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SKIN DELIVERY OF PROPYLTHIOURACIL FROM VESICULAR SYSTEMS

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ศุภกาญจน์ ศรีเพ็ชร์ : การนำส่งโพรพิลไธโอยูเรซิลทางผิวหนังจากระบบเวซิเคิล (SKIN DELIVERY OF PROPYLTHIOURACIL FROM VESICULAR SYSTEMS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก : ผศ.ภญ.ดร.นนทิมา วรรธนะภูติ , อ.ที่ปรึกษาวิทยานิพนธ์ร่วม : รศ.ภญ.ดร.วราภรณ์ สุวกูล, 126 หน้า.

ระบบเวซิเคิลสามารถเพิ่มการนำส่งยาทางผิวหนังสำหรับยาหลายชนิดที่มีคุณสมบัติในการละลายแตกต่างกัน อย่างไรก็ตามยังไม่มีข้อสรุปที่ชัดเจนเกี่ยวกับกลไกที่ระบบเวซิเคิลใช้ในการนำส่งยาและกลไกอาจขึ้นกับคุณสมบัติของตัวยาที่นำส่ง การศึกษานี้จึงมีวัตถุประสงค์เพื่อศึกษาการนำส่งยาต้นแบบที่ไม่ชอบตัวทำละลายทางผิวหนังด้วยระบบเวซิเคิล โดยเน้นที่กลไกของระบบเวซิเคิลในการนำส่งยาทางผิวหนังและปัจจัยที่มีผลต่อการซึมผ่านผิวหนัง ในการศึกษานี้ใช้โพรพิลไธโอยูเรซิล (พีทียู) เป็นตัวแทนยาชนิดที่ไม่ชอบตัวทำละลาย โดยเตรียมระบบเวซิเคิลได้แก่ ลิโพโซม นิโอโซม และเอโธโซมที่บรรจุพีทียูขึ้นและศึกษาคุณลักษณะเฉพาะของระบบเวซิเคิลที่เตรียมได้ในด้านของ ขนาดและการกระจายขนาด ประสิทธิภาพการกักเก็บยา อุณหภูมิการเปลี่ยนเฟส และการปลดปล่อยตัวยา รวมทั้งศึกษาการนำส่งพีทียูทางผิวหนังจากระบบเวซิเคิลโดยใช้เซลล์สำหรับการศึกษาการแพร่แบบฟรานซ์ชนิดดัดแปลงและใช้ผิวหนังลูกหมูแรกเกิดเป็นเมมเบรน ผลการศึกษาแสดงให้เห็นว่าขนาดเวซิเคิลและประสิทธิภาพการกักเก็บยาของระบบเวซิเคิลที่บรรจุพีทียูขึ้นกับส่วนประกอบของระบบและระบบเวซิเคิลที่บรรจุพีทียูอยู่ในสถานะผลึกเหลว ณ อุณหภูมิในการทดลองทุกระบบ การศึกษาการปลดปล่อยยาแสดงให้เห็นว่าการปลดปล่อยพีทียูจากระบบเวซิเคิลเกิดขึ้นอย่างช้า ๆ และเข้าได้กับจลนศาสตร์อันดับที่หนึ่ง ได้เปอร์เซ็นต์การปลดปล่อยพีทียูสะสมที่ 24 ชั่วโมงสูงสุดถึงประมาณ 90 เปอร์เซ็นต์ ในการศึกษาการซึมผ่านผิวหนังภายใต้สภาวะแบบเปิด ระบบลิโพโซมไม่เพิ่มการซึมผ่านผิวหนังลูกหมูแรกเกิดของพีทียู ขณะที่ระบบนิโอโซมและเอโธโซมมีแนวโน้มในการเพิ่มการนำส่งพีทียูทางผิวหนังได้ ในการศึกษาระบบเอโธโซม พบว่าปัจจัยด้านสภาวะการทามีผลอย่างมากต่อการซึมผ่านผิวหนังของพีทียู เมื่อคัดเลือกระบบเอโธโซมมาศึกษาต่อในเรื่องปัจจัยที่มีผลต่อการซึมผ่านผิวหนัง พบว่าปัจจัยด้านการมีโครงสร้างแบบเวซิเคิลต่อการซึมผ่านของพีทียูมีอิทธิพลน้อยกว่าผลในการเสริมฤทธิ์เพิ่มการแทรกผ่านของส่วนประกอบในตำรับและตัวทำละลายที่ใช้ในการศึกษา การวิเคราะห์ข้อมูลจากการศึกษาคุณลักษณะเฉพาะและการซึมผ่านผิวหนังของพีทียูจากเอโธโซมภายใต้สภาวะแบบปิดแสดงให้เห็นว่ากลไกการแพร่ของตัวยาอิสระ การผสมกันของเวซิเคิลกับไขมันของผิวหนังและการเพิ่มการแทรกผ่านโดยการเปลี่ยนแปลงคุณสมบัติของผิวหนังเป็นกลไกที่อาจเกิดขึ้นได้ทั้งสามกลไก ดังนั้นในการนำส่งพีทียูทางผิวหนังจากระบบเอโธโซมจึงมีหลายกลไกเข้ามาเกี่ยวข้อง

ภาควิชา วิทยาการเภสัชกรรมและเภสัชอุตสาหกรรม	ลายมือชื่อนิสิต.....
สาขาวิชา เภสัชกรรม	ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์หลัก.....
ปีการศึกษา 2552	ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์ร่วม.....

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KEYWORDS: Liposomes / Niosomes / Ethosomes / Propylthiouracil / Skin delivery

SUPPAKARN SRIPETCH: SKIN DEVERY OF PROPYLTHIOURACIL FROM VESICULAR SYSTEMS. THESIS ADVISOR: ASST. PROF. NONTIMA VARDHANABHUTI, Ph.D., THESIS CO-ADVISOR: ASSOC. PROF. WARAPORN SUWAKUL, Ph.D., 126 pp.

Vesicular systems are known to improve skin delivery of many drugs with diversity in solubility. The mechanism of skin delivery of these systems is inconclusive and likely to depend on the drug delivered. The aim of this present study was to investigate the skin delivery of a model lyophobic drug from vesicular systems. The study focused on skin delivery mechanism and the factors affecting skin permeation from vesicular systems. In this study, propylthiouracil (PTU) was used as a model lyophobic drug. Three vesicular systems containing PTU, i.e. liposomes, niosomes, and ethosomes, were prepared and characterized with regard to size and size distribution, drug entrapment efficiency, phase transition temperature, and drug release. The skin delivery of PTU from vesicular systems was studied using modified Franz diffusion cells. The newborn pig skin was used as a membrane. The results demonstrated that the vesicle size and the drug entrapment efficiency of PTU vesicular systems depended on the composition of the system. All PTU vesicular systems were in liquid crystalline-state at the temperature used in the study. Drug release studies revealed that all vesicular systems could sustain the release of PTU and the release was consistent with the first order kinetics. The 24-hour cumulative percent release of PTU up to 90% was achieved. In skin permeation study under the non-occlusive condition, the liposomal systems did not enhance PTU permeation through the skin, while the niosomal system and the ethosomal systems were likely to improve PTU skin delivery. The application condition had a considerable impact on skin permeation of PTU from the ethosomal systems. The ethosomal systems were thus selected for further investigation under the occlusive condition. The effect of existence of vesicular structure on PTU permeation from ethosomes was less remarkable than the synergistic enhancing effect of the ethosomal components and the solvent used in the study. Analysis of the data from characterization and skin permeation studies under the occlusive condition indicated that the diffusion of free drug, the mixing of the vesicles with the skin lipids, and the penetration enhancement were all possible to occur in skin permeation of PTU from the ethosomal systems. Therefore, skin delivery of PTU from the ethosomal systems appeared to involved several mechanisms that operated simultaneously.

Department: Pharmaceutics and Industrial Pharmacy Student's Signature .....

Field of Study: Pharmaceutics Advisor's Signature .....

Academic Year: 2009 Co-Advisor's Signature .....

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5-FU	=	5-fluorouracil
A_0	=	initial amount of PTU in the dispersion
ANOVA	=	analysis of variance
AR	=	analytical reagent
A_t	=	cumulative released amount of PTU at a particular time
$^{\circ}C$	=	degree Celsius
C_d	=	drug concentration in the donor compartment
CF	=	carboxyfluorescein
CH	=	cholesterol
CLSM	=	confocal laser scanning microscopy
cm	=	centrimeter
cm^2	=	squared centimeter
DPPC	=	dipalmitoylphosphatidylcholine
DSC	=	differential scanning calorimeter
EE	=	entrapment efficiency
EF	=	enhancement factor
EF of Q_{24}	=	enhancement factor of Q_{24}
EF of Q_s	=	enhancement factor of Q_s
EPC	=	egg yolk lecithin
HPLC	=	high pressure liquid chromatography
J_{max}	=	transepidermal maximum flux
J_{ss}	=	steady state flux
LUV	=	large unilamellar vesicle
mg	=	milligram
min	=	minute
ml	=	milliliter
MLV	=	multilamellar vesicle
mm	=	millimeter

MWCO	=	molecular weight cut-off
nm	=	nanometer
PBS	=	phosphate buffered saline
PC	=	phosphatidylcholine
PCL	=	phosphatidylcholine liposomes
PCCHL	=	phosphatidylcholine:cholesterol liposomes
PCE	=	phosphatidylcholine ethosomes
PCCHE	=	phosphatidylcholine:cholesterol ethosomes
PE	=	phosphatidylethanolamine
PG	=	propylene glycol
P_s	=	permeability coefficient
PTU	=	propylthiouracil
Q_{24}	=	cumulative PTU amount in the receiver at 24 hours
Q_s	=	PTU amount in the skin
RF	=	relative flux
rpm	=	revolution per minute
SC	=	stratum corneum
SD	=	standard deviation
SEM	=	standard error of means
SN	=	Span®20 niosomes
SUV	=	small unilamellar vesicle
µg	=	microgram
µl	=	microliter
µm	=	micrometer
M	=	molar
v/v	=	volume by volume
w/w	=	weight by weight

CHAPTER I

INTRODUCTION

Recently, the skin has become an increasingly popular route of drug administration, owing to several advantages over other routes (Roberts, Cross, and Pellett, 2002; Honeywell-Nguyen and Bouwstra, 2005; El Maghraby, Williams, and Barry, 2006). Firstly, transdermal and dermal delivery circumvents the hepatic first pass metabolism and the gastrointestinal tract side effects. Secondly, it avoids the variables that could influence gastrointestinal absorption such as pH, food intake and gastric emptying. Furthermore, transdermal route provides a more-controlled delivery, thus reducing the variation in plasma drug concentration. Finally, it can improve the patient compliance because of the reduced frequency of administration as well as the noninvasiveness in its nature. Unfortunately, not all drugs can enter the body via this route due to the barrier nature of skin.

There are many techniques to improve transdermal and dermal delivery. One of these techniques is using lipid vesicles as drug carriers (Barry, 2001). Vesicles or vesicular systems have been investigated for better delivery of drugs through/into the skin for a long time. Mezei and Gulasekharam (1980) first reported that liposomes increased the deposition of triamcinolone in the skin and decreased the percutaneous absorption of the drug. Subsequently, many research works with respect to vesicles were published. Examples of success are ubiquitous. For example, the skin lipid liposomes provide effective transdermal delivery of incorporated triamcinolone acetonide (Yu and Liao, 1996). In addition, Fang et al., (2001) found that the optimized liposomes and niosomes enhanced delivery of enoxacin across the skin. Touitou et al. (2000) introduced ethosomes as a novel vesicular carrier system. Ethosomes increase delivery of many drugs via the skin in terms of both quantity and depth of drug deposition (Dayan and Touitou, 2000; Lodzki et al., 2003; Dubey, Mishra, and Jain, 2007; Dubey et al., 2010).

In general, vesicles could be classified according to their main components as phospholipid-based vesicles and nonionic surfactant-based vesicles. As drug

delivery systems, the vesicles may be recognized, according to their composition and/or expected function, as liposomes, niosomes, ethosomes, and Transfersome®. These vesicular systems have different efficiencies in facilitating drug delivery to the skin, which may be due to differences in their physicochemical properties. Many factors regarding vesicular systems are known to affect permeation of drug into the skin: for example, lipid composition, transition state of the bilayer, size, charge, and lamellarity (Sinico et al., 2005; Elsayed et al., 2007). These factors have to be optimized to yield suitable skin permeation when a vesicular formulation is under development. In several studies, the vesicles were successfully designed for an effective skin delivery but the mechanism of enhancing skin permeation of these carriers was hardly investigated.

Most of the previous works that involved skin delivery from vesicular systems only suggest the possible mechanisms without further investigation on any particular one (El Maghraby, Williams, and Barry, 1999; Honeywell-Nguyen and Bouwstra, 2003). One reason for this lack of scientific evidence is that many parameters may be involved. The information on delivery mechanism of vesicles is likely to depend on the physicochemical properties of the entrapped drug, the physicochemical properties of the vesicular system, and the experimental conditions including the skin model and the application condition. Some reported mechanisms of vesicles to improve drug delivery via the skin are diffusion of free drug, penetration enhancement, mixing of vesicles with the skin lipids, skin penetration by intact vesicles, and mechanism involving the transepidermal osmotic gradient (El Maghraby, Barry, and Williams, 2008). Understanding how the vesicles work is beneficial, however, for further optimization and development of the product as well as in application of these systems to deliver other active moieties to the skin. For example, the penetration enhancement is a relatively non-specific mechanism. If it is the major mechanism, the vesicular systems can be reasonably expected to be applicable to other drugs with a high success rate. On the other hand, if the mixing of the vesicles with the skin lipid matrix is the predominant mechanism, other modification may be needed. In this case, the parameters that might affect drug entrapment, as well as the choice of vesicular lipid composition, must be carefully considered during the development and the production processes.

To investigate the formulation factors and the mechanisms involved in drug delivery via the skin with vesicular systems, the model drug used in this study was propylthiouracil (PTU). PTU is an antithyroid drug with an antiproliferative activity. It has been used in the treatment of psoriasis, both orally and topically (Elias et al., 1993; Elias et al., 1994). The problem of PTU is its low solubility in various solvents. Thus, it is difficult to formulate PTU into an effective preparation for skin delivery using conventional dosage forms. The vesicles can serve as solvent for the solubilization of poorly soluble drugs due to their amphiphilic structure. There are some previous studies regarding PTU formulation and successful delivery of the drug by vesicular systems. Phospholipid-based liposomes successfully deliver PTU into various cell types. Rattana Rattanatraiphop (2000) found that phosphatidylcholine (PC) liposomes containing PTU could inhibit BALB/c mouse 3T3 fibroblast proliferation better than the drug solution. On the other hand, Puapermpoonsiri, Lipipun and Vardhanabhuti (2005) reported that co-incubation of empty PC liposomes and PTU solution synergistically retarded the human histiocytic U-937 cell growth. In addition, PTU niosomes was introduced by Suwakul, Ongpipattanakul, and Vardhanabhuti (2006). The niosomal formulations were reported to significantly improved PTU permeation into the newborn pig skin (Waraporn Suwakul, 2005). The possible mechanisms of niosomes in enhancing PTU permeation were also proposed.

Ethosomes would be a good candidate to deliver PTU into/through the skin. Based on the previous works on PTU vesicular systems, ethosomes were expected be able to increase PTU solubility as well as to enhance PTU skin permeation. Ethosomal systems have been used as drug carriers for both hydrophobic and hydrophilic drugs (Dayan and Touitou, 2000; Ainbinder and Touitou, 2005; Mura, Maestrelli et al., 2007; Fang et al., 2009). A few studies elucidated the mechanism of ethosomal systems in drug transport to the skin (Godin and Touitou, 2004; Elsayed et al., 2006). However, there is no study on ethosomal systems as a carrier system for skin delivery of lyophobic drugs such as PTU. Therefore, the present study focused on investigating skin delivery of PTU by ethosomes in comparison with other widely known vesicular systems such as liposomes and niosomes. The mechanisms by which the vesicular systems operated in delivery of PTU into and through the skin were also investigated. The outcomes obtained from

this study could expand our understandings in the mechanism of skin delivery by vesicular systems. In addition, the information was also useful in further development of PTU vesicular systems for clinical use.

Objectives

The specific objectives of this study were as follows:

1. To prepare and characterize PTU liposomes, niosomes, and ethosomes
2. To study skin delivery of PTU from liposomes, niosomes, and ethosomes
3. To study the effects of vesicular structure on PTU skin delivery of vesicular systems with superior delivery characteristics
4. To propose the probable mechanism(s) of PTU skin delivery for the selected vesicular systems.

CHAPTER II

LITERATURE REVIEW

1. Vesicular systems as drug delivery systems for the skin

The problem with skin delivery is the natural barrier of the skin. An important barrier is the stratum corneum (SC). The SC is the outermost layer of the skin to protect the body from the environment. The SC, which is composed of corneocytes and intercellular lipids, has a low permeation for drugs. There are many approaches to increase drug permeation into and across the SC. These include modification of the SC, circumvention of the SC, use of vesicles and particles as delivery systems, and electrically assisted delivery techniques (Barry, 2001). Vesicles or vesicular systems are spherical particulate dispersion, typically consisting of amphiphilic molecules that can form concentric bilayers alone or with cholesterol (CH) in an excess of solvent (water or an aqueous medium). In general, vesicles are classified according to their main components as phospholipid-based vesicles and nonionic surfactant-based vesicles. As drug delivery systems, the vesicles may be recognized, according to their compositions and/or expected functions, as liposomes, niosomes, ethosomes, and Transfersome®. Use of vesicular systems is a controversial approach because their efficiencies in facilitating drug delivery to the skin are rather different. Some reports attribute success of the vesicles in skin delivery to a localizing effect whereby vesicular systems accumulate drugs in SC or other upper skin layers (Mezei and Gulasekharam, 1980; Egbaria and Weiner, 1990; Michel et al., 1992; Touitou et al., 1994; Fresta and Puglisi, 1997; Sinico et al., 2005; Manconi et al., 2006). On the other hand, many investigations reported that the vesicular systems transported drugs through the skin (Guo et al., 2000; Agarwal, Katare, and Vyas, 2001; Carafa, Santucci, and Lucania, 2002; Fočo, Gašperlin, and Kristl, 2005). The difference in drug delivery efficiency of vesicular systems is due largely to the physicochemical characteristics of the vesicular systems and of the drugs studied. The compositions of vesicular systems affect the physicochemical properties of the

vesicles and consequently influence the skin delivery. A suitable composition of vesicular system is necessary to achieve an optimal skin delivery of drugs.

1.1 Dermal delivery

Vesicular systems can improve drug deposition within the skin at the site of action, where the goal is to reduce systemic absorption and thus minimize side effects. The first report on topical administration of vesicular systems was published in 1980. Mezei and Gulasekharam (1980) reported that topical application of triamcinolone acetonide-loaded liposomes resulted in the drug deposition in epidermis and dermis four times higher than that obtained with a control ointment. The drug concentration decreased in the internal organs, the site of its adverse effects. Afterwards, a large number of researchers studied the vesicular systems as drug carriers for topical delivery. The vesicular systems are used to deliver both lipophilic and hydrophilic compounds into the skin. For example, the lipophilic drugs delivered include tocopherol nicotinate (TN), hydrocortisone, betamethasone, triamcinolone acetonide, tretinoin, and minoxidil (Michel et al., 1992; Fresta and Puglisi, 1997; Sinico et al., 2005; Manconi et al., 2006; Mura, Pirot et al., 2007). A few examples of hydrophilic drugs are caffeine and 5-fluorouracil (5-FU) (Touitou et al., 1994; El Maghraby, Williams, and Barry, 2001a).

Several lines of scientific evidence indicate that vesicular systems can improve skin delivery of hydrophobic compounds. Liposomal systems can increase skin deposition of hydrophobic compounds better than conventional formulations such as gels or ointments (Michel et al., 1992; Fresta and Puglisi, 1997). Similarly, tretinoin and minoxidil depositions in the skin are also raised when applied in the form of drug-loaded liposomes (Sinico et al., 2005; Mura, Pirot et al., 2007). Besides the liposomal systems, the niosomal systems are able to facilitate tretinoin deposition in the skin (Manconi et al., 2006).

Enhanced dermal delivery of hydrophilic drugs is also reported. Touitou et al. (1994) studied skin permeation of caffeine from liposomal systems. They found that the liposomal systems, especially small vesicles with high PC:CH ratio, enhanced caffeine accumulation in the skin. In addition, El Maghraby et al. (2001a) investigated in vitro skin delivery of 5-FU from ultradeformable and various traditional liposomes.

They reported that the skin deposition of 5-FU from ultradeformable liposomes was greater than that from all traditional liposomes and the corresponding control solution.

The evidence described above indicates that vesicular systems are efficient drug delivery systems for dermal delivery.

1.2 Transdermal delivery

Though the skin can be a route for drug administration, it is sometimes not the target site of action. Many research works have been aimed at improving systemic absorption of drug by transdermal delivery. Although most studies dealing with conventional liposomes focus on enhancement of drug deposition in the skin, some studies indicate improvement in transdermal delivery with vesicular systems. Agarwal et al. (2001) evaluated skin permeation of dithranol entrapped in liposomal and niosomal systems. They found that both vesicular systems could enhance dithranol delivery through mouse skin. Carafa et al. (2002) also investigated in vitro skin permeation of lidocaine and lidocaine hydrochloride from Tween®20 niosomes or PC liposomes. They reported that lidocaine hydrochloride permeation through mouse abdominal skin from Tween®20 niosomes showed a higher flux and a shorter lag time relative to PC liposomes. Lidocaine permeation rates, on the contrary, were quite similar between niosomes and liposomes. Moreover, Fočo et al. (2005) determined the enhancement effect of liposomes on sodium ascorbyl phosphate percutaneous absorption. Liposomal systems consisting either of hydrogenated PC or of non-hydrogenated PC with CH could enhance permeation of sodium ascorbyl phosphate through the human abdominal epidermis.

Recently, novel vesicular systems that are superior to conventional vesicular systems have been introduced. There are many studies comparing the improvement in transdermal delivery between conventional and novel vesicular systems such as ultradeformable liposomes and ethosomes. Cevc and Blume (1992) introduced a new class of ultradeformable liposomes that have been termed Transfersome®. They claimed that ultradeformable liposomes could penetrate intact into the deeper layers of the skin and might progress far enough to reach the systemic circulation when applied under the non-occlusive condition. Guo et al. (2000) compared in vitro and in vivo skin delivery of cyclosporin A between flexible

(ultradeformable) and conventional liposomes. The flexible liposomes comprised of sodium cholate and PC transported cyclosporin A into the blood while conventional liposomes failed to do so. In addition, elastic surfactant vesicles composed of sucrose laurate ester, octaoxyethylene laurate ester, and sulfosuccinate (L-595:PEG-8-L:sulfosuccinate) show higher efficiency in transdermal delivery of pergolide (Honeywell-Nguyen and Bouwstra, 2003). Similarly, ethosomes can improve transdermal delivery of several drugs and active molecules. These compounds include minoxidil (Touitou et al., 2000; López-Pinto, González-Rodríguez, and Rabasco, 2005), trihexyphenidyl HCl (Dayan and Touitou, 2000), cannabidiol (Lodzki et al., 2003), bacitracin (Godin and Touitou, 2004), testosterone (Ainbinder and Touitou, 2005), erythromycin (Godin et al., 2005), ammonium glycyrrhizinate (Paolino et al., 2005), ketotifen (Elsayed et al., 2006), melatonin (Dubey et al., 2007), methotrexate (Dubey, Mishra, Dutta et al., 2007), salbutamol sulfate (Bendas and Tadros, 2007), lamivudine (Jain et al., 2007), benzocaine (Mura, Maestrelli et al., 2007), Hepatitis B surface antigen (Mishra et al. 2008), 5-aminolevulinic acid (Fang et al. 2008), and fluconazole (Bhalaria, Naik, and Misra, 2009). Besides improving transdermal delivery of these drugs, ethosomal systems can also increase skin accumulation of some drugs such as melatonin, metrotrexate, and 5-aminolevulinic acid (Dubey et al., 2007; Dubey, Mishra, Dutta et al., 2007; Fang et al. 2008).

Liposomal systems with low concentrations of ethanol in the formulation also enhance both transdermal and dermal drug delivery (Verma and Fahr, 2004). The systems composed of NAT 8539, a commercial lipid mixture, with various ratios of ethanol (3.3-20%) were evaluated for a potential to improve skin delivery of cyclosporin A. Increase in cyclosporin A delivery through/into the human stratum corneum was evident.

2. Factors affecting skin delivery

Although the use of vesicular systems with proper components should increase drug delivery into and through the skin, many questions still arise about factors that can affect skin delivery of these formulations. Therefore, much effort has been put toward investigating the factors that can affect drug delivery to and across the skin from vesicular systems. Some factors that have been studied are the

thermodynamic state of vesicles, vesicle size and lamellarity, application condition, and the existence of vesicular structure.

2.1 Thermodynamic state of vesicles

The thermodynamic state of vesicles depends on the composition of the vesicular system, especially the bilayer components. The vesicular systems may be classified according to the thermodynamic state as liquid crystalline, gel or rigid, and elastic or ultradeformable. Many studies have compared the potential of vesicular systems with different thermodynamic states in delivering drugs through and/or into the skin. To evaluate the skin delivery by vesicles with different thermodynamic states, researchers often directly compare drug permeation through and/or into the skin (Hofland, van der Geest et al., 1994; El Maghraby et al., 1999; Fang et al., 2001). Investigation of vesicle-skin interaction is sometimes carried out to elucidate underlying mechanisms (van Kuijk-Meuwissen, Junginger, and Bouwatra, 1998; van den Bergh, Salomons-de Vries, and Bouwstra, 1998; Touitou et al., 2000). To compare drug permeation, several methods can be used such as in vitro transport study using Franz diffusion cells and skin stripping (du Plessis, Ramachandran et al., 1994; du Plessis, Weiner, and Müler, 1994; Sinico et al., 2005; Manconi et al., 2006). To detect vesicle-skin interaction, techniques such as transmission electron microscopy, freeze fracture electron microscopy, confocal laser scanning microscopy (CLSM), small-angle X-ray scattering, and resonance energy transfer are useful (Kirjavainen et al., 1996; van Kuijk-Meuwissen et al., 1998; van den Bergh et al., 1998; Kirjavainen et al., 1999; Coderch et al., 2000).

In general, vesicles in the liquid crystalline state are superior in delivering drugs through/into the skin when compared to those in the gel state. Hofland, van der Geest et al. (1994) studied in vitro permeation of estradiol from niosomes with various thermodynamic states through human stratum corneum. The gel state niosomes were composed of $C_{18}EO_3$ and the liquid crystalline vesicles consisted of $C_{12}EO_3$ and $C_{9=9}EO_{10}$. All formulations were saturated with estradiol, making the thermodynamic activity equal among these formulations. They reported that the effect of niosomes on estradiol transport through the skin could be either impairment (gel state vesicles) or enhancement (liquid state vesicles). Yu and Liao (1996) reported

that permeation of triamcinolone acetonide from liposomes across rat skin was higher than that from a commercial ointment. They also reported that the permeation of triamcinolone from the liquid crystalline PC liposomes was significant higher than that from the gel-state dipalmitoylphosphatidylcholine (DPPC) liposomes. Agarwal et al. (2001) reported that the gel-state Span®60 niosomes decreased dithranol delivery across the skin compared with the PC-based liposomes. Fang et al. (2001) also found that the liquid crystalline liposomal systems composed of soybean or egg PC not only improved transdermal delivery of enoxacin but also increased enoxacin accumulation in nude-mouse skin compared with the gel-state dimyristoylphosphatidylcholine liposomes.

From the above-mentioned studies, it seems very clear that the thermodynamic state of the bilayer of the vesicles plays an important role in the performance of vesicular system on drug delivery through/to the skin, both in vitro and in vivo. However, the thermodynamic state of the bilayer might not be the only concern for skin delivery by vesicular systems. Other factors may confound the effect of thermodynamic state on skin delivery from vesicles. For example, liposomes consisting of skin lipids in the gel state significantly may enhance drug permeation better than other types of liposomes due to its easy miscibility with skin lipids. Skin lipid liposomes provide superior corticosteroids accumulation in the human skin compared with phospholipid-based liposomes (Fresta and Puglisi, 1997). The enhancement effect of skin lipids has been attributed to better mixing of skin lipids with the intercellular lipid matrix of the skin. Fang et al. (2001) also found that the gel-state, Span®-based niosomes increased enoxacin delivery both through and into the skin. The increase in enoxacin delivery might come from the enhancing effect of the non-ionic surfactant, which was the major component of the niosomes.

In the last decade, elastic vesicular systems and fluidized vesicular systems have been of interest to researchers. Besides being in the liquid crystalline state, these vesicles contain surfactants that can act as edge activators. Edge activators allow the vesicles to be more malleable under pressure. These elastic vesicular systems are thought to carry drug molecules across the skin as intact vesicles. Some studies examined the drug transport to the skin from vesicular systems in liquid crystalline, gel, and elastic states (El Maghraby et al., 1999; El Maghraby et al., 2001a). The

researchers investigated transport of estradiol and 5-fluorouracil as model compounds for lipophilic and hydrophilic drugs, respectively. They found that the elastic vesicles provided the greatest drug permeation through the skin for both compounds. In vivo studies also support the superiority in skin delivery of elastic vesicles. Guo et al. (2000) investigated transdermal delivery of cyclosporin A from lecithin vesicular systems with and without sodium cholate. They assessed the enhancing effect of these vesicular systems on the penetration of cyclosporin A by both in vitro and in vivo studies. The in vitro study revealed that the deformable vesicles transported cyclosporin A through and into mouse skin, but the traditional vesicles only increased cyclosporin A deposition in the skin when compared with the control solution. Furthermore, the in vivo studies indicated that the deformable vesicles could transfer cyclosporin A into the blood while the traditional vesicles could not.

Vesicles in the liquid crystalline state can be further modified for better skin delivery. The presence of ethanol increases the fluidity of the lipid bilayer (Touitou et al., 2000). Ethanol containing vesicles (termed ethosomes by the inventors) improve the transdermal and dermal delivery of various drugs. The results of several studies indicate that ethosomes are superior to conventional liposomes in skin delivery, both in the gel state and in the liquid crystalline state. López-Pinto et al. (2005) studied minoxidil skin permeation from gel-state DPPC liposomes and from ethosomes containing the same lipid components. They found that the ethosomal systems were much more efficient at delivering minoxidil through the skin than either the liposomal systems or the control hydroalcoholic solution. Similar results were obtained from other research groups that studied skin permeation from liquid crystalline state liposomes and ethosomes. Dubey, Mishra, Dutta et al. (2007) investigated dermal and transdermal delivery of an anti-psoriatic agent via ethosomes. They reported that the PC ethosomal systems could enhance methotrexate delivery through the human cadaver skin and increase skin deposition compared with PC liposomes and the corresponding reference solution. Bhalaria et al. (2009) carried out sequential studies comparing in vitro skin permeation and clinical efficacy of dermatological gels containing fluconazole-loaded ethosomes or liposomes. The results clearly revealed that the ethosomal system was superior to the other tested preparations, including a marketed fluconazole cream.

2.2 Vesicle size and/or lamellarity

Many researchers studied the effect of vesicle size and/or lamellarity on skin delivery. du Plessis, Ramachandran et al. (1994) showed that smaller size (60 nm) liposomes prepared from PC did not result in higher levels of radiolabelled cyclosposin A and cholesteryl sulfate in the deeper skin layer of hairless mouse, hamster, and pig skins. However, they found that intermediate size (300 nm) liposomes provided both the highest reservoir in the deeper skin layer of hairless mouse and hamster skin and the highest drug concentration in the receiver compartment of the diffusion cells. They suggested that the follicular route might be responsible in drug transfer into and through the skin. Similarly, Hofland, van der Geest et al. (1994) also found that multilamellar vesicles (MLV, > 1 μm) prepared from $C_{9=9}EO_{10}$ gave higher fluxes of estradiol across human skin than small unilamellar vesicles (SUV, 100-200 nm) did. The effects of vesicle size and lamellarity are also supported by other studies. Sinico et al. (2005) studied the effect of vesicle size on skin delivery of tretinoin from PC-based liposomal systems. They found that large unilamellar vesicle (LUV, < 300 nm) was better than MLV (> 500 nm) in increasing skin accumulation of tretinoin. Manconi et al. (2006) also found that LUV (80-230 nm) niosomes enhanced tretinoin delivery into newborn pig skin better than MLV (200-400 nm) niosomes did. Verma et al. (2003a) showed the effect of vesicle size on permeation of hydrophilic and lipophilic fluorescent compounds from deformable vesicles consisting of PC and sodium cholate across human skin using the skin stripping and the CLSM method. The results revealed that permeation of both compounds was inversely related to the size.

In contrast, Yu and Liao (1996) found that the size of MLV (0.2-1 μm) liposomes prepared from DPPC or PC did not show any significant effect in skin permeation and retention of triamcinolone acetonide. Touitou et al. (1994) also found that caffeine permeation through the skin obtained from small (40 nm) and large unilamellar liposomal vesicles composed of PC and CH (600 nm) were comparable. However, the composition as well as the size might also play a role in skin deposition of caffeine in this case since the results revealed the effect of size only when the CH content was low. At a low CH content, small vesicles seemed to deposit the drug into the skin better than larger ones. In addition, the physicochemical properties of the

active compounds may supersede the effect of size. Michel et al. (1992) investigated the effect of liposomal size on in vivo penetration of TN and 2-(t-butyl)-4-cyclohexylphenylnicotinate N-oxide (L44O) from drug-loaded liposomes that were incorporated into Carbopol® gels using the skin stripping method. For TN, SUV (50.6 nm) was superior to MLV (242.4 nm). For L44O, penetration from SUV (22.6 nm) and MLV (122.9 nm) was comparable.

2.3 Application condition

When the liposomal system was first used as drug carrier for the skin, it was applied under the non-occlusive condition (Mezei and Gulasekharam, 1980). However, hydration of stratum corneum is known to increase drug permeation through/into the skin (Barry, 2001). Thus, the effect of application condition on skin delivery of vesicular systems drew interest from several researchers. The result seems to depend largely on the type of vesicular systems studied.

Michel et al. (1992) evaluated the effect of application condition on in vivo penetration of TN from liposomal gels. They reported that the occlusive condition yield a much higher degree of saturation of stratum corneum with TN. Most researchers, however, worked with conventional vesicular systems under the non-occlusive condition (du Plessis, Ramachandran et al., 1994; Fresta and Puglisi, 1997; van Kuijk-Meuwissen et al., 1998; Tabbakhian et al., 2006).

The scenario is rather different with elastic/deformable vesicular systems. Cevc and Blume (1992), who introduced the deformable vesicles under the name of Transfersomes®, suggested that these deformable vesicles be applied under the non-occlusive condition in order to result in the systemic absorption of the incorporated compound. Some other researchers have also studied drug permeation from elastic or ultradeformable vesicular systems under the non-occlusive and the occlusive conditions. For ultradeformable liposomes, Guo et al. (2000) studied the effect of hydration on cyclosporin A skin delivery from liposomes consisting of PC and sodium cholate. They applied the ultradeformable liposomes containing cyclosporin A onto the pre-hydrated mouse skin and untreated skin. They found that the ultradeformable liposomes failed to transport the drug through the pre-hydrated mouse skin but could improve skin deposition of the drug. In addition, El Maghraby

et al. (2001a) investigated in vitro skin delivery of 5-FU from ultradeformable and various traditional liposomes under the non-occlusive and the occlusive conditions. They reported that under the non-occlusive condition both ultradeformable and traditional liposomes improved estradiol skin delivery, but ultradeformable liposomes were superior. On the contrary, the occlusive condition reduced skin delivery from both types of vesicular systems. For elastic niosomes, Honeywell-Nguyen and Bouwstra (2003) examined skin penetration of pergolide under the non-occlusive condition compared with the occlusive condition. The elastic niosomes improved the skin delivery of pergolide under the non-occlusive condition compared with the control solution. Under the occlusive condition, however, the enhanced delivery from the elastic niosomes was nullified. Pergolide transport from the control solution was much improved due to excessive skin hydration from occlusion. Thus, for elastic and deformable vesicular systems, the osmotic gradient across the skin under the non-occlusive condition is proposed to be the driving force for drug transport (Cevc and Blume, 1992).

For ethosomes, however, the effect of application condition is inconclusive. The enhancement in skin transport of different drugs was reported under both the non-occlusive and the occlusive conditions (Dayan and Touitou, 2000; Lodzki et al., 2003; Ainbinder and Touitou, 2005; Paolino et al., 2005; López-Pinto et al., 2005; Elsayed et al., 2006; Dubey, Mishra, and Jain, 2007; Fang et al., 2008). Thus, the mechanism of skin penetration enhancement of ethosomes is proposed to be different from that of the elastic/deformable vesicular systems (Touitou et al., 2000).

2.4 Existence of vesicular structure

The existence of vesicular structure is generally crucial in enhancing drug penetration into and/or through the skin. Most studies agree that the vesicular system is better than the mixture of the vesicular components in dermal and transdermal delivery of active compounds.

Two groups of researchers reported similar results with regard to the importance of vesicular structure in skin delivery. El Maghraby, Williams, and Barry (2000) studied the necessity of liposomal structure in permeation of estradiol across the human skin by comparing drug permeation from lipid solution in 90% w/w

propylene glycol (PG) with that from the corresponding liposomes. They found that all liposomes in the vesicular form gave greater relative fluxes than the corresponding lipid solution. They concluded that it was important to prepare the phospholipid as vesicles for efficient estradiol skin delivery. Fang et al. (2001) studied transport of enoxacin across nude mouse skin from soybean PC liposomes, Span®60 niosomes, and physical mixtures of the components from corresponding vesicular systems. The results demonstrated that the Span®60 physical mixture had a significantly lower permeation than the Span®60 niosomes. The permeation of enoxacin from soybean PC physical mixture, however, was comparable to that from the corresponding liposomes. In addition, vesicles seem to be a better skin delivery system than some other colloidal structures such as micelles. Guo et al. (2000) compared cyclosporin A deposition in the mouse skin from vesicular systems and micelles. The lipid phase of the vesicular systems were composed of PC and PC:sodium cholate. The micelles were formulated with sodium cholate both at the same concentration as that in the vesicles and at a higher concentration. They found that both formulas of micelles did not enhance the skin penetration of cyclosporin A with respect to the control solution. The retained amounts of cyclosporin A in the skin from micelles were considerably lower than the amounts obtained from the vesicular systems. Similarly, Carafa et al. (2002) showed that the permeation rates of lidocaine and lidocaine hydrochloride from drug dispersions in the micellar form were lower than the rate from the corresponding liposomes and niosomes.

All these findings indicate that the existence of vesicular structure of liposomes and niosomes are necessary in skin delivery of several drugs. However, such scientific evidence is still lacking for ethosomes.

3. Mechanisms in dermal and transdermal delivery from vesicular systems

There are many studies attempting to investigate the mechanisms involved in skin transfer of active compounds. The mechanism regarding dermal and transdermal delivery of vesicular systems is system specific. Several probable mechanisms have been proposed. These include penetration of intact vesicles into the skin, diffusion of free drug into and through the skin, mixing of the vesicles with the intercellular lipid matrix, and penetration enhancement by the vesicles (Schreier and

Bouwstra, 1994; Choi and Maibach, 2005; El Maghraby et al., 2006; Elsayed et al., 2007; El Maghraby et al. 2008).

3.1 Penetration of intact vesicles into the skin

The mechanism of dermal and transdermal delivery from vesicular systems involving penetration of drug-loaded vesicles intact into the stratum corneum and deeper skin layers has been suggested since early 1980. However, the arguments about this hypothesis have also arisen due to the doubt about penetration of large lipid vesicles into densely packed stratum corneum. According to this mechanism, the smaller vesicle size should result in an increase in drug transport through/into the skin (El Maghraby et al., 2006). However, at least for conventional vesicles, there is scientific evidence that indicates otherwise. du Plessis, Ramachandran et al. (1994) reported that the smaller liposomal particle size did not result in higher cyclosporin A levels in the deeper skin strata of any of the skin species studied (hairless mouse, hamster, and pig skin). On the contrary, the bigger sizes resulted in the highest reservoir in the deeper skin strata as well as the highest drug concentration in the receiver. Thus, they concluded that the mechanism for topical liposomal drug delivery did not involve the passage of intact liposomes into the skin. Though in some other studies vesicles with smaller sizes resulted in better topical skin delivery (Sinico et al., 2005; Manconi et al., 2006), there has been no evidence to support that the vesicles can penetrate the skin intact. However, the species of the experimental animal and the physicochemical properties of the model compound used in these studies might disconcert the interpretation of their results.

On the other hand, this mechanism of skin delivery may be plausible for elastic/deformable vesicles. Cevc and Blume (1992) proposed that ultradeformable liposomes could pass through the stratum corneum as intact structures. To prove that the intact vesicle of deformable liposomes could penetrate the skin, El Maghraby et al. (1999) compared the transepidermal maximum flux (J_{max}) of estradiol permeation from large MLV and SUV deformable liposomes. They found that there was no significant difference in the relative J_{max} between SUV and large MLV. This finding does not support the hypothesis that the intact deformable liposomes could pass the stratum corneum. Verma et al. (2003a) investigated the influence of deformable

liposomal size on transport of hydrophilic and lipophilic fluorescent compounds into the human skin using the Franz diffusion cells and CLSM. The smaller size of deformable liposomes facilitated skin delivery of both hydrophilic and lipophilic fluorescent compounds, but still with no clear evidence to support that the liposomes could pass intact into deeper layers of the skin.

Touitou et al. (2000) have proposed the mechanism of ethosomal systems. According to the proposed scenario, ethanol disturbs the organization of the stratum corneum lipid bilayers and enhances its fluidity. The flexible ethosomal vesicles then penetrate the disturbed stratum corneum bilayers and even forge a pathway through the skin by virtue of their particulate nature. Godin and Touitou (2004) later investigated the dermal delivery of bacitracin, an antibiotic peptide, from an ethosomal system. They used the fluorescent-labeled bacitracin and PC to clarify the mechanism. They reported the co-localization of the drug and the phospholipid in the skin.

In light of the above reports, there are not sufficient evidences that the vesicular systems can pass into the skin as an intact structure.

3.2 Diffusion of free drug

Ganesan et al. (1984) and Ho et al. (1985) demonstrated that neither liposomes nor phospholipid molecules diffused through intact skin of hairless mice. They suggested three probable mechanisms for skin delivery from vesicles (Figure 1). The first mechanism involved release of liposome-entrapped solutes and percutaneous absorption of the free solutes. The other one was this first mechanism coupled with direct liposome/skin solute transfer. The last one focused only on liposome/skin solute transfer. They also concluded that the first mechanism would be applicable to hydrophilic drugs entrapped in the aqueous phase of the liposomes such as glucose. This proposed scheme was based on the evidence that permeation of glucose depended on release rate. For lipophilic drugs associated with the lipid bilayers, such as progesterone, a direct liposome/skin transfer mechanism was proposed, based on the very slow release rate of these compounds. For lipophilic drugs with relatively high release rates like hydrocortisone, both mechanisms would operate simultaneously.

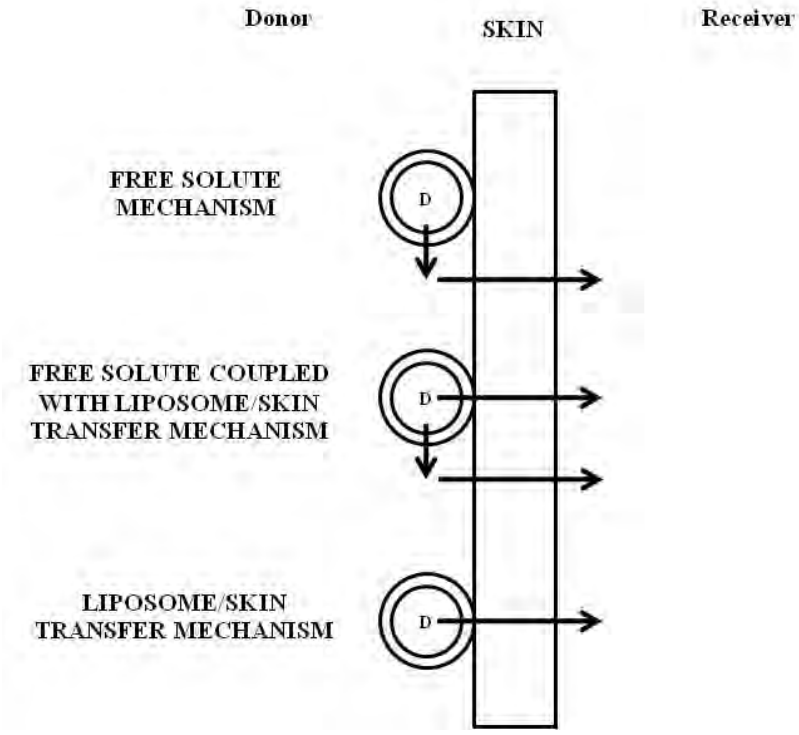

Figure 1: Schematic description of various mechanisms in the skin permeation of drug molecules from liposomes, D = drug (source: Ganesan et al., 1984)

If the mechanism of a vesicular system is solely the diffusion of free solute mechanism (see Figure 1), the rate limiting step of skin transport is then the release rate. However, there are some reports on the lack of relationship between the drug release rate and skin permeation. El Maghraby et al. (1999) found that the peak flux of estradiol from deformable liposomes through human skin occurred at a time during which drug release was negligible. They concluded that the diffusion of free drug mechanism did not operate for all liposomal formulations. Similarly, Montenegro et al. (1996) compared the release of retinoic acid from DPPC liposomes with its permeation across human skin. They reported that the skin permeation from each of various DPPC liposomes and an alcoholic solution was significantly lower than the total amount of the drug released from that formulation.

3.3 Mixing of the vesicles with the intercellular lipid matrix

Hofland, Bouwstra et al. (1994) investigated interaction of liposomes and niosomes with human skin. Human stratum corneum was treated in vitro with various formulas of liposomes and niosomes. The effects of vesicular systems on human stratum corneum were monitored using freeze-fracture electron microscopy, small-angle X-ray scattering, and CLSM. They found that liposomes and niosomes, depending on their composition, interacted with human stratum corneum from a strong to a very mild degree. According to their findings, there are two types of interaction between the stratum corneum and vesicles. First, adsorption and fusion of drug-loaded vesicles onto the surface of the skin leads to a high thermodynamic activity gradient of the drug at the dispersion-stratum corneum interface. Second, the effect of vesicles on the deeper layer of the stratum corneum may lead to changes in drug permeation kinetics due to an impaired barrier function of the stratum corneum for the drug. This interaction might be caused either by an alteration of the intercellular lipid bilayers of the stratum corneum or by inclusion of vesicular components from the interface down into the lipid bilayer region. The first interaction is often seen with gel-state vesicles. For liquid crystalline vesicles, both the first and the second interactions can operate, either separately or simultaneously.

Several research works were focused on skin-vesicle interaction of vesicles with different thermodynamic states and of vesicles with various lipid components (Kirjavainen et al., 1996; van Kuijk-Meuwissen et al. 1998; van den Bergh et al., 1998). The scientific evidence from these studies indicates that the liquid crystalline state liposomes or the flexible liposomes can fuse and mix with the intercellular lipid of the stratum corneum and sometimes mix with the intracellular region. The interaction provides the change of the deeper skin layer and facilitates skin delivery of the drug. In addition, Egbaria et al. (1990) reported that deposition of interferon in the deeper skin strata from liposomes prepared from lipids with a composition similar to the stratum corneum lipid was much higher than the skin deposition seen with liposomes prepared from phospholipids. Fresta and Puglisi (1997) also found that the skin lipid liposomes improved corticosteroid accumulation in the epidermis and the dermis of human skin compared with phospholipid-based liposomes and a conventional ointment. They attributed their finding to the better

mixing of the skin lipid liposomes with the intercellular lipid matrix of the skin due to similar lipid compositions of the liposomes and the skin. Sinico et al. (2005) studied the vesicle-skin interaction between tretinoin liposomes and newborn pig skin by transmission electron microscopy. The electron micrographs revealed different vesicle-skin interactions between the two types of liposomes studied. The Soy PC liposomes that were spreaded onto the corneocyte surface fused with the stratum corneum. On the contrary, the liposomal vesicles from hydrogenated soy PC were still intact on the SC surface. Both types of liposomes, however, caused the ultrastructure changes of the pig skin. El Maghraby et al. (1999) also investigated the interaction of vesicles with the skin by determination of drug uptake in the skin after dipping the skin into the vesicular dispersion and an aqueous solution. They reported that the uptake ratios of the vesicles and the solution were not significantly different among corresponding formulations. They also concluded that one possible mechanism of vesicular skin delivery was adhesion of liposomes onto the skin and subsequent fusion or mixing of liposomes with the skin lipids.

Interaction of any specific vesicular component with the skin is also possible in vesicular delivery via the skin. In a study, when ethanol was present in the donor with egg yolk lecithin (EPC), permeability values of model drugs were substantially increased (Kirjavainen et al., 1999). Confocal microscopy revealed that EPC did not penetrate into the skin from solutions in water, but it penetrated deeply into the stratum corneum from hydroalcoholic solutions. Touitou et al. (2000) suggested that liposomes with high concentrations of ethanol could penetrate the disturbed stratum corneum bilayers. The fluorescence-based methods and microscopy methods have been used to study interactions between ethosomal systems and the skin. Generally, the ethosomal system containing hydrophilic and lipophilic fluorescent probes are prepared. The hydrophilic fluorescent-labeled probe is used to monitor the hydrophilic substance contained in the aqueous core of the vesicles. The lipophilic one is used to monitor the lipophilic substance intercalated in the lipid bilayer of the vesicles. The phospholipids are often labeled to locate the vesicles in the skin. Ethosomal systems successfully improve skin permeation of both hydrophilic and lipophilic compounds (Dayan and Touitou, 2000; Bendas and Tadros, 2007; Jain et al., 2007; Dubey et al., 2007; Dubey, Mishra, Dutta et al., 2007; Mura,

Maestrelli et al., 2007). In most cases, co-localization of the fluorescent probes in the deeper skin lipid region are evident (Touitou et al., 2000; Godin and Touitou, 2004; Dubey et al., 2007; Dubey, Mishra, Dutta et al., 2007; Jain et al., 2007). These findings indicate that ethosomes fuse and mix with the skin lipids.

3.4 Penetration enhancement

According to this mechanism, the vesicular system plays a role as a penetration enhancer in facilitating drug transport through/into the skin. If the vesicular systems act as penetration enhancers, drug permeation can be increased from either pretreatment of the skin with empty vesicles or co-treatment of the skin with empty vesicles and drug solution. Thus, many researchers investigated this mechanism by comparing drug permeation from saturated solution after pretreatment of the skin with empty vesicles with drug permeation from the drug-loaded vesicles. Alternatively, some researchers employed the co-treatment of empty vesicles and drug solution. If drug permeation parameters from both conditions are comparable, penetration enhancement of the vesicles or the lipid components may be the predominant mechanism of skin delivery.

du Plessis, Weiner et al. (1994) reported that hydrocortisone deposition from pretreatment of the hairless mice skin with empty PC-based liposomes or empty skin lipid liposomes negated the advantage of incorporating the drug into liposomes. They found that the amount of hydrocortisone in the receiver from pretreatment of the skin with empty liposomes was much higher than that from drug-loaded liposomes. On the contrary, Hofland, van der Geest et al. (1994) reported that pretreatment of human stratum corneum with empty niosomes provided much lower permeation of estradiol than with direct application of the drug loaded vesicles. Similarly, estradiol permeation through the human skin from traditional and deformable liposomes was higher than that from solution after pretreatment of the skin with empty vesicles (El Maghraby et al., 1999). A similar result was also seen in a study on skin permeation of pergolide from elastic niosomes (Honeywell-Nguyen and Bouwstra, 2003). The researchers found that direct application of elastic vesicles better enhanced drug permeation across human skin in relative to pretreatment with empty vesicles.

The results from co-treatment studies, on the other hand, support the role of vesicles as penetration enhancer. Kim et al. (2002) studied skin permeation of caffeine from treatment of caffeine loaded PC liposomes and co-treatment of empty PC liposomes and caffeine solution. Skin permeations of caffeine from both conditions were comparable. Their results indicate that the PC liposomes can act as a penetration enhancer. This finding is consistent with the report of Elsayed et al. (2006) who found that co-treatment of empty deformable liposomes and ketotifen solution greatly increased skin permeation of the drug. On the contrary, Verma et al. (2003b) investigated human skin penetration of entrapped and non-entrapped hydrophilic carboxyfluorescein (CF) from deformable liposomes. They found that loading the compound into the liposomes enhanced penetration of CF into the human stratum corneum significantly. Similar results were noted by Paolino et al. (2005) who studied in vitro percutaneous permeation of ethosomes containing either methylnicotinate or ammonium glycyrrhizinate through human skin. Ethosomes containing the tested drug were better than the co-treatment of empty ethosomes with drug solution in delivering the drug through the human skin. Elsayed et al. (2006) also reported that PC ethosomes were not able to improve skin delivery of non-entrapped ketotifen.

The results of these studies indicate that the role of vesicles as penetration enhancer depends on the vesicular system as well as the drug to be delivered. It is worth noting that the experimental condition (pretreatment versus co-treatment) can also confound the investigation of this mechanism.

CHAPTER III

MATERIALS AND METHODS

Materials

1 Acetonitrile, HPLC grade (Lab-scan Analytical sciences, Thailand)

2 Ammonium molybdate (crystals) (Mallinckrodt AR® , USA, Lot no. 3420X12465)

3 Chloroform, AR grade (Lab-scan Analytical Sciences, Thailand)

4 Cholesterol (Fluka® Analytical, Japan, Lot no. 1324049)

5 Disodium hydrogen phosphate, analytical reagent (UNIVAR, Australia, Lot no. F2F136)

6 Ethanol, AR grade (Merck, Germany)

7 Fiske-Subbarow Reducer (Fluka BioChemika, Sigma-Aldrich, USA, Lot no. 1195556 and Fluka® Analytical, Sigma-Aldrich, USA, Lot no. 1396442)

8 Hydrogen peroxide (Merck, Germany, Lot no. K32656587 345)

9 Isopropanol, AR grade (Lab-scan Analytical sciences, Thailand)

10 Methanol, HPLC grade (Lab-scan Analytical sciences, Thailand)

11 Potassium dihydrogen phosphate (Merck, Germany, Lot no. A476973)

12 Propylthiouracil, pharmaceutical grade (a gift from Sriprasit Pharma Co. Ltd., Thailand, Lot no. 07052117)

13 Sodium chloride (Merck, Germany, Lot no. TA419536)

14 Solulan® C24 (Amerchol, UK)

15 Soybean phosphatidylcholine (Phospholipon ®90, Nattermann Phospholipid GmbH, Cologne, Germany, Lot no. 770991)

16 Span® 20 (EAC Chemical, Lot no. 16790)

17 Sulphuric acid (J.T. Baker, USA, Lot no. C40029)

18 Theophylline, DMSc reference standard (a gift from the Bureau of Drug and Narcotic, Department of Medical Sciences, Ministry of Public Health, Thailand, Control no. 349019)

19 Ultrapure® water (Elgastat Maxima UF, Elga, England)

Equipment

1 Analytical balances (AX105 DeltaRange®, Mettler Toledo, Switzerland)
2 AL-Crucibles, 40 µl (Mettler Toledo, Switzerland)
3 Dialysis membrane tubing (regenerated cellulose tubular membrane, MWCO = 12000-14000) (CelluSep® T4, Membrane Filtration Products, USA, Lot no. 8764)
4 Differential scanning calorimeter (DSC 822^e, Mettler Toledo, Switzerland)
5 Dry bath incubator (Boekel Scientific, Japan)
6 High performance liquid chromatography system equipped with
   - Prominence degasser (DGU-20A$_3$, Shimadzu, Japan)
   - Prominence liquid chromatography pump (LC-20AD, Shimadzu, Japan)
   - Prominence auto sampler (SIL-20AC HT, Shimadzu, Japan)
   - Prominence diode array detector (SPD-M20A, Shimadzu, Japan)
   - HPLC precolumn insert (µBondapack™ C18, 10 µm, 125 A°, Guard-Pak™, Ireland, Lot no. 020235347A)
   - HPLC precolumn holder (Water Corporation, Ireland)
   - HPLC column (Hypersil® BDS C18, 300 x 4.6 mm, 5 µm, ThermoHypersil, UK, Lot no. 5/120/5826)
7 Hot air incubator (Memmert, Germany)
8 Light microscopes (Eclipse E200, Nikon, and IX51, Olympus, Japan)
9 Magnetic stirrer (M6, Schott, Germany)
10 Membrane filters (Nylon 47 mm, 0.45 µm) (Vertical™, Thailand, Lot no. 50113)
11 Modified Franz diffusion cells (Science Service, Thailand)
12 Mini orbital shaker (S05, Stuart Scientific, UK)
13 Particle size analyzer (Mastersizer 2000, Malvern Instruments, UK)
14 pH meter (420A, Orion, USA)
15 Centrifuge tubes (Polyallomer Bell-top Quick-Seal® 13 x 25 mm) (Beckman, USA, Lot no. P7-05-23, P7-01-29, and P8-09-04)
16 Refrigerated incubator (FOC 225i, VELP® Scientifica, Italy)
17 Rotary evaporator (Buchi, Switzerland)
18 Sonicator (275DAE, Crest, Malaysia)

19 Syringe filters (Nylon membrane, 13 mm, 0.45 µm) (VertiPure™, Thailand, Lot no. 50113)

20 Ultracentrifuge (Optima™ L-100XP, Beckman Coulter, USA)

21 Ultrasonic bath (TP 680 DH, Elma, Germany)

22 UV-Visible spectrophotometer (UV-1601, Shimadzu, Japan)

23 Vacuum pump (Water model D0A-V130-VN, Millipore, USA)

24 Vortex mixer (G560E, Vortex-Genie2, USA)

25 Water baths (Memmert, Germany, and ThermoNESLAB, USA)

Methods

1 Preparation of vesicular systems

1.1 Preparation of blank vesicular systems

1.1.1 Preparation of blank liposomes

Liposomes were prepared by the film-hydration method (New, 1997). The lipid phase of liposomes was composed of soybean phosphatidylcholine (PC) with and without cholesterol (CH). The total lipid concentration was 132 μmoles/ml. The molar ratio of PC:CH was 7:3. The aqueous phase of liposomes was water. Briefly, PC (501 mg) or PC (351 mg) with CH (76.5 mg) was dissolved in 8 ml of chloroform and the solution was transferred to a 1000-ml round-bottomed flask. The organic solvent was evaporated to form a thin lipid film using a rotary evaporator. The thin lipid film was hydrated with 5 ml of the aqueous phase. The hydration time was at least 2.5 hours to allow liposomal vesicles to form completely.

1.1.2 Preparation of blank niosomes

The modified sonication method (Suwakul et al., 2006) was used to prepare niosomes consisting of Span® 20, CH and Solulan® C24 as the lipid phase. The weight ratio of Span® 20:CH:Solulan® C24 in the lipid phase was 28.5:18.6:2.48. This ratio was selected from a previous work (Waraporn Suwakul, 2005) since it had given the best permeation profile of all the formulations studied. The total lipid concentration of niosomes was also kept at 132 μmoles/ml. The components of the lipid phase (28.5 mg of Span® 20, 18.6 mg of CH and 2.48 mg of Solulan® C24) were accurately weighed, mixed in a 10 ml glass tube, and then melted in a dry bath incubator at 130 °C. An aliquot of the aqueous phase (1 ml), which had previously been warmed and kept at 70 °C, was added to the melted lipid mixture. The mixture was immediately sonicated at 70 °C for 10 minutes using an ultrasonic bath at 140% power and then vortexed for 1 minute. The resultant niosomal dispersion was allowed to cool down at room temperature.

1.1.3 Preparation of blank ethosomes

The lipid phase of ethosomes was composed of either PC or PC:CH (7:3), similar to that of liposomes. The aqueous phase was 30% w/w ethanol. Different methods of preparation were used for the two compositions of ethosomes. The method modified from earlier works on ethosomes (Touitou et al., 2000; Paolino et

al., 2005) was used for the PC-only ethosomes. Because CH could not sufficiently dissolve in ethanol, the film-hydration method was applied for the PC:CH ethosomes.

1.1.3.1 PC ethosomes

PC ethosomes were prepared by a rapid injection of the water phase (668 µl of water) into an ethanolic solution of PC (100 mg PC in 360 µl of absolute ethanol) with continuous stirring at 800 rpm, 35 °C for 10 minutes in a closed water-jacketed glass cell (see Appendix A). The final concentration of ethanol in the system was 30% w/w.

1.1.3.2 PC:CH ethosomes

PC:CH ethosomes were prepared by the film-hydration method modified from López-Pinto et al. (2005). PC (70.2 mg) and CH (15.6 mg) were dissolved in chloroform (0.6 ml) and the solution was transferred into a water-jacketed glass cell. Chloroform was removed with a vacuum pump for 45 minutes, during which the glass cell was being shaken on an orbital shaker at 150 rpm to aid solvent evaporation. The dried lipid film was hydrated with 30% w/w ethanol with continuous stirring at 800 rpm, 35 °C for 10 minutes.

1.2 Determination of saturation solubility of propylthiouracil (PTU)

1.2.1 Saturation solubility of PTU in 30% w/w ethanol

The solubility of PTU in 30% w/w ethanol was determined by continuous shaking of an excess amount of PTU in 30% w/w ethanol at ambient temperature. The samples were withdrawn at 1, 4, 6, and 9 days and filtered through syringe filters (0.22 µm) to eliminate drug crystals. The filtrate was appropriately diluted and analyzed by UV spectrophotometry at 275 nm.

1.2.2 Saturation solubility of PTU in the vesicular systems

The maximum amount of PTU that could be incorporated in each vesicular system was determined by titration. PTU at various amounts was added to the vesicular preparations. The maximum amount of PTU that could be added to the preparation, when a saturated PTU solution was used as the aqueous phase, was considered the saturation solubility of PTU in the system.

Briefly, the vesicular systems were prepared as described under Section 1.1. For liposomes and ethosomes, PTU was incorporated into both the lipid phase

and the aqueous phase. The concentration of 1.23 mg/ml of PTU in water was used as the saturation solubility of PTU in water at ambient temperature as previously determined (Suwakul et al., 2006). The concentration of PTU in the aqueous phase was fixed at 1.23 mg/ml (100% saturation solubility), but the drug concentration in the lipid phase was varied from 1.0 mg to 2.4 mg/132 µmoles of total lipid for liposomes and from 1.0 to 1.2 mg/132 µmoles of total lipid for ethosomes.

For niosomal preparations, however, PTU was added only in the aqueous phase because PTU could not be dissolved in the lipid phase at any appreciable amounts. The concentration of PTU in water was varied from 2.0 mg/ml to 3.0 mg/ml. The concentrations higher than 1.23 mg/ml could be used during the preparation of niosomes since these preparations were prepared at 70 $^{\circ}$C.

After preparation, all the vesicular dispersions containing PTU were kept in a refrigerated incubator at 26 $^{\circ}$C for 2 days. The temperature was fixed at 26 $^{\circ}$C because the ambient temperature in the laboratory fluctuated between 26-29 $^{\circ}$C during the preliminary study (data not shown). The vesicular dispersions were then monitored for drug crystals under a light microscope at 400x magnification. PTU concentration in the dispersion with highest drug loading in which the PTU crystal was absent was considered saturation solubility of PTU for that system.

1.3 Preparation of PTU vesicular systems

The PTU vesicular systems were prepared as described under Section 1.1. The total PTU amount added to each vesicular system was at 80% saturation of that system in order to establish equal thermodynamic activities among the systems studied.

All vesicular preparations were routinely examined under a light microscope at 400x magnification to verify decency of each preparation. Any preparation with irregular vesicular structures and/or with excessive lipid remnants was discarded. The decent vesicular preparations were left at ambient conditions overnight before characterization/use in the subsequent experiments.

2 Characterization of PTU vesicular systems

The PTU vesicular systems were characterized for size and size distribution, entrapment efficiency, phase transition temperature, and drug release.

2.1 Determination of size and size distribution

Size and size distribution of the vesicles in the dispersion were examined by the laser diffraction technique using a Malvern Mastersizer® 2000 and expressed as D[4,3] as recommended by the manufacturer of the instrument. The experiment was performed using at least three batches of each preparation and the measurement was done in triplicate.

2.2 Determination of PTU entrapment efficiency

To determine the entrapment efficiency of PTU, the PTU vesicular dispersions were separated into the supernatant containing the free drug and the pellet containing the entrapped drug by ultracentrifugation (see below). The PTU content in the pellet was assayed by UV spectrophotometry at 275 nm and was used to calculate the entrapment efficiency. PTU in the supernatant was also assayed for routine monitoring of the analytical recovery. The experiment was done in triplicate with three batches of the vesicular dispersion.

2.2.1 Separation of the pellet

An aliquot (2 ml) of the vesicular dispersion was carefully packed into a polyallomer Bell-top Quick-Seal® centrifuged tube and subjected to centrifugation at 85,000 rpm, at 25 °C for 6 hours in an ultracentrifuge (Optima™ L-100XP, Beckman Coulter). The supernatant was carefully separated from the pellet. The PTU contents in the pellet and in the supernatant were assayed by the following assay protocol.

2.2.2 Quantitative analysis of PTU in the pellet

The pellet separated from the 2 ml aliquot of the vesicular dispersion was dissolved in isopropanol in a 25 ml volumetric flask, and the solution was adjusted to volume. This solution was appropriately diluted and assayed by UV spectrophotometry at 275 nm. The UV spectrophotometric assay method of PTU was verified for specificity, linearity, accuracy, and precision (see Appendix F).

2.2.3 Quantitative analysis of PTU in the supernatant

The supernatant was transferred to a 10 ml volumetric flask and the solution was adjusted to volume with isopropanol. The solution was appropriately diluted and analyzed by UV spectrophotometry at 275 nm.

2.2.4 Assay of PC

The amount of PC in the pellet was determined by the standard Bartlett assay (New, 1997). The pellet was dissolved in isopropanol. The solution was further diluted with isopropanol to give a concentration of approximately 1 mg/ml of phospholipid before being subjected to further assay procedure (see Appendix B).

2.2.5 Calculation of the entrapment efficiency (Pérez-Cullell, Coderch, and Estelrich, 2000)

The entrapment efficiency of PTU entrapment of each formulation was calculated from the following equation:

$$\text{Entrapment efficiency (\% by mole)} = \frac{\text{Amount of PTU in pellet (µmole) x 100}}{\text{Amount of total lipid in pellet (µmole)}} \quad (1)$$

2.3 Determination of phase transition temperature

The calorimetric analysis was performed to determine phase transition temperature of both the blank and the PTU vesicular systems. The vesicular dispersion was centrifuged as described under Section 2.2.1 to collect the pellet. The differential scanning calorimetric (DSC) apparatus was calibrated using indium as the calibration standard. An accurately weighed amount (15-20 mg) of the pellet was packed in a sealed aluminum crucible with a vent. The sample was heated at the rate of 5 $^{\circ}$C/min in the temperature range of -40-60 $^{\circ}$C and 180-225 $^{\circ}$C. Between 60 $^{\circ}$C and 180 $^{\circ}$C, the heating rate was 10 $^{\circ}$C/min. The measurement was done under a nitrogen atmosphere. The empty aluminum crucible with a lid was used as the reference. The experiment was carried out in triplicate with one batch of the preparation.

2.4 Study of drug release

To characterize the in vitro release profiles of the three vesicular systems, modified Franz diffusion cells were used. The solutions of PTU in water and in 30% w/w ethanol at 80% saturation were used as references. Ultrapure® water or 30% w/w

ethanol was used as a receptor medium, corresponding to the composition of the PTU vesicular system being tested.

The internal diameter of the modified Franz diffusion cells ranged from 1.70-1.75 cm, corresponding to an effective permeable surface area of 2.27-2.40 cm^2. The receptor compartment contained 13.66-14.34 ml (from calibration) of the receptor medium. The receptor compartment was equipped with a magnetic stirring bar rotating at 600 ± 5 rpm. The temperature of the cell was kept constant at 37 $^{\circ}C$ by circulating water through a jacket surrounding the cell body throughout the experiments. The donor and the receptor compartments were separated by a cellulose acetate membrane with a molecular cut-off of 12,000-14,000. The membrane was soaked in Ultrapure® water overnight before use. The membrane was cut into a circular shape with a diameter of 3 cm. Before mounting the circular cellulose membrane onto a diffusion cell, the membrane was rinsed with boiling water to wash off any soluble contaminants. The membrane was then soaked in water or 30% w/w ethanol for 30 minutes and clamped in place between the donor and the receptor compartments of the cell. The receptor medium and the membrane in the Franz diffusion cells were left to reach the desired temperature for 30 minutes. After equilibration, the sample (1 ml of the saturated PTU solution or the vesicular dispersion) was carefully placed on the membrane surface of each cell and the cell was then covered completely and tightly with Parafilm®. An aliquot (3 ml) of the receptor medium was removed at appropriate time intervals and replaced with an equal volume of the pre-warmed fresh medium. The sample taken was diluted to an appropriate concentration with isopropanol and assayed by spectrophotometry at 275 nm.

The percent of PTU released was calculated using the following equation:

$$\%\text{PTU released} = (A_t/A_0) \times 100 \qquad (2)$$

where A_t is the cumulative released amount of PTU at a particular time; A_0 is the initial amount of PTU in the dispersion.

The release study for each formulation was performed in triplicate using three batches of the formulation.

3 Permeation studies

The permeation of PTU from the vesicular systems was studied using modified Franz diffusion cells. The abdominal skin of newborn pigs was used as the model skin membrane.

3.1 Preparation of newborn pig skin membrane

To prepare a full-thickness abdominal skin membrane, the skin of newborn pigs was carefully excised from the animal carcasses. The subcutaneous fat and extraneous tissues were completely removed using forceps, scissors, and surgical blades. The separated skin was cleaned by rinsing with purified water. The hair (if any) was carefully clipped with a pair of scissors. The clean skin was then wrapped in aluminum foil and stored in a freezer (-20 $^{\circ}$C) until it was used. Immediately before the permeation study, the frozen skin was thawed at ambient conditions. Rehydration of the skin was done by immersion of the skin in phosphate buffered saline (PBS), pH 7.4, at room temperature for about one hour. The skin was cut into a circular shape with a diameter of about 3 cm and was mounted onto the diffusion cell.

3.2 Permeation study

The experimental setup for permeation study was similar to that of the determination of drug release described under Section 2.4. The receptor medium was PBS, pH 7.4. Briefly, the receptor medium of the diffusion cells was left to equilibrate to 37 $^{\circ}$C. The excised pig skin was set in place with the stratum corneum facing the donor compartment and the dermal side facing the receptor compartment. The mounted skin was allowed to reach the desired temperature for 30 minutes. After temperature equilibration of the skin, an aliquot (150 µl/cm^2) of the PTU vesicular dispersion or the corresponding reference solution was carefully placed on the membrane surface of each cell. Samples (about 1.0 ml) were withdrawn from the receptor compartment at appropriate time intervals for up to 24 hours. The receptor medium was replaced with an equal volume of the fresh, pre-warmed PBS after each sampling. Replacement of the receptor medium was performed with great care to avoid air trapping beneath the dermis that could severely distort the permeation profile. The samples were kept refrigerated until they were analyzed by the high-performance liquid chromatographic (HPLC) method. The analysis was carried out

within 24 hours after the samples were taken. Each treatment was done with at least six diffusion cells.

3.3 Determination of PTU in the skin

At the end of the permeation study, the skin surface and the donor cap were washed 3-5 times with methanol. The methanolic solution from skin rinsing was collected for further assay of PTU remaining in the donor compartment by the HPLC method. The skin was then removed and blotted dry with filter paper. The skin was then cut into small pieces and extracted with methanol (3 ml) by vortexing for 5 minutes, sonicating for 5 minutes, shaking on an orbital shaker at ambient temperature for 2 hours, and filtering through a membrane filter (0.45 µm). The filtrate was analyzed for the amount of PTU accumulated in the skin, Qs, by the HPLC method.

3.4 Assay of PTU by the HPLC method

The chromatographic system and condition modified from *USP 25* (The United States Pharmacopoeia Convention, 2002) were as follows:

Column	:	BDS Hypersil® C18, 5 µm, 300 x 4.6 mm
Precolumn	:	µBondapack C18, 10 µm, 125 A°
Mobile phase	:	0.025 M phosphate buffer pH4.6:acetonitrile (85:15 v/v)
Injection volume	:	20 µm
Flow rate	:	1 ml/min
Detector	:	UV detector at 272 nm
Temperature	:	ambient
Internal standard	:	theophylline (5.0 µg/ml)

The analytical procedure was verified for specificity, linearity, accuracy, and precision (see Appendix G).

3.5 Data treatment

For each membrane specimen, the cumulative amount of PTU permeated per diffusion area was plotted against time. The observed steady state flux (J_{ss}) was obtained from the slope of the plot. The permeation coefficient (P_s) was calculated using Equation 3.

$$P_s = J_{ss}/C_d \quad (3)$$

where C_d is the drug concentration in the donor compartment.

The relative flux (RF) of the formulation was defined as in Equation 4.

$$RF = (J_{ss} \text{ of the formulation})/(J_{ss} \text{ of the reference}) \quad (4)$$

The enhancement factor, EF, of the formulation based on the permeability coefficient was defined as in Equation 5.

$$EF = (P_s \text{ of the formulation})/(P_s \text{ of the reference}) \quad (5)$$

The enhancement factor of the formulation based on the PTU amount in the skin (Q_s) was defined as in Equation 6.

$$\text{EF of } Q_s = (Q_s \text{ of the formulation})/(Q_s \text{ of the reference}) \quad (6)$$

where Q_s is the percent of PTU amount in the skin, which was calculated from Equation 7.

$$Q_s = \frac{\text{PTU amount in the skin}}{\text{Initial amount of PTU in the donor}} \times 100 \quad (7)$$

The enhancement factor of the formulation based on the cumulative amount of PTU in the receptor medium at 24 hours (Q_{24}) was defined as in Equation 8.

$$\text{EF of } Q_{24} = (Q_{24} \text{ of the formulation})/(Q_{24} \text{ of the reference}) \quad (8)$$

where Q_{24} is the percent of cumulative PTU amount in the receiver at 24 hours, which was calculated from Equation 9.

$$Q_{24} = \frac{\text{Cumulative PTU amount in the receiver at 24 hours}}{\text{Initial amount of PTU in the donor}} \times 100 \quad (9)$$

Formulations with an EF of more than one were selected for further investigation.

4 Factors affecting drug permeation into/through the skin

4.1 Effect of application condition

To examine the effect of the application condition, the PTU permeation parameters from the selected formulations under non-occlusive and occlusive conditions were determined and compared. The occlusive condition was created by wrapping the donor compartment of the diffusion cells tightly with Parafilm®.

4.2 Effect of vesicular structure

The permeation of PTU from the physical mixture of the components of each selected preparation dispersed in 90% v/v propylene glycol in water was studied using the method described under Section 3. The experiment was carried out under the occlusive condition. The permeation parameters of PTU from the vesicular dispersions were compared with those from the physical mixtures.

5 Elucidation of possible mechanism(s) of PTU permeation from vesicular systems

5.1 Diffusion of free drug

For the free-drug diffusion mechanism, the drug is expected to be released from the vesicles and independently permeated the skin. Thus, if this is the sole mechanism of drug delivery by the vesicles, the rate-limiting step of skin permeation will be the release rate. To investigate this mechanism, the correlation trend between permeation parameters of PTU and in vitro release rate constants of relevant formula was estimated.

5.2 Mixing of the vesicles with skin lipids

If mixing of the vesicles entrapping the drug with the skin lipid is the major mechanism of drug delivery, the permeation of drug through/into the skin should be correspondingly high with the formulations containing high drug entrapment efficiency. To assess the possibility of this mechanism, the correlation trend between entrapment efficiency and permeation parameters of PTU of the same vesicular dispersion was determined.

5.3 Penetration enhancement of the vesicles

In order to explore this mechanism, the effect of skin pretreatment with empty vesicles on PTU permeation from an aqueous solution at 80% saturation was conducted using modified Franz diffusion cells as described under Section 3. The empty vesicular systems (400 µl/cell) were carefully placed on the skin and the cell was then covered completely and tightly with Parafilm® (the occlusive condition). At the end of 4 hours, the empty vesicles were removed from the skin and the skin was

washed with water and dried gently with cotton buds. The permeation of PTU from the aqueous solution at 80% saturation through the skin pretreated with the empty vesicles was then performed under the occlusive condition. The reference cells were pretreated with water or 30% w/w ethanol in the same manner. EF of permeation of PTU, EF of PTU in the skin (EF of Q_s), EF of PTU in the receiver (EF of Q_{24}) and relative flux (RF) were calculated from Equations 10-13.

$$\text{EF} = \frac{P_s \text{ after pretreatment with empty vesicle}}{P_s \text{ after pretreatment with aqueous phase}} \quad (10)$$

$$\text{EF of } Q_s = \frac{Q_s \text{ after pretreatment with empty vesicle}}{Q_s \text{ after pretreatment with aqueous phase}} \quad (11)$$

$$\text{EF of } Q_{24} = \frac{Q_{24} \text{ after pretreatment with empty vesicle}}{Q_{24} \text{ after pretreatment with aqueous phase}} \quad (12)$$

$$\text{RF} = \frac{J_{ss} \text{ after pretreatment with empty vesicle}}{J_{ss} \text{ after pretreatment with aqueous phase}} \quad (13)$$

The data were evaluated to determine whether the empty vesicles could act as penetration enhancers.

6 Statistical analysis

The data were presented as means ± standard error of the mean or means ± standard deviation as appropriate. The validity of assumptions for ANOVA was tested on pooled data by using Kolmogorov-Smirnov normality test and Levene's test of homogeneity of variances. If the distribution of data did not significantly deviate from normality, the one-way ANOVA with Tukey HSD or Dunnett T3 as a post hoc comparison was used. The Student's t-test was used to compare two treatment mean. The level of significance was chosen at the probability of 0.05. The ANOVA was used mostly for comparison of the parameters from the in vitro release and the permeation studies. The Student's t-test was used mostly for comparison of parameters from the studies of factors and mechanisms in PTU skin delivery.

CHAPTER IV

RESULTS AND DISCUSSION

1 Preparation of PTU vesicular systems

Blank vesicular systems were prepared to evaluate the feasibility of vesicular preparation at a total lipid concentration of 132 µmoles/ml. This lipid concentration was chosen from preliminary experiments as an optimal concentration at which all vesicular systems of interest could be prepared. The lipid compositions of liposomes and ethosomes were selected from literature review and from preliminary experiments. For niosomes, the lipid composition was selected from a previous work due to the ability of the system to increase PTU skin permeation without any modification (Waraporn Suwakul, 2005). The ratio of PC:CH in liposomes and ethosomes was varied at 60:40% and 70:30% by mole. Ethosomes could form only at the ratio of 70:30. Thus, the ratio of PC:CH used in this study was 70:30 for both liposomes and ethosomes. A total of five vesicular formulas were successfully prepared (Table 1). The saturation solubility of PTU in 30% w/w ethanol experimentally determined in this study was 5.38 ± 0.10 mg/ml. The compositions of all vesicular systems and saturation solubilities of PTU in these systems are shown in Table 1. The total PTU amount loaded in each formulation was at 80% of the saturation solubility of PTU in that system.

Table 1: Compositions of vesicular systems successfully prepared

Type of vesicular system	Formula code	Lipid phase	Aqueous phase	100% saturation of PTU		
				Lipid phase (mg)	Aqueous phase (mg)	Dispersion (mg/ml)
Liposome	PTU/PCL	PC	Water	2.00	1.23	3.23
	PTU/PCCHL	PC:CH	Water	1.20	1.23	2.43
Niosome	PTU/SN	Span®20:CH: Solulan®C24	Water	-*	2.50	2.50
Ethosome	PTU/PCE	PC	30% w/w Ethanol	1.00	5.38	6.38
	PTU/PCCHE	PC:CH	30% w/w Ethanol	-*	5.27	5.27

*PTU could not be added in any appreciable amounts in the lipid phase.

2 Characteristics of PTU vesicular systems

All PTU vesicular systems were characterized for size and size distribution, entrapment efficiency, phase transition temperature, and drug release.

2.1 Size and size distribution

The size and size distribution of PTU vesicular systems are shown in Table 2. The SPAN index is the measurement of the width of the distribution. The rank order of PTU vesicle sizes was PTU/PCE ~ PTU/SN < PTU/PCCHE < PTU/PCCHL < PTU/PCL (Table 2). The vesicle sizes of the ethosomal systems were smaller than those of the liposomal systems were. The rank order of vesicle sizes observed under a light microscope was consistent with size measurement by laser diffraction. The photomicrographs of vesicular systems are shown in Figure 2. However, since the preparation methods were not the same, the differences in size could not be attributed solely to the difference in the vesicular composition. The SPAN indexes indicate a wide size distribution of these vesicles. After preparation, the vesicular systems were used without further size reduction.

Table 2: Sizes and size distribution of PTU vesicular systems (Mean ± SD, n = 3)

Formula	Size (µm)	SPAN index
PTU/PCL	10.41 ± 0.10	2.29 ± 0.07
PTU/PCCHL	7.34 ± 0.01	1.98 ± 0.01
PTU/SN	2.58 ± 0.02	2.54 ± 0.01
PTU/PCE	2.14 ± 0.01	1.59 ± 0.12
PTU/PCCHE	5.47 ± 0.11	1.23 ± 0.06

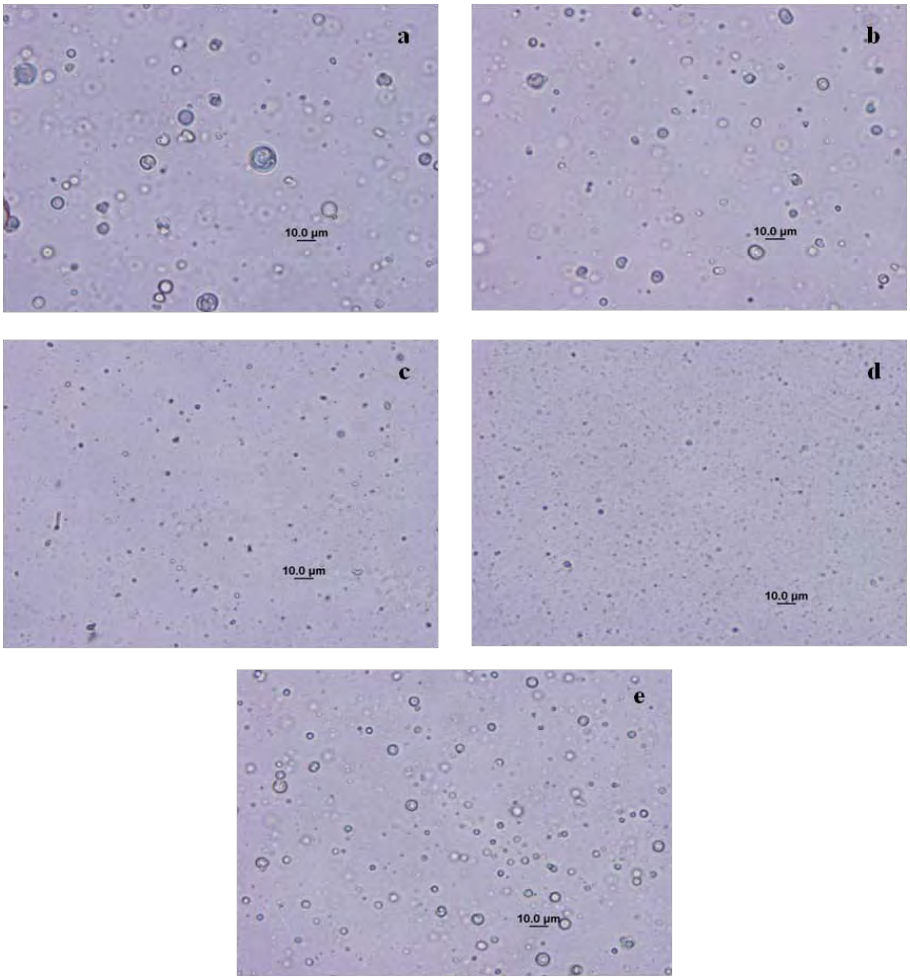

Figure 2: Photomicrographs of PTU vesicular systems (x400): a = PTU/PCL, b = PTU/PCCHL, c = PTU/SN, d = PTU/PCE, and e = PTU/PCCHE

The vesicle size of PTU/PCL was significantly greater than the size of PTU/PCCHL. The presence of CH in the bilayer caused the vesicle size to become

smaller. The effect of CH inclusion on vesicular size has been reported previously. Fang et al. (2008) investigated phosphatidylethanolamine (PE) liposomes with (PE:CH) and without CH. The PE:CH liposomes was smaller in size than the PE liposomes. On the contrary, the increase in size of liposomes due to the increasing CH concentration or the presence of CH in the bilayer of liposomes was reported in other previous studies (El Maghraby et al., 1999; López-Pinto et al., 2005; Bendas and Tadros, 2007; Bhardwaj and Burgess, 2010). The reason for this contrast might be the difference in the main lipid component (phospholipids) and the CH content. For ethosomes, however, the difference in vesicular size could not be attributed conclusively to the presence of CH in the bilayer since the methods of preparation were not the same.

2.2 PTU Entrapment efficiency

Figure 3 shows the entrapment efficiency (EE) of PTU vesicular systems. The entrapment efficiencies of all PTU vesicular systems were significantly different ($p < 0.05$). Among the vesicular systems using water as the aqueous phase, the EE of the PTU/PCL system was the highest. For the systems using 30% w/w ethanol as the aqueous phase, the EE of PTU/PCE was greater than that of the PTU/PCCHE system. For systems with the same aqueous phase, the difference in the EE could be attributed to the different PTU saturation solubilities in the lipid phase of these systems. The saturation solubility of PTU in PTU/PCL was the highest among the three formulations with water as the aqueous phase (PTU/PCL, PTU/PCCHL and PTU/SN, see Table 1). Likewise, the PTU saturation solubility of the PTU/PCE was higher than that of the PTU/PCCHE system. The saturation solubility of PTU depended largely on the composition of the vesicular system. The saturation solubility in the lipid phase consequently affected the EE of the system since it reflected the ability of the bilayer to accommodate PTU molecules. This study agrees with many previous research works where the composition of the vesicle influences the EE of the vesicular system (Ratana Rattanatraiphop, 2000; Suwakul et al., 2006; Bhardwaj and Burgess, 2010). Ratana Rattanatraiphop (2000) found that surface charge, pH, and CH content interactively affected PTU entrapment in PC-based liposomes. Entrapment of PTU in niosomes also depends on the bilayer composition (Suwakul et al., 2006). The acyl

chain length of phosphatidylcholine also affects drug entrapment in liposomes. Dexamethasone encapsulation of non-extruded liposomes decreases with an increase in phospholipid acyl chain length (Bhardwaj and Burgess, 2010). However, the entrapment of some drugs may not depend on the vesicular lipid composition. For example, Montenegro et al. (1996) studied the entrapment of retinoic acid in various liposomal formulations. They found no difference in retinoic acid entrapment among different liposomal formulations.

Effect of the aqueous phase on the EE of PTU was also detected in this present study. The EE of PTU ethosomes was significantly better than the EE of PTU liposomes (Figure 3, $p < 0.05$) due to the higher solubility of PTU in 30% w/w ethanol. Evidently, the higher concentration of PTU in the aqueous phase of ethosomes contributed largely to the overall entrapment of these vesicular systems. As previously reported, drug entrapment in ethosomal systems is usually higher than that in liposomal systems for most drugs (López-Pinto et al., 2005; Paolino et al., 2005; Dubey, Mishra, Dutta et al., 2007; Bendas and Tadros, 2007).

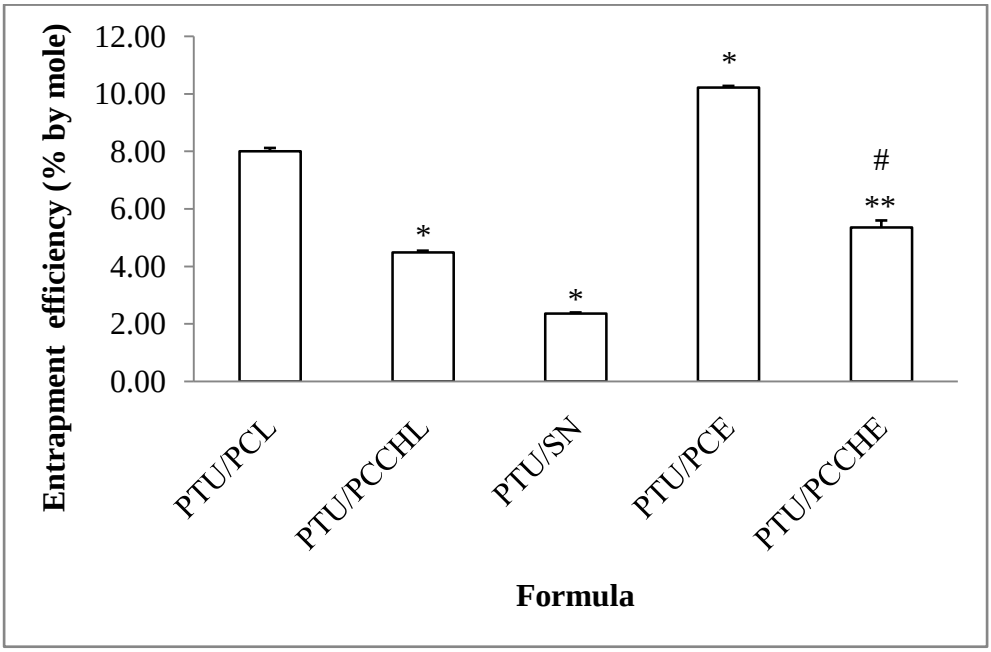

Figure 3: Entrapment efficiency of PTU vesicular systems (Mean ± SEM, n = 3)

*$p < 0.05$ compared with PTU/PCL

**$p < 0.05$ compared with PTU/PCE

#$p < 0.05$ compared with PTU/PCCHL

Inclusion of CH in the lipid bilayer of liposomes and ethosomes resulted in a decrease in PTU entrapment efficiency (Figure 3). CH molecules fill in the hydrophobic region of the bilayer (New, 1997). The capacity to entrap the lipophilic form of the drug in this region of the bilayer was likely to decrease in the presence of CH molecules due to steric competition between CH and the drug molecules. Similar results have been reported with several liposomal systems including estradiol liposomes (El Maghraby et al., 1999) and dexamethasone liposomes (Bhardwaj and Burgess, 2010). The CH content of the bilayer also affects the entrapment of hydrophilic compounds. For example, the increase in CH content causes the reduction in encapsulation of fluorescein sodium in liposomes (Coderch et al., 2000).

2.3 Phase transition temperature

The information from differential scanning calorimetric (DSC) study can be used for compound identification, compound interaction, or in an estimation of purity. The phase transition temperatures of the blank and the corresponding PTU vesicular systems in Table 3 show comparable endothermic melting peaks with no melting peaks of individual components. This implies that the presence of drug crystals and lipid remnants was negligible. All PTU vesicular systems displayed the transition peak below the room temperature (storage temperature). This means that they were in the liquid crystalline state (Cevc, ed., 1993).

Table 3: Phase transition temperatures of the blank and the corresponding PTU vesicular systems (Mean ± SD, n = 3)

Formula	Peak temperature (°C)	
	Blank vesicles	**PTU vesicles**
PCL	1.50 ± 0.49	1.60 ± 0.16
PCCHL	1.95 ± 0.11	1.99 ± 0.11
SN	1.45 ± 0.34	1.28 ± 0.11
PCE	-13.88 ± 0.56	-16.62 ± 0.92
PCCHE	-16.23 ± 1.33	-17.17 ± 0.97

As expected, the ethosomal vesicles had much lower transition temperatures than the liposomal vesicles despite the fact that the lipid components were similar. The data verified the presence of ethanol in the bilayer of PTU ethosomes since ethanol is known to increase fluidity of lipid bilayers. These results are in good agreement with the results from previous investigations by Touitou et al. (2000) and Esposito, Menegatti, and Cortesi (2004).

2.4 Drug release

The release profiles of PTU from solutions and from vesicular systems are illustrated in Figures 4 and 5. The diffusion of PTU from solutions was nearly complete (>90%) within 6 hours. The release of free drug from solutions was highly reproducible. These could ensure that the dialysis membrane did not hinder PTU diffusion during the release study. All vesicular systems, except the niosomal system, resulted in the slower PTU release than their reference solutions. These findings are in good agreement with previous studies by Yoshioka, Sternberg, and Florence (1994), Montenegro et al. (1996), Fočo et al. (2005), and Nounou et al. (2006). These researchers found that vesicles could prolong the drug release. Although the PTU release from all vesicular systems was slower than that from the solutions, the PTU release profile of the niosomal system was much closer to that of its reference solution. The most probable reason would be the viscosity of the dispersion. Both liposomal dispersions were more viscous than the niosomal dispersion at the same molar lipid concentration. Viscosity of the medium is a known factor that affects diffusion of drug molecules. The velocity of the solute molecules decreases with increasing viscosity of the medium (Sinko, ed., 2006).

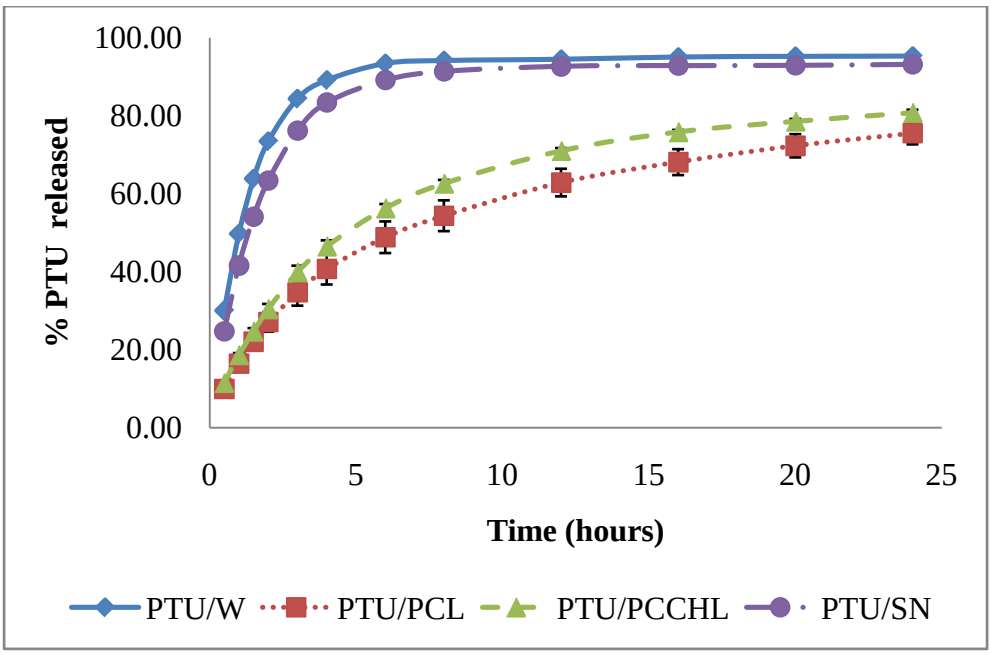

Figure 4: Release profiles of PTU from aqueous solution, liposomes, and niosomes (Mean ± SEM, n = 3)

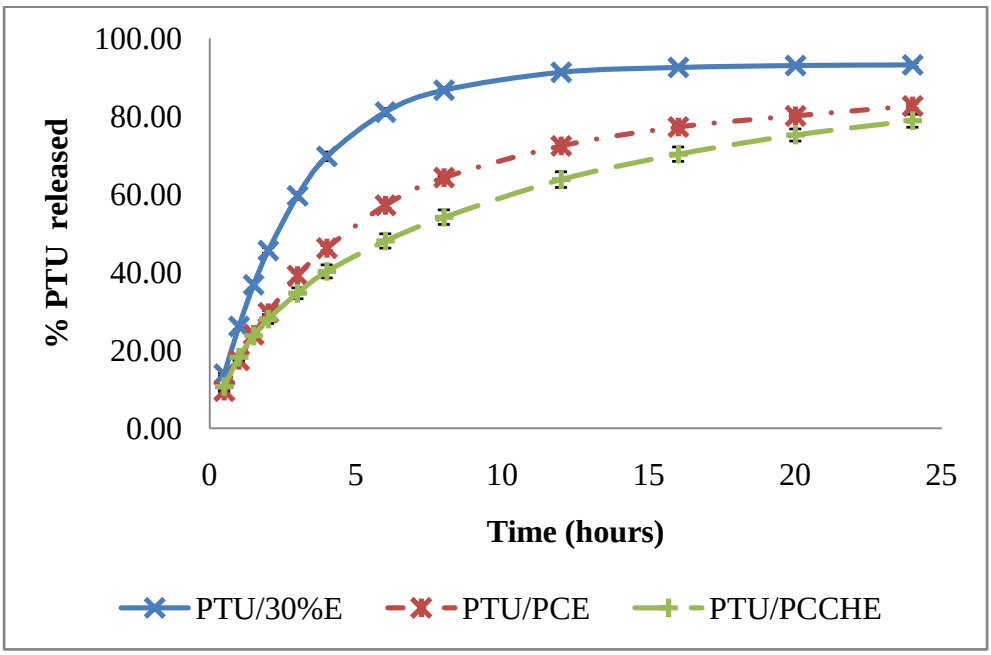

Figure 5: Release profiles of PTU from hydroalcoholic solution and ethosomes (Mean ± SEM, n = 3)

The release of PTU from vesicular systems was consistent with the first-order kinetics. This result agrees well with many previous reports on vesicles (Montenegro et al., 1996; Manconi et al., 2002; Suwakul et al., 2006). Table 4 shows the release rate constants of the vesicular systems obtained from the slope of the first order plot between percent of drug remaining against time. The release rate constants of all vesicular dispersions were significantly different from those of their corresponding reference solutions ($p < 0.05$).

Table 4: Release rate constants of the vesicular systems and their reference solutions (Mean ± SEM, n = 3)

Formula	Release rate constant (hr^{-1})
PTU/W	0.71 ± 0.01
PTU/PCL	0.19 ± 0.03
PTU/PCCHL	0.23 ± 0.01
PTU/SN	0.58 ± 0.01
PTU/30%E	0.36 ± 0.01
PTU/PCE	0.21 ± 0.01
PTU/PCCHE	0.16 ± 0.00

Drug release rates from vesicular systems can often be associated to drug entrapment. In the present study, there was also a trend of negative relationship between drug EE and drug release (Figure 6). The PTU release from the vesicular systems with water as the aqueous phase was low when the EE of PTU in that vesicular system was high (Figure 3 and Table 4). For a formulation with lower drug entrapment, a high amount of the drug would exist as free drug in the aqueous phase. The free drug could diffuse freely through the dialysis membrane as soon as the formulation was placed on the membrane. Therefore, the initial release rate was fast. After the burst release of free drug, the slow release of the entrapped drug from the vesicles was observed (Figure 4). The vesicles gradually released the entrapped PTU over 24 hours. The release of PTU was about 75-93% within 24 hours. In a previous study, niosomes with higher PTU entrapment also release the drug more slowly when

compared to those with lower entrapments (Suwakul et al., 2006). Guinedi et al. (2005) also found that the release of acetazolamide from niosomes was in inverse proportion to the entrapment efficiency.

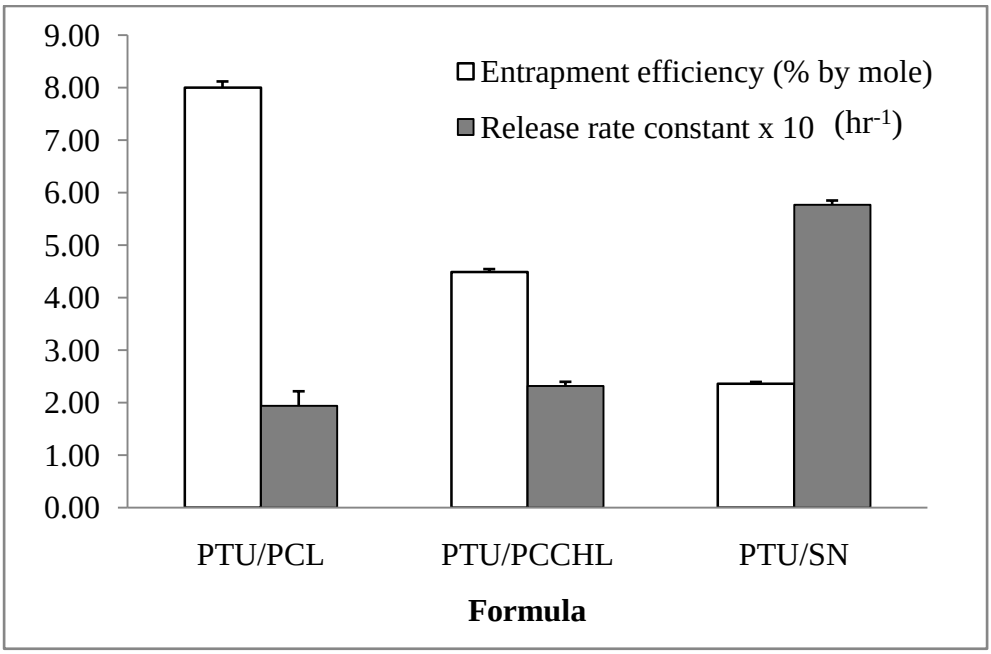

Figure 6: Entrapment efficiencies and release rate constants of PTU/PCL, PTU/PCCHL, and PTU/SN (Mean ± SEM, n = 3)

However, the effect of PTU EE on the PTU release was not found in the ethosomal systems. The ethosomes with higher PTU entrapment (PTU/PCE) also had a higher release rate (Figure 3 and Table 4). This finding might be explained in terms of the effects of drug location and of ethanol. According to its physicochemical properties, besides being intercalated within the lipid bilayer, PTU molecules should be present in the aqueous core of the vesicle as well as in the external phase of the dispersion. During the release study, ethanol molecules in the vesicular bilayer could diffuse into the external phase. Ethanol causes an increase in lipid bilayer fluidity (Touitou et al., 2000; Esposito et al., 2004). Once ethanol left the bilayer, the lipid bilayer fluidity would be reduced. Without ethanol, the bilayer of the PTU/PCCHE system was more resistant to PTU diffusion from the aqueous core than that of the PTU/PCE system due to the effect of CH. As a consequence, the release of PTU from PTU/PCCHE was slower than that from PTU/PCE (Figure 5).

3 Permeation studies

To investigate the skin delivery of PTU from all vesicular systems, modified Franz diffusion cells and newborn pig skin were used. The permeation studies were done under the non-occlusive condition. The non-occlusive condition was used because it is known to improve the flux (Manconi et al., 2006). Besides, it mimics the application condition of most topical preparations. The corresponding reference solution was PTU in water for liposomes and niosomes. For ethosomes, PTU in 30% w/w ethanol was used. J_{ss}, P_s, Q_s, and Q_{24} were defined in this study as steady state flux, permeability coefficient, PTU accumulated in the skin, and cumulative amount of PTU in the receptor compartment at 24 hours, respectively. These parameters described the absolute performances of the formulations themselves. On the other hand, RF, EF, EF of Q_s, and EF of Q_{24} were parameters comparing PTU permeation from vesicular systems with that from the corresponding reference. They were defined as relative flux, enhancement factor of P_s, enhancement factor of Q_s, and enhancement factor of Q_{24}, respectively. There was no statistically significant difference in these parameters among the vesicular systems (Table 5, $p > 0.05$). For clarity, RF, EF, EF of Q_s, and EF of Q_{24} are shown graphically in Figures 7-10, respectively.

Table 5: Permeation parameters of PTU solutions and PTU vesicular systems under the non-occlusive condition (Mean ± SEM, n = 5)

Permeation parameter	Formula						
	PTU/W	**PTU/PCL**	**PTU/PCCHL**	**PTU/SN**	**PTU/30%E**	**PTU/PCE**	**PTU/PCCHE**
J_{ss} x 10^2	10.26 ± 2.80	17.40 ± 8.37	7.78 ± 3.94	21.20 ± 9.46	9.66 ± 2.91	8.16 ± 1.57	11.44 ± 2.60
P_s x 10^5	10.28 ± 2.74	7.22 ± 3.52	4.21 ± 2.12	10.26 ± 4.49	2.16 ± 0.65	1.53 ± 0.30	2.84 ± 0.63
Q_s (%)	2.42 ± 0.55	1.44 ± 0.26	2.90 ± 0.73	1.33 ± 0.28	1.18 ± 0.57	0.79 ± 0.27	0.69 ± 0.17
Q_{24} (%)	1.10 ± 0.31	0.74 ± 0.32	0.45 ± 0.20	1.09 ± 0.44	0.21 ± 0.06	0.16 ± 0.02	0.28 ± 0.05
RF	1	1.49 ± 0.36	0.96 ± 0.39	2.34 ± 0.91	1	1.16 ± 0.34	1.54 ± 0.61
EF	1	0.61 ± 0.15	0.51 ± 0.21	1.13 ± 0.43	1	0.96 ± 0.27	1.68 ± 0.63
EF of Q_s	1	0.67 ± 0.14	1.23 ± 0.29	0.64 ± 0.16	1	1.08 ± 0.35	1.14 ± 0.42
EF of Q_{24}	1	0.62 ± 0.15	0.55 ± 0.21	1.19 ± 0.47	1	0.98 ± 0.23	1.78 ± 0.64

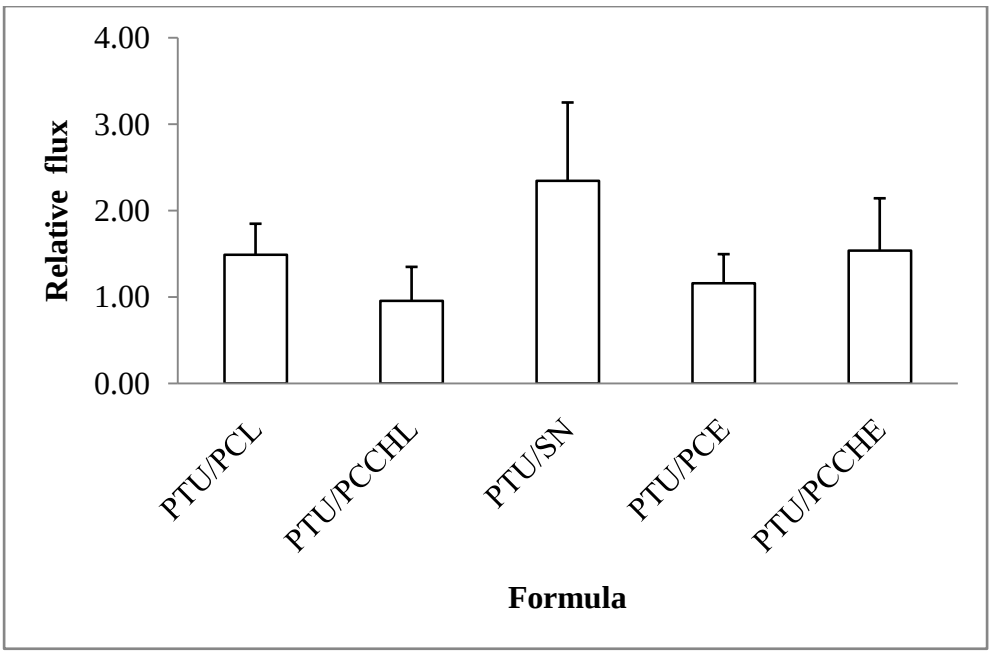

Figure 7: Relative fluxes of PTU from vesicular systems (Mean ± SEM, n = 5)

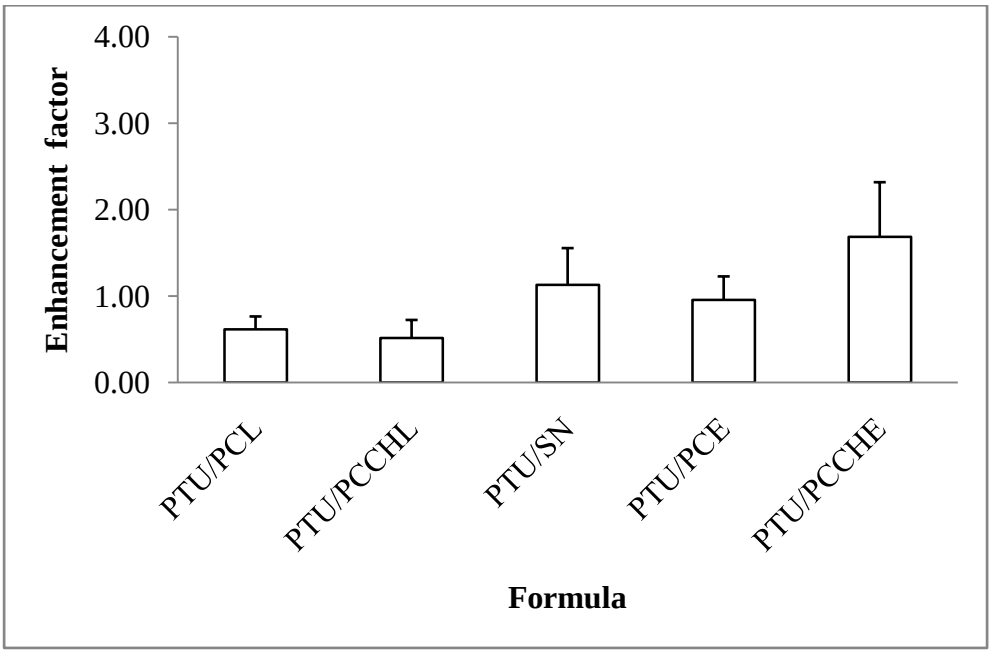

Figure 8: Enhancement factors of PTU from vesicular systems (Mean ± SEM, n= 5)

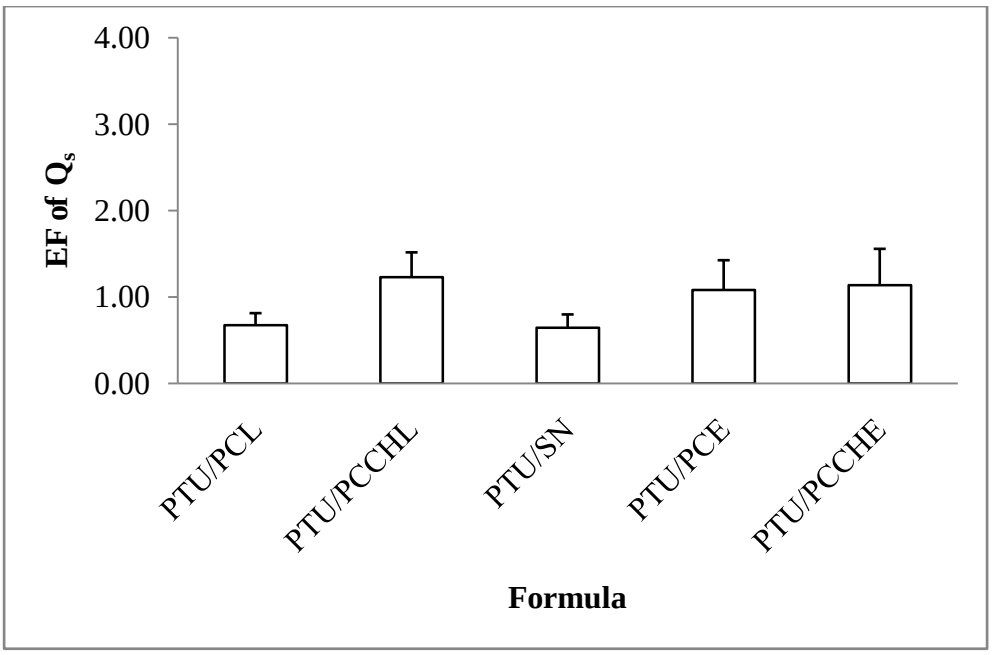

Figure 9: Enhancement factors of Q_s of PTU from vesicular systems (Mean ± SEM, n= 5)

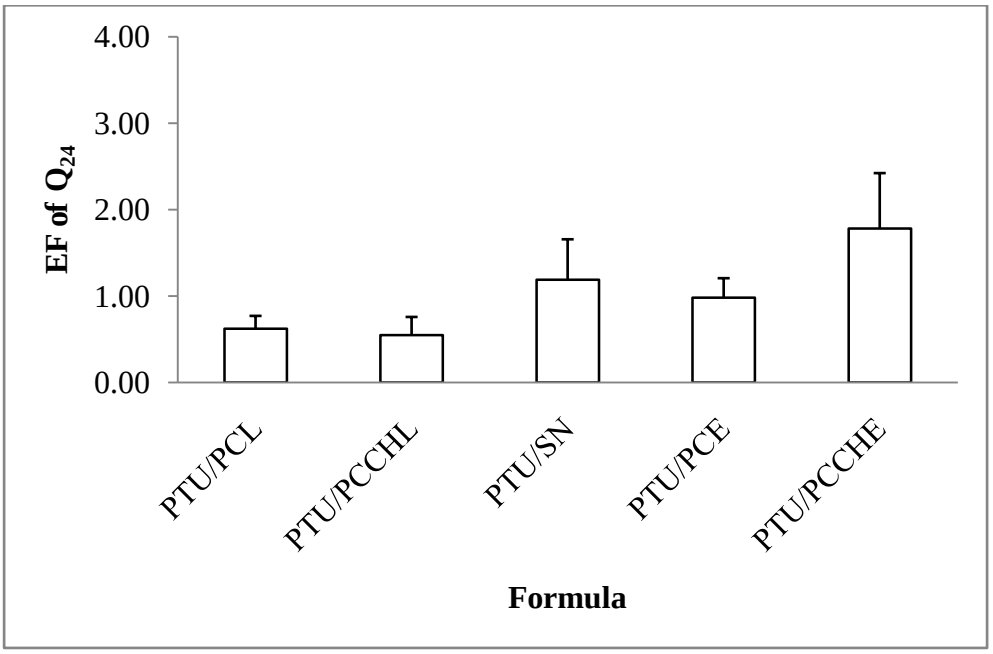

Figure 10: Enhancement factors of Q_{24} of PTU from vesicular systems (Mean ± SEM, n= 5)

Although the EF values of all vesicular systems were not significantly different, the EF values of PTU/SN and PTU/PCCHE were more than one (Table 5

and Figure 8). This result indicated that PTU/SN and PTU/PCCHE could increase the skin permeation of PTU under the non-occlusive condition. In addition, the PTU/SN system, which improved transdermal delivery of PTU, gave lower skin deposition of the drug as can be seen from Q_s and EF of Q_s. In another study on enoxacin PC-based liposomes and Span®-based niosomes, however, both vesicular systems enhance enoxacin permeation in terms of both transdermal and dermal delivery (Fang et al., 2001). Thus, it would be possible for vesicular systems to target drug delivery for both topical and transdermal purposes. The physicochemical properties of the drug might be a significant factor in skin permeation from vesicular systems.

In this present study, both liposomal systems did not enhance PTU permeation across the skin. Their EF and EF of Q_{24} values were less than one. This result was different from the liposomal delivery that was seen with some other drugs in previous research works (El Maghraby et al., 1999; Fang et al., 2001). El Maghraby et al. (1999) found that PC-based liposomes not only enhanced estradiol dermal delivery but also improved its transdermal delivery. Fang et al. (2001) reported that the soybean PC liposomes allowed higher enoxacin amount in both the skin and the receiver compared to the free form of enoxacin. On the other hand, Guo et al. (2000) investigated lecithin vesicular carriers for transdermal delivery of cyclosporine A. They found that PC liposomes failed to transfer cyclosporine A into the receiver, but the drug was found accumulated in the skin. However, PTU deposition in the skin obtained from PTU/PCCHL was greater when compared to that from the reference solution, the PTU/PCL or the PTU/SN. Thus, liposomes composed of PC:CH increased PTU deposition in the skin (Table 5 and Figure 9). Similar results were seen in skin transport studies of caffeine and tretinoin by Touitou et al. (1994) and Manconi et al. (2006), respectively.

From the results described above, the niosomal systems might be more suitable for transdermal delivery of PTU than the liposomal systems. On the other hand, the liposomal systems, especially the PTU/PCCHL, might be useful as a dermal PTU delivery system.

PTU delivery parameters from both ethosomal dispersions were comparable. RF, EF, EF of Q_s, and EF of Q_{24} of the two systems were not significantly different ($P > 0.05$) (Table 5 and Figures 7-10). Surprisingly, ethosomal

systems delivered negligible amounts of PTU to the skin. There are several reports supporting the enhancement role of ethosomes in skin permeation of various drugs (Touitou et al., 2000; Godin and Touitou, 2004; López-Pinto et al., 2005; Dubey et al., 2007; Fang et al., 2009). In this present study, ethosomes did not provide better skin delivery than liposomes or niosomes. The reason for this finding could be dehydration of the dispersions on the skin. During skin permeation studies under the non-occlusive condition, the hydroalcoholic formulations dried out rather quickly compared to the other systems. The PTU crystals appeared on the newborn pig skin treated with PTU/30%E and the PTU-containing ethosomal dispersions within 4-5 hours after application. When topical formulations lose the aqueous phase, drug delivery usually stops (Mura, Pirot et al., 2007). Since the non-occlusive condition might not be the suitable condition for hydroalcoholic formulations, the skin permeation of PTU ethosomes was performed again under the occlusive condition.

PTU/PCE, PTU/PCCHE, and their reference solution (PTU/30%E) were evaluated using the procedure described under Section 3.2 and under the occlusive condition. The permeation parameters of the PTU hydroalcoholic solution and the ethosomes are depicted in Table 6. Most permeation parameters from the occlusive permeation study were higher than the parameters from the non-occlusive study (Table 5). J_{ss}, P_s, and Q_{24} values of the PTU ethosomes and the PTU reference solution from the occlusive study were significantly higher than those from the non-occlusive study, except for the Q_s were. Figures 11-14 illustrate the relative parameters obtained under the non-occlusive and occlusive conditions of the two PTU ethosomal dispersions. These parameters clearly showed that the ethosomal systems enhanced PTU delivery through the skin under the occlusive condition. This data confirmed that the application condition affected transdermal and dermal delivery of vesicular systems. On the contrary, Godin and Touitou (2004) reported that occlusion had no effect on the permeation of bacitracin through human cadaver skin from ethosomes in vitro. Furthermore, El Maghraby, William, and Barry (2001b) found that occlusion reduced the delivering efficiency of both ultradeformable and conventional liposomes. Honeywell-Nguyen and Bouwstra (2003) observed a similar result. They found that occlusion reduced the action of elastic vesicles, but could increase pergolide transport from the reference solution since water was a good

penetration enhancer for this particular drug. Most investigators, however, explored the skin delivery of various drugs from ethosomes under the occlusive condition to avoid the evaporation process (López-Pinto et al., 2005; Paolino et al., 2005; Dubey et al., 2007; Dubey, Mishra, Dutta et al., 2007; Fang et al., 2008).

Table 6: Permeation parameters of PTU hydroalcoholic solution and PTU ethosomal systems under the occlusive condition (Mean ± SEM, n = 6)

Permeation parameter	Formula		
	PTU/30%E	**PTU/PCE**	**PTU/PCCHE**
J_{ss} x 10^2	23.77 ± 4.57	143.90 ± 30.07	71.13 ± 21.29
P_s x 10^5	5.06 ± 0.97	25.46 ± 5.60	17.58 ± 5.17
Q_s (%)	1.91 ± 0.69	1.68 ± 0.11	2.04 ± 0.46
Q_{24} (%)	0.61 ± 0.12	2.18 ± 0.54	1.45 ± 0.36
RF	1	7.62 ± 2.64	2.95 ± 0.50
EF	1	6.21 ± 2.06	3.44 ± 0.57
EF of Q_s	1	1.29 ± 0.27	1.45 ± 0.31
EF of Q_{24}	1	4.32 ± 1.34	2.43 ± 0.30

The J_{ss}, P_s, and Q_{24} values of PTU/PCE were significantly different from those of the reference solution ($p < 0.05$) but the Q_s value was not ($p > 0.05$). The relative parameters (RF, EF, EF of Q_s, and EF of Q_{24}) of PTU/PCE were not significantly different ($p > 0.05$) compared with the reference solution due to the large variation in the data. The permeation parameters of PTU/PCCHE (J_{ss}, P_s, Q_s, and Q_{24}) were not significantly different ($p > 0.05$) from those of the reference solution. However, its relative parameters (RF, EF, and EF of Q_{24}) were significantly different ($p < 0.05$) from those of the reference solution. Although the statistical testing on the two ethosomal dispersions did not show any statistically significant difference ($p > 0.05$), the permeation parameters of PTU/PCE (except Q_s and EF of Q_s) were greater compared to those of PTU/PCCHE. The Q_s and EF of Q_s values of PTU/PCCHE were slightly higher than those of PTU/PCE. These results support the tendency that PTU/PCE might improve PTU permeation through the skin, while PTU/PCCHE

might enhance PTU deposition in the skin. CH, which is a membrane stabilizer, might change the interaction between the vesicles and the skin, resulting in the different profiles in PTU skin delivery seen here.

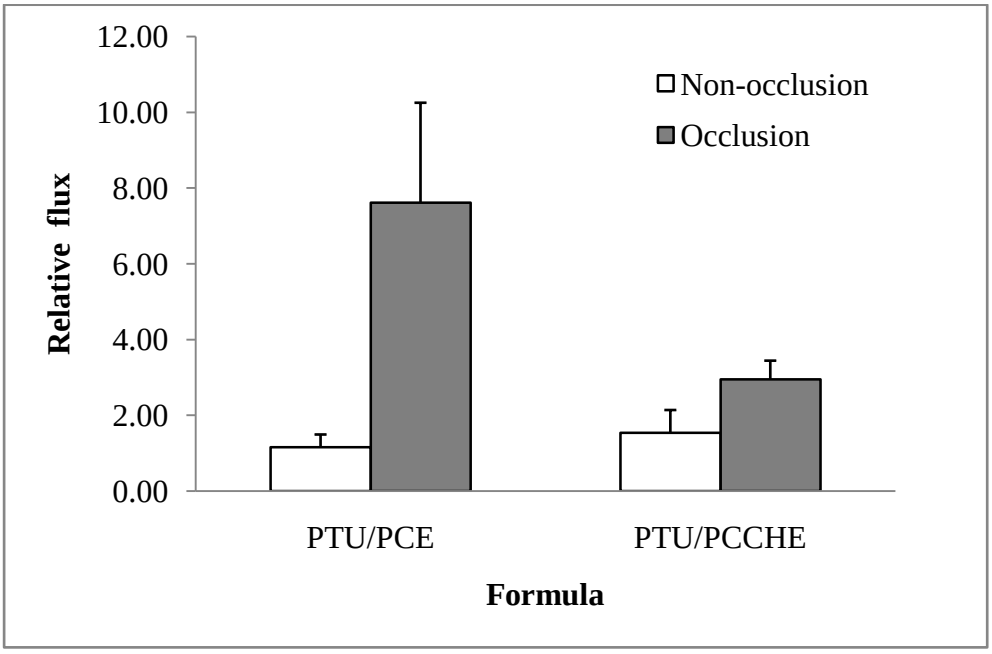

Figure 11: Relative fluxes of PTU from ethosomes under the non-occlusive and the occlusive conditions (Mean ± SEM, n = 5-6)

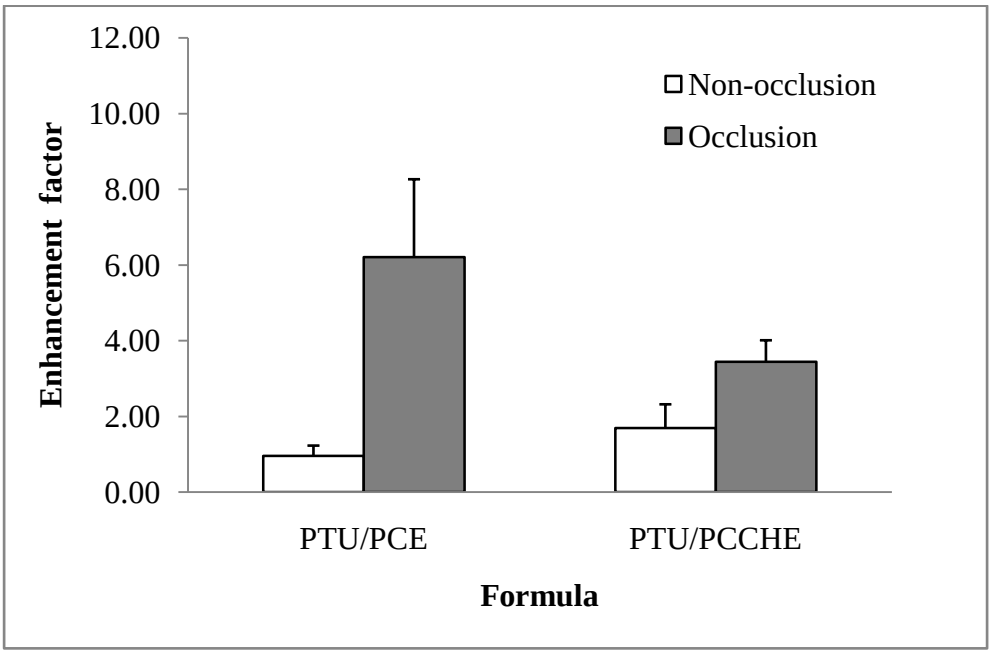

Figure 12: Enhancement factors of PTU from ethosomes under the non-occlusive and the occlusive conditions (Mean ± SEM, n = 5-6)

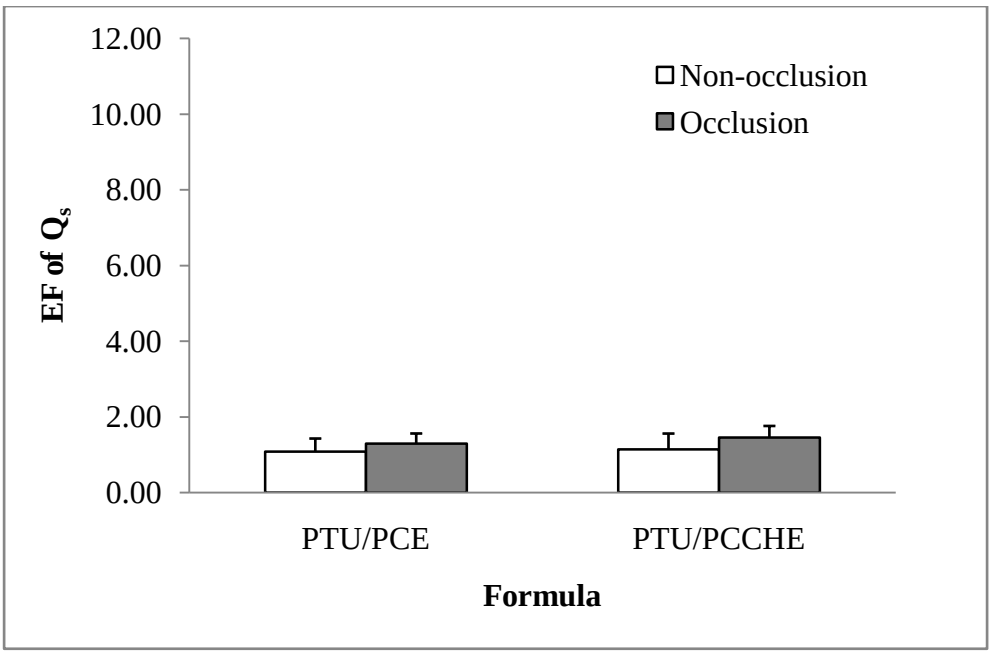

Figure 13: Enhancement factors of Q_s of PTU from ethosomes under the non-occlusive and the occlusive conditions (Mean ± SEM, n = 5-6)

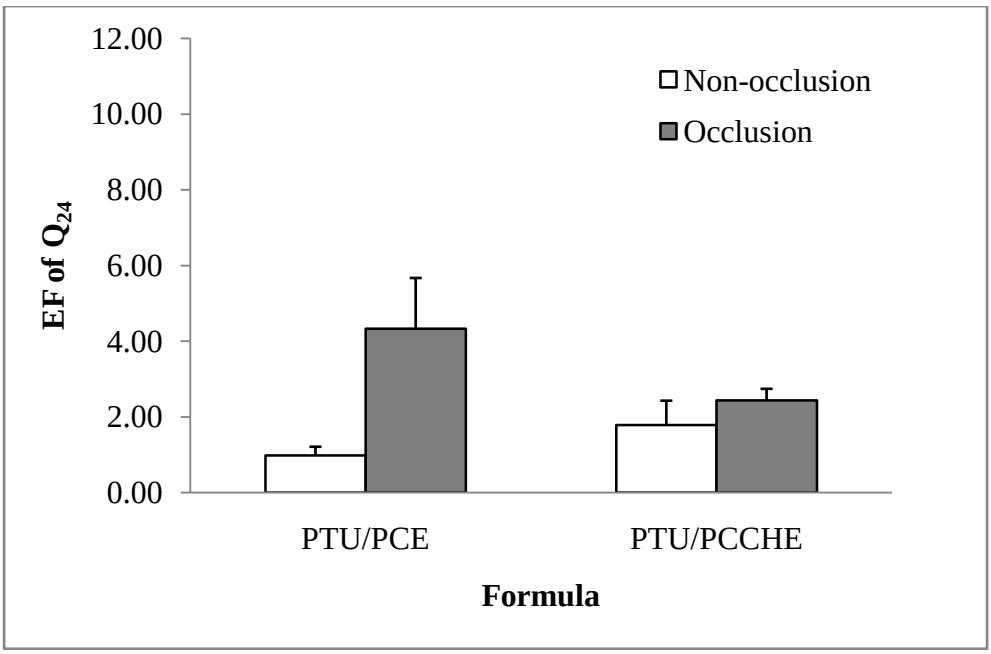

Figure 14: Enhancement factors of Q_{24} of PTU from ethosomes under the non-occlusive and the occlusive conditions (Mean ± SEM, n =5-6)

Both ethosomal systems were selected for further investigation on the effect of vesicular structure and the possible mechanism(s) of PTU permeation from ethosomal systems because of their high efficiency in PTU skin delivery.

4 Effect of vesicular structure

The permeation study under the occlusive condition revealed that the ethosomal systems increased PTU permeation through/into the skin. There was still a question whether it would be necessary to apply these formulations in the form of vesicles. To study the effect of vesicular structure on the delivery of PTU to the skin, a permeation study using a mixture containing the same components as the corresponding vesicular system in a suitable solvent was performed under the occlusive condition. Propylene glycol (PG) is widely used as an additive in pharmaceutical products and its enhancing effect on skin permeation arising from structural changes is marginal (Yamane, Williams, and Barry, 1995). Therefore, 90% v/v PG in water was selected as a solvent for the components of the vesicles. The solubility of PTU in 90% v/v PG in water at ambient temperature was 28.49 ± 0.78 mg/ml (Waraporn Suwakul, 2005). To keep a constant thermodynamic activity, PTU concentration in 90% v/v PG in water was used at 80% saturation. The permeation parameters of PTU from the solution in 90% v/v PG at 80% saturation and from the physical mixtures of ethosomal components are shown in Table 7.

Table 7: Permeation parameters of PTU from PTU solution in 90% PG and from physical mixtures of ethosomal components in 90% PG (Mean ± SEM, n = 6)

Permeation parameter	PTU/PG*	PTU+PC+E/PG**	PTU+PC+CH+E/PG***
J_{ss} x 10^2	5.17 ± 1.04	145.48 ± 25.15	50.22 ± 12.18
P_s x 10^5	0.21 ± 0.04	6.00 ± 1.04	2.08 ± 0.51
Q_s (%)	0.39 ± 0.11	0.27 ± 0.04	0.31 ± 0.04
Q_{24} (%)	0.03 ± 0.01	0.47 ± 0.08	0.21 ± 0.04
RF	1	30.74 ± 4.53	9.47 ± 1.42
EF	1	30.77 ± 4.53	9.51 ± 1.44
EF of Q_s	1	1.03 ± 0.31	1.11 ± 0.29
EF of Q_{24}	1	19.04 ± 2.89	6.30 ± 1.48

*PTU/PG = PTU solution in 90% v/v PG in water

**PTU+PC+E/PG = mixture of PTU and components of PCE in 90% v/v PG in water

***PTU+PC+CH+E/PG = mixture of PTU and components of PCCHE in 90% v/v PG in water

The RF, EF, EF of Q_s, and EF of Q_{24} values of the ethosomal vesicles compared with those of the ethosomal components in 90% v/v PG in water are illustrated in Figures 15-18. The RF, EF, and EF of Q_{24} values of both ethosomal vesicles were significantly different from those of the corresponding physical mixtures in PG ($p < 0.05$). On the other hand, the EF of Q_s of ethosomal vesicles was not different from that of the physical mixture in PG. This finding indicated that the vesicular structure was not essential to PTU skin delivery by ethosomal systems, in contrast to the previous works by El Maghraby et al. (2000) and Fang et al. (2001). El Maghraby et al. (2000) studied the importance of liposomal structure in permeation of estradiol across the human skin. They compared the transepidermal fluxes of estradiol from four formulas of liposomes (PC, PC and sodium cholate, PC and Span®80, and PC and oleic acid) with that from the lipid solutions in 90% w/w PG in water. They found that the vesicular forms were more efficient in delivering the drug than the solution forms. Fang et al. (2001) reported that the permeation of enoxacin from soybean PC liposomes was higher than that from the soybean PC physical mixture. The necessity for the vesicular structure was also seen with the Span®60 niosome system in their study.

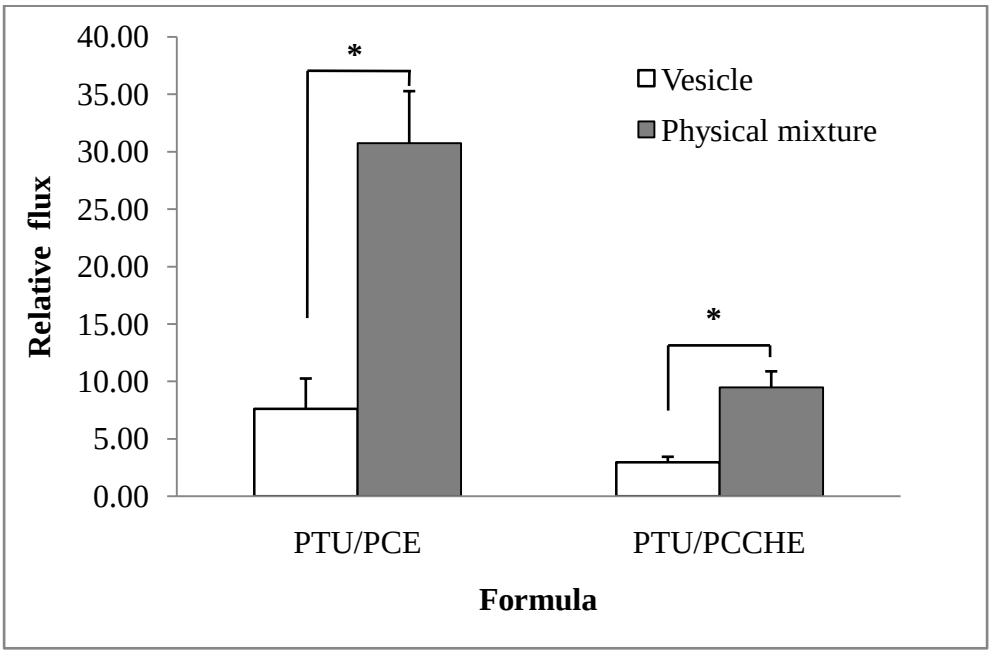

Figure 15: Relative fluxes of PTU from ethosomal systems and physical mixtures of ethosomal components in 90% v/v PG (Mean ± SEM, n = 6)

*$p < 0.05$

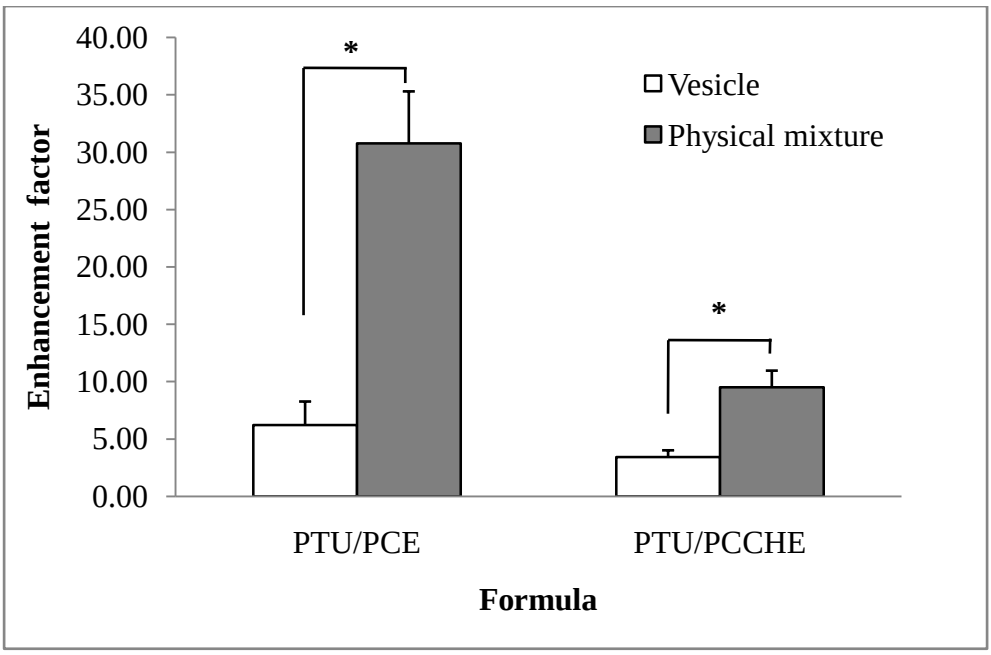

Figure 16: Enhancement factors of PTU from ethosomal systems and physical mixtures of ethosomal components in 90% v/v PG (Mean ± SEM, n = 6) *p < 0.05

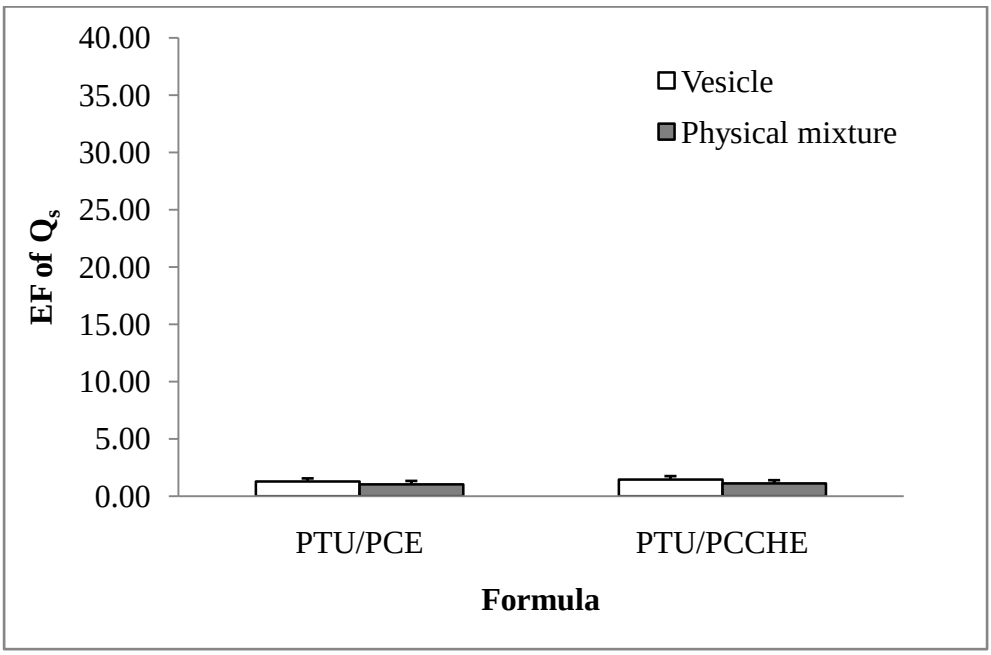

Figure 17: Enhancement factors of Q_s of PTU from ethosomal systems and physical mixtures of ethosomal components in 90% v/v PG (Mean ± SEM, n = 6)

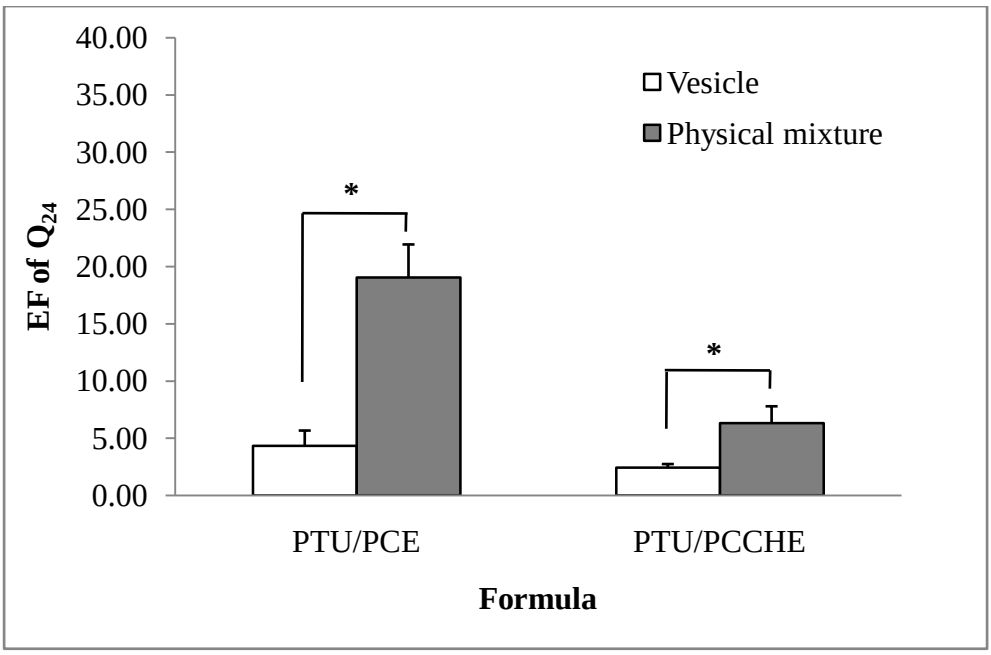

Figure 18: Enhancement factors of Q_{24} of PTU from ethosomal systems and physical mixtures of ethosomal components in 90% v/v PG (Mean ± SEM, n = 6) *$p < 0.05$

Ethanol, PG, and PC are well-known penetration enhancers that have been used for skin delivery for a long time (Williams, 2003). Ethanol and PG are used as a vehicle either alone or as a co-solvent. Levang, Zhao, and Singh (1999) studied the effect of ethanol and PG on the in vitro transport of aspirin through porcine epidermis. They found that all ratios of ethanol and PG solvent mixture gave higher fluxes of aspirin than each pure solvent solution. Other works also support the synergistic effect of these solvents as penetration enhancers (Yokomizo and Sagitani, 1996; Valjakka-Koskela et al., 1998). A dramatic enhancement of PTU skin permeation was seen with ethosomal components in 90% v/v PG in water, especially with PC+E/PG. The enhancing effect was likely to be from the synergistic effect of the three penetration enhancers. In addition, PC+E/PG increased PTU permeation through the skin better than PC+CH+E/PG did ($p < 0.05$) (Table 8). One of the possible causes of the difference seen in this study might be the lower PC concentration in the PC+CH+E/PG system since PC can also act as a permeation enhancer (Yokomizo and Sagitani, 1996). Another possible explanation was the obstruction of PTU diffusion by CH crystals. Since CH was not soluble in 90% v/v PG in water, abundant CH

crystals were clearly observed on the skin under a light microscope in this present study.

Table 8: Permeation parameters of PTU from physical mixtures of ethosomal components (Mean ± SEM, n = 6)

Permeation parameter	PTU+PC+E/PG	PTU+PC+CH+E/PG	p-value
J_{ss} x 10^2	145.48 ± 25.15	50.22 ± 12.18	0.03
P_s x 10^5	6.00 ± 1.04	2.08 ± 0.51	0.03
Q_s (%)	0.27 ± 0.04	0.31 ± 0.04	0.94
Q_{24} (%)	0.47 ± 0.08	0.21 ± 0.04	0.05
RF	30.74 ± 4.53	9.47 ± 1.42	0.00
EF	30.77 ± 4.53	9.51 ± 1.44	0.00
EF of Q_s	1.03 ± 0.31	1.11 ± 0.29	0.85
EF of Q_{24}	19.05 ± 2.89	6.30 ± 1.48	0.00

Robinson et al. (1991) reported that 90% PG/water showed only minimal irritation to rabbit skin in primary irritation test. However, the use of high concentration of penetration enhancer could promote skin irritation in diseased skin. Although PC+E/PG enhanced the PTU permeation through the skin, it might not be an appropriate vehicle for psoriatic skin. Besides, PG is allowed in topical preparations only at concentrations less than 80% v/v (Rowe, Sheskey, and Owen, eds., 2006). Thus, it might not be feasible to use PG at this high concentration for PTU delivery. Ethosomes might be a better delivery system for PTU since these vesicles lack of irritation potential (Dubey et al., 2007).

Nevertheless, despite the results of the above study, the role of vesicular structure in PTU skin delivery could not be ruled out due to the confounding effect of PG. In order to clarify this, further studies such as the co-treatment of the blank ethosomes with PTU solution might be of value. If the co-treatment was much better than the physical mixture in delivery of PTU, the vesicular structure would still be necessity for ethosomes.

5 Elucidation of possible mechanism(s) of PTU permeation from ethosomal systems

5.1 Diffusion of free drug in the aqueous medium and/or vesicle/skin solute transfer

Drug molecules associated with vesicular formulations could penetrate the skin by first freely diffusing from the vesicles into the aqueous medium and then through the skin. Alternatively, the drug molecules could penetrate the skin by diffusing directly from the lipid bilayer into the skin (Weiner et al., 1989). The latter is referred to as the vesicle/skin solute transfer.

Based on free drug diffusion, Ganesan et al. (1984) suggested three probable skin delivery mechanisms of dipalmitoyl phosphatidylcholine (DPPC) liposomes containing glucose, hydrocortisone, and progesterone. Firstly, the solute is released from liposomes before diffusing though the skin. Thus, drug release is the rate-limiting step. Secondly, the liposome-entrapped solute directly transfers or partitions from liposomes to the skin. Thirdly, the solute is delivered by both mechanisms simultaneously. The mechanism most likely to take place depends on the physicochemical properties of the drug entrapped in liposomes. The first mechanism is applied mostly to hydrophilic drugs entrapped in the aqueous phase of the vesicles such as glucose. The second one is appropriate for lipophilic drugs that are associated in the lipid bilayer such as progesterone. For drugs that are not hydrophilic but somewhat less hydrophobic than progesterone, both mechanisms operate simultaneously with liposome/skin solute transfer being predominate. This last scenario is applied to drugs such as hydrocortisone. For PTU ethosomes, the drug could exist both as free drug molecules in the aqueous phase and intercalating with the lipid bilayer. Thus, both the free drug diffusion and the vesicle/skin solute transfer mechanisms seemed plausible.

If the diffusion of free drug from the aqueous phase into the skin operated as the principal mechanism of delivery, the formulation with a faster release rate would permeate the skin faster. To investigate this mechanism, the correlation between permeation parameters of PTU and in vitro release rate constants of relevant formulas was explored. Figures 19 and 20 show the release rate constants of PTU ethosomal systems and EF of PTU from the ethosomal systems, respectively. They

demonstrated a correlation trend between the release rate constant and the EF. The PTU/PCE with a significantly faster release rate delivered PTU through the skin better. Thus, the free drug diffusion through the aqueous phase mechanism applicable to most hydrophilic drugs might also operate on PTU permeation from the ethosomal systems. However, PTU is a lyophobic drug with a log P (octanol/water) of 1.0 (Moffat, Osselton, and Widdop, eds., 2004). Considering the physicochemical properties of the drug, log P of PTU is not much different from that of hydrocortisone (log P = 1.53) (Ho et al., 1985). Therefore, the mechanism of PTU permeation from the ethosomal systems might as well be the free drug diffusion through the external aqueous phase coupled with the vesicle/skin solute transfer mechanism as proposed for hydrocortisone. In the previous study by Ganesan et al. (1984), hydrocortisone-entrapped liposomes slowly released the drug followed by a fast permeation of the drug through the skin. The release of hydrocortisone was the rate-limiting step in skin permeation. Partitioning of the drug from liposomes to the external aqueous phase was slow due to its lipophilic property. For PTU ethosomal systems, however, both the faster release and the fast penetration into the skin were evident. PTU located both in the bilayer and in the aqueous phase of the ethosomes. Ethanol in the external phase of the preparation facilitated the release of PTU from the vesicles and could act as a penetration enhancer. Once PTU was released from the vesicles, it could permeate across the skin freely. In the hydrocortisone case, only 1% of skin permeation was attributed to hydrocortisone diffusion through the external phase. PTU solubility in 30% w/w ethanol (5.38 ± 0.10 mg/ml) is much higher than hydrocortisone solubility in normal saline. Thus, the free drug diffusion through the external phase was expected to be considerable in PTU skin permeation relative to the hydrocortisone case.

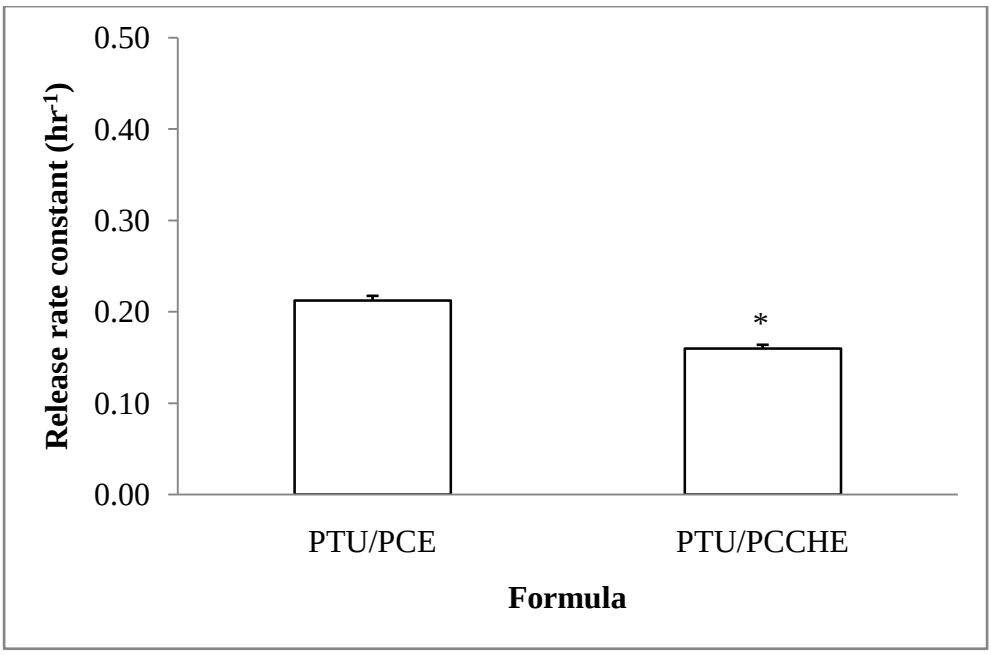

Figure 19: Release rate constants of PTU ethosomal systems (Mean ± SEM, n = 3)
*p < 0.05 compared with PTU/PCE

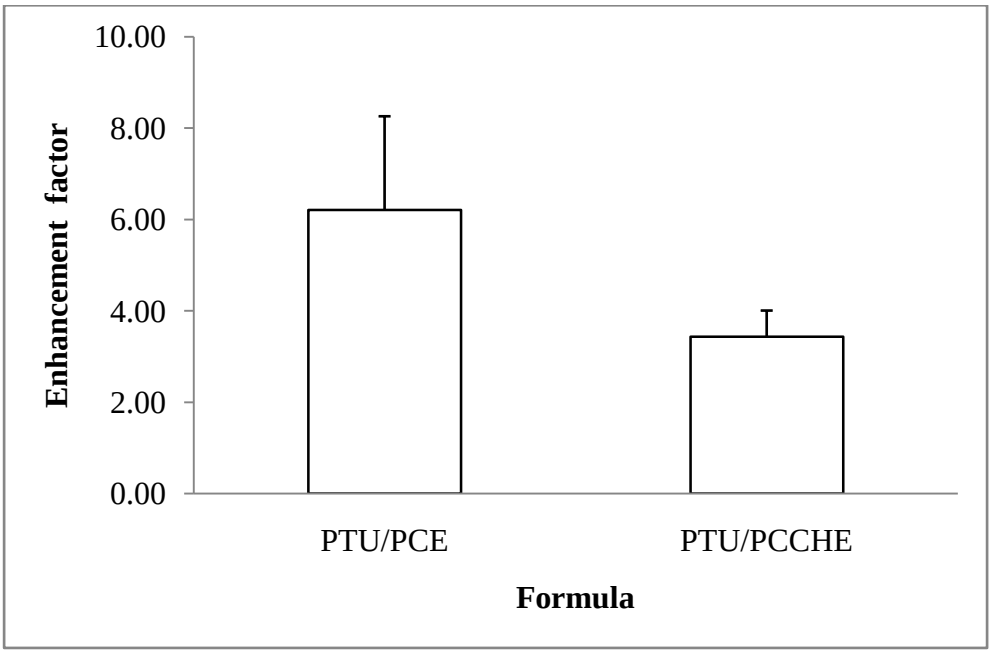

Figure 20: Enhancement factors of PTU from ethosomal systems under the occlusive condition (Mean ± SEM, n = 6)

This proposed plausible mechanism for PTU ethosomes was different from the reported mechanism for PTU niosomes (Waraporn Suwakul, 2005) and for some liposomes containing lipophilic drugs such as retinoic acid (Montenegro et al. 1996) and estradiol (El Maghraby et al., 1999). Waraporn Suwakul (2005) found that free drug mechanism was not the predominate mechanism in skin permeation of PTU from niosomes. PTU permeation parameters (EF) from Span®20 and L-595 niosomes, which displayed comparable release rates, were significantly different. Montenegro et al. (1996) explored in vitro retinoic acid release and skin permeation from different DPPC liposomal formulations. The skin permeation of retinoic acid from both the DPPC liposomes and the alcoholic solution was significantly lower than the amount of the drug released from the same formulations. El Maghraby et al. (1999) reported that the peak flux of estradiol through the skin occurred at a time during which drug release was negligible. Thus, the mechanism involving free drug diffusion into the external aqueous phase was not likely for estradiol skin permeation from liposomes. These findings indicate that mechanism of skin permeation from vesicles depends on the properties of the drug as well as the vesicular system.

Therefore, the mechanism involving diffusion of free drug could be proposed as a possible mechanism for PTU skin permeation from the ethosomal systems. This proposed mechanism for PTU ethosomes coupled the permeation of the drug that was first released into the external phase with the direct vesicle/skin drug transfer.

5.2 Mixing of the vesicles with skin lipids

If mixing of the vesicles entrapping the drug with the skin lipids is the major mechanism of drug delivery, the permeation of drug through/into the skin should be correspondingly high with the formulations having high drug entrapment efficiency. To assess the possibility of this mechanism, the correlation between entrapment efficiency and EF of PTU should be established. However, the correlation plot could not be constructed in this present study since there were only two formulations involved. Alternatively, the entrapment efficiency and EF of PTU permeation of PTU ethosomes were plotted side by side to explore the correlation trend of these two parameters. The plot is shown in Figure 21. The rank orders of the

entrapment efficiency of PTU ethosomal systems and of the skin permeation of PTU of these formulas were similar. The EE of PTU/PCE was significantly higher than that of PTU/PCCHE. These results suggested that the mechanism involving mixing of the vesicles with skin lipids might also operate for PTU ethosomes.

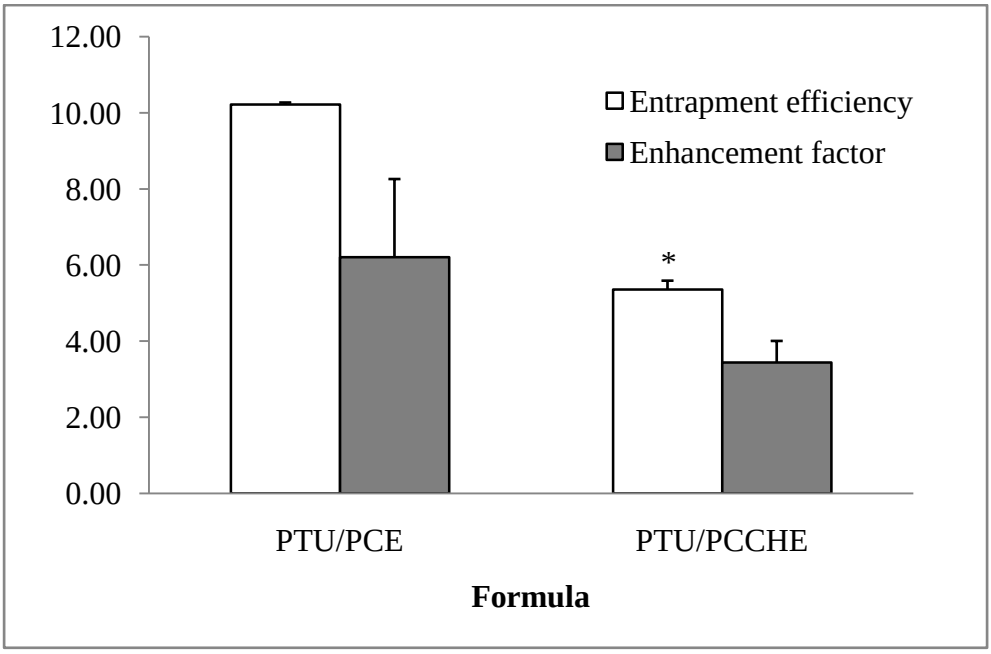

Figure 21: Entrapment efficiencies of PTU ethosomal systems (Mean ± SEM, n = 3) and enhancement factors of PTU from ethosomal systems under the occlusive condition (Mean ± SEM, n = 6), $p < 0.05$ compared with PTU/PCE

Recently, many researchers have studied the interaction of lipid vesicles and the skin. The liquid crystalline-state vesicles can mix and fuse with the stratum corneum (intracellular and intercellular regions) (Kirjavainen et al., 1996; van Kuijk-Meuwissen et al., 1998; van den Bergh et al., 1998). Godin and Touitou (2000) investigated the penetration of bacitracin-entrapped ethosomal systems into human cadaver skin using confocal laser scanning microscopy. They found that the two fluorescent probes, which were used to label the drug and the phospholipids, co-localized in the skin. It indicates that the drug and the ethosomal carrier could penetrate into the skin together. In addition, Touitou et al. (2000) have proposed that

ethosomes penetrate and mix with the disturbed stratum corneum lipid bilayers after ethanol disturbs the organization of the skin lipids.

In the present study, when the ethosomal vesicles loaded with a high amount of PTU mixed with the skin lipids, they conveyed PTU into the skin at a correspondingly high amount. Therefore, the mixing of the vesicles with skin lipids might be involved in PTU transport by the ethosomal systems.

5.3 Penetration enhancement of the vesicles

An important mechanism with respect to the vesicle and skin interaction is the penetration enhancement. Vesicles play a role as a penetration enhancer in the mechanism in which vesicles modify the stratum corneum and subsequently facilitate penetration of free drug molecules into and across the stratum corneum (Honeywell-Nguyen and Bouwstra, 2003). In order to explore this mechanism, the effect of skin pretreatment with empty vesicles on PTU permeation from an aqueous solution at 80% PTU saturation was conducted. The permeation parameters of all formulas are summarized in Table 9. Most parameters of both ethosomal systems were significantly different ($p < 0.05$) from those of the corresponding reference (blank 30%E). The exception was seen with Q_s and EF of Q_s ($p > 0.05$). All parameters of both ethosomal systems were, on the other hand, comparable ($p > 0.05$). These data indicated that both empty ethosomal systems had a remarkable penetration enhancing effect. Skin penetration enhancement has also been reported with niosomes. Hofland, van der Geest et al. (1994) found that human stratum corneum pretreated with empty niosomes allowed higher estradiol fluxes compared with the untreated stratum corneum. El Maghraby et al. (1999) also studied the effect of skin pretreatment with empty liposomes on epidermal permeability to estradiol from the saturated solution. They found that the pretreatment with blank pure PC liposomes provided a greater enhancement ratio of estradiol than the treatment with estradiol-entrapped PC liposomes. Fang et al. (2001) studied enoxacin permeation across nude-mouse skin by pretreatment of the skin with empty PC liposomes and Span®60 niosomes. The total amount of enoxacin permeated from both types of empty vesicles was better than that from the corresponding control. However, some other earlier studies resulted in a different finding (du Plessis, Weiner et al., 1994; Honeywell-Nguyen and Bouwstra,

2003). In these studies, the tested vesicles did not display the penetration enhancing effect. Thus, the penetration enhancement mechanism might also depend on the physicochemical properties of both the drug and the vesicles.

Table 9: Permeation parameters of PTU from an aqueous solution at 80% saturation after pretreatment of the skin with blank hydroalcoholic solution and blank ethosomal systems

Permeation parameter	Pretreated formula		
	Blank 30%E	**Blank PCE**	**Blank PCCHE**
J_{ss} x 10^2	5.47 ± 0.63	17.65 ± 3.31	11.41 ± 1.41
P_s x 10^5	5.30 ± 0.63	17.12 ± 3.26	11.06 ± 1.39
Q_s (%)	1.91 ± 0.66	1.95 ± 0.37	2.59 ± 0.53
Q_{24} (%)	0.78 ± 0.10	2.45 ± 0.43	1.59 ± 0.20
RF	1	3.55 ± 0.82	2.16 ± 0.27
EF	1	3.56 ± 0.82	2.17 ± 0.27
EF of Q_s	1	1.59 ± 0.52	2.24 ± 0.76
EF of Q_{24}	1	3.49 ± 0.78	2.12 ± 0.25

Figures 22-25 present the RF, EF, EF of Q_s, and EF of Q_{24} of PTU from the PTU ethosomes and the PTU aqueous solution after pretreating the skin with empty ethosomes. No significant differences in these parameters were found between the treatment with PTU ethosomes and the pretreatment with empty ethosomes. Nevertheless, it is worth noting that the RF, EF, and EF of Q_{24} values of PTU from PTU-loaded ethosomes were higher when compared to those from the pretreatment with empty ethosomes (Figures 22-25). It indicated that PTU should be entrapped in the ethosomal system to obtain high skin permeation. This result is consistent with previous studies in that the drug should be incorporated in vesicular systems for more effective skin delivery. Kim et al. (2002) found that the skin permeation of caffeine from caffeine-loaded liposomes was higher than that from co-treatment of empty liposomes and caffeine solution. Similar results were noted by Paolino et al. (2005) who investigated in vitro percutaneous permeation through human skin of various

ethosomes containing either methyl-nicotinate or ammonium glycyrrhizinate. The ethosomes containing the tested drug was better than empty ethosomes with drug solution in terms of permeation through human skin. Elsayed et al. (2006) also reported that PC ethosomes was not able to improve skin delivery of non-entrapped ketotifen.

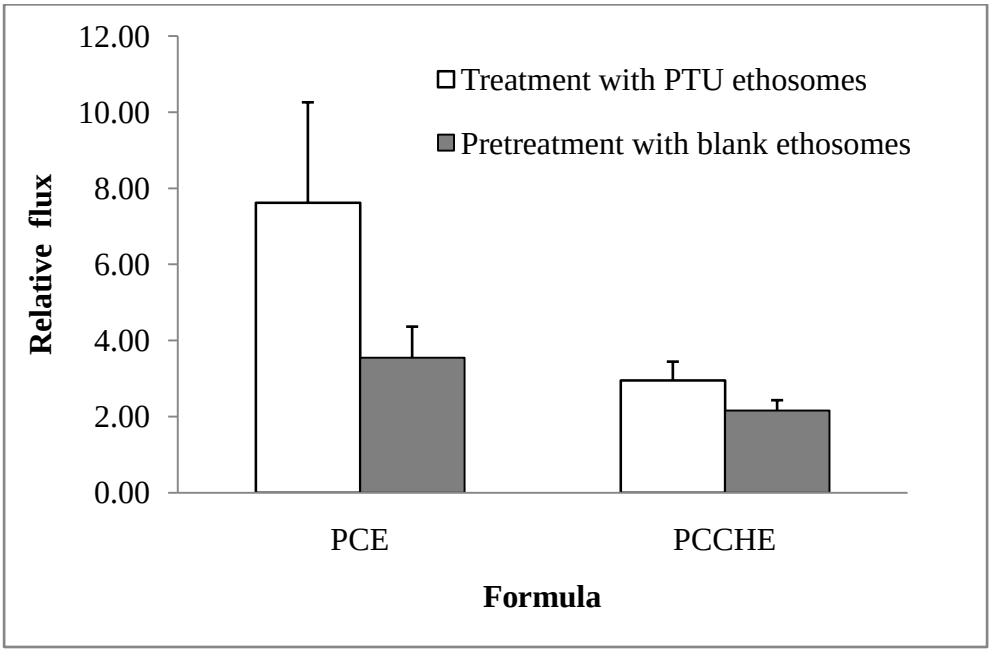

Figure 22: Relative fluxes of PTU from ethosomal systems and of PTU from aqueous solution at 80% saturation after pretreatment with blank ethosomal systems (Mean ± SEM, n = 6)

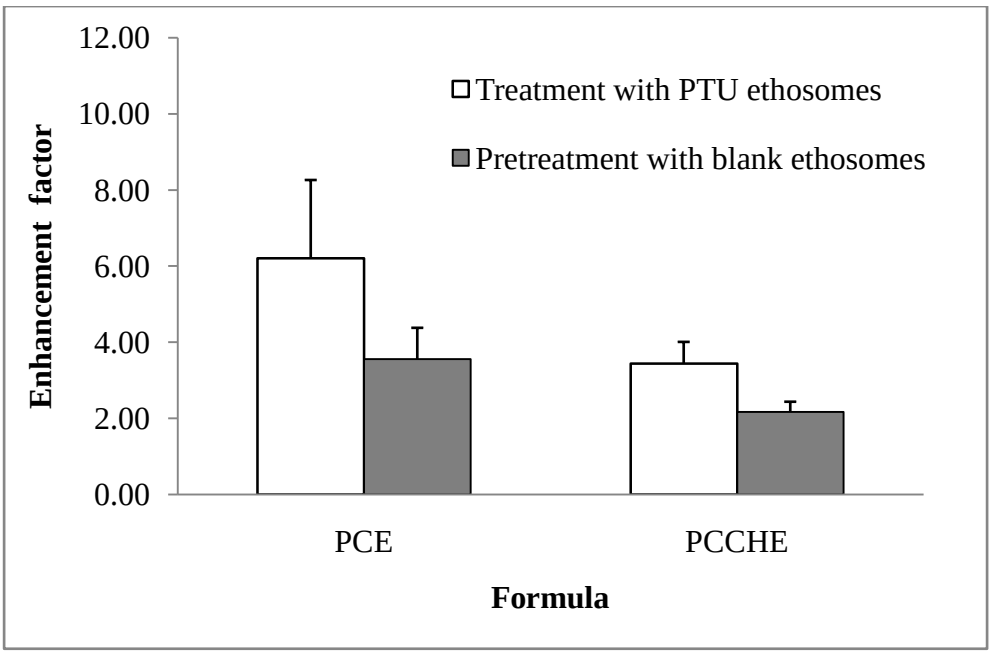

Figure 23: Enhancement factors of PTU from ethosomal systems and of PTU from aqueous solution at 80% saturation after pretreatment with blank ethosomal systems (Mean ± SEM, n = 6)

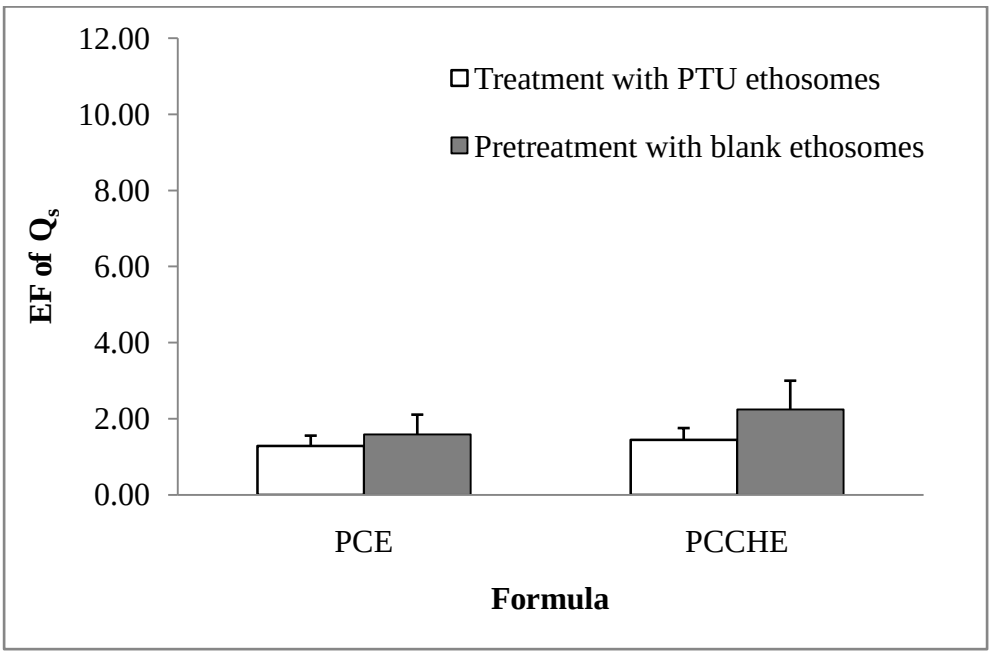

Figure 24: Enhancement factors of Q_s of PTU from ethosomal systems and of PTU from aqueous solution at 80% saturation after pretreatment with blank ethosomal systems (Mean ± SEM, n = 6)

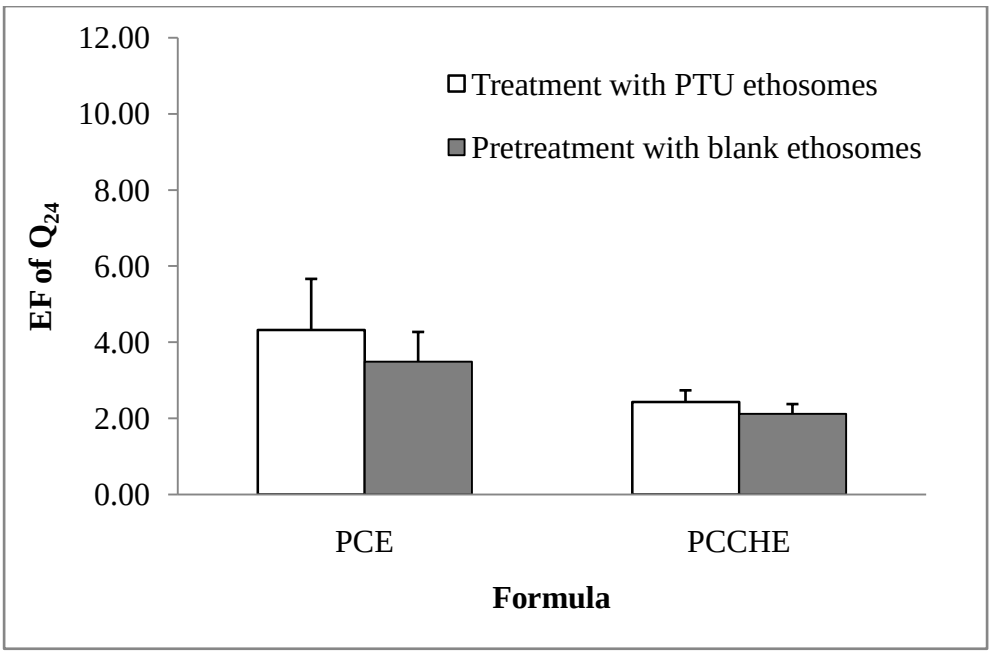

Figure 25: Enhancement factors of Q_{24} of PTU from ethosomal systems and of PTU from aqueous solution at 80% saturation after pretreatment with blank ethosomal systems (Mean ± SEM, n = 6)

The result obtained from comparing permeation parameters from the treatment of the skin with PTU ethosomes to those from the pretreatment with empty ethosomes implied that the penetration enhancement also involved in the PTU skin penetration.

Overall results from the mechanistic studies indicated that no sole mechanism operated on the enhancement of PTU delivery to the skin by the ethosomal systems. The data suggested that all three mechanisms might operate concurrently. Since the pretreatment with empty ethosomes gave EF as high as two thirds of that obtained with PTU-loaded ethosomes, the penetration enhancement might be the predominant mechanism for PTU skin delivery by the ethosomal systems.

CHAPTER V

CONCLUSIONS

This present study investigated the skin delivery of PTU from various vesicular systems. Formulation factors affecting skin permeation of PTU and underlying mechanisms for PTU skin delivery of the most efficient vesicular systems were also explored.

Three types of vesicular systems, i.e. liposomes, niosomes, and ethosomes, were prepared. The liposomal and ethosomal preparations were composed of PC with or without CH at a molar ratio of 7:3. The niosomal preparation was constructed from Span®20:CH:Solulan®C24 at a weight ratio of 28.5:18.6:2.48. PTU was incorporated into each preparation at 80% of its saturation solubility in order to establish equal thermodynamic activities among the systems studied.

The vesicular systems were characterized for size and size distribution, entrapment efficiency, phase transition temperature, and drug release. The size of the vesicle depended on the composition of vesicles and the method of preparation. The vesicular systems were heterogeneous in size. The PTU entrapment efficiency depended on the composition of the vesicles. Inclusion of CH in the lipid bilayer of both liposomes and ethosomes decreased PTU entrapment efficiency due to competition in intercalation of CH and PTU in the bilayer. The phase transition temperatures indicated that all vesicular systems were in liquid crystalline state. The phase transition temperatures of both ethosomal systems were lower in comparison with the corresponding liposomal systems. The release studies demonstrated that the vesicular systems studied could sustain the release of PTU and the release rate was consistent with the first order kinetics. These vesicular systems released PTU up to 90% in 24 hours.

Under the non-occlusive condition, the liposomal systems did not enhance PTU permeation through the newborn pig skin, while the niosomal system and the ethosomal systems were likely to improve PTU delivery to the newborn pig skin. However, solvent evaporation seemed to limit PTU delivery from these vesicular

systems, especially from the ethosomal systems. The permeation study under the occlusive condition revealed the prominent effect of the application condition on skin permeation of PTU from the ethosomal systems. Under the occlusive condition, PTU permeation of ethosomal systems was much improved. On the other hand, the necessity of the vesicular structure of ethosomes on PTU permeation seemed to be negligible when compared to the synergistic penetration enhancing effect of PC, ethanol, and PG, which was used as a co-solvent for PTU and the lipid components in the study.

A correlation trend between some characteristics (release rate and entrapment efficiency) of the ethosomes and EF of PTU permeation was evident. The diffusion of free drug and the mixing of the vesicles with skin lipids might involve in permeation of PTU from the ethosomal systems. Moreover, penetration enhancement might also be a probable mechanism of PTU delivery from ethosomes.

The overall results of this study indicated that PTU delivery from various vesicular systems depended on the type of the vesicles as well as the application condition. The best PTU delivery through the skin was seen with ethosomes under the occlusive condition. The diffusion of free drug, the mixing of the vesicles with the skin lipids, and the penetration enhancement might operate together on the PTU skin permeation. The penetration enhancement might be the main probable mechanism of PTU delivery from the ethosomal systems.

However, this research work did not investigate interaction of the ethosomal systems with the skin lipids. Such information would be useful in further development of PTU ethosomes for clinical use. In addition, if co-treatment of the blank ethosomes with PTU solution could provide the enhancement effect as well as, or better than the pretreatment could, the necessity to entrap PTU in the vesicles might be eliminated. Therefore, further studies such as the investigation of vesicle-skin interaction and the co-treatment of the blank ethosomes with PTU solution should be performed.

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APPENDICES

APPENDIX A

Closed water-jacketed glass cell for preparation of ethosomes

Figure A1: A closed water-jacketed glass cell for preparation of ethosomes

APPENDIX B

Bartlett assay (New, 1997)

Standard preparation

Table B1: Preparation of phosphate standard solutions

Stock solution	Transferred volume (µl)	Adjusted volume (ml)	Concentration of standard solutions (µmole/ml)
Phosphorus 6.4 µmole/ml	100	10	0.064
	150		0.096
	200		0.128
	250		0.160
	300		0.192
	350		0.224
	400		0.256

Sample preparation

1. For vesicular dispersions

Vesicular dispersions were diluted with distilled water to obtain 1 mg/ml of phospholipid.

2. For phospholipid solutions

Lipid solutions were diluted to obtain 1 mg/ml of phospholipid.

Assay procedure

Standards: Standard solutions 0.5 ml of each concentration

Blank: Double-distilled water 0.5 ml

Sample: 50 µl of sample were dried down and resuspended in 0.5 ml of distilled water.

Pipette standards, blank, or samples into separated test tube with cap

↓

Add 0.4 ml of sulphuric acid reagent to each tube

↓ Cover and incubate in hot air oven at 180-200 °C for an hour

↓ Cool by standing at room temperature

Add 0.1 ml of diluted hydrogen peroxide to each tube

↓ Incubate in hot air oven at 180-200 °C for 30 minutes to achieve clear solution

Assay procedure (continued)

↓ Cool by standing at room temperature

Add 4.6 ml of acid-molybdate solution to each tube and mix

↓

Add 0.2 ml of Fiske&Subbarow reducer to each tube and mix

↓

Cover and place them in a boiling water bath for 7 minutes

↓ Cool by standing at room temperature

Measure absorbance of all tubes against blank at 800 nm

APPENDIX C

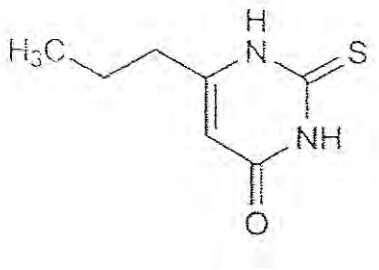
Molecular structure and physical properties of propylthiouracil (PTU)

(Aboul-Enein, 1977; Moffat et al., eds., 2004)

1. **Molecular structure**

1.1 Empirical: $C_7H_{10}N_2OS$

1.2 Structural:



1.3 Molecular weight: 170.23

2. **Physical properties**

2.1 Melting range: 219-221 °C

2.2 Log P: 1.0

2.3 Solubility:

PTU is sparingly soluble in water (1:900 at 20 °C), soluble in 100 parts of boiling water, in 60 parts of ethanol, in 60 parts of acetone, practically insoluble in ether, chloroform, benzene, freely soluble in aqueous solutions of ammonia and alkali hydroxide. A saturated aqueous solution is neutral or slightly acidic to litmus.

2.4 Ultraviolet spectrum:

PTU in neutral methanol absorbs ultraviolet radiation at 275 nm (molar absorptivity = 15800) and at 214 nm (molar absorptivity = 15600). In alkaline medium, it shows maxima at 315.5 nm (molar absorptivity = 10900), 260 nm (molar absorptivity = 10700) and at 207.5 nm (molar absorptivity = 15400).

2.5 Stability:

PTU is a relatively stable compound at room temperature. It is recommended that it should be kept in a well-closed container protected from light.

APPENDIX D

Molecular structures of PC, CH, Span®20, and Solulan®C24

(Cevc, ed., 1993; Graham and Higglin, 1997; Uchegbu and Vyas, 1998; Kibbe, ed., 2000; Sigma-Aldrich, 2009)

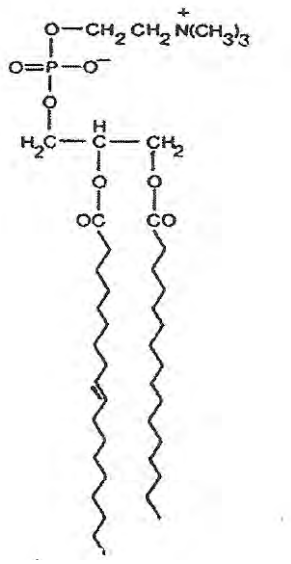
Phosphatidylcholine

Synonym: 1, 2-diacyl-sn-glycerol-3-phosphoryl choline

Empirical: -

Molecular weight: 760-780

Structure:



(From Graham and Higglin, 1997)

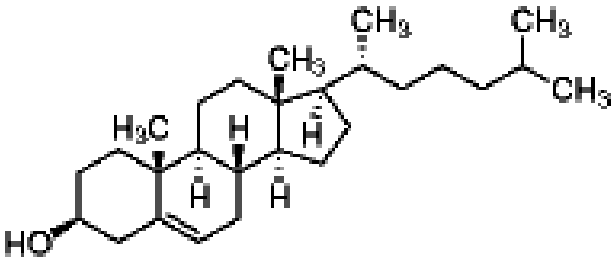
Cholesterol

Synonym: 3β-Hydroxy-5-cholestene, 5-Cholesten-3β-ol

Empirical: $C_{27}H_{46}O$

Molecular weight: 386.65

Structure:



(From Sigma-Aldrich, 2009)

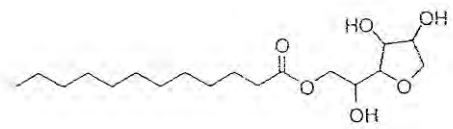
Span®20

Synonym: Sorbitan laurate; Sorbitan monododecanoate

Empirical: $C_{18}H_{34}O_6$

Molecular weight: 346

Structure:



(From Kibbe, ed., 2000)

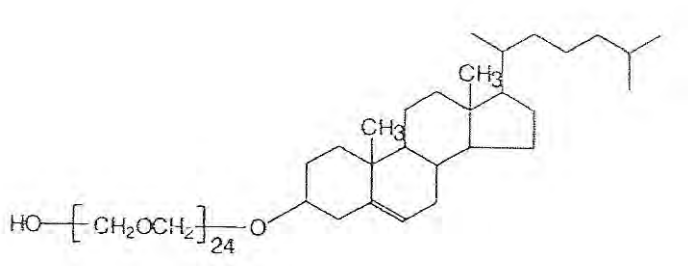
Solulan®C24

Synonym: Cholesteryl poly-24-oxyethylene ether

Empirical: -

Molecular weight: 1443

Structure:



(From Kibbe, ed., 2000)

APPENDIX E

Permeation parameters of PTU from various systems

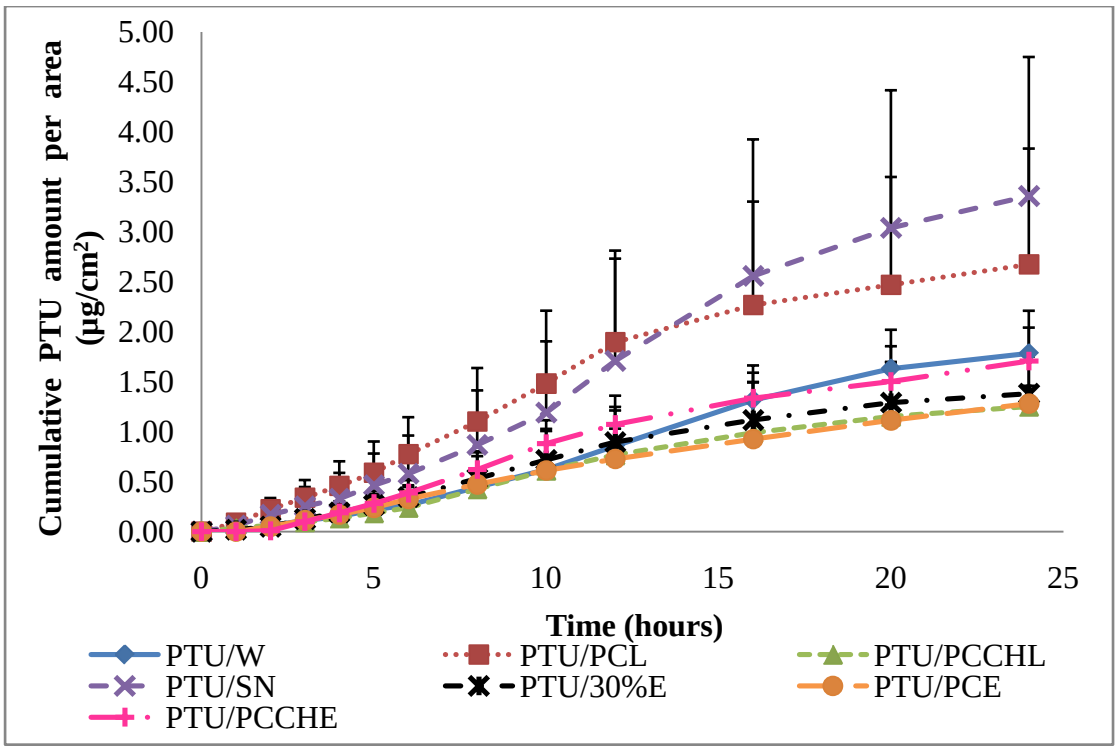


Figure E1: Permeation profiles of PTU solutions and PTU vesicular systems under the non-occlusive condition (Mean ± SEM, n = 5)

Table E1: Permeation parameters of PTU solutions and PTU vesicular systems under the non-occlusive condition (Mean ± SEM, n = 5)

Permeation parameter	Formula						
	PTU/W	**PTU/PCL**	**PTU/PCCHL**	**PTU/SN**	**PTU/30%E**	**PTU/PCE**	**PTU/PCCHE**
J_{ss} x 10^2	10.26 ± 2.80	17.40 ± 8.37	7.78 ± 3.94	21.20 ± 9.46	9.66 ± 2.91	8.16 ± 1.57	11.44 ± 2.60
P_s x 10^5	10.28 ± 2.74	7.22 ± 3.52	4.21 ± 2.12	10.26 ± 4.49	2.16 ± 0.65	1.53 ± 0.30	2.84 ± 0.63
Q_s (%)	2.42 ± 0.55	1.44 ± 0.26	2.90 ± 0.73	1.33 ± 0.28	1.18 ± 0.57	0.79 ± 0.27	0.69 ± 0.17
Q_{24} (%)	1.10 ± 0.31	0.74 ± 0.32	0.45 ± 0.20	1.09 ± 0.44	0.21 ± 0.06	0.16 ± 0.02	0.28 ± 0.05
RF	1	1.49 ± 0.36	0.96 ± 0.39	2.34 ± 0.91	1	1.16 ± 0.34	1.54 ± 0.61
EF	1	0.61 ± 0.15	0.51 ± 0.21	1.13 ± 0.43	1	0.96 ± 0.27	1.68 ± 0.63
EF of Q_s	1	0.67 ± 0.14	1.23 ± 0.29	0.64 ± 0.16	1	1.08 ± 0.35	1.14 ± 0.42
EF of Q_{24}	1	0.62 ± 0.15	0.55 ± 0.21	1.19 ± 0.47	1	0.98 ± 0.23	1.78 ± 0.64
Lag time (h)	4.76 ± 0.73	1.75 ± 1.20	3.03 ± 1.49	5.09 ± 1.09	3.86 ± 1.50	2.38 ± 0.78	2.92 ± 0.70
Analytical recovery (%)	104.53 ± 1.52	82.75 ± 2.11	77.57 ± 1.06	95.88 ± 1.15	99.63 ± 2.56	96.77 ± 1.19	93.06 ± 1.73

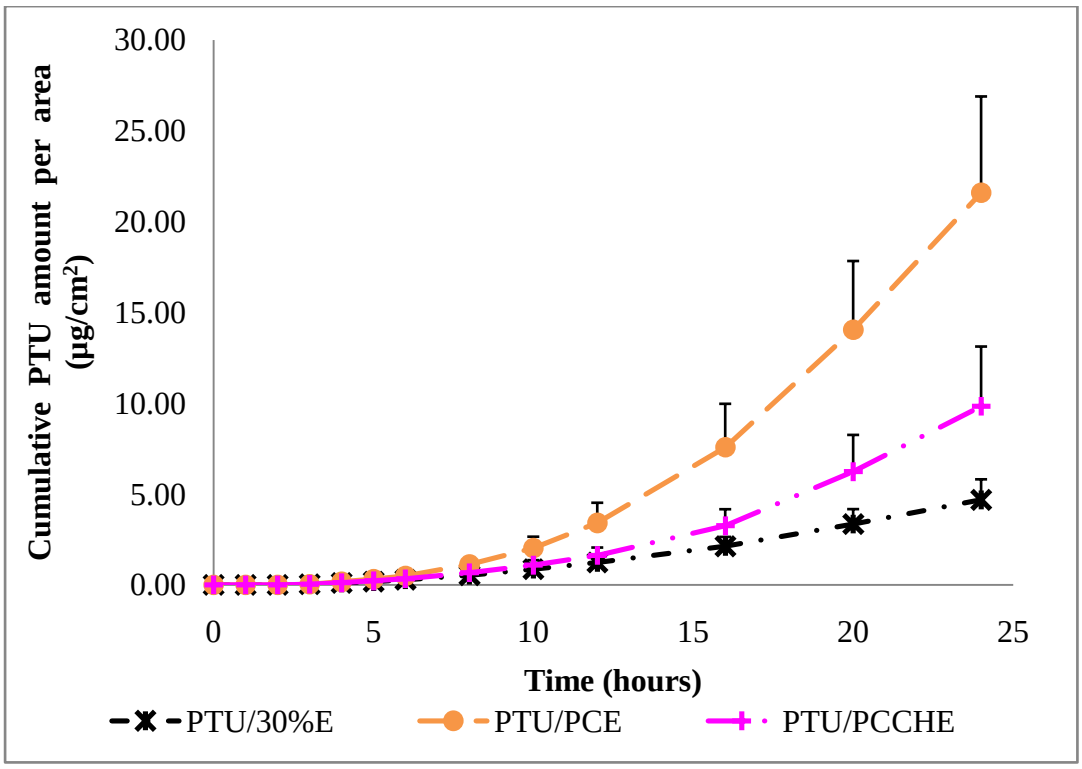


Figure E2: Permeation profiles of PTU hydroalcoholic solution and PTU ethosomal systems under the occlusive condition (Mean ± SEM, n = 4)

Table E2: Permeation parameters of PTU hydroalcoholic solution and PTU ethosomal systems under the occlusive condition (Mean ± SEM, n = 6)

Permeation parameter	Formula		
	PTU/30%E	**PTU/PCE**	**PTU/PCCHE**
J_{ss} x 10^2	23.77 ± 4.57	143.90 ± 30.07	71.13 ± 21.29
P_s x 10^5	5.06 ± 0.97	25.46 ± 5.60	17.58 ± 5.17
Q_s (%)	1.91 ± 0.69	1.68 ± 0.11	2.04 ± 0.46
Q_{24} (%)	0.61 ± 0.12	2.18 ± 0.54	1.45 ± 0.36
RF	1	7.62 ± 2.64	2.95 ± 0.50
EF	1	6.21 ± 2.06	3.44 ± 0.57
EF of Q_s	1	1.29 ± 0.27	1.45 ± 0.31
EF of Q_{24}	1	4.32 ± 1.34	2.43 ± 0.30
Lag time (h)	7.15 ± 0.29	11.22 ± 0.86	11.03 ± 0.92
Analytical recovery (%)	96.51 ± 0.91	93.82 ± 0.57	94.94 ± 1.24

Table E3: Permeation parameters of PTU hydroalcoholic solution and PTU ethosomal systems under the non-occlusive and the occlusive conditions (Mean ± SEM, n = 5-6)

Permeation parameter	Non-occlusion			Occlusion		
	PTU/30%E	**PTU/PCE**	**PTU/PCCHE**	**PTU/30%E**	**PTU/PCE**	**PTU/PCCHE**
J_{ss} x 10^2	9.66 ± 2.91	8.16 ± 1.57	11.44 ± 2.60	23.77 ± 4.57	143.90 ± 30.07	71.13 ± 21.29
P_s x 10^5	2.16 ± 0.65	1.53 ± 0.30	2.84 ± 0.63	5.06 ± 0.97	25.46 ± 5.60	17.58 ± 5.17
Q_s (%)	1.18 ± 0.57	0.79 ± 0.27	0.69 ± 0.17	1.91 ± 0.69	1.68 ± 0.11	2.04 ± 0.46
Q_{24} (%)	0.21 ± 0.06	0.16 ± 0.02	0.28 ± 0.05	0.61 ± 0.12	2.18 ± 0.54	1.45 ± 0.36
RF	1	1.16 ± 0.34	1.54 ± 0.61	1	7.62 ± 2.64	2.95 ± 0.50
EF	1	0.96 ± 0.27	1.68 ± 0.63	1	6.21 ± 2.06	3.44 ± 0.57
EF of Q_s	1	1.08 ± 0.35	1.14 ± 0.42	1	1.29 ± 0.27	1.45 ± 0.31
EF of Q_{24}	1	0.98 ± 0.23	1.78 ± 0.64	1	4.32 ± 1.34	2.43 ± 0.30
Lag time (h)	3.86 ± 1.50	2.38 ± 0.78	2.92 ± 0.70	7.15 ± 0.29	11.22 ± 0.86	11.03 ± 0.92
Analytical recovery (%)	99.63 ± 2.56	96.77 ± 1.19	93.06 ± 1.73	96.51 ± 0.91	93.82 ± 0.57	94.94 ± 1.24

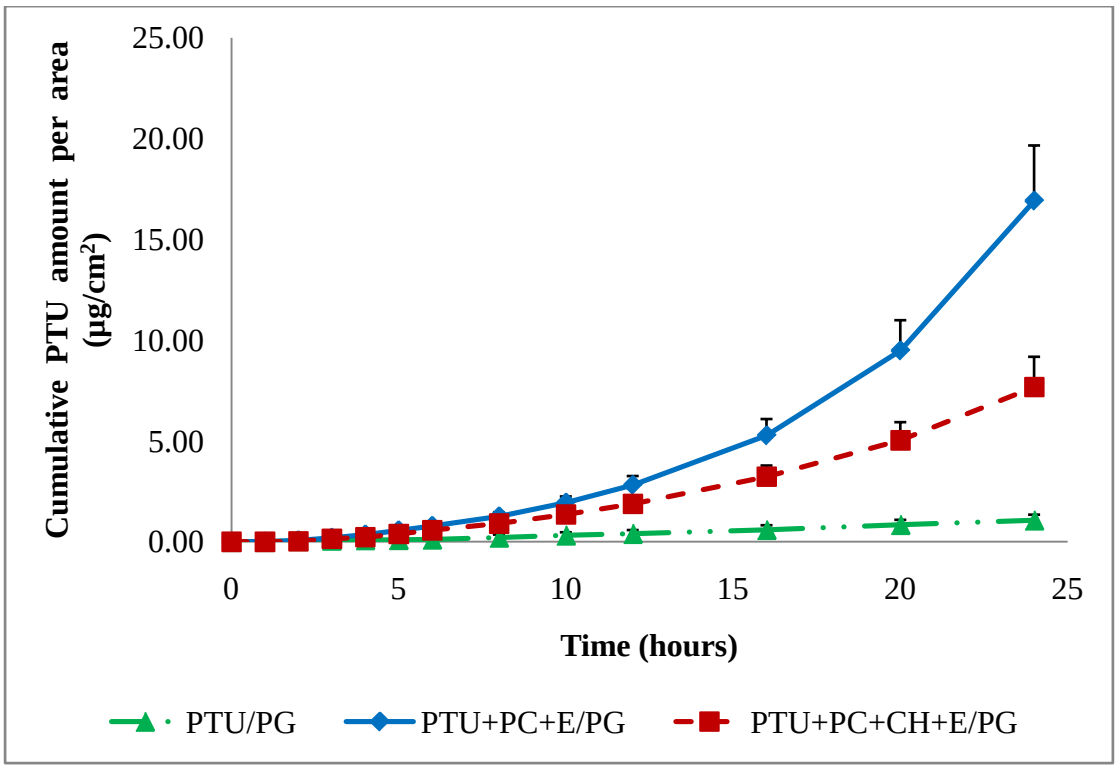


Figure E3: Permeation profiles of PTU permeation from physical mixtures of ethosomal components in 90% (v/v) PG under the occlusive condition (Mean ± SEM, n = 6)

Table E4: Permeation parameters of PTU from PTU solution in 90% (v/v) PG and from physical mixtures of ethosomal components in 90% (v/v) PG (Mean ± SEM, n = 6)

Permeation parameter	PTU/PG	PTU+PC+E/PG	PTU+PC+CH+E/PG
J_{ss} x 10^2	5.17 ± 1.04	145.48 ± 25.15	50.22 ± 12.18
P_s x 10^5	0.21 ± 0.04	6.00 ± 1.04	2.08 ± 0.51
Q_s (%)	0.39 ± 0.11	0.27 ± 0.04	0.31 ± 0.04
Q_{24} (%)	0.03 ± 0.01	0.47 ± 0.08	0.21 ± 0.04
RF	1	30.74 ± 4.53	9.47 ± 1.42
EF	1	30.77 ± 4.53	9.51 ± 1.44
EF of Q_s	1	1.03 ± 0.31	1.11 ± 0.29
EF of Q_{24}	1	19.04 ± 2.89	6.30 ± 1.48
Lag time (h)	5.29 ± 0.85	12.59 ± 0.36	8.23 ± 0.90
Analytical recovery (%)	99.65 ± 0.38	95.39 ± 0.49	95.68 ± 2.01

Table E5: Permeation parameters of PTU from solutions, vesicles and physical mixtures of ethosomal components in 90% (v/v) PG under the occlusive condition (Mean ± SEM, n = 6)

Permeation parameter	Solutions		Vesicles		Physical mixture in 90% PG	
	PTU/30%E	PTU/PG	PTU/PCE	PTU/PCCHE	PTU+PC+E/PG	PTU+PC+CH+E/PG
J_{ss} x 10^2	23.77 ± 4.57	5.17 ± 1.04	143.90 ± 30.07	71.13 ± 21.29	145.48 ± 25.15	50.22 ± 12.18
P_s x 10^5	5.06 ± 0.97	0.21 ± 0.04	25.46 ± 5.60	17.58 ± 5.17	6.00 ± 1.04	2.08 ± 0.51
Q_s (%)	1.91 ± 0.69	0.39 ± 0.11	1.68 ± 0.11	2.04 ± 0.46	0.27 ± 0.04	0.31 ± 0.04
Q_{24} (%)	0.61 ± 0.12	0.03 ± 0.01	2.18 ± 0.54	1.45 ± 0.36	0.47 ± 0.08	0.21 ± 0.04
RF	1	1	7.62 ± 2.64	2.95 ± 0.50	30.74 ± 4.53	9.47 ± 1.42
EF	1	1	6.21 ± 2.06	3.44 ± 0.57	30.77 ± 4.53	9.51 ± 1.44
EF of Q_s	1	1	1.29 ± 0.27	1.45 ± 0.31	1.03 ± 0.31	1.11 ± 0.29
EF of Q_{24}	1	1	4.32 ± 1.34	2.43 ± 0.30	19.04 ± 2.89	6.30 ± 1.48
Lag time (h)	7.15 ± 0.29	5.29 ± 0.85	11.22 ± 0.86	11.03 ± 0.92	12.59 ± 0.36	8.23 ± 0.90
Analytical recovery (%)	96.51 ± 0.91	99.65 ± 0.38	93.82 ± 0.57	94.94 ± 1.24	95.39 ± 0.49	95.68 ± 2.01

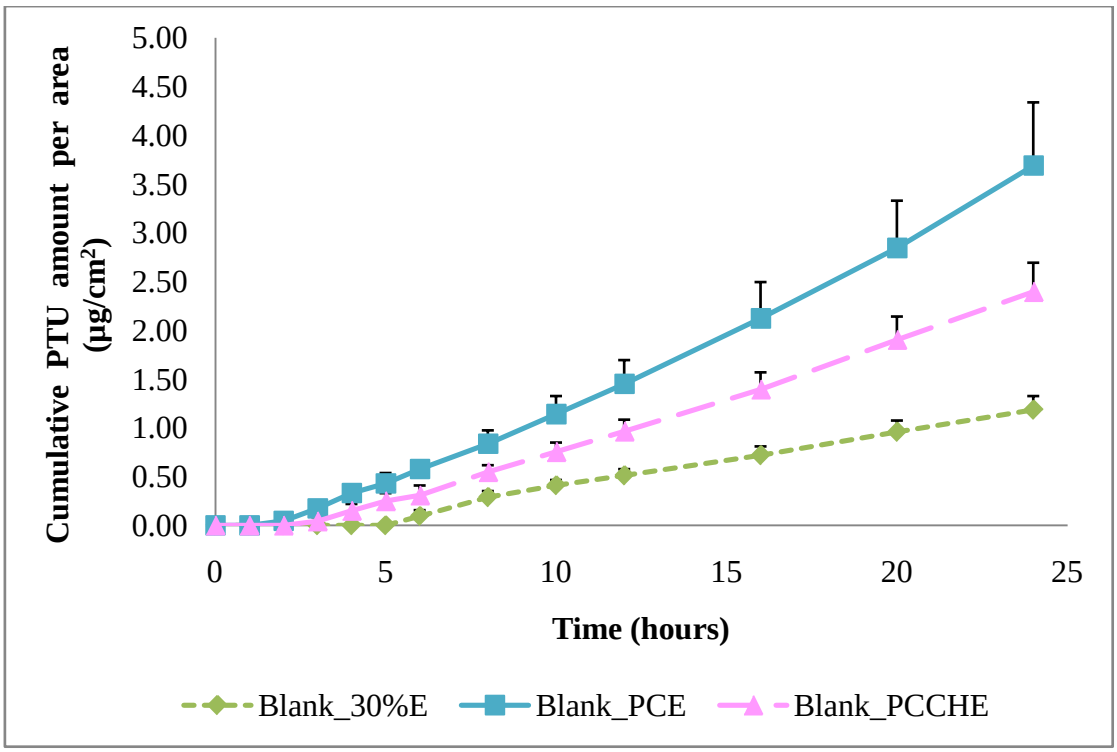


Figure E4: Permeation profiles of PTU from aqueous solution at 80% saturation after pretreatment of the skin with blank hydroalcoholic solution and blank ethosomal systems (Mean ± SEM, n=6)

Table E6: Permeation parameters of PTU from aqueous solution at 80% saturation after pretreatment of the skin with blank hydroalcoholic solution and blank ethosomal systems (Mean ± SEM, n=6)

Permeation parameter	Pretreated formula		
	Blank 30%E	**Blank PCE**	**Blank PCCHE**
J_{ss} x 10^2	5.47 ± 0.63	17.65 ± 3.31	11.41 ± 1.41
P_s x 10^5	5.30 ± 0.63	17.12 ± 3.26	11.06 ± 1.39
Q_s (%)	1.91 ± 0.66	1.95 ± 0.37	2.59 ± 0.53
Q_{24} (%)	0.78 ± 0.10	2.45 ± 0.43	1.59 ± 0.20
RF	1	3.55 ± 0.82	2.16 ± 0.27
EF	1	3.56 ± 0.82	2.17 ± 0.27
EF of Q_s	1	1.59 ± 0.52	2.24 ± 0.76
EF of Q_{24}	1	3.49 ± 0.78	2.12 ± 0.25
Lag time (h)	2.36 ± 0.47	3.34 ± 0.56	3.31 ± 0.23
Analytical recovery (%)	100.69 ± 1.11	89.24 ± 4.34	91.70 ± 3.87

Table E7: Permeation parameters of PTU hydroalcoholic solution, PTU ethosomal systems, and PTU aqueous solution at 80% saturation after pretreatment of the skin with blank hydroalcoholic solution and blank ethosomal systems (Mean ± SEM, n = 6)

Permeation parameter	Treated formula			Pretreated formula		
	PTU/30%E	PTU/PCE	PTU/PCCHE	Blank 30%E	Blank PCE	Blank PCCHE
$J_{ss} \times 10^2$	23.77 ± 4.57	143.90 ± 30.07	71.13 ± 21.29	5.47 ± 0.63	17.65 ± 3.31	11.41 ± 1.41
$P_s \times 10^5$	5.06 ± 0.97	25.46 ± 5.60	17.58 ± 5.17	5.30 ± 0.63	17.12 ± 3.26	11.06 ± 1.39
Q_s (%)	1.91 ± 0.69	1.68 ± 0.11	2.04 ± 0.46	1.91 ± 0.66	1.95 ± 0.37	2.59 ± 0.53
Q_{24} (%)	0.61 ± 0.12	2.18 ± 0.54	1.45 ± 0.36	0.78 ± 0.10	2.45 ± 0.43	1.59 ± 0.20
RF	1	7.62 ± 2.64	2.95 ± 0.50	1	3.55 ± 0.82	2.16 ± 0.27
EF	1	6.21 ± 2.06	3.44 ± 0.57	1	3.56 ± 0.82	2.17 ± 0.27
EF of Q_s	1	1.29 ± 0.27	1.45 ± 0.31	1	1.59 ± 0.52	2.24 ± 0.76
EF of Q_{24}	1	4.32 ± 1.34	2.43 ± 0.30	1	3.49 ± 0.78	2.12 ± 0.25
Lag time (h)	7.15 ± 0.29	11.22 ± 0.86	11.03 ± 0.92	2.36 ± 0.47	3.34 ± 0.56	3.31 ± 0.23
Analytical recovery (%)	96.51 ± 0.91	93.82 ± 0.57	94.94 ± 1.24	100.69 ± 1.11	89.24 ± 4.34	91.70 ± 3.87

APPENDIX F

Verification of the UV spectroscopic method for PTU entrapment and release studies

Verification for the quantitative determination of PTU in isopropanol by UV spectroscopy

1. Specificity

Under the UV absorption spectrophotometric method used, the absorbance of PTU must not be interfered by the absorbance of other components in the sample. The spectra of blank vesicular dispersions (without PTU) and corresponding PTU vesicular dispersions was compared with the spectra of the PTU.

2. Linearity

Eight standard solutions of PTU ranging from 1.0 to 8.0 µg/ml were prepared and analyzed. Linear regression analysis of the absorbance versus the corresponding concentration was performed. The linearity was determined from the coefficient of determination.

3. Accuracy

Three sets of mixtures of vesicular components (in equivalent amounts to those present in 1 ml vesicular dispersion) spiked with PTU solutions were prepared to obtained the PTU concentration at 1.5, 4.5, and 7.5 µg/ml. Each individual sample was analyzed for PTU by UV spectrophotometry at 275 nm. The analytical recovery of each sample was calculated and compared with the amount of PTU added.

4. Precision

The within run precision was evaluated by analyzing five replicates of three sets of the solutions of PTU in the same day. The mean, standard deviation (SD), and the coefficient of variation (% CV) at each concentration were determined.

Results from verification for the quantitative determination of PTU solution in isopropanol by UV Spectrophotometry

The analytical parameters used for the assay verification were specificity, linearity, accuracy, and precision.

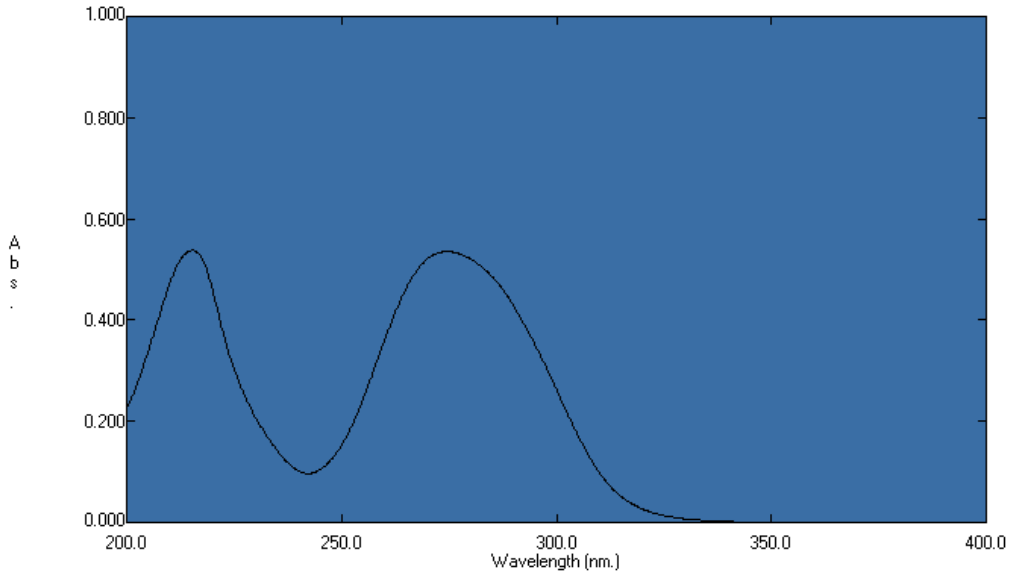


Figure F1: Absorption spectrum of PTU in water diluted with isopropanol

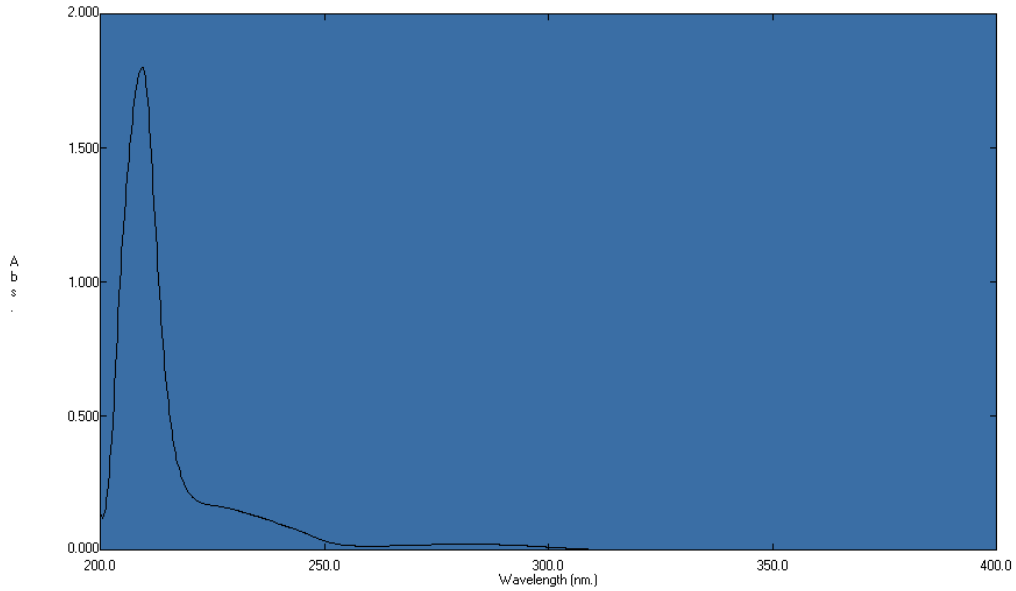


Figure F2: Absorption spectrum of PC in water diluted with isopropanol

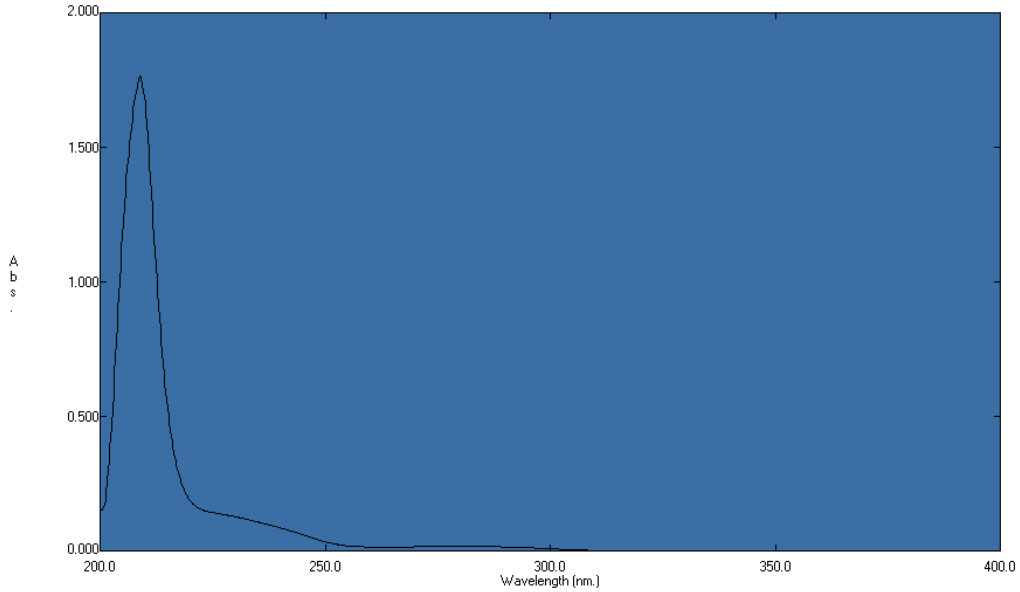


Figure F3: Absorption spectrum of PC and CH in water diluted with isopropanol

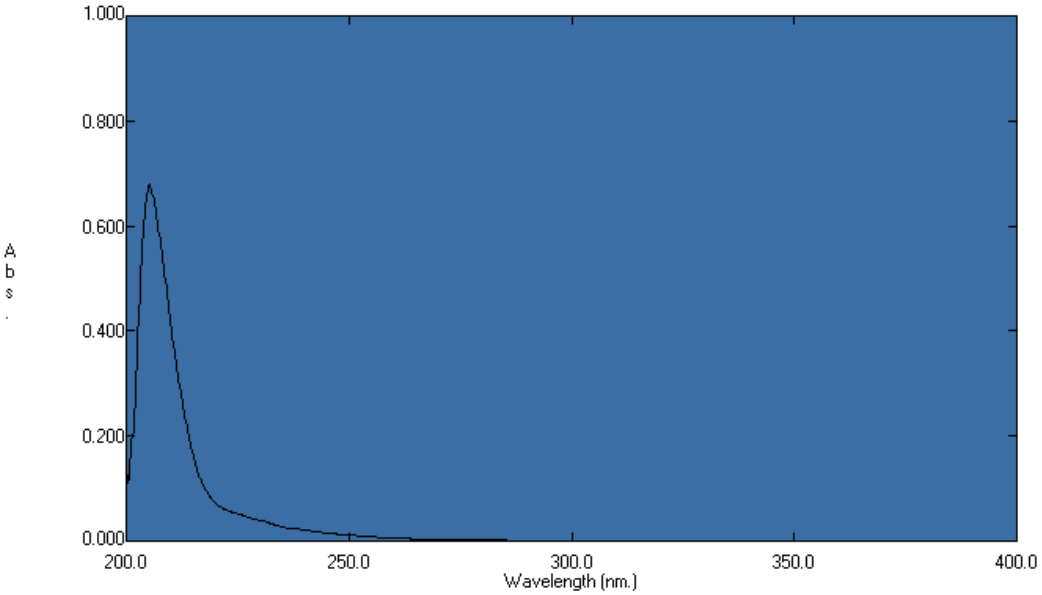


Figure F4: Absorption spectrum of Span®20:CH:Solulan®C24 in water diluted with isopropanol

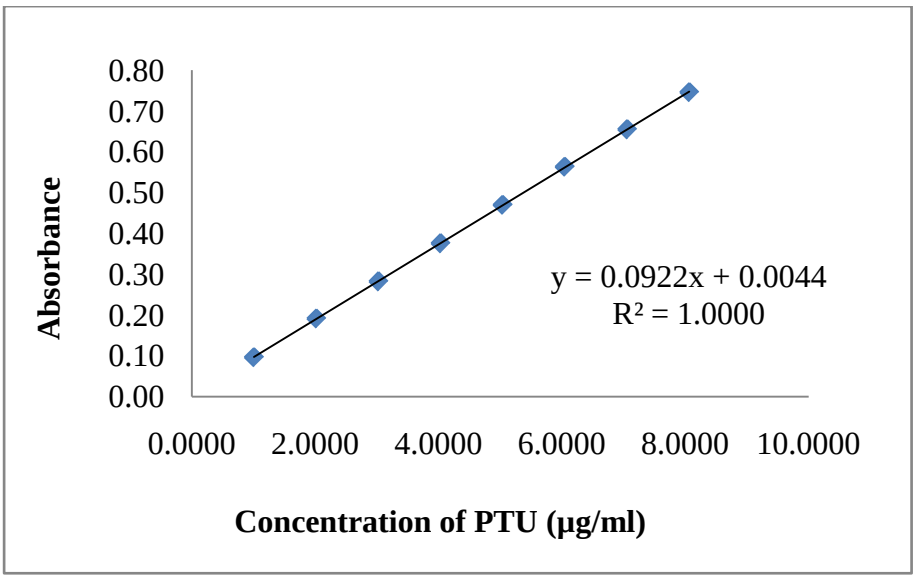


Figure F5: A representation of standard calibration lines of PTU in water diluted with isopropanol

Table F1: The percentages of analytical recovery of PTU diluted with isopropanol by UV spectrophotometric method

Actual concentration of PTU (µg/ml)	Calculated concentration of PTU (µg/ml)	% Analytical recovery
1.5060	1.5586	103.49
1.5060	1.5358	101.98
1.5060	1.5412	102.34
1.5060	1.5293	101.55
1.5060	1.5401	102.27
4.5180	4.6486	102.89
4.5180	4.6171	102.19
4.5180	4.6117	102.07
4.5180	4.5857	101.50
4.5180	4.6020	101.86
7.5300	7.7668	103.14
7.5300	7.6898	102.12
7.5300	7.6985	102.24
7.5300	7.6833	102.04
7.5300	7.6725	101.89

Mean % Analytical recovery = 102.24

SD = 0.55

% CV = 0.54

Table F2: Data for the within run precision of PTU diluted with isopropanol by UV spectrophotometric method

Conc. (µg/ml)	Calculated Conc. (µg/ml)					Mean	SD	% CV
	1	2	3	4	5			
1.5060	1.5586	1.5358	1.5412	1.5293	1.5401	1.5410	0.0109	0.7063
4.5180	4.6486	4.6171	4.6117	4.5857	4.6020	4.6130	0.0232	0.5030
7.5300	7.7668	7.6898	7.6985	7.6833	7.6725	7.7022	0.0374	0.4851

In conclusion, the analysis of PTU solution in isopropanol by UV spectrophotometric method verified in this study showed good specificity, linearity, accuracy, and precision. Thus, this method could use for the quantitative determination of PTU in the entrapment efficiency and release studies.

APPENDIX G

Verification of the HPLC method for permeation studies

Verification for the quantitative determination of PTU by the HPLC method

1. Specificity

Under the HPLC method used, the chromatographic peak of PTU must not be interfered by the chromatographic peaks of other components in the sample. The chromatogram of the blank vesicular dispersion (without PTU) was compared with the chromatogram of the PTU solution.

2. Linearity

Eight standard solutions of PTU (ranging from 0.05 to 10.0 µg/ml for the PBS system or from 0.10 to 10.0 µg/ml for the methanol system) were prepared and analyzed. Linear regression analysis of the absorbance versus the corresponding concentration was performed. The linearity was determined from the coefficient of determination.

3. Accuracy

Three sets of PTU solutions at 1.5, 5.0, and 8.5 µg/ml for the PBS system and 1.5, 4.0, and 9.0 µg/ml for the methanol system were prepared. Each individual sample was analyzed by the HPLC method. The analytical recovery of each PTU concentration was calculated.

4. Precision

The within run precision was evaluated by analyzing five replicates of the three standard solutions of PTU (at the same concentrations used for the accuracy determination) in the same day. The mean, standard deviation (SD), and the coefficient of variation (% CV) at each concentration were determined.

Results from verification for the quantitative determination of PTU by the HPLC method

The analytical parameters used for the assay verification were specificity, linearity, accuracy, and precision.

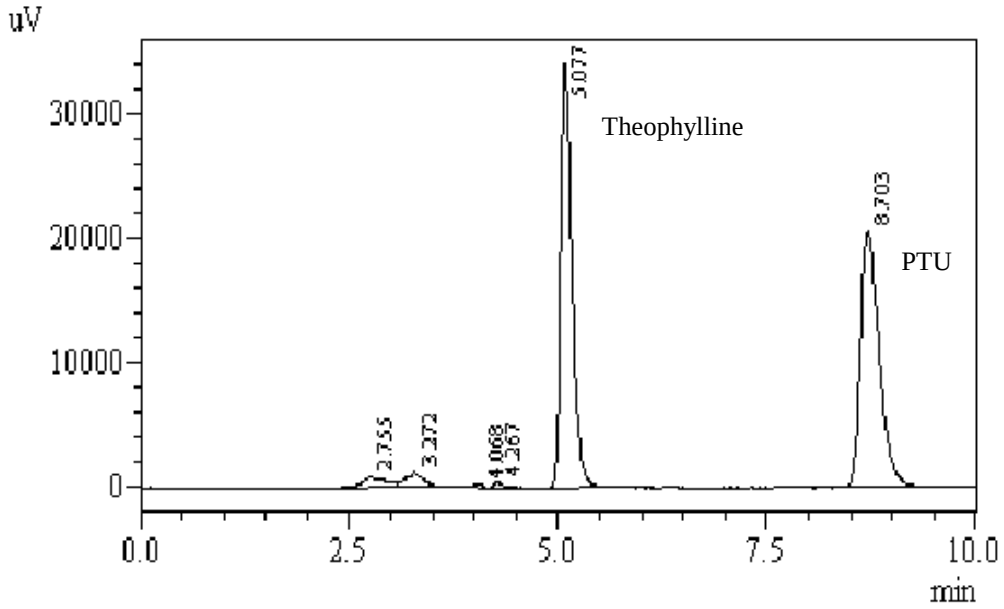


Figure G1: A representation of HPLC chromatograms of PTU and theophylline in PBS pH 7.4

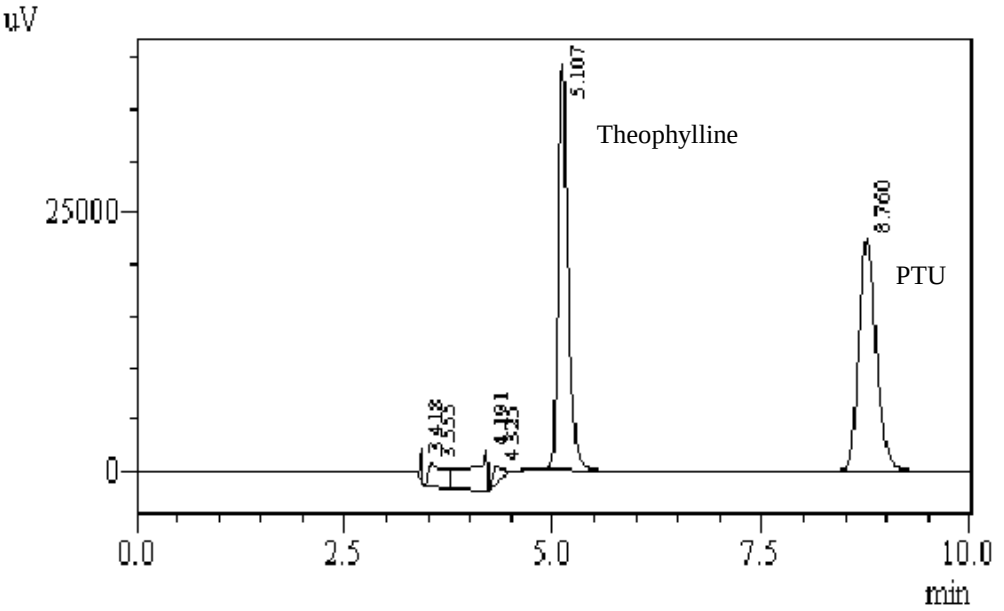


Figure G2: A representation of HPLC chromatograms of PTU and theophylline in methanol

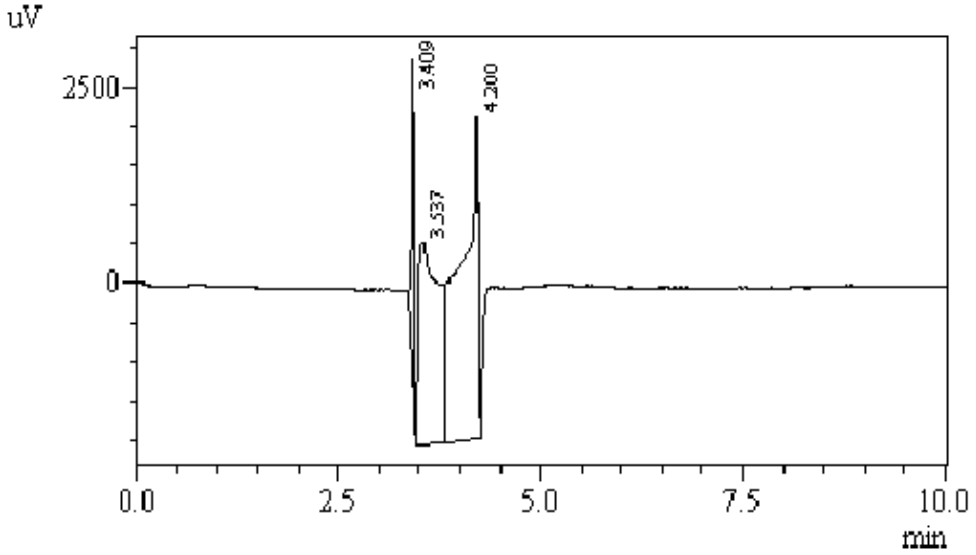


Figure G3: A representation of HPLC chromatogram of blank PCE in methanol

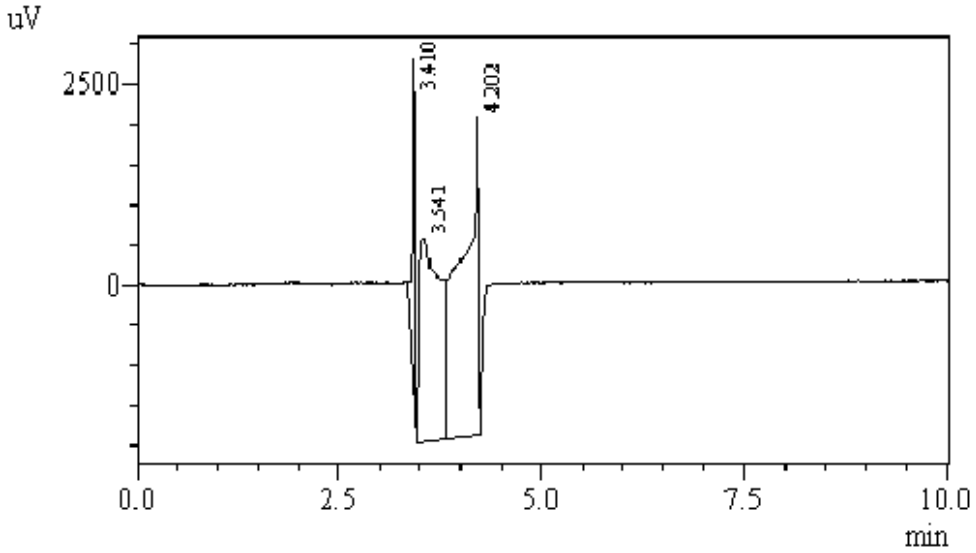


Figure G4: A representation of HPLC chromatogram of blank PCCHE in methanol

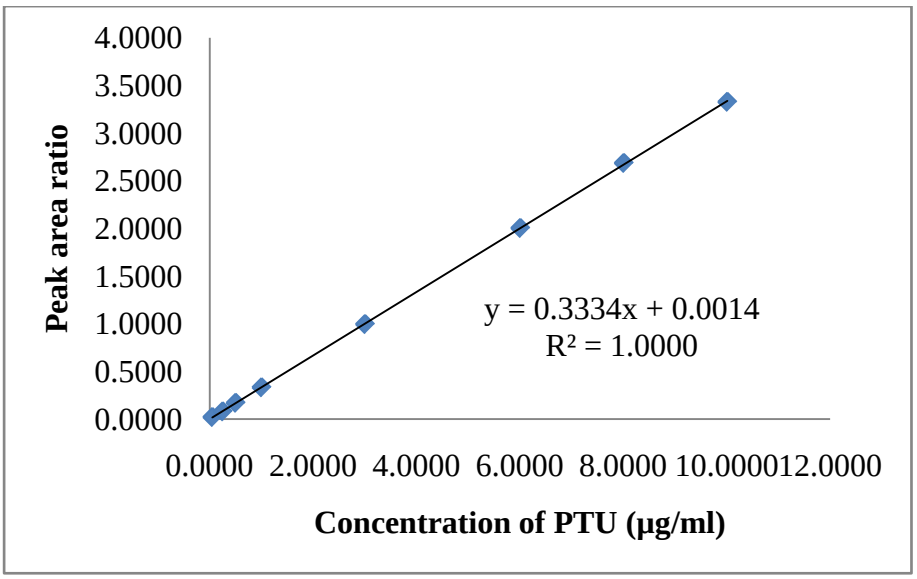


Figure G5: A representation of standard calibration lines of PTU diluted with PBS pH 7.4

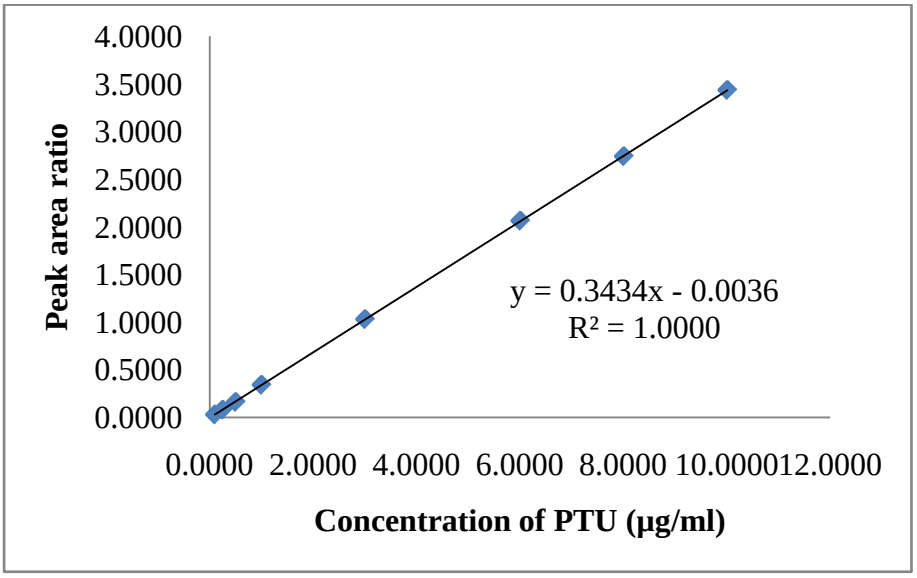


Figure G6: A representation of standard calibration lines of PTU diluted with methanol

Table G1: The percentages of analytical recovery of PTU in water diluted with PBS pH 7.4

Actual concentration of PTU (µg/ml)	Calculated concentration of PTU (µg/ml)	% Analytical recovery
0.1502	0.1492	99.35
0.1502	0.1520	101.25
0.1502	0.1473	98.09
0.1502	0.1474	98.18
0.1502	0.1525	101.53
5.0050	4.9503	98.91
5.0050	4.9905	99.71
5.0050	5.0277	100.45
5.0050	4.9684	99.27
5.0050	5.0215	100.33
8.5085	8.4223	98.99
8.5085	8.4504	99.32
8.5085	8.4554	99.38
8.5085	8.4742	99.60
8.5085	8.4662	99.50

Mean % Analytical recovery = 99.59

SD = 0.97

% CV = 0.98

Table G2: Data for the within run precision of PTU diluted with PBS pH 7.4 by HPLC method

Conc. (µg/ml)	Calculated Conc. (µg/ml)					Mean	SD	%CV
	1	2	3	4	5			
0.1502	0.1492	0.1520	0.1473	0.1474	0.1525	0.1497	0.0025	1.6493
5.0050	4.9503	4.9905	5.0277	4.9684	5.0215	4.9917	0.0333	0.6678
8.5085	8.4223	8.4504	8.4554	8.4742	8.4662	8.4537	0.0199	0.2349

Table G3: The percentages of analytical recovery of PTU in water diluted with methanol

Actual concentration of PTU (µg/ml)	Calculated concentration of PTU (µg/ml)	% Analytical recovery
0.1512	0.1540	101.83
0.1509	0.1540	102.03
0.1502	0.1508	100.46
0.1499	0.1555	103.78
0.1508	0.1551	102.91
4.0320	4.1237	102.27
4.0240	4.0890	101.62
4.0040	4.0735	101.73
3.9960	4.1345	103.47
4.0200	4.1323	102.79
9.0720	9.2001	101.41
9.0540	9.1539	101.10
9.0090	9.1641	101.72
8.9910	9.2868	103.29
9.0450	9.3107	102.94

Mean % Analytical recovery = 102.22

SD = 0.94

% CV = 0.92

Table G4: Data for the within run precision of PTU diluted with methanol by HPLC method

Conc. (µg/ml)	Calculated Conc. (µg/ml)					Mean	SD	% CV
	1	2	3	4	5			
0.1499-0.1512	0.1540	0.1540	0.1508	0.1555	0.1551	0.1539	0.0018	1.1953
3.9960-4.0320	4.1237	4.0890	4.0735	4.1345	4.1323	4.1106	0.0276	0.6726
8.9910-9.0720	9.2001	9.1539	9.1641	9.2868	9.3107	9.2231	0.0717	0.7770

In conclusion, the analysis of PTU in PBS and methanol by the HPLC method verified in this study showed good specificity, linearity, accuracy, and precision. Thus, this method could use for the quantitative determination of PTU in the permeation studies.

APPENDIX H

DSC thermograms of vesicular systems

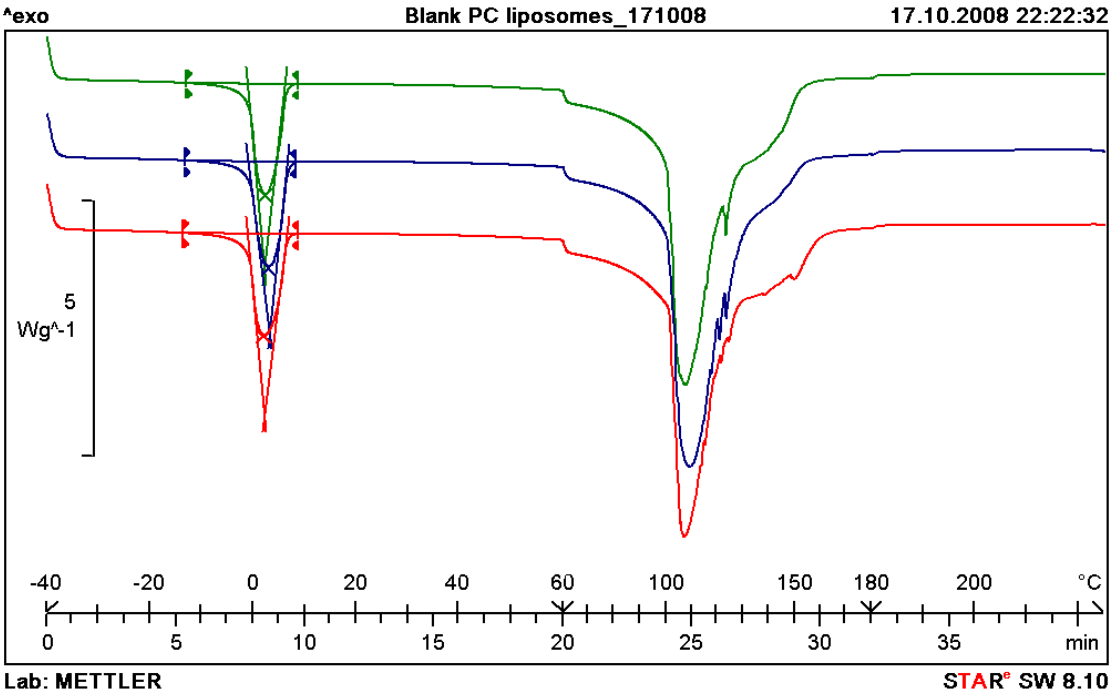


Figure H1: DSC thermograms of blank PCL. The three graphs were obtained from different samples taken from a single batch of the formulation.

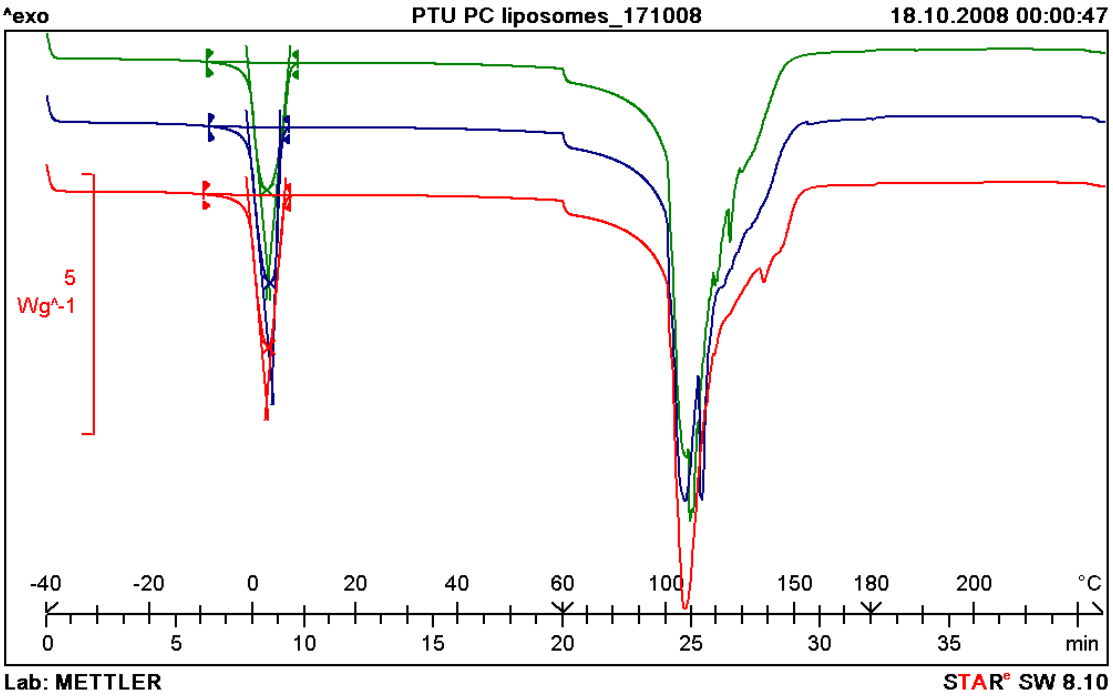


Figure H2: DSC thermograms of PTU/PCL. The three graphs were obtained from different samples taken from a single batch of the formulation.

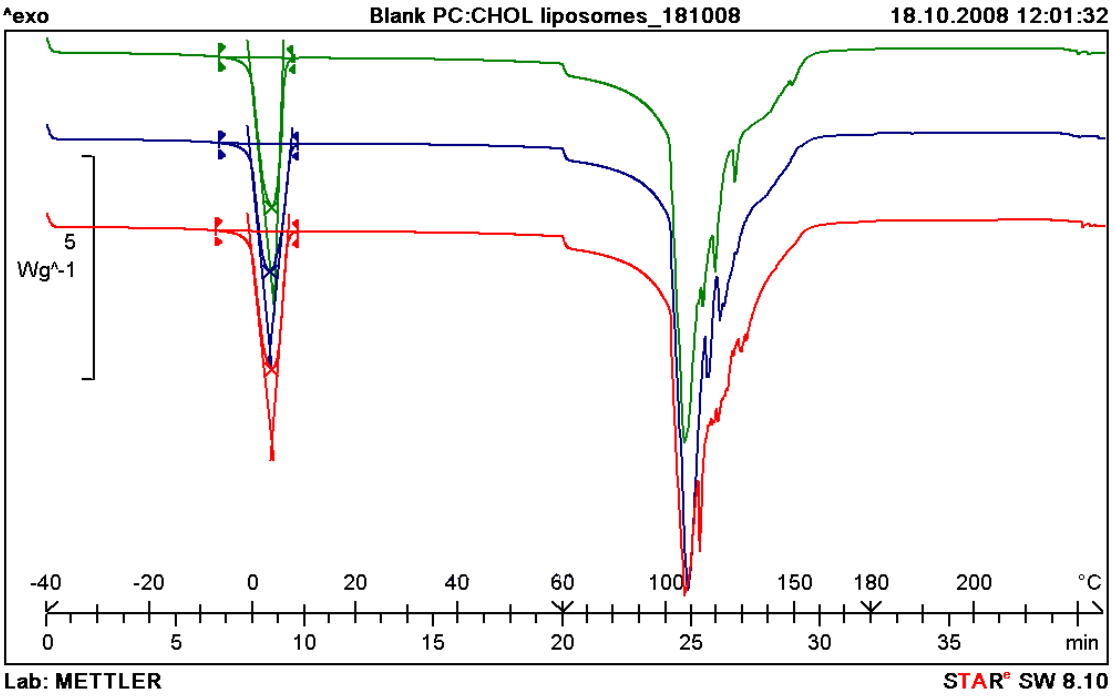


Figure H3: DSC thermograms of blank PCCHL. The three graphs were obtained from different samples taken from a single batch of the formulation.

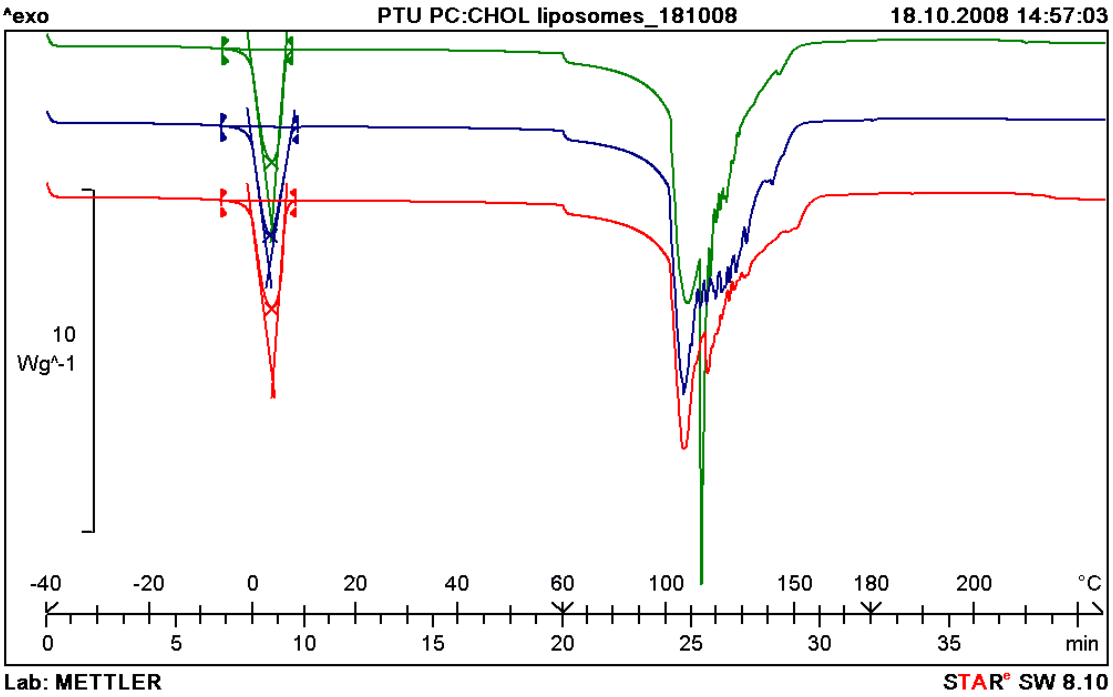


Figure H4: DSC thermograms of PTU/PCCHL. The three graphs were obtained from different samples taken from a single batch of the formulation.

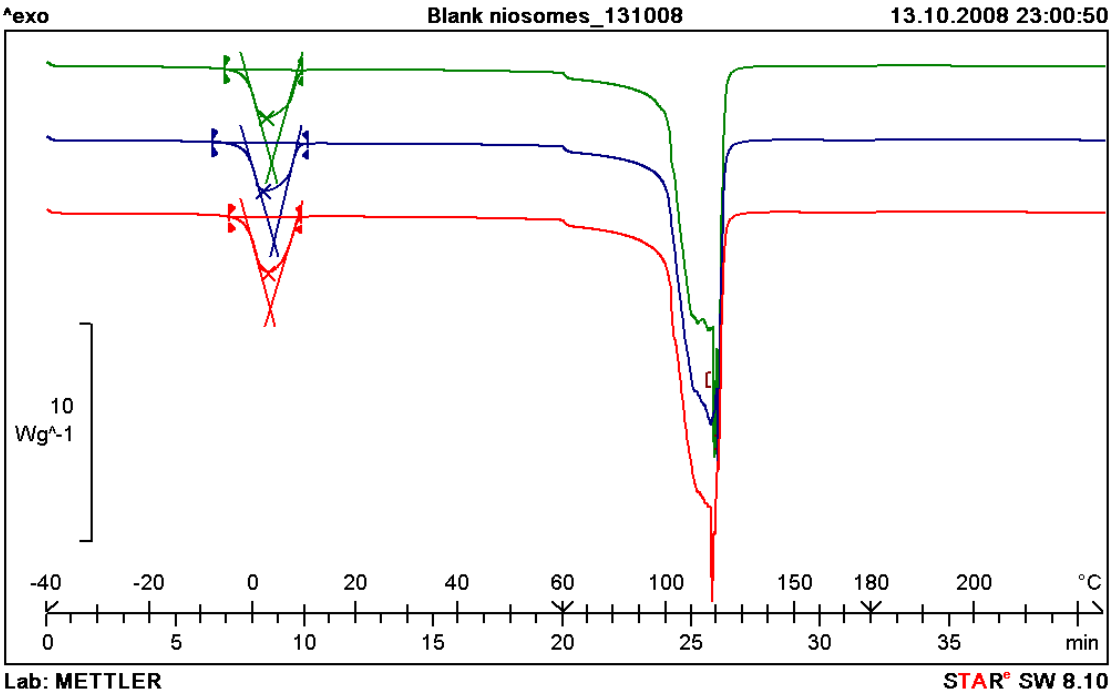


Figure H5: DSC thermograms of blank SN. The three graphs were obtained from different samples taken from a single batch of the formulation.

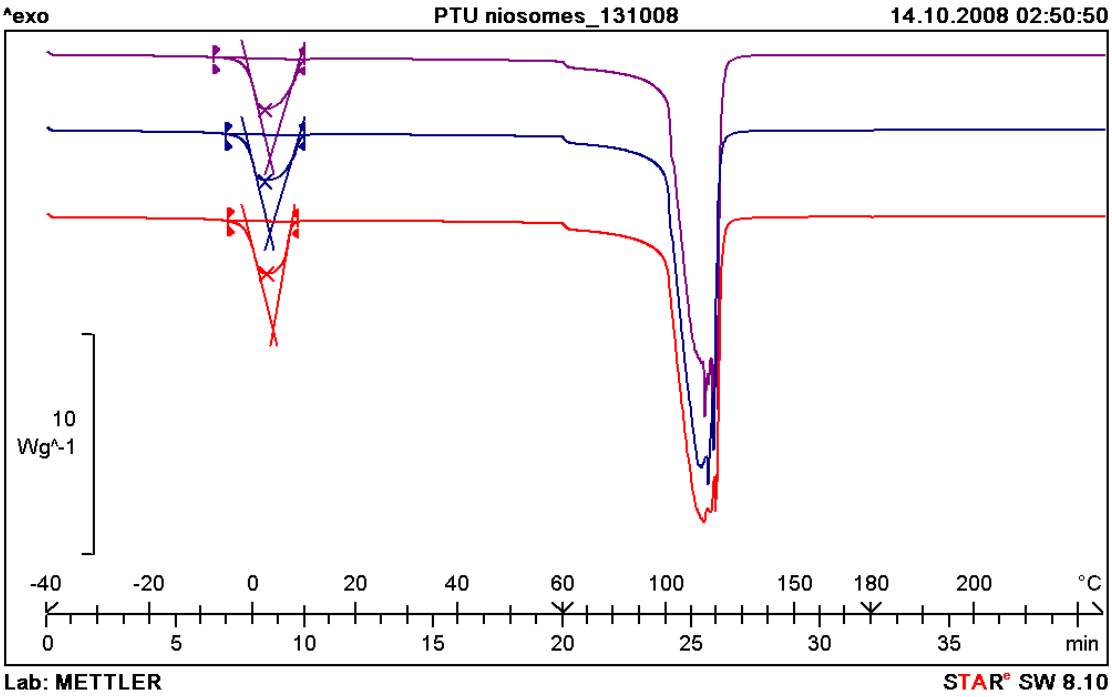


Figure H6: DSC thermograms of PTU/SN. The three graphs were obtained from different samples taken from a single batch of the formulation.

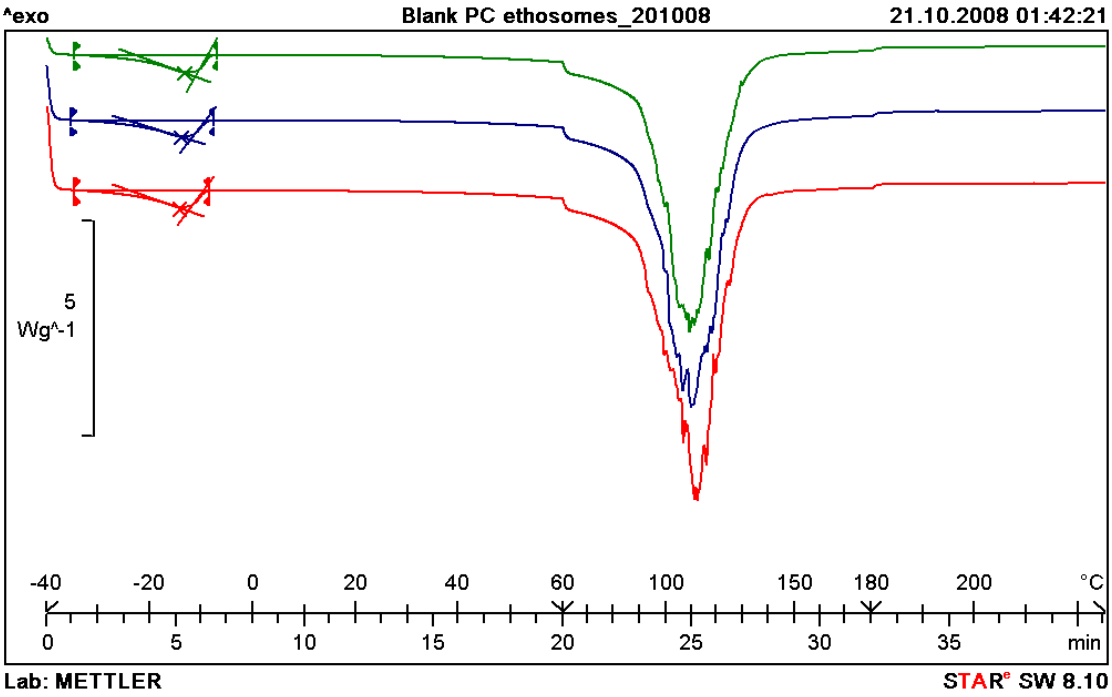


Figure H7: DSC thermograms of blank PCE. The three graphs were obtained from different samples taken from a single batch of the formulation.

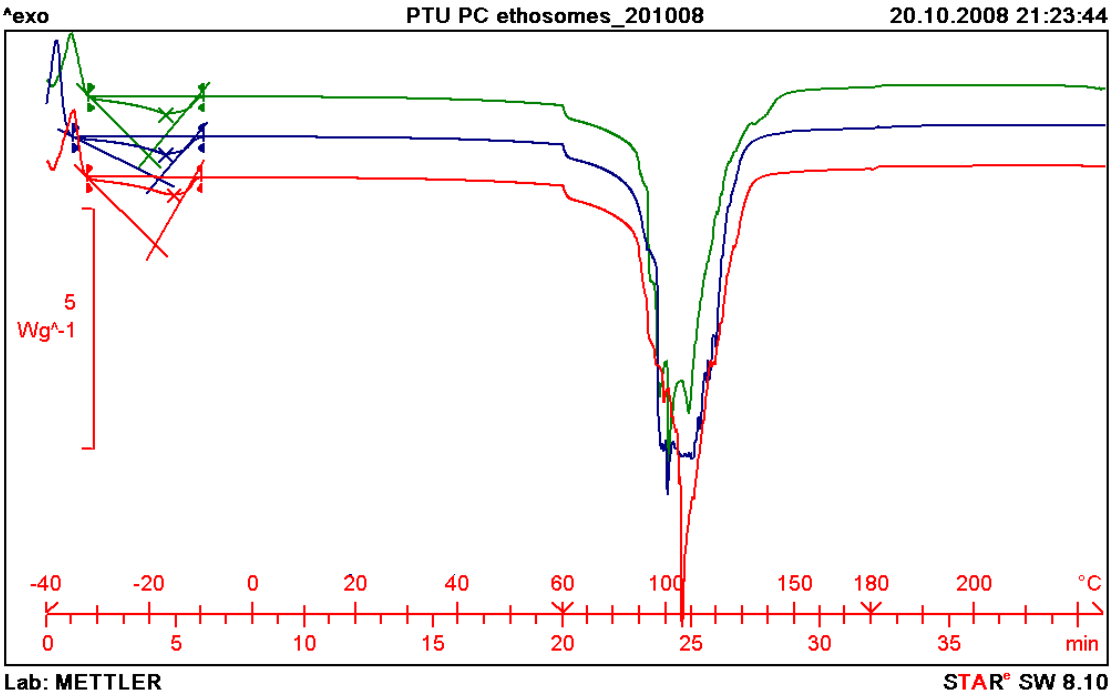


Figure H8: DSC thermograms of PTU/PCE. The three graphs were obtained from different samples taken from a single batch of the formulation.

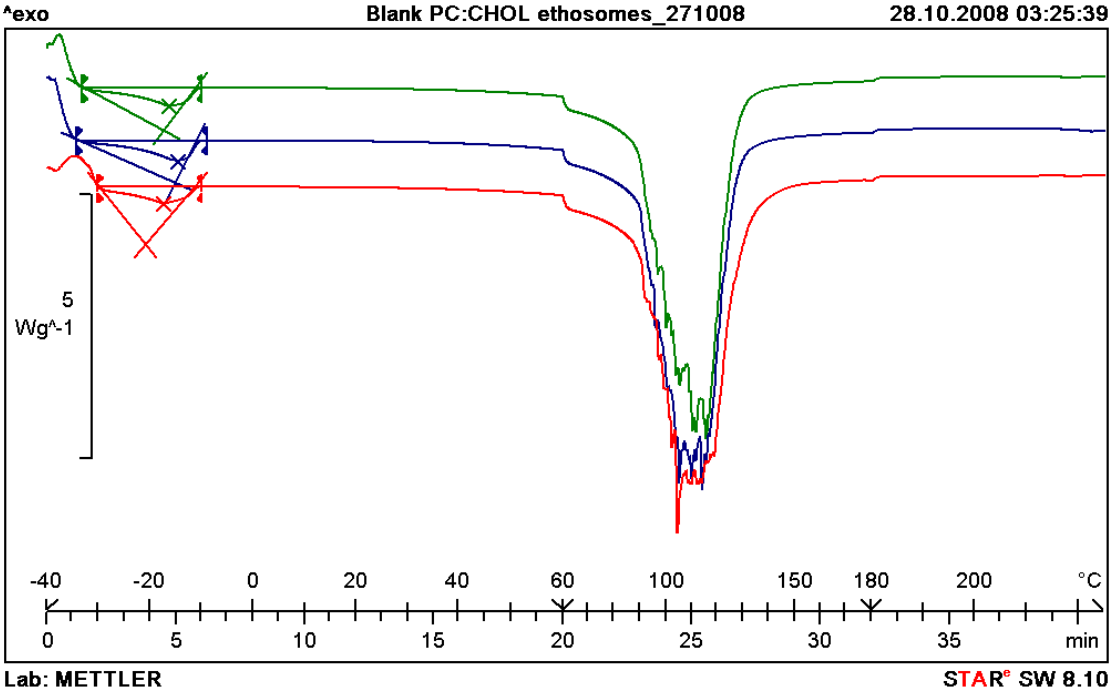


Figure H9: DSC thermograms of blank PCCHE. The three graphs were obtained from different samples taken from a single batch of the formulation.

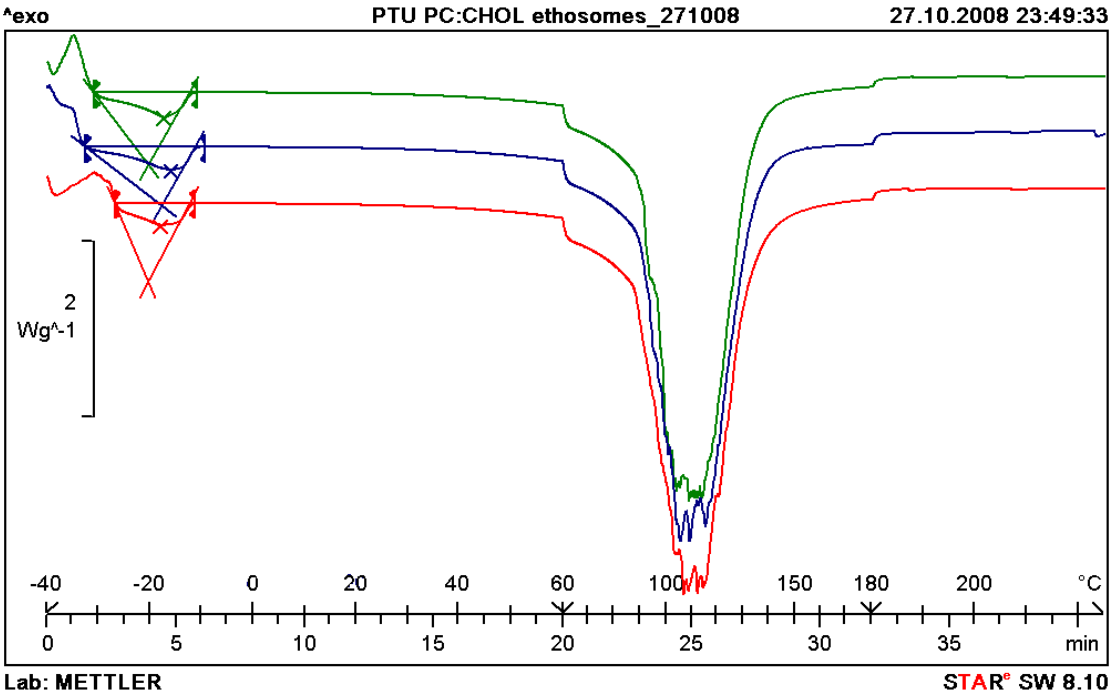


Figure H10: DSC thermograms of PTU/PCCHE. The three graphs were obtained from different samples taken from a single batch of the formulation.

VITA

Miss Suppakarn Sripetch was born on May 15, 1981 in Saraburi, Thailand. She received the Bachelor's degree in Pharmaceutical Science from Chulalongkorn University in 2004. Since graduation, she has worked at the Narcotic section, Bureau of Drug and Narcotic, Department of Medical Sciences, Ministry of Public Health, Nonthaburi. She entered the master's degree program in Pharmaceutics at Chulalongkorn University in 2006.