การพัฒนาอิลาสติกลิโพโซมของคาเฟอีนสำหรับการรักษาเซลลูไลต์

นางสาวชุณห์กฤดา เก้าเอี้ยน

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาเภสัชศาสตรมหาบัณฑิต
สาขาวิชาเภสัชกรรม ภาควิชาวิทยาการเภสัชกรรมและเภสัชอุตสาหกรรม
คณะเภสัชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2559

DEVELOPMENT OF ELASTIC LIPOSOMES OF CAFFEINE FOR THE TREATMENT OF CELLULITE

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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science in Pharmacy Program in Pharmaceutics
Department of Pharmaceutics and Industrial Pharmacy
Faculty of Pharmaceutical Sciences
Chulalongkorn University
Academic Year 2016

Thesis Title	DEVELOPMENT OF ELASTIC LIPOSOMES OF CAFFEINE FOR THE TREATMENT OF CELLULITE
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Accepted by the Faculty of Pharmaceutical Sciences, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

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ชุณห์กฤดา เก้าเอี้ยน : การพัฒนาอิลาสติกลิโพโซมของคาเฟอีนสำหรับการรักษาเซลลูไลต์ (DEVELOPMENT OF ELASTIC LIPOSOMES OF CAFFEINE FOR THE TREATMENT OF CELLULITE) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. ภญ. ดร.นนทิมา วรรธนะภูติ, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ. ภญ. ดร.วราภรณ์ สุวกูล, 112 หน้า.

การศึกษานี้เป็นการพัฒนาอิลาสติกลิโพโซมของคาเฟอีนเพื่อรักษาเซลลูไลต์โดยการใช้สารลดแรงตึงผิวร่วมกับเอทานอล อิลาสติกลิโพโซมประกอบด้วยฟอสฟาทิดิลคอลีนเป็นโครงสร้างไขมัน สแปน 80 หรือทวีน 80 ที่มีอัตราส่วนโดยโมลเท่ากัน (5-10% และ 15-25% โดยน้ำหนักของไขมันทั้งหมด ตามลำดับ) เป็นเอจแอติเวเตอร์ และเอทานอลความเข้มข้นต่างๆ (5-25% โดยปริมาตร) ผู้วิจัยศึกษาผลของชนิดและความเข้มข้นของสารลดแรงตึงผิวและเอทานอลต่อสมบัติทางกายภาพของลิโพโซม (เช่น การเกิดเวซิเคิล ขนาดและการกระจายขนาด และความยืดหยุ่น) และเลือกสูตรตำรับอิลาสติกลิโพโซมของสารลดแรงตึงผิวแต่ละชนิดที่มีความยืดหยุ่นสูงสุดมาเตรียมอิลาสติกลิโพโซมที่บรรจุคาเฟอีน รวมทั้งได้ศึกษาคุณสมบัติทางกายภาพต่าง ๆ ประสิทธิภาพในการเก็บกัก และความคงตัวทางกายภาพของอิลาสติกลิโพโซมที่บรรจุคาเฟอีน และศึกษาการนำส่งคาเฟอีนผ่านผิวหนังแบบนอกกายโดยใช้เซลล์สำหรับศึกษาการแพร่แบบฟรานซ์ดัดแปลงและหนังสุกรแรกเกิดภายใต้สภาวะเปิด ผลการศึกษาพบว่าเกิดเวซิเคิลสมบูรณ์ที่ความเข้มข้นเอทานอลต่ำ (5-15% โดยปริมาตร) ขนาดของลิโพโซมที่มีสแปน 80 และทวีน 80 เป็นส่วนประกอบอยู่ในช่วง 5.84-8.08 ไมโครเมตร และ 3.73-5.27 ไมโครเมตร ตามลำดับ การใช้สารลดแรงตึงผิวร่วมกับเอทานอลมีผลอย่างมีนัยสำคัญต่อคุณสมบัติทางกายภาพของอิลาสติกลิโพโซม ความยืดหยุ่นของลิโพโซมที่มีสแปน 80 และทวีน 80 เท่ากับ 27.07-79.86% และ 8.44-15.74% ตามลำดับ ผู้วิจัยเลือกตำรับ S7.5_5 ที่มีสแปน 80 7.5% โดยน้ำหนักของไขมันทั้งหมดและเอทานอล 5% โดยปริมาตร และ T20_5 ที่มีทวีน 80 20% โดยน้ำหนักของไขมันทั้งหมดและเอทานอล 5% โดยปริมาตร เพื่อนำมาบรรจุคาเฟอีนเนื่องจากมีค่าความยืดหยุ่นสูงสุดคุณสมบัติทางกายภาพของอิลาสติกลิโพโซมที่มีคาเฟอีนคล้ายกับลิโพโซมเปล่า อย่างไรก็ตาม การเติมคาเฟอีนทำให้ความยืดหยุ่นของอิลาสติกลิโพโซมลดลงจากอิลาสติกลิโพโซมเปล่าถึงประมาณ 20-30% ค่าความยืดหยุ่นของตำรับ S7.5_5 และ T20_5 คือ 53.46±8.63% และ 11.93±0.46% ตามลำดับ ประสิทธิภาพในการเก็บกักคาเฟอีนของสูตร S7.5_5 มีค่าน้อยกว่าสูตร T20_5 ซึ่งมีค่าเท่ากับ 35.05±0.60 และ 41.31±3.32% โดยน้ำหนัก ตามลำดับ การซึมผ่านผิวจากสูตร S7.5_5 และ T20_5 สูงกว่าสารละลายแอลกอฮอล์ที่ใช้เป็นเฟสน้ำในการเตรียมลิโพโซมอย่างมีนัยสำคัญ อย่างไรก็ตาม ไม่พบว่ามีความแตกต่างอย่างมีนัยสำคัญของพารามิเตอร์ในการซึมผ่านผิวของคาเฟอีนระหว่างอิลาสติกลิโพโซมทั้งสองสูตรแม้ว่าค่าความยืดหยุ่นจะแตกต่างกันอย่างชัดเจน ยกเว้นปริมาณคาเฟอีนในผิวหนังของตำรับ S7.5_5 ซึ่งมีค่าเปรียบเทียบกับสารละลายสูงกว่าตำรับ T20_5 เกือบ 2 เท่า

ภาควิชา วิทยาการเภสัชกรรมและเภสัชอุตสาหกรรม ลายมือชื่อนิสิต .....

สาขาวิชา เภสัชกรรม ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ปีการศึกษา 2559 ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

5576240733 : MAJOR PHARMACEUTICS

KEYWORDS: ELASTIC LIPOSOMES / SURFACTANT / ETHANOL / ELASTICITY / CAFFEINE / ANTICELLULITE

CHUNGRIDA KAO-IAN: DEVELOPMENT OF ELASTIC LIPOSOMES OF CAFFEINE FOR THE TREATMENT OF CELLULITE. ADVISOR: ASST. PROF. NONTIMA VARDHANABHUTI, Ph.D., CO-ADVISOR: ASSOC. PROF. WARAPORN SUWAKUL, Ph.D., 112 pp.

In this study, caffeine elastic liposomes were developed for cellulite treatment by using the combination of surfactant and ethanol. The elastic liposomes studied consisted of phosphatidylcholine as the structural lipid, Span® 80 or Tween® 80 of the same molar ratio (5-10% w/w and 15-25% w/w of total lipid, respectively) as the edge activator and various concentrations of ethanol (5-25% v/v). The effects of type and amount of surfactants and concentration of ethanol on physical properties i.e., vesicle formation, size and size distribution and elasticity of blank liposomes were investigated. The blank liposome formulations with highest elasticity of each surfactant were selected to prepare the caffeine-containing elastic liposomes. These elastic liposomes were characterized for physical properties including entrapment efficiency and physical stability. The skin delivery of caffeine from the chosen elastic liposome formulations were carried out *in vitro* with modified Franz diffusion cells under the non-occlusive condition using newborn pig skin as the model membrane. The results showed that the complete vesicle formation was observed at low ethanol concentrations (5-15% v/v). The vesicle size of liposomes composed of Span® 80 and Tween® 80 was in the range of 5.84-8.08 μm and 3.73-5.27 μm, respectively. The combination of surfactant and ethanol showed significant effects on physical properties of elastic liposomes. The elasticity of liposomes containing Span® 80 and Tween® 80 was in the range of 27.07-79.86% and 8.44-15.74%, respectively. The formulations with highest elasticity, S7.5_5 with Span® 80 7.5% w/w and ethanol 5% v/v and T20_5 with Tween® 80 20% w/w and ethanol 5% v/v, were chosen to load caffeine. In general, physical properties of caffeine elastic liposomes were similar to the corresponding blank liposomes. However, caffeine loading decreased the elasticity of caffeine-entrapped elastic liposome formulations by approximately 20-30% compared to that of the blank elastic liposomes. The elasticity values of the caffeine-loaded elastic liposome formulations S7.5_5 and T20_5 were 53.46±8.63% and 11.93±0.46%, respectively. The entrapment efficiency values of S7.5_5 was lower than that of T20_5 (35.05±0.60 and 41.31±3.32% w/w, respectively). Skin permeation from S7.5_5 and T20_5 were significantly higher than that from the hydro-alcoholic solution used as the aqueous phase of these elastic liposomes. However, despite the profound difference in elasticity between the 2 formulations, no significantly differences were seen in most permeation parameters studied. The only significant difference detected was the enhancement factor of caffeine amount in the skin, the mean of which was almost doubled for the S7.5_5 formulation when compared to the T20_5 formulation.

Department:	Pharmaceutics and Industrial Pharmacy	Student's Signature	.....
		Advisor's Signature	.....
Field of Study:	Pharmaceutics	Co-Advisor's Signature	.....
Academic Year:	2016		

ACKNOWLEDGEMENTS

I would like to express my sincere thanks and gratitude to my advisor, Assistant Professor Nontima Vardhanabhuti, Ph.D., for her scientific guidance, encouragement, suggestion, invaluable advice and discussion throughout this study. I would also like to express my grateful thanks to Associate Professor Waraporn Suwakul, Ph.D., my co-advisor, for her support, suggestion, scientific guidance, and helpful discussion.

I would also like to thank the thesis committee for their helpful discussion and valuable suggestions. I sincerely thank Professor Garnpimol C. Ritthidej, Ph.D., of the Department of Pharmaceutics and Industrial Pharmacy for providing the Mastersizer 2000 and Assistant Professor Vichien Jongbunprasert of the Department of Pharmacognosy and Pharmaceutical Botany for the access to the optical microscopes. Director of Scientific and Technological Research Equipment Centre and Miss Keaw Kajornchaiyakul, of the Scientific and Technological Research Equipment Centre, are also highly appreciated for the use of small volume dispersion unit for size analysis. Mr. Wichpong Kao-ian is highly recognized for his advice and technical support on computer program for elasticity analysis.

I also wish to express my gratitude to Assistant Professor Dachrit Nilubol, Ph.D., of the Department of Veterinary Microbiology, Faculty of Veterinary Science, Chulalongkorn University for his help and assistance in providing the newborn pig skin. Special thanks go to staff members in Jareonpol Farm, Ratchaburi, Thailand, for providing the newborn pigs dying from natural cause. I am also greatful to all the faculty and staff members in the Department of Pharmaceutics and Industrial Pharmacy for their support. In addition, I would like to thank all my friends in this department and to other persons whose names have not been mentioned for their friendship and valuable support.

My sincere thanks also go to the members of the Bureau of Cosmetics and Hazardous substances, Department of Medical Sciences, Ministry of Public Health for encouraging me and providing the High Performance Liquid Chromatography facility for this research.

Above all, greatest thanks are directed to my family for their unconditional encouragement, care, endless love and continued support during the course of my education.

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LIST OF ABBREVIATIONS

ANOVA	=	analysis of variance
°C	=	degree Celsius
C_d	=	drug concentration in the donor compartment
cm	=	centrimeter
CMIT	=	methylchloroisothiazolinone
cm^2	=	squared centimeter
CV	=	coefficient of variation
EF	=	enhancement factor
EF of Q_{24}	=	enhancement factor of Q_{24}
EF of Q_s	=	enhancement factor of Q_s
HLB	=	hydrophilic-lipophilic balance
HPLC	=	high pressure liquid chromatography
HSD	=	Honest Significant Difference
J_{ss}	=	steady state flux
LOD	=	limit of detection
LOQ	=	limit of quantitation
mg	=	milligram
ml	=	milliliter
mm	=	millimeter
nm	=	nanometer
PBS	=	phosphate buffered saline
PC	=	phosphatidylcholine
P_s	=	permeability coefficient
Q_{24}	=	cumulative caffeine amount in the receiver at 24 hours
Q_s	=	caffeine amount in the skin
RF	=	relative flux
rpm	=	revolution per minute
SD	=	standard deviation

SPC	=	soybean phosphatidylcholine soy lecithin
µg	=	microgram
µl	=	microliter
µm	=	micrometer
M	=	molar
v/v	=	volume by volume
w/w	=	weight by weight

CHAPTER I

INTRODUCTION

Caffeine is a xanthine compound. It is known to be effective in reducing cellulite and has been used in various anti-cellulite products (CBSNEWS, 2007; Conry, 2015). Cellulite is a condition with atypical roughness of the skin that is often described as an orange-peel look. It is caused by the accumulation of fat in the subcutaneous tissue coupled with the retraction of the septum. Hence, the dermis is forced and the skin looks dimpled and bumpy (Khan et al., 2010). Several anti-cellulite mechanisms are attributed to caffeine. One mechanism is inhibition of α-adrenergic receptor to prevent fat accumulation. The inhibition of phosphodiesterase that consequently increases cyclic adenosine monophosphate (cAMP) level is also proposed for anti-cellulite mechanism of caffeine. The increase in cAMP will stimulate hormone-sensitive lipase, causing fat breakdown to free fatty acids and glycerol in adipocytes. Caffeine also stimulates the lymphatic system in adipose tissue. The elimination of fat, toxin or other substances formed by the breakdown of fat through the lymphatic system is thus increased (Herman and Herman, 2013).

Caffeine is a water-soluble substance. Its solubility in water is 1 g per 50 ml (Zubair, Hassan, and Al-Meshal, 1986). The octanol/water partition coefficient (log P) of caffeine is -0.091 to -0.07 (Leo, Hansch, and Elkins, 1971; Sigma-Aldrich, 2014). Generally, substances with reasonable passive skin permeation should have log P in the range of 1-3 (Walters and Brain, 2009). Hence, the skin absorption of caffeine is very low (Boucaud et al., 2001). New delivery approaches to enhance caffeine absorption through the skin are of interest.

Various approaches have been reported to improve caffeine skin delivery. Chemical structure modification is one of such approaches. Siloxanetriol alginate

caffeine was developed as a prodrug for caffeine (Velasco et al., 2008). The prodrug is converted to caffeine by hydrolysis. The drawback of the prodrug approach is that the synthesis process is often cumbersome in terms of time and resources (Stella, 2007). The other approaches to delivery caffeine are physical methods. High frequency sound waves (ultrasound) has been combined with caffeine gel to delivery caffeine for cellulite treatment (Pires-de-Campos et al., 2008). This method requires specific tools and must be administered by a specialist. It is inconvenient because self-administration is not plausible. For conditions, such as cellulite, a delivery method that can be self-administered should be preferable.

The effectiveness of chemical penetration enhancers on caffeine delivery from solutions was also assessed. Benzakonium chloride, α-terpineol and oleyl alcohol were used as penetration enhancers and their effects were compared to that of the high frequency sound waves. The results showed that the penetration enhancers were more effective in enhancing caffeine permeability through the skin than the physical method (Monti et al., 2001). In another study, the skin permeability of caffeine solution was improved with the combination of 20% Transcutol® and 10% oleic acid (Touitou et al., 1994). However, the use of permeation enhancers in high doses can cause skin irritation (Effendy and Maibach, 1995; Williams and Barry, 2004).

Bolzinger et al. (2008) conducted a comparative study on emulsion, gel and microemulsion systems containing caffeine. The highest caffeine delivery was observed with the microemulsion system. However, the microemulsion used in this study contained a combination of surfactants at a concentration as high as 24% w/w. In another study, the surfactant content as high as 27% w/w of the formula was used (Zhang and Michniak-Kohn, 2011). A nanoemulsion system containing 30% Transcutol® HP and 10% isopropanol by weight was developed (Shakeel and Ramadan, 2010). The concentration of the surfactant used was too high for cosmetic applications. The content of Transcutol® HP in cosmetic products recommended by the Scientific

Committee on Consumer Safety (SCCS) was limited to not more than 5% by weight (SCCS, 2013). The nanoemulsion formulation was water in oil type, which contained a high amount of oil, as 40% of Lauroglycol® 90. The high oil content could cause a sticky feeling when applied to the skin in a wide area. Vesicular systems have also been reported on caffeine delivery. Conventional caffeine liposomes showed low skin absorption because caffeine was accumulated in the epidermis (Touitou et al., 1994). From the research mentioned above, different types of techniques and delivery systems were developed to enhance caffeine delivery through the skin, but they still had several disadvantages.

Elastic liposomes are vesicular systems with flexible bilayers developed from conventional liposomes. These vesicular systems have been used to deliver molecules with different physicochemical properties into and through the skin. Success in skin delivery has been reported with elastic liposomes. Elastic liposomes have been used for large hydrophilic molecules such as insulin (Cevc and Blume, 1992) plasmid DNA (Lee et al., 2005) and peptide antigen (Rattanapak et al., 2012). Lipophilic substances that were delivered through the skin via elastic liposomes include oestradiol (El Maghraby, Williams, and Barry, 2000a), triamcinolone acetonide (Cevc and Blume, 2003), catechins (Fang et al., 2006), ketotifen (Elsayed et al., 2006), retinol (Oh et al., 2006), asiaticoside (Paolino et al., 2012) and voriconazole (Song et al., 2012). Various small hydrophilic substances have also been formulated into elastic liposomes. These include carboxyfluorescein (Verma et al., 2003a), propranolol hydrochloride (Mishra et al., 2007), diclofenac sodium (El Zaafarany et al., 2010), methotrexate, acyclovir, idoxuridine (Ita et al., 2007), colchicine (Singh et al., 2009b), calcein (Bahia et al., 2010) and bleomycin hydrochloride (Hiruta et al., 2006).

Elastic liposomes are different from conventional liposomes. They are highly flexible and capable to deliver many substances into the deeper skin layer (Cevc and Blume, 1992). The elastic liposomes usually consist of two components. These two

main components are phosphatidylcholine, which is the main lipid structure, and the surfactant that acts as edge activator. Two groups of surfactants have been used as edge activator. The anionic surfactants that have been used include sodium cholate (Cevc et al., 1998; Guo, Ping, and Zhang, 2000; Verma et al., 2003a; Lee et al., 2005; Hiruta et al., 2006; Ita et al., 2007; Paolino et al., 2012), sodium deoxycholate (Ita et al., 2007), deoxycholic acid (Fang et al., 2006). Several nonionic surfactants have also been used. Among those reported were Tween® 80 (Lee et al., 2005; El Zaafarany et al., 2010), Tween® 20 (Oh et al., 2006), Span® 60 (Ita et al., 2007), Span® 80 (Mishra et al., 2007) and Span® 85 (El Zaafarany et al., 2010). For the surfactants that can act as edge activators, anionic surfactants are more toxic to cells (Paolino et al., 2012) and are at higher prices than non-ionic surfactants (Sigma-Aldrich, 2016).

Another type of liposomes for skin delivery is ethosomes. Besides phosphatidylcholine, ethosomes also contain ethanol at high concentrations (20-50% by volume) as a crucial component (Touitou, 1996). Ethosomes render better skin permeation than traditional liposomes (Dayan and Touitou, 2000). They can deliver a variety of compounds such as minoxidil (Touitou et al., 2000), acylclovir (Horwitz et al., 1999), trihexylphenidyl hydrochloride (Dayan and Touitou, 2000), testosterone (Ainbinder and Touitou, 2005), melatonin (Dubey, Mishra, and Jain, 2007), simvastatin (An et al., 2011), ketoprofen (Chourasia, Kang, and Chan, 2011) and caffeine (Touitou, 1996) through the skin. The high ethanolic contents in the aqueous phase of ethosomes may cause skin irritation (Ophaswongse and Maibach, 1994).

Since surfactants and ethanol have different mechanisms in making the liposomal bilayers soft and malleable, the combination of these two components might have a synergistic effect. Thus, reduction in concentrations of both surfactant and ethanol might be possible when they were combined. This could lead to less skin irritation. The combination of ethanol and surfactant has been used with success in elastic liposomes to deliver lipophilic substances, namely catechin and voriconazole.

These systems contained 15% or 30% ethanol by volume of the water phase, respectively (Fang et al., 2006; Song et al., 2012). The surfactant used for catechin-containing elastic liposomes was deoxycholate at 5% by weight of the total lipid, whereas deoxycholate or Tween® 80 at 14% by weight of the total lipid was used for voriconazole elastic liposomes. The combination of surfactant and ethanol has also been reported for delivery of water-soluble substances such as caffeine. Unfortunately, the skin permeation of caffeine from these liposomes was not different from that of its control solution (Ascenso et al., 2015).

The present study was aimed at developing elastic liposomes for delivery of caffeine to the skin. The combination of surfactant and ethanol was used in order to minimize the concentrations of individual components used. In this study, nonionic surfactants were selected to combine with ethanol at various concentrations. Both ethanol and surfactants increase flexibility and elasticity of the bilayer and act as permeation enhancers. The developed system might improve caffeine delivery into the subcutaneous layer, which is its targeting area. In addition, the combination of nonionic surfactant and ethanol might result in reducing skin irritation due to lower surfactant and ethanol concentrations in the preparation. Moreover, the type and amount of surfactant and the amount of ethanol affects the physical properties of elastic liposomes, such as particle size, elasticity, entrapment efficacy and skin permeation (El Maghraby et al., 2000a; Touitou et al., 2000; Lee et al., 2005; López-Pinto, González-Rodríguez, and Rabasco, 2005; Hiruta et al., 2006; Ita et al., 2007; El Zaafarany et al., 2010). Thus, in this study the effects of type and amount of surfactant and ethanol concentration on physical properties of the resultant elastic liposomes were monitored. Selected formulations were also evaluated for their ability to deliver caffeine across the skin.

Objectives

The specific objectives of this study were as follows:

1. To formulate and characterize caffeine elastic liposomes

2. To study the *in vitro* skin permeation of selected caffeine elastic liposome formulations.

CHAPTER II
LITERATURE REVIEW

Caffeine

Caffeine, a xanthine compound, is a central nervous system stimulant. It is a substance commonly used as a mild stimulant and is found in various dietary sources such as coffee, tea, and cocoa. Since caffeine is a water-soluble substance (1 g per 50 ml) (Zubair et al., 1986), it is often used as a hydrophilic model molecule in research papers (Boucaud et al., 2001; Dreher et al., 2002; Garland et al., 2012; Schlupp et al., 2014; Abd, Roberts, and Grice, 2016). Caffeine is used in the treatment of cellulite with a variety of mechanisms such as to prevent lipid accumulation and to stimulate lipid breakdown in adipocytes (Herman and Herman, 2013). Nakabayashi et al. (2008) reported that caffeine and its metabolites could reduce intracellular lipid accumulation as well as glucose uptake stimulated by insulin in adipocytes. Su et al. (2013) also reported that caffeine had antiadipogenic properties.

Cellulite is a typical roughness of the skin, which is dimpled like an orange peel or cottage cheese. It is primarily observed in areas with excess adipose tissue such as breasts, upper arms, buttocks, and thighs (Draelos, 2005). The appearance of cellulite is caused by the herniated fat lobules which protrude from subcutaneous layer through the inferior surface at the dermo-hypodermal interface (Nürnberger and Müller, 1978).

The target site of caffeine for cellulite treatment is at the subcutaneous layer. Drug permeation through the skin depends largely on physicochemical properties of the drug. Physicochemical properties affecting drug permeation include lipophilicity, partition coefficient, molecular size, solubility and degree of ionization. To render skin permeation, the molecular weight of the drug should be less than 500 Da. The

solubility in skin lipids and degree of ionization is dependent on chemical structure of the drug. The drug solubility and degree of ionization have crucial influences on partitioning of the drug from the vehicle to the stratum corneum as well as the passage of the drug through the viable epidermis. An optimum partition coefficient is also necessary for drug permeability through the skin. Substances that can passively penetrate/permeate into and through the skin should have log P values in the range of 1-3 (Walters and Brain, 2009). Log P of caffeine is in the range of -0.091 to -0.07 (Leo et al., 1971; Sigma-Aldrich, 2014) So, the ability of caffeine to penetrate the skin is low. Consequently, several approaches and delivery systems have been developed to improve this limitation (Touitou, 1996; Monti et al., 2001; Bolzinger et al., 2008; Pires-de-Campos et al., 2008; Velasco et al., 2008).

Techniques for improving caffeine skin permeation

The outer most layer of the skin, the stratum corneum, acts as the main barrier for many compounds to transport into and through the skin. Various techniques have been applied to improve transdermal delivery of the active compounds. These include use of chemical penetration enhancers, chemical structure modification and optimization, skin structure disruption by physical means and use of delivery systems including vesicular delivery systems such as liposomes, niosomes, and ethosomes. These approaches have also been used to improve caffeine skin delivery.

Velasco et al. (2008) compared caffeine and the prodrug siloxanetriol alginate caffeine (SAC) effect on fatty tissues by monitoring diameter and number of adipocytes. Emulsions and gels of caffeine, caffeine with sodium benzoate or SAC were formulated and tested on mice dorsal skin. The results indicated that both diameter and number of adipocytes were decreased in the SAC emulsion group. On the other hand, when the caffeine emulsion was used, only the diameter of adipocytes decreased. No significant differences from the control vehicles were seen with caffeine and SAC gels.

However, the prodrug approach is usually associated with the synthesis process that is cumbersome and resource consuming (Stella, 2007). The safety profile of the prodrug may be different from that of the active compound and must be evaluated. The prodrug also needs to be converted into the active form by an enzymatic or a chemical reaction (Sloan and Wasdo, 2007), resulting in a different pharmacokinetic profile from its active metabolite. Thus, a prodrug must be approved by the regulatory authority before it can be used.

The physical enhancement methods to improve skin permeation such as pressure, high frequency sound waves (ultrasound), use of weak electrical current (iontophoresis), and the microneedles have also been applied. The effect of massage on caffeine permeation was investigated by Treffel et al. (1993). They applied 0.25 bar over the atmospheric pressure on excised human skin mounted on Franz diffusion cells. When the pressure was applied, the flux from caffeine solution in acetone was 1.8 times of the flux seen without the applied pressure. The authors suggested that the enhancing effect of increased pressure was probably due to the transappendageal route or the higher filling-up of the preparation in the stratum corneum. However, the authors did not carry out any further experiments to confirm their suggestions. Enhanced skin permeation of caffeine solution by ultrasound was reported by Mitragotri et al. (1995) and Boucaud et al. (2001). The experiments were performed *in vitro* using heat-stripped human cadaver skin (Mitragotri et al., 1995) and human skin and hairless rat skin (Boucaud et al., 2001) as the model membranes. The ultrasound was also used in combination with caffeine gel (Pires-de-Campos et al., 2008). The authors conducted the experiment *in vivo*, on the dorsal areas of pigs. They reported reduction in the thickness of the subcutaneous tissue and decrease in the number of adipocytes. Monti et al. (2001) also found that low frequency ultrasound treatment, but not the high frequency one, could increase transdermal flux of caffeine through hairless mouse skin *in vitro*. The increase in skin permeability and the enhanced

cumulative amount of caffeine delivered through porcine ear skin by iontophoresis have been reported (Marra et al., 2008). Although the physical enhancement method has been shown to enhance transdermal delivery of a variety of drugs including caffeine, these methods require specific tools and must be administered by a specialist. Thus, they are not suitable for self-administration and might not be practical for cosmeceutical products.

The ability of chemical penetration enhancers to enhance caffeine delivery from solution has also been assessed. The effects of benzakonium chloride, **α**-terpineol or oleyl alcohol in propylene glycol solution were compared to that of propylene glycol solution *in vitro*, using hairless mouse skin (Monti et al., 2001). Enhanced fluxed were found with all chemical enhancers used in the study. In another study, Touitou et al. (1994) reported that the skin permeability of caffeine solution was improved with the combination of 20% Transcutol® and 10% oleic acid. The authors compared fluxes and cumulative amounts of caffeine in hairless mouse skin *in vitro*. Caffeine (3% w/v) were tested in five different carrier systems: polyethylene glycol solution, conventional liposomes, aqueous solution, combination of polyethylene glycol and enhancing mixture and combination of aqueous solution and enhancing mixture. The enhancing mixture was a mixture of 20% Transcutol® and 10% oleic acid. The conventional liposomes and polyethylene glycol failed to deliver caffeine in any appreciable amounts. On the contrary, the caffeine flux from the combination of aqueous solution and enhancing mixture was 40 times higher than that from the corresponding aqueous solution. However, the use of permeation enhancers in high doses may cause skin irritation (Effendy and Maibach, 1995; Williams and Barry, 2004).

Various delivery systems have been employed to deliver caffeine through the skin. Bolzinger et al. (2008) conducted a comparative permeation study from caffeine-containing emulsion, gel and microemulsion. The highest caffeine delivery was observed with the microemulsion formulation. Microemulsions were also studied by

Zhang and Michniak-Kohn (2011). Three microemulsion formulations with different water (20-70% w/w) and surfactant/co-surfactant (27-72% w/w) contents. All microemulsion formulations gave higher fluxes than that of the control. However, microemulsions usually contain high surfactant contents that may cause skin irritation (Effendy and Maibach, 1995; Bolzinger et al., 2008; Zhang and Michniak-Kohn, 2011).

A w/o nanoemulsion formulation developed by Shakeel and Ramadan (2010) contained 30% Transcutol® HP and 10% isopropanol by weight. Skin permeation of caffeine was studied *in vitro* using Franz diffusion cells on full-thickness rat abdominal skin. The enhancement ratio seen was 17.37 when compared to the aqueous solution of caffeine. The researchers did not notice any erythema of the skin nor edema in the dermis of rats in a single-dose treatment. The Scientific Committee on Consumer Safety (SCCS), however, recommended that Transcutol® HP should not be used at a concentration higher than 5% in cosmetic products (SCCS, 2013). In addition, the formulation was water-in-oil type, which contained a high amount of oil. The high oil content can cause a sticky feeling when applied to the skin in a wide area.

Very recently, elastic liposomes have been studied for delivering caffeine into the skin. Abd et al. (2016) compared caffeine delivery from 'transferosomes' composed of PC, decyl polyglucoside and cholesterol with niosomes, conventional liposomes, and conventional liposomes with penetration enhancer. The steady flux from the transferosome preparation was higher than those from a control solution and all conventional liposome preparations. On the other hand, niosomes seemed to be as good as transferosomes in delivery of caffeine.

Elastic liposomes

Liposomes are colloidal systems comprising phospholipid bilayers. Topical administration of early conventional liposomes retains the substance at the outer skin layer (Mezei and Gulasekharam, 1980; Vermorken et al., 1984). There are many

liposome application for various indications such as acne (Škalko, Čajkovac, and Jalšenjak, 1992), psoriasis (Agarwal, Katare, and Vyas, 2001) and topical anesthetic (Foldvari, Gesztes, and Mezei, 1990). Liposomes have been further modified to improve their functions in delivering active compounds into the skin (Cevc and Blume, 1992; Dayan and Touitou, 2000; El Maghraby, Williams, and Barry, 2004; Abd et al., 2016). Success of liposomes in targeting skin delivery depends on the type and composition of liposomes as well as the application protocol (Dayan and Touitou, 2000; Verma et al., 2003a; El Maghraby et al., 2004; Fang et al., 2006; Elsayed et al., 2007; Mishra et al., 2007; El Zaafarany et al., 2010; Paolino et al., 2012; Song et al., 2012; Liu et al., 2013).

Elastic liposomes were first reported by Cevc and Blume (1992) as a new type of liposomes termed Transfersomes™. Transfersomes™ were claimed to have high as well as self-optimizing deformability (Cevc, Schätzlein, and Blume, 1995). The improvement in insulin skin delivery was shown with these elastic vesicles (Cevc and Blume, 1992; Cevc et al., 1998; Jain et al., 2008; El Zaafarany et al., 2010). Thus, elastic liposomes are different from conventional liposomes. They are highly flexible and capable to deliver many substances into the deeper skin layers (Cevc and Blume, 1992; Jain et al., 2008; El Zaafarany et al., 2010). These liposomes are composed mainly of the structural lipid and an edge activator. The combination renders the vesicles elasticity or deformability.

Material used in the preparation of elastic liposomes

The first reported deformable vesicles, Transfersomes®, were composed of phosphatidylcholine and sodium cholate. From the literature review, elastic liposomes consist of 2 main components: the structural lipid and the edge activator. The most commonly used structural lipid in elastic liposomes is phosphatidylcholine (Guo, Ping, Sun, et al., 2000; Trotta et al., 2002; Fang et al., 2006; Ita et al., 2007; El Zaafarany et

al., 2010). The surfactants used as an edge activator in the preparations of elastic liposomes can be divided into two groups:

(1) Anionic surfactants such as sodium cholate (Cevc et al., 1998; Guo, Ping, Sun, et al., 2000; Ita et al., 2007; Paolino et al., 2012), sodium deoxycholate (Ita et al., 2007), deoxycholic acid (Fang et al., 2006) and di-potassium glycyrrhizinate (Trotta et al., 2002)

(2) Nonionic surfactants such as Tween® 80 (Lee et al., 2005; El Zaafarany et al., 2010), Tween® 20 (Oh et al., 2006), Span® 40 (Jain et al., 2008), Span® 60 (Ita et al., 2007; Jain et al., 2008), Span® 80 (Mishra et al., 2007; Garg et al., 2008; Jain et al., 2008) and Span® 85 (El Zaafarany et al., 2010).

Alcohol at low concentrations (5-7% v/v) was also used in elastic liposomes in combination with surfactants such as sodium cholate (Paolino et al., 2012), Tween® 80 (Elsayed et al., 2006) or Span® 80 (El Maghraby et al., 2000a). Alcohol was used mainly to increase drug solubility (Fang et al., 2006; Tsai et al., 2015). In some certain cases, alcohol used in elastic liposomes might increase physical stability of the preparation due to the increased zeta potential (Badran, Shalaby, and Al-Omrani, 2012). Oxidation of phospholipid might also be reduced in the presence of ethanol (Fang et al., 2006).

Characterization of elastic liposomes

Characterization of elastic liposomes is necessary to confirm that all properties of the vesicular structure formed are suitable for their intended used. Elastic liposomes preparation is usually characterized in terms of morphology, size and size distribution, entrapment efficiency and elasticity. These parameters possibly affect drug permeation (Guo, Ping, and Zhang, 2000; Elsayed et al., 2006; Fang et al., 2006; Mishra et al., 2007; Garg et al., 2008).

1. Morphology

The morphology and microstructure of elastic liposomes can be examined by optical microscopes (Garg et al., 2008), transmission electron microscopy (TEM) (Guo, Ping, Sun, et al., 2000; Jain et al., 2008; Singh et al., 2009a) and scanning electron microscopes (SEM) (Hiruta et al., 2006).

2. Size and size distribution

The method for determining the size of liposomes with high accuracy is electron microscopic examination. This method permits one to view each individual liposomes, but it is very time-consuming and requires sophisticated equipment. In contrast, another method is laser light scattering, which is very simple and rapid to perform (New, 1990). From the previous studies, the particle size and size distribution of vesicular formulations were generally measured by dynamic light scattering (Cevc et al., 1995; Hiruta et al., 2006; El Zaafarany et al., 2010; Song et al., 2012).

3. Entrapment efficiency

Entrapment efficiency describes drug loading in elastic liposomes. The important process is the separation method to remove un-entrapped solute from the vesicle before determining the quantity of drug in the vesicles. Ultracentrifugation was a method chosen to use for removing un-entrapped materials in preparations that contained a volatile component such as ethanol (Touitou et al., 2000; López-Pinto et al., 2005; Fang et al., 2006). The gel filtration is another separation method for elastic liposomes without ethanol (Guo, Ping, and Zhang, 2000; Jain et al., 2008; El Zaafarany et al., 2010). However, gel filtration has been used for separation of un-entrapped material in some elastic liposomes with ethanol (Garg et al., 2008; Singh et al., 2009a). In this case, loss of ethanol and changes in vesicular composition may result. Moreover, dialysis (Verma et al., 2003a) and membrane filtration (Song et al., 2012) have also been used to remove un-entrapped materials from elastic liposomes.

4. Elasticity

A main functional property of elastic liposomes is elasticity. This parameter indicates the physical property of the vesicles that may be related to the permeation property of the elastic liposomes (Mishra et al., 2007; Garg et al., 2008; Jain et al., 2008; Singh et al., 2009a). Elasticity can be defined in many different ways as follows:

(1) Permeation capability: The permeation capability is calculated as permeability of vesicles relative to that of water (%). The flux of liposomes suspension and water through a known pore size membrane is driven by an external pressure and measured as a function of time. The changing of vesicle size is controlled by comparing the size and size distribution between before and after extrusion (Cevc et al., 1995).

(2) Elasticity value: van den Bergh et al. (2001) proposed the calculation of elasticity value as $J_{flux} \times (r_v/r_p)^2$; where J_{flux} is the rate of vesicle penetration through a permeability barrier, r_v is the size of vesicles after extrusion and r_p is the pore size of the barrier. This parameter has been used by several research groups (Hiruta et al., 2006; Mishra et al., 2007; Garg et al., 2008; Singh et al., 2009a; El Zaafarany et al., 2010). The recovery of PC in sample after extrusion was also determined by Hiruta et al. (2006).

(3) Degree of deformability: Another term used to describe elasticity is the degree of deformability. This parameter is determined by comparing the sizes of vesicles before and after extrusion through the polycarbonate membrane filter, the pore size of which was smaller than the size of the starting vesicles (Goindi et al., 2013). The degree of deformability was calculated as %deformity (the change in size after extrusion/ the size before extrusion).

5. Stability

The stability of any pharmaceutical products is defined as the capacity of the formulation to remain within the specified limits for its shelf life (USP 35-NF30, 2012b).

Both chemical and physical stability are usually investigated. Problems associated with stability of vesicular preparations include loss of entrapment, change in vesicular structure, changes in particle size and particle size distribution, aggregation and chemical instability of the entrapped drug (Lee et al., 2005; Fang et al., 2006; Hiruta et al., 2006; El Zaafarany et al., 2010).

Factors affecting the properties of elastic liposomes

1. Type of surfactant

Type of surfactant can affect some properties of elastic liposomes such as size and elasticity. Elsayed et al. (2007) and Liu et al. (2013) reported that the size of elastic liposomes containing Tween® 80 was smaller than that of the corresponding conventional liposomes. On the contrary, larger sizes were seen with elastic liposomes containing bile salts or bile acids such as sodium cholate (Elsayed et al., 2007; Liu et al., 2013), sodium deoxycholate (Elsayed et al., 2007) and deoxycholic acid (Fang et al., 2006).

The effects of surfactant type on the size of elastic liposomes may be attributed partly to the hydrophilic-lipophilic balance (HLB) of the surfactant. Size of elastic liposomes increased with the increased HLB of Tween® and Span®. El Zaafarany et al. (2010) reported that elastic liposomes prepared from Tween® 80 (HLB = 15) had a larger particle size than that of liposomes prepared from Span® (HLB = 4.3). Span® 85 (HLB = 1.8) yielded the smallest vesicles of all the three non-ionic surfactants studied. However, an opposite result was seen between Tween® 80 (HLB = 15) and Span® 20 (HLB = 8.6) in another study (Liu et al., 2013). In the latter case, the polyoxyethylene group on Tween® 80 was attributed to the increase in curvature of the vesicle, resulting in a smaller vesicle size. In addition, the effect of HLB could not explain the results seen when Tween® 80 was compared with ionic surfactants. Elastic

liposomes containing Tween® 80 resulted in larger vesicles than those containing sodium cholate or sodium deoxycholate (Lee et al., 2005; El Zaafarany et al., 2010).

The type of surfactant used as edge activator in elastic liposomes also affect the vesicles elasticity. In one study, Tween® 80 provided the highest elasticity compared with sodium cholate, sodium deoxycholate, Span® 80 and Span® 85 when used at the same concentration (El Zaafarany et al., 2010). The researchers suggested that bulkiness of the molecular structure might be responsible for the difference in vesicle elasticity. When compared between Span® 80 and Span® 85, the tri-ester Span® 85 resulted in less flexible vesicles due to the more bulkiness of its structure. The similar results were seen when Tween® 80 was compared with sodium cholate and sodium deoxycholate.

2. Surfactant contents

The elasticity of vesicle seems to be affected by the surfactant contents. Elasticity is increased with the amount of surfactant in the vesicle until an optimum concentration is reached. Elasticity of the vesicle decreases with the excess of surfactant (Mishra et al., 2007; Garg et al., 2008; Jain et al., 2008; El Zaafarany et al., 2010). A study using electron spin resonance revealed that surfactant molecules intercalated into the lipid structure to create the elastic property. However, further increase in surfactant concentration led to pore formation in the bilayer, leading to poor coordination of the vesicle structure (van den Bergh et al., 2001).

Amount of surfactant in the vesicle seems to also affect entrapment efficiency. Excess surfactant contents decrease the entrapment efficacy (Mishra et al., 2007; Jain et al., 2008; Goindi et al., 2013). Differential scanning calorimetry was used to show that incorporation of surfactant into lipid vesicles disturb the arrangement and coordination of lipid structure (El Maghraby et al., 2000a). With continuing increase in

surfactant concentration, the vesicles may be changed to mixed micelles (El Maghraby et al., 2004). The structural changes could result in reduction in entrapment efficiency.

Elastic liposomes as a topical drug delivery carrier

Elastic liposomes are deformable vesicles and can pass through pores smaller than their average size without any appreciable changes in the size. They could improve skin delivery of several drugs. When applied to the skin under a non-occlusive condition, elastic liposomes permeate through stratum corneum by the osmotic gradient and hydration force caused by the difference of water concentration between the skin surface and the deep skin (Cevc and Gebauer, 2003). Moreover, their components such as surfactant or alcohol can also act as a penetration enhancer to increase skin permeation (Williams and Barry, 2004).

Various substances were successfully delivered through the skin by elastic liposomes. These molecules comprise a variety of structures and physicochemical properties. Large hydrophilic molecules with better delivery using elastic liposomes include insulin (Cevc et al., 1998), plasmid DNA (Lee et al., 2005) and peptide antigen (Rattanapak et al., 2012). Lipid-soluble substances that were delivered using elastic liposomes include diclofenac (Cevc and Blume, 2001), oestradiol (El Maghraby, Williams, and Barry, 2000b), triamcinolone acetonide (Cevc and Blume, 2003), catechins (Fang et al., 2006), ketotifen (Elsayed et al., 2007), retinol (Oh et al., 2006), asiaticoside (Paolino et al., 2012) and voriconazole (Song et al., 2012). Several water-soluble substances have also been delivered in elastic liposomes with success. These water-soluble molecules include carboxyfluorescein (Verma et al., 2003a), propranolol hydrochloride (Mishra et al., 2007), diclofenac sodium (El Zaafarany et al., 2010), colchicine (Singh et al., 2009a), calcein (Bahia et al., 2010) and bleomycin hydrochloride (Hiruta et al., 2006). Thus, elastic liposomes can provide targeted delivery. Additionally,

they can enhance transdermal drug delivery and, thus, increase drug concentrations in blood circulation.

The first elastic liposome preparation was reported in 1992 by Cevc and Blume (Cevc and Blume, 1992). This new type of liposomes was termed Transfersomes™ and was claimed for their high and "self-optimizing" deformability (Cevc et al., 1995). Transfersomes™ was prepared by 8.7% w/w of soybean phosphatidylcholine and 1.3% w/w of sodium cholate as well as approximate 8.5% v/v of ethanol in triethanolamine hydrochloride buffer, pH 6.5. Transfersomes™ provided improvement in insulin skin delivery. The effect seen with Transfersomes™ was comparable to that of subcutaneous injection *in vivo* (in mice and in human) (Cevc et al., 1995; Cevc et al., 1998). The other elastic liposome preparation with improved hypoglycemic effect in Kunming mice was prepared with 3.5% w/w of soy lecithin and 1% w/w of sodium cholate in 0.9% NaCl without alcohol (Guo, Ping, and Zhang, 2000).

The efficiency of plasmid DNA transfection was reported in elastic liposomes containing sodium cholate or sodium deoxycholate (Lee et al., 2005). Elastic liposomes prepared with soybean phosphatidylcholine and sodium cholate increased skin permeation of carboxyfluorescein (CF). Interestingly, CF skin permeation was increased regardless of whether CF was entrapped within the vesicles or not. However, when CF was not entrapped in the vesicles the increased permeation ceased within the stratum corneum layer (Verma et al., 2003a). These findings indicate that even blank elastic liposomes can act as a penetration enhancer.

El Maghraby et al. (2000a) reported comparable results in skin delivery of oestradiol through abdominal human skin when Span® 80, Tween® 80 or sodium cholate was used as an edge activator, provided that the molar ratio was optimized for each surfactant. Propranolol hydrochloride and acyclovir sodium could be delivered systematically with elastic liposomes using Span® 80 as edge activator (Mishra et al., 2007; Jain et al., 2008). The elastic liposomes composed of soybean

phosphatidylcholine and Span® 80 with 7% v/v ethanol increased skin deposition with sustained delivery and improved the site specificity of colchicine (Singh et al., 2009a). Elastic liposomes were shown to improve the *in vitro* permeation of rizatriptan across rat abdominal skin at about 20 times of that of solution and 5 times of that of conventional liposomes (Garg et al., 2008).

Trotta et al. (2002) formulated anti-inflammatory elastic liposomes using the active ingredient dipotassium glycyrrhizinate as edge activator. The preparation gave higher skin deposition of the drug in pig ear skin when compared to the corresponding drug solution.

Factors affecting the skin permeation of elastic liposomes

The elastic liposomes enhance skin permeation and deliver drug into deeper skin layers. The properties of the elastic liposomes can affect skin permeation of the drug from elastic liposomes.

1. Vesicle size

The effects of vesicle size on entrapment efficiency and transdermal flux of elastic liposomes were studied by Mahmood, Taher, and Mandal (2014). The authors reported that different particle sizes (191±11 nm and 113±6 nm) of raloxifene hydrochloride-loaded elastic liposomes produced similar entrapment efficiencies and transdermal fluxes. In another study, the effect of vesicle size on the penetration of carboxyfluorescein into the human skin was reported. Verma et al. (2003b) found that liposomes with a size of 120 nm, when compared to larger liposomes (810 nm), resulted in higher accumulation of carboxyfluorescein in the stratum corneum, deeper skin layers, and receptor compartment of the Franz diffusion cell.

2. Elasticity

Liposomes with high elasticity can increase skin permeation better than liposomes with lower elasticity regardless of surfactant concentration (Mishra et al.,

2007). The results from the previous study indicated that the increased skin permeation was a result from elasticity of the vesicles rather than from the penetration-enhancing effect of the surfactant. In general, vesicles with higher elasticity can better enhance skin permeation of active compounds (Guo, Ping, and Zhang, 2000; Garg et al., 2008; Singh et al., 2009a; Song et al., 2012; Ascenso et al., 2015).

3. Entrapment efficiency

High entrapment efficiency is usually associated with better skin delivery. In one study, carboxyfluorescein elastic liposomes prepared with soybean phophatidylcholine and sodium cholate increased CF skin penetration into stratum corneum and viable epidermis of both entrapped and non-entrapped CF *in vitro* (Verma et al., 2003a). However, the entrapped CF was much better delivered to the viable epidermis than the non-entrapped CF. In many other studies the skin permeation was also increased when the entrapment efficacy increased (Mishra et al., 2007; Garg et al., 2008; Singh et al., 2009a).

4. Composition

The type and concentration of edge activator is important to improve skin permeation of elastic liposomes. Ita et al. (2007) investigated effect of surfactant on skin permeation of several hydrophilic drug (metrotrexate, acylclovir and idoxuridine) from elastic liposomes prepared with sodium deoxycholate, sodium cholate, Span® 20, Span® 40, Span® 60 and Span® 80. The higher skin permeation was observed in elastic liposomes containing sodium cholate compared with those containing sodium deoxycholate and the non-ionic surfactants. However, no significant differences were observed among the sorbitan ester nonionic surfactants with different hydrocarbon chain length and condensed packing.

Ethosomes

Ethosomes constitute another type of liposomes with high alcohol contents intended to use as a skin delivery carrier. They consist of high ethanol contents to increase lipid membrane fluidity. Ethosomes show better skin permeation than the traditional liposomes (Dayan and Touitou, 2000). They act by penetrating into intercellular lipids, increasing lipid fluidity and decreasing the density of the intercellular lipid matrix. Ethosomes usually contain 20-50%v/v of ethanol (Touitou, 1996). Ethosomes are able to deliver both hydrophilic and hydrophobic substances into and through the skin. Better drug delivery with ethosomes has been reported with drugs such as minoxidil (Touitou et al., 2000), acylclovir (Horwitz et al., 1999), trihexylphenidyl hydrochloride (Dayan and Touitou, 2000), testosterone (Ainbinder and Touitou, 2005), melatonin (Dubey et al., 2007), simvastatin (An et al., 2011) and ketoprofen (Chourasia et al., 2011).

Roles of ethanol in elastic liposomes

The combination of ethanol and surfactant have been used in elastic liposomes to deliver a few lipophilic substances. Fang et al. (2006) used 15% v/v of ethanol combined with 0.25% w/v of deoxycholic acid to deliver catechin. Song et al. (2012) used ethanol (7 and 30% v/v) and Tween® 80 (0.53% w/v) for enhanced skin delivery of voriconazole. Ethanol was added into these preparations to improve solubility of the hydrophobic substances or to enhance skin permeation. The flux of catechin in deoxycholic acid-containing liposomes without 15% ethanol was the same as that found with the conventional liposomes. The higher catechin flux was achieved in the presence of ethanol (Fang et al., 2006). Similarly, Song et al. (2012) reported that the permeation rate and the cumulative permeated amount of voriconazole were increased when the ethanol contents were increased from 7 to 30% v/v. Recently, the combination of ethanol and surfactant was also used in caffeine elastic liposomes to

increase caffeine permeation. Ascenso et al. (2015) used 30% v/v of ethanol combined with 2.3% w/v of sodium cholate or 1.8% w/v of Span® 20 in their so-called "transethosomes" preparation and tested in a pig ear skin model. Unfortunately, they found that the control solution was better in terms of stratrum corneum retention and epidermis and dermis retention than their transethosome preparation. Caffeine skin permeation was comparable between transethosomes and the control solution.

CHAPTER III

MATERIALS AND METHODS

Materials

1. Absolute Ethanol (Merck, Germany, Lot no. K377560483731)
2. Amonium molybdic acid (Mallinckrodt AR, Germany, Lot no. 3420X12465)
3. Caffeine (Sigma-aldrich, USA, Lot no. BCBQ5381V)
4. Chloroform (Labscan analytical science, Thailand, Lot no.09041093)
5. Disodium hydrogen phosphate (Riedel-deHean, Germany, Lot no.50490)
6. Fiske-Subbarow reducer (Fluka, USA, Lot no. BCBF0620)
7. High purity nitrogen gas (Thai Industrial Gas)
8. Hydrogen peroxide (Merck, Germany, Lot no. 39266387845)
9. Isopropanol (Scharlau, EU, Lot no. AL03212500)
10. Methylchloroisothiazolinone (Dr. Ehrenstorfer, Germany, Lot no. 30912)
11. Potassium chloride (Merck, Germany, Lot no. TA658736001)
12. Potassium dihydrogen phosphate (Merck, Germany, Lot no. A476973328)
13. Sodium chloride (Ajax Finechem Pty Ltd, Australia, Lot no. 1403164044)
14. Soy Phosphatidylcholine (Epikuron® 200) (Cargill, USA, Lot no.129020)
15. Span® 80 (Croda, Singapore, Lot no.30447)
16. Sulfuric acid (Labscan analytical science, Thailand, Lot no.15010153)
17. Tween® 80 (B. L. Hua, Thailand, Lot no.101859)
18. Ultrapure™ water (Elgastat Maxima UF, Elga, England)

Equipment

1. Analytical balance (AG285, Mettler Toledo, Switzerland), (XP205, Mettler Toledo, Switzerland)
2. Analytical balance (PG403-S, Mettler Toledo, Switzerland)

3. Hand-held extruder (LipoFast, Avestin, Canada)
4. High Performance Liquid Chromatography System (HPLC) (Waters, USA) equipped with
    - HPLC-system (Alliance 2695, Waters, USA)
    - Column (Xterra RP18, 5 µm, 4.6 x 250 mm, Waters, USA)
    - Guard column (Xterra RP18, 5 µm, 3.9 x 20 mm, Waters, USA)
    - Photodiode Array (PDA) Detector (2998, Waters, USA)
5. Horizontal shaker (HS500 Janke& Kunkel, IKA, Germany)
6. Hot air incubator (Memmert, Germany)
7. Light microscopes (Eclipse E200, Nikon, Japan and Zeiss, Germany)
8. Lipex ™ Extruder (Northern Lipid, Canada)
9. Magnetic bar (Schott, Germany)
10. Modified Franz diffusion cells (Science Service, Thailand)
11. Particle size analyzer (Mastersizer 2000, Malvern Instrument, UK)
12. pH meter (Orion 903, Thermo, USA)
13. Refrigerated incubator (FOC 225i, VELP® Scientifica, Italy)
14. Rotary evaporator (Buchi Rotavapor R-215, Buchi Rotavapor R-210, Switzerland)
15. Ultracentrifuge (Optima™ L-100XP, Beckman Coulter, USA)
16. UV-Visible spectrophotometer (UV-1601, Shimadzu, Japan)
17. Vortex mixer (G 560E, Vortex-genie®2, USA)

Supplies

1. Centrifuge tubes (Thin wall konical™ Polyallomer Tubes 1.5 ml 11x35 mm) (Beckman, USA)
2. Disposable syringe filters (Nylon 13 mm, 0.45 µm) (Vertical™, Thailand)
3. Disposable syringe filters (Nylon 13 mm, 0.22 µm) (National scientific, USA)

4. Membrane filters (Nylon 47 mm, 0.45 μm) (Vertical™, Thailand)

Methods

1. Development of caffeine elastic liposomes

Liposomes were prepared with approximately 57 mg/ml of the lipid phase that contained soybean phosphatidylcholine (SPC) with 15-25% w/w Tween® 80 or 5-10% w/w Span® 80 as an edge activator. The concentration of total lipid used was determined in the preliminary study. These compositions resulted in the same surfactant-to-lipid molar ratio for both Tween® 80 and Span® 80. Ethanol at 5-25% v/v in water was used as the aqueous phase.

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 (SPC) and a surfactant. The total lipid concentration was approximately 57 mg/ml. The percentage of Span® 80 in the lipid phase was 5, 7.5 and 10% w/w, whereas the percentage of Tween® 80 in the lipid phase was 15, 20 and 25% w/w. The aqueous phase of liposomes was solution of 5, 15, 20 and 25% v/v of ethanol in Ultrapure™ water. The compositions of blank liposomes are described in Table 1. The mixture of SPC and surfactant (approximately 200 mg) was placed in a 1000-ml round bottom flask and dissolved with approximately 10 ml of chloroform. Chloroform was removed by rotary evaporation at 45 °C under vacuum to make a thin lipid film. The dry lipid film was hydrated with 3.5 ml of the ethanol solution until liposomal vesicles were formed completely. Resultant liposomes were subsequently extruded through polycarbonate membranes with a pore diameter of 5 μm to obtain preparations devoid of unreasonably large liposomes. All vesicular preparations were routinely examined under a light microscope 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 protected from light and left at 4 ºC overnight before characterization/use in the subsequent experiments. All liposomal formulations were kept at 4 ºC until use.

Table 1: Compositions of blank liposomes

Formulation code	Composition of lipid mixture (% w/w)			Composition of hydrating solution
	SPC	Span® 80	Tween® 80	Ethanol in water (% v/v)
S5_5	95	5	0	5
S5_15	95	5	0	15
S5_20	95	5	0	20
S5_25	95	5	0	25
S7.5_5	92.5	7.5	0	5
S7.5_15	92.5	7.5	0	15
S7.5_20	92.5	7.5	0	20
S7.5_25	92.5	7.5	0	25
S10_5	90	10	0	5
S10_15	90	10	0	15
S10_20	90	10	0	20
S10_25	90	10	0	25
T15_5	85	0	15	5
T15_15	85	0	15	15
T15_20	85	0	15	20
T15_25	85	0	15	25
T20_5	80	0	20	5
T20_15	80	0	20	15
T20_20	80	0	20	20
T20_25	80	0	20	25
T25_5	75	0	25	5
T25_15	75	0	25	15
T25_20	75	0	25	20
T25_25	75	0	25	25

1.2 Determination of saturation solubility of caffeine in the aqueous phase of liposome formulation at 4 °C

An aliquot (0.050 g) of caffeine was placed in a glass test tube. The alcohol solution that was used as the aqueous phase for liposome preparation was added to the test tube in small portions (100 µl at a time). The tightly-closed test tube with its contents was vortexed and observed for caffeine crystals. The solvent was added until caffeine was completely dissolved. After leaving in the ambient for 3-4 hours, the solution was assayed for caffeine by UV-spectrophotometry at 273 nm using a partially validated method described in Appendix D. A caffeine solution at 70% of the assayed concentration was then prepared and kept in a refrigerator at 3-6 °C. Samples were taken at intervals during days 1-8 and the caffeine content was determined. To avoid sampling of caffeine crystals, the samples were filtered through syringe filters (0.22 µm) immediately after the samples were taken. The experiment was run in triplicate. The average of caffeine concentrations between consecutive days of sampling, where no statistical difference in concentration was detected, was used as the saturation solubility of caffeine in the aqueous phase at 4 °C.

1.3 Preparation of caffeine elastic liposomes

The compositions of caffeine elastic liposomes were selected from blank formulations in Section 1.1 after physical characterization. Formulations that resulted in complete vesicle formation and the highest elasticity were selected for each type of surfactant used. The aqueous phase was caffeine at its 80% saturation solubility in the ethanol solution at 4 °C determined from the experiment in Section 1.2. The method of preparation was the same as that described in Section 1.1. All liposomal formulations were protected from light and kept at 4 °C until further use.

Percentage of yield for caffeine-loading elastic liposomes was calculated from the ratio of PC after extrusion through the polycarbonate membrane and the initial amount of PC in the preparation.

2. Characterization of liposomes

The physical appearance of all preparation was observed first with naked eyes. The decent preparation displayed a homogenous, milky dispersion without any precipitates. Blank liposomes were further characterized for morphology, size and size distribution and elasticity. Caffeine elastic liposomes were characterized for morphology, size and size distribution, elasticity, entrapment and loading efficiencies, and physical stability.

2.1 Morphology

2.1.1 Optical microscopy

The overall quality of the preparation was assessed with an optical microscope. Three samples were taken from each preparation. The samples were monitored for complete, round vesicles and for lipid remnants.

2.1.2 Polarized light microscopy

Polarized light microscopy can be used to verify existence of vesicular bilayers in the preparation (Bangham and Horne, 1964). A drop of sample was placed on a glass slide and examined between two crossed-polarizing filters under an optical microscope. The polarized-light photomicrographs were recorded using a digital camera.

2.2 Determination of size and size distribution

Size and size distribution of vesicles were determined by laser diffraction technique using a Mastersizer 2000 (Malvern Instruments, UK). The refractive index was set at 1.5 (Chong and Colbow, 1976). Size and size distribution were expressed as D [4, 3] and span value as recommended by the manufacturer of the instrument. Samples were diluted to suitable concentrations with Ultrapure™ water. The experiment was done in triplicate with pooled samples from 2-3 batches of liposomes.

2.3 Determination of vesicle elasticity

2.3.1 Measurement of vesicle elasticity

The method used was modified from the method described by Goindi et al. (2013). The elasticity of liposomes was determined by extruding the formulation through polycarbonate membranes with 2 µm-pore diameter. The extrusion was performed on a hand-held extruder (LipoFast™, Avestin). The particle size distribution profiles before and after extrusion were compared. Percentage of elasticity of liposomes (E) was calculated using the following formula:

$$E = \frac{A \times 100}{B} \quad (1),$$

where A= the overlapping area of size distribution profiles obtained before and after extrusion and B= area under the curve of the size distribution profile before extrusion (Figure 1).

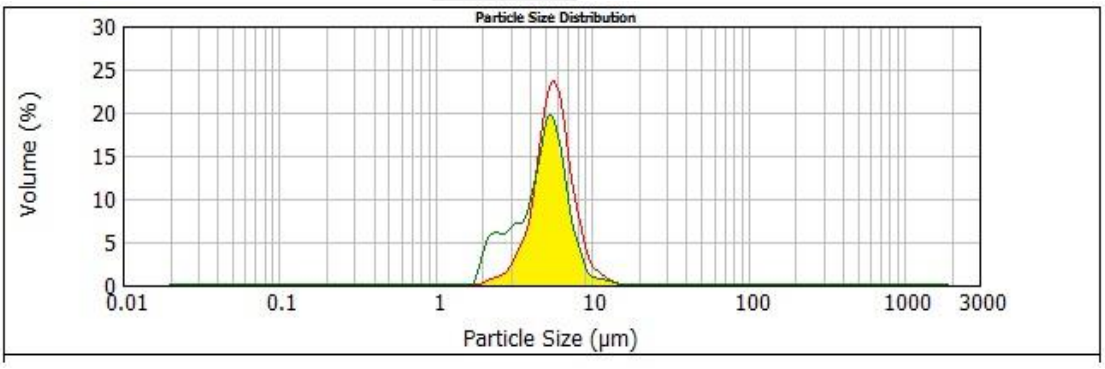

Figure 1: Particle size distribution profiles of S7.5_5 before (▬) and after (▬) extrusion through 2 µm-pore diameter polycarbonate membrane

▬ Overlapping area of the profile after extrusion through 2µm-pore diameter polycarbonate membrane compared to the profile before extrusion.

2.3.2 Measurement of PC

This assay was conducted to ascertain that most of the preparation passed through the extruder (LipoFast™) membrane after extrusion so that the area comparison would be valid. The amounts of PC in the liposome suspension before

and after extrusion through 2 μm-pore diameter polycarbonate membranes were compared. PC contents were determined by the standard Bartlett assay as described in Appendix C (New, 1990).

2.4 Determination of caffeine entrapment and loading efficiencies

To determine the entrapment and loading efficiencies of caffeine, the caffeine dispersions were separated into the supernatant containing the free caffeine and the pellet containing the entrapped drug by ultracentrifugation. The caffeine contents in the supernatant and the pellet were assayed by UV spectrophotometry at 273 nm. The partial validation of the assay methods for specificity, linearity and range, accuracy, and precision was described in Appendix D. The phospholipid content was assayed as described in Section 2.3.2. The total lipid (PC plus surfactant) was calculated from the phospholipid content. Caffeine in the supernatant was also assayed for routine monitoring of the analytical recovery. The experiment was done in triplicate with pooled samples from at least 6 batches of liposomes.

2.4.1 Separation of the pellet

An aliquot (1.25 ml) of the vesicular dispersion was pipetted into a polyallomer konical™ centrifuge tube and subjected to centrifugation at 59,000 rpm, at 4 °C for 5.5 hours in an ultracentrifuge (Optima™ L-100XP, Beckman Coulter, SW60Ti Rotor). The supernatant was carefully separated from the sediment using a syringe with small needle. The supernatant and the pellet were further assayed for the caffeine contents.

2.4.2 Quantitative analysis of caffeine in the supernatant

The supernatant was transferred to a 10 ml volumetric flask and the solution was adjusted to volume with Ultrapure™ water. The sample was appropriately diluted with water and analyzed by UV spectrophotometry at 273 nm.

2.4.3 Quantitative analysis of caffeine in the pellet

The separated pellet from Section 2.4.1 was dissolved with 4:1 of isopropanol:water in a 10 ml volumetric flask. The solution was adjusted to volume with the same medium. This solution was mixed, appropriately diluted and assayed by UV spectrophotometry at 273 nm.

2.4.4 Quantitative analysis of total lipid

The total lipid in the finished liposome dispersion was calculated from the PC amount that was determined by the standard Bartlett assay (New, 1990) (see Appendix D). The amount of total lipid of each liposome preparation was calculated from the following equation:

$$\text{Amount of total lipid (mg)} = \frac{\text{Amount of PC from the assay (mg)}}{\text{Fraction of PC in the lipid phase}} \quad (2)$$

2.4.5 Calculation of the percentage of entrapment (New, 1990)

The percentage of caffeine entrapment of each formulation was calculated from the following equation:

$$\text{Percentage of entrapment} = \frac{\text{Amount of caffeine in the pellet (mg)} \times 100}{\text{Amount of total caffeine (mg)}} \quad (3)$$

2.4.6 Calculation of the percentage of loading (Ascenso et al., 2015)

The percentage of caffeine loading of each formulation was calculated from the following equation:

$$\text{Percentage of loading} = \frac{\text{Amount of caffeine in the pellet (mg)} \times 100}{\text{Amount of total lipid (mg)}} \quad (4)$$

The calculation was based on an assumption that total lipid in the preparation existed exclusively as liposomes in the pellet.

2.5 Physical stability of caffeine elastic liposomes

All preparations of caffeine elastic liposomes were studied for stability by monitoring aggregation, changes in color, presence of drug crystals, size and size distribution, changes in entrapment efficiency, and elasticity for 7 days at day 1, 3, and

7. All preparations were protected from light and kept at 4 ºC. The experiments were performed in triplicate with pooled samples from at least 6 batches of liposomes.

3. Caffeine skin permeation from elastic liposomes

Modified Franz diffusion cells were used to study *in vitro* permeation of caffeine from different formulations. The abdominal skin of a newborn pig who died of natural causes was used as the model skin membrane.

3.1 Preparation of newborn pig skin membrane

The full-thickness abdominal skin of newborn pig was prepared. The subcutaneous fat and other tissues were carefully and completely removed from the skin using appropriate surgical tools. The hair was clipped with a pair of scissors with care. The separated skin was washed with purified water to get rid of tissue debris. The skin was blotted dry with paper towel and wrapped in a sheet of aluminum foil. The prepared skin was kept frozen at -20 °C until use. The frozen skin was used within 2 weeks after it was prepared. On the day of the experiment, the frozen skin was thawed in the ambient and rehydrated by being immersed in phosphate buffered saline (PBS), pH 7.4, at room temperature for about one hour before use. The hydrated skin was cut into a circular shape with a diameter of about 3 cm and mounted onto the diffusion cell. The skin was allowed to equilibrate to the temperature set for the diffusion cell for at least 30 minutes.

3.2 Caffeine permeation from elastic liposomes

Formulations used in this study were:

1. A caffeine solution at 80% saturation solubility (8.6 mg/ml of caffeine in 5% v/v of ethanol in water)
2. Formulation S7.5_5 (7.5% w/w of Span® 80 and 5% v/v of ethanol) containing 8.6 mg/ml of caffeine
3. Formulation T20_5 (20% w/w of Tween® 80 and 5% v/v of ethanol) containing 8.6 mg/ml of caffeine

The two caffeine-containing elastic liposome preparations were selected based on the highest elasticity for each edge activator. The caffeine solution at the same caffeine concentration was used as a reference formulation. PBS, pH 7.4, was used as the receptor medium. Modified Franz diffusion cells were used to study permeation of caffeine from the elastic liposomes and the caffeine solution. The internal diameter of the 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 PBS, pH 7.4, as the receptor medium. The receptor compartment was equipped with a magnetic stirring bar rotating at 280-350 rpm. The temperature of the water bath was kept controlled constant at 37 °C throughout the experiment. This setting rendered the skin temperature be controlled at 32 °C. The donor and the receptor compartments were separated by the abdominal newborn pig's skin. 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 receptor fluid and the skin was equilibrated to reach the preset temperature for 30 minutes. After equilibration, an aliquot (150 µl/cm^2) of the caffeine elastic liposome dispersion or the reference solution was pipetted onto the membrane surface of each cell. Samples (about 1 ml) were withdrawn from the receptor compartment at 1, 1.5, 2, 2.5, 3, 4, 5, 6, 8, 10.5, 13, 16, 22, and 24 hours. After each sample was taken, the receptor medium was replaced with an equal volume of the fresh, warm (37° C) medium. Air trapping beneath the dermis that could severely distort the permeation profile was avoided during the medium replacement. The samples were kept frozen (-20 °C) until they were analyzed by the high-performance liquid chromatographic (HPLC) method. The HPLC analytical method was validated using the guidelines in USP 35 (USP 35-NF30, 2012a). The validation results for specificity, linearity and range, accuracy, and precision are shown in Appendix E. Each treatment was done with at

least six diffusion cells. The allocation of the formulation on the diffusion cells was designed to avoid the influence of cell position.

3.3 Caffeine retention in the skin (modified from Sripetch (2009))

At the end of permeation study, the skin surface and the donor cap were washed 3-5 times with Ultrapure™ water, which was later assayed by the HPLC method for caffeine remaining in the donor compartment. The skin was un-mounted from the diffusion cell and blotted dry with filter paper. The skin was then cut into small pieces with a pair of scissors and placed in a glass tube. An aliquot (2 ml) of Ultrapure™ water was added into the tube to extract caffeine from the skin by vortexing for 5 minutes, sonicating for 15 minutes, shaking with shaker at ambient temperature for 2 hours. The extracted solution was filtered through a membrane filter (0.45 µm) and analyzed for caffeine amount in the skin, Q_s, by the validated HPLC method.

3.4 HPLC system and conditions for caffeine assay (modified from Duracher et al. (2009) and Bolzinger et al. (2008))

The concentration of caffeine in the samples from Sections 3.2-3.3 was determined by HPLC method (Waters, USA). The chromatographic system and conditions were as follows.

Column	:	Xterra RP18, 5 µm, 4.6 x 250 mm
Guard column	:	Xterra RP18, 5 µm, 3.9 x 20 mm
Mobile phase	:	water: acetonitrile (85:15 v/v)
Injection volume	:	20 µL
Flow rate	:	1 ml/min
Detector	:	PDA detector at λ 273 nm
Temperature	:	ambient
Internal Standard	:	methylchloroisothiazolinone (5.0 µg/ml)

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

Concentrations of caffeine were back calculated from a calibration line constructed from the peak area ratio of caffeine to the internal standard against standard caffeine concentrations. A calibration line was constructed for each and every run of the assay.

3.5 Data treatment

The cumulative amount of permeated caffeine per cm^2 was plotted against time in hr. The observed steady state flux (J_{ss}) was obtained from the slope by regression of the linear portion of the cumulative amount-time profile. The permeation coefficient (P_s) was calculated using Equation 5.

$$P_s = \frac{J_{ss}}{C_d} \quad (5),$$

where C_d is the caffeine concentration in the donor compartment.

The enhancement factor, EF, of the formulation based on the permeability coefficient was defined as

$$EF = \frac{P_s \text{ of the formulation}}{P_s \text{ of the reference}} \quad (6),$$

The enhancement factor of the formulation based on the caffeine amount in the skin (Q_s) was defined using Equation 7.

$$EF \text{ of } Q_s = \frac{Q_s \text{ of the formulation}}{Q_s \text{ of the reference}} \quad (7),$$

where Qs is the percentage of caffeine amount in the skin, which was calculated from Equation 8.

$$Q_s = \frac{\text{Caffeine amount in the skin} \times 100}{\text{Initial amount of caffeine in the donor}} \quad (8)$$

The enhancement factor of the formulation based on the cumulative amount of caffeine in the receptor medium at 24 hours (Q_{24}) was calculated using Equation 9.

$$EF \text{ of } Q_{24} = \frac{Q_{24} \text{ of the formulation}}{Q_{24} \text{ of the reference}} \quad (9),$$

where Q_{24} is the percent of cumulative caffeine amount in the receptor at 24 hours, which was calculated from Equation 10.

$$Q_{24} = \frac{\text{Cumulative caffeine amount in the receptor at 24 hours x 100}}{\text{Initial amount of caffeine in the donor}} \quad (10)$$

The relative flux (RF) of the formulation was defined as follows.

$$RF = \frac{J_{ss} \text{ of the formulation}}{J_{ss} \text{ of the reference}} \quad (11)$$

Total caffeine delivered (Q_s+Q_{24}) was calculated by combining the caffeine amount in the skin with the cumulative caffeine amount in the receptor at 24 hours.

Analytical recovery of permeation studies was monitored. The total caffeine amounts in the receptor, the donor and the skin were added up and compared with the amount of caffeine loaded in each diffusion cell.

4. Statistical analysis

Statistical analysis to compare treatment means was performed on SPSS version 22. The validity of assumptions for ANOVA was tested using Levene's test of homogeneity of variances. Tukey's HSD or Dunnett T3 was used for a post hoc comparison, accordingly. The Student's t-test was used to compare two treatment means. The level of significance was chosen at probability of 0.05.

CHAPTER IV
RESULTS AND DISCUSSION

In this present study, elastic liposomes were developed as a delivery system for caffeine to treat cellulite. The elastic liposomes studied consisted of phosphatidylcholine as the structural lipid, a surfactant as edge activator and ethanol. The concurrent use of surfactant and ethanol was aimed to substitute high concentrations of the individual penetration enhancers in caffeine skin delivery.

1. Development of caffeine elastic liposomes

1.1 Blank liposomes

Blank liposomes were prepared to evaluate the feasibility of liposomal preparation at a total lipid concentration of 57 mg/ml. This lipid concentration was chosen from preliminary experiments as an optimal concentration at which all liposomes of interest could be prepared. Ethanol at 5, 15, 20 or 25 percent by volume was used. The ethanol concentrations were selected to be in the lower limit of that used in ethosomes and less. Span® 80 or Tween® 80 was used as an edge activator. These surfactants were selected based on literature review on surfactants successfully used for elastic liposomes that differ in HLB, but with the same chain length. The percentage of edge activator in lipid phase was varied at 5, 7.5 and 10 % by weight of Span® 80 and 15, 20 and 25 % by weight of Tween® 80 in the lipid phase. In a previous study, Paolino et al. (2012) recommended surfactant molar ratio of ≤ 0.2 to avoid decreases in drug entrapment due to structural changes from vesicles to micelles at higher surfactant concentrations. The corresponding molar ratio was 0.1, 0.15 and 0.2 for both Span® 80 at 5, 7.5 and 10% w/w and Tween® 80 at 15, 20 and 25% w/w.

1.2. Characteristics of blank liposomes

Twenty four preparations of blank liposomes were prepared. The blank liposomes were prepared from the compositions shown in Table 1 in Chapter 3. The acceptance criteria for completeness of liposome formation included being homogeneous milky dispersion, spherical vesicles, absence of lipid crystals, Brownian movement of vesicles and being birefringent of the lamellar structure under the cross-polarized light.

1.2.1 Physical appearance

The physical appearance of all preparations was shown in Table 2. Upon inspection with naked eyes, no lipid remnants were observed in any of the formulations. All liposome formulations were homogeneous and turbid with white to pale yellow color.

1.2.2 Morphology under light and polarized-light microscopes

All 24 blank liposomes were viewed under an optical microscope to observe the vesicle shape and the presence of lipid remnants. The liposomes appearing under the light microscope varied in size. Lipid remnants were found in some of liposome formulations (see Appendix F). Formulations with lipid remnants were not further used. The birefringence of the lamellar structure can be seen under polarized-light microscope. The formation and arrangement of bilayer have been proven using this technique (Bangham and Horne, 1964). The representative photographs of formulations that formed complete liposomes are shown in Appendix F.

Photographs of blank liposomes composed of Span® 80 and Tween® 80 at the same molar ratio under optical microscopes are displayed in Appendix F (Figure F1). In general, under the optical microscope, the size of liposomes composed of Span®

80 was bigger than the size of liposomes composed of Tween® 80. Thus, the corresponding cross-polarized images of liposomes from Span® 80 showed more clearly the lamellar pattern than those of the liposomes from Tween® 80. In some formulations with very small vesicle size, however, the birefringence could be observed only at a low magnification (x100). The birefringence could not be detected at a higher magnification (x400) due to background light interference.

Table 2: Physical appearance of the blank liposome formulations

Formulation code	Physical appearance	Formulation code	Physical appearance
S5_5	Turbid, white milky dispersion	T15_5	Turbid, pale yellow milky dispersion
S5_15	Turbid, white milky dispersion	T15_15	Turbid, pale yellow milky dispersion
S5_20	Turbid, pale yellow milky dispersion	T15_20	Translucent, pale yellow dispersion
S5_25	Turbid, pale yellow milky dispersion	T15_25	Translucent, pale yellow dispersion
S7.5_5	Turbid, white milky dispersion	T20_5	Turbid, pale yellow milky dispersion
S7.5_15	Turbid, white milky dispersion	T20_15	Translucent, pale yellow dispersion
S7.5_20	Turbid, pale yellow milky dispersion	T20_20	Translucent, pale yellow dispersion
S7.5_25	Less turbid, pale yellow milky dispersion	T20_25	Translucent, yellow dispersion
S10_5	Turbid, pale yellow milky dispersion	T25_5	Turbid, pale yellow milky dispersion
S10_15	Turbid, pale yellow milky dispersion	T25_15	Less turbid, pale yellow milky dispersion
S10_20	Less turbid, pale yellow milky dispersion	T25_20	Translucent, pale yellow dispersion
S10_25	Less turbid, yellow milky dispersion	T25_25	Translucent, yellow dispersion

Table 3 summarizes the completeness of vesicle formation of the blank liposomes. A total of 13 liposome preparations were completely formed. All formulations containing 5 and 15% v/v of ethanol formed complete vesicles, regardless of type and concentration of the surfactant. At a higher ethanol concentration (20%), complete liposome formation was seen only with the

formulation with 25% Tween® 80 (T25_20). Complete vesicle formation was not seen with any formulations at 25% v/v of ethanol. The phospholipid might be solubilized at high ethanol and surfactant concentrations (Touitou et al., 2000). In addition, surfactant solubility might also be increased at high alcohol concentrations (Yeh, Yang, and Chang, 2005; Wu et al., 2007). Moreover, mixed micelles could form instead of vesicles when surfactant concentration was increased (López et al., 1998). Thus, the formation of vesicles could be disturbed when the concentration of either surfactant or ethanol was too high. Only formulations that gave complete vesicle formation were further characterized for size and size distribution and elasticity.

Table 3: Completeness of vesicle formation of the liposome formulations

Surfactant concentration	Ethanol concentration (% v/v)			
	5	15	20	25
S5	Y	Y	N	N
S7.5	Y	Y	N	N
S10	Y	Y	N	N
T15	Y	Y	N	N
T20	Y	Y	N	N
T25	Y	Y	Y	N

Y = Complete formation N = Incomplete formation

S5 = 5% w/w of Span® 80, S7.5 = 7.5% w/w of Span® 80, S10 = 10% w/w of Span® 80,

T15 = 15% w/w of Tween® 80, T20 = 20% w/w of Tween® 80 and T25 = 25% w/w of Tween® 80

1.2.3 Size and size distribution

Only the 13 completely formed blank liposomes from Table 3 were characterized for size and size distribution. The average sizes (D [4, 3]) and span values

are shown in Table 4. The vesicle size of liposomes composed of Span® 80 and Tween® 80 was in the range of 5.84-8.08 μm and 3.73-5.27 μm, respectively. Statistically, the vesicle size of blank liposomes tended to decrease with increasing ethanol contents, except for the formulations with 7.5 and 10% Span® 80. Thus, the effects of ethanol contents on vesicle size seems to be inconclusive in this study. Vesicle size reduction with increasing ethanol contents have been previously reported (Touitou et al., 2000; Elsayed et al., 2007; Chourasia et al., 2011). Modification of system net charge and steric stabilization of phospholipid bilayer with increasing ethanol contents was also attributed to the smaller vesicle size (López-Pinto et al., 2005). Mishra et al. (2007) and Jain et al. (2008) reported that increased concentration or decreased HLB of surfactant decreased the vesicle size. As shown in Table 4, the effect of surfactant concentration was not clearly seen in this present study. Besides, the average size of liposomes composed of Tween® 80 (HLB = 15) was smaller than that of liposomes composed of Span® 80 (HLB = 4.3). Decreasing vesicle size with increasing HLB was previously reported by Liu et al. (2013), who compared Tween® 80 with Span® 20 (HLB = 8.6). They attributed the small vesicle size and the higher curvature of vesicles seen with Tween® 80 to hydrophilicity of liposome surface produced by the surfactant polyoxyethylene chain.

Table 4: Average sizes (in μm) and span values (in parentheses) of vesicles prepared from various compositions. The data are shown as mean ± SD, n = 3.

Surfactant concentration	Ethanol concentration (% v/v)			
	5	15	20	25
S5	7.21 ± 0.05 (1.02 ± 0.06)	6.77 ± 0.02 (1.04 ± 0.01)	N/A	N/A
S7.5	5.84 ± 0.01 (0.73 ± 0.00)	7.67 ± 0.09 (1.00 ± 0.03)	N/A	N/A
S10	6.32 ± 0.03 (0.78 ± 0.00)	8.08 ± 0.02 (0.95 ± 0.03)	N/A	N/A
T15	5.27 ± 0.08 (0.81 ± 0.01)	4.40 ± 0.17 (1.13 ± 0.00)	N/A	N/A
T20	4.79 ± 0.03 (1.07 ± 0.04)	4.56 ± 0.15 (1.07 ± 0.03)	N/A	N/A
T25	5.19 ± 0.02 (0.93 ± 0.01)	3.73 ± 0.05 (1.22 ± 0.03)	4.16 ± 0.12 (1.13 ± 0.02)	N/A

N/A = not available,

S5 = 5% w/w of Span® 80, S7.5 = 7.5% w/w of Span® 80, S10 = 10% w/w of Span® 80,

T15 = 15% w/w of Tween® 80, T20 = 20% w/w of Tween® 80 and T25 = 25% w/w of Tween® 80

The differences ($p < 0.05$) are significant among all formulations except between T15_15 and T20_15.

In terms of size distribution, Span® 80 gave liposomes with better homogeneity than Tween® 80. In Figures 2a and 2b, the particle size distribution profile of S7.5_5 shows a single, relatively sharp peak, whereas T20_5 shows a profile with polydispersity. This was seen at all surfactant and ethanol concentrations where complete liposome formation occurred. At higher ethanol concentrations, however, preparations containing Span® 80 resulted in broader peaks (Appendix G). On the other

hand, preparations containing Tween® 80 resulted in polydispersity with more small vesicles detected. The polydispersity seen in preparations containing Tween® 80 seemed to be responsible for their apparent smaller average sizes expressed as D [4, 3].

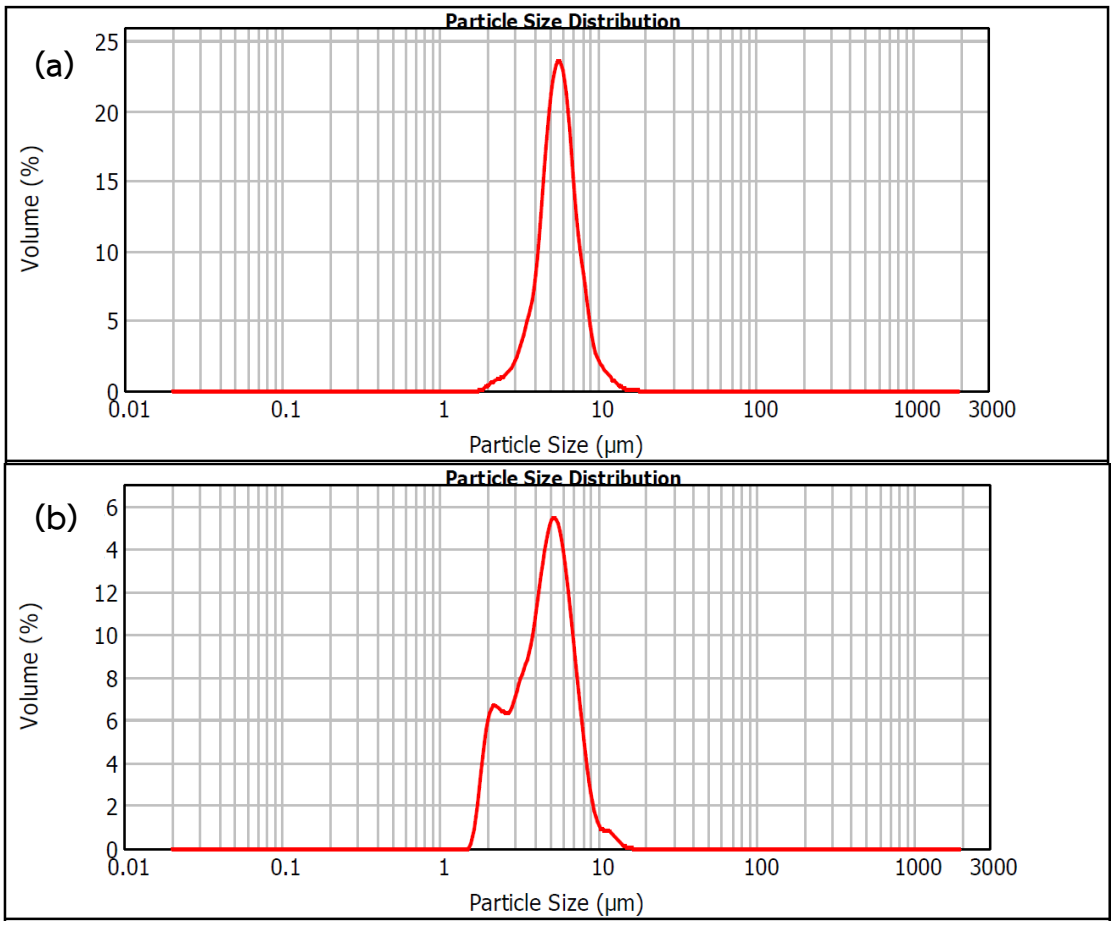

Figure 2: (a) Size distribution profile of the vesicles prepared from 7.5% w/w of Span® 80 with 5% v/v of ethanol

(b) Size distribution profile of the vesicles prepared from 20% w/w of Tween® *80* with 5% v/v of ethanol

It is worth noting that, though vesicle sizes of these liposomes in this present study were statistically different, the differences seen did not seem to be pronounced enough to be practically meaningful.

1.2.4 Elasticity of the vesicles

The elasticity of liposome formulations containing Span® 80 and Tween® 80 was in the range of 27.07-79.86% and 8.44-15.74%, respectively. The difference in the measured amounts of PC between before and after the extrusion was small. The recovery of PC after extrusion was more than 90% in all cases as shown in Table 5. The results indicated that the whole preparation passed through the membrane and thus the size distribution profile could be used to estimate the elasticity of the preparation.

Table 5: Phospholipid contents before and after extrusion through 2.0 μm membrane filters in the determination of elasticity

Formulation code	Phospholipid contents (mg/ml)		PC recovery (%)
	before extrusion*	after extrusion	
S5_5	56.55±2.57	54.41±0.73	96.21
S7.5_5	51.78±6.95	51.05±2.59	98.59
S10_5	51.09±0.73	48.20±1.30	94.33
S5_15	54.18±1.39	51.28±1.44	94.66
S7.5_15	53.08±1.42	52.83±3.02	99.53
S10_15	54.34±1.21	54.31±1.87	99.96
T15_5	47.96±1.09	48.78±1.45	101.71
T20_5	45.90±1.174	45.29±6.94	98.69
T25_5	43.71±1.01	40.41±0.25	92.46
T15_15	46.71±1.03	43.07±1.16	92.22
T20_15	43.57±1.14	41.99±1.99	96.37
T25_15	40.34±1.65	37.90±1.39	93.96
T25_20	40.90±2.33	39.24±1.23	95.95

* Liposome preparations were prepared using LipoFast™.

In this study, the elasticity of vesicles depended on surfactant type, surfactant concentration and ethanol contents. The elasticity of preparations containing Span® 80 is shown in Figure 3. The elasticity of preparations containing Tween® 80 is shown in Table 6. At 5% v/v of ethanol, the elasticity increased and then decreased with increasing surfactant concentrations for both surfactants. Thus, only at a suitable

surfactant concentration, the highest elasticity would be resulted. Similar findings have been published with vesicles without ethanol or with low concentration (7% v/v) of ethanol (Mishra et al., 2007; Garg et al., 2008; Jain et al., 2008; El Zaafarany et al., 2010). When the concentration of ethanol was increased to 15% v/v in vesicles with Span® 80, elasticity consistently decreased with the increasing concentrations of the surfactant. Vesicles containing Tween® 80 seemed to lose their elasticity completely at 15% v/v of ethanol. In ethosomes, the incorporation of ethanol in the phospholipid bilayer increases the fluidity of the bilayer, making the vesicles soft and flexible (Touitou et al., 2000). In elastic liposomes, however the presence of ethanol might interfere with solubility and/or mobility of surfactant in the membrane. Thus, elasticity was reduced at high concentrations of ethanol.

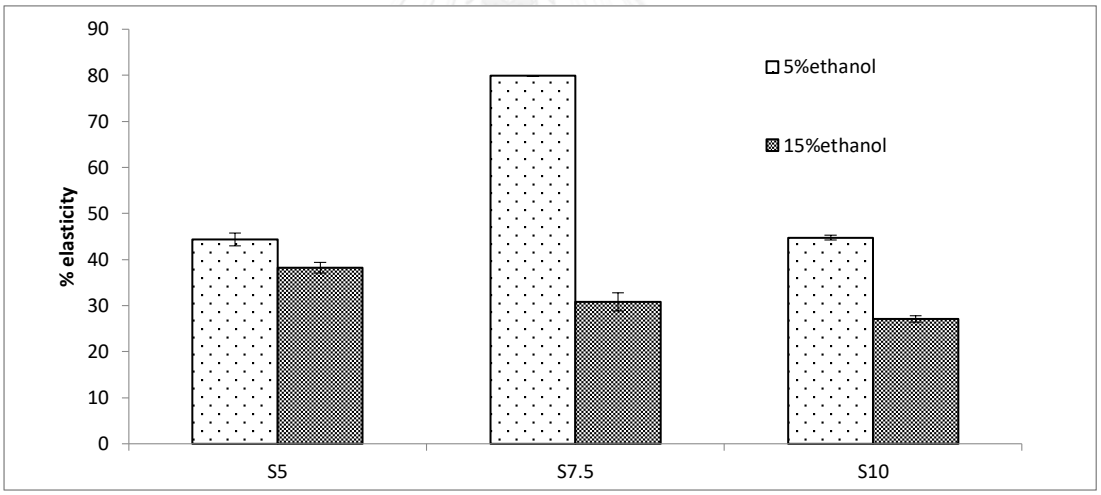

Figure 3: Effects of ethanol and surfactant on the elasticity of formulations composed of Span® 80. The data are shown as mean ± SD, n = *3.* Significant differences were seen among all formulations ($p < 0.05$*).*
(S5= 5% w/w of Span® 80, S7.5 = 7.5% w/w of Span® 80, S10 = 10% w/w of Span® 80)

Table 6: Effects of ethanol and surfactant concentrations on the elasticity of formulations composed of Tween® 80. The data are shown as mean ± SD, n = 3.

Tween®80 concentration (% w/w)	Ethanol concentration (% v/v)		
	5	15	20
15	8.44 ± 0.60	0	N/A
20	15.74 ± 0.38	0	N/A
25	11.11 ± 0.59	0	0

Significant differences were seen among all formulations ($p < 0.05$).

Figure 4 displays the overlay of distribution profiles of vesicles containing Span® 80 (S7.5_5) before and after extrusion through polycarbonate membranes. A similar representation for vesicles containing Tween® 80 (T20_5) is shown in Figure 5. These preparations were those that displayed the highest elasticity for each surfactant type. Vesicles containing Span® 80 had much more elasticity than those containing Tween® 80, as could be seen from the overlapping areas of the profiles before and after extrusion. Span® 80 has a higher ability to incorporate into phospholipid bilayer than Tween® 80 because it is more hydrophobic (El Maghraby et al., 2000b). Thus, higher elasticity was seen in liposomes composed of Span® 80.

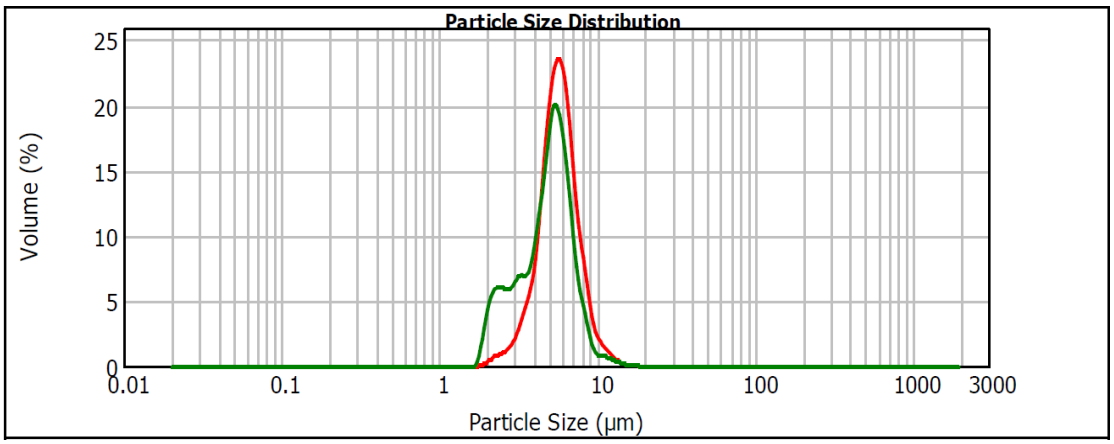

Figure 4: Overlay of the size distribution profiles of vesicles prepared from 7.5% w/w of Span® 80 with 5% v/v of ethanol before ( ) and after ( ) extrusion through 2 µm pore size membrane

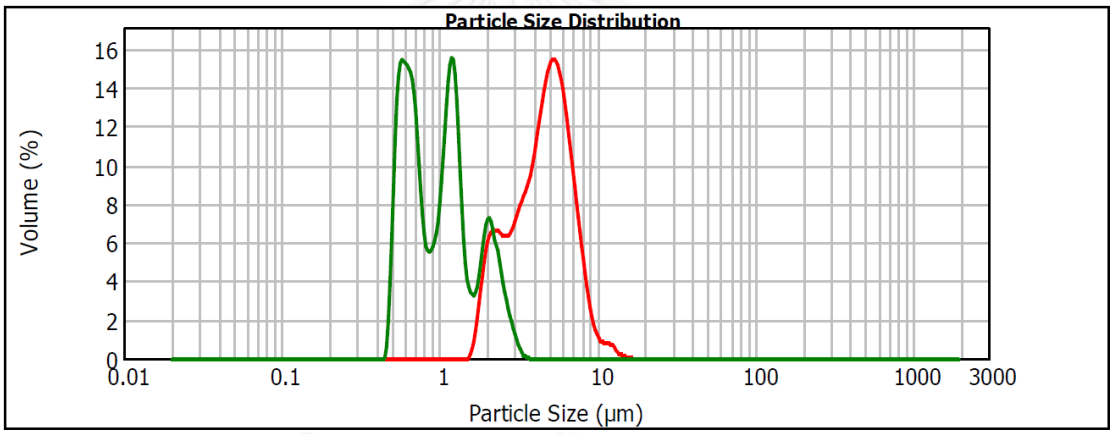

Figure 5: Overlay of the size distribution profiles of vesicles prepared from 20% w/w of Tween® 80 with 5% v/v of ethanol before ( ) and after ( ) extrusion through 2 µm pore size membrane

1.3 Caffeine elastic liposomes

Two formulations of blank elastic liposomes with highest elasticity were chosen to prepare caffeine entrapped elastic liposomes, one for each type of surfactant. They were S7.5_5 and T20_5.

1.3.1 Saturation solubility of caffeine at 4° C

The saturation solubility of caffeine in ethanol solutions used in the preparation of blank liposomes was determined. Figure 6 shows the solubility profiles of caffeine in various solvents at 4 °C. Upon refrigeration, the solubility of caffeine initially decreased and became stable after 3 days. The caffeine solubility in each solvent was expressed as the average of the concentrations on those consecutive days where no statistically significant difference in caffeine concentration was detected. The results are shown in Table 7. The solubility values of caffeine in 5, 15, 20 and 25% v/v of ethanol at 4° C were 10.75±0.43, 11.84±1.26, 11.03±0.86, and 12.09±1.44 mg/ml, respectively. Caffeine solution at 80%, 90% and 100% of its saturation solubility in each ethanol concentration were prepared and kept in refrigerator at 4° C to select the optimum concentration to be used. Caffeine crystals were detected in all solutions at 90 and 100% saturation within 2 weeks. None was detected at 80% caffeine saturation for as long as 28 days. Thus, caffeine solution at 80% saturation was selected for further study. In 5% v/v ethanol, caffeine concentration at 80% saturation was 8.6 mg/ml. This concentration was used for the aqueous phase to prepare elastic liposomes.

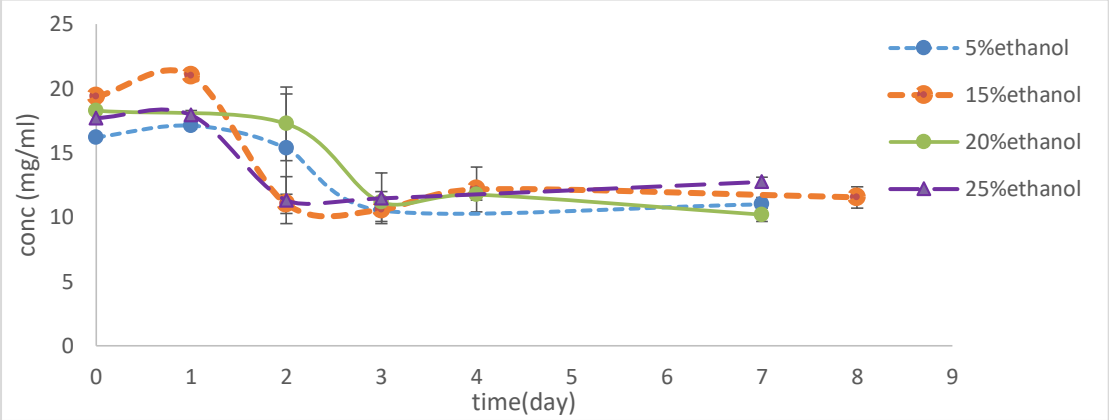

Figure 6: Solubility profiles of caffeine in alcoholic solution at 4 °C

Table 7: Concentrations of caffeine (mg/ml) in various ethanolic solutions at 4 ºC. The data are shown as mean ± SD, n = 3.

Sampling time (day)	Ethanol concentration (% v/v)			
	5	15	20	25
1	17.10±0.28	21.0±0.32	-	17.92±0.37
2	15.35±4.22	11.02±0.74	17.26±2.87	11.31±1.82
3	10.51±0.14	10.56±0.90	11.16±0.9	11.45±1.96
4	- *	12.15±1.73 *	11.74±0.40 *	- *
7	10.99±0.52	-	10.19±0.52	12.73±0.37
8	-	11.53±0.83	-	-
Solubility	10.75±0.43	11.84±1.26	11.03±0.86	12.09±1.44

* The concentrations used in calculation of caffeine solubility

1.4 Characteristics of caffeine elastic liposomes

Caffeine-entrapped elastic liposomes were prepared from compositions described in Section 1.3 using Lipex ™ extruder. The yield of S7.5_5 and T20_5 were 73.78±2.78 and 100±2.44%, respectively. Both formulations were further characterized in terms of physical appearance, morphology, size and size distribution, elasticity, entrapment efficiency, and physical stability.

1.4.1 Physical appearance

Both formulations were homogeneous and turbid and had the similar physical appearance as their corresponding blank liposomes. The formulation with Span® 80 was more viscous than the formulation with Tween® 80.

1.4.2 Morphology under optical and polarized-light microscopes

The complete formation of liposomes was confirmed under optical and polarized-light microscopes. Morphology of caffeine-entrapped elastic liposomes was similar to that of the corresponding blank liposomes.

1.4.3 Size and size distribution

The vesicle sizes of caffeine-entrapped S7.5_5 and T20_5 elastic liposomes were 5.87±0.45 and 5.36±0.77 µm, respectively. The size distribution profiles of the 2 formulations were similar to their corresponding blank liposomes. The vesicle sizes of both caffeine-entrapped elastic liposomes were similar to those of the blank elastic liposomes as shown in Table 9. No significant difference in vesicle size between caffeine-entrapped elastic liposomes containing Span® 80 and those containing Tween® 80 was detected (p-value = 0.29). Figure 7 displays size distribution profiles of the two preparations of caffeine-entrapped elastic liposomes.

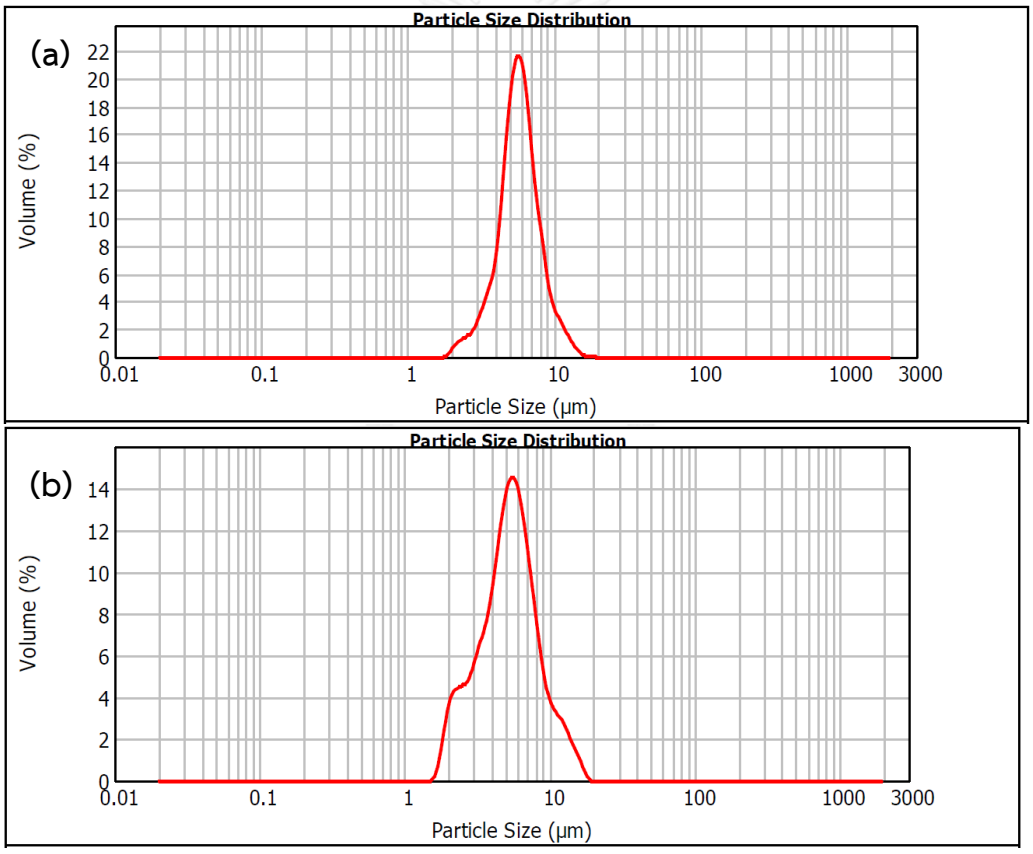

Figure 7: (a) Size distribution profile of the caffeine-entrapped vesicles prepared from 7.5% w/w of Span® 80 with 5% v/v of ethanol

(b) Size distribution profile of the caffeine-entrapped vesicles prepared from 20% w/w of Tween® 80 with 5% v/v of ethanol

1.4.4 Vesicle elasticity

The elasticity of caffeine S7.5_5 and T20_5 elastic liposomes were 53.46±8.63% and 11.93±0.46% respectively. The recovery of phospholipid contents are shown in Table 8. The elasticity was decreased by approximately 20-30% caffeine-entrapped elastic liposomes when compared with those of blank liposomes (Table 9). This finding was similar to the results obtained in a previous study where caffeine was entrapped in elastic liposomes composed of 23% w/w of sodium cholate and 30% v/v of ethanol (Ascenso et al., 2015). The reason for these observations was not known. Caffeine is a water-soluble compound and should be protonated at the pH of the aqueous phase used in this study. Thus, minimum interaction with the bilayer was expected.

Table 8: Phospholipid contents before and after extrusion through 2.0 μm membrane filters in the determination of elasticity of caffeine-entrapped elastic liposomes. The data are shown as mean ± SD, n = 6

Formulation code	Phospholipid contents (mg/ml)		PC recovery (%)
	before extrusion*	after extrusion	
S7.5_5	39.00±1.47	36.55±0.93	93.72
T20_5	45.71±1.11	46.06±1.01	100.77

* Liposome preparations were prepared using Lipex^{TM} extruder

Table 9: Comparison of physical properties of blank and caffeine-entrapped elastic liposomes composed of Span® 80 and Tween® 80. The data are shown as mean ± SD.

Physical property	S7.5_5			T20_5		
	Blank (n=3)	Caffeine-entrapped (n=4)	p-value	Blank (n=3)	Caffeine-entrapped (n=4)	p-value
Size (μm)	5.84±0.01	5.87±0.45	0.91	4.79±0.03	5.36±0.77	0.26
% Elasticity	79.86±0.12	53.46±8.63	0.004	15.74±0.38	11.93±0.46	0

1.4.5 Caffeine entrapment and loading efficiencies

In this study, the entrapment and loading efficiencies were calculated by comparing caffeine in the vesicles with total caffeine and with total lipid. The data were expressed as percentage of entrapment and percentage of loading. In this study, there was statistical difference between Tween® 80-containing and Span® 80-containing elastic liposomes in percentage of entrapment. The caffeine entrapment efficiencies of the S7.5_5 formulation and the T20_5 formulation were 35.05±2.60 and 41.31±3.32%, respectively. On the other hand, these was no statistical difference in percentage of loading. The caffeine loading efficiencies were 7.10±0.65 and 6.11±0.61% for S7.5_5 and T20_5, respectively. Caffeine is hydrophilic. In general, entrapment of hydrophilic molecules increases as the vesicle size increases (Schieren et al., 1978; Szoka and Papahadjopoulos, 1978; Thompson, Couchoud, and Singh, 2009). However, the entrapment efficiencies of these caffeine-entrapped elastic liposomes were much different despite of their similar sizes. The high entrapment seen with T20_5 could come from the higher yield in liposome production (100±2.44%) compared with that of S7.5_5 (73.78±2.78%). The physical properties of caffeine-entrapped elastic liposomes are summarized in Table 10.

Table 10: Physical properties of caffeine-entrapped elastic liposomes composed of Span® 80 and Tween® 80. The data are shown as mean ± SD, n = 4.

Physical property	Caffeine elastic liposomal formulation		p-value
	S7.5_5	T20_5	
Size (μm)	5.87±0.45	5.36±0.77	0.290
% Elasticity	53.46±8.63	11.93±0.46	0.002
% Entrapment	35.05±2.60	41.31±3.32	0.025
% Loading	7.10±0.65	6.11±0.61	0.068

1.4.6 Stability of caffeine elastic liposomes

The stability of caffeine vesicular dispersions was monitored for aggregation, size and size distribution, changes in color, presence of drug crystals, changes in entrapment efficiency and elasticity for 7 days at 4 °C. Samples were taken on days 1, 3, and 7. The results are shown in Table 11.

By visual inspection, no observable changes were detected over the period of 7 days. The changes in color and physical appearance were not evident. Aggregation and drug crystals were not observed under a microscope. Despite the statistical differences, changes in vesicle size were practically negligible. Size of liposomes composed of Span® 80 slightly (less than 5%) increased, while the size of liposomes composed of Tween® 80 decreased (10-15%) with time.

A 14% reduction in entrapment efficiency was seen with the preparation containing Span® 80. The entrapment efficiency was practically unchanged for the preparation containing Tween® 80. A 13.5% and 5.5% reduction in loading efficiency was seen with preparations containing Span® 80 and Tween® 80 upon the 7-day storage. At day 7, elasticity of the preparation containing Span® 80 was reduced by approximately 20%. In conclusion, at day 3, no crucial changes in entrapment efficiency

or elasticity were observed though some statistically significant changes could be detected. Thus, these preparations were used for permeation study within 2 days after preparation.

Table 11: Stability of caffeine-entrapped elastic liposomes composed of Span® 80 and Tween® 80. The data are shown as mean ± SD, n = 3.

Formulation code	Physical parameter	Day1	Day3	Day7
S7.5_5	Size (μm)	6.10±0.07	6.36±0.05 *	6.40±0.02 *
		(0.88±0.09)	(1.01 ± 0.01)	(1.00 ± 0.00)
	% Elasticity	49.22±1.97	51.17±0.94	39.14±1.68 *
	% Entrapment	36.33±0.53	35.44±0.34	31.51±1.18 *
	% Loading	7.41±0.02	7.51±0.15	6.40±0.28 *
T20_5	Size (μm)	5.74±0.04	5.11±0.04 *	4.95±0.04 *
		(1.24±0.05)	(1.08 ± 0.01)	(0.93 ± 0.01)
	% Elasticity	12.13±0.30	14.77±0.57 *	13.20±1.47
	% Entrapment	42.96±0.63	41.16±0.70 *	42.94±1.18
	% Loading	6.41±0.03	6.48±0.18	6.06±0.08 *

* $p < 0.05$

2. Permeation studies

The skin delivery of caffeine from the two elastic liposome formulations were carried out with Franz diffusion cells under the non-occlusive condition. Newborn pig skin was used as the model membrane. Elastic liposomes are known to be most efficient under the non-occlusive condition because they penetrate into the intact skin by osmotic gradients and hydration force (Cevc and Blume, 1992). The aqueous solution of caffeine in 5% v/v of ethanol was used as a reference formulation in this study. All formulations contained caffeine at 80% of its saturation solubility at 4 °C

The permeation profiles of both liposome formulations and the reference formulation are shown in Figure 8. The caffeine permeation profile, however, seemed to display two phases of caffeine permeation. The latter slower phase might be resulted because the preparation in the donor started to dry. The formulation drying out was seen with all formulations studied since the experiment was set under the non-occlusive condition.

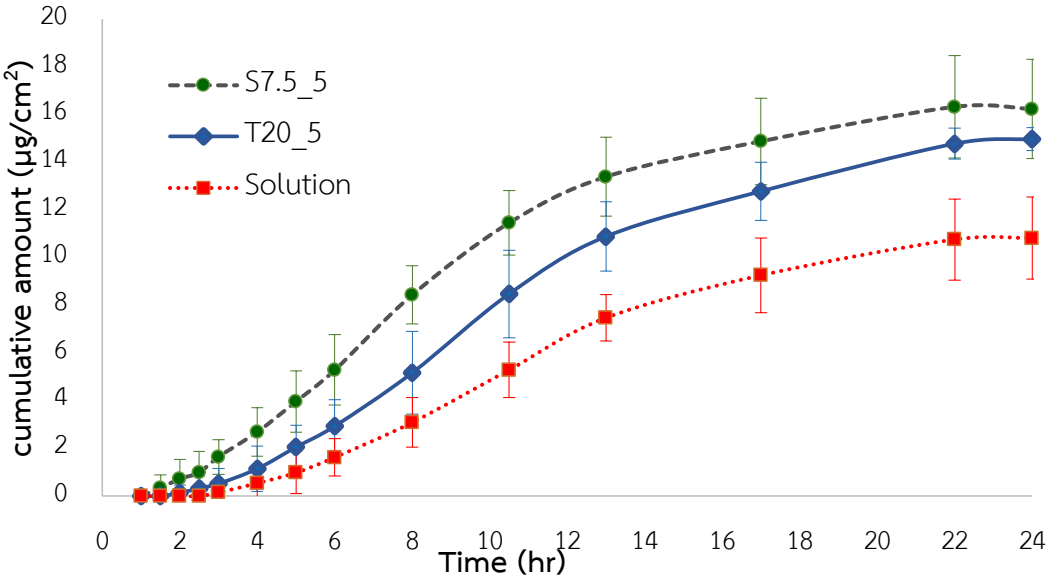

Figure 8: Permeation profiles of caffeine delivery from caffeine-entrapped elastic liposomes and aqueous solution containing 5% v/v of ethanol (the reference formulation). Data are shown as mean ± SD, n=6.

The lag time, steady state flux (J_{ss}), permeation coefficient (P_s), caffeine amount in the skin (Q_s) and cumulative amount of caffeine in the receptor medium at 24 hours (Q_{24}) were calculated. In addition, relative flux (RF), enhancement factor (EF), enhancement factor of the formulation based on Q_s (EF of Q_s) and enhancement factor of the formulation based on Q_{24} (EF of Q_{24}) were calculated by comparing with the reference formulation and were used to compare between Span® 80-containing and Tween® 80-containing elastic liposomes. These permeation parameters are shown in Table 12.

Table 12: Permeation parameters of caffeine from different caffeine formulations (mean ± SD, n = 6)

Permeation parameter	S7.5_5	T20_5	Solution	p-value
Steady state flux (J_{ss})[#]	1.32±0.21	1.10±0.15	0.86±0.10	0.001 *
Permeability coefficient (P_s)[##] x10^4	5.16±1.1	4.37±1.07	3.07±0.51	0.005 *
Q_s (%)	0.32±0.10	0.18±.10	0.39±0.15	0.027 *
Q_{24} (%)	1.30±0.17	1.21±0.04	0.83±0.13	0.000 * *
Total caffeine delivered at 24 h (Q_s+Q_{24}) (%)	1.62±0.27	1.39±0.13	1.22±0.17	0.011 *
Lag time (hr)	1.79±0.94	3.06±0.85	4.06±1.31	0.007 *
EF	1.68±0.36	1.43±0.35	1	0.237
EF of Q_s	0.82±0.26	0.47±0.25	1	0.038 *
EF of Q_{24}	1.56±0.20	1.45±0.05	1	0.206
Relative flux	1.53±0.24	1.27±0.18	1	0.057
Analytical recovery (%)	92.42±6.42	94.15±5.36	95.07±5.36	-

[#] μg/cm^2hr, [##] cm/hr, * $p < 0.05$,

These was no statistically significant difference in lag time, J_{ss} and P_s between Tween® 80-containing elastic liposomes and the hydro-alcoholic solution. The difference was seen only with Q_{24}. In contrast, Span® 80-containing elastic liposomes gave higher J_{ss}, P_s, and Q_{24} with lower lag time than the reference formulation. However, RF, EF, and EF of Q_{24} were not significantly different between the two elastic liposome formulations. This result indicated that Span® 80-containing elastic liposomes was better than the hydro-alcoholic solution to improve the permeation of caffeine through the skin. The permeation enhancement could be attributed to the vesicular structure and the elasticity of the Span® 80-containing elastic liposomes. As previously reported, liposome vesicular structure was important for skin delivery of various drugs (El Maghraby et al., 2000b; Ita et al., 2007; Mishra et al., 2007; Jain et al., 2008; Badran et al., 2012; Ascenso et al., 2015; Abd et al., 2016). Moreover, Span® 80 can also act as a penetration enhancer (Williams and Barry, 2004). In addition, the lowest lag time seen with elastic liposomes composed of Span® 80 agreed well with results from previous studies (Mishra et al., 2007; Jain et al., 2008). In the previous studies, vesicles with higher elasticity also resulted in lower lag time for hydrophilic drugs. On the other hand, Q_{24} was the only one permeation parameter of Tween® 80-containing elastic liposomes that showed better permeation than solution because of their vesicular structure and penetration enhancing effect of the surfactant.

From previous studies, the elastic liposomes with higher elasticity usually have higher potential to enhance skin permeation (Mishra et al., 2007; Garg et al., 2008; Paolino et al., 2012; Song et al., 2012). However, despite the much higher elasticity of the Span® 80-containing elastic liposomes (53.46% versus 11.93%), no significant difference in caffeine delivery (in terms of J_{ss}, P_s, and total caffeine delivered) was seen between the two elastic liposome formulations. The entrapment of Tween® 80-containing elastic liposomes was higher than that of Span® 80-containing elastic liposomes. The difference in entrapment could be attributed to difference in lipid

contents of the two preparations. The total lipid content of the Tween® 80-containing elastic liposomes was higher than that of the Span® 80-containing elastic liposomes (57.14 mg/ml versus 42.16 mg/ml). The total lipid contents, which were back-calculated from PC contents, of the two formulation were different because of the loss during the preparation process. The difference in the caffeine entrapment could offset the difference in elasticity of the two elastic liposome formulations. Consequently, no significant difference in caffeine delivery could be detected, despite the much higher elasticity of the preparation containing Span® 80. Thus, in this present study, the effect of HLB on skin delivery of caffeine could not be demonstrated.

Tween® 80-containing elastic liposomes gave lower Q_s than the reference solution. The reason behind this finding was unknown. The EF of Q_s of the Tween® 80-containing elastic liposomes was also lower than that of Span® 80-containing elastic liposomes. These results indicated the retaining property of Span® 80-containing elastic liposomes in the skin. The HLB values of Span® 80 and Tween® 80 are 4.3 and 15, respectively. Thus, the more hydrophobic Span® 80-containing elastic liposomes were better retained in the skin when compared with the Tween® 80-containing elastic liposomes.

However, another factor that could affect skin delivery of caffeine was ethanol. Ethanol has permeation enhancing property that can increase transdermal permeability of various drugs (Narasimha Murthy and Shivakumar, 2010). In this study, though all formulations including the reference solution were prepared using 5% v/v of ethanol, ethanol contents in Span® 80 and Tween® 80-containing elastic liposomal formulation could be less than expected due to the loss during the production process. This could explain partly why the more pronounced difference between formulations was not detected.

CHAPTER V
CONCLUSIONS

In this study, elastic liposomes were developed as a delivery system for caffeine to treat cellulite. The elastic liposomes consisted of phosphatidylcholine as the structural lipid, Span® 80 or Tween® 80 as an edge activator and a low content of ethanol in the hydrating medium. The concurrent use of surfactant and ethanol was aimed to substitute high concentrations of the individual penetration enhancers in caffeine skin delivery. The differences in HLB of surfactant and concentration of surfactant and ethanol were investigated on the formation and physical properties of the resultant liposomes.

Three concentrations (0.1, 0.15 and 0.2 molar ratio) of each edge activator were used with 4 different concentrations of ethanol in water (5-25% v/v) as the hydrating solution. For blank liposomes, a total of 13 out of 24 formulations resulted in completely formed liposomes. At ethanol concentrations lower than 20% v/v, liposomes could formed at all surfactant concentrations. At the high ethanol concentration, the complete vesicle formation was not seen possibly due to high solubility of the structural lipid in ethanol. The size of blank liposomes depended on the composition. However, though vesicle sizes of these liposomes were statistically different, the difference seen was not pronounced enough to be practically meaningful. Elasticity of the resultant liposomes varied with the type and concentration of the edge activator. Ethanol seemed to decrease elasticity in these elastic liposomes. In fact, liposome preparations containing Tween® 80 lost all their elasticity when ethanol contents in the formulation were higher than 5% v/v. Overall results indicated that optimum concentrations of both the edge activator and ethanol were needed to produce high elasticity of blank liposomes.

The formulations with highest elasticity for each type of surfactant were chosen to prepare caffeine-entrapped elastic liposomes. Caffeine solutions at 80% of its saturation solubility was incorporated into each preparation in order to establish equal thermodynamic activities among the systems studied. Caffeine entrapment was higher in preparation containing Tween® 80 due to the higher liposome yield from the preparation process. Caffeine incorporation did not seem to affect general physical properties of liposomes such as size and size distribution and morphology when compared to the corresponding blank liposomes. However, caffeine imposed approximately 30% changes in elasticity to the liposomes. Elasticity of both Span® 80- and Tween® 80-containing liposomes decreased in the presence of caffeine. The underlying mechanism behind these findings was not known. Nevertheless, elasticity was still higher in the Span® 80-containing liposomes. The Tween® 80-containing preparation also showed higher caffeine entrapment efficiency than the Span® 80-containing preparation. On the other hand, the vesicle sizes were comparable between liposomes containing Span® 80 and those containing Tween® 80.

When the two caffeine-entrapped elastic liposome preparations were evaluated for their ability to deliver caffeine into and through the skin, both preparations significantly increased amount of caffeine delivered through the newborn pig skin. Only Span® 80–containing preparation significantly increased flux of caffeine when compared to the reference hydro-alcoholic solution. When comparing between the preparations with different edge activators, Span® 80–containing preparation could deposit more caffeine into the skin. Fluxes and the cumulative amounts of caffeine delivered through the skin were not different between the 2 preparation, but with a much shorter lag time for the Span® 80–containing preparation. Tween® 80-containing preparation had higher caffeine entrapment. It was possible that the high entrapment could counteract the effect of vesicle elasticity. Thus, the difference in caffeine delivery was not detected between the two preparations.

The overall results of this present study showed that it was possible to formulate a hydrophilic drug such as caffeine into elastic liposomes containing both surfactant as edge activator and ethanol. The bilayer composition and the aqueous medium played an important role on physicochemical properties of the resultant elastic liposomes. Elastic liposomes with concurrent use of surfactant and ethanol could enhance caffeine permeation into and across the model skin. This information should be used for further development of caffeine elastic liposomes. However, further studies are still required. Optimization of the preparation could be done with various other factors including total lipid contents. In order to better modify the preparation, the underlying mechanisms of caffeine permeation enhancement by elastic liposomes should be further studied. Stability improvement as well as practical large-scale preparation methods should also be further focused on. In addition, irritation potential of caffeine elastic liposomes should also be assessed.

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APPENDICES

APPENDIX A

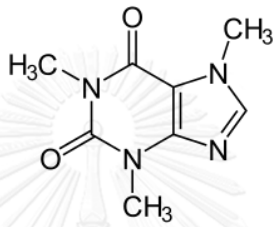
Molecular Structure and Physical Properties of Caffeine

(Leo et al., 1971; Zubair et al., 1986; Moffat, Osselton, and Widdop, 2012; Sigma-Aldrich, 2014)

1. Molecular structure

1.1 Empirical: $C_8H_{10}N_4O_2$

1.2 Structural:



1.3 Molecular weight: 194.2

2. Physical properties

2.1 Melting point: 235-237.5 ºC

2.2 Log P: -0.091 to -0.07

2.3 **pK_a:** 14.0 (25 ºC), 10.4 (40 ºC)

2.3 Solubility

1 g of anhydrous caffeine dissolves in about 50 ml water, 6 ml water at 80 ºC, 75 ml alcohol, about 25 ml alcohol at 60 ºC, about 6 ml chloroform.

2.4 Ultraviolet spectrum

The UV spectrum of caffeine in methanol and ethanol exhibited a λ_{max} at 270 and 273 nm respectively.

2.5 Stability

Caffeine is stable compound at room temperature. It is recommended that it should be kept in well closed containers protected from light.

APPENDIX B

Molecular Structure and Physical Properties of Some Selected Materials

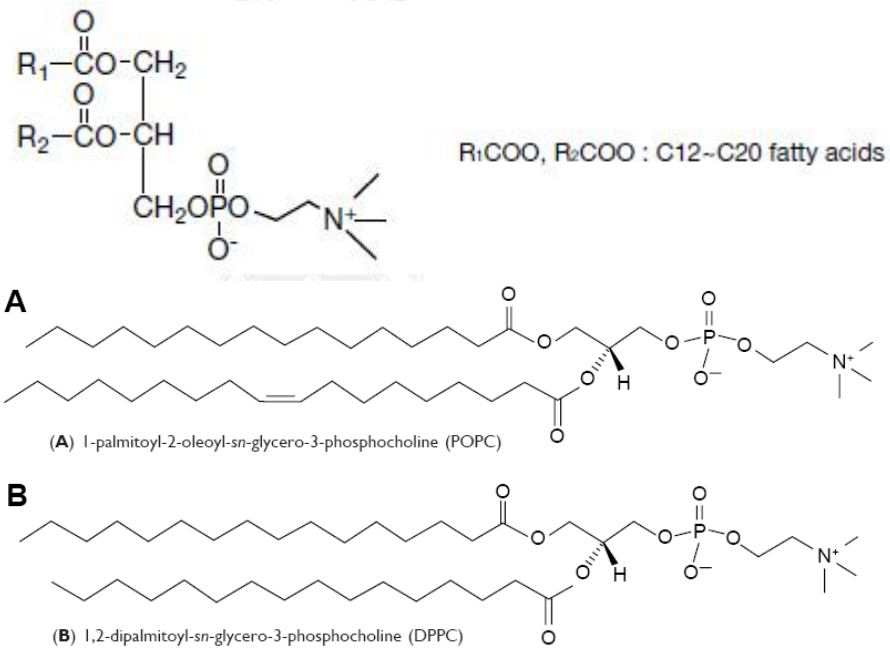
(New, 1990; Rowe, Sheskey, and Quinn, 2009)

1. Phosphatidylcholine

Synonym: 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine

Empirical: -

Structural:



(Modified from Santhosh et al. (2015))

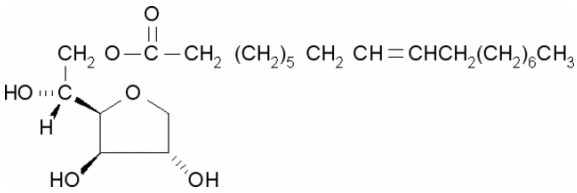
Molecular weight: 760-780

2. Span® 80

Synonym: Sorbitan monooleate

Empirical: $C_{24}H_{44}O_6$

Structural:



(Sigma-Aldrich, 2016)

Molecular weight: 428.6

HLB: 4.3

3. Tween® 80

Synonym: Polysorbate 80

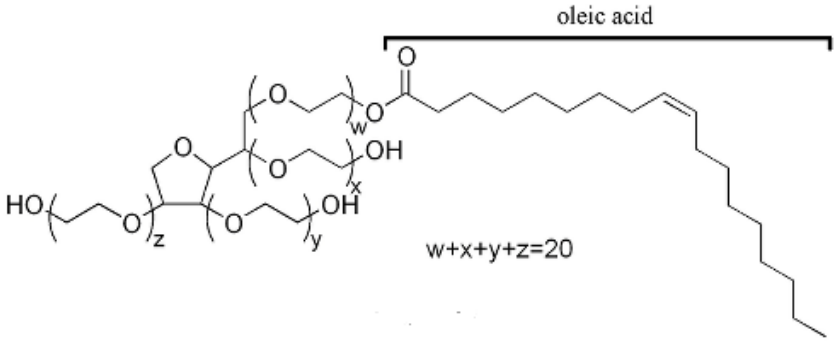
Polyoxyethylene (20) Sorbitan monooleate

Polyethylene glycol sorbitan monooleate

Polyoxyethylenesorbitan monooleate,

Empirical: $C_{64}H_{124}O_{26}$

Structural:



(Johnson, 2013)

Molecular weight: 1310

HLB: 15.0

APPENDIX C

Bartlett assay (New, 1990)

1. Preparation of phosphate standard solution

1.1 Preparation of standard stock solution

Dry the solid anhydrous potassium dihydrogen phosphate at 105 ºC for 4 hours in vacuum oven. Accurately weigh 43.55 mg and transfer to volumetric flask 100 ml. Dissolve and make up to volume with double-distilled water. Mix well and label as stock phosphate solution. The final concentration should be phosphorous 3.2 µmol/ml.

1.2 Preparation of standard solution for calibration curve

1.2.1 Using positive displacement pipette, pipette 200, 300, 400, 500, 600 and 700 µl of stock phosphate solution in 1.1 into each volumetric flask 10 ml, respectively.

1.2.2 Make up to volume with double-distilled water and mix well. The concentrations of working standard solutions are 0.064, 0.096, 0.128, 0.160, 0.192 and 0.224 µmol/ml, respectively.

2. Preparation of sample solution

Dilute the dispersion with double-distilled water to obtain 1 mg/ml of phospholipid.

3. Preparation of reagent

3.1 Preparation of 5 Molar Sulphuric acid reagent

3.1.1 Add 140 ml of concentrated sulphuric acid to 300 ml of distilled water in a beaker in ice bath. Stir carefully.

3.1.2. Transfer to cylinder 500 ml. Make up to volume with distilled water and mix well.

3.2 Preparation of Ammonium molybdate-sulphuric acid solution

3.2.1 Add 5 ml of 5 M sulphuric acid reagent to approximately 50 ml of distilled water.

3.2.2. Add 0.44 g of ammonium molybdate in 3.2.1 and mix well.

3.2.3 Make up to 200 ml with distilled water and mix well.

3.3 Preparation of 1-Amino 2-naphthyl 4-sulphonic acid reagent

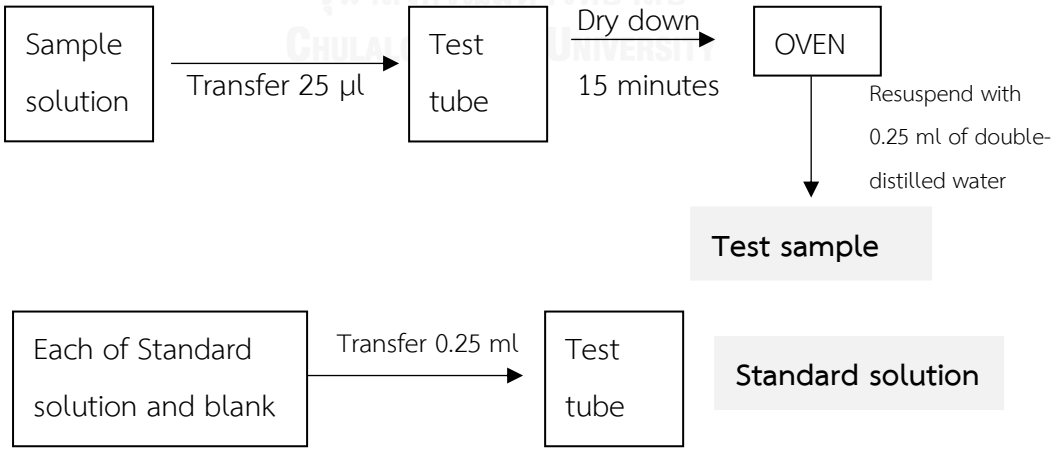
Use Fiske & Subbarrow reducer (Sigma chemical Co.) and freshly prepare on day of use. Weigh out 0.32 g, dissolving in 2 ml of distilled water and mix well.

3.4 Preparation of 10% Hydrogen peroxide

Add 1 ml of 30% hydrogen peroxide to 2 ml of distilled water and mix well. Prepare fresh immediately before use.

4. Assay procedure

Equilibrate oven (about 200° C) 30 minutes before experiment.



Test sample and **Standard solution**

↓ + 5M H_2SO_4 (0.2ml)

↓ Cover and incubate in hot air oven for 1 hour.

↓ Cool down at room temperature

↓ + 10% H_2O_2 solution (50 µl)

↓ Incubate at 180-200° C for 30 minutes (hot air oven)

Clear solution — Repeat addition and heating until solution is clear.

↓ Cool down at room temperature.

↓ + Acid molybdate solution (2.3 ml)

↓ Mix by vortexing.

↓ + Fiske & Subbarrow (0.1 ml)

↓ Mix by vortexing

↓ Cover the tubes and place in boiling water bath for 7 minutes.

↓ Cool down

UV spectrophotometry — Measure absorbance of all tubes against blank at 800 nm.

APPENDIX D

Partial validation of UV Spectroscopy

(USP 35-NF30, 2012a)

1. Partial validation for the quantitative determination of caffeine by UV spectroscopy

This validation of analytical method was the process of demonstrating that established analytical method is suitable for determine caffeine in the pellets and supernatant which separated from liposomes dispersion. In this study, there are 2 systems for analysis of caffeine in water and in solvent. The caffeine in supernatant were determined by dissolving supernatant in water and caffeine in pallet were determined by dissolving pellet in 4:1 of isopropanol:water. These analytical methods quantified the sample at a high concentration. The requirement of performance characteristics which need to meet is specificity, linearity and range, accuracy and precision.

1. Specificity

Other components must not be interfered the absorbance of caffeine under the UV absorption spectrophotometric method used. For the solvent system, spectrum of the maximum content of each composition mixture which equivalent to liposome 2 ml were diluted to working concentration (dilution factor = 1000) and compared with the spectrum of lowest concentration of caffeine. In detail, the spectrum of caffeine in 4:1 of isopropanol: water was compared with PC, Span® 80 and Tween® 80 4:1 of isopropanol:water under UV absorption spectrophotometric method for determining caffeine in pellets and the spectrum of caffeine in water was compared with Span® 80 and Tween® 80 under UV absorption spectrophotometric method for determining caffeine in supernatant.

2. Linearity and range

Five standard solutions of caffeine ranging from 4 to 20 µg/ml in water and 5 to 20 µg/ml in 4:1 of isopropanol:water were prepared and analyzed with each system. Linear regression analysis of the absorbance versus the caffeine concentration was performed. The linearity shown ability of analytical procedure to obtain by UV absorption directly proportion to the caffeine concentration.

3. Accuracy

Spike solutions for each method were prepared. The mixture of vesicular components (in equivalent amounts to those maximum presents in 2 ml vesicular dispersion) spiked with caffeine solutions were triplicate prepared to obtained the caffeine concentration at 6, 12, and 18 µg/ml in 4:1 of isopropanol: water. The mixture of Span® 80 and Tween® 80 spiked with caffeine solutions were triplicate prepared to obtain the caffeine concentration at 6, 12, and 18 µg/ml in water. Each individual sample were analyzed for caffeine by UV spectrophotometry at 273 nm. The blank mixture of vesicular components was also analyzed for caffeine in 4:1 of isopropanol:water. The analytical recovery of each sample was calculated and compared with the amount of caffeine added.

4. Precision

Three replicates of three concentration level of the of caffeine solutions with the mixtures of vesicular components in the same day of each analytical system was evaluated as within run precision and the three replicates of these three-concentration level with the mixtures of vesicular components in the different day of each analytical system was evaluated as between run precision. The mean, standard deviation (SD), and the coefficient of variation (% CV) at each concentration were determined.

2. Results from partial validation for the quantitative determination of caffeine in water by UV Spectrophotometry

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

1. Specificity

The UV absorption spectra in water (Figure D1-D4) indicated that the wavelength 273 nm was the optimal wavelength giving the highest sensitivity without interference of Span® 80 and Tween® 80 which show no absorbance at wavelength 225-400 nm.

2. Linearity and range

The standard curve of caffeine in water was shown in figure D5. The standard curve was found to be linear with coefficient of determination 0.9998. This result indicated that UV spectrophotometric method was acceptable for quantitative analysis of caffeine in the range studied. The equations of standard curve according to Beer's Law were used for calculating the concentration of caffeine.

3. Accuracy

The percentage of analytical recovery of each caffeine concentration in water is shown table D1. All percentage analytical recovery of all drug concentrations in water with a mean and a %CV of percentage analytical recovery indicated high accuracy of this method. Thus, it could be used for analysis of caffeine in all concentration used.

4. Precision

The precision of analysis of caffeine in water by UV spectrophotometric method was determined both within run precision and between run precision as illustrated in Table D2-D3. All percentage coefficient of variation value of within run precision was

low, indicating that of the UV spectrophotometric method used were precise for quantitative analysis of caffeine on the range studied.

In conclusion, the analysis of caffeine in water by UV spectrophotometric method developed in this study showed good specificity, linearity, accuracy and precision. Thus, this method was used for determination of the content of caffeine in water.

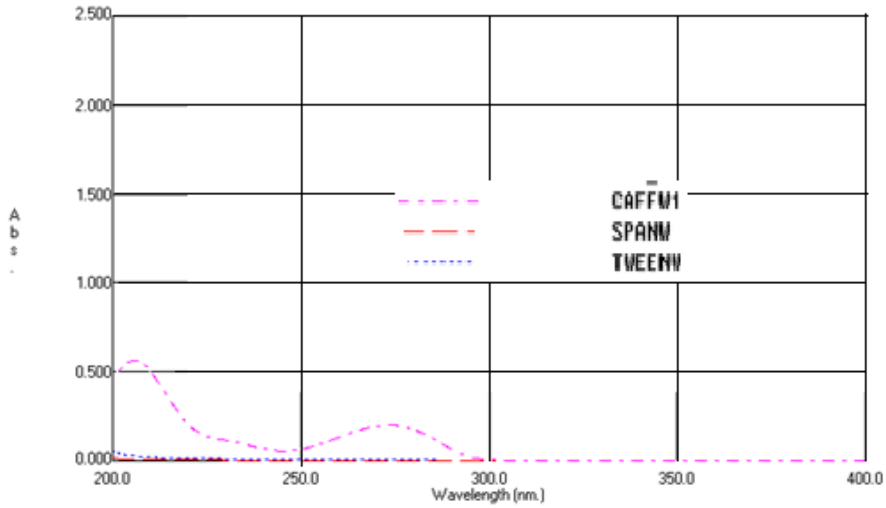


Figure D1: Overlay of spectrum of substances in supernatant of caffeine elastic liposomes (caffeine, Span® 80 and Tween® 80) in water

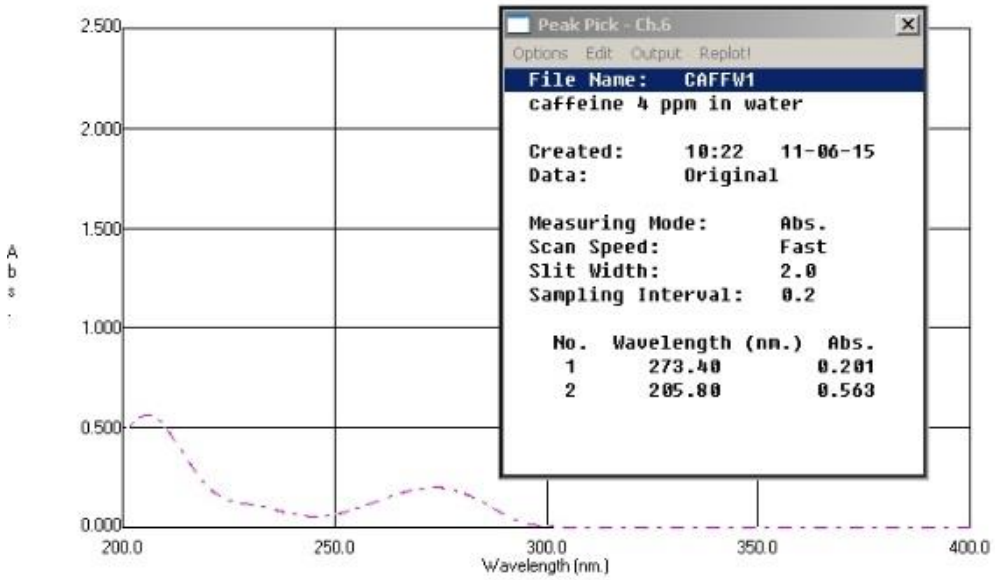


Figure D2: Spectrum of caffeine in water

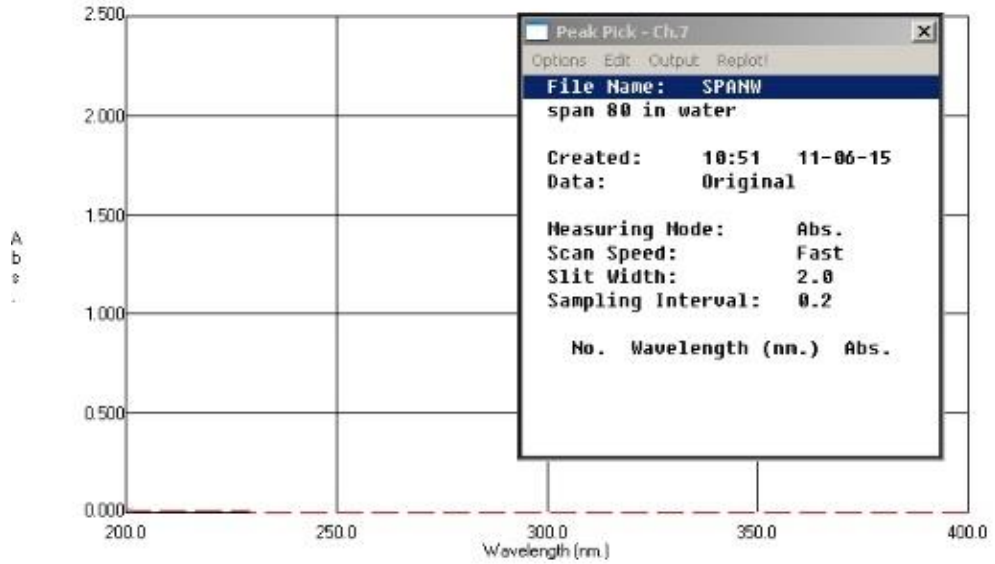


Figure D3: Spectrum of Span® 80 in water

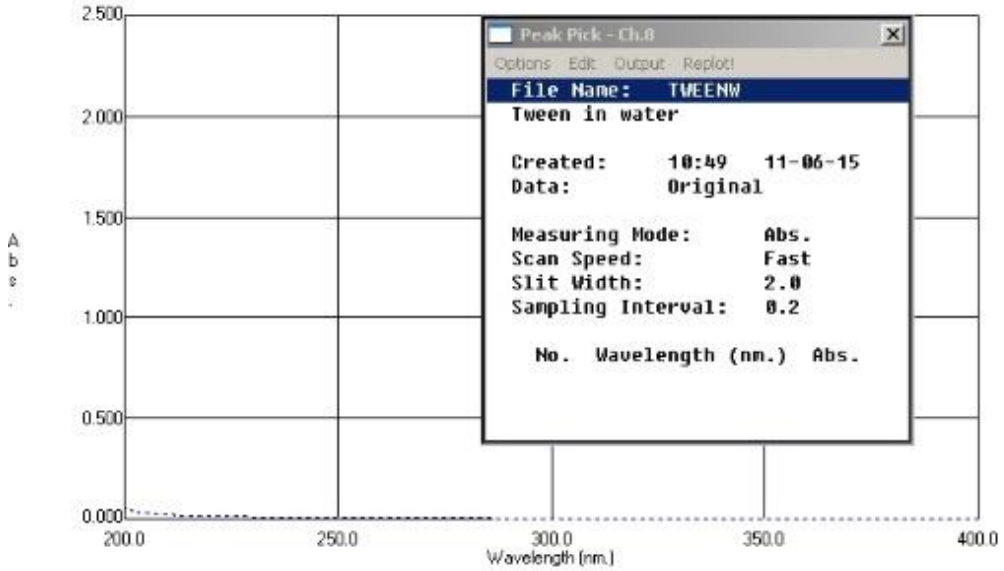


Figure D4: Spectrum of Tween® 80 in water

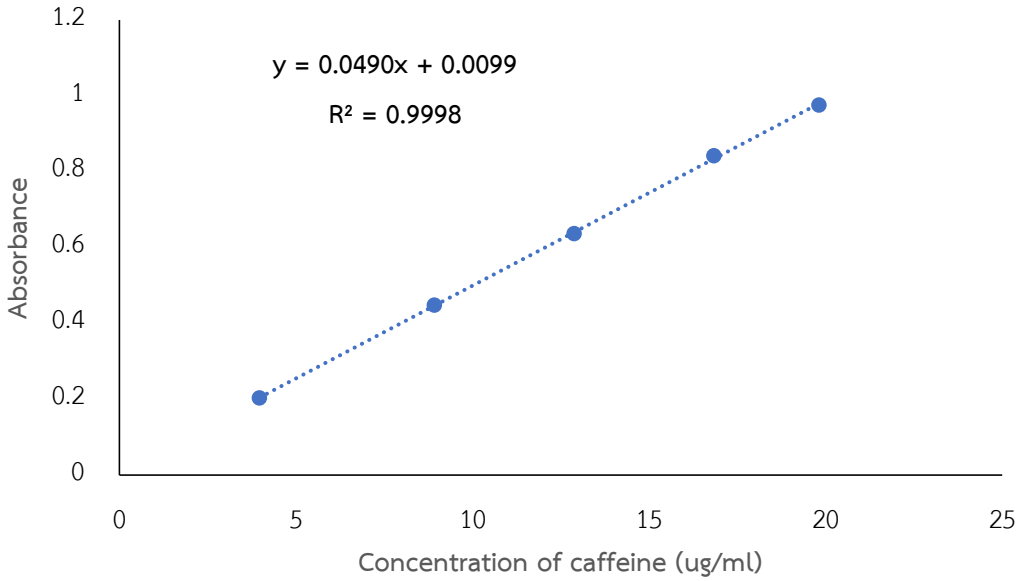


Figure D5: A Standard calibration line of caffeine in water

Table D1: The percentages of analytical recovery of caffeine in water by UV spectrophotometric method

Actual concentration of caffeine (µg/ml)	Calculated concentration of caffeine (µg/ml)	% Analytical recovery
5.8893	5.8116	98.68
5.8893	5.8094	98.64
5.8893	5.8630	99.55
11.7786	11.7281	99.57
11.7786	11.8351	100.48
11.7786	11.6060	98.53
17.6679	18.2698	101.50
17.6679	18.1842	101.02
17.6679	18.2784	101.55

Mean % Analytical recovery = 99.95,

SD = 1.22, % CV = 1.23

Table D2: Data for the within run precision of caffeine in water by UV spectrophotometric method

Conc. (µg/ml)	Calculated Conc. (µg/ml)			Mean	SD	% CV
	1	2	3			
5.8893	5.8116	5.8094	5.8630	5.8280	0.03	0.52
11.7786	11.7281	11.835	11.6060	11.7231	0.11	0.98
17.6679	18.2698	18.1842	18.2784	18.2441	0.05	0.29

Table D3: Data for the between run precision of caffeine in water by UV spectrophotometric method

Conc.	day							
(µg/ml)	1	2	3	4	5	Mean	SD	% CV
	5.8116	6.2102	5.9503	5.9503	6.1123			
5.8893-	5.8094	6.1224	5.8509	5.9781	6.1705			
6.1747	5.8630	6.1837	5.8628	5.9841	6.2037			
Mean	5.8280	6.1721	5.8880	5.9708	6.1622	6.0042	0.1572	2.6177
SD	0.0303	0.0450	0.0543	0.0180	0.0463			
% CV	0.5200	0.7292	0.9218	0.3021	0.7514			
	11.7281	12.1612	11.9344	11.6501	12.3222			
11.7786-	11.8351	12.2122	11.8767	11.7495	12.5301			
12.3494	11.6060	12.3633	12.1471	11.7575	12.4948			
Mean	11.7231	12.2456	11.9861	11.7190	12.4491	12.0246	0.3123	2.5970
SD	0.1146	0.1051	0.1424	0.0598	0.1112			
% CV	0.9779	0.8580	1.1881	0.5104	0.8936			
	18.2698	18.1510	17.8032	17.6859	18.9189			
17.6679-	18.1842	18.3408	17.9483	17.8767	18.4511			
18.5242	18.2784	18.1184	18.0835	17.8946	18.2973			
Mean	18.2441	18.2034	17.9450	17.8191	18.5558	18.1535	0.2863	1.5770
SD	0.0521	0.1201	0.1402	0.1157	0.3238			
% CV	0.2856	0.6599	0.7812	0.6493	1.7448			

3. Results from partial validation for the quantitative determination of caffeine in pellets in 4:1 of isopropanol:water by UV Spectrophotometry

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

1. Specificity

The UV absorption spectra in 4:1 of isopropanol:water (Figure D6-D11) indicated that the wavelength 273 nm was the optimal wavelength giving the highest sensitivity without interference of composition in liposomes which are PC, Span® 80 and Tween®

80. They show no absorbance at wavelength 225-400 nm. In Figure D7-D8 displayed no sample interference because no difference in the spectrum of caffeine and spike solution of caffeine in blank liposome.

2. Linearity and range

The standard curve of caffeine in 4:1 of isopropanol:water was shown in Figure D12. The standard curve was found to be linear with coefficient of determination 0.9999. This result indicated that UV spectrophotometric method was acceptable for quantitative analysis of caffeine in the range studied. The equations of standard curve according to Beer's Law were used for calculating the concentration of caffeine.

3. Accuracy

The percentage of analytical recovery of each caffeine concentration in 4:1 of isopropanol:water is shown Table D4. The small absorbance of blank mixture of liposomes did not effect on the accuracy of this method at the working concentration.

All percentage analytical recovery of all drug concentrations in 4:1 of isopropanol:water with a mean and a %CV of percentage analytical recovery indicated high accuracy of this method. Thus, it could be used for analysis of caffeine in all concentration used.

4. Precision

The precision of analysis of caffeine in 4:1 of isopropanol:water by UV spectrophotometric method was determined both within run precision and between run precision as illustrated in Table D5-D6. All percentage coefficient of variation value of within run precision was low, indicating that of the UV spectrophotometric method used were precise for quantitative analysis of caffeine on the range studied.

In conclusion, the analysis of caffeine in 4:1 of isopropanol:water by UV spectrophotometric method developed in this study showed good specificity, linearity, accuracy and precision. Thus, this method was used for determination of the content of caffeine in the pellet.

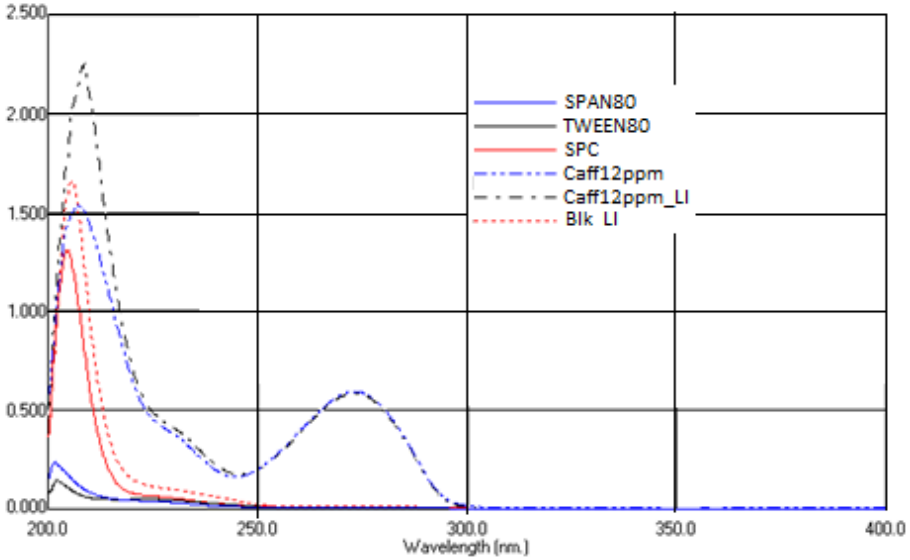


Figure D6: Overlay of spectrum of substances in pellet of caffeine elastic liposomes and caffeine-spiked samples (Span® 80, Tween® 80, SPC, caffeine, caffeine spiked sample and blank liposomes) diluted with 4:1 of isopropanol:water

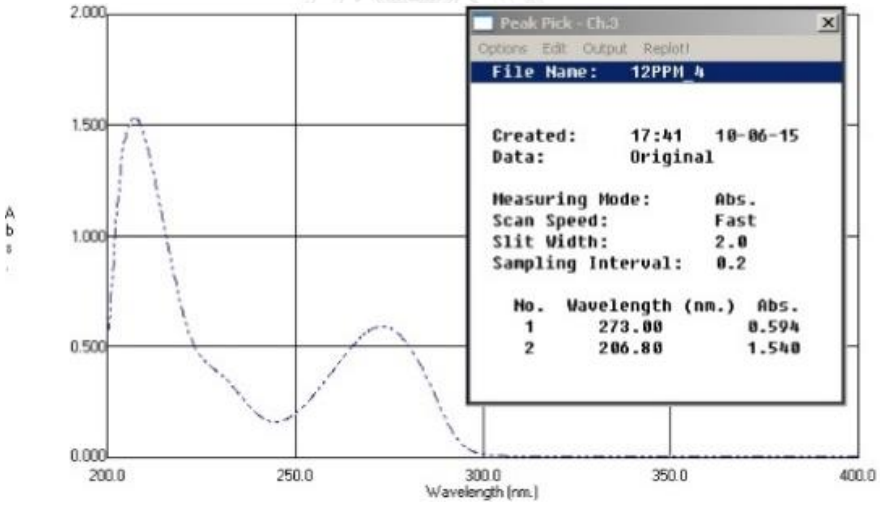


Figure D7: Spectrum of caffeine (12 µg/ml) in 4:1 of isopropanol:water

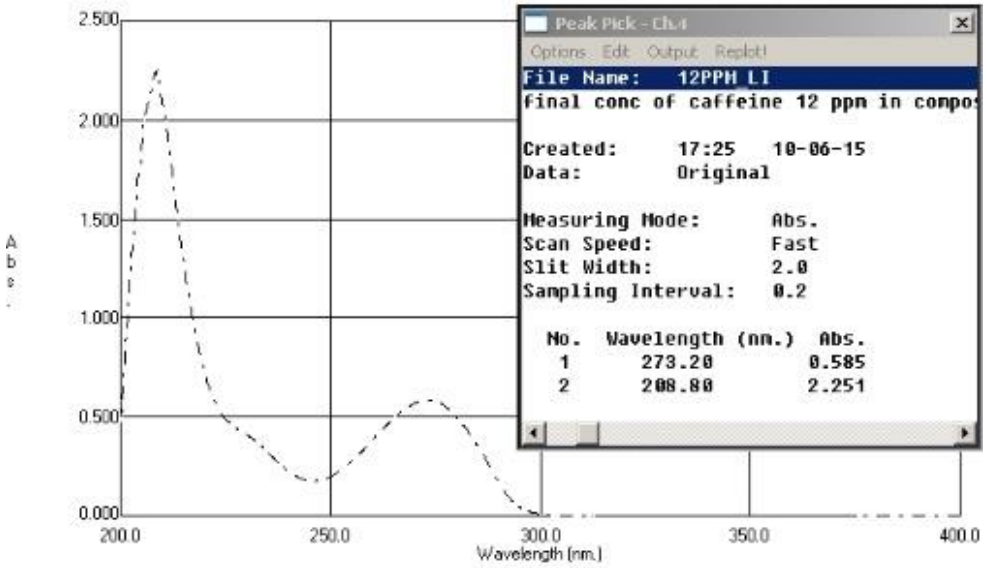


Figure D8: Spectrum of caffeine in elastic liposomes at 12 µg/ml in 4:1 of isopropanol:water

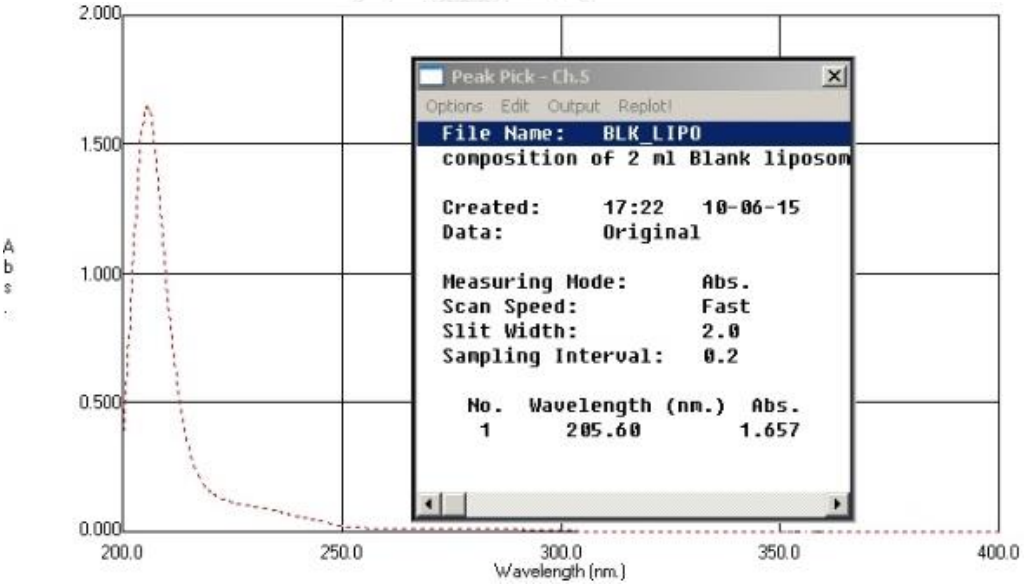


Figure D9: Spectrum of blank liposomes in 4:1 of isopropanol:water

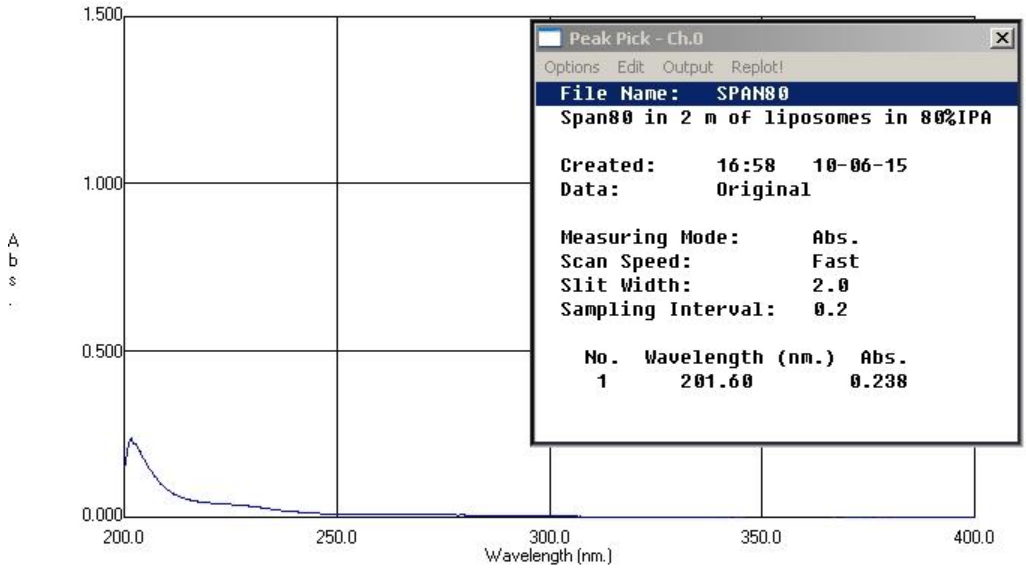


Figure D10: Spectrum of Span® 80 in 4:1 of isopropanol:water

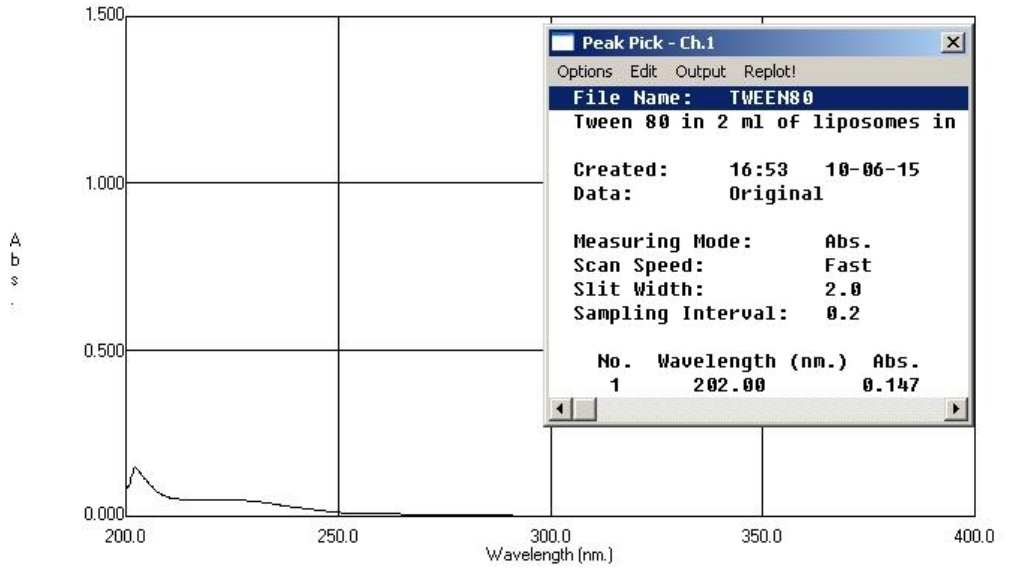


Figure D11: Spectrum of Tween® 80 in 4:1 of isopropanol:water

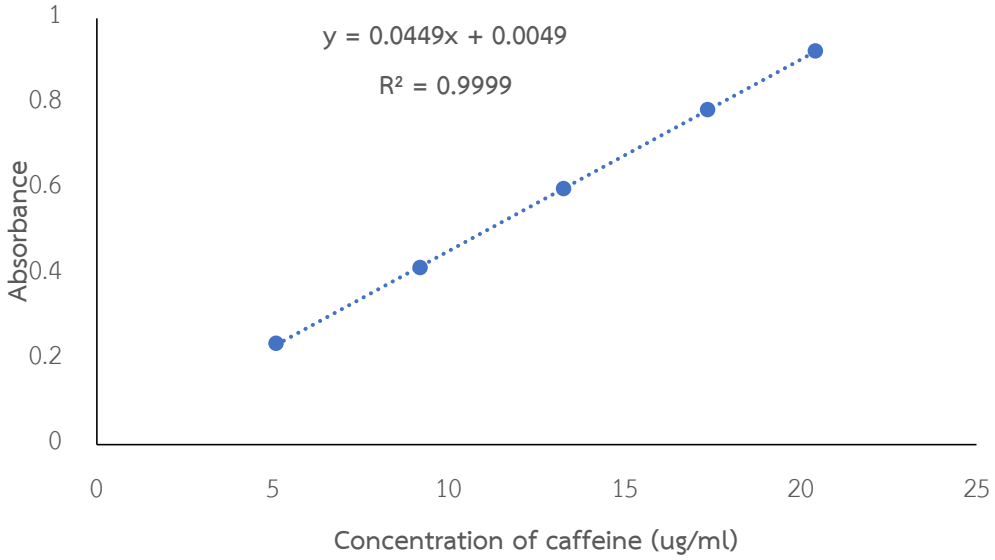


Figure D12: A Standard calibration line of caffeine in the pellet in 4:1 of isopropanol:water

Table D4: The percentages of analytical recovery of caffeine in the pellet in 4:1 of isopropanol:water by UV spectrophotometric method

Actual concentration of caffeine (µg/ml)	Calculated concentration of caffeine (µg/ml)	% Analytical recovery
6.0702	6.1402	101.15
6.0702	6.0833	100.22
6.0702	6.1728	101.69
12.1404	12.1118	99.76
12.1404	11.9553	98.48
12.1404	12.1524	100.10
18.2106	17.9451	99.70
18.2106	17.9492	99.72
18.2106	17.9085	99.49

Mean % Analytical recovery = 100.03

SD = 0.94, % CV = 0.94

Table D5: Data for the within run precision of caffeine in the pellet in 4:1 of isopropanol:water by UV spectrophotometric method

Conc.	Calculated Concentration (µg/ml)					
(µg/ml)	1	2	3	Mean	SD	% CV
0	0.6728	0.7053	0.6992	0.6924	0.02	2.50
5.8893	6.1402	6.0833	6.1728	6.1321	0.05	0.74
11.7786	12.1118	11.9553	12.1524	12.0732	0.10	0.86
17.6679	17.9451	17.9492	17.9085	17.9343	0.02	0.12

Table D6: Data for the between run precision of caffeine in the pellet in 4:1 of isopropanol:water UV spectrophotometric method

Conc.	day							
(µg/ml)	1	2	3	4	5	Mean	SD	% CV
	6.1402	6.2211	6.3808	6.1538	6.3683			
6.0702-	6.0833	6.4263	6.5033	6.2727	6.5610			
6.4119	6.1728	6.2032	6.4321	6.2940	6.5503			
Mean	6.1321	6.2835	6.4388	6.2398	6.4932	6.3175	0.1476	2.3362
SD	0.0453	0.124	0.0615	0.0761	0.1083			
% CV	0.7382	1.9728	0.9555	1.2200	1.6680			
	12.1118	12.4781	12.7840	12.4429	12.9936			
12.1404-	11.9553	12.4522	12.9488	12.5377	12.8501			
12.8238	12.1524	12.5199	13.0223	12.4217	12.9143			
Mean	12.0732	12.4834	12.9183	12.4674	12.9193	12.5723	0.3377	2.6859
SD	0.1041	0.0342	0.1220	0.0618	0.0719			
% CV	0.8622	0.2738	0.9447	0.4956	0.5563			
	17.9451	19.1866	18.1002	18.4292	18.0600			
18.2106-	17.9492	18.2327	17.9243	17.9399	17.6767			
20.1884	17.9085	18.2369	17.7327	18.1524	18.3019			
Mean	17.9343	18.5521	17.9191	18.1738	18.0128	18.1184	0.2626	1.4495
SD	0.0224	0.5495	0.1838	0.2453	0.3153			
% CV	0.1248	2.9620	1.0257	1.3500	1.7503			

In conclusion, the analysis of caffeine in water and 4:1 of isopropanol:water by UV spectrophotometric method verified in this study showed good specificity, linearity, accuracy, and precision. Thus, these methods could use for quantify caffeine content in supernatant and pellets in the entrapment efficiency determination.

APPENDIX E

Partial validation of HPLC method for permeation study

(USP 35-NF30, 2012a)

1. Partial validation for the quantitative determination of caffeine by HPLC method

This validation of analytical method was the process of demonstrating that established analytical method is suitable for determine caffeine in 2 matrixes e.g. donor fluid from the blank liposomes and receptor fluid from the control vehicle in the skin permeation experiment. The requirement of performance characteristics which need to meet is specificity, linearity and range, accuracy and precision.

1. Specificity

The other components must not be interfered the peak of caffeine and internal standard under the HPLC used. The chromatogram of donor fluid, receptor fluid from the control vehicle and PBS, pH7.4, were compared with the chromatogram of the caffeine solution and internal standard, methylchloroisothiazolinone (CMIT).

2. Detection limit

Limit of detection (LOD) is the lowest concentration of caffeine in sample that can be detected. The comparison of the signal-to-noise ratio from blank liposomes with known caffeine concentration solution was done. The minimum concentration which had signal-to-noise ratio between 3 or 2:1 was estimated as LOD.

3. Quantitation limit

Limit of quantitation (LOQ) is the lowest concentration of caffeine in sample that can be quantified with acceptable precision and accuracy. The comparison of the signal-to-noise ratio from blank liposomes with known caffeine concentration solution

was done. The minimum concentration which had signal-to-noise ratio between 10:1 was estimated as LOQ.

4. Linearity and range

Eight standard solutions of caffeine (ranging from 0.15 to 6 µg/ml) with CMIT 5 µg/ml were prepared and analyzed. Linear regression analysis of the peak area ratio between caffeine and CMIT versus the caffeine concentration was performed.

5. Accuracy

Three sets of caffeine solutions at 0.35, 2.5, and 5.5 µg/ml in donor and receptor fluid from the control vehicle were prepared. Each individual sample was analyzed by the HPLC method. The analytical recovery of caffeine in each sample was calculated and compared with the amount of caffeine added.

6. Precision

Three replicates of three concentration level of the of caffeine solutions at 0.35, 2.5, and 5.5 µg/ml in donor and receptor fluid from the control vehicle were evaluated as within run precision and three replicates of these three-concentration level of the of caffeine solutions in blank liposomes and receptor fluid from the control vehicle in the different run was evaluated as between run precision. The mean, standard deviation (SD), and the coefficient of variation (% CV) at each concentration were determined.

2. Results from partial validation for the quantitative determination of caffeine by HPLC

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

1. Specificity

The chromatograms (Figure E1-E6) indicated that the conditions used was the optimal condition giving the highest sensitivity without interference of Span® 80, Tween® 80, SPC and other composition in PBS and receptor fluid from the control

vehicle. They show no peak chromatograms at the peak of internal standard and caffeine. The retention time of caffeine and CMIT were 5.5 and 6.3 minutes, respectively. Thus, these two peaks were completely separated from each other.

2. Detection limit

The HPLC chromatogram in Figure E7 show the peak area and peak height of caffeine 0.05 µg/ml was about 3 times of noise signal which display matched UV absorbance spectrum with caffeine. This indicated that LOD of this HPLC method was 0.05 µg/ml.

3. Quantitation limit

The chromatogram in Figure E8 show the peak area of caffeine 0.15 µg/ml was about 10 times of noise signal which display matched UV absorbance spectrum with caffeine. This indicated that LOQ of this HPLC method was 0.15 µg/ml.

4. Linearity and range

The standard curve of caffeine was shown in Figure E9. The standard curve was found to be linear with coefficient of determination 0.9999. This result indicated that HPLC method was acceptable for quantitative analysis of caffeine in the range studied. The equations of standard curve according to Beer's Law were used for calculating the concentration of caffeine.

5. Accuracy

The percentage of analytical recovery of each caffeine concentration in donor and receptor fluid are shown Table E1-E2, respectively. All percentage analytical recovery of all drug concentrations in donor and receptor fluid with a mean and a %CV of percentage analytical recovery indicated high accuracy of this method. Thus, it could be used for analysis of caffeine in all concentration used.

6. Precision

The precision of analysis of caffeine in donor and receptor fluid by HPLC method were determined both within run precision and between run precision as

illustrated in Table E3-E6. All percentage coefficient of variation value of within run precision was lower than 2%, indicating that of the HPLC method used were precise for quantitative analysis of caffeine on the range studied.

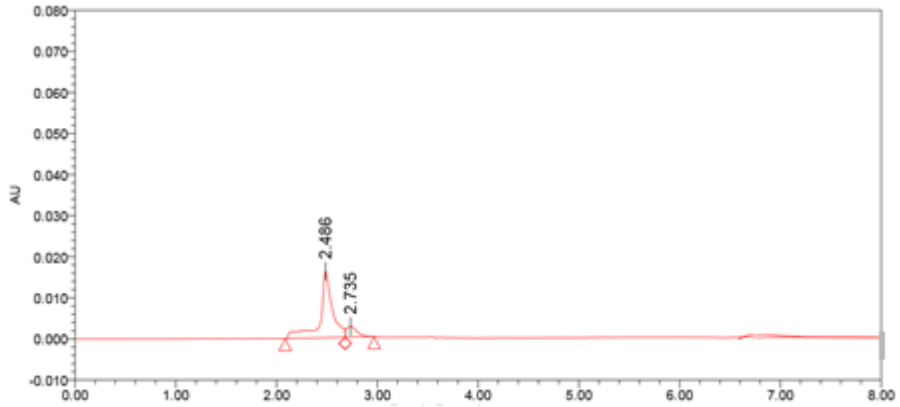


Figure E1: A representation of HPLC chromatograms of PBS, pH 7.4

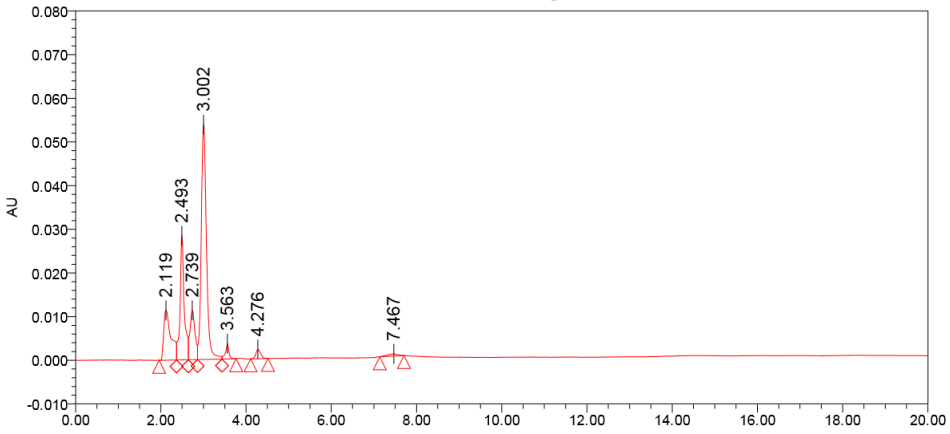


Figure E2: A representation of HPLC chromatograms of the receptor fluid from the control vehicle

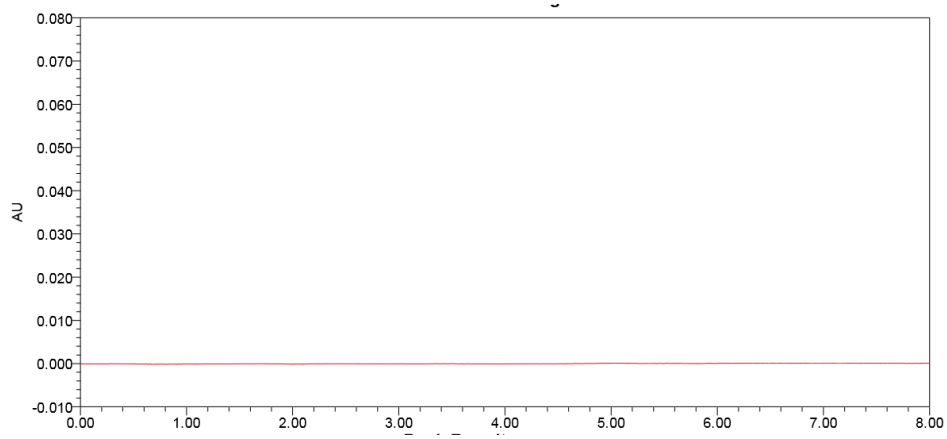


Figure E3: A representation of HPLC chromatograms of blank liposomes

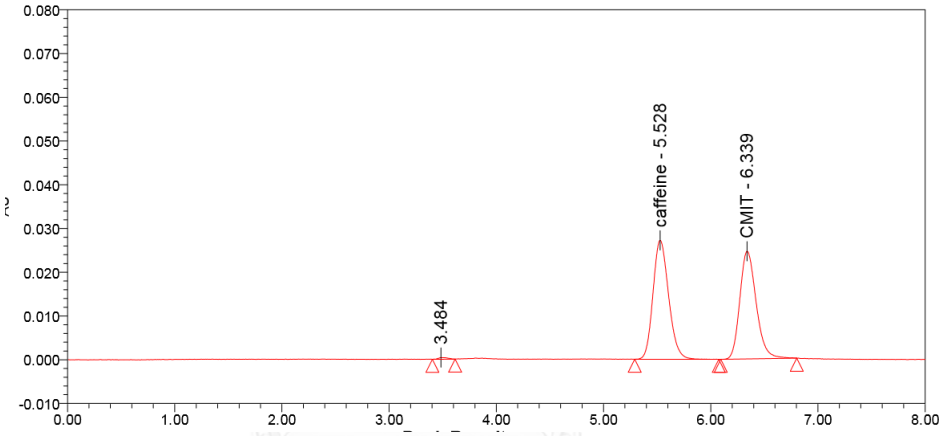


Figure E4: A representation of HPLC chromatograms of caffeine and CMIT in blank liposomes

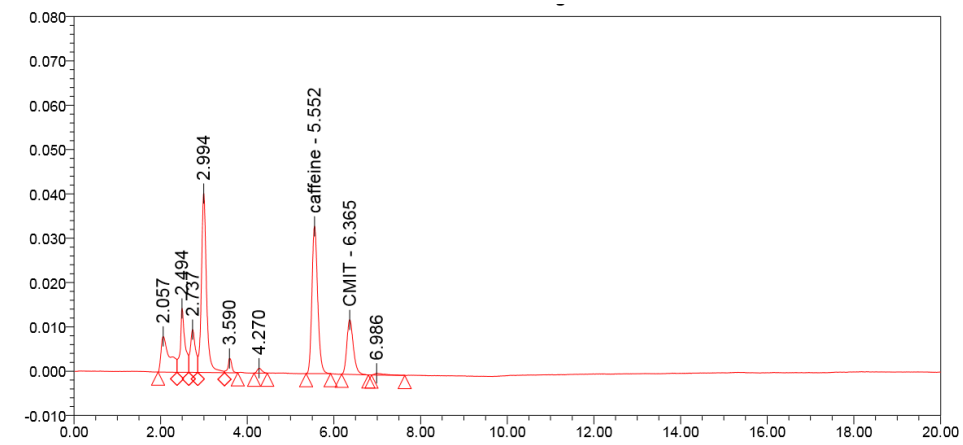


Figure E5: A representation of HPLC chromatograms of caffeine and CMIT in the receptor fluid from the control vehicle

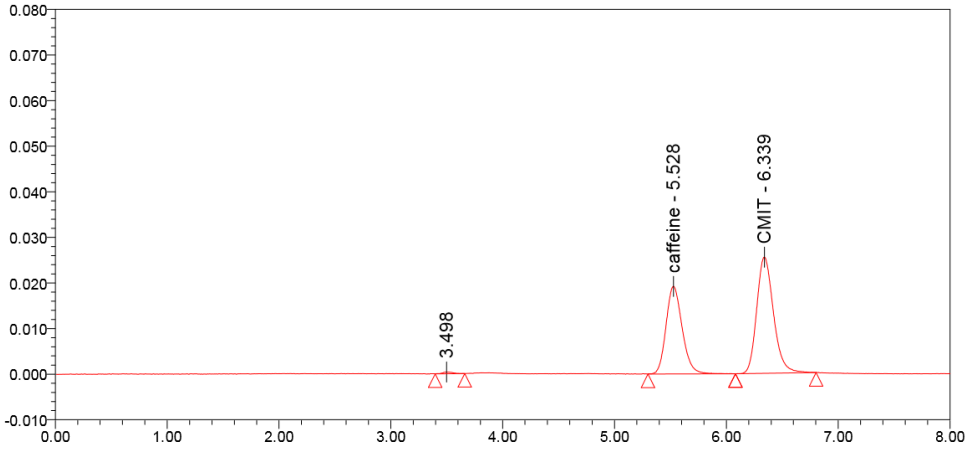
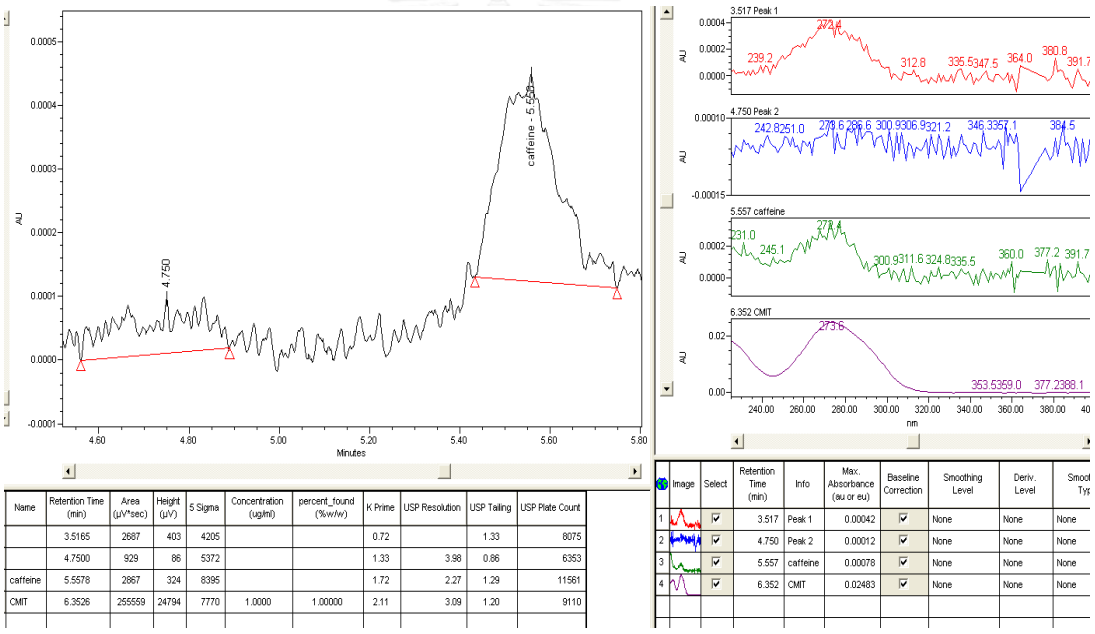


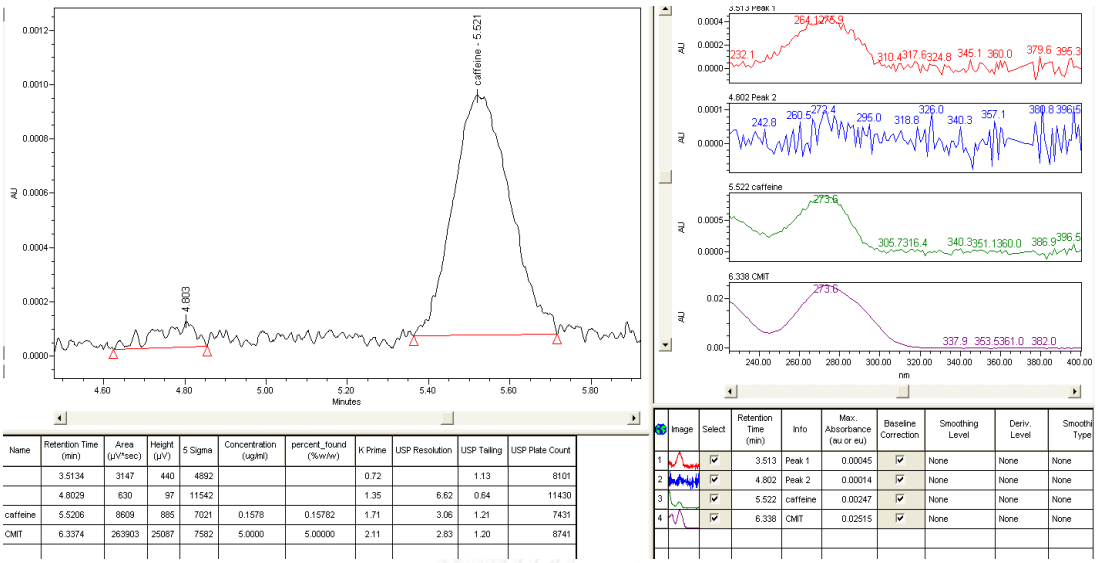
Figure E6: A representation of HPLC chromatograms of caffeine and CMIT in the mobile phase



Name	Retention Time (min)	Area (µV*sec)	Height (µV)	5 Sigma	Concentration (ug/ml)	percent_found (%w/w)	K Prime	USP Resolution	USP Tailing	USP Plate Count
	3.5165	2687	403	4205			0.72		1.33	8075
	4.7500	929	86	5372			1.33	3.98	0.86	6353
caffeine	5.5578	2867	324	8395			1.72	2.27	1.29	11561
CMIT	6.3526	255559	24794	7770	1.0000	1.00000	2.11	3.09	1.20	9110

	Image	Select	Retention Time (min)	Info	Max. Absorbance (au or eu)	Baseline Correction	Smoothing Level	Deriv. Level	Smoo Typ
1		☑	3.517	Peak 1	0.00042	☑	None	None	None
2		☑	4.750	Peak 2	0.00012	☑	None	None	None
3		☑	5.557	caffeine	0.00078	☑	None	None	None
4		☑	6.352	CMIT	0.02483	☑	None	None	None

Figure E7: A representation of HPLC chromatograms for LOD (peak of caffeine 0.05 µg/ml and noise signal)



Name	Retention Time (min)	Area (µV*sec)	Height (µV)	S Sigma	Concentration (ug/ml)	percent_found (%w/w)	K Prime	USP Resolution	USP Tailing	USP Plate Count
	3.5134	3147	440	4892			0.72		1.13	8101
	4.8029	630	97	11542			1.35	6.62	0.64	11430
caffeine	5.5206	8609	885	7021	0.1578	0.15782	1.71	3.06	1.21	7431
CMIT	6.3374	263903	25087	7582	5.0000	5.00000	2.11	2.83	1.20	8741

Image	Select	Retention Time (min)	Info	Max. Absorbance (au or eu)	Baseline Correction	Smoothing Level	Deriv. Level	Smoothing Type
1	☑	3.513	Peak 1	0.00045	☑	None	None	None
2	☑	4.802	Peak 2	0.00014	☑	None	None	None
3	☑	5.522	caffeine	0.00247	☑	None	None	None
4	☑	6.338	CMIT	0.02515	☑	None	None	None

Figure E8: A representation of HPLC chromatograms for LOQ (peak of caffeine 0.15 µg/ml and noise signal)

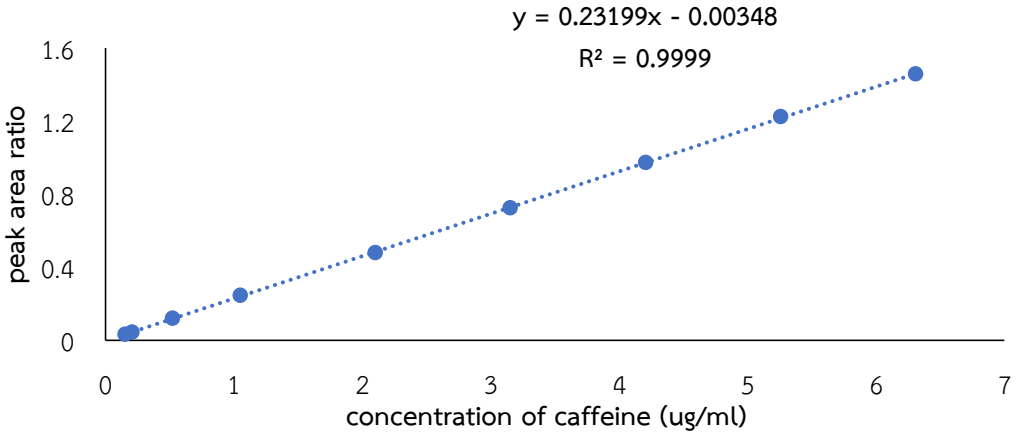


Figure E9: A representation of standard calibration lines of caffeine diluted with mobile phase (water:acetronitrile, 85:15)

Table E1: The percentages of analytical recovery of caffeine in blank vesicular dispersion

Actual concentration of caffeine (µg/ml)	Calculated concentration of caffeine (µg/ml)	% Analytical recovery
0.3501	0.3493	99.77
0.3501	0.3450	98.57
0.3501	0.3533	100.92
0.3501	0.3493	99.80
0.3501	0.3431	98.01
2.4994	2.5036	100.17
2.4994	2.4897	99.61
2.4994	2.5212	100.88
2.4994	2.5194	100.80
2.4994	2.4784	99.16
5.5028	5.4880	99.73
5.5028	5.4998	99.95
5.5028	5.5272	100.44
5.5028	5.4634	99.28
5.5028	5.5260	100.42

Mean % Analytical recovery = 99.83

SD = 0.84, % CV = 0.84

Table E2: The percentages of analytical recovery of caffeine in receptor fluid from the control vehicle

Actual concentration of caffeine (µg/ml)	Calculated concentration of caffeine (µg/ml)	% Analytical recovery
0.3499	0.3503	100.11
0.3499	0.3470	99.17
0.3499	0.3481	99.49
0.3499	0.3493	99.82
0.3499	0.3487	99.66
2.5029	2.5394	101.46
2.5029	2.5520	101.96
2.5029	2.5150	100.48
2.5029	2.4980	99.81
2.5029	2.5388	101.43
5.5063	5.5415	100.64
5.5063	5.5877	101.48
5.5063	5.4678	99.30
5.5063	5.5606	100.99
5.5063	5.5217	100.28

Mean % Analytical recovery = 100.41

SD = 0.89, % CV = 0.88

Table E3: Data for the within run precision of caffeine in blank vesicular dispersion

Actual concentration of caffeine (µg/ml)	Calculated concentration of caffeine (µg/ml)					mean	SD	%CV
	1	2	3	4	5			
0.3499	0.3493	0.3450	0.3533	0.3493	0.3431	0.3480	0.004	1.15
2.5029	2.5036	2.4897	2.5212	2.5194	2.4784	2.5025	0.019	0.74
5.5063	5.4880	5.4998	5.5272	5.4634	5.5260	5.5009	0.027	0.49

Table E4: Data for the between run precision of caffeine in blank vesicular dispersion

Actual concentration of caffeine (µg/ml)	Calculated concentration of caffeine (µg/ml)							
	run 1	run 2	run 3	run 4	run 5	mean	SD	%CV
0.035	0.3532	0.3505	0.3487	0.3478	0.3480	0.3496	0.002	0.65
0.25	2.4921	2.5027	2.4982	2.4997	2.5025	2.4991	0.004	0.17
0.55	5.5540	5.5091	5.5136	5.4614	5.5009	5.5078	0.033	0.60

Table E5: Data for the within run precision of caffeine in receptor fluid from the control vehicle

Actual concentration of caffeine (µg/ml)	Calculated concentration of caffeine (µg/ml)					mean	SD	%CV
	1	2	3	4	5			
0.3499	0.3503	0.3470	0.3481	0.3493	0.3487	0.3487	0.0012	0.35
2.5029	2.5394	2.5520	2.5150	2.4980	2.5388	2.5287	0.0217	0.86
5.5063	5.5415	5.5877	5.4678	5.5606	5.5217	5.5359	0.0452	0.82

Table E6: Data for the between run precision of caffeine in receptor fluid from the control vehicle

Actual concentration of caffeine (µg/ml)	Calculated concentration of caffeine (µg/ml)							
	run 1	run 2	run 3	run 4	run 5	mean	SD	%CV
0.35	0.3564	0.3550	0.3501	0.3483	0.3485	0.3517	0.004	1.08
2.50	2.5326	2.5194	2.5005	2.5158	2.5355	2.5208	0.014	0.56
5.50	5.5486	5.4995	5.5245	5.5084	5.5359	5.5234	0.020	0.36

In conclusion, the analysis of caffeine in receptor fluid from the control vehicle and in blank vesicular dispersion by the HPLC method partial validated in this study showed good specificity, linearity, accuracy, and precision. Thus, this method could use for the quantitative determination of caffeine in the permeation studies with LOD and LOQ were 0.05 and 0.15 µg/ml, respectively.

APPENDIX F

Microscopic images of vesicles

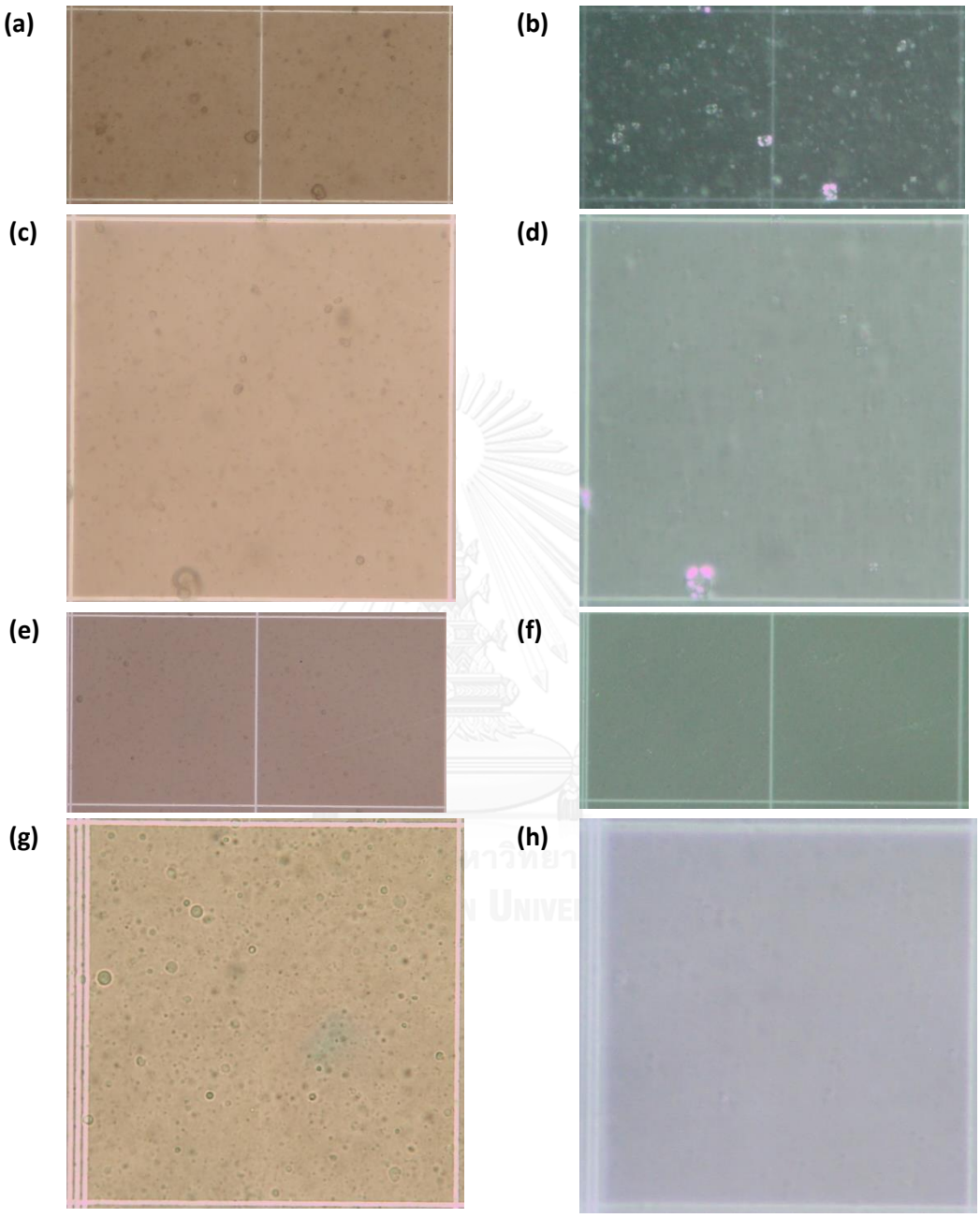
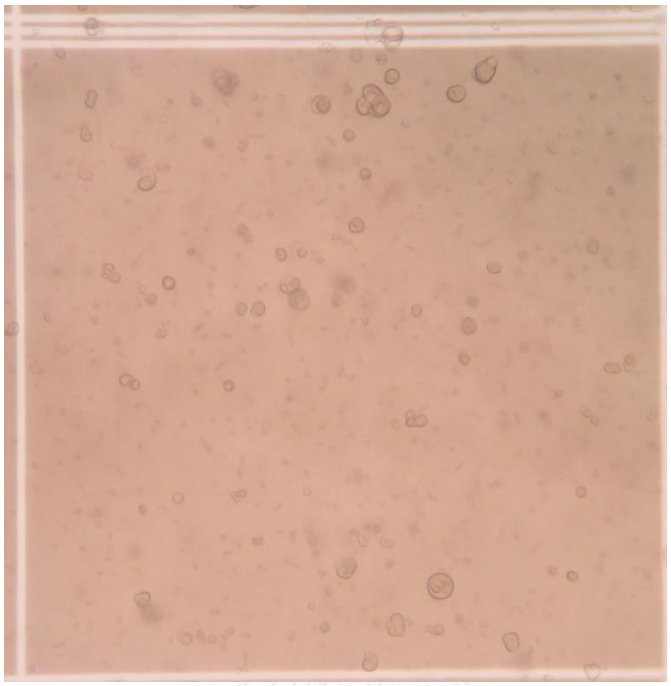


Figure F1: Photographs of blank liposomes

(a) under optical microscope (x 100), (b) under polarized-light microscope (x 100), (c) under optical microscope (x 200), (d) under polarized-light microscope (x 200) of S7.5_5 (e) under optical microscope (x 100), (f) under polarized-light microscope (x 100), (g) under optical microscope (x 200), (h) under polarized-light microscope (x 200) of T20_5

(a)



(b)

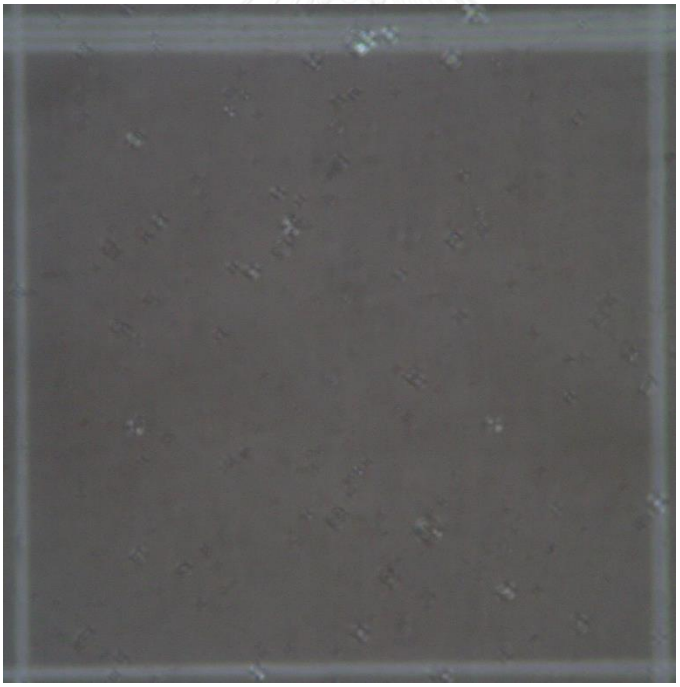


Figure F2: Photographs showing complete formation of S5_5

(a) Photograph of S5_5 under microscope (x 400)

(b) Photograph of S5_5 under polarized-light microscope (x 400)

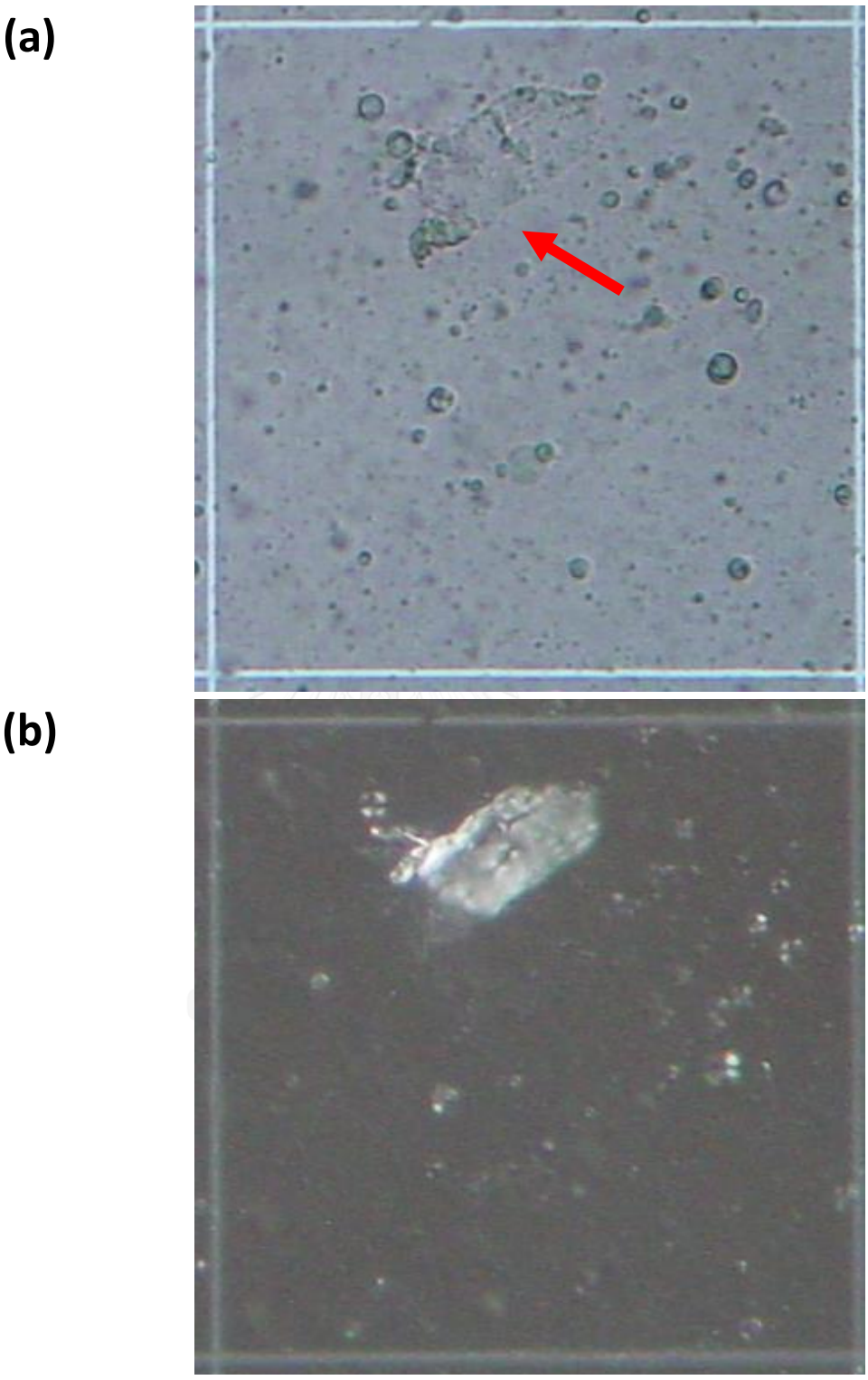


Figure F3: Photographs showing a lipid remnant in S7.5_25

(a) under optical microscope (x 100)

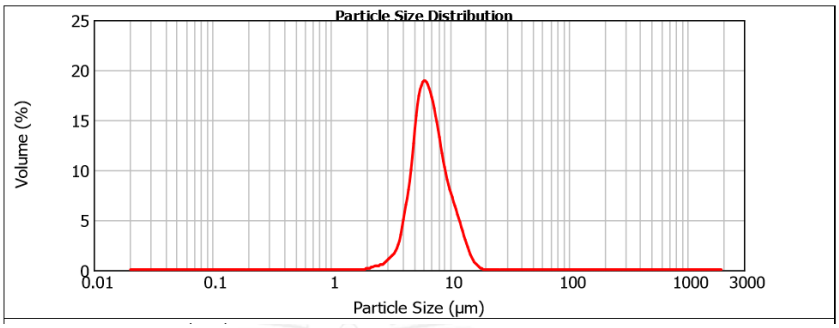
(b) under polarized-light microscope (x 100)

APPENDIX G

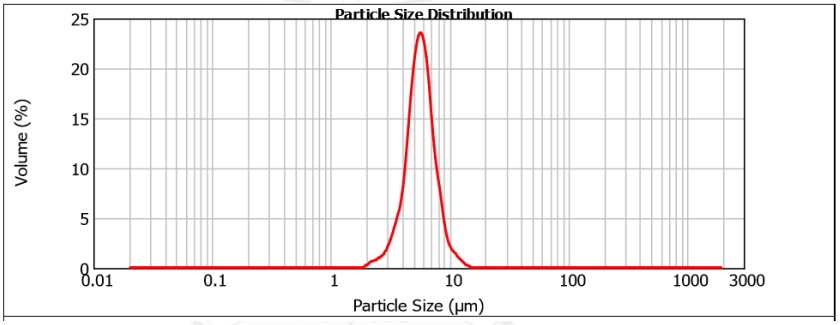
Size distribution of vesicles

Size distribution profile of completely formed blank liposomes is displayed below with the formulation code as describes at Table 1 in Chapter 3.

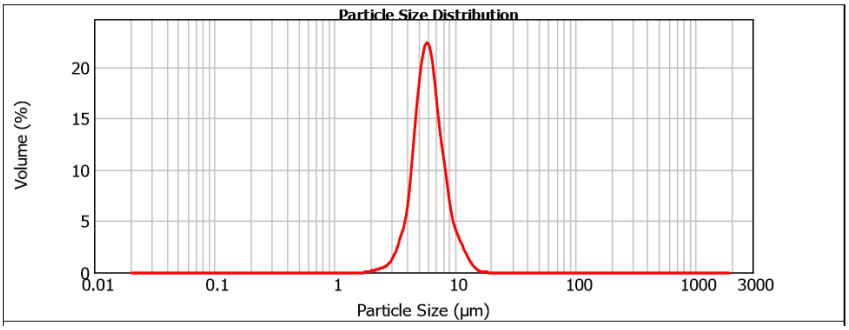
S5_5



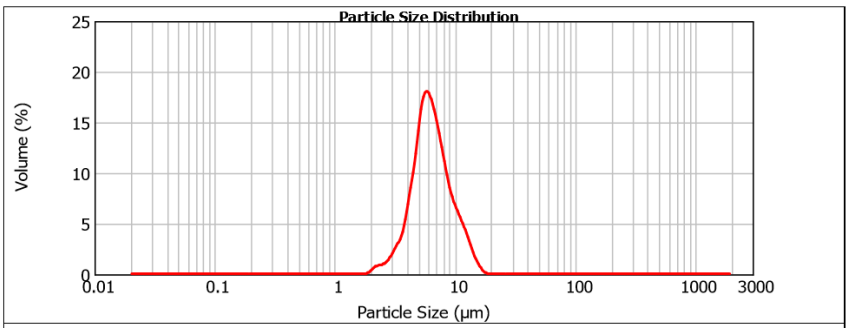
S7.5_5



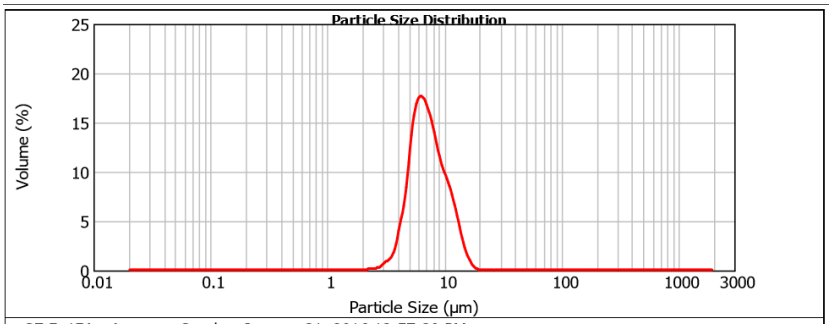
S10_5



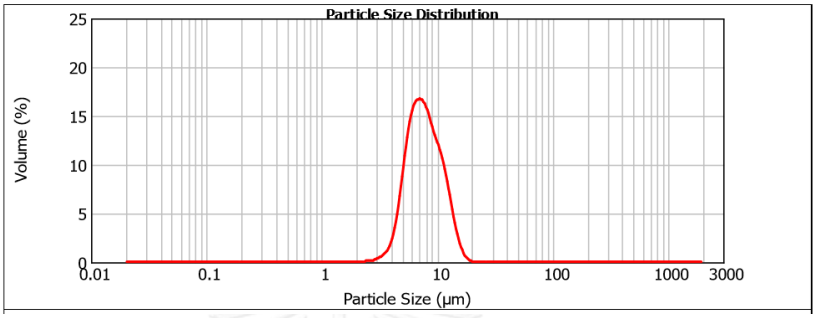
S5_15



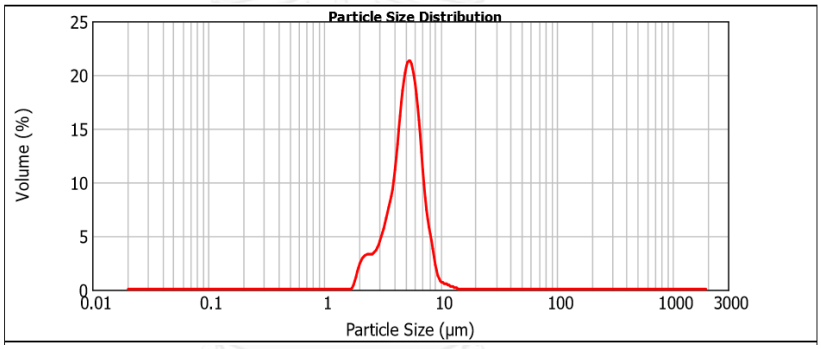
S7.5_15



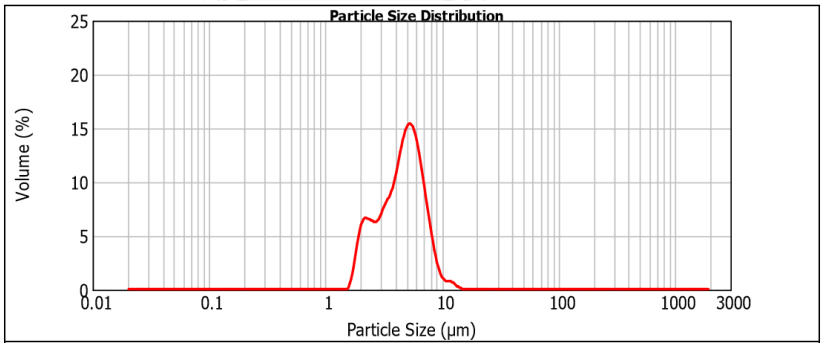
S10_15



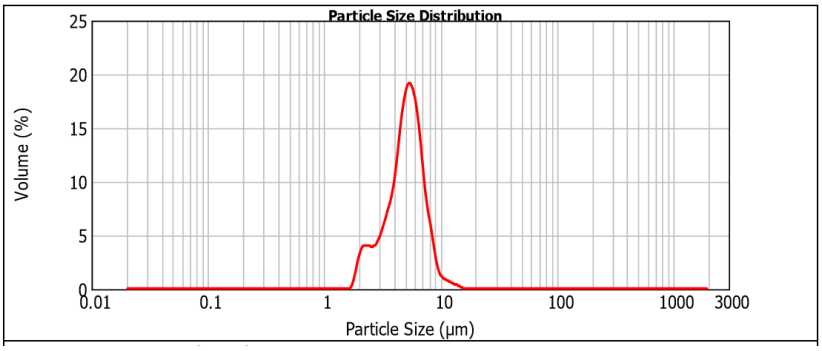
T15_5



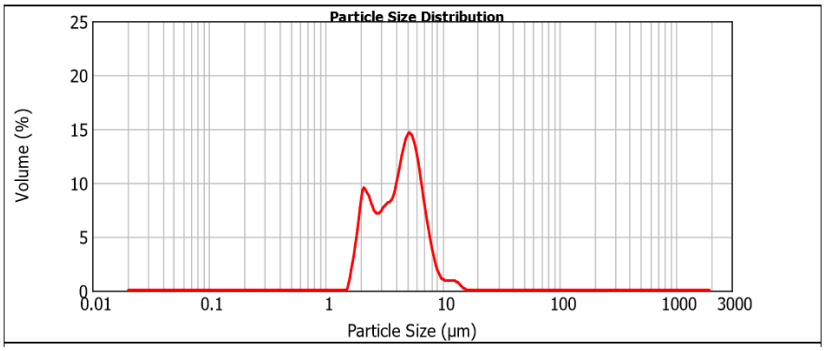
T20_5



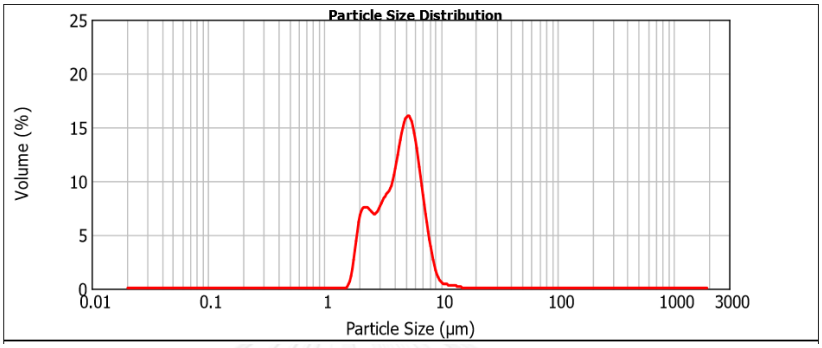
T25_5



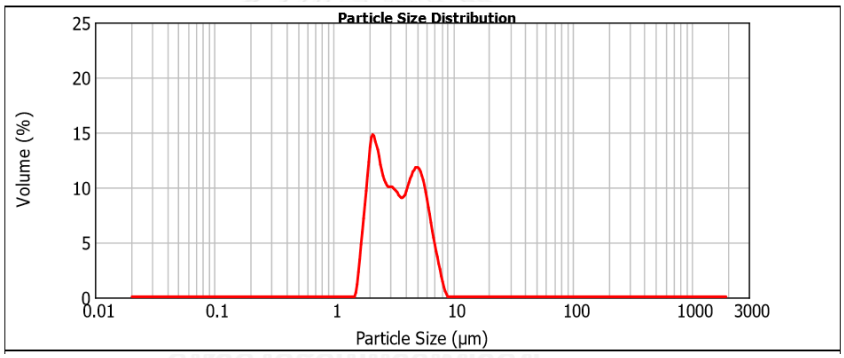
T15_15



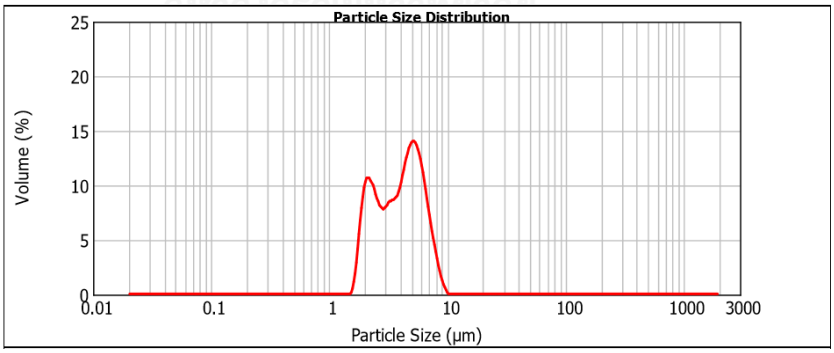
T20_15



T25_15



T25_20



VITA

Miss Chungrida Kao-ian was born on October 22, 1982 in Trang, Thailand. She received the Bachelor's degree in Pharmaceutical Science from Prince of Songkla University in 2006. She has worked at the cosmetic section, Bureau of Cosmetics and Hazardous substances, Department of Medical Sciences, Ministry of Public Health, Nonthaburi. She entered the master's degree program in Pharmaceutics at Chulalongkorn University in 2012.