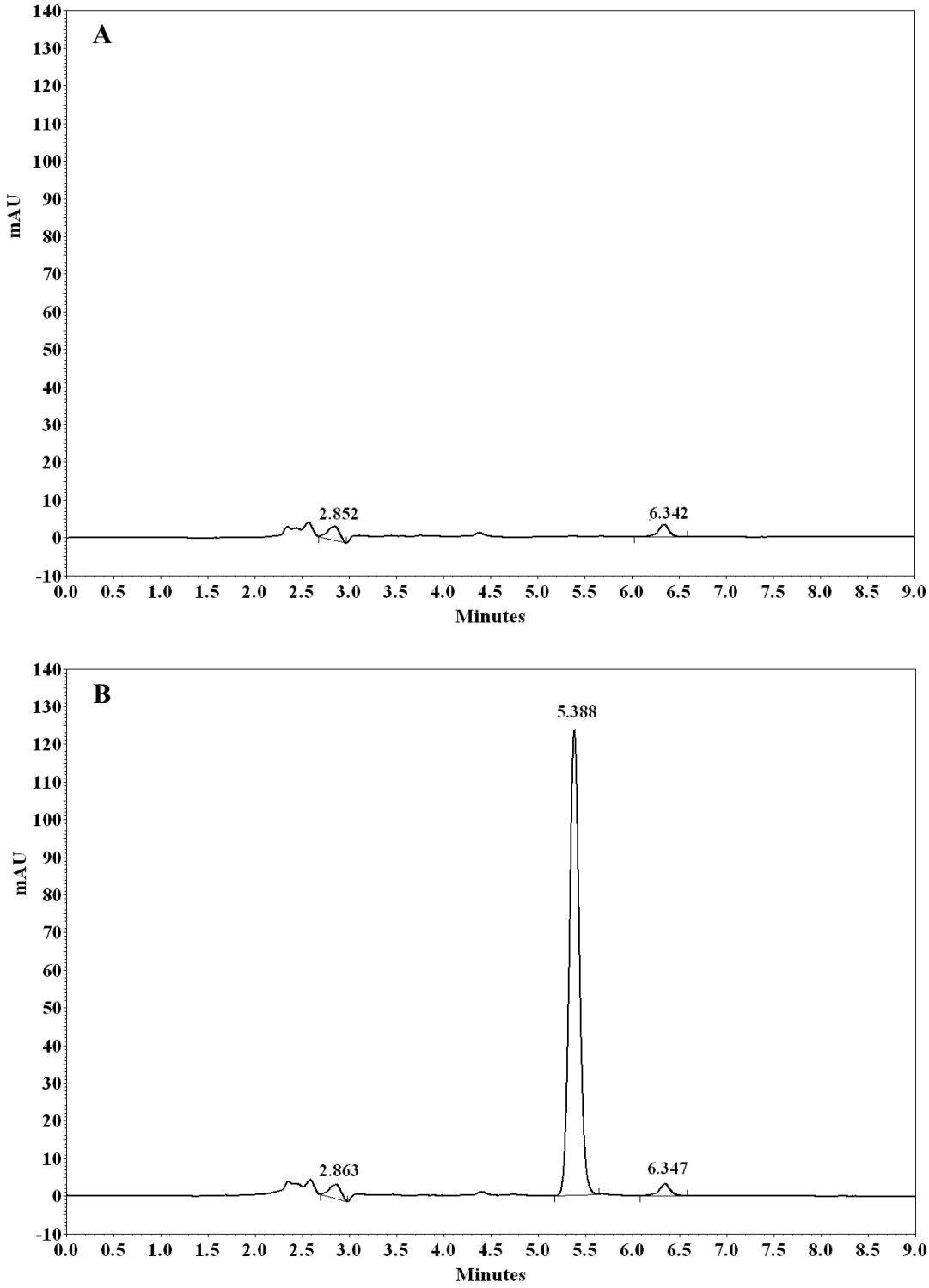

Figure 2 Chromatogram obtained from the analysis of blank ethosome (A) and MPA spiked ethosome (B) in a study of the formulation and preparation of ethosome containing MPA using a HPLC Thermo LC (Massachusetts, United States)

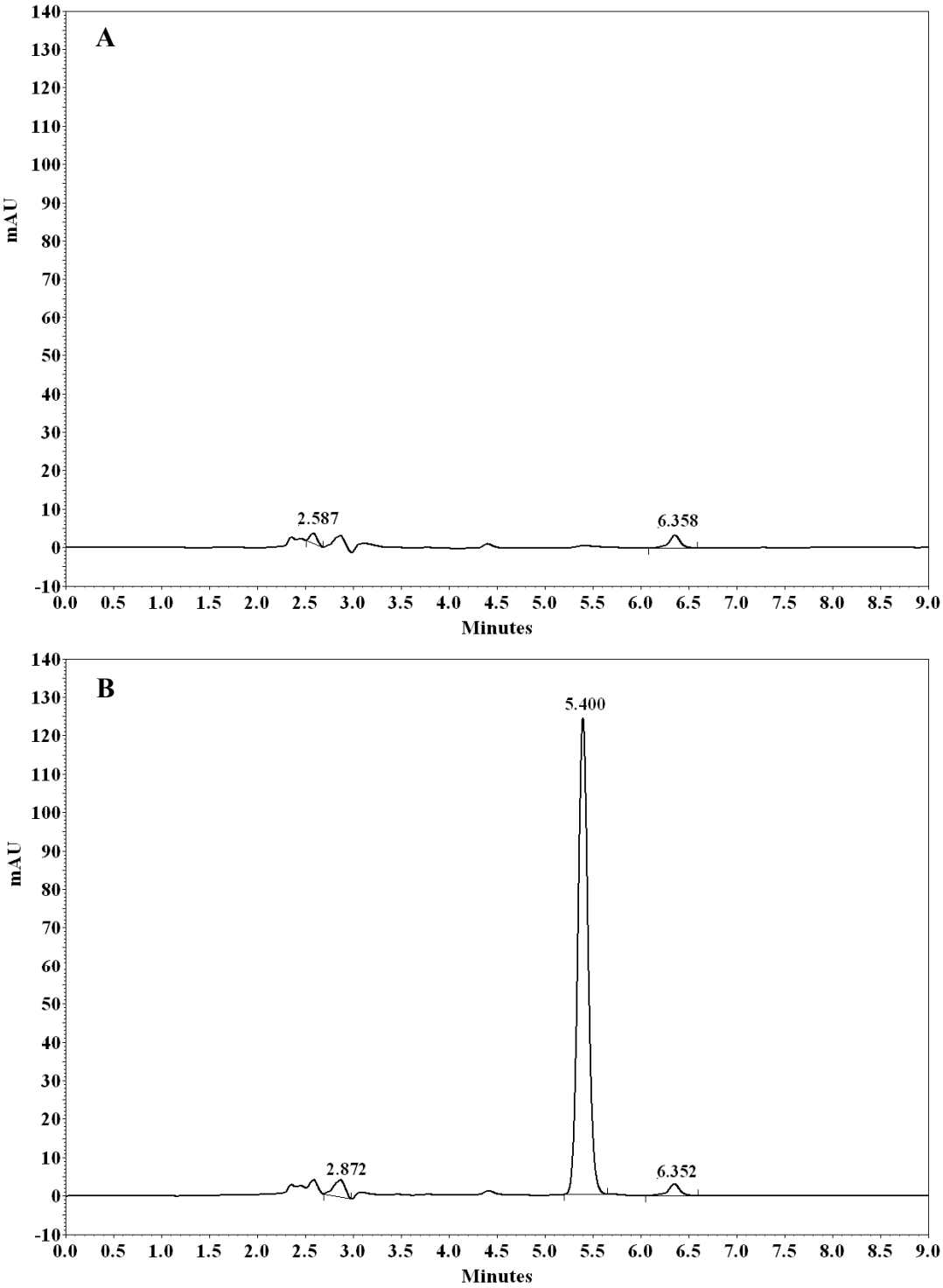

Figure 3 Chromatogram obtained from the analysis of blank ethosome (A) and MPA spiked ethosome (B) in a study to determine the amount of MPA at the remaining in the donor compartment by using a HPLC Agilent 1100 series (Waldbronn, Germany)

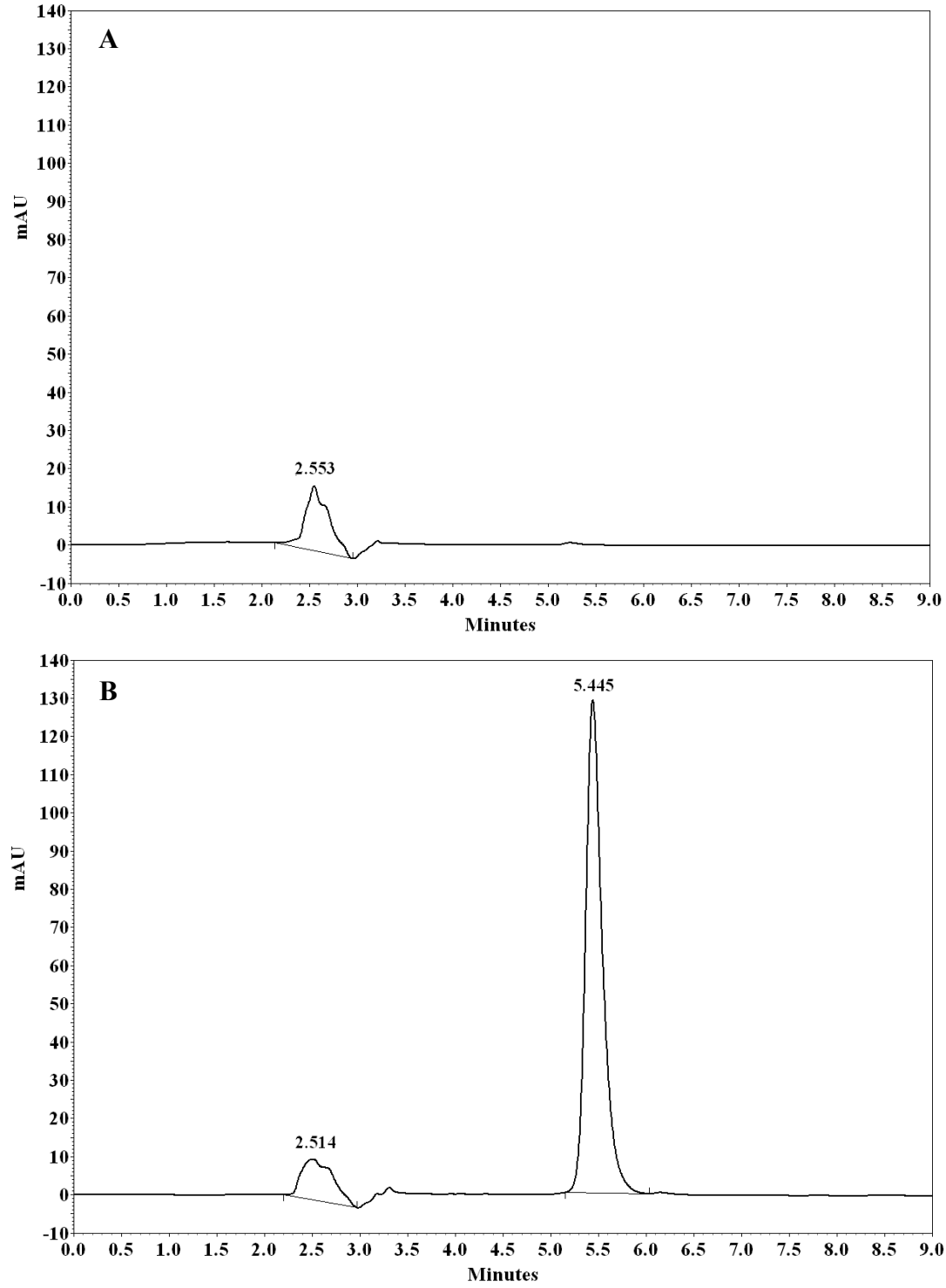

Figure 4 Chromatogram obtained from the analysis of blank membrane pig (A) and MPA spiked membrane pig after extraction with methanol (B) in a study to determine the amount of MPA retained in the skin by using a HPLC Agilent 1100 series (Waldbronn, Germany)

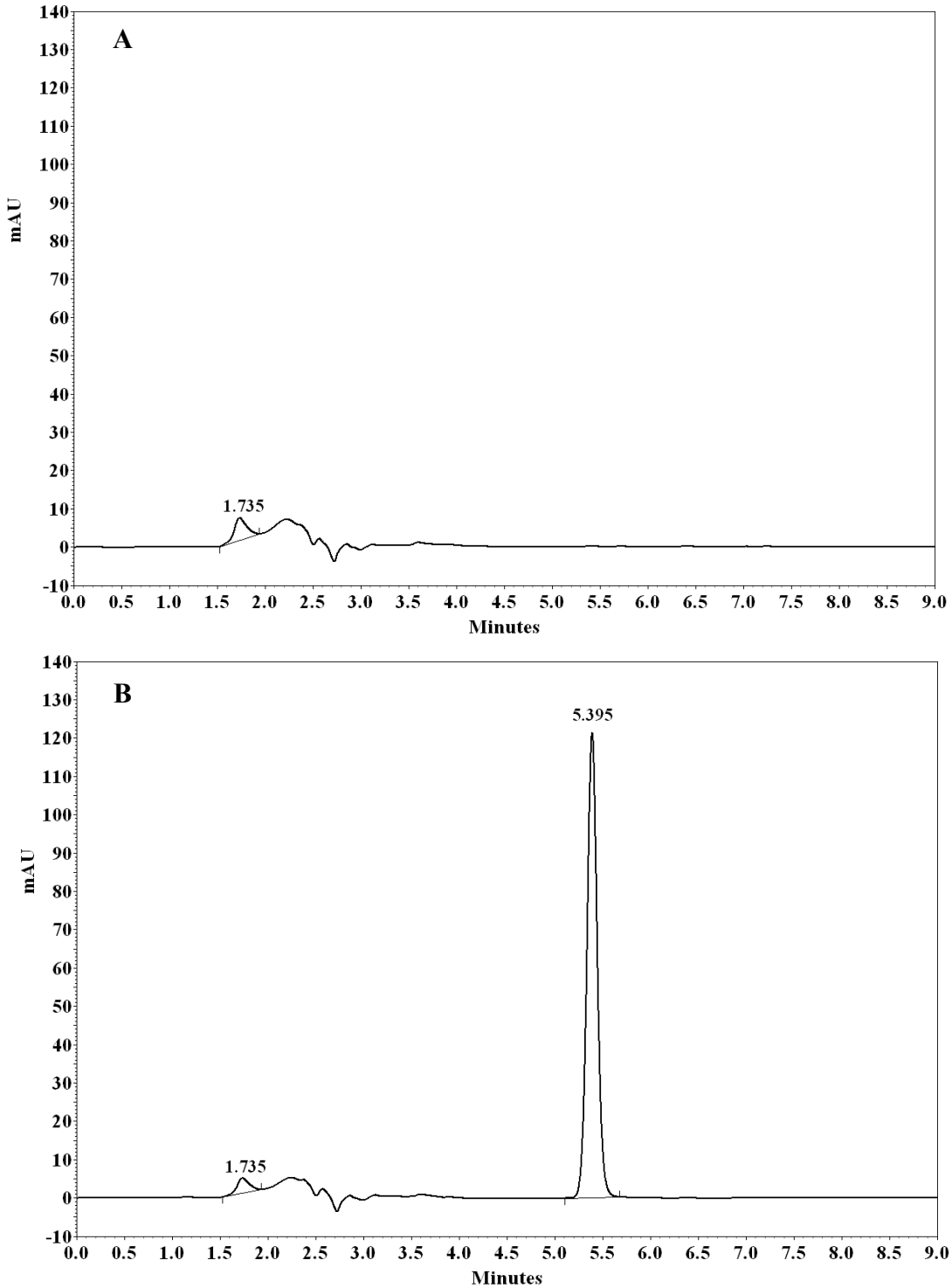

Figure 5 Chromatogram obtained from the analysis of blank PBS (A) and MPA spiked PBS (B) in a study to determine the amount of MPA permeated through the skin into the receptor compartment by using a HPLC Agilent 1100 series (Waldbronn, Germany)

Linearity

The calibration curves was evaluated by plotting the average of peak area (y) against the concentration range (x) 3-40 μg/ml for the analysis of MPA in ethosome formulations, 1-40 μg/ml for the analysis of MPA in the donor compartments, 0.2-20 μg/ml for the analysis of MPA in membrane pig and 0.2-30 μg/ml for the analysis of MPA in receptor compartments as shown in Figure 6A, B, C and D, respectively.

The linearity of these methods was determined by the linear regression equation and correlation coefficient (R^2), which were summarized in Table 1. All calibration curves showed correlation coefficient greater than 0.999, this indicated that all methods could be applied in the analysis of MPA.

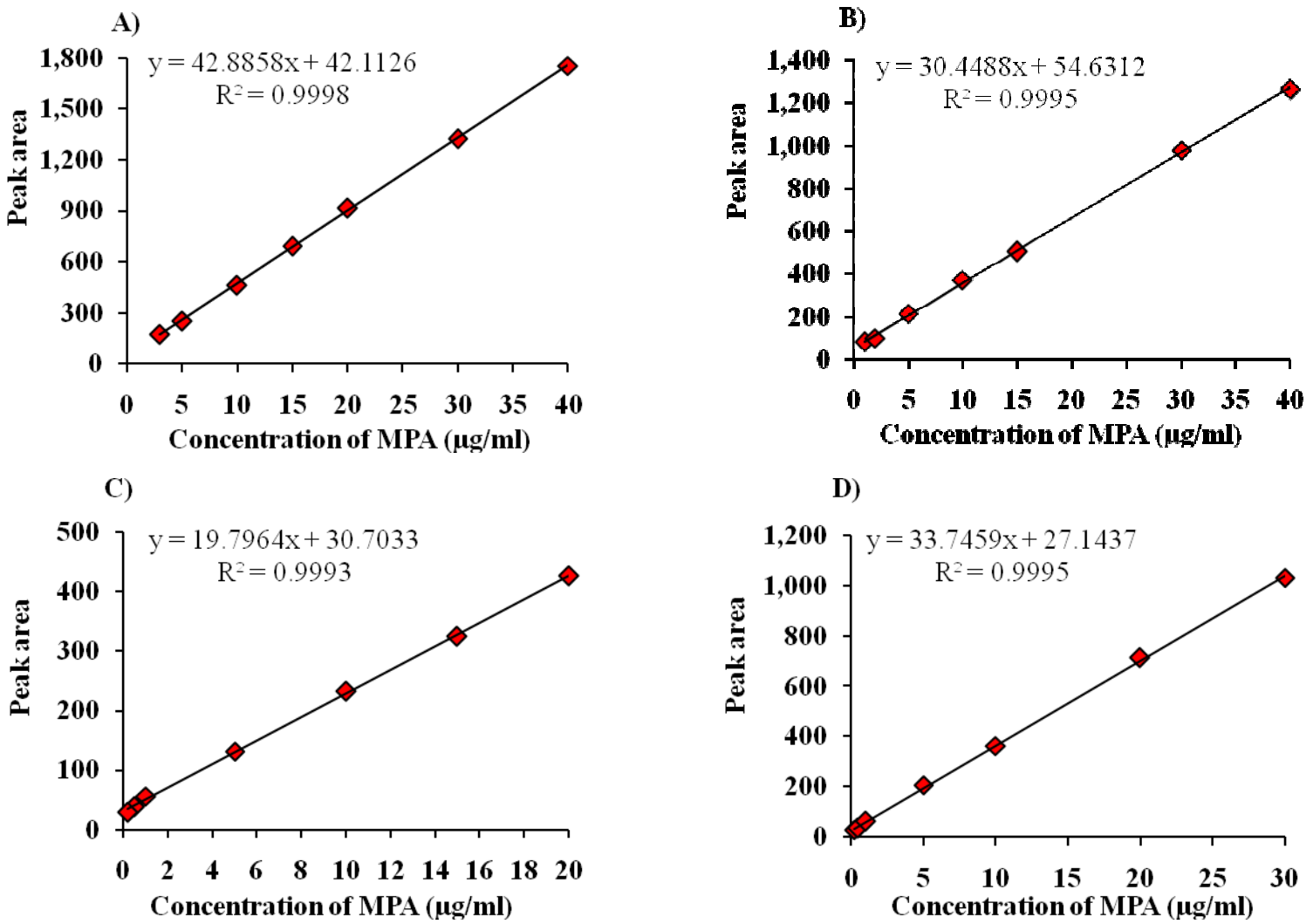

Figure 6 A standard calibration curve of MPA for the analysis of MPA in ethosome formulations (A), in the donor compartments (B), in membrane pig (C) and in receptor compartments (D). The plotted data are mean ±SD (n = 5). Solid line is a linear regression fit to the data

Table 1 The summary results of linear range, linear regression equation, correlation coefficient, limit of detection (LOD) and limit of quantitation (LOQ)

Method	Linear Range (µg/ml)	Linear regression equation*	Correlation coefficient (R^2)	LOD (µg/ml)	LOQ (µg/ml)
The analysis of MPA in ethosome formulation					
	3.0 - 40.0	y = 42.88x + 42.11	0.9998	0.09	0.30
The analysis of MPA in skin permeation study					
- Donor	1.0 - 40.0	y = 30.45x + 54.63	0.9995	0.04	0.13
- Membrane pig	0.2 - 20.0	y = 19.80x + 30.70	0.9993	0.03	0.10
- Receptor	0.2 - 30.0	y = 33.74x + 27.14	0.9995	0.04	0.13

*y = the average of peak area; x = the concentration range (µg/ml)

The plotted data are mean ±SD (n = 5)

Limit of detection and Limit of quantification

The LOD was calculated to be 0.09, 0.04, 0.03 and 0.04 µg/ml. The LOQ was calculated to be 0.30, 0.13, 0.10 and 0.13 µg/ml for the analysis of MPA in ethosome formulations, in the donor compartments, in membrane pig and in receptor compartments, respectively (Table 1). All results indicated that the established HPLC method was sufficiently sensitive for determination of MPA in various formulations.

Accuracy and precision

Table 2 shows the intra-day reproducibility of the MPA assay. At each concentration, the intra-day RSD varied between 0.44-2.40%. The accuracy of the average measured concentration for each day, which was reported in terms of % recovery was between 85.40-101.51%. The inter-day reproducibility of the MPA assay, given in Table 3, showed that the inter-day RSD varied between 1.14 - 1.90% and the recovery range of 86.81- 100.60%. These values were within acceptable limits of AOAC (1993) for both recovery (80-110%) and RSD (less than 7.3%). These results demonstrated that these methods gave both acceptable accuracy and precision. Therefore, these methods were suitable to analyze the amount of MPA from various formulations.

Table 2 Intra-day variability of MPA analysis

Method	Spiked conc. (mg/ml)	Day	*Measured conc. (mg/ml)	Average recovery (%)	RSD (%)
The analysis of MPA in ethosome formulation					
		1	7.43 ± 0.14	99.07	1.87
	7.49	2	7.51 ± 0.07	100.12	0.95
		3	7.56 ± 0.05	100.74	0.73
		1	9.92 ± 0.17	99.24	1.71
	10.00	2	9.97 ± 0.19	99.71	1.88
		3	9.91 ± 0.20	99.14	1.99
		1	12.40 ± 0.17	99.23	1.34
	12.50	2	12.43 ± 0.13	99.41	1.04
		3	12.52 ± 0.21	100.13	1.64
The analysis of MPA in the donor compartments					
		1	7.36 ± 0.14	98.12	1.90
	7.50	2	7.44 ± 0.12	99.26	1.56
		3	7.52 ± 0.08	100.27	1.01
		1	9.51 ± 0.13	95.05	1.99
	10.00	2	9.51 ± 0.13	95.05	1.99
		3	9.95 ± 0.17	99.51	1.75
		1	12.36 ± 0.18	98.88	1.43
	12.50	2	12.58 ± 0.19	100.63	1.52
		3	12.69 ± 0.21	101.51	1.66
The analysis of MPA in pig skin					
	7.51	1	6.55 ± 0.13	87.24	1.67
		2	6.67 ± 0.14	88.80	1.94
		3	6.65 ± 0.17	88.58	1.72
	10.01	1	8.55 ± 0.04	85.40	0.44
		2	8.72 ± 0.19	87.12	1.95
		3	8.80 ± 0.19	87.91	1.93
	12.50	1	11.17 ± 0.22	89.33	1.70
		2	11.43 ± 0.25	91.42	1.92
		3	11.37 ± 0.22	90.97	1.71
The analysis of MPA in the receptor compartments					
	7.50	1	7.43 ± 0.14	99.03	1.83
		2	7.46 ± 0.15	99.32	2.40
		3	7.52 ± 0.14	100.23	0.71
	10.00	1	9.98 ± 0.19	99.68	1.92
		2	10.11 ± 0.07	101.01	0.72
		3	10.12 ± 0.11	101.12	0.50
	12.50	1	12.39 ± 0.17	99.15	0.56
		2	12.49 ± 0.04	99.94	1.52
		3	12.52 ± 0.19	100.21	1.59

*All values were mean ± S.D. (n = 3)

Table 3 Inter-day variability of MPA analysis

Method	Spiked conc. (mg/ml)	*Measured conc. (mg/ml)	Average recovery (%)	RSD (%)
The analysis of MPA in ethosome formulation				
	7.49	7.50 ± 0.11	99.98	1.42
	10.00	9.94 ± 0.18	99.36	1.81
	12.50	12.45 ± 0.17	99.59	1.37
The analysis of MPA in the donor compartments				
	7.50	7.44 ± 0.11	99.21	1.49
	10.00	9.64 ± 0.17	96.81	1.52
	12.50	12.54 ± 0.19	100.34	1.54
The analysis of MPA in pig skin				
	7.51	6.62 ± 0.14	88.20	1.78
	10.01	8.69 ± 0.14	86.81	1.44
	12.50	11.32 ± 0.23	90.57	1.78
The analysis of MPA in the receptor compartments				
	7.51	7.47 ± 0.14	99.53	1.90
	10.01	10.07 ± 0.12	100.60	1.24
	12.50	12.67 ± 0.18	99.77	1.14

*All values were mean ± S.D. (n = 3)

2. Preparation of isotonic phosphate buffer pH 7.4

Isotonic phosphate buffer pH 7.4 was prepared by mixing two stock solutions, 200 ml of a solution containing 10.4 g of monobasic sodium phosphate ($NaH_2PO_4.2H_2O$) per liter and 800 ml of a solution containing 9.47 g of dibasic sodium phosphate (Na_2HPO_4) per liter. Then it was adjusted with respect to tonicity by adding 4.4 g of sodium chloride (NaCl). The obtained solution was filtered through a 0.45 μm nylon membrane and degassed by sonication before use.

VITAE

Name Miss Tunyaluk Limsuwan

Student ID 5210720009

Educational Attainment

Degree	Name of Institution	Year of Graduation
Bachelor of Science (Chemistry)	Prince of Songkla University	2009

Scholarship Awards during Enrolment

Research Assistantships in Pharmaceutical Sciences, Prince of Songkla University, 2009-2011

List of Publication and Proceedings

Tunyaluk Limsuwan, Chalermkiat Songkram and Thanaporn Amnuaikit. 2012. *In vitro* skin permeation study of ethosome containing mycophenolic acid. *Isan Journal of Pharmaceutical Sciences*. 8(1): 210-218.

Tunyaluk Limsuwan and Thanaporn Amnuaikit. 2012. Development of Ethosomes Containing Mycophenolic Acid. *Procedia Chemistry* (*In Press)*.

Tunyaluk Limsuwan and Thanaporn Amnuaikit. 2011. Development of Ethosomes Containing Mycophenolic Acid. *Innovation in Polymer Science and Technology 2011 (IPST2011) Conference*, *Workshop & Exhibition*, Asian Polymer Association (APA) and Indonesian Polymer Association (HPI). Sanur Paradise Plaza Hotel, Sanur, Denpasar, Bali, Indonesia. 28 November-2 December 2011. (Poster presentation).

Tunyaluk Limsuwan, Chalermkiat Songkram and Thanaporn Amnuaikit. 2012. *In vitro* skin permeation study of ethosome containing mycophenolic acid. *The 4th Annual Northeast Pharmacy Research conference 2012 "Pharmacy Profession in Harmony"*, Faculty of Pharmaceutical Sciences, Khon Kaen University, Ubon Ratchathani University and Mahasarakham University. Faculty of Pharmaceutical Sciences, Khon Kaen University. 11-12 February 2012. (Poster presentation).