การพัฒนานิโอโซมของกรดไกลโคลิกเพื่อนำส่งเข้าสู่ผิวหนัง

นางสาวศศิวิมล กลิ่นหอม

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาเภสัชศาสตรมหาบัณฑิต
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DEVELOPMENT OF GLYCOLIC ACID NIOSOMES FOR SKIN DELIVERY

Miss Sasiwimol Klinhom

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Thesis Title	DEVELOPMENT OF GLYCOLIC ACID NIOSOMES FOR SKIN DELIVERY
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Field of Study	Pharmaceutics
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อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ดร.วราภรณ์ สุวกูล, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ผศ. ดร.นนทิมา วรรธนะภูติ, 131 หน้า.

กรดไกลโคลิกนิยมใช้ในผลิตภัณฑ์เครื่องสำอางหลายชนิดเพื่อช่วยผลัดเซลล์ผิวและเพิ่มความชุ่มชื้นให้แก่ผิว เมื่อใช้ในความเข้มข้นสูงจะทำให้เกิดการระคายเคืองผิวหนัง ในการวิจัยนี้จึงมีวัตถุประสงค์เพื่อเตรียมและศึกษาคุณลักษณะเฉพาะของนิโอโซมของกรดไกลโคลิกเพื่อเพิ่มการนำส่งเข้าสู่ผิวหนังและลดการระคายเคือง ในการศึกษานี้ได้เตรียมนิโอโซมซึ่งประกอบด้วยสารลดแรงตึงผิวไม่มีประจุชนิดต่าง ๆ เช่น โพลิออกซิเอธิลีนอัลคิล อีเธอร์ (บริจ®52 และ บริจ®76) ซอร์บิแทน แฟตตีแอซิด เอสเทอร์ (สแปน®20 สแปน®40 และ สแปน®60) กลีเซอริลไดสเตียเรท (จีดีเอส) และ ซูโครส ลอเรทเอสเทอร์ (เอล-595) โดยวิธีการใช้คลื่นเสียงความถี่สูงที่ไม่ใช้ตัวทำละลายอินทรีย์ จากนั้นศึกษาคุณลักษณะเฉพาะของนิโอโซม สังเกตลักษณะรูปร่างของเวซิเคิลโดยใช้กล้องจุลทรรศน์และแสงโพลาไรซ์ วัดขนาดและการกระจายขนาดอนุภาคด้วยวิธีไดนามิกไลท์สแคทเทอริง ประเมินประสิทธิภาพการกักเก็บกรดไกลโคลิกโดยใช้วิธีอัลตราเซนทริฟิวเกชันและวิธีโครมาโทกราฟีแบบของเหลวสมรรถนะสูง ศึกษาความคงตัวทางกายภาพโดยสังเกตการเปลี่ยนแปลงของลักษณะรูปร่าง ขนาดและการกระจายขนาด ประสิทธิภาพการกักเก็บและพีเอชในแต่ละสูตรตำรับ ศึกษาการปลดปล่อยผ่านเยื่อซึมผ่านชนิดเซลลูโลสอะซีเตทและการซึมผ่านผิวหนังลูกหมูแรกเกิดของกรดไกลโคลิกโดยใช้เซลล์สำหรับศึกษาการแพร่แบบฟรานซ์ชนิดดัดแปลง นอกจากนี้ได้ศึกษาความเป็นไปได้ในการก่อการระคายเคืองของนิโอโซมโดยการทดสอบกับเม็ดเลือดแดงของแกะ ผลการศึกษานี้แสดงให้เห็นว่าสามารถเตรียมนิโอโซมที่มีรูปร่างกลมได้สมบูรณ์ ขนาดและประสิทธิภาพการกักเก็บขึ้นอยู่กับส่วนประกอบของนิโอโซมและโครงสร้างของสารลดแรงตึงผิว นิโอโซมทุกตำรับที่เตรียมได้มีความคงตัวทางกายภาพเมื่อเก็บไว้ที่อุณหภูมิห้องเป็นเวลา 2 เดือน การปลดปล่อยของกรดไกลโคลิกจากระบบนิโอโซมที่คัดเลือกมาทำการศึกษาเกิดขึ้นอย่างช้า ๆ และเป็นไปตามจลนศาสตร์อันดับที่หนึ่ง อัตราการปลดปล่อยคงที่ขึ้นกับประสิทธิภาพการกักเก็บและสถานะทางอุณหพลศาสตร์ของผนังเวซิเคิล นิโอโซมสามารถเพิ่มการซึมผ่านผิวหนังของกรดไกลโคลิกโดยขึ้นอยู่กับโครงสร้างของสารลดแรงตึงผิวและสถานะทางอุณหพลศาสตร์ของผนังเวซิเคิล นอกจากนี้นิโอโซมของกรดไกลโคลิกทุกตำรับสามารถลดการระคายเคืองได้มากกว่ารูปแบบสารละลาย โดยขึ้นอยู่กับส่วนประกอบของนิโอโซม โครงสร้างของสารลดแรงตึงผิวและสถานะทางอุณหพลศาสตร์ของผนังเวซิเคิล ดังนั้นผลการศึกษานี้ชี้ให้เห็นว่าสามารถพัฒนานิโอโซมเพื่อเป็นระบบนำส่งเฉพาะที่ของสารที่ละลายน้ำ เช่น กรดไกลโคลิก เพื่อใช้ประโยชน์ด้านเครื่องสำอางได้

ภาควิชา……..วิทยาการเภสัชกรรมและเภสัชอุตสาหกรรม...ลายมือชื่อนิสิต……
สาขาวิชา……เภสัชกรรม……...ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์หลัก……..…..
ปีการศึกษา….2551…….ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์ร่วม.....

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KEYWORDS: GLYCOLIC ACID/ NIOSOMES / CHARACTERIZATION / PERMEATION / SKIN DELIVERY / IRRITATION POTENTIAL

SASIWIMOL KLINHOM: DEVELOPMENT OF GLYCOLIC ACID NIOSOMES FOR SKIN DELIVERY. ADVISOR: ASSOC. PROF. WARAPORN SUWAKUL, Ph.D., CO-ADVISOR: ASST. PROF. NONTIMA VARDHANABHUTI, Ph.D., 131 pp.

Glycolic acid (GA) is popularly used in many cosmetic products as exfoliant and moisturizer. Unfortunately high glycolic acid concentration has high potential for skin irritation. The aim of this study was to prepare and characterize glycolic acid niosomes in order to increase skin delivery and reduce its irritation effect. For this purpose niosomes which contain of various classes of nonionic surfactant such as polyoxyethylene alkyl ethers (Brij®52 and Brij®76), sorbitan fatty acid esters (Span®20, Span®40, and Span®60), glyceryl distearate (GDS), and sucrose laurate ester (L-595®) were prepared by sonication method that was devoid of organic solvent. Characterization of GA niosomes was then performed. Morphology of vesicle was observed by optical and polarized light microscopy. Size and size distribution were characterized by dynamic light scattering. Entrapment of glycolic acid was evaluated by ultracentrifugation and HPLC method. Physical stability was investigated by the change of morphology, size and size distribution, entrapment, and pH in each formulation. Release of GA through cellulose acetate membrane and permeation of GA across newborn pig skin were studied using modified Franz diffusion cells. Furthermore, irritation potential of niosomes was investigated by sheep red blood cell test. The results revealed that niosomes completely formed spherical shape. Niosomal size and entrapment depended on niosomal compositions and surfactant structure. All niosomal formulations were physical stable within two months of storage at ambient temperature. The release of GA from some selected niosomal systems was sustained and followed the first order kinetics. The release rate constants depended on entrapment and thermodynamic state of bilayers. Most of niosomal formulations enhanced GA permeability across the skin based on surfactant structure and thermodynamic state of bilayers. Moreover, all GA niosomes much reduced irritation than that from aqueous solution and depended on niosomal compositions, surfactant structure, and thermodynamic state of bilayers. Consequently, the results of this study indicate that niosomes could be developed as topical delivery of water soluble substances such as glycolic acid for cosmetic uses.

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

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

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LISTS OF ABBREVIATIONS

ANOVA	=	analysis of variance
°C	=	degree Celsius
CHO	=	cholesterol
Conc.	=	concentration
cm	=	centimeter
cm^2	=	square centimeter
CV	=	coefficient of variation
df	=	degree of freedom
EE	=	entrapment efficiency
EF	=	enhancement factor
EO	=	polyoxyethylene group
et al.	=	*et alii*, 'and others'
g	=	gram
GA	=	glycolic acid
GDS	=	glyceryl distearate
HLB	=	hydrophile-lipophile balance
hr	=	hour
HPLC	=	high performance liquid chromatography
i. e.	=	id est
L	=	liter
L-595	=	sucrose laurate ester
mg	=	milligram
min	=	minute
mL	=	milliliter
MLVs	=	multilamellar vesicles
mm	=	millimeter
MP	=	melting point
MW	=	molecular weight
n	=	sample size
nm	=	nanometer

PAE	=	polyoxyethylene alkyl ethers
PBS	=	phosphate buffer saline
PEG-8-L	=	octaoxyethyleneglycol-8-laurate
pH	=	the negative logarithm of the hydrogen ion concentration
P_s	=	permeability coefficient
Q_{24}	=	GA in PBS at 24 hours
Q_s	=	GA in skin
r^2	=	coefficient of determination
RF	=	relative flux
rpm	=	round per minute
SD	=	standard deviation
SEM	=	standard error of the mean
SPSS	=	statistical package for the social sciences
SUVs	=	small unilamellar vesicles
TEM	=	Transmission Electron Microscopy
USP/NF	=	The United States Pharmacopoeia/National Formulary
UV	=	ultraviolet
v/v	=	volume by volume
w/w	=	weight by weight
µg	=	microgram
µL	=	microliter
µm	=	micrometer

CHAPTER I

INTRODUCTION

The use of cosmetic compounds on the skin surface has the main purpose for protection and preservation of the normality of the skin. In the last twenty years many people have also become concerned with their appearance. This certainly arises in great part from different information sources that exploit several methods, including the use of cosmetic products. Recently, skin care products become more important in protecting the skin from environments. They have great benefit to improve the skin structure and function such as reducing wrinkles and hyperpigmentation and increasing skin moisturization.

Glycolic acid is a cosmetic compound in the alpha hydroxy acids (AHAs) or fruit acids group. This acid is found mostly in sugarcane juice (Pierard, Franchimont, and Hermanns-Le, 2000; Ramos-E-Silva et al., 2001). Glycolic acid is widely used in many cosmetic products as an exfoliant, moisturizer, and emollient. Dermatologists have used glycolic acid to treat various skin disorders, including photoaging (Johnson, 2002; Ditre, 2005), acne (Bordat and Chesnoy, 2005), acne scar (Erbagci and Akcali, 2000), hyperpigmentation (Burns et al., 1997), melasma (Lim, 1999), xerosis (Berardesca, 2001), wrinkles (Johnson, 2002; Ditre, 2005), ichthyosis (Pierard et al., 2000; Johnson, 2002), seborrheic dermatitis (Ditre, 2005), keratose (Pierard et al., 2000; Ditre, 2005), and other hyperkeratotic conditions (Pierard et al., 2000; Ramos-E-Silva et al., 2001; Johnson, 2002; Ditre, 2005). Because of its effective effects on the skin, such as increasing cell turnover in stratum corneum (Berardesca, 2001), glycolic acid has become increasingly popular for cosmetic use. It has been incorporated into a variety of conventional dosage forms such as creams (Erbagci and Akcali, 2000), gels (Lim, 1999), solutions (Fartasch, Teal, and Menon, 1997; Kim et al., 2001), lotions (Bernstein et al., 2001), and emulsions (Bordat and Chesnoy, 2005) for several uses. Glycolic acid is a weak acid and the pH of the system depends on its concentration. The pH decreases when concentration increases. Glycolic acid solutions in water at 0.5, 5, and 10% have pH values of 2.5, 1.91, and 1.73,

respectively (Budavali et al., 1996). Accordingly, the use of conventional dosage forms containing high glycolic acid concentrations is limited due to skin irritation such as burning, stinging, tingling, and erythema (Kraeling and Bronaugh, 1999; Ramos-E-Silva et al., 2001; Johnson, 2002; Ditre, 2005). So there has been real concern about the skin compatibility of glycolic acid products. The irritation effect is related to the susceptibility of the individual and the strength of the glycolic acid (Johnson, 2002). Although glycolic acid has multiple effects on the skin related to pH and concentration, with respect to irritation, the Cosmetic Ingredient Review (CIR) concluded that glycolic acid is safe for use in cosmetic products at concentration equal to or less than 10%, at a final formulation pH equal to or greater than 3.5, when formulated to avoid increasing sun sensitivity or when directions for use include the daily use of sun protection (Pierard et al., 2000; Berardesca, 2001; Johnson, 2002). The irritation issue is recognized. There has been much work by the cosmetic industries to develop products that retain the benefits of glycolic acid without the sensory irritation, but so far with only modest success.

The natural function of the skin is to protect for unwanted influences from environment. The main barrier of the skin is located in the outermost layer of the skin, the stratum corneum. The major obstacle for topical drug delivery is the low permeation rate of drug across the stratum corneum. Several methods have been assessed to increase the permeation rate of drugs temporarily such as the use of chemical enhancers, microneedles, iontophoresis, electrophoresis, ultrasound, and vesicles (liposomes and niosomes) (Schreir and Bouwstra, 1994; Barry, 2001). One of the most controversial methods is the use of vesicular formulations as skin delivery systems (Honeywell-Nguyen and Bouwstra, 2005). The vesicular benefits include in increasing drug permeation to the different skin layers (Honeywell-Nguyen et al., 2002; Honeywell-Nguyen, Arenja, and Bouwstra, 2003) and perturbations of the skin ultrastructure (Bouwstra and Honeywell, 2002), controlling the release of drugs (Suwakul, Ongpipattanakul, and Vardhanabhuti, 2006), increasing the duration of drug localized in the skin (Manconi et al., 2006), skin softener and moisturization (Egbaria and Weiner, 1991), capability of entrapment both hydrophilic and hydrophobic substances (du Plessis, Weiner, and Muller, 1994; Yang et al., 2007),

decreasing toxicity and irritation of entrapped drugs (Lasch and Wohlrab, 1986; Guinedi et al., 2005; Manconi et al., 2006; Lakshmi et al., 2007; Paolino et al., 2007), safety, and biocompatibility (Hofland et al., 1991; Hofland et al., 1992).

Niosomes or non-ionic surfactant vesicles are analogous to liposomes as they assume a bilayer vesicular structure. Niosomes have been prepared from several classes of non-ionic surfactants such as sorbitan ester (Span®), polyoxyethylene ether (Brij®), glycerol diester, and sucrose laurate ester (Bouwstra and Hofland, 1994; Uchegbu and Florence, 1995; Honeywell-Nguyen et al., 2002; Honeywell-Nguyen, Arenja et al., 2003; Suwakul et al., 2006). Normally, cholesterol, charged molecules and membrane stabilizers are intercalated in the bilayers in order to increase the physical stability (Uchegbu and Florence, 1995; Uchegbu and Vyas, 1998). The advantages of niosomes for drug delivery are higher chemical and physical stability of the surfactants than that of phospholipids, which are used in the preparation of liposomes (Bouwstra and Hofland, 1994; Guinedi et al., 2005), bearing in acid condition (Rieger, 1997), low cost, ease of production and no special precautions or storage conditions (Baillie et al., 1985; Guinedi et al., 2005; Girigoswami, Das and De, 2006). In addition, niosomes have been proposed as system capable of protecting the toxicity of irritating drugs (Guinedi et al., 2005; Manconi et al., 2006; Lakshmi et al., 2007; Paolino et al., 2007; Yang et al., 2007). There are several preparation methods of niosomes such as sonication (Baillie et al., 1985), hand-shaking (Baillie et al., 1985; Nasseri and Florence, 2003), thin-film hydration (Guinedi et al., 2005; Girigoswami et al., 2006), ether injection (Baillie et al., 1985; Devaraj et al., 2002), and reverse-phase evaporation (Perugini et al., 2000; Guinedi et al., 2005).

Thus, the development of glycolic acid niosomes may be used to increase skin permeation and solve irritation problems. A vesicular drug delivery system such as niosomes may direct the drug to its site of action more efficiently than the conventional dosage forms. Since drugs encapsulated in niosomes may penetrate into the skin with higher permeation rate, so formulators may be able to decrease the glycolic acid concentration in the dosage forms. Accordingly, the formulations would

have low skin irritation due to the low acid concentration. Scientific evidence is necessary to decide whether niosomes can entrap glycolic acid into vesicles and prevent the direct contact between skin and this irritating compound. This type of delivery system seems to be a good alternative for topical delivery. Thus, development of glycolic acid into niosomal preparation should improve permeation of glycolic acid into the skin and reduce its irritation effect to the skin.

Therefore, the aim of this present study was to develop glycolic acid niosomes using various classes of commercially available non-ionic surfactants and cholesterol as structural lipids. The resultant niosomal systems were investigated in terms of morphology, size and size distribution, entrapment, release, short-term (2 months) physical stability, skin permeation, and irritation potential. The information obtained will be helpful in developing glycolic acid, as well as other compounds with similar solubility and acid property, niosomes into practical formulations for topical skin delivery.

Objectives

The specific objectives of this study were as follows:

1. To formulate glycolic acid niosomes
2. To characterize glycolic acid niosomes in terms of morphology, size and size distribution, entrapment, release, and short-term (2 months) physical stability
3. To study the in vitro skin permeation of glycolic acid from niosomes
4. To estimate the irritation potential of glycolic acid niosomes

CHAPTER II

LITERATURE REVIEW

Glycolic acid

Alpha hydroxy acids (AHAs) are a group of organic acid found in natural foods. They called fruit acids because of their abundance in common fruits such as sugarcane (glycolic acid), apples (malic acid), citrus fruits (citric and ascorbic acid), grape (tartaric acid), and milk (lactic acid) (Johnson, 2002). A particular AHAs, glycolic acid was used in the first AHAs facial moisturizers and remains the most widely used in cosmetic products. Among AHAs family, the different acids differ in the length of molecules. Glycolic acid is the smallest one because it contains two carbons (Figure 1). This structure makes glycolic acid particular easy to handle for dermatological application since it can permeate through the skin layers (Cotellessa, Peris, and Chimenti, 1995). Glycolic acid has distinctive pharmacologic properties that recommend it for topical use in a range of skin disorders such as photoaging (Johnson, 2002; Ditre, 2005), acne (Bordat and Chesnoy, 2005), acne scar (Erbagci and Akcali, 2000), hyperpigmentation (Burns et al., 1997), melasma (Lim, 1999), xerosis (Berardesca, 2001), wrinkle (Johnson, 2002; Ditre, 2005), ichthyosis (Pierard et al., 2000; Johnson, 2002), seborrheic dermatitis (Ditre, 2005), keratose (Pierard et al., 2000; Ditre, 2005), and other hyperkeratotic conditions (Pierard et al., 2000; Ramos-E-Silva et al., 2001; Johnson, 2002; Ditre, 2005).

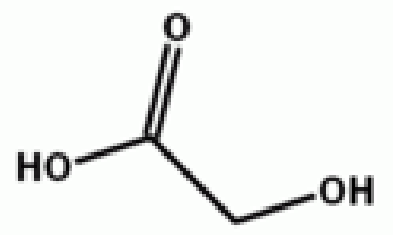

Figure 1 Chemical structure of glycolic acid (Johnson, 2002)

Mechanism of action of glycolic acid (Kraeling and Bronaugh, 1999; Berardesca, 2001; Ramos-E-Silva et al., 2001; Johnson, 2002)

Glycolic acid has a variety of different action on skin depending on its pH and concentration. The exact mechanism of action of glycolic acid is still unknown, however, it can be hypothesized via multiple effects on the skin.

1. On stratum corneum: low concentration of glycolic acid diminishes corneocyte cohesion. The effect occurs at the lower levels of the stratum corneum and may involve in a particular step of keratinization. Glycolic acid facilitates shedding of the outer layer of epidemis by interfering in intercellular ionic bond, thereby reducing corneocyte cohesion at lower level of the stratum corneum. The effect is clinically evident as a sheetlike separation of the stratum corneum, commonly referred to as exfoliation. It stimulates the growth of new skin, resulting in a rejuvenated and refresher complexion. Another mechanism induced by glycolic acid is the enzymatic inhibition of the reaction of sulphate transferase, phosphotransferase, and kinases which leads to fewer electronegative sulphate and phosphate groups on the outer wall of corneocytes resulting in diminishment of cohesion forces. On the contrary, retinoids reduce intercorneocyte cohesion by breaking down already formed sulphate and phosphate bonds via induction or activation of sulphatase or phosphatase.

2. On epidermis: glycolic acid stimulates epidermal proliferation possibly by improving energy and redox status of keratinocytes. Changes detected on normal skin after treatment with glycolic acid are similar to those noted during wound healing, in the rebound period after steroid-induced atrophy, and in retinoic acid-treated skin. Increasing in the overall thickness of viable epidermis as well as in the number of granular layer suggested a stimulation of epidermal turnover. Glycolic acid reduces the calcium ion concentration in the epidermis and removes calcium ions from the cell adhesions by chelation. This causes a loss of calcium ions from the cadherins of the desmosomes and from other type of junctions, resulting in a disruption of the adherence, which results in sheding. Another mechanism is increasing epidermal ceramide and hyalulonic acid biosymthesis, resulting in the proper moisture level of the skin. Moreover, GA might work on pigmented lesions by accelerating epidermal

turnover and by directly inhibiting melanin formation in melanocytes, resulting in improvement of the appearance in hyperpigmentation.

3. On dermis: at high concentration and in an appropriate vehicle, glycolic acid impacts on the papillary dermis and reticular dermis that can lead to dermal changes such as increasing dermal perfusion and the synthesis of new collagen. Glycolic acid might turn on the biosynthesis of dermal glycosaminoglycans and other intercellular substances that could be responsible for eradication of fine wrinkles. It has also been speculated that glycolic acid might promote collagen synthesis in human skin.

Skin compatibility and safety of glycolic acid

There has been real concern about the skin compatibility of glycolic acid products. The reality is that glycolic acid induces sensory irritation (chemosensory irritability) such as burning, stinging, tingling, and erythema (Pierard et al., 2000; Johnson, 2002). The effect is related to the susceptibility of the individual and the strength of the acid. When applied to the skin in high concentration, glycolic acid causes necrosis and detachment of keratinocytes leading to epidermolysis (van Scott and Yu, 1989). Such injury is a chemical peeling depending primarily upon the disruption of the skin pH. The farther away from the physiological pH has the greater caustic effect and the risk of side effects, but more likely to the patients have more benefits of the peeling agents. A tolerable sense of burning itch is often experienced by patients (Pierard et al., 2000). There was much data from which to conclude that glycolic acid are not mutagenic or carcinogenic, are not reproductive or developmental toxins, and are not skin sensitizers. The Cosmetic Ingredient Review (CIR) identified for particular consideration were the irritation potential of glycolic acid and the exfoliating effect of glycolic acid that could potentially enhance penetration of other ingredients and/or increase the sensitivity of the skin to the sun. The Expert Panel concluded that glycolic acid are safe for use in cosmetic products at concentrations equal or less than 10%, at final formulation pH equal or greater than 3.5 or pH of the formulation ± 0.5 pH units of the pK_a (3.83), when formulated to

avoid increasing sun sensitivity or when directions for use include the daily use of sun protection (Johnson, 2002).

Vesicular carrier for skin delivery

Transdermal and dermal drug delivery is problematic because the skin acting as a natural barrier. The most important reason for this is the low permeability of drugs into the stratum corneum, the outermost layer of the skin acting as the main barrier of the skin. The structure of the stratum corneum is often compared with a brick wall, with the corneocytes as the bricks surrounded by the motar of the intercellular lipid lamellae (Figure 2). It has been generally accepted that the highly organized crystalline lipid lamellae play an essential role in the barrier properties of the stratum corneum. Many techniques have been aimed to disrupt and weaken the highly organized intercellular lipids in as attempt to enhance drug transport across the intact skin or to increase the driving force for permeation of drugs across this skin barrier. One of the most controversial methods is the use of vesicle formulations as skin delivery systems (Honeywell-Nguyen and Bouwstra, 2005).

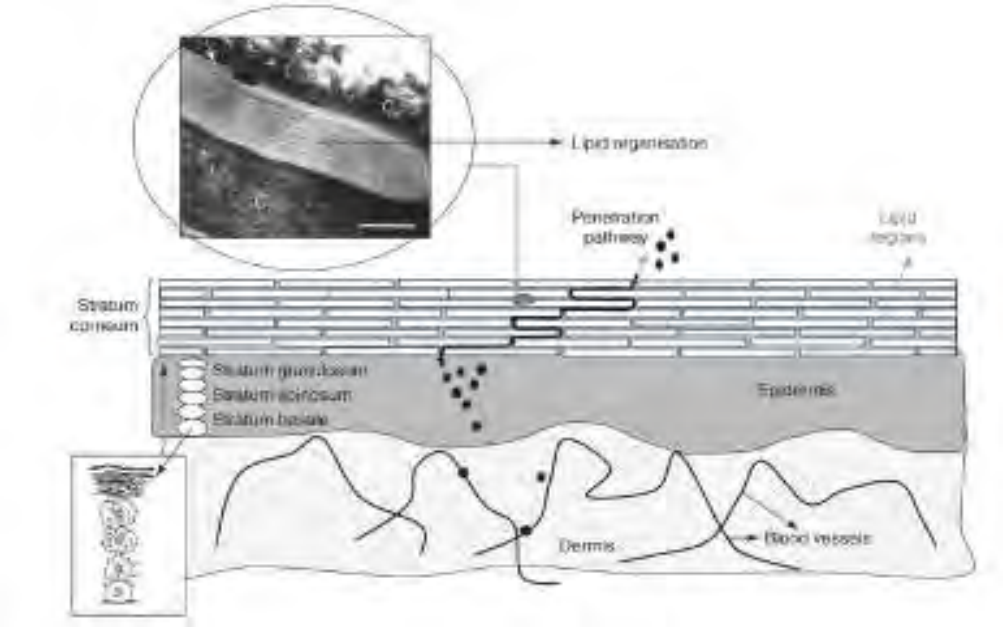

Figure 2 A schematic drawing of a skin cross-section. The corneocytes are embedded in lipid lamellar regions. Substances permeate mainly along the tortuous pathway in the intercellular lamellar regions (Honeywell-Nguyen and Bouwstra, 2005).

Niosomes

Non-ionic surfactant based vesicles (niosomes) are formed from the self-assembly of non-ionic amphiphiles in aqueous media resulting in closed bilayer structures. The assembly into closed bilayers is rarely spontaneous and usually involves some input of energy such as physical agitation or heat. The result is an assembly in which the hydrophobic parts of the molecule are shielded from the aqueous solvent and the hydrophilic head groups enjoy maximum contact with the same. These structures are analogous to phospholipid vesicles (liposomes) and are able to encapsulate aqueous solutes and serve as drug carriers. The low cost, greater stability and resultant ease of storage of non-ionic surfactants has lead to the exploitation of these compounds as alternative to phospholipids. Niosomes were first reported in the seventies as a feature of the cosmetic industry but have been studied as drug targeting agents (Uchegbu and Vyas, 1998). Drugs with various degree of lipophilic can be encapsulated in niosomes. Hydrophilic drugs can be entrapped into the internal aqueous compartment, whereas amphiphilic, lipophilic and charged hydrophilic drugs can be associated with the vesicle bilayer by hydrophobic and/or electrostatic interaction (Figure 3) (Honeywell-Nguyen and Bouwstra, 2005).

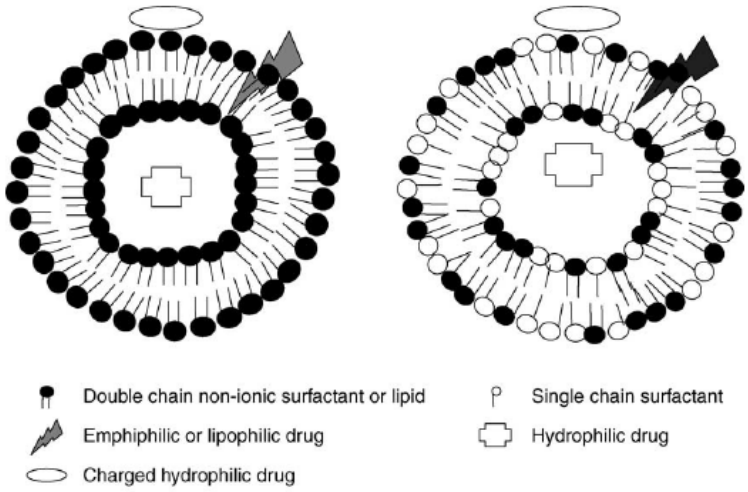

Figure 3 Charged hydrophilic, amphiphilic and lipophilic drug molecules can be associated with the bilayers of the vesicles, whereas hydrophilic substances can also be entrapped in the vesicles (Honeywell-Nguyen and Bouwstra, 2005).

Furthermore, niosomes have advantages for skin delivery such as increasing drug permeation to the different skin layers (Honeywell-Nguyen et al., 2002; Honeywell-Nguyen, Arenja et al., 2003), controlling the release of drugs (Suwakul et al., 2006), increasing the duration of drug localized in the skin (Manconi et al., 2006), reducing drug toxicity and irritation (Lasch and Wohlrab, 1986), safety and biocompatibility (Hofland et al., 1991; Hofland et al., 1992). Niosomes are classified by their size and number of bilayers into multilamellar vesicles (MLVs: several bilayers, size 0.1-20 μm), small unilamellar vesicles (SUVs: single bilayer, size 0.01-0.1 μm), and large unilamellar vesicles (LUVs: single bilayer, size 0.1-1 μm) (Sharma and Sharma, 1997).

Materials used in the preparation of niosomes

Niosomes are vesicles mainly consisting of non-ionic surfactants. They have been prepared from several classes of non-ionic surfactant. Normally cholesterol, charged molecules, and membrane stabilizers are intercalated in the bilayers in order to increase their stability (Uchegbu and Vyas, 1998). In brief, the commonly used components of niosomes use as follows (Roson, 1989; Kibbe, 2000):

1. Non-ionic surfactants

Non-ionic surfactants possess a wide variety of structures. They are usually prepared with the presence of CHO, form both multilamellar and unilamellar vesicles. The balance between hydrophobicity and hydrophilicity (HLB number) and forces of attraction and repulsion of both hydrophobic and hydrophilic parts are crucial in determining the type of aggregate formed in aqueous environments, whether spherical or asymmetrical micelle, mesophase, or vesicle. Besides, the optimum of head group area, hydrocarbon chain volume and hydrocarbon chain length of non-ionic surfactants are also important. Polyoxyethylene alkyl ethers (PAE) and long chain carboxylic acid ester are non-ionic surfactants most commonly used in niosome preparations. In addition, other non-ionic surfactants that form vesicles are the following: alkyl polyglucosides, alkyl methylglucamides, alkyl polyglycerol ethers,

stearoidal esters, and hexadecyl diglycerol (Florence, 1993). The properties of some of these commonly used surfactants are as follows:

1.1 Polyoxyethylene alkyl ethers (Kibbe, 2000)

Polyoxyethylene alkyl ethers (PAE) are non-ionic surfactants widely used in topical pharmaceutical formulations and cosmetics. PAE are series of polyoxyethylene glycol ethers of n-alcohols (lauryl, myristyl, cetyl, and stearyl alcohol). It can be produces by the polyethoxylation of linear fatty alcohols. These products tend to be mixtures of polymers of slightly varying molecular weights, and the numbers used to describe polymer lengths are average values. The most common synonyms or trade names applicable to PAE are Brij® and Steareth®. For example, polyoxyl 10 stearyl ether has the other name of Brij®76 (POE-10) or Steareth®10. This structure formula is $CH_3(CH_2)_x(OCH_2CH_2)_yOH$, where (x+1) is the number of carbon atoms in the alkyl chain and y is the number of ethylene oxide groups in the hydrophilic chain, typically 10-60. PAE are chemically stable in strongly acidic or alkaline conditions.

1.2 Long-chain carboxylic acid esters

In this long chain carboxylic acid esters group, polyoxyethylene sorbitan fatty acid esters and sorbitan fatty acid esters are usually used in niosome preparation.

1.2.1 Polyoxyethylene sorbitan fatty acid esters

Polyoxyethylene sorbitan fatty acid esters are series of fatty acid esters of sorbitol, its anhydrides copolymerizes with approximately 20 moles of ethylene oxide for each mole of sorbitol and its anhydrides. Their synonyms are Tween® and polysorbate. These compounds are hydrophilic non-ionic surfactants widely used in cosmetics and food products. The most commonly used derivatives to prepare niosomes are Tween®20 and Tween®80 (Carafa et al., 1998; Ruckmani, Jayakar, and

Ghosal, 2000). Polysorbates are stable to weak acids and bases. Gradual saponification occurs with strong acids and bases.

1.2.2 Sorbitan fatty acid esters

Sorbitan fatty acid esters are series of mixtures of partial esters of sorbitol and its mono- and di-anhydrides with fatty acids. Sorbitan esters are widely used in cosmetics, food products, and pharmaceutical formulations as lipophilic non-ionic surfactant (Roson, 1989; Kibbe, 2000). Their synonym is Span®. The commonly used ones in niosome preparation are Span®20 (Yoshioka, Sternberg, and Florence, 1994; Namdeo and Jain, 1999; Suwakul et al., 2006), Span®40 (Uchegbu and Duncan, 1997; Namdeo and Jain, 1999; Hao et al., 2002; Suwakul et al., 2006; Pardakhty, Varshosaz, and Rouholamini, 2007), Span®60 (Ruckmani et al., 2000; Manconi et al., 2002; Suwakul et al., 2006; Khazaeli, Pardakhty, and Shoorabi, 2007), Span®80 (Hao et al., 2002; Shahiwala and Misra, 2002), and Span®85 (Yoshioka et al., 1994; Shahiwala and Misra, 2002).

1.3 Glycerol diesters

Glycerol diesters are diesters of glycerol and lipophilic non-ionic surfactants with HLB of 4-6. They are used in foodstuff, medicine and cosmetic industries as an emulsifier, stabilizer, deformer, and thickener. Glycerol diesters used in niosomes are glyceryl distearate (GDS) and glyceryl dilaurate (GDL). GDS and GDL are used as vesicle forming agents for many drugs (Margalit et al., 1992; Niemiec, Ramachandran, and Weiner, 1995; Ohta, Ramachandran, and Weiner, 1996).

1.4 Sucrose fatty acid esters

Sucrose fatty acid esters are non-ionic surfactants with a sucrose substituent as the polar head group. They are nontoxic and biodegradable surfactants approved by WHO as food additives. Since they are nonirritant to the skin, they are also suitable for therapeutic and cosmetic applications. Sucrose fatty acid esters used in

dermatological preparations in the forms of liquid crystals and microemulsions are sucrose laurate and sucrose ricinoleate. Sucrose laurate ester (L-595) used in niosome preparation is usually used with octaoxyethyleneglycol-8-laurate ester (PEG-8-L) (Honeywell-Nguyen et al., 2002; Honeywell-Nguyen, Arenja et al., 2003; Honeywell-Nguyen and Bouwstra, 2003; Honeywell-Nguyen, Groenink et al., 2003).

2. Cholesterol (CHO)

CHO abolishes the gel to liquid crystalline phase transition of liposomal and niosomal systems. In liposomal systems, CHO has been shown to have a remarkable effect on the release of the entrapped solutes and the stability of the systems in vitro and in vivo. Similar effects have also seen with niosomes (Uchegbu and Vyas, 1998). CHO can reduce the fluidity of membranes above the phase transition temperature, with a corresponding reduction in permeability to aqueous solutes. Consequently, inclusion of CHO into unsaturated membranes is often essential in order to achieve sufficient stability. On the other hand, CHO increases the fluidity of membranes below the phase transition temperature. Its inclusion in saturated membranes, which are usually in the gel phase at ambient temperature, may result in a reduction in stability. CHO is usually included at a 1:1 molar ratio in most liposomal and niosomal formulation (Uchegbu and Vyas, 1998).

3. Other additives

Additives are often added to liposomal and niosomal systems to control the properties of the vesicles. Additives such as those giving steric and electrostatic stabilization can improve entrapment efficiency (EE) of the vesicles by preventing flocculation, and hence fusion, resulting in less leakage of the entrapped molecules. These additives also increase the entrapment of water-soluble substances by increasing the thickness of the aqueous layers between the lipid bilayers (Rose, Ribier, and Vanlerberghe, 1993).

3.1 Solulan®C24

Solulan®C24 (poly-24-oxyethylene cholesteryl ether) is one of additive stabilizers used in vesicle preparation for antiaggregation by steric hindrance. Solulan®C24 has been used as a stabilizer in many niosomal formulations (Uchegbu and Florence, 1995; Arunothayanun et al., 2000; Suwakul et al., 2006). Solulan®C24 has been used at a concentration of 5% by weight without toxicity (Dimitrijevic et al., 1997). Addition of Solulan®C24 in niosomes influences niosomal properties such as entrapment efficiency, viscosity, stability, and toxicity.

3.2 Dicetylphosphate (DCP)

Dicetylphosphate (DCP) is a negative charged lipid and it prevents vesicle aggregation by electrostatic repulsion. It has been widely used in both liposomes and niosomes (Yoshioka et al., 1994; Carafa et al., 1998; Uchegbu and Vyas, 1998; Namdeo and Jain, 1999; Carafa, Santucci, and Lucania, 2002). Inclusion of DCP in niosomal formulation affects drug entrapment and stability.

3.3 Micelle-forming agents

Niosomes prepared from L-595 form rigid vesicles. Incorporation of a micelle forming agent surfactant, PEG-8-L, into vesicle bilayers would also result in partial solubilization of the bilayer and thereby increasing the elasticity of the vesicular system. Therefore, the series of vesicles can be obtained, ranging from very rigid to very elastic by changing the ratio of the vesicle forming and the micelle forming agents. Previous reports concluded that increasing the PEG-8-L content increased vesicle elasticity, vesicle stability, and drug solubility but a further increase in the PEG-8-L content resulted in a decrease in drug solubility and vesicle stability. The elastic vesicles consisting of L-595 and PEG-8-L in the molar ratio of 50:50 gave the most elastic vesicles. (van den Bergh et al., 2001; Honeywell-Nguyen et al., 2002).

Niosomes preparation methods (Uchegbu and Vyas, 1998)

The formation of vesicular assemblies requires the input of some form of energy and all the surveyed experimental methods consist of the hydration of a mixture of the surfactant/lipid at elevated temperature follow by optimal size reduction to obtain a colloidal dispersion. Most methods used for liposomes may be applied to niosomes. In addition, since non-ionic surfactants are heat stable, methods devoid of organic solvents are also possible. The hydration temperatures are usually above the gel to liquid transition temperature of the system. In case of hydrophilic drugs, separation of the free drug molecules from the entrapped molecule is usually required. This can be done by various methods such as centrifugation, gel filtration, or dialysis. From a pharmaceutical point of view, this can be wasteful if the entrapment efficiency is low. For hydrophobic molecules, the amount of drug in the formulation is usually predetermined, without free drug left in the final preparation. The preparation methods commonly used for niosomes are as follows:

1. Sonication (Baillie et al., 1985; Florence, 1993; Suwakul et al., 2006)

A surfactants/lipids mixture was melted. Then preheated aqueous phase was added into the surfactants/lipids mixture. These simple mixing methods do not require the use of organic solvents. Formation of vesicles can be facilitated by applying some energy to the system. Sonication has been employed this purpose.

2. Thin-film hydration (Baillie et al., 1985; Nasseri and Florence, 2003; Guinedi et al., 2005; Girigoswami et al., 2006)

The most commonly used method is film hydration method. A surfactants/lipids film is formed on a smooth surface by evaporation of an organic solution of the surfactants/lipids under vacuum. Hydrophobic drugs can be included in the film. The film is then hydrated, with appropriate agitation, with preheated aqueous phase. If the drug to be incorporated is hydrophilic, it can be dissolved in the aqueous phase prior to hydration. This method is particular suitable for water-soluble drug that sensitive to organic solvents since direct contact with the organic solvent can be avoided.

3. Ether injection (Baillie et al., 1985; Devaraj et al., 2002)

An organic solution of surfactants/lipids is slowly injected in an aqueous phase, which is heated above the boiling point of the organic solvent. The drug can be in either the organic phase or the aqueous phase, depending on the nature of drug.

4. Reverse-phase evaporation (Perugini et al., 2000; Guinedi et al., 2005)

An oil in water emulsion is formed from an organic solution of surfactants/lipids and an aqueous phase. The organic solvent is evaporated under vacuum to yield niosome dispersion. Niosome prepared from this method has high entrapment efficiency.

5. Mixing of melted surfactants/lipids with the aqueous phase (Florence, 1993)

Niosomes can be formed by injecting melted surfactants/lipids into a highly agitated preheated aqueous phase. Alternatively, the preheated aqueous phase can be added to the melted surfactants/lipids. In some instances, the warmed aqueous phase can be added to a mixture of the solid surfactants/lipids to form niosomes.

6. Enzymatic conversion (Chopineau, Lesieur, and Ollivon, 1994)

Niosomes may be formed from a mix micellar solution with the use of enzymes. Polyoxyethylene cholesteryl sebatate diester (PCSD) can be cleaved by esterases to yield polyoxyethylene, sebacic acid, and CHO. CHO in combination with non-ionic surfactant ($C_{16}G_2$) and DCP can subsequently form vesicles. The mix micellar solution of PCSD/$C_{16}G_2$ was reported to convert into niosomal suspension upon incubation with esterase.

7. The bubble method (Talsma et al., 1994)

This is another method where the use of organic solvents is avoided. In this method, a surfactants/lipids mixture is homogenized, followed by the bubbling of nitrogen gas through the mixture. The homogenization step may be omitted from the procedure if a longer bubbling time is allowed.

Usually size of niosomes prepared as described above are in the micron range. Often a size reduction step must be incorporated into the niosome production procedure, subsequent to the initial hydration step because vesicle size has an important bearing on vesicle biodistribution. A reduction in vesicle size may be achieved by a number of methods such as probe sonication, extrusion, combination of sonication and filtration, use of microfluidizer, and high-pressure homogenization (Uchegbu and Vyas, 1998).

Characterization of niosomes (Florence, 1993)

Characterization of niosomes is necessary to confirm that properties of the structure formed are suitable for their intended used. Niosomal preparations are usually characterized in terms of morphology, size and size distribution, entrapment efficiency, lamellarity, as well as physical stability. Some of these parameters such as size and size distribution are routinely used for batch-to-batch quality control.

1. Morphology

The vesicle formulations can be examined by freeze fracture electron microscopy (Yoshioka et al., 1994; Carafa et al., 2002), transmission electron microscopy (TEM) (Arunothayanun at al., 2000; Guinedi et al., 2005; Manconi et al., 2006), scanning electron microscopy (SEM) (Touitou et al., 2000; Manconi et al., 2006), and cryo-TEM (Honeywell-Nguyen et al., 2002) to characterize the morphology and microstructure.

2. Size and size distribution

The particle size and size distribution of vesicular formulations are measured by photon correlation spectroscopy or dynamic light scattering (Arunothayanun et al., 2000; Agarwal, Katare, and Vyas, 2001; Guinedi et al., 2005; Suwakul et al., 2006), and electron microscopy (Ruckmani et al., 2000).

3. Entrapment efficiency

Entrapment efficiency (EE) describes drug loading in niosomes and it thus crucial in application of niosomes as delivery systems. There are several ways to remove unentrapped solute from the vesicles before determining the quantity of drug in the vesicles. The methods that have been used for the removal of unentrapped materials include:

1. Exhaustive dialysis (Baillie et al., 1985; Namdeo and Jain, 1999; Ruckmani et al., 2002; Manosroi et al., 2003)
2. Gel filtration (Manconi et al., 2003; Carafa et al., 2004)
3. Centrifugation (Uchegbu and Vyas, 1998)
4. Ultracentrifugation (Arunothayanun et al., 2000; El Maghraby, Williams, and Barry, 2000; Fang, Hong et al., 2001; Guinedi et al., 2005; Suwakul et al., 2006)

4. Lamellarity

Lamellarity is the number of layers making up the shell of the vesicles. It can be determined by electron microscopy or nuclear magnetic resonance spectroscopy (Touitou et al., 2000).

5. Physical stability

The stability of any pharmaceutical product is usually defined as the capacity of the formulation to remain within defined limits for a predetermine period of times (shelf life of the product). The stability of the vesicular products should preferably meet the standard of conventional pharmaceutical products. Both chemical and physical stability aspects are involved. Problem of stability include loss of entrapped drug, change in the vesicular structure, particle size distribution, aggregation, fusion, and chemical instability of entrapped drug (Gianasi et al., 1997; Carafa et al., 1998; Fang, Hong et al., 2001; Guinedi et al., 2005; Khazaeli et al., 2007; Manosroi et al., 2008).

Factors affecting niosomes characteristics

1. Factors affecting niosomes size

1.1 Surfactant structure

The vesicle size depended on alkyl chain length of surfactants. Increasing hydrocarbon chain length of surfactant monomers lead to smaller vesicle size (Yoshioka et al., 1994). Several studies reported that vesicle size was dependent on the HLB of surfactant used. The higher HLB gave the larger vesicle size (Yoshioka et al., 1994; Ruckmani et al., 2000; Manconi et al., 2006; Khazaeli et al., 2007). Khazaeli et al. (2007) reported that the mean size of caffeine loaded niosomes are followed the rank order of Span®20 > Span®40 > Span®60 > Span®80. Similarly, this result was observed from study of Yoshioka et al. (1994) who reported that vesicle sizes prepared using Span® series was dependent on HLB of Span® used. On the contrary, some studies revealed that the higher HLB gave the smaller vesicle sizes. Manconi et al. (2002) reported that tretinoin niosomes prepared from Span®40 by film hydration method was smaller than Span®60 niosomes. Size of Span® niosomes with higher HLB was smaller than niosomes with lower HLB (Uchegbu and Duncan, 1997; Guinedi et al., 2005). In case of Brij® niosomes, size depended on both alkyl chain length and polyoxyethylene unit. This result was observed from the study of Stafford, Baillie, and Florence (1988). They reported that niosomes composed of $C_{18}EO_5$/CHO were larger that those niosomes of $C_{16}EO_5$/CHO prepared by dehydration-rehydration technique. It is obvious that when polyoxyethylene head group is constant and alkyl chain length is increased both micellar volume and vesicle size prepared by sonication method (Hofland et al., 1993) or film hydration method (Pardakhty et al., 2007) will be increased. Moreover, Yoshida et al. (1992) reported that the size of the niosomes prepared from C_nEO_3 was smaller than C_nEO_7. It is likely that when alkyl chain length is constant, vesicle size was directly dependent on the number of polyoxyethylene head group.

1.2 Membrane additives

Membrane additives may affect the vesicle size. The effect of CHO and DCP on vesicle size depended on surfactant type. Increasing the amount of CHO content from 1 to 5 molar ratio reduced the volume diameter of $C_{16}EO_2$ (Brij®52) significantly. This effect was also observed in $C_{18}EO_2$ (Brij®72) vesicles when the molar ratio of CHO was increased from 0 to 3. As CHO increase the chain order, stabilizes bilayer of vesicles, especially the small ones. It is expected that the vesicles with relatively high CHO content be smaller than vesicles with low amounts of CHO (Pardakhty et al., 2007). Bouwstra et al. (1997) reported that addition of CHO and DCP in C_nEO_m niosomes prepared by sonication method reduced the vesicle size. The reason for this result is the effect of CHO in decreasing tendency of the surfactant to aggregate at elevated temperatures, while DCP might increase the curvature of the bilayer and effect on the electrostatic repulsion between ionized head group, thus increasing hydrophilic surface area. Similar results were obtained in vesicle composed of $C_{16}EO_2$/CHO, $C_{18}EO_2$/CHO (Pardakhty et al., 2007), Span®20, Span®40 Span®60, Span®80 (Khazaeli et al., 2007), and C_nEO_3, C_nEO_7 (Yoshida et al., 1992). However, Agarwal et al. (2001) concluded that no effect of DCP on vesicle size was observed in dithranol loaded niosomes prepared by film hydration method. Furthermore, Suwakul et al. (2006) revealed that average size of propylthiouracil loaded Span® and Brij® niosomes were either reduced or practically unchanged when Solulan®C24 was used as stabilizer. This result was the ability of Solulan®C24 in preventing aggregation of the vesicles.

1.3 Method of preparation

The method of preparation can also alter size of vesicular drug carriers. Thus, niosomes prepared from different methods have different properties. Size of water soluble drug such as carboxyfluorescein is allowed the rank in order of hand-shaking > ether injection > sonication (Baillie et al., 1985). Florence (1993) reported that $C_{16}G_3$ niosomes of carboxyfluorescein prepared by hand shaking method were larger than those of niosomes prepared by reverse phase evaporation method. On the

other hand, for water insoluble drug, PK1, polyoxyethylene alkyl ether niosomes prepared by dehydration rehydration method were larger than those of niosomes prepared by thin-film hydration method (Uchegbu and Duncan, 1997). Therefore, it is often difficult to compare results from different laboratories, especially when sizes are reported by different measurements.

2. Factors affecting niosomes entrapment

2.1 Surfactant structure

Surfactant structure of niosome membrane may be manipulated to increase drug entrapment by altering the nature of the hydrophilic part and/or hydrophobic part. Some researchers found that the entrapment efficiency of niosome directly depended on the hydrophobic alkyl chain length. The drug loading of 5-fluorouracil and colchicine increased with increasing alkyl chain length (Hao et al., 2000). Manconi et al. (2002) reported that tretinoin niosomes prepared from Span®60 by film hydration method gave higher entrapment than that of Span®40 niosomes. When a series of Span® and Brij® were examined, similar results were obtained (Uchegbu and Duncan, 1997; Manosroi et al., 2003; Suwakul et al., 2006). In another study Yoshioka et al. (1994) reported that the entrapment efficiency of carboxyfluorescein in niosomes prepared by hand shaking method varied with vesicle membrane composition. They reported that Span®60 and Span®40 gave higher entrapment than Span®20 and Span®80 and that Span®60 were the least leaky due to the highest phase transition temperature. In contrast, Manconi et al. (2006) revealed that entrapment efficiency of tretinoin loaded polyalkylglucoside niosomes depended on HLB of surfactant used. Increasing HLB values gave higher drug entrapment. However, the study of van Hal et al. (1996) who prepared lidocaine loaded polyoxyethylene alkyl ether niosomes found that neither an influence of hydrocarbon chain length nor an influence in the number of oxyethylene units could be detected.

2.2 Surfactant/lipid concentration

Surfactant/lipid concentration affects the drug entrapment of the vesicles. Increasing surfactant/lipid concentration resulted in increase drug loading since the number of vesicles formed is increased. Shahiwala and Misra (2002) reported that encapsulation capacity of nimesulide in Span® niosomes depended on total lipid concentration. High entrapment efficiency was observed when the vesicles were prepared with a high total surfactant or lipid concentration. This result is in accordance with those of Yoshioka et al. (1994) who concluded that Span®80 niosomes of carboxyfluorescein prepared from higher total lipid concentration gave higher drug entrapment than those prepared from lower total lipid concentration. Similarly, entrapment efficiency of 5-fluorouracil in Span® niosomes prepared by various methods was linearly increased when the total lipid was increased (Namdeo and Jain, 1999).

2.3 Membrane additives

CHO abolishes phase transition of the system and affects fluidity of the membrane. Addition of CHO may alter the physical structure of niosomes as well as drug entrapment. Yoshioka et al. (1994) revealed that entrapment efficiency for Span®60 and Span®40 niosomes of carboxyfluorescein prepared by hand shaking method increases with increasing CHO content. Dithranol entrapment in niosome prepared from Span®60 by film hydration method was increased when CHO content was increased due to CHO decreased the drug leakage (Agarwal et al., 2001). These results agree well with those of Hao et al. (2002) who reported that colchicine niosome prepared from Span®60 by evaporation method, entrapment capacity was increased with increasing CHO concentration. However, Namdeo and Jain (1999) reported that incorporation of CHO in Span®40 niosomes decreases 5-fluorouracil loading in the vesicles since the encapsulation volume is decreased. Whereas, Pardakhty et al. (2007) reported that inclusion of CHO into niosomes was not influenced on the entrapment of insulin.

2.4 Method of preparation

Method of preparation affects drug entrapment in niosomes. Acetazolamide entrapment of Span®60 and Span®40 niosomes prepared by film hydration method were higher than those of niosomes prepared by reverse phase evaporation method (Guinedi et al., 2005). Similarly, the rank order of tretinoin entrapment in Span®40 niosome was from film hydration (MLVs) > extrusion (LUVs) > sonication (SUVs) method (Manconi et al., 2002). On the other hand, entrapment efficiency of carboxyfluorescein are followed the rank order of ether injection > reverse phase evaporation > film hydration methods and the different may be due to the encapsulation volume and physical nature of vesicles (Baillie et al., 1985).

2.5 Physicochemical properties of the drug

Physicochemical properties of the drug govern the entrapment efficiency in niosome. For hydrophobic drugs, lipid packing seems to be major determinant. On the contrary, ionic interaction seems to play a major role for ionizable drugs (Philippot and Schuber, 1995). Hao et al. (2002) compared drug entrapment between 5-fluorouracil and colchicine in Span®60 niosomes. They reported that the entrapment efficiency of 5-fluorouracil (5-FU) and colchicines were similar due to the difference in the interaction between drug and membrane. Although 5-FU has a small molecular weight, it possesses two amides, while large molecular weight colchicine has only one amido group. Therefore, interaction between 5-FU and membrane is stronger than that of colchicine. Considering the molecular weight and interaction between drug and membrane, so entrapment efficiency of 5-FU and colchicine were similar. For ionizable drugs, chemical form of drug (acidic, basic, or salt form) and environmental pH conditions affect entrapment of the drug. Carafa et al. (2002**)** reported that entrapment efficiency of charged lidocaine in Tween®20 niosomes was higher than that from uncharged lidocaine. Lidocaine has pK_a of 7.8. At pH 5.5, most of lidocaine is positively charged while at pH 8.6, most of lidocaine is uncharged. Amount of lidocaine entrapped at pH 5.5 was higher than at pH 8.6, which lidocaine entrapped was negligible.

2.6 Temperature

The hydration temperature has an influence on encapsulation. The hydration temperature used to make niosomes should usually be above the gel to liquid phase transition temperature of the system. Hao et al. (2002) reported that when the hydration of colchicine entrapment in Span® niosome was performed at room temperature, the drug loaded was less than that at 60 °C. Method of preparation of PK1 loaded Span®60 niosomes involved hydration of lipid films with a solution of PK1 at 60 °C. However, they reported that PK1 precipitated from aqueous solution at 55 °C and this leads to a drastically reduced incorporation of PK1 into niosomes when high concentration of PK1 was used to hydrate lipid films (Uchegbu and Duncan 1997).

3. Factors affecting niosomes release

3.1 Surfactant structure

In vitro drug release is generally used to evaluate drug delivery from topical products. Previous studies reported that surfactant structure was influenced on drug release from niosomes. In general, short chain non-ionic surfactants produce more fluid membrane (liquid state bilayer) than membrane of long chain surfactants (gel state bilayer) and unsaturation in the hydrocarbon chain can lead to a more permeable membrane. Ruckmani et al. (2000) reported about the release of cytarabine hydrochloride from niosomes prepared from Span®60 and Span®80. They found that drug release from Span®60 niosomes was slower than that from Span®80 niosomes. In another study, niosomes prepared from Span®60 gave slower release than those prepared from Span®40 (Guinedi et al., 2005). This result agrees well with those of Yoshioka et al. (1994) who reported that the release of carboxyfluorescein from Span®60 and Span®40 was slower than that from Span®20, Span®80, and Span®85 niosomes. Accordingly, the release of drug from niosomes depended on surfactant structure or thermodynamic state of bilayer.

3.2 Membrane additives

CHO content affects membrane fluidity. If CHO decreases membrane fluidity, the drug release will be decreased when CHO content is increased. Uchegbu and Florence (1995) reported that increasing CHO content in $C_{16}G_2$-stearylamine niosomes of doxorubicin reduced drug release. This result agrees well with those of Namdeo and Jain (1999) and Guinedi et al. (2005). Namdeo and Jain (1999) reported that addition of CHO and DCP in Span®40 niosomes of 5-fluorouracil reduced drug release. Guinedi et al. (2005) found that increasing CHO content in Span®60 and Span®40 niosomes of acetazolamide also reduced drug release.

3.3 Physicochemical properties of the drug

The nature of solute affects the rate of solute release. In the experiments to quantify the release of carboxyfluorescein and doxorubicin from $C_{16}G_2$ niosomes, they found that doxorubicin gave greater release than carboxyfluorescein. This may be due to carboxyfluorescein would be fully ionized at working pH and thus its passage through the membrane would be high energy process, when compared to the passage of the partially ionized doxorubicin molecule through the membrane (Uchegbu and Florence, 1995). Ho et al. (1985) reported that the ability of entrapped glucose, hydrocortisone and progesterone to diffuse out of the liposomes decreases with increasing lipophilicity. However, the release of 5-fluorouracil from niosomes is the same as that of colchicine. The molecular weight and interaction between them and membrane also play an important role in release experiments (Hao et al., 2002).

4. Factors affecting niosomes stability

The stable niosome dispersion must exhibit a constant particle size and a content level of entrapped drug. There must be no precipitation of membrane components, which are to a large extent not insoluble in aqueous media. There are several methods to stabilize niosomal dispersion. The addition of cholesterol, charged molecules, and membrane stabilizers in the bilayer have been used to stabilized

niosomes. The incorporation of cholesterol into the systems can decrease the leakiness of the membrane. The inclusion of a charged molecule and membrane stabilizer also prevent niosome aggregation (Uchegbu and Yvas, 1998). Various factors can affect niosome stability. These include the following:

4.1 Surfactant structure

The chemical structure of membrane surfactant determines the nature of the membrane and thus also affects stability of the system. In making a choice of surfactant, the higher phase transition surfactants appear to yield more desirable stability and toxicity profiles. The details are as displayed in Figure 4.

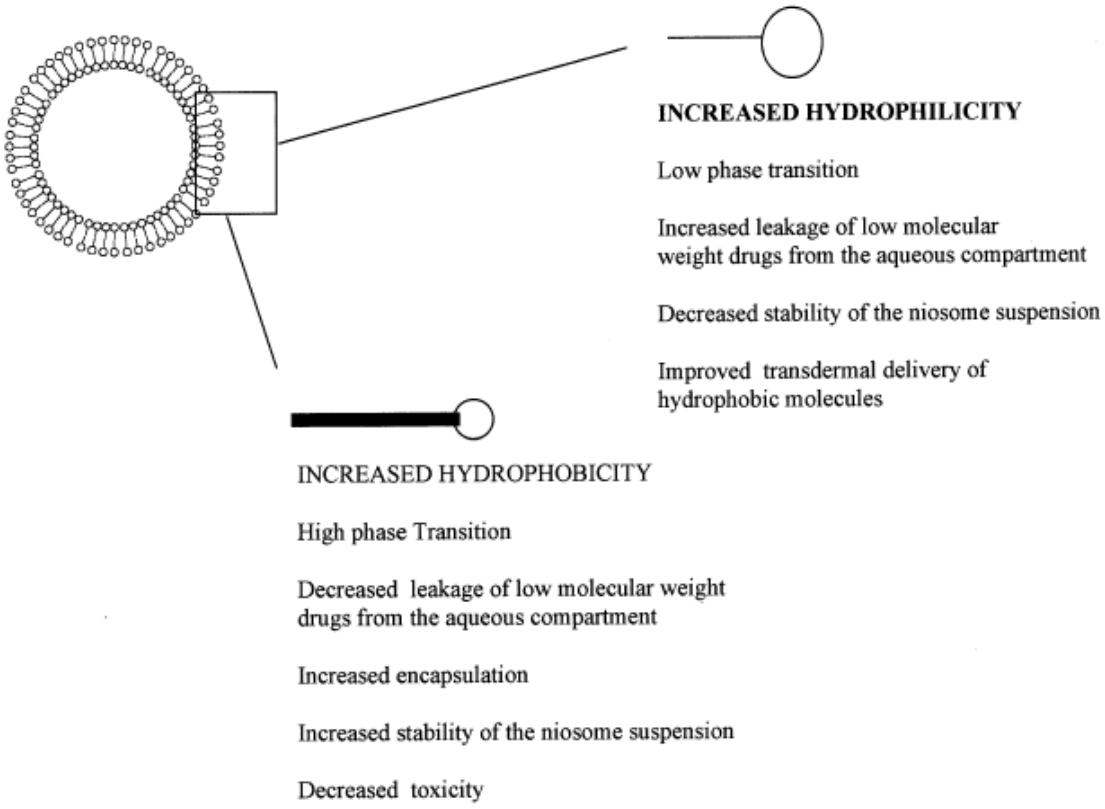

Figure 4 The effect of the choice of niosome forming surfactant on the properties of the niosomal dispersion (Uchegbu and Vyas, 1998)

Niosomes prepared from solid-state surfactant (Span®40 and Span®60) could better maintain the entrapped caffeine under storage conditions and were stable morphologically. On the other hand, liquid state vesicles from Span®20 changed entrapment efficiency of drug during the storage time. These significant alterations in stability markers may result from the fluidity of bilayers and leading to more leaky and unstable structure (Khazaeli et al., 2007). Moreover, Yoshioka et al. (1994) reported that the rank of the leakiness of carboxyfluorescein from niosomes prepared from a series of Span® surfactants was Span®80 > Span®20 > Span®40 > Span®60. Thus, surfactant structure affects the stability of niosomal system.

4.2 Membrane additives

Membrane additives may also affect stability of niosomes. Incorporation of charged molecule in the bilayer shifts the electrophoretic mobility making it positive with the inclusion of stearylamine and negative with the inclusion of DCP and also prevents niosome aggregation and led to prevention of creaming or sedimentation of niosomes due to electrostatic stability. In addition, the entrapment of hydrophobic drugs or macromolecule prodrugs (Gianasi et al., 1997) also increases the stability of these dispesions. In some systems, incorporation of polyoxyethylene carring addition such as Solulan®C24 causes steric stabilization (Uchegbu and Duncan, 1997). However, the destruction of $C_{16}G_2$ niosomes by high concentration of Solulan®C24 appears to solubilized and converted niosomes into mix micelles (Uchegbu, Bouwstra, and Florence, 1992).

4.3 Physicochemical properties of the drug

The entrapped drug could be the major determinant of niosome stability in terms of drug leakage. Acetazolamide was retained in niosome formulations for a relative long period of time (Guinedi et al., 2005). Similar results were obtained from nimesulide (Shahiwala and Misra, 2002) and cytarabine hydrochloride niosomes (Ruckmani et al., 2000). The entrapped polymer conjugate, PK1, was thought to lead a more stable system since the membrane was sufficiently

impermeable to the macromolecule (Gianasi et al., 1997). In choosing a suitable drug to be delivery by niosomes, it should be born in mind that niosomes encapsulating hydrophobic drugs and macromolecule are more stable than niosomes encapsulating low molecular weight drugs. In contrast, hydrophilic drugs can easily leak from niosomes and decrease the stability of niosomal dispersions. Encapsulation usually increases with amphiphilic drugs. Accordingly, transdermal drug delivery appears possible with hydrophobic or hydrophilic molecules as described in Figure 5.

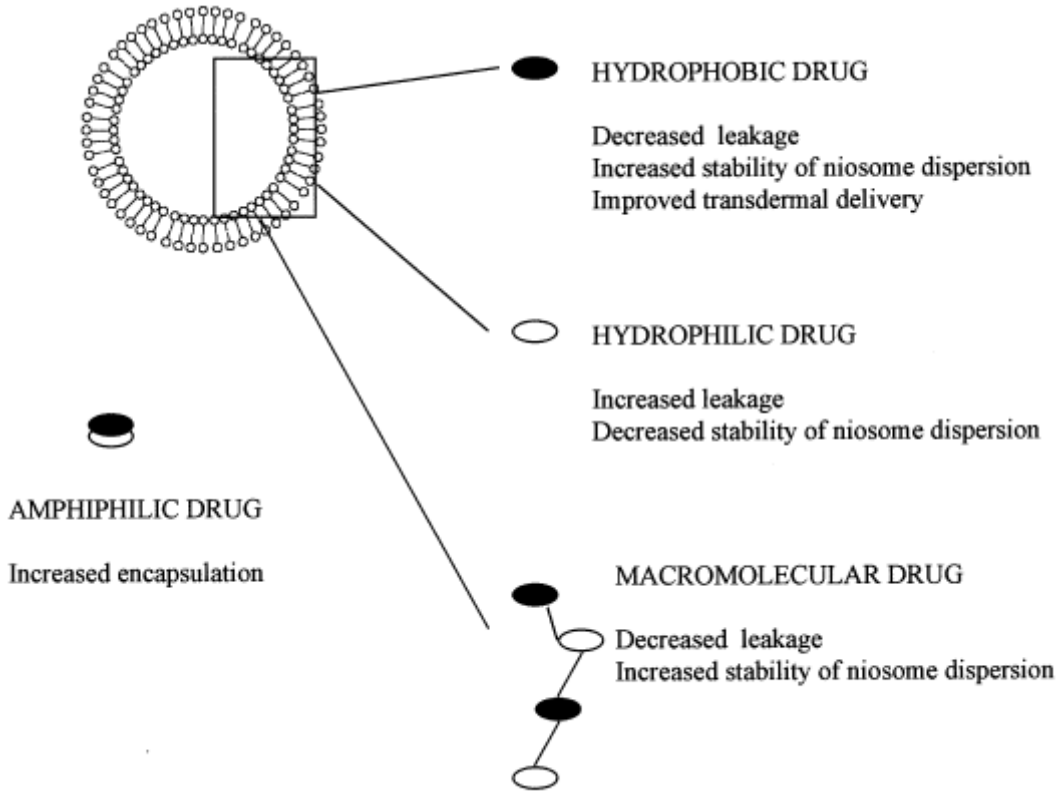

Figure 5 The effect of the nature of encapsulated drug on the properties of the niosomal dispersion (Uchegbu and Vyas, 1998)

4.4 Temperature

The temperature of storage may affect niosomes stability. Thus, storage temperature must be controlled. Changes in the temperature of the niosomal system often lead to a change in fundamental nature of the system, including drug leakage (Uchegbu and Vyas, 1998).

Niosomes as a topical drug delivery carrier

Liposomes and niosomes have been studied as potential carriers for topical skin delivery. Both hydrophilic and hydrophobic drugs can be incorporated in niosomal vesicles. Many hydrophobic drugs have been encapsulated in niosomes for topical applications such as piroxicam (Reddy and Udupa, 1993), enoxacin (Fang, Hong et al., 2001), levonorgestrel (Fang, Yu et al., 2001), dithranol (Agarwal et al., 2001), ketoconazole (Satturwar and Fulzele, 2002), lidocaine (Carafa et al., 2002), ketolorac (Ibrahim and Ahmed, 2004), and tretinoin (Manconi et al., 2006). Some hydrophilic drugs also have been incorporated in niosomes such as glycolic and glycerol (Ohta et al., 1996), 5-FU (Namdeo and Jain, 1999), cytarabine hydrochloride (Ruckmani et al., 2000), colchicine (Hao et al., 2002), insulin (Pardakhty et al., 2007), ammonium glycyrrhinate (Paolino et al., 2007), and ferulic acid (Muzzalupo et al., 2008). Niosomes have been reported to enhance penetration of many drugs through skin. Hofland et al. (1994) studied estradiol permeation from polyoxyethylene alkyl ether niosomes. It was concluded that direct contact between liquid-state MLVs niosomes and skin was imperative to exert the highest effect on drug transport. Niemiec et al. (1995) reported that niosomes prepared using glyceryl dilaurate, cholesterol, and polyoxyethylene-10-stearyl ether enhanced the topical delivery of peptide drugs into pilosebaceous units in the hamster ear model. Span®60 flurbiprofen niosomes in a hydroxypropyl methyl cellulose semi-solid base when applied transdermally gave a higher area under plasma level time curve than when administered orally in a saline suspension. It is likely that the use of hydroxypropyl methyl cellulose in this formulation may affect drug penetration and it is unclear as what effect the presence of this semi-solid base had on niosome integrity (Reddy and Udupa, 1993). Ohta et al. (1996) also demonstrated that glyceryl dilaurate niosomes delivered high amount of glycolic acid and glycerol into stratum corneum and the living skin strata while retarding systemic absorption. In conclusion, the effects of niosomes on drug transport through the skin can be enhancement, depending on the drug molecules and the vesicle compositions. Thus, niosomes appear to have potential as a drug carrier system for both dermal and transdermal delivery of drugs.

Vesicles affect drug transport across skin (Bouwstra and Honeywell-Nguyen, 2002)

One of the most controversial methods to increase drug transport across the skin is the use of vesicles. It has been generally accepted that the use of vesicles with proper composition should result in increase drug transport across the skin. Several studies were carried out to investigate whether niosome composition affects skin penetration of drugs. Hofland et al. (1994) studied the in vitro permeation of estradiol from niosomes in various thermodynamic stated through human stratum corneum. The gel state niosomes were composed of $C_{18}EO_3$ and the liquid crystalline vesicles consisted of $C_{12}EO_3$ and Brij96 ($C_{9=9}EO_{10}$). All formulations were saturated with estradiol making the thermodynamic activity equal among these formulations. They reported that the estradiol incorporated in gel state non-ionic surfactant resulted in a low drug transport rate through human stratum corneum compared to estradiol in liquid state bilayers. They also revealed that a drug applied in liquid state vesicles resulted in higher penetration rates than when applied in a phosphate buffered saline solution.

In another study, Tabbakhian et al. (2006) investigated the in vitro permeation of ^{3}H-finasteride from niosomes in various thermodynamic states through hamster flank skin. The liquid state niosomes were composed of Brij®97 and Brij®76:Brij®97 (3.5:3.5 in molar ratio). The gel state niosomes were consisted of Span®40, Brij®72, and Brij®76. They reported that the fluxes of finasteride from liquid state Brij®76:Brij®97 and Brij®97 niosome were higher, where each compared with the flux of drug from gel state Span®40 niosome. Differences in effect on drug transport between the liquid state and the gel state vesicles may be explained by the differences in skin-formulation interactions, either due to the extent of interaction between vesicles and the stratum corneum or differences in partitioning of the drug between the vesicles and the stratum corneum (Ganesan et al., 1984). The surfactant molecules of liquid state nature are thought to permeate into the intercellular lipid bilayers, thereby reduce the crystallinity of the intercellular lipid bilayers and thus increase the permeability of these bilayers. In contrast, surfactant molecules forming rigid gel state

bilayers can not penetrate into the stratum corneum, and thus they are not able to induce a penetration enhancer effect (Hofland et al., 1994). Similarly, the packing nature of unsaturated fatty acids (i.e Brij®97) can change the fluidity of stratum corneum lipid structure and facilitate the skin permeation of drug (Fang, Hong et al., 2001). Thus, transfer of drug from the lipid bilayers into skin can occur as long as the bilayers are in a liquid crystalline state.

As reported by du Plessis et al. (1994), the effect of vesicle size and lamellarity on drug deposition was minimal suggesting that intact vesicles transport does not occur. They concluded that intact penetration of liposome does not occur. It seems that the physical parameters as vesicle size and lamellarity are less important than the thermodynamic state of the bilayers and the application method.

In vitro permeation studies

The in vitro study of drug skin permeability plays an essential role in the selection of candidates for the development of transdermal dosage forms. Such experiments are generally performed by using a diffusion cell whose donor and receiver compartments are separated by a membrane as described in Figure 6.

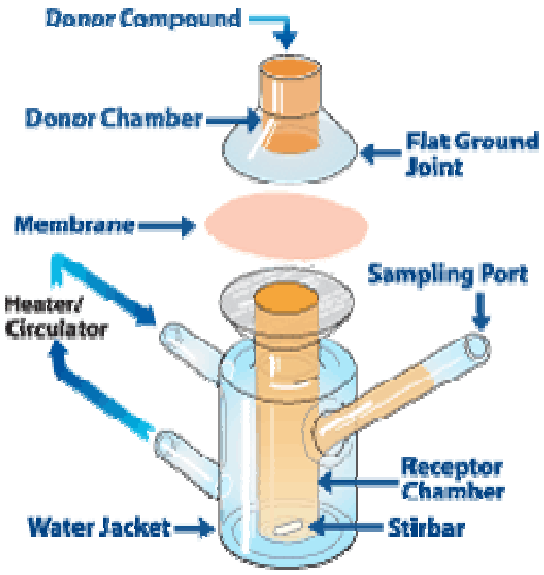

Figure 6 A schematic drawing of Franz diffusion cell (http://www.permegear.com)

Human skin, either excised from surgical reduction or obtained from a cadaver, is the membrane of choice, but it is not readily available. Thus, in the last decades many efforts have been made to individuate a suitable alternative using synthetic and biological membranes. The former can not be used to replace human skin in an experimental context. In the setting of animal models for transdermal permeation studies, the characteristics of excised skin from mice, rats, rabbits, and pigs were thoroughly investigated and widely used. However, animal skin is different from human skin in several features. Indeed, the main barrier to drug permeation through skin is the stratum corneum, which has been reported to differ in terms of lipid composition, water content, and morphological characteristics (thickness, number of pores and follicles) on the basis of species. Pig stratum corneum is the most similar to human stratum corneum in terms of lipid composition, but it presents a marked difference in terms of thickness (Hammond, Tsonis, and Sellins, 2000). On the other hand, the thickness of newborn pig skin stratum corneum is considerable thinner than that of adult pig and more similar to that of human skin, even if the number of hair follicles is higher than that of human or adult pig skin (Cilurzo, Paola, and Chiara, 2007).

The frequently employed technique to test the relative permeability of topical drug involves the in vitro use of excised skin mounted in diffusion chambers. In this case, the release of drug is controlled by membrane and the Fick's first law is applicable because the absorption mechanism is usually a passive diffusion (Martin, 1993). An equation of an amount (M) of material flowing through a unit cross-section (A) of a barrier in unit time (t) is known as the flux (J). Therefore, the Fick's first law of diffusion is derived as follows:

$$J = \frac{dM}{A.\,dt} \qquad (1)$$

If a membrane separates the two compartments of a diffusion cell with cross-sectional area (A) and thickness (h), and if the concentrations in the membrane on the donor and receptor chamber are C_1 and C_2, respectively, equation (1) may be written as:

$$J = \frac{D(C_1 - C_2)}{h} \quad (2)$$

in which $(C_1 - C_2)/h$ approximates dC/dx. The gradient $(C_1 - C_2)/h$ within membrane must be assumed to be constant for a quasi-stationary state to exist. Equation (2) presumes that the aqueous boundary layers on both sides of the membrane do not significantly affect the total transport process.

The concentration C_1 and C_2 within the membrane ordinary are not known but can be replaced by the partition coefficient (K) multiplied by the concentration in the donor side (C_d) or the receiver side (C_r). The partition coefficient (K) is given by

$$K = \frac{C_1}{C_d} = \frac{C_2}{C_r} \quad (3)$$

Therefore, equation (2) can be written as:

$$\frac{dM}{dt} = \frac{DAK(C_d - C_r)}{h} \quad (4)$$

and, if sink conditions hold in the receptor compartment or $C_r = 0$,

$$\frac{dM}{dt} = \frac{DAKC_d}{h} = PAC_d \quad (5)$$

in which

$$P = \frac{DK}{h} \quad (6)$$

where P is a permeation coefficient (cm/sec). Eventually, the amount of drug permeating into a sink bears the following relationship to the time:

$$M = PA\, C_d t \quad (7)$$

The cumulative amount of drug presented in the receptor compartment during the *n* th sampling (Q_n) was estimate by

$$Q_n = C_n \times V + V_s \times \sum_{i=1}^{n-1} C_i \quad (8)$$

where C is the measured concentration in the n th sample, V is the volume of receptor solution, and V_s is the volume of sampling. Permeation profile is constructed by plotting the cumulative amount of drug permeation per diffusion area against time. The steady-state flux (J_{ss}) of drug permeation is determined from the slope of the permeation profile. The permeability coefficient (P) can be then obtained from the steady-state flux dividing by C_d, based on the fact that drug concentration in the receptor compartment is negligible compared with that in the donor compartment (C_d) (Yu and Liao, 1996), equation may be written as:

$$P = J_{ss}/C_d \quad (9)$$

Toxicity and irritation studies

An important aspect of the safety assessment process for any topical product is determination of their capacity to produce adverse skin effects such as irritation. An assessment of skin irritation is required during the product development process for skin care products, and prior to manufacturing and marketing of new products, in order to help ensure worker and consumer safety. Neither the length of the polyoxyethylene chain or alkyl chain had any influence on the skin toxicity of alkyl polyoxyethylene niosomes as assessed by cell proliferation of human keratinocytes in vitro (Hofland et al., 1991; Hofland et al., 1992). However, the nature of the linkage in the surfactant molecule was a determining factor in this model, and the more labile ester bond was found to be more toxic than the ether bond.

Hemocompatibility studies are often used to evaluate toxicity of parenteral preparation. $C_{16}G_2$ and Span®60 niosomes containing 10 mol% Solulan®C24 caused less than 5% hemolysis of rat erythrocytes after 5 hours of incubation. This level of hemolysis is not considered significant since both $C_{16}G_2$ and Span®60 niosomes were

rapidly cleared from the plasma after dosing (Uchegbu and Duncan, 1997). Solulan®C24 was found to be toxic to Caco-2 cells in vitro. However, when incorporated into niosomes at 10 mol%, its toxicity was dramatically reduced. There was an increase in toxicity when the level of Solulan®C24 in niosomes was increased above 10 mol%. At above 10 mol%, Solulan®C24 is not completely incorporated into the membrane of $C_{16}G_2$ niosomes and is thus present in solution as monomers or micelles (Uchegbu and Vyas, 1998).

The cholesterol content does not appear to have any effect on the proliferation of the keratinocytes, implying lack of toxicity of the lipid. The toxicity of alkyl polyoxyethylene niosomes on the nasal mucosa revealed that as increase in alkyl chain length was accompanied by a decrease in toxicity, whereas an increase in the polyoxyethylene chain length caused an increase in ciliotoxicity (Hofland et al., 1991; Hofland et al., 1992). The toxic effect of two niosomal preparations (Span®80:CHO:Solulan®C24 and $C_{16}G_2$:CHO:Solulan®C24) were thought to be principally a result of the amount of free surfactants present in the niosomal suspensions. In a previous report, free Solulan®C24 at a level of 0.1% w/v was very toxic to Caco-2 cells, whereas the same concentrations of Solulan®C24 in niosomes had no effect on the cell viability, using MTT test. However, some toxicity was observed at higher level of Solulan®C24 in niosomal form (Dimitrijevic et al., 1997). Degree of toxicity varies, depending on individual surfactants. In one report using rabbit skin tests, polysorbate caused more irritation than sorbitans, and polyoxyethylene ethers caused the greatest irritation (Mezei et al., 1966).

The evaluation of the irritation potential of chemicals in vivo is traditionally conducted in animals, particularly in rabbits using Draize test method (Singh and Maibach, 1998). However, due to increasing concern over animal use and in lights of its potential ban in the near future, alongside with the obvious ethical implications of using directly human subjects, in vitro alternative methods now be encouraged (Martinez et al., 2006). In vitro skin irritation test methods can address two key needs: (1) to provide initial screening or confirmatory data prior to human skin exposures to a new ingredient or formulation, within classes of materials for which the in vitro

methods are validated; and (2) to meet specific regulatory requirement for skin safety data (Pape et al., 1999). Different in vitro approaches have been proposed for assessing skin irritation as an alternative to the Draize eye irritation test, including the use of cultured cell such as human epidermal keratinocytes (Hofland et al., 1991; Hofland et al., 1992; Martinez et al., 2006), fibroblast cell line and macrophage cell line (Pape and Hoppe, 1991), lactate dehydrogenase assay, MTT assay, and neutral red assay (Welss, Basketter, and Schroder, 2004). The red blood cell (RBC) lysis assay has also been introduced as in vitro reliable alternative model (Pape, Pfannenbecker, and Hoppe, 1987; Pape and Hoppe, 1991; Pape et al., 1999).

An in vitro red blood cell lysis assay presented sensitivity and specificity exceeded 80% and showed the least discordance with the Draize test in different validation studies (Pape et al., 1987; Pape and Hoppe, 1990; Pape et al., 1999). This assay allows the estimation of the irritation potential of surfactants and surfactant-containing materials. The estimation is based on the fact that surfactants interact strongly with cellular membranes. This effect measured photometrically via the use of an inherent native dye, oxyhemoglobin. Though the procedure was not designed primarily for skin irritation, it should be able to rank the irritation potentials of the test products, if a suitable positive control is present.

The protocol of the in vitro red blood cell assay describes an approach based on the use of red blood cells to quantify adverse effects of surfactants and detergent products on the cytoplasmic membrane (hemolysis). This can be sensitively detected by following changes in the photometrical absorbance of oxyhemoglobin, an indicator of the process. Generally, safety testing of potential products is primarily related to the injury of accidental applications, in particular to mucous membranes. The predominant interest is therefore direct to the first step of its elicitation, which is known to be the damage of cellular membranes. The red blood cell assay can be used routinely to assess irritancy in safety evaluation of surfactant and tensidoactive consumer goods. The assay is inexpensive, does not require special equipment, and needs only one hour per sample. The test can also be used as a rapid screening assay in a first-order in vitro test battery for the assessment of acute eye irritation potential.

Hemoglobin release is an excellent end-point of cytoplasmic membrane integrity. Oxyhemoglobin is also denatured by surfactants, therefore to take this into account, measurements are made at 575 and 540 nm to monitor the spectral changes of the protein as a result of tenside denaturation. The red blood cell test is not proposed as a global alternative to the Draize test, as part of a practical in vitro test battery. It is far less expensive than other cell culture test and commercial systems. On the contrary, the hemolysis has been designed as a test for chemicals, for which lysis of membranes constitutes their mechanism of action. Thus, red blood cell test is proposed as a bioassay for predicting the hemolytic and damaging effect of tensides or surfactants at large on the plasma membrane. The hemolytic potency alone may not be sufficient to characterize fully the irritation potential of tensides. However, it can sufficiently rank the chemical of interest among the known tenside entities if a suitable standard is also included in the test.

CHAPTER III

MATERIALS AND METHODS

Materials

1. Acetic acid (Merck, Germany, Lot no. K35040963 533)
2. Brij®52 (EAC Chemical, Thailand, Lot no. 11930)
3. Brij®76 (EAC Chemical, Thailand, Lot no. 11935)
4. Cholesterol (Sigma, USA, Lot no. 072K5313)
5. Chloroform (Lab-Scan, Ireland, Lot no. 05111278)
6. Dialysis membrane (Regenerated cellulose tubular membrane, MWCO 12,000-14,000 (CelluSep® T4, Canada, Lot no. 8764)
7. Disodium hydrogen phosphate (Merck, Germany, Lot no. F1021786 125)
8. Glyceryl distearate (Stepan Company, USA, Lot no. 7067373)
9. Glycolic acid (Fluka, USA, Lot no. 1300322 14306165)
10. Hydrochloric acid (Merck, Germany, Lot no. K29322217 121)
11. Isopropanol (Lab-Scan, Ireland, Lot no. 08070032)
12. Methanol (Lab-Scan, Ireland, Lot no. 07070007)
13. Polyoxyethyleneglycol-8-laurate (Stepan Company, USA, Lot no. 7015816)
14. Potassium dihydrogen phosphate (Merck, Germany, Lot no. A315973 127)
15. Phosphoric acid (J.T.Baker, USA, Lot no. 7664382)
16. Sodium acetate (Merck, Germany, Lot no. TA914067 203)
17. Sodium chloride (Merck, Germany, Lot no. K32104204 324)
18. Sodium hydroxide (Merck, Germany, Lot no. UN106498)
19. Solulan®C24 (Amerchol, UK)
20. Span®20 (EAC Chemical, Thailand, Lot no. 16790)
21. Span®40 (EAC Chemical, Thailand, Lot no. 11036)
22. Span®60 (EAC Chemical, Thailand, Lot no. 16794)
23. Sucrose laurate ester (Mitsubishi-Kagaku Foods corporation, Japan, Lot no. 44021111)

24. Sheep whole blood (Animal Husbandry Department, Faculty of Veterinary, Chulalongkorn University, Thailand)
25. Newborn pig skin (Ratchaburi Farm, Thailand)

Equipment

1. Analytical balance (AG285, Mettler Toledo, Switzerland)
2. Analytical balance (UMTZ, Mettler Toledo, Switzerland)
3. Centrifuge (ALC® Centrifugette 4206, Italy)
4. Digital camera (Nikon Coolpix X 5400, Japan)
5. Disposable syringe filter (nylon 13 mm, 0.45 µm) (Chrom Tech, USA)
6. Dry bath incubator (Boekel Scientific, Japan)
7. High performance liquid chromatography system equipped with
    - Automatic sample injector (SIL-10A, Shimadzu, Japan)
    - Communications bus module (CBM-10A, Shimadzu, Japan)
    - Column (BDS Hypersil® C18, 5 µm, 250 x 4.6 mm, Lot no. 8323)
    - Liquid chromatograph pump (LC-10AD, Shimadzu, Japan)
    - Precolumn (µBondapak C18, 10 µm, 125 Aº, Water, Ireland)
    - UV-VIS detector (SPD-10A, Shimadzu, Japan)
8. Light microscope (Nikon Eclipse E 200, Japan)
9. Microcentrifuge (Hermle Z230 MA, Germany)
10. Micropipette (Gilson, France)
11. Microplate reader (Anthos htl, Anthos Labtec Instrument, Austria)
12. Modified Franz Diffusion cells (Science Service, Thailand)
13. Orbital shaker (S05, Stuart Scientific, UK)
14. pH meter (Sartorious, USA)
15. Polycarbonate ultracentrifuge bottles (Beckman, USA, Lot no. A60519)
16. Sonicator (Trans-sonic Digital, Elma, Germany)
17. Ultracentrifuge (L80, Beckman, USA)
18. Vortex mixer (G 560 E, Vortex-genie, USA)
19. Water bath (Memmert, Germany)

Methods

1. Development of glycolic acid (GA) niosomes

1.1 Preparation of blank niosomes at pH 4.0

Niosomes were prepared by sonication method that was devoid of organic solvents. The method used was modified from that of Baillie et al. (1985). The total lipid concentration (surfactant plus cholesterol and/or Solulan®C24) used in all formulations was 100 mg/mL. The non-ionic surfactants used to form niosomes in this study were Span®20, Span®40, Span®60, Brij®52, Brij®76, surcrose laurate ester (L-595), and glyceryl distearate (GDS). The compositions (weight ratio) of non-ionic surfactant, cholesterol, with or without Solulan®C24 chosen from a previous work (Suwakul et al., 2006) were as follows:

1) Span®20:CHO (60:40)
2) Span®40:CHO (70:30)
3) Span®60:CHO (60:40)
4) Brij®52:CHO (70:30)
5) Brij®76:CHO (50:50)
6) L-595:PEG-8-L (50:50)
7) GDS:CHO:Brij®76 (45:15:40)
8) Span®20:CHO:Solulan®C24 (57.5:37.5:5)
9) Span®40:CHO:Solulan®C24 (67.5:27.5:5)
10) Span®60:CHO:Solulan®C24 (57.5:37.5:5)
11) Brij®52:CHO:Solulan®C24 (67.5:27.5:5)
12) Brij®76:CHO:Solulan®C24 (47.5:47.5:5)

A mixture of surfactant and cholesterol was accurately weighed and melted in a 10 mL glass tube in a dry bath incubator at 130 °C. The aqueous phase, acetate buffer pH 4.0, which had previously been warmed and kept at 70 °C, was then added to the melted mixture. The mixture was immediately sonicated at 70 °C for 10 minutes using an ultrasonic bath (Elma Transsonic Digital type 680 DH) at 140%

power (40 kHz) and then vortexed for 1 minute. The resultant preparation was left to cool down at room temperature. The niosomal suspension was assessed by eyes and under microscopic observation (at 400x and 1000x) for completeness of vesicle formation and lipid remnants. The acceptance criteria was milky suspension, homogeneity, spherical particles, birefringence of bilayers, and absence of surfactant, CHO, and Solulan®C24 crystals. The experiment was done at least in triplicate before the formulation was accepted as feasible for vesicle formation in acetate buffer pH 4.0.

1.2 Preparation of GA niosomes

The compositions of blank formulations with complete vesicle formation were selected to prepare niosomes containing glycolic acid. The aqueous phase for GA niosomes used in this experiment was 60 mg/mL GA in acetate buffer pH 4.0. This concentration was below the saturation solubility of GA in water (1.6 g/mL) at ambient temperature (≥ 25 °C). The effective concentration of GA most widely used in cosmetic products is 4 to 10% glycolic acid at pH 3.8-4.0 (Johnson, 2002). The method of preparation and acceptance criteria were the same as described in Section 1.1. Additionally, the pH of individual GA niosomal suspensions was measured. All preparations were regularly monitored for physical stability. Care was taken to detect any aggregation of vesicles, changes in color and pH, and presence of surfactant, CHO, and Solulan®C24 crystals under the microscope. The experiment was done at least in triplicate before the formulation was accepted as GA niosomes.

2. Characterization of GA niosomes

GA niosomes were characterized in terms of morphology, size and size distribution, entrapment efficiency, and short-term (2 months) physical stability.

2.1 Morphology

2.1.1 Optical microscopy

In this study, the overall quality of the preparation such as vesicle shape and presence of lipid crystals were monitored with an optical microscope (Nikon Eclipse E 200, Japan) at 400x and 1000x magnifications.

2.1.2 Polarized light microscopy

Polarized light microscopy can be used to verify existence of vesicular bilayers in the preparation (Manosroi et al., 2003). A drop of sample was placed on a glass slide and examined between two crossed-polarizing filters under a light microscope. The polarized light photomicrographs were recorded using a digital camera (Nikon Coolpix X 5400, Japan).

2.2 Determination of size and size distribution

Size and size distribution of the vesicles were determined by laser diffraction technique (Guinedi et al., 2005) (Mastersizer 2000, Malvern Instruments, UK). The refractive index was set at 1.60 with polystylene nature. Size and size distribution were expressed as D [4,3] as recommended by the manufacturer of the instrument. The experiment was done in triplicate with pooled samples.

2.3 Determination of GA entrapment efficiency

After preparation, the vesicular suspension was left at room temperature overnight to allow complete annealing and equilibrating of GA between the lipid and the aqueous phases. The suspension was then separated into supernatant containing free drug and the pellet containing the entrapped drug by ultracentrifuge. The GA content in the pellet was analyzed and used to calculate the percentage of entrapment and entrapment efficiency (EE) by using equations 10 and 11. GA in the supernatant was also assayed for routine monitoring of total recovery. The HPLC assay of GA was carried out using the modified method described in Gomis (1992) and Couch and Howard (2002). The analytical method was validated using the guidelines in USP 29

(The United States Pharmacopieal Convention, 2006). The validation results for accuracy, precision, linearity, and specificity are shown in Appendix C. The experiment was done in triplicate with pooled vesicles from at least three batches of each formulation.

2.3.1 Separation of pellets

Five milliliters of the aqueous phase was added to an aliquot (1 mL) of vesicular suspension to aid the centrifugation process. The suspension was then centrifuged (L80, Beckman, USA) at 65,000 rpm at 25 °C for 4-8 hours. The supernatant was carefully separated from the pellet. GA contents in the pellet and the supernatant were determined.

2.3.2 Quantitative analysis of GA in pellets

The niosomal pellet was dissolved in 95% isopropanol in a 25 mL volumetric flask and the solution was adjusted to volume. One hundred microliters of this solution was further diluted with the mobile phase in a 5 mL volumetric flask. The final solution was assayed by HPLC method at 210 nm (see below). The presence of surfactant and/or cholesterol in the pellets did not interfere with the assay.

2.3.3 Quantitative analysis of GA in supernatants

The supernatant was collected and diluted with water in a 25 mL volumetric flask and the solution was adjusted to volume. Thirty microliters of this solution was further diluted with the mobile phase in a 5 mL volumetric flask. The final solution was assayed by HPLC method at 210 nm (see below).

2.3.4 Calculation of the percentage of entrapment and entrapment efficiency

The percentage of GA entrapment of each preparation was determined from the following equation:

$$\% \text{ Entrapment} = \frac{\text{Amount of GA in pellet x 100}}{\text{Total loading amount of GA}} \quad (10)$$

Entrapment efficiency was defined as the fraction of GA found in niosomal pellets and expressed as milligram of GA in the pellet per milligram of total lipid (Uchegbu and Vyas, 1998).

$$\text{Entrapment efficiency} = \frac{\text{Amount of GA in pellets (mg/mL)}}{\text{Amount of total lipid (mg/mL)}} \quad (11)$$

2.3.5 Quantitative analysis of GA by HPLC method

2.3.5.1 HPLC conditions

The concentration of GA was determined by HPLC method. The HPLC conditions modified from Gomis (1992) and Couch and Howard (2002) were as follows:

Column	:	BDS Hypersil® C18, 5 µm, 250 X 4.6 mm
Precolumn	:	µBondapak C18, 10 µm, 125 Aº
Mobile phase	:	0.025 M phosphate buffer, pH 2.25
Injection volume	:	20 µL
Flow rate	:	0.8 mL/min
Detector	:	UV detector at 210 nm
Temperature	:	ambient
Run time	:	12 min
Internal standard	:	citric acid (20.0 µg/mL)

2.4 Physical stability of GA niosomes

All accepted preparations of GA niosomes from Section 1.2 were prepared by sonication method and were kept in glass bottles tightly sealed with cap closures and paraffin films. All preparations were kept at ambient temperature in the form of

niosomes without further separation. The physical stability was studied by monitoring aggregation, changes in color and pH, morphology, size and size distribution, entrapment at 0, 1, and 2 months (Vora, Khopade, and Jain, 1998). The experiments were performed in triplicate with pooled samples.

3. Release studies

The release of GA from the selected formulations was studied utilized modified Franz diffusion cell (Manconi et al., 2006; Suwakul et al., 2006). The dialysis membrane was used to separate the donor and the receptor compartments.

3.1 Preparation of dialysis membrane

A dialysis membrane (cellulose tubular membrane, Cellu-Sep®) with a molecular weight cut-off of 12,000-14,000 separated the donor and the receptor compartments. The membrane was cut into a circular shape, with a diameter of 3 cm, and soaked in purified water overnight. Before being mounted onto a diffusion cell, the membrane was rinsed with boiling water to wash off any soluble contaminants. The membrane was then soaked in acetate buffer pH 4.0 for at least 30 minutes before used.

3.2 Release studies

A GA solution (60 mg/mL in acetate buffer pH 4.0) and five formulations of GA niosomes: 1) Span®20:CHO:Solulan®C24 (57.5:37.5:5) 2) Span® 40:CHO:Solulan®C24 (67.5:27.5:5) 3) GDS:CHO:Brij®76 (45:15:40) 4) Brij® 52:CHO:Solulan®C24 (67.5:27.5:5) 5) Brij®76:CHO:Solulan®C24 (47.5:47.5:5) were selected to study. The choices of formulations were based on high GA entrapment of the vesicles and good physical stability in 2 months. The solution of GA in acetate buffer pH 4.0 at the same GA concentration was also tested as a reference. Acetate buffer pH 4.0 was used as the receptor fluid.

Modified Franz diffusion cells consisting of the donor and the receptor compartments were used to study in vitro release of GA from different niosomal formulations and GA solution. The internal diameter of the cell ranged from 1.70-1.75 cm, corresponding to an effective permeable surface area of 2.27-2.41 cm^2. The receptor compartment was equipped with a magnetic stirring bar rotating at 800 rpm and the temperature was kept constant at 37 °C by circulating water through a jacket surrounding the cell body throughout the experiment. The receptor compartment contained 14.00-14.48 mL (from calibration) of acetate buffer pH 4.0 as the release medium. The soaked membrane was clamped in place between the donor and the receptor compartments of the cell. The receptor fluid and the membrane in Franz diffusion cell were equilibrated to the desired temperature for 30 minutes. After equilibration, 1 mL of the formulation was carefully placed on the membrane surface of each cell and the cell was then covered completely and tightly with paraffin film. The receptor fluid (3 mL) was removed at 0.5, 1, 1.5, 2, 3, 4, 6, 8, 10, 12, 14, 16, and 24 hours and replaced with an equal volume of fresh medium. The sample taken was diluted as appropriate and the final solution was assayed by HPLC method at 210 nm. The release study for each formulation was done in quadruplicate.

3.3 Data treatment

The percent of GA released was calculated by the following equation:

$$\% \text{ GA released} = (A_t/A_o) \times 100$$

where A_t is the cumulative amount of GA released at a particular time; A_o is the initial amount of GA in the formulation.

3.4 Quantitative analysis of GA by HPLC method

The concentrations of GA in niosomal suspension and in receiver medium were determined by HPLC method. The HPLC conditions were the same as described under Section 2.3.5.

4. Permeation studies

The permeation of GA from the formulations was studied utilizing modified Franz diffusion cell. The abdominal skin of a newborn pig was used as the membrane (Cilurzo et al., 2007).

4.1 Preparation of newborn pig skin membranes

The abdominal newborn pig skin was completely separated from the subcutaneous fat and tissue using scissors and forceps. The separated skin was cleaned in purified water and then was wrapped in aluminium foil and stored in a freezer (-20 °C) until used. The frozen skin was thawed and immersed in phosphate buffer saline (PBS), pH 7.4, at room temperature for about one hour before used. The hydrated skin was cut to a circular shape with a diameter of about 3 cm.

4.2 Permeation studies

Modified Franz diffusion cells were used to study in vitro permeation from different formulations (Alsarra et al., 2005; Manconi et al., 2006). The selected formulations and the procedure of the study were the same as that of the release study in Section 3 except that the membrane used in this study was newborn pig skin and the receptor medium was PBS, pH 7.4. 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 were equilibrated to the desired temperature for 30 minutes. After equilibration, 176 $\mu L/cm^2$ of the GA niosomes or control solution (GA 60 mg/mL in acetate buffer pH 4.0) was carefully placed on the membrane surface of each cell and the system was run under the non-occlusive condition. Samples (about 1 mL) were withdrawn from the receptor compartment at 2, 4, 6, 8, 10, 12, 14, 20, and 24 hours and replaced with an equal volume of pre-thermostated (37 °C) fresh PBS. Addition of fresh PBS to the receptor compartment was performed with great care to avoid air trapping beneath the dermis. Then samples were analyzed at the day of permeation experiment by HPLC method at 210 nm. The analytical method was validated using the guidelines in USP 29 (The United States

Pharmacopieal Convention, 2006). The validation results for accuracy, precision, linearity, and specificity are shown in Appendix C. Each set of experiments was performed with six diffusion cells.

4.3 Skin retention of GA

At the end of permeation study the skin surface and the donor cap were washed 3 times with 95% isopropanol. The isopropanolic solution containing GA remaining in the donor compartment was appropriately diluted and analyzed by HPLC method at 210 nm. The skin was then removed from the receptor compartment. The skin was cut into small pieces and extracted with 95% isopropanol (2 mL) by shaking at ambient temperature for 4 hours followed by sonicating for 30 minutes and filtering through a membrane filter (0.45 μm). The filtrate was assayed for GA accumulated in the skin by HPLC method.

4.4 Data treatment

For each permeation experiment, the cumulative permeated amount of GA per diffusion area was plotted against time (Hofland et al., 1994). The observed steady state flux (J_{ss}) was obtained from the slope. Permeation parameters were calculated using the following equations:

Permeation coefficient (P_s) = J_{ss}/C_d

Enhancement factor (EF) = (P_s of the formulation)/(P_s of control)

Enhancement factor of Q_s = (Q_s of the formulation)/(Q_s of control)

Enhancement factor of Q_{24} = (Q_{24} of the formulation)/(Q_{24} of control)

Where C_d is GA concentration in the donor compartment

Q_s is GA accumulated amount in the skin

Q_{24} is cumulative amount of GA in the receptor medium at 24 hours

control is GA 60 mg/mL in acetate buffer pH 4.0

4.5 Quantitative analysis of GA by HPLC method

The concentrations of GA in the donor compartment, the skin, and the receptor medium were determined by HPLC method. The HPLC condition was the same as described under Section 2.3.5.

5. Estimation of the irritation potential of GA niosomes

The GA niosomal suspension was aimed for reducing irritation when compared to conventional dosage forms such as solution. As a topical product, its potential to cause severe irritation to mucous membranes should be estimated before efficacy studies in animals or human can be carried out. Niosomes were selected (as previously described in Section 3.2) to be investigated for irritation potential as follows by the INVITTOX protocol No.37 (Pape et al., 1987; Pape and Hoppe, 1990).

5.1 Preparation of phosphate buffered isotonic saline (PBS), pH 7.4

PBS, pH 7.4, was prepared according to the formula that follows (http//www.ecvam.jrc.it):

Na_2HPO_4	22.2	mmol/L
KH_2PO_4	5.6	mmol/L
NaCl	123.3	mmol/L
Glucose	10.0	mmol/L

The vehicle used was Ultrapure® water. The buffer was stored at 4 °C and used within one week.

5.2 Preparation of red blood cells (RBC)

Blood sample (10 g) were weighed and centrifuged at 4,000 rpm for 12 minutes (ATC® Centrifugette 4206, Italy). The supernatant was carefully separated from the RBC. The RBC was washed four times with PBS, pH 7.4. This washing procedure removed the bulk of the white cells, any traces of plasma, and the buffy

coat. Red blood cells were counted with a hematocytometer. The RBC suspension was appropriately diluted to contain about 8 x 10^9 cells/mL.

5.3 Hemolysis study (Monchida Kanjanapadit, 2005)

5.3.1 Preparation of standard solutions

A stock solution of sodium dodecyl sulfate (SDS) was prepared by accurately weighing 10.0 mg of SDS into a 10 mL volumetric flask. SDS was dissolved and the solution was adjusted to volume with PBS. This solution had a final concentration of 1 mg/mL. The following volumes of the stock solution were pipetted into reaction vials: 0, 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 µL. Each vial was filled up to 900 µL with PBS. An aliquot (100 µL) of RBC suspension containing about 8 x 10^9 cells/mL was added into the reaction vials and the vials were shaken for 30 seconds. The mixture was incubated for 10 minutes on an orbital shaker (S05, Staurt Scientific, UK) at 150 rpm at room temperature. The incubation period was terminated by centrifugation at 13,000 rpm for 1 minute in a microcentrifuge (Hermle Z230 MA, Germany). This removed intact cells and debris from the medium. After centrifugation, aliquots of the supernatant (100 µL) were immediately separated and placed into a 96-well plate and the absorbance monitored photometrically at 570 nm against the blank using microplate reader (Anthos htl, Anthos Labtec Instrument, Austria). The blank consisted of the sample diluted with PBS without RBC.

5.3.2 Preparation of negative and positive controls (0% and 100% hemolysis)

Spontaneous hemolysis was monitored by adding 100 µL of RBC suspension to 900 µL of PBS. This gave zero hemolysis value (negative control). Another aliquot 100 µL of RBC suspension was added to 900 µL of distilled water to give the 100% hemolysis value (positive control). The procedure was similar to that described under Section 5.3.1.

5.3.3 Preparation of test samples

5.3.3.1 GA niosomes and GA solution

GA niosomes were prepared as describes under Section 1. The GA solution (60 mg/mL) was prepared in acetate buffer pH 4.0. The procedure was the same as that described under Section 5.3.1. The percentages of GA niosomes and solution used were as follows: 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10% v/v in PBS pH 7.4. These amounts corresponded to the detection limit of the assay. However, since niosomal vesicles were much smaller than RBC and could interfere to the assay due to turbidity, the samples containing niosomes were centrifuged at 65,000 rpm at 25 °C for 2 hours in an ultracentrifuge after the intact RBC and cell debris had been removed.

5.3.3.2 Blank niosomes and acetate buffer pH 4.0

Blank niosomes and acetate buffer pH 4.0 were prepared. The percentages of blank niosomes and acetate buffer used were as follows: 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10% v/v in PBS pH 7.4. These amounts corresponded to the detection limit of the assay and were compared to GA niosomes and solution. Degree of RBC hemolysis was determined as described under Section 5.3.1.

All hemolysis experiments were done in triplicate. Graphs were constructed by plotting the absorbance at 570 against the concentration of the test substance. The concentrations of the test substances that cause 50% hemolysis (HC_{50}) were compared to assess the irritation potential with that of SDS, which was used as the standard tenside.

6. Statistical analysis

Statistically analysis of mean differences was performed on SPSS version 13.0. The validity of assumptions for analysis of variance (ANOVA) was tested on pooled data. If the distribution of data did not significantly deviate from normality, ANOVA, with either Tukey's HSD or Dunnett T3 test as a post hoc comparison, was used. The level of significance was chosen at 0.05 probability.

CHAPTER IV

RESULTS AND DISCUSSION

1. Development of GA niosomes

Previous studies reported that glycolic acid has multiple effects on the skin related to pH and concentration. With respect to irritation, the Cosmetic Ingredient Reviews (CIR) concluded that glycolic acid is safe for use in cosmetic products at a concentration equal to or less than 10%, at a final formulation pH equal to or greater than 3.5 or pH of the formulation ± 0.5 pH unit of the pK_a (pK_a of glycolic acid = 3.83) when formulated to avoid increasing sun sensitivity or when directions for use include the daily use of sun protection (Pierard et al., 2000; Berardesca, 2001; Johnson, 2002). Accordingly, the development of GA niosomes was selected at 6% (60 mg/mL) glycolic acid in acetate buffer pH 4.0 as aqueous medium.

In this study, various formulations of niosomes selected from those reported by Suwakul et al. (2006) were prepared using the sonication method, which was modified from Baillie et al. (1985). The total lipid concentration was kept at 100 mg/mL. The lipid was hydrated with acetate buffer pH 4.0 for blank niosomes or with glycolic acid (60 mg/mL) in acetate buffer pH 4.0 for GA niosomes.

From Table 1, blank and GA niosomes were completely formed with all compositions except for the formulations composed of Span® and CHO without Solulan®C24 (Span®20:CHO (60:40), Span®40:CHO (70:30), and Span®60:CHO (60:40)). These formulations immediately precipitated and separation between the lipid phase and the aqueous phase took place. Span® is sorbitan fatty acid esters. Thus, the acid hydrolysis of ester bonds can occur readily at pH 4.0 (Rieger, 1997). On the contrary, when Solulan®C24 (a membrane stabilizer) was added, Span®20, Span®40, and Span®60 formed stable vesicles. Solulan®C24 provides a steric barrier on the vesicle surface and thus protects the ester bonds of Span® from acid hydrolysis by steric effect (Arunothayanun et al., 2000).

Table 1 Compositions of lipid in the formulations that formed complete vesicles in acetate buffer pH 4.0

Formulation	% by weight ratio
Brij®52:CHO (B52)	70:30
Brij®76:CHO (B76)	50:50
Brij®52:CHO:Solulan®C24 (B52S)	67.5:27.5:5
Brij®76:CHO:Solulan®C24 (B76S)	47.5:47.5:5
Span®20:CHO:Solulan®C24 (S20S)	57.5:37.5:5
Span®40:CHO:Solulan®C24 (S40S)	67.5:27.5:5
Span®60:CHO:Solulan®C24 (S60S)	57.5:37.5:5
GDS:CHO:Brij®76 (GDS)	45:15:40
L-595:PEG-8-L (L595)	50:50

2. Characterization of GA niosomes

The GA niosomes were prepared from lipid compositions shown in Table 1 and were characterized regarding morphology, size and size distribution, entrapment efficiency, and short-term (2 months) physical stability.

2.1 Morphology

2.1.1 Optical microscopy

All batches of GA niosomes were viewed under an optical microscope to observe the shape of the vesicles and overall nature of the preparation. All compositions of niosomes formed spherical vesicles. The vesicles appearing under the light microscope varied in size. Surfactant, cholesterol, and Solulan®C24 crystals were not found in all niosomal preparations (see Appendix D).

2.1.2 Polarized light microscopy

All batches of GA niosomes were viewed under polarized light microscope for the birefringence of the lamellar structure. Vesicle formation and the arrangement of bilayer have been proven under this technique (Mura et al., 2007). The birefringence of the lamellar structure has been used to identify niosomal structure as well as other closed bilayers (Manosroi et al., 2003). Photographs in Appendix D show polarized light microscope images of vesicles from all formulations that formed vesicles completely. The size of vesicles composed of Brij®52 and Span® surfactants were smaller than the size of vesicles composed of Brij®76 surfactant. Thus, the corresponding cross-polarized images of vesicles from Brij®52 and Span® surfactants showed less clearly interference patterns than the vesicles from Brij®76 surfactant. Moreover, the nature of L-595:PEG-8-L vesicles was translucent when observed under light microscope. Hence, the birefringence images of vesicles from L-595:
PEG-8-L surfactant did not show the interference patterns due to the translucent nature of the vesicles.

2.2 Size and size distribution

The size distribution of all niosomal formulations is shown as the SPAN index in Table 2. Most of niosomal formulations showed a small SPAN index, which indicates a relatively narrow size distribution of these vesicles (see example in Figure 7). However, Brij®52 with Solulan®C24 and L-595 niosomes showed bimodal distribution (Figures 8 and 9) with large SPAN indices. This might indicate the effect of vesicular composition on vesicle size or the inappropriate preparation method resulting in the heterogeneity of the preparations.

Table 2 Average sizes of GA vesicles prepared from various compositions (Mean±SEM, n = 3)

Formulation	Size (μm)	SPAN index
Brij®52:CHO 70:30	5.48 ± 0.09	1.89 ± 0.04
Brij®76:CHO 50:50	8.35 ± 0.04	1.31 ± 0.01
Brij®52:CHO:Solulan®C24	4.38 ± 0.02	3.14 ± 0.07
Brij®76:CHO:Solulan®C24	8.15 ± 0.01	1.44 ± 0.04
Span®20:CHO:Solulan®C24	4.98 ± 0.02	1.37 ± 0.03
Span®40:CHO:Solulan®C24	5.89 ± 0.08	1.79 ± 0.06
Span®60:CHO:Solulan®C24	7.30 ± 0.04	1.99 ± 0.00
GDS:CHO:Brij®76	6.61 ± 0.06	1.79 ± 0.06
L-595:PEG-8-L 50:50	4.01 ± 0.12	3.45 ± 0.02

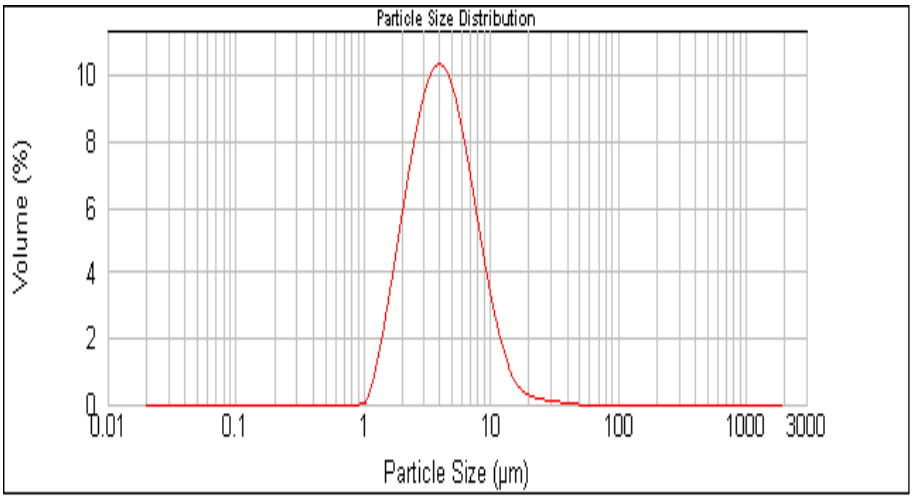

Figure 7 Size distribution of the vesicles prepared from Brij®52 without Solulan®C24

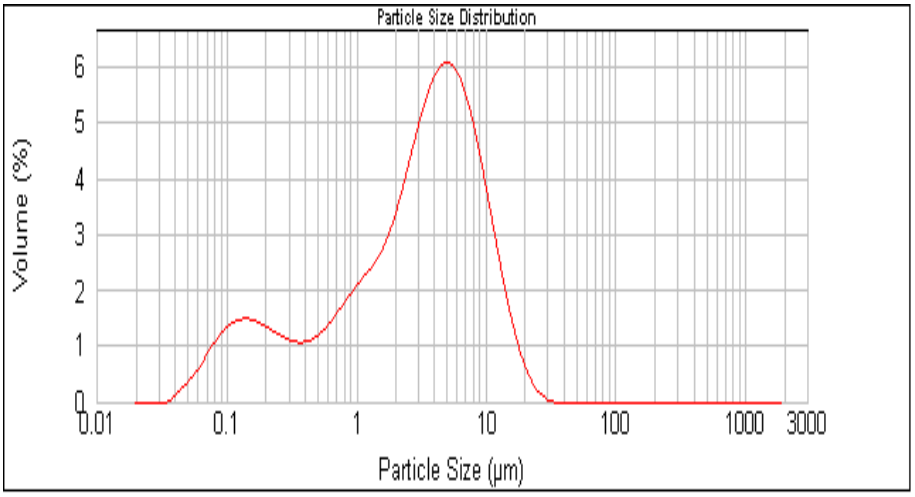

Figure 8 Size distribution of the vesicles prepared from Brij®52 with Solulan®C24

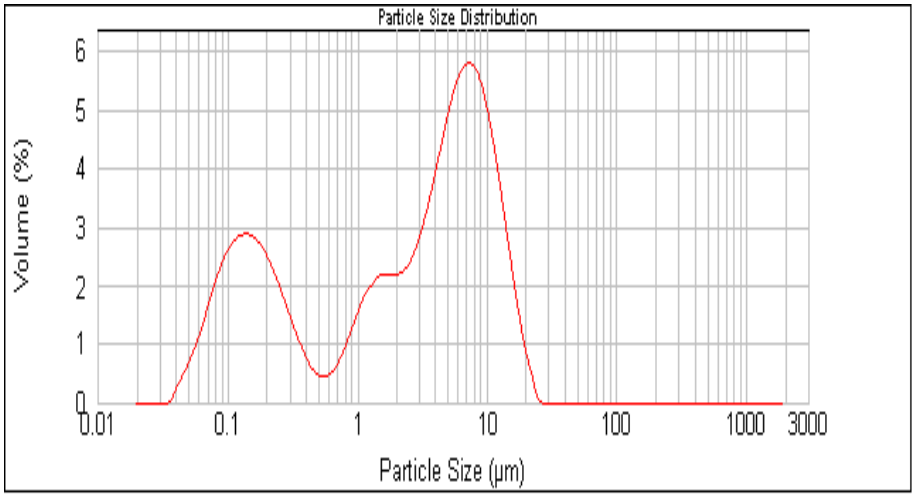

Figure 9 Size distribution of the vesicles prepared from L-595:PEG-8-L 50:50

GA niosome sizes ranged from 4-8 μm (Table 2). Particle sizes of GA niosomes were greater than those of the corresponding blank niosomes ($p < 0.05$) except GDS (Figure 10). At pH 4.0, 50% of the GA is in unionized form, which might be interacted with the vesicle surface and/or the bilayer. Degree of interaction between GA and each surfactant is different. Moreover, in the size measurement studies, niosomes diluted with water. Thus, the swelling of GA vesicles due to osmotic pressure could not be ruled out.

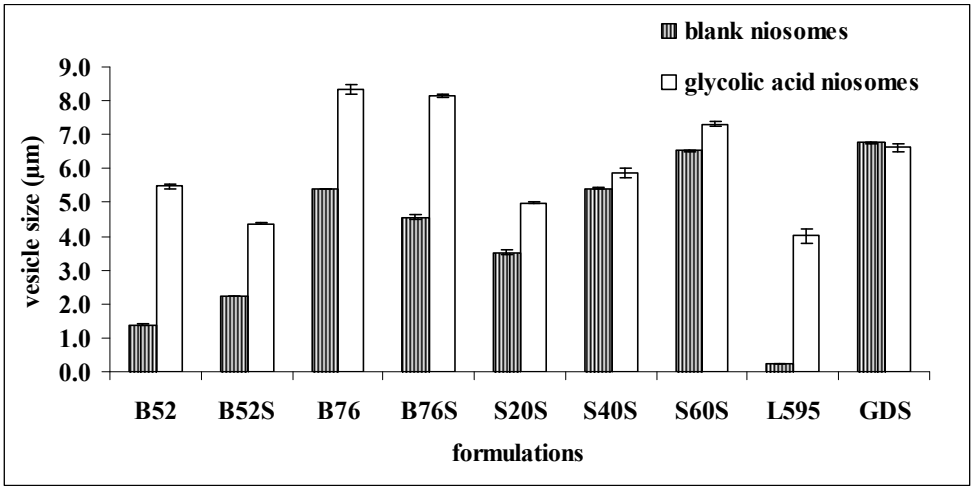

Figure 10 Average sizes of blank and glycolic acid niosomes (Mean ± SEM, n = 3)

This observation is consistent with many previous reports in which the particle sizes of niosomes/liposomes containing hydrophilic drugs were larger than those of the corresponding empty niosomes/liposomes (Stafford et al., 1988; Florence, 1993; Betz et al., 2001; Manosroi et al., 2008). Betz et al. (2001) reported that liposomal sizes increased in the presence of water soluble heparin. They explained that these results may be due to the interaction of heparin molecules onto the surface of the liposomes. Foco, Gasperlin, and Krist (2005) also investigated liposomes containing sodium ascobyl phosphate (SAP), a water soluble compound. Liposomes containing SAP were larger in size than those without SAP, presumably reflecting the ineraction of ascobyl phosphate on liposomes surface. On the contrary, the size of ammonium glycyrrhizinate-loaded Bola niosomes was slightly lower than that of the empty niosomes. The difference is probably due to the interaction between the steroid ring of ammonium glycyrrhizinate and the acyl chains of the synthetic surfactant bilayer

(Paolino et al., 2007). Furthermore, Paolino et al. (2008) found that 5-FU-loaded Bola niosomes was slightly smaller than empty niosomes. This may be explained by a different interaction between the polar heads of Bola surfactant and the carbonyl groups of the anticancer drug. However, the study of Trotta et al. (2004) revealed that the presence of water soluble methotrexate did not significantly alter the size of liposomes. This result could be due to methotrexate being entrapped in the hydrophilic core and interaction between drug and vesicle did not occur. Thus, the different results depended on physicochemical properties of loaded drugs.

The rank order of the GA vesicle sizes in the Span® surfactant group was Span®60 (C18) > Span®40 (C16) > Span®20 (C12) ($p < 0.05$) (Table 2). The chemical structure of Span® is sorbitan fatty acid ester. Different types of Span® vary in the acyl chain length with the constant head group. The lower HLB gave vesicles with a larger size. The increasing hydrocarbon chain length of the surfactant monomer could extend the length of the hydrophobic portion of the vesicles. The result is in accordance with the study of Guinidi et al. (2005) who reported that acetazolamide niosomes prepared using Span®60 (HLB 4.7) were larger in size than niosomes prepared using Span®40 (HLB 6.7). Similarly, tretinoin niosomes prepared by film hydration method from Span®60 were larger than Span®40 niosomes (Manconi et al., 2002). Therefore, the vesicle size of the Span® series depended on alkyl chain length of the surfactants. Increasing hydrocarbon chain length of surfactant monomer led to larger vesicles (Uchegbu and Duncan, 1997; Manosroi et al., 2003; Guinedi et al., 2005). However, there are several studies which reported that vesicle sizes were directly dependent on HLB of surfactant used where the higher HLB gave the larger size of vesicles (Yoshioka et al., 1994; Ruckmani et al., 2000; Khazaeli et al., 2007). The discrepancy is probably due to the different physicochemical properties of loaded drugs. Yoshioka et al. (1994) reported about carboxyfluorescein-loaded niosomes which were composed of sorbitan monoesters (Span®20, Span®40, Span®60, and Span®80) and sorbitan triester (Span®85). The mean size of niosomes increased with the progressive increase in the HLB values of these sorbitan ester surfactants. The similar result was observed by Khazaeli et al. (2007) who reported that the mean volume diameter of caffeine loaded niosomes increased with increasing HLB from

Span®80 (HLB = 4.3) to Span®20 (HLB = 8.6). These results might be anticipated since surface free energy decreases with increasing hydrophobicity (Yoshioka et al., 1994).

GA vesicle sizes from Brij® surfactants followed the rank order of Brij®76 ($C_{18}EO_{10}$) > Brij®52 ($C_{16}EO_2$) both with or without Solulan®C24 ($p < 0.05$) (see Table 2). The particle size directly depended on HLB values of surfactants used. Increasing HLB value resulted in a larger vesicle size. Brij® is classified as polyoxyethylene alkyl ether surfactants. Different types of Brij® vary in the alkyl chain length and the number of oxyethylene unit in the surfactant structure. Brij®76 (HLB = 12.4) has higher HLB value than Brij®52 (HLB = 5.3). Hence, the larger vesicle sizes of Brij®76 niosomes were evident when compared to Brij®52 niosomes. This result is in accordance with an observation by Suwakul et al. (2006) who reported that the vesicle size of propylthiouracil-loaded niosomes prepared from Brij®76 was larger than that of niosomes prepared from Brij®52. In contrast, the study of Tabbakhian et al. (2006) showed that finasteride-loaded Brij®76 niosomes were smaller than drug-loaded Brij®52 niosomes. The discrepancy may resulted from the different preparation methods or the effect of membrane additives on vesicle size. However, Pardakhty et al. (2007) revealed that when polyoxyethylene head group is constant and alkyl chain length is increased, both micellar volume and vesicle size will be increased. This finding was also seen by Uchegbu and Duncan (1997) who reported that niosomes composed of $C_{18}EO_5$/CHO were larger that those niosomes of $C_{16}EO_5$/CHO when prepared by dehydration-rehydration technique.

When Solulan®C24 was used as membrane stabilizer, the average sizes of niosomes composed of Brij®52 with Solulan®C24 were decreased when compared to those without Solulan®C24 ($p < 0.05$). However, the bimodal distribution was seen in the system composed of Brij®52 with Solulan®C24 (Figure 8), whereas Brij®52 without Solulan®C24 showed small unimodal distribution (Figure 7). This was almost in contrast to the ability of Solulan®C24 in preventing aggregation of the vesicles (Dimitrijevic et al., 1997; Uchegbu and Vyas, 1998; Arunothayanun et al., 2000). The mechanism behind this is still unclear.

2.3 Entrapment efficiency

The ability of colloidal drug delivery carriers to entrap and retain an active agent is very important to evaluate the potential therapeutic use of a certain drug carrier. This is also true when the drug carrier has to be used for topical application. Therefore, an important parameter to be evaluated for the niosomal formulation is its loading capacity. Table 3 summarizes %GA entrapment and entrapment efficiency prepared from various niosomal formulations. GA niosomes from GDS:CHO:Brij®76 (45:15:40), Span®40:CHO:Solulan®C24 (67.5:27.5:5), Span®20:CHO:Solulan®C24 (57.5:37.5:5) and Brij®52:CH:Solulan®C24 (67.5:27.5:5) showed high percent entrapment of about 50-52%. This value was higher than that expected by drug encapsulation in the aqueous compartment of a vesicular delivery system. Since at the pH studied, GA can be present in significant amount in the unionized form, this finding was probably due to significant interaction of the unionized glycolic acid molecules with the bilayer.

Table 3 %GA entrapment and entrapment efficiency prepared from various formulations (Mean±SEM, n = 3)

Formulation	%Entrapment	Entrapment efficiency (% by weight)
Brij®52:CHO 70:30	39.90 ± 0.45	24.07 ± 0.27
Brij®76:CHO 50:50	34.44 ± 0.12	20.63 ± 0.07
Brij®52:CHO:Solulan®C24	49.54 ± 0.23	29.89 ± 0.14
Brij®76:CHO:Solulan®C24	31.44 ± 0.02	18.97 ± 0.02
Span®20:CHO:Solulan®C24	50.68 ± 0.19	30.41 ± 0.12
Span®40:CHO:Solulan®C24	50.79 ± 0.14	30.48 ± 0.09
Span®60:CHO:Solulan®C24	24.16 ± 0.13	14.50 ± 0.08
GDS:CHO:Brij®76	52.39 ± 0.42	31.55 ± 0.25
L-595:PEG-8-L 50:50	10.23 ± 0.03	6.16 ± 0.02

These results are in good agreement with Paolino et al. (2008) who reported that 5-FU loaded Bola niosomes showed great loading capacity about 45%. This finding could be due to a significant interaction of 5-FU with the niosomal membrane. Similarly, Rogerson et al. (1987) investigated that adriamycin is located not only at the aqueous interface of the lamellar surface but also associated with the vesicle forming material. Such an interaction would be a likely explanation for the increase in entrapment efficiency. Hao et al. (2002) also reported similar results. They have shown that hydrophilic 5-FU and colchicine loaded Span® niosomes showed high encapsulation efficiency of about 72-99%. This high entrapment efficiency could be a result of significant interaction between amido group of 5-FU and colchicine with hydroxyl group of Span® via hydrogen bonding. However, Pardakhty et al. (2007) reported that inclusion of negatively charged molecules, dicetylphosphate (DCP), in Brij® niosomes led to decrease in insulin encapsulation efficiency. This was due to negatively charged nature of insulin molecule in PBS pH 7.4 that has less affinity to negatively charged DCP in the niosomal bilayer because of electrostatic repulsion. Thus, the differences in entrapment efficiency of various drugs loaded in vesicular systems may probably be attributed to the different physiochemical properties of the drugs and the vesicle compositions.

The results in Table 3 indicated that entrapment of the formulation with Span®60 was lower than those of the formulations with Span®20 and Span®40 ($p < 0.05$). Span®60 (C18) has longer saturated alkyl chain than Span®20 (C12) and Span®40 (C16). It was possible that there might be some difference in arrangement of surfactant molecules into bilayers. The longer alkyl chain is most likely to result in increased vesicle size and bilayer thickness (Yoshioka et al., 1994). Entrapment efficiency of GA might depend on interaction of GA on the vesicle surface and/or the bilayer. Therefore, the larger vesicle sizes of Span®60 should result in smaller surface area when compared to other formulations. Consequently, the interaction of GA on Span®60 vesicles surface was smaller and resulting in lower encapsulation efficiency than those with Span®20 and Span®40 systems. In case of Span®20 and Span®40 niosomes, entrapment efficiency was not significantly different ($p > 0.05$). This result

was probably due to the comparable vesicle sizes. Thus, this result implies that vesicle size of Span® system also influenced the entrapment efficiency.

The same results were also seen in systems with Brij®. Entrapment efficiency of Brij®76 ($C_{18}EO_{10}$) was lower than those of formulations of Brij®52 ($C_{16}EO_2$), both with or without Solulan®C24 (Table 3). Brij®76 formulations, both with or without Solulan®C24, showed larger vesicle sizes than those of Brij®52 systems. Hence, the interaction of GA on Brij®76 niosomes was smaller than that on Brij®52 systems. In the L-595:PEG-8-L (50:50) system, entrapment efficiency was the lowest. The elasticity of vesicle might result in weaker interaction between GA molecules and the bilayer as well as the leakage of GA from the aqueous phase of niosomes due to the high membrane permeability (Honeywell-Nguyen et al., 2002; Honeywell-Nguyen and Bouwstra, 2003).

Since several factors involved in entrapment efficiency of GA such as vesicle composition, surfactant structure, membrane stabilizer, and vesicle elasticity, the effect of size on entrapment was not clearly seen when the data of all niosomal preparations were put together. Figure 11 shows relation between percentage of entrapment and vesicle size. GA entrapment does not give good correlation to average size. Pearson correlation coefficient of the system was only -0.058.

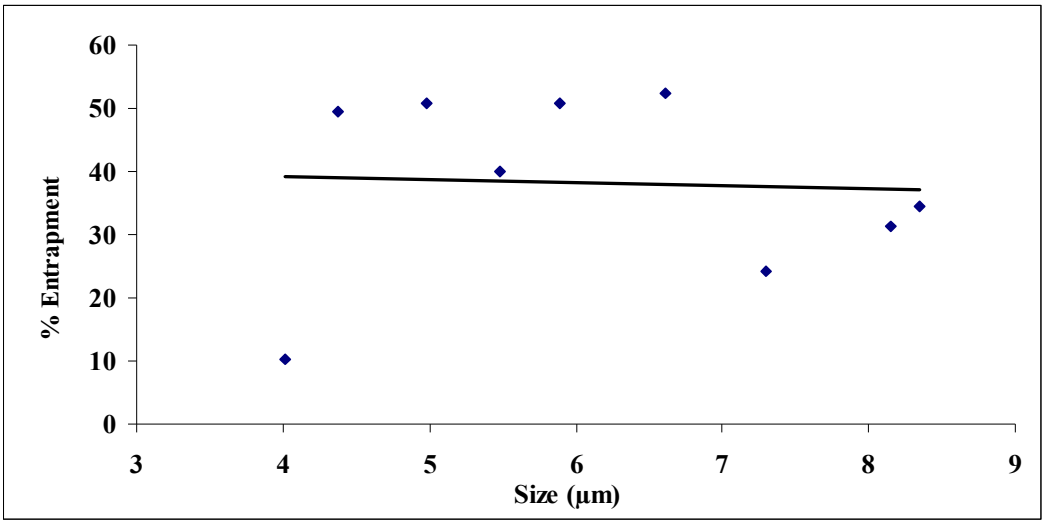

Figure 11 Relation between percent entrapment and size of GA niosomes

2.4 Physical stability

To estimate physical stability of niosomal preparations, GA niosomes contained in glass bottles tightly sealed with cap closures and paraffin films were stored at ambient conditions for two months. The influence of formulation factors on physical properties of niosomes, including the ability of niosomes to retain the entrapped GA was determined. The percentage of entrapment efficiency of GA in niosomes was analyzed by HPLC method at 0, 1, and 2 months, respectively.

By visual inspection, the color changes and phase separation of all formulations were not evident during the period of two months. There was no gross precipitation in any of the niosomal preparations when inspected visually. The pH of these suspensions was unchanged during the storage time (Table 4). Under microscopic observation, morphology of the vesicles at 30 days and 60 days was the same as that at 1 day. No surfactant and cholesterol crystals were found in any preparations over the two months of study.

Table 4 pH of GA vesicular suspensions at 1, 30, and 60 days of storage at ambient temperature (Mean±SEM, n = 3)

Formulation	pH		
	Time (Days)		
	1	30	60
B52	4.06 ± 0.01	4.08 ± 0.00	4.08 ± 0.01
B76	4.12 ± 0.00	4.12 ± 0.00	4.12 ± 0.01
B52S	4.06 ± 0.00	4.08 ± 0.00	4.08 ± 0.00
B76S	4.12 ± 0.00	4.11 ± 0.01	4.12 ± 0.01
S20S	4.03 ± 0.00	4.07 ± 0.00	4.09 ± 0.00
S40S	4.04 ± 0.00	4.07 ± 0.01	4.08 ± 0.01
S60S	4.06 ± 0.01	4.09 ± 0.00	4.11 ± 0.01
GDS	4.09 ± 0.00	4.04 ± 0.01	4.10 ± 0.01
L595	4.06 ± 0.00	4.08 ± 0.01	4.07 ± 0.01

Table 5 shows average sizes of the GA vesicles at 1, 30, and 60 days. The size and size distribution at 60 days was the same as those at 1 day except for the formulation consisting of Brij®52 without Solulan®C24, the vesicle size of which slightly increased after two months. This may be due to the aggregation or fusion of vesicles since the formulation lacked of membrane stabilizer. These results show that niosomal formulations were stable at ambient temperature for at least two months.

Table 5 Average sizes of GA vesicles at 1, 30, and 60 days of storage at ambient temperature (Mean±SEM, n = 3)

Formulation	Size (μm)		
	Time (Days)		
	1	30	60
B52	5.48 ± 0.15	7.77 ± 0.27	7.49 ± 0.07
B76	8.35 ± 0.06	8.38 ± 0.24	8.44 ± 0.06
B52S	4.38 ± 0.03	5.02 ± 0.05	4.92 ± 0.04
B76S	8.15 ± 0.02	8.17 ± 0.34	8.38 ± 0.04
S20S	4.98 ± 0.03	5.00 ± 0.13	5.70 ± 0.08
S40S	5.89 ± 0.14	6.09 ± 0.40	6.24 ± 0.12
S60S	7.30 ± 0.06	7.66 ± 0.33	8.36 ± 0.16
GDS	6.61 ± 0.11	6.64 ± 0.26	6.56 ± 0.10
L595	4.01 ± 0.20	3.47 ± 0.34	3.62 ± 0.12

Table 6 shows percent entrapment of GA niosomes when they were stored at ambient temperature for two months. Analysis of data indicates that GA entrapment values of all niosomal formulations at 0, 1, and 2 months were not statistically different with respect to storage time, using one-way analysis of variance followed by Dunnett T3 test ($p = 0.05$). This implies that there was no severe destruction in niosomal structure during the storage intervals. Thus, all niosomal formulations showed good physical stability in two months.

Table 6 %Entrapment of GA vesicles at 1, 30, and 60 days of storage at ambient temperature (Mean±SEM, n = 3)

Formulation	%Entrapment		
	Time (Days)		
	1	30	60
B52	39.90 ± 0.78	38.92 ± 0.47	38.88 ± 0.24
B76	34.44 ± 0.22	34.56 ± 0.08	34.41 ± 0.18
B52S	49.54 ± 0.39	49.40 ± 0.01	49.26 ± 0.19
B76S	31.44 ± 0.04	30.54 ± 0.54	30.52 ± 0.52
S20S	50.68 ± 0.34	50.67 ± 0.78	50.66 ± 0.03
S40S	50.79 ± 0.24	50.65 ± 0.29	50.54 ± 0.13
S60S	24.16 ± 0.22	24.04 ± 0.69	24.10 ± 0.18
GDS	52.39 ± 0.72	52.11 ± 0.29	52.00 ± 0.77
L595	10.23 ± 0.06	10.22 ± 0.09	10.23 ± 0.02

3. Release Studies

In vitro release is generally used in evaluation of drug delivery from topical formulations. The results from such experiment can predict the behavior of drug release to the skin in vivo. In this study, five formulations of GA niosomes: 1) Span®20:CHO:Solulan®C24 (57.5:37.5:5), 2) Span® 40:CHO:Solulan®C24 (67.5:27.5:5), 3) GDS:CHO:Brij®76 (45:15:40), 4) Brij® 52:CHO:Solulan®C24 (67.5:27.5:5), and 5) Brij®76:CHO:Solulan®C24 (47.5:47.5:5) were selected. The choices of formulations were based on high GA entrapment of the vesicles and good physical stability in two months. The solution of GA in acetate buffer pH 4.0 at the same concentration was also tested as references.

The release profiles of GA from niosomal systems and from aqueous solution were constructed from the plot between the percentage of cumulative amounts of drug released and time as shown in Figure 12. The rate of drug release through cellulose membrane from all niosomal systems was slower than that from the solution. The diffusion of GA from solution was complete in 6 hours, while the vesicles released about 82-99% of GA within 24 hours.

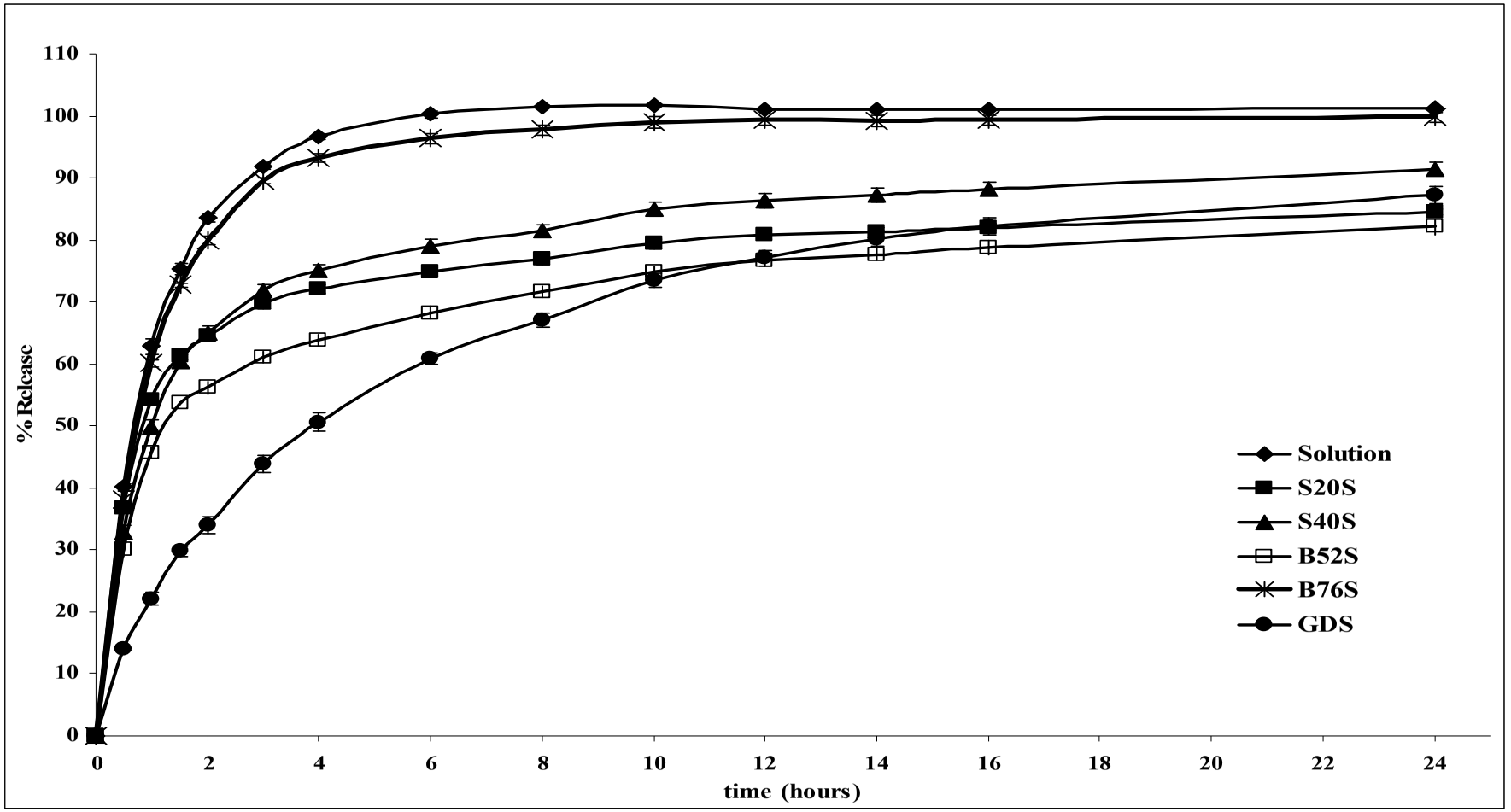

Figure 12 Release profiles of GA niosomes and aqueous solution (Mean±SEM, n = 4)

B52S = Brij®52:CHO:Solulan®C24 67.5:27.5:5, B76S = Brij®76:CHO:Solulan®C24 47.5:47.5:5, S20S = Span®20:CHO: Solulan®C24 57.5:37.5:5, S40S = Span®40:CHO:Solulan®C24 67.5:27.5:5, GDS = GDS:CHO:Brij®76 45:15:40

The release of drug was sustained with encapsulation in niosomal formulations except that of the Brij®76 system. Several studies reported similar controlled release of both hydrophilic and hydrophobic compounds from niosomes and liposomes. Examples of hydrophilic molecules include carboxyfluorescein (Yoshioka et al., 1994), 5-FU (Namdeo and Jain, 1999; Paolino et al., 2008), colchicine (Hao et al., 2002), atenolol (Betageri and Parsons, 1992), insulin (Pardahkty et al., 2007), and cytarabine hydrochloride (Ruckmani et al., 2000) and those of hydrophobic drugs were caffeine (Khazaeli et al., 2007), enrofloxacin (Sezer, Akbuga, and Bas, 2007), and retinoic acid (Montenegro et al., 1996). Effluxes of GA from niosomes were of a biphasic pattern, with an initial faster release for 1-2 hours, followed by a period of slow release. This biphasic release pattern of water soluble molecules seems to be the characteristic of bilayered vesicles (Baillie et al., 1985; Betageri and Parsons, 1992; Namdeo and Jain, 1999). The rapid initial phase may be originated from permeation of free GA and desorption of the compound from the surface of niosomes and the slower phase was related primarily to the diffusion of GA through the bilayers. This kind of release profile has been observed for 5-FU loaded niosomes (Namdeo and Jain, 1999), cytarabine hydrochloride delivery from niosomes (Ruckmani et al., 2000), GA delivery from liposomes (Perugini et al., 2000), and insulin encapsulated in niosomes (Pardahkty et al., 2007).

The release of GA from vesicles follows the first order kinetics. This result is in accordance with several previous reports (Tsukada, Ueda, and Okada, 1984; Lichtenstein and Margalit, 1995; Lopes de Menezes and Vargha-Butler, 1996; Manconi et al., 2002; Suwakul et al., 2006). From the semi-logarithmic plot of the percent drug remaining against time, values of release rate constant were obtained from the slope of the first order plot. Table 7 shows the release rate constants of niosomal formulations. There were statistically significant differences in the release rate constants among the formulations tested ($p < 0.05$).

Table 7 Release rate constants of various formulations (Mean±SEM, n = 4)

Formulation	Release rate constants (hr^{-1})	r^2
Aqueous solution	0.79 ± 0.02	0.9982
Brij®76:CHO:Solulan®C24 47.5:47.5:5	0.75 ± 0.02	0.9964
Span®20:CHO:Solulan®C24 57.5:37.5:5	0.69 ± 0.01	0.9569
Span®40:CHO:Solulan®C24 67.5:27.5:5	0.63 ± 0.01	0.9746
Brij®52:CHO:Solulan®C24 67.5:27.5:5	0.56 ± 0.01	0.9344
GDS:CHO:Brij®76 45:15:40	0.29 ± 0.01	0.9978

The results from Figure 12 and Table 7 show that the release rate seems to be related to the entrapment efficiency. The formulation with lower entrapment had a higher release rate. Brij®76 formulation, which had the lowest GA entrapment, gave the fastest rate of release. This result indicates that free GA had rapid diffusion in the aqueous phase (outside the vesicle) of the formulations. The similar inverse relation between drug entrapment and drug release was previous reported with GA liposomes (Perugini et al., 2005) and 5-FU niosomes (Muzzalupo et al., 2007). Other niosomal formulations (Span®20, Span®40, Brij®52, and GDS) showed similar amounts of entrapped GA with no statistically difference ($p > 0.05$). They were expected to have comparable rates of release. In contrast, the release rate of GDS system was slowest compared to Brij®52, Span®40, and Span®20 systems (Table 7). This finding may probably due to the higher viscosity of GDS niosomal suspensions when compared to other formulations. Hence, the release rate of the GDS formulation was retarded by its viscosity (Monchida Kanjanapadit, 2005).

Based on the comparable entrapment efficiencies, the state of bilayer also influenced the release rate. There were statistically significant differences between release rates of the gel state (Brij®52 and GDS) and the liquid crystalline state (Span®20) systems ($p < 0.05$). The liquid crystalline state vesicles showed significant higher rate of release than the gel state vesicles. The release rate of GA from Span®20 formulation was higher than that from Span®40 formulation ($p > 0.05$) (Table 7). The

difference can be attributed to phase transition temperatures of the vesicular compositions. Phase transition temperature of Span®20 is 5.1 °C (Suwakul et al., 2006). Hence, Span®20 niosomes was in the liquid crystalline state at the temperature used (32 °C in donor compartment) resulting in increased permeability of the bilayer. On the other hand, phase transition temperature of Span®40 is 42.4 °C (Suwakul et al., 2006). Span®40 formulation was in the gel state with less permeable bilayers, resulting a higher resistance to drug release. This explanation could also apply to GDS and Brij®52 formulations, which possess phase transition temperatures of about 48.5 °C and 33 °C, respectively (Suwakul et al., 2006). Many researchers have also reported similar results that release rate of liquid crystalline state vesicles was faster than those of gel state vesicles (Betageri and Parsons, 1992; Yoshioka and Florence, 1994; Yoshioka et al., 1994; Namdeo and Jain, 1999; Suwakul et al., 2006; Bandyopadhyay and Johnson, 2007; Khazaeli et al., 2007; Pardahkty et al., 2007).

4. Permeation Studies

The in vitro study of skin permeability plays an essential role in the selection of candidates for the development of transdermal dosage forms. The experiments were performed by using modified Franz diffusion cells whose donor and receiver compartments were separated by newborn pig skin. In these studies five formulations of GA niosomes were selected based on high entrapment and good physical stability in two months. The solution of GA in acetate buffer pH 4.0 was tested as references.

Flux (J_{ss}), P_s, Q_s, and Q_{24} were defined in this study as steady state flux, permeability coefficient, GA accumulated in the skin, and cumulative amount of GA in the receptor compartment at 24 hours, respectively. These parameters were of the formulations themselves. EF, EF of Q_s, EF of Q_{24}, and RF, which were parameters of GA permeation from the niosomal formulations as compared with that from the aqueous control, were defined as enhancement factor of P_s, enhancement factor of Q_s, enhancement factor of Q_{24}, and relative flux, respctively.

The permeation profiles of GA from niosomal systems and aqueous solution were constructed from plots between the cumulative amounts of drug permeated and time as shown in Figure 13. The permeation parameters are summarized in Table 8. All permeation parameters of the formulations themselves were not statistically significant different ($p > 0.5$) due to the great variation among formulations tested except for the Q_s. The Q_s of the Brij®52 system was higher than that of the GDS system ($p < 0.05$). This result implies that Brij®52 niosomes was a better carrier, which possessed a great accumulation of GA in the skin. From the results in Figure 13, it is evident that GDS, Span®20, and Brij®76 systems were more effective for the transport of GA across the skin than other vesicular formulations (Brij®52 and Span®40) and the aqueous control. Among GDS, Span®20, and Brij®76 formulations, all permeation parameters were similar with no significant differences ($p > 0.05$). All permeation parameters of GDS, Span®20, and Brij®76 formulations were higher than the parameters of Brij®52, Span®40, and aqueous solution ($p > 0.05$). This finding could be due to the effect of surfactant structures on the skin. GDS vesicles consisted of glyceryl distearate 45 parts and Brij®76 40 parts. Chemical structure of GDS is a diester. Its formula is $C_{39}H_{76}O_5$ with lowest HLB value of 2.4 compared to other surfactants. Hydrophobic surfactants have a strong affinity to the skin (Dalvi and Zatz, 1981; Endo, Yamamoto, and Ijuin, 1996). Hence, GDS might partition into the stratum corneum better than Span®40 and Brij®52 vesicles, which have higher HLB values. Suwakul (2005) studied in vitro cutaneous delivery of propylthiouracil from Span®40 and GDS vesicles across newborn pig skin. The author found the similar results that GDS vesicles (HLB = 2.4) gave higher permeation rate and were more potent penetration enhancer than Span®40 (HLB = 6.7) vesicles. In the same way, Manconi et al. (2006) investigated in vitro permeation of tretinoin from the vesicles prepared from alkyl polyglucosides. They also revealed that Oramix®NS10 vesicles (HLB = 11) showed lower permeation rate and higher skin accumulation than Brij®30 vesicles (HLB = 9.7). Hydrophilic surfactants with high HLB are not able to penetrate into stratum corneum. However, when permeation occurs, it can strongly interact with skin lipids (Junginger, Hofland, and Bouwstra, 1991).

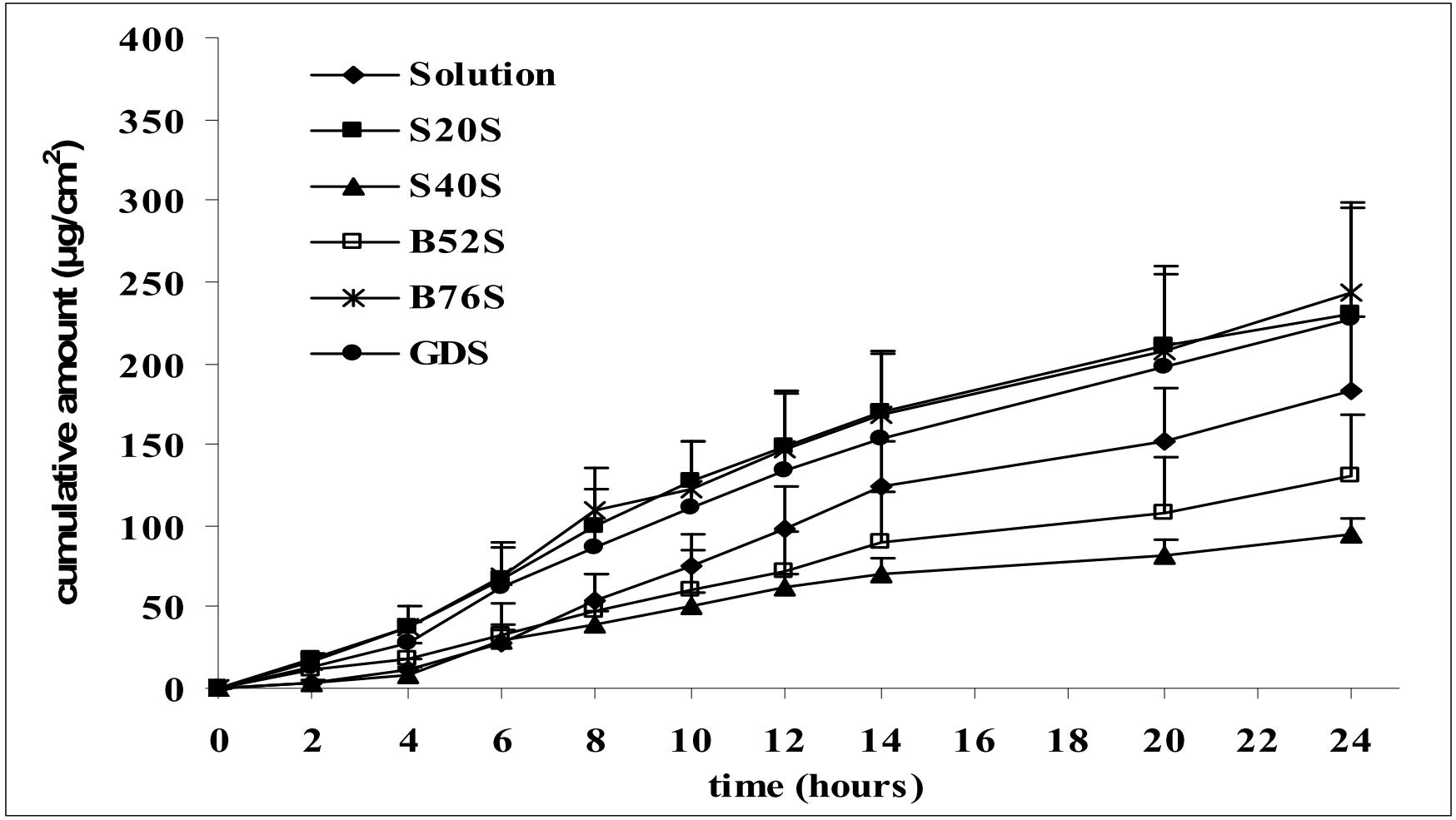

Figure 13 Permeation profiles of GA niosomes and aqueous solution (Mean±SEM, n = 6)

B52S = Brij®52:CHO:Solulan®C24 67.5:27.5:5, B76S = Brij®76:CHO:Solulan®C24 47.5:47.5:5, S20S = Span®20:CHO:Solulan®C24 57.5:37.5:5, S40S = Span®40:CHO:Solulan®C24 67.5:27.5:5, GDS = GDS:CHO:Brij®76 45:15:40

Table 8 Permeation parameters of GA niosomes and aqueous solution (Mean±SEM, n = 6)

Permeation parameter	Span®20:CHO:Solulan®C24 57.5:37.5:5	Span®40:CHO:Solulan®C24 67.5:27.5:5	Brij®52:CHO:Solulan®C24 67.5:27.5:5	Brij®76:CHO:Solulan®C24 47.5:47.5:5
Flux (J_{ss})	9.51 ± 3.39	3.97 ± 0.33	5.47 ± 1.22	9.76 ± 2.44
P_s x 10^{-4}	4.39 ± 1.56	1.83 ± 0.15	2.34 ± 0.52	4.38 ± 1.09
Q_s (%)	5.61 ± 1.50	4.99 ± 1.36	8.95 ± 0.85	6.25 ± 0.71
Q_{24} (%)	2.79 ± 0.87	0.99 ± 0.10	0.96 ± 0.30	2.48 ± 0.54
EF	1.01 ± 0.23	0.58 ± 0.14	0.77 ± 0.22	1.11 ± 0.27
EF of Q_s	1.89 ± 0.73	1.13 ± 0.21	3.36 ± 1.21	2.50 ± 0.96
EF of Q_{24}	2.18 ± 0.80	0.85 ± 0.22	1.09 ± 0.54	1.71 ± 0.58
RF	0.88 ± 0.20	0.51 ± 0.12	0.73 ± 0.21	1.00 ± 0.24
Recovery	84.56 ± 1.59	82.61 ± 0.99	81.98 ± 0.80	84.05 ± 1.41

Table 8 (continued) Permeation parameters of GA niosomes and aqueous solution (Mean±SEM, n = 6)

Permeation parameter	GDS:CHO:Brij®76 45:15:40	Aqueous solution
Flux (J_{ss})	9.69 ± 2.68	9.44 ± 1.57
P_s x 10^{-4}	4.33 ± 1.20	3.82 ± 0.64
Q_s (%)	3.28 ± 1.01	4.58 ± 1.34
Q_{24} (%)	1.57 ± 0.39	1.68 ± 0.41
EF	1.10 ± 0.29	1
EF of Q_s	0.81 ± 0.15	1
EF of Q_{24}	1.21 ± 0.54	1
RF	1.00 ± 0.27	1
Recovery	96.08 ± 1.44	96.39 ± 1.21

In conclusion, the possible explanations about the better GA permeation across the skin from GDS vesicles when compared to Brij®52 and Span®40 vesicles were the better partition of GDS into the skin and its potent penetration enhancer effect. The exception was seen with Brij®76 vesicles with an HLB value of 12.4, which gave higher permeation parameters. As mention before, the chemical structure of surfactants could affect on the interaction with the membrane. The polar moiety of the surfactant plays the major role with respect to interaction with skin lipids. Brij®76 ($C_{18}EO_{10}$) with its very hydrophilic head group is not able to penetrate significantly into the stratum corneum. However, when penetration occurs, its highly polar head might be able to strongly perturb the stratum corneum intercellular lipid bilayer giving rise to a facilitated pathway for the drug that can reach the dermis and receiver compartment more easily and in a greater amount (Manconi et al., 2006). The author reported that Oramix®CG110 niosomes (HLB = 16) with the very strong hydrophilic head group showed higher fluxes. Moreover, the other explanation could be that Brij®76 formulation had lower entrapment efficiency than other formulations tested. Therefore, free GA was present in a larger amount and resulting in much readily absorption.

In comparison of the effect of one variable, the other variables should be fixed. Thus, in comparison of the thermodynamic state on permeation, the formulation should be composed of the same surfactant class. The GA permeation across the pig skin of vesicles prepared using Span®20 (liquid crystalline state) was higher than that from Span®40 (gel state) (Figure 13 and Table 8). However, all permeation parameters of the liquid crystalline state vesicles were not significantly different from those of the gel state vesicles ($p > 0.05$). It is generally explained that gel state vesicles aggregate, fuse and adhere on the stratum corneum surface, thereby depositing stacks of lamellar sheets and forming lipid bilayer networks and can not induce ultrastructural changes in the skin. On the other hand, liquid crystalline state vesicles might act not only in the stratum corneum surface but may also induce ultrastructural changes in the deeper layers of the stratum corneum (van den Bergh, de Vries, and Bouwstra, 1998; van den Bergh et al., 1999). The superior mode of action of liquid crystalline state vesicles for skin interaction is the most possible explanation

for their better effectiveness in enhancing drug transport into and through the skin. Several in vitro permeation studies reported similar results for both hydrophobic and hydrophilic drugs. Examples of hydrophilic drugs were glycolic acid and glycerol (Ohta et al., 1996), 5-FU (El Maghraby, Williams, and Barry, 2001), and methotrexate (Trotta et al., 2004) and those of hydrophobic drugs were estradiol (Hofland et al., 1994) and finasteride (Tabbakhian et al., 2006).

From Figure 13, GA aqueous solution showed higher permeation rate than Brij®52 and Span®40 vesicular suspensions. However, all permeation parameters were not statistically significant ($p > 0.05$). GA aqueous solution contained more free GA in acetate buffer pH 4.0, which could be readily absorbed compared to niosomal suspensions. Hence, the much greater absorption of GA from solution was seen. Furthermore, the percutaneous absorption of GA is dependent on the pH of the formulation since the ionized molecule is more polar and therefore less readily absorbed. The effect of pH on the ionization of GA ($pK_a = 3.83$) can be calculated from the Henderson-Hasselbach equation. At formulation pH of 4.0, 50% of the GA is in the unionized form. Therefore, the unionized form of GA has the capability to penetrate through the stratum corneum. This result is in good agreement with the work of Kraeling and Bronaugh (1997) who investigated the effect of ionization of GA on skin penetration. The much greater absorption of GA was seen at pH 3.0 than at pH 7.0. At pH 3.0, the GA remains mostly unionized (87%) and thus much readily absorbed. Similarly, the percutaneous absorption of GA through animal skin has previously been reported from an aqueous solution. Absorption values of 0.7% and 0.9% were reported in 8 hours through minipig and hairless mouse skin, respectively, from a pH 3.8 aqueous solution (Goldstein and Brucks, 1994).

5. Estimation of the irritation potential of GA niosomes

This study employed the principle of red blood cell (RBC) hemolysis to determine the potential of niosomal formulations in causing irritation. The RBC test is a cell-based cytotoxicity assay, which allows assessing membrane damage of erythrocytes (hemolysis) and changes of the hemoglobin configuration. To determine

hemolysis, the hemoglobin leakage is measured spectrophotometrically. The RBC test is proposed as an alternative to the Draize rabbit eye test as an inexpensive, rapid assay with reliable results and good reproducibility (Pape et al., 1987). Pape and co-workers showed that substances that induce hemolysis always cause some extent of eye irritation in the Draize rabbit eye test (Pape et al., 1987; Pape and Hoppe, 1990). Therefore, this assay is currently employed to analyze surfactant and surfactant-based formulations. It is routinely used by industry as part of a test battery for screening purposes (Gettings et al., 1996; Pape et al., 1999). It is interesting that vesicular drug delivery systems may reduce drug toxicity and irritation by encapsulation drug into vesicles and prevent the direct contact between the irritating drug and the skin.

The 50% hemolysis concentration of tested samples are shown in Table 9. The data reveals that GA solution (60 mg/mL in acetate buffer, pH 4.0) was slightly lower irritating than sodium dodecyl sulphate (1 mg/mL in PBS, pH 7.4), which is a moderately irritating anionic surfactant. This result confirms that GA is an irritating substance. Moreover, GA solution (in acetate buffer pH 4.0) was more irritating than acetate buffer pH 4.0. This finding indicates that irritation effect of GA resulted mainly from its properties, not from the pH of the medium. As illustrated in Table 9, blank niosomes were less irritating than GA niosomes. This implies that niosomal compositions and concentrations used in this study could not eradicate the irritation potential of GA. It is also evident that GA itself caused the irritation. Furthermore, all GA niosomal formulations were less irritating than GA solution. Thus, niosomes used in this study still had a potential to reduce drug toxicity and irritation. Several studies reported the same results that vesicular delivery systems can reduce toxicity and irritation of drugs (Agarwal et al., 2001; Guinedi et al., 2005; Lakshmi et al., 2007; Paolino et al., 2007).

The effect of surfactant type on irritation was also investigated. Among these, GA loaded Brij® niosomes were more irritating than Span® and GDS niosomes (Table 9). Monchida (2005) also reported similar results that minoxidil loaded Brij® niosomes were more irritating than Span® niosomes. The nature of chemical linkage

(ester or ether) is also a determining factor in this model. Ester bonds may be easily hydrolyzed by acidic environment.

Table 9 Concentration of GA niosomes and other corresponding components that caused 50% hemolysis

Component	50% Hemolysis (%v/v)
Glycolic acid niosomes	
Span®20:CHO:Solulan®C24 57.5:37.5:5	8
Span®40:CHO:Solulan®C24 67.5:27.5:5	> 10
Brij®52:CHO:Solulan®C24 67.5:27.5:5	5
Brij®76:CHO:Solulan®C24 47.5:47.5:5	6
GDS:CHO:Brij®76 45:15:40	> 10
Blank niosomes	
Span®20:CHO:Solulan®C24 57.5:37.5:5	> 10
Span®40:CHO:Solulan®C24 67.5:27.5:5	> 10
Brij®52:CHO:Solulan®C24 67.5:27.5:5	> 10
Brij®76:CHO:Solulan®C24 47.5:47.5:5	> 10
GDS:CHO:Brij®76 45:15:40	> 10
Glycolic acid in acetate buffer pH 4.0 (60 mg/mL)	2
Acetate buffer pH 4.0	6.5
Sodium dodecyl sulphate (1 mg/mL)	1.2

Therefore, the lower toxicity of the ester surfactants may be explained by their chemical instability. These results are in good agreement with Hofland et al. (1992) and Kadir et al. (1992). Hofland et al. (1992) investigated the influence of non-ionic surfactant vesicles prepared from either oleyl-EO_5 ether or oleyl-EO_5 ester on the toxicity to tissue culture. The concentration of the ether-surfactant that inhibited cell

proliferation by 50% was about one-sixteenth that of the ester-surfactant. Similarly, Kadir et al. (1992) found ten times more inhibition of cell proliferation for ether compared to the ester compound.

The hemolysis potential of GA niosomes showed tendency to decrease with an increase in hydrophobic chain length. Brij®76 (C18) niosomes were less irritating than Brij®52 (C16) niosomes and Span®40 (C16) niosomes were less irritating than Span®20 (C12) niosomes (Table 9). The hemolytic actions in this study are in line with those previous reported with polyoxyethylene alkyl ether surfactants in transformed keratinocytes (SVK14) (Hofland et al., 1991). Hofland et al. (1992) also reported in vitro studies on a ciliotoxicity model to estimate the toxicity of alkyl polyoxyethylene niosomes on the nasal mucosa. Their studies revealed that an increase in alkyl chain length was accompanies by a decrease in toxicity to nasal mucosa, while an increase in the polyethylene chain length caused an increase in ciliotoxicity.

From Table 9, it was also observed that the gel state vesicles (Span®40) were less irritating than the liquid state vesicles (Span®20). The surfactant molecules that are present in the liquid state bilayers can be readily exchanged with the environment. Therefore, they can be more easily incorporated in the membrane of target cells, leading to more severe effects on biological tissues (Hofland et al., 1992). Moreover, it is worthwhile to note that the HLB number of surfactants had no influence on the irritation potential of the compounds. Thus, the overall results indicate that GA niosomes were less irritating and seemed to be safer to use than the GA aqueous solution.

CHAPTER V

CONCLUSIONS

GA is an alpha hydroxy acid used in many cosmetic products as exfoliant and moisturizer. High GA concentration has high potential to cause skin irritation. A niosomal preparation may be an appropriate delivery system for reducing its irritation. This study focused on the development of GA niosomes for skin delivery. In the development of GA niosomes, various classes of non-ionic surfactants and cholesterol with and without Solulan®C24 were used to form niosomal vesicles. The resultant niosomal systems were investigated in terms of morphology, size and size distribution, entrapment, release, short-term (2 months) physical stability, skin permeation, and irritation potential. Conclusions can be drawn from the study as follows:

1. It was feasible to prepare GA niosomes from some commonly available non-ionic surfactants by sonication method that was devoid of the use of organic solvent. These non-ionic surfactants were Span® (20, 40, and 60) with Solulan®C24, Brij® (52 and 76) with and without Solulan®C24, GDS, and L-595.

2. The morphology of GA niosomes was spherical in shape. Birefringence of the lamellar structure was displayed with polarized light microscope.

3. Size, size distribution, and entrapment efficiency of GA niosomes depended on niosomal compositions and the surfactant structure.

4. All formulations of GA niosomes were physically stable within two months of storage at ambient temperature.

5. The release of GA from five formulations selected based on high entrapment efficiency and good physical stability:
1) Span®20:CHO:Solulan®C24 (57.5:37.5:5), 2) Span® 40:CHO:Solulan®C24 (67.5:27.5:5), 3) GDS:CHO:Brij®76 (45:15:40), 4) Brij® 52:CHO:Solulan®C24

(67.5:27.5:5), and 5) Brij®76:CHO:Solulan®C24 (47.5:47.5:5), was sustained and followed the first order kinetics except that of the formulation containing Brij®76. The release rate constant depended on the entrapment efficiency and the thermodynamic state of the bilayers.

6. The permeation of GA across newborn pig skin depended on the surfactant structure and the thermodynamic state of the bilayers. Formulations containing GDS, Span®20, and Brij®76 gave higher GA permeation than other formulations.

7. All GA niosomes were less irritating than the GA aqueous solution. The degree of irritation depended on the niosomal composition, the surfactant structure, and the thermodynamic state of the bilayers.

In conclusion, the results of this present study show that it was possible to prepare niosomes containing the hydrophilic compound, GA, at an acid pH. The vesicular composition played an important role in characteristic of resultant niosomes. Thermodynamic state of the bilayers also affected the release and skin permeation of the compound from niosomal vesicles. Most of GA niosomes could enhance GA permeation across the skin and decrease GA irritation compared to the aqueous solution. Niosomal formulations containing GDS, Span®20, and Brij®76 showed high potential for increasing GA permeation and reducing irrtitation. Consequently, GA niosomes might be a good topical preparation for cosmetic uses of GA. Further studies such as in vitro comparison between permeation of GA niosomes and currently available products on the market and in vivo study should be performed. The information obtained from this present study will be helpful in developing GA formulations, as well as other compounds with similar properties, into practical formulations for topical skin delivery.

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APPENDICES

APPENDIX A

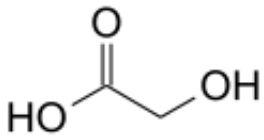
Molecular structure and physical properties of glycolic acid

(Budavali et al., 1996; Johnson, 2002)

1. Molecular structure

1.1 Empirical : $C_2H_4O_3$

1.2 Structural :



1.3 Molecular weight : 76.05

2. Physicochemical properties of glycolic acid

2.1 Melting range : 75-80 °C

2.2 Log P : -1.1

2.3 Solubility :

Glycolic acid is freely soluble in water, methanol, alcohol, acetone, acetic acid, ether, and ethyl acetate.

2.4 Stability :

Glycolic acid is a extremely stable compound at room temperature and not subject to thermal decomposition. It is recommended that it should be kept in a well-closed containers protected from moisture.

2.5 Dissociation constant : 3.83

2.6 pH of aqueous solution :

2.5 (0.5%)

2.33 (1.0%)

2.16 (2.0%)

1.91 (5.0%)

1.73 (10.0%)

2.7 Ultraviolet spectrum :

Glycolic acid absorbs ultraviolet radiation at 210 nm.

APPENDIX B

Molecular structure and physical properties of some selected materials

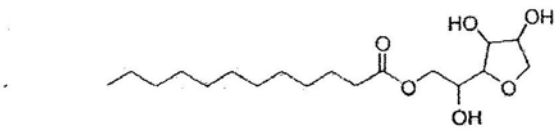
(Kibbe, 2000)

Properties of some selected materials

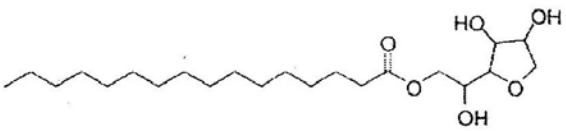
Material	Formula	Property
Cholesterol	$C_{27}H_{46}O$	MW: 386.67 MP: 174-150 °C BP: 360 °C
Span®20	$C_{18}H_{34}O_6$	MW: 346 MP: 5.1 °C HLB: 8.6
Span®40	$C_{22}H_{42}O_6$	MW: 403 MP: 44-48 °C HLB: 6.7
Span®60	$C_{24}H_{46}O_6$	MW: 431 MP: 53-57 °C HLB: 4.7
Brij®52	$C_{22}H_{45}O_3$	MW: 357 MP: 33 °C HLB: 5.3
Brij®76	$C_{38}H_{78}O_{11}$	MW: 710 MP: 38 °C HLB: 12.4
Solulan®C24	-	MW: 1,443 HLB: 8-9 Clound point: 88-95 °C
Glyceryl distearate	$C_{39}H_{76}O_5$	MW: 636 MP: 2.4 HLB: 55-60 °C
Sucrose laurate ester (L-595)	-	HLB: 5.0
PEG-8-L	$C_{29}H_{58}O_{10}$	MW: 552 MP: 12 °C HLB: 13

The structure of Span®20, Span®40, Span®60, Brij®52, Brij®76, Solulan®C24, glyceryl distearate (GDS), sucrose laurate ester (L-595), and PEG-8-L (Suwakul et al., 2006)

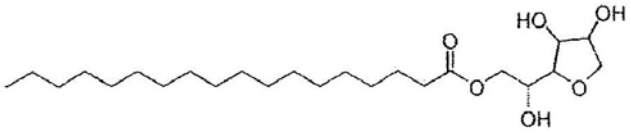
1. Span®20



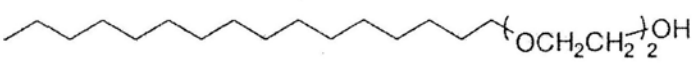
2. Span®40



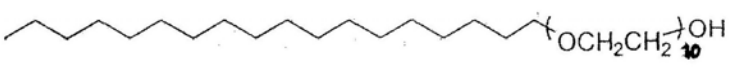
3. Span®60



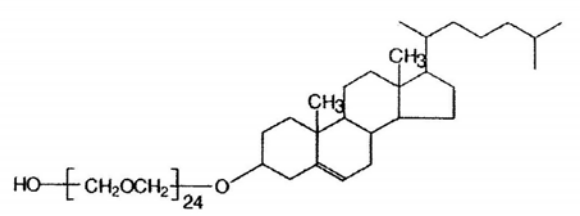
4. Brij®52



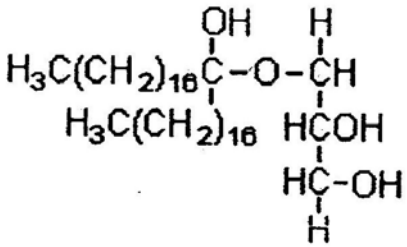
5. Brij®76



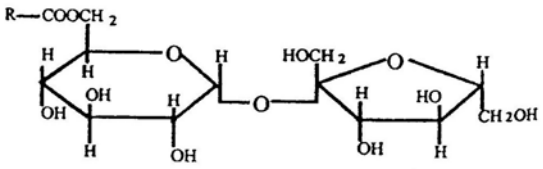
6. Solulan®C24



7. Glyceryl distearate (GDS)

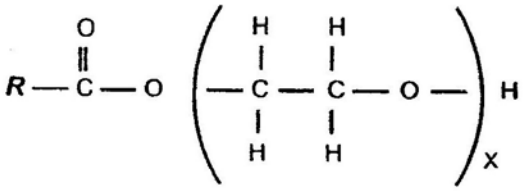


8. sucrose laurate ester (L-595)



R = C_{12}

9. PEG-8-L



R = C_{12} and X = 8

APPENDIX C

Validation of HPLC method

(The United State Pharmacopieal Convention, 2006)

Validation for the quantitative determination of GA in mobile phase and isopropanol/mobile phase systems by HPLC method

1. Specificity

Under the HPLC method used, the peak chromatogram of GA must not be interfered by the peak chromatogram of other components in the sample. The blank vesicular suspension (without GA) and GA vesicular suspension were prepared. The chromatogram of the blank vesicular suspension was compared with chromatogram of the GA vesicular suspension.

2. Linearity

Seven standard concentrations of GA ranging from 5.0 to 50.0 µg/mL in mobile phase and isopropanol/mobile phase systems were prepared and analyzed. Linear regression analysis of the peak area ratio versus their concentrations was performed. The linearity was determined from the coefficient of determination.

3. Accuracy

GA concentrations at 12.5, 27.5, and 42.5 ug/mL in mobile phase and isopropanol/mobile phase systems were prepared. Three sets of each concentration. Each individual sample was analyzed by HPLC method, and percent analytical recovery of each sample was calculated.

4. Precision

4.1 Within Run Precision

The within run precision was evaluated by analyzing five sets of the three standard solution of GA in five intervals of time in the same day. The mean, standard deviation (SD) and the coefficient of variation (%CV) of each standard solution were determined.

4.2 Between Run Precision

The between run precision was evaluated by comparing each concentration of five sets of standard solutions were prepared and analyzed in different days. The mean, standard deviation (SD) and the coefficient of variation (%CV) of each standard solution were determined.

Validation for the quantitative determination of GA in mobile phase and isopropanol/mobile phase systems by HPLC method

The validation of the analytical method is the process by which performance characteristics of the method are established to meet the requirements of the intended analytical parameters. The analytical parameters used for the assay validation were specificity, linearity, accuracy, and precision.

1. Specificity

The specificity of an analytical method is its ability to measure the given analyte accurately and specificity in the presence of other components in the sample. The chromatograms (Figure C1-C16) indicated that the conditions used was the optimal condition giving the highest sensitivity without interference of surfactants, cholesterol, and Solulan®C24 which showed no peak chromatograms at the peak of GA and internal standard (citric acid). The retention time of GA and citric acid were about 4.2 and 9.9 min, respectively. Thus, these two peaks were completely separated from each other.

2. Linearity

The linearity of analytical method is its ability to elicit test results that are directly or by a well defined mathematical transformation, proportional to the concentration of the analyte in samples within a given range. The linearity is usually expressed in term of the variance around the slope of regression line calculated according to an established mathematical relationship from the test results obtained by the analysis of samples with varying concentration of analyte.

The standard curves of GA solution diluted with mobile phase and isopropanol/mobile phase were shown in Figure C17-C18, respectively. The standard curves were found to be linear with coefficient of determination 0.9999 and 0.9999, respectively. These results indicated that HPLC method was acceptable for quantitative analysis of GA in the range studied. The equations of standard curves according to Beer's Law were used for calculating the concentration of GA.

3. Accuracy

The accuracy of an analytical method is the closeness of test results obtained by the method to the true value. Accuracy may often be expressed as percent recovery by the assay of known, added amount of analyte. The percentages of analytical recovery of GA concentration in mobile phase and isopropanal/mobile phase systems were shown in Table C1 and C2. All the percentage analytical recovery of all drug concentrations in both system, indicated the high accuracy of this method. Thus, it could be used for analysis of GA in all concentrations used.

4. Precision

The precision of an analytical method is the degree of agreement among individual test results when the procedure is applied repeatly to multiple samplings of a homogeneous sample. The precision of an analytical method is usually expressed as the standard deviation or relative standard deviation (coefficient of variation).

The precision of the analysis of GA in mobile phase and isopropanol/mobile phase systems by HPLC method was determined both within run precision and between run precision as illustrated in Table C3-C6. All percentage coefficient of variation values were lower than 2.00%, indicating that of the HPLC method used were precise for quantitative analysis of GA in the range studied.

In conclusion, the analysis of GA in mobile phase and isopropanal/mobile phase systems by HPLC method developed in this study showed good specificity, linearity, accuracy, and precision. Thus, this method was used for assay of content of GA.

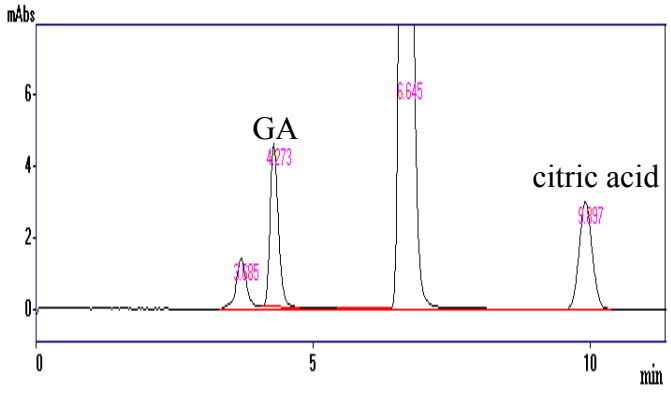


Figure C1 HPLC chromatogram of GA (35 µg/mL) and citric acid in mobile phase

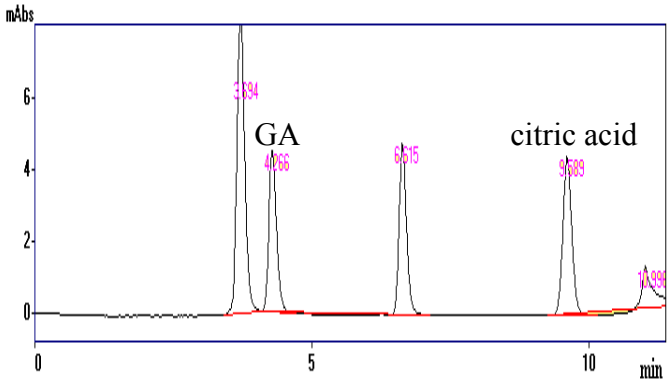


Figure C2 HPLC chromatogram of GA (35 µg/mL) and citric acid in isopropanol/ mobile phase

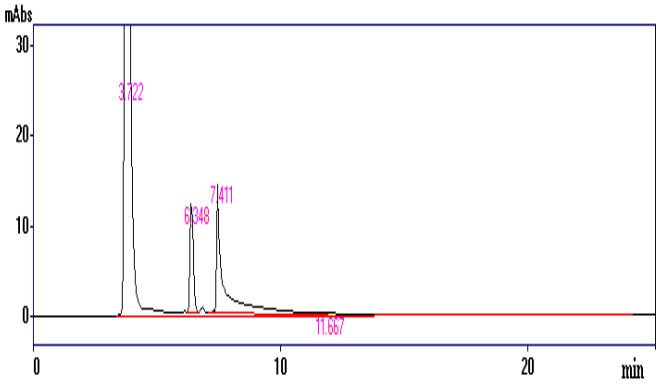


Figure C3 HPLC chromatogram of Span®20:CHO:Solulan®C24 in isopropanol/ mobile phase

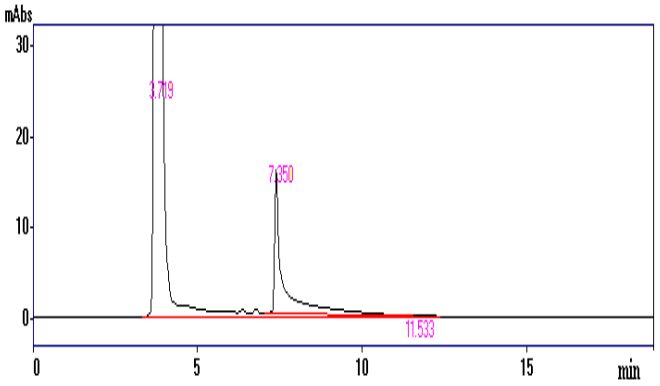


Figure C4 HPLC chromatogram of Span®40:CHO:Solulan®C24 in isopropanol/ mobile phase

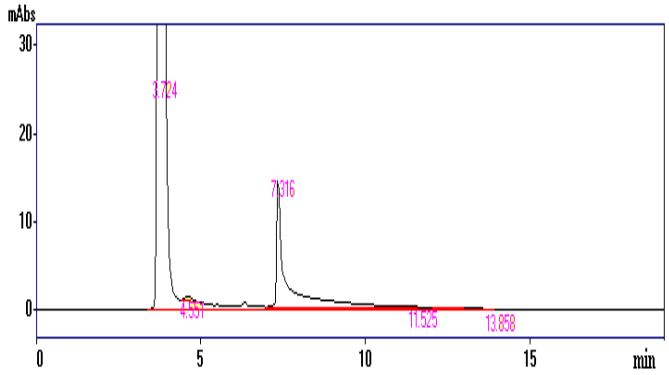


Figure C5 HPLC chromatogram of Span®60:CHO:Solulan®C24 in isopropanol/ mobile phase

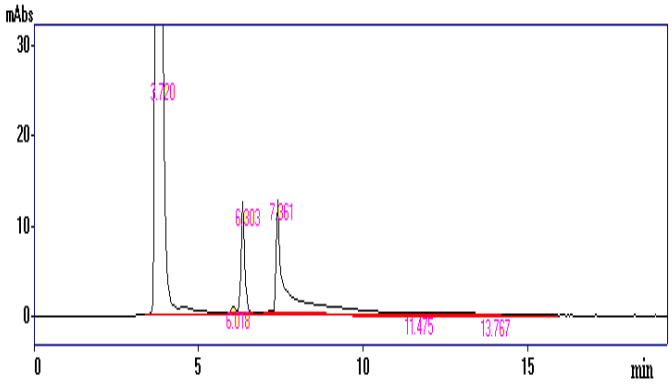


Figure C6 HPLC chromatogram of Brij®52:CHO:Solulan®C24 in isopropanol/ mobile phase

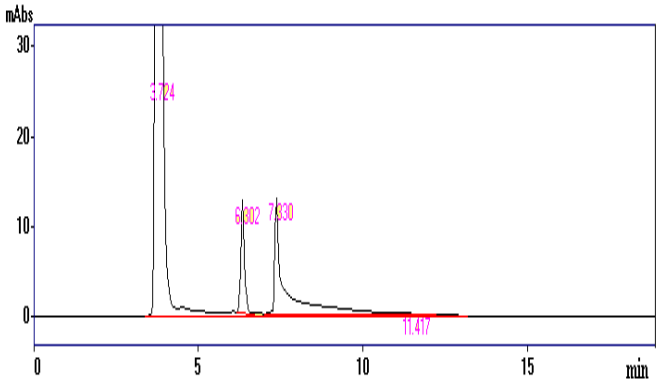


Figure C7 HPLC chromatogram of Brij®76:CHO:Solulan®C24 in isopropanol/ mobile phase

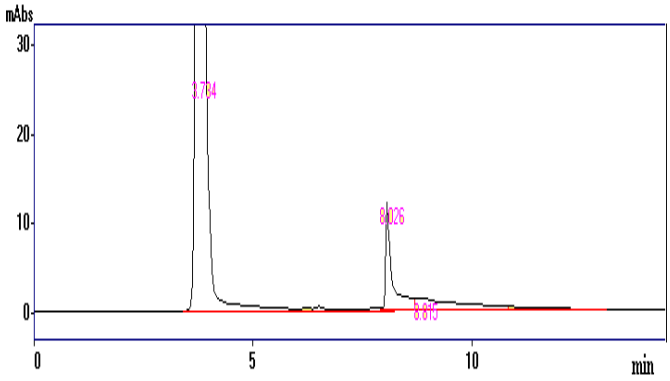


Figure C8 HPLC chromatogram of GDS:CHO:Brij®76 in isopropanol/mobile phase

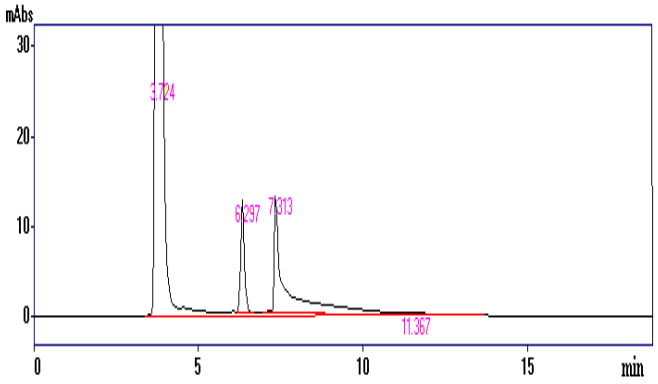


Figure C9 HPLC chromatogram of L-595:PEG-8-L in isopropanol/ mobile phase

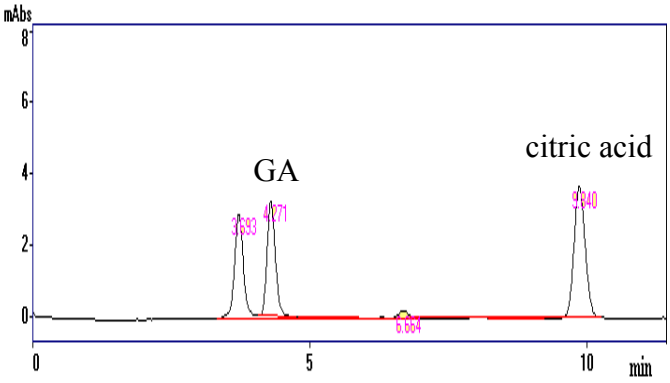


Figure C10 HPLC chromatogram of GA in Span®20:CHO:Solulan®C24 in isopropanol/mobile phase

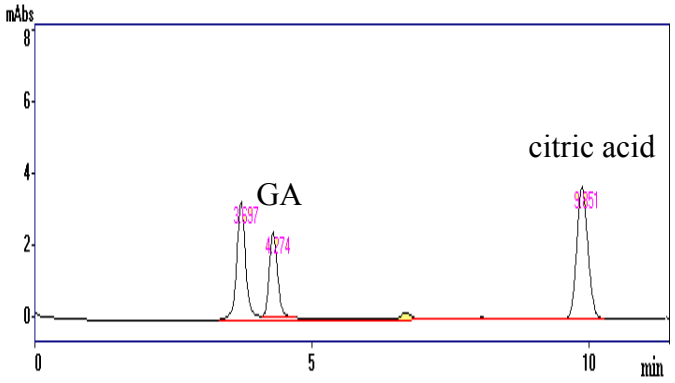


Figure C11 HPLC chromatogram of GA in Span®40:CHO:Solulan®C24 in isopropanol/mobile phase

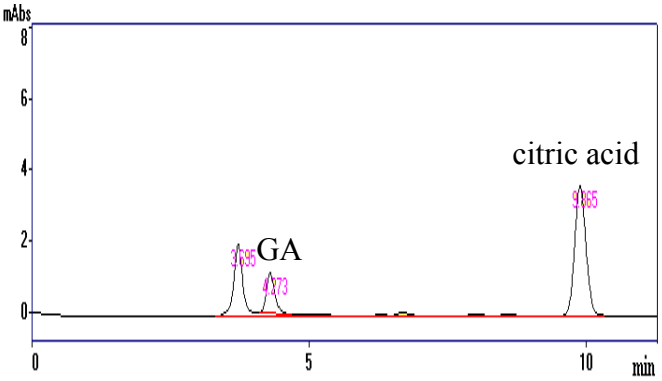


Figure C12 HPLC chromatogram of GA in Span®60:CHO:Solulan®C24 in isopropanol/mobile phase

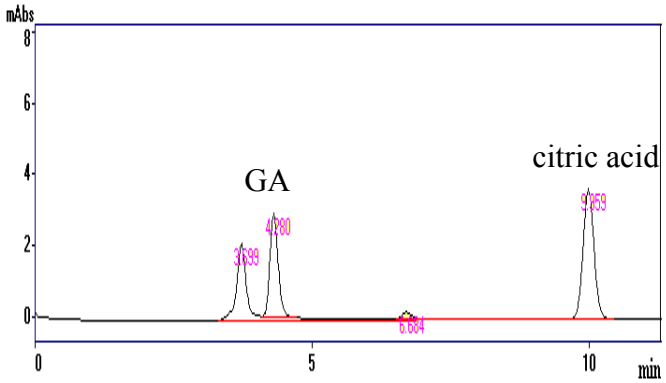


Figure C13 HPLC chromatogram of GA in Brij®52:CHO:Solulan®C24 in isopropanol/mobile phase

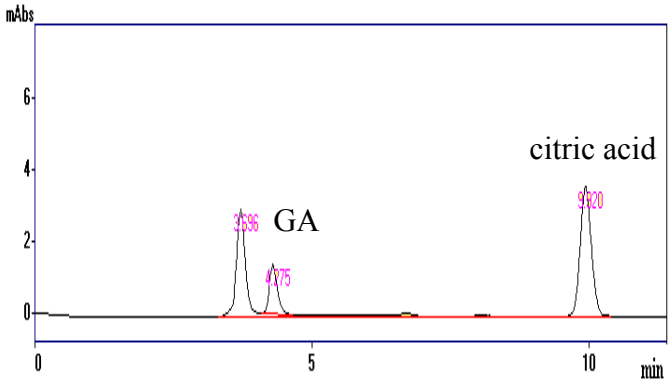


Figure C14 HPLC chromatogram of GA in Brij®76:CHO:Solulan®C24 in isopropanol/mobile phase

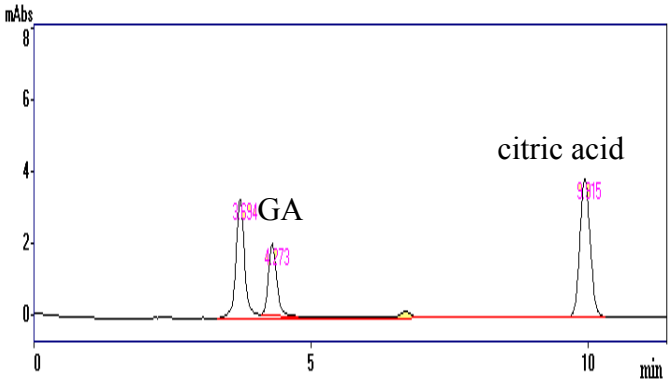


Figure C15 HPLC chromatogram of GA in GDS:CHO:Brij®76 in isopropanol/ mobile phase

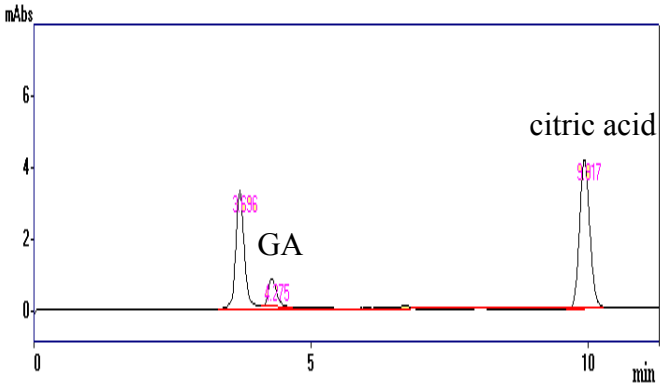


Figure C16 HPLC chromatogram of GA in L-595:PEG-8-L in isopropanol/ mobile phase

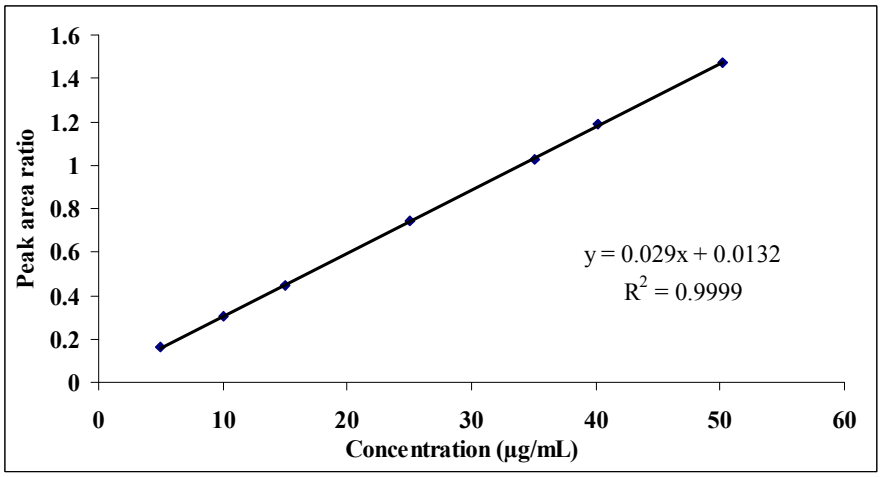


Figure 17 A representative of standard curve of GA solution diluted with mobile phase

Where y = 0.0290x + 0.0132; r^2 = 0.9999

y = peak area ratio, x = concentration (µg/mL)

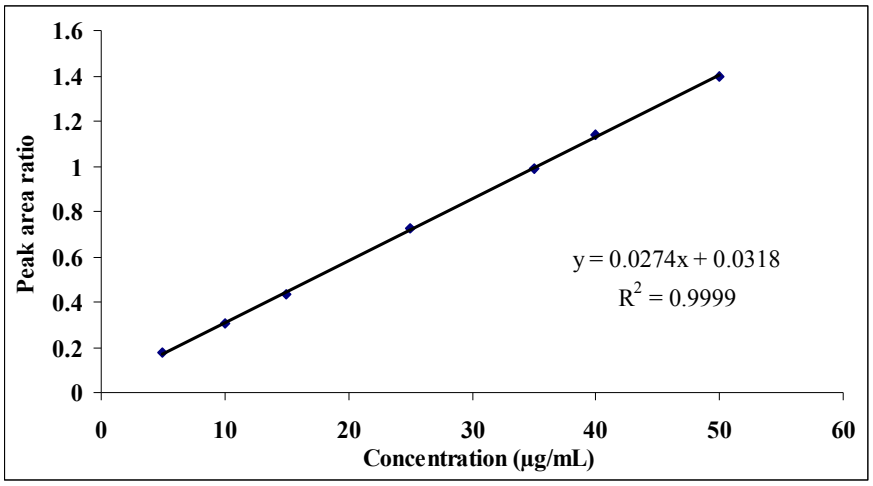


Figure 18 A representative of standard curve of GA solution diluted with isopropanol/mobile phase

Where y = 0.0274x + 0.0318; r^2 = 0.9999

y = peak area ratio, x = concentration (µg/mL)

Table C1 The percentages of analytical recovery of GA solution diluted with mobile phase

Actual concentration of GA (µg/mL)	Calculated concentration of GA (µg/mL)	% Analytical recovery
12.5	12.521	100.16
	12.665	101.32
	12.400	99.20
27.5	27.505	100.02
	27.519	100.07
	27.417	99.70
42.5	42.595	100.22
	42.460	99.91
	42.450	99.88

Mean % Recovery = 100.05

SD = 0.57

% CV = 0.57

Table C2 The percentages of analytical recovery of GA solution diluted with isopropanol/mobile phase

Actual concentration of GA (µg/mL)	Calculated concentration of GA (µg/mL)	% Analytical recovery
12.5	12.610	100.72
	12.672	101.21
	12.573	100.42
27.5	27.954	101.49
	27.568	100.09
	27.790	100.89
42.5	42.674	100.25
	43.565	102.34
	42.716	100.35

Mean % Recovery = 100.86

SD = 0.72

% CV = 0.72

Table C3 The within run precision of GA solution diluted with mobile phase

Conc.	Calculated Conc.(µg/mL)					Mean	SD	%CV
(µg/mL)	1	2	3	4	5			
12.5	12.521	12.399	12.375	12.666	12.277	12.448	0.149	1.201
27.5	27.505	27.520	27.417	27.707	27.183	27.467	0.190	0.693
42.5	42.460	42.450	42.595	42.767	42.296	42.514	0.177	0.416

Table C4 The within run precision of GA solution diluted with isopropanol/mobile phase

Conc.	Calculated Conc.(µg/mL)					Mean	SD	%CV
(µg/mL)	1	2	3	4	5			
12.5	12.610	12.572	13.008	13.026	12.972	12.838	0.226	1.761
27.5	27.568	27.954	27.790	28.005	28.221	27.908	0.245	0.877
42.5	42.674	42.716	43.565	43.659	43.731	43.269	0.528	1.220

Table C5 The between run precision of GA solution diluted with mobile phase

Conc. (μg/mL)	Calculated Conc.(μg/mL) Day1	Day2	Day3	Day4	Day5	Mean	SD	%CV
12.5	12.521	12.375	12.517	12.403	12.571	12.477	0.084	0.673
27.5	27.520	27.707	27.507	27.361	27.955	27.610	0.229	0.828
42.5	42.595	42.767	42.303	42.794	42.111	42.514	0.298	0.702

Table C6 The between run precision of GA solution diluted with isopropanol/mobile phase

Conc. (μg/mL)	Calculated Conc.(μg/mL) Day1	Day2	Day3	Day4	Day5	Mean	SD	%CV
12.5	12.572	12.610	13.032	13.057	13.014	12.857	0.243	1.895
27.5	27.568	27.789	28.005	27.954	28.321	27.927	0.278	0.997
42.5	42.673	42.715	43.731	43.659	43.565	43.269	0.527	1.219

APPENDIX D

Microscopic images of GA niosomes

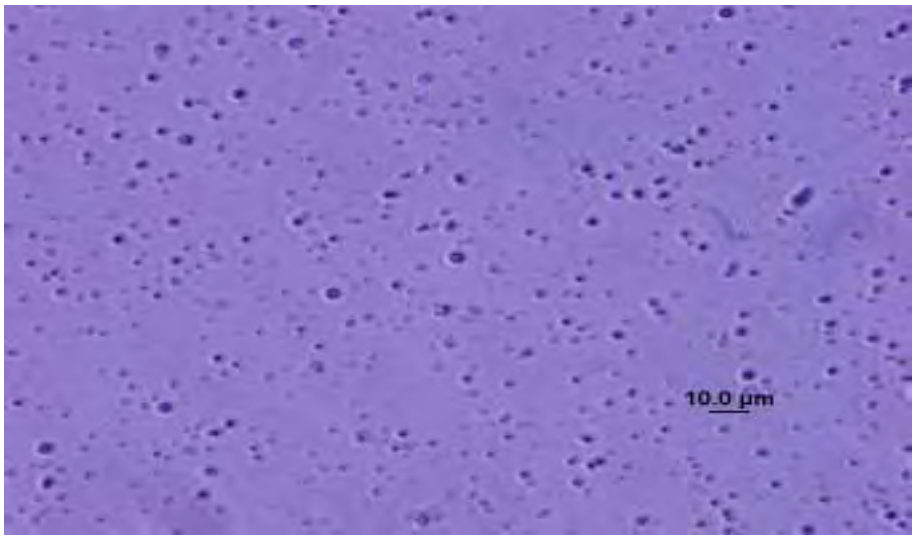


Figure D1 Photograph of Brij®52:CHO 70:30 (400X)

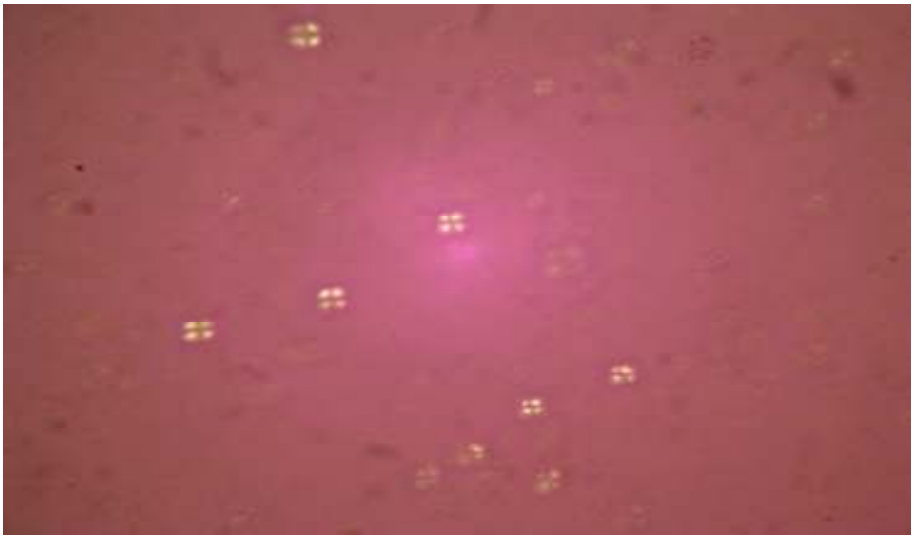


Figure D2 Polarized light microscopic image of Brij®52:CHO 70:30 (1000X)

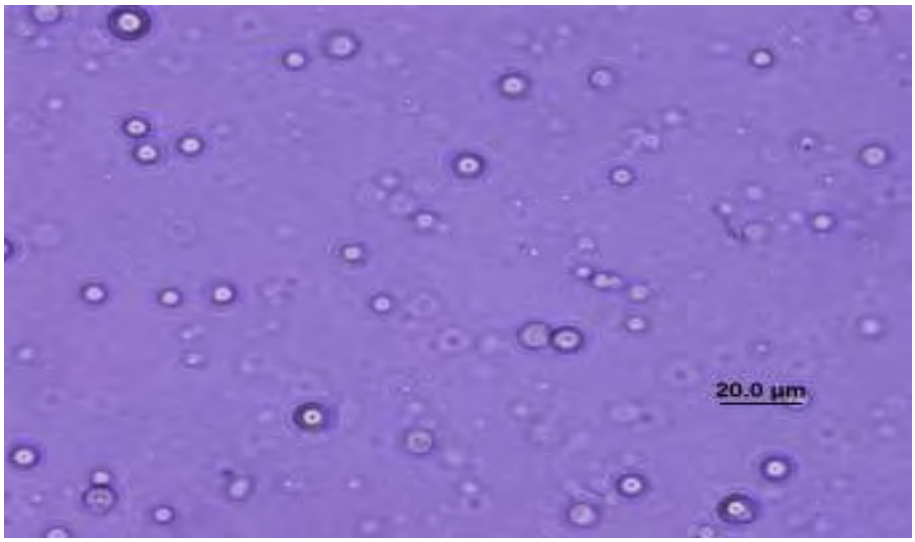


Figure D3 Photograph of Brij®76:CHO 50:50 (400X)

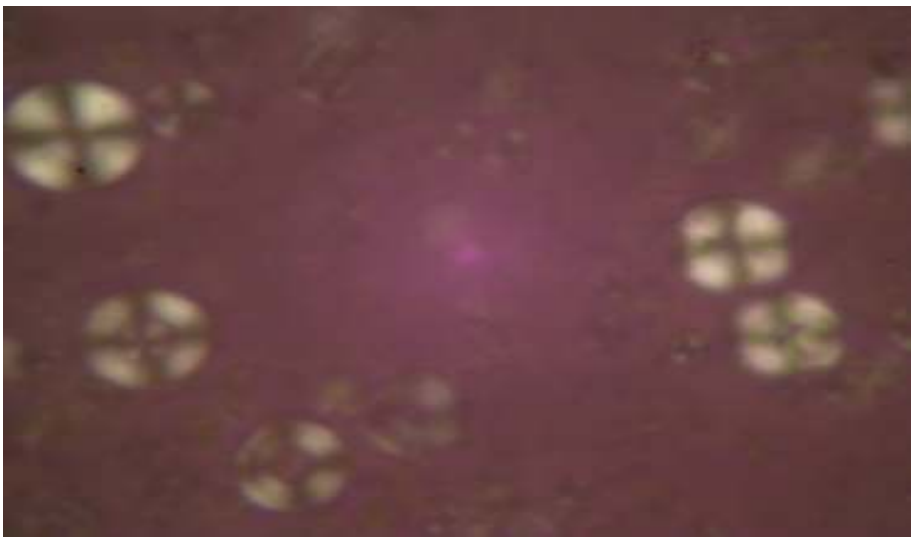


Figure D4 Polarized light microscopic image of Brij®76:CHO 50:50 (1000X)

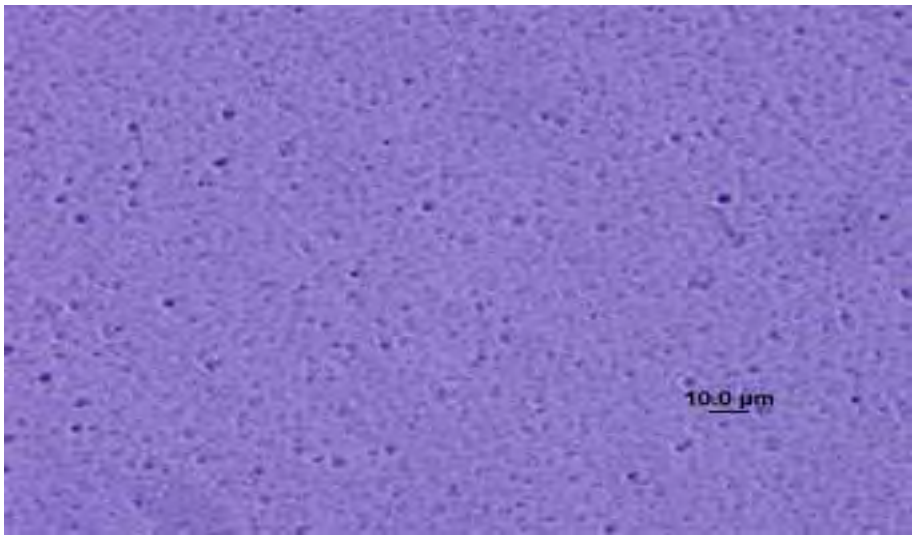


Figure D5 Photograph of Brij®52:CHO:Solulan®C24 67.5:27.5:5 (400X)

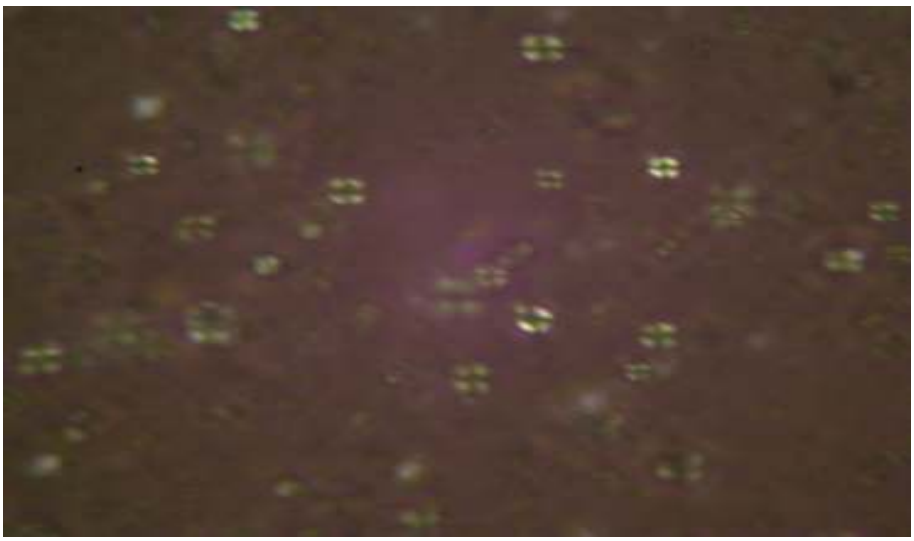


Figure D6 Polarized light microscopic image of Brij®52:CHO:Solulan®C24 67.5:27.5:5 (1000X)

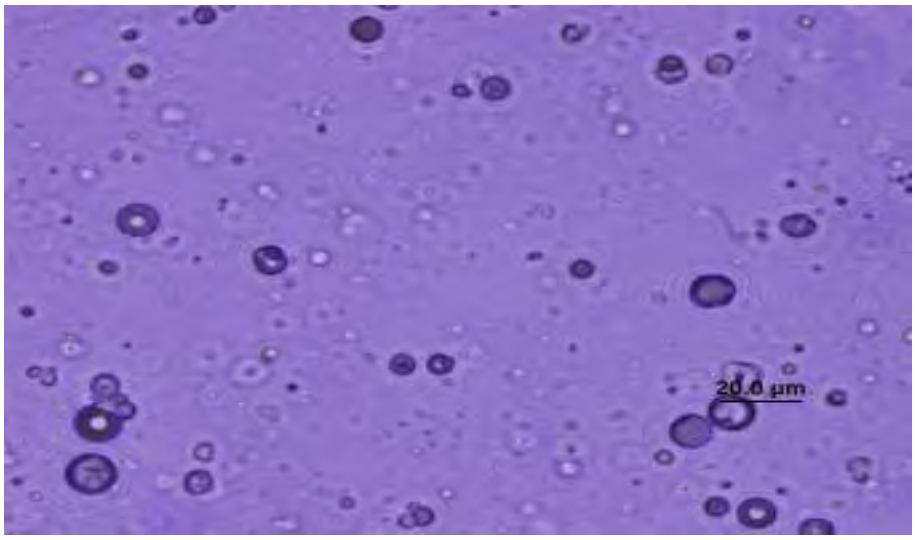


Figure D7 Photograph of Brij®76:CHO:Solulan®C24 47.5:47.5:5 (400X)

Figure D8 Polarized light microscopic image of Brij®76:CHO:Solulan®C24 47.5:47.5:5 (1000X)

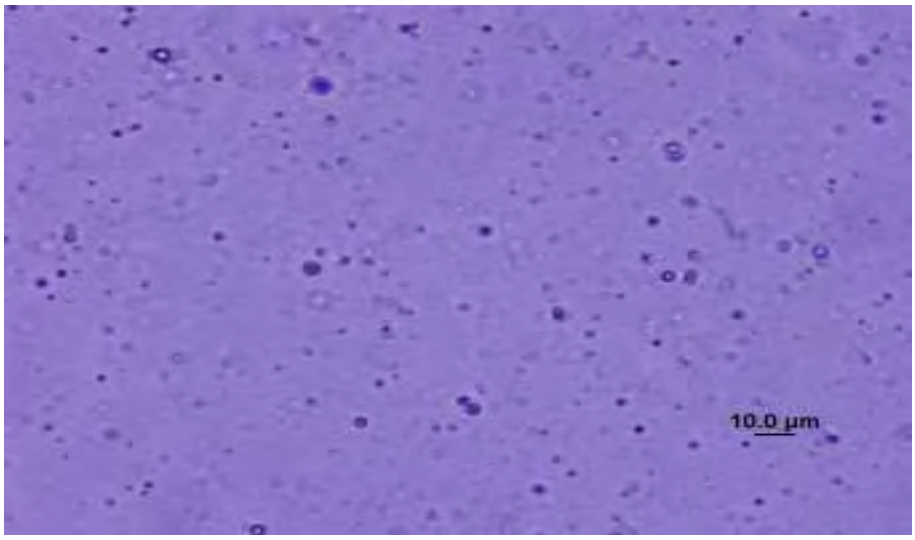


Figure D9 Photograph of Span®20:CHO:Solulan®C24 57.5:37.5:5 (400X)

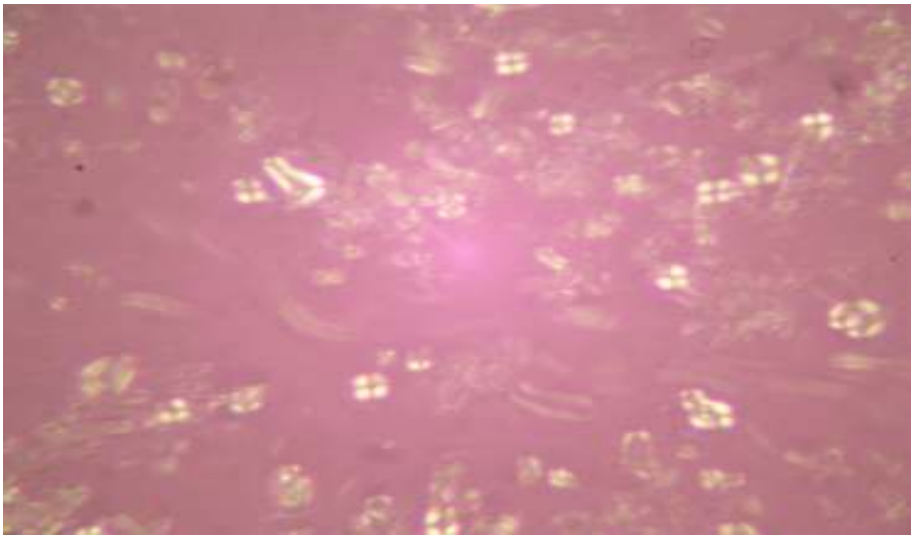


Figure D10 Polarized light microscopic image of Span®20:CHO: Solulan®C24 57.5:37.5:5 (1000X)

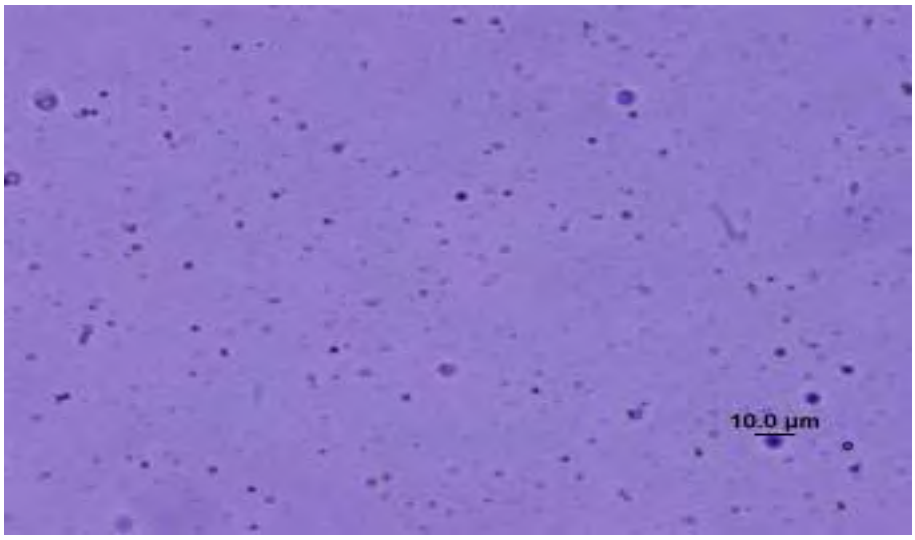


Figure D11 Photograph of Span®40:CHO:Solulan®C24 67.5:27.5:5 (400X)

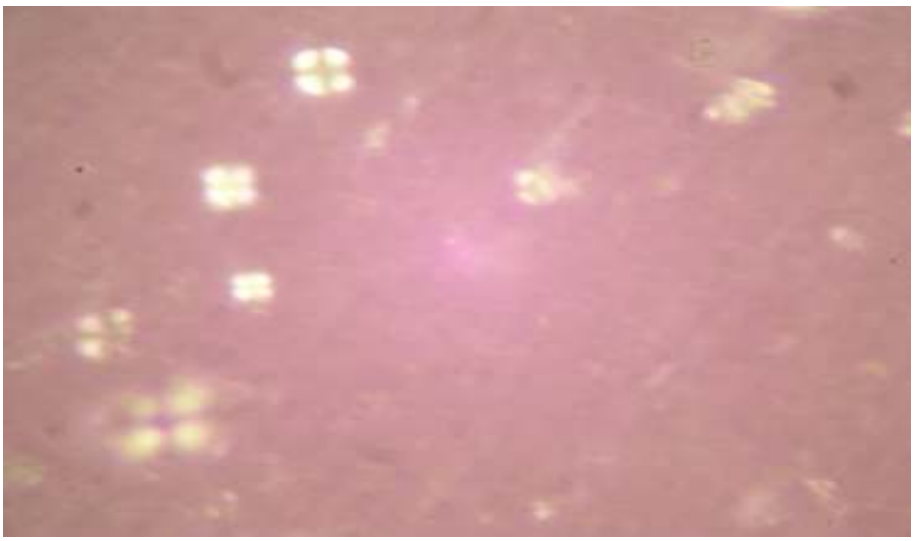


Figure D12 Polarized light microscopic image of Span®40:CHO: Solulan®C24 67.5:27.5:5 (1000X)

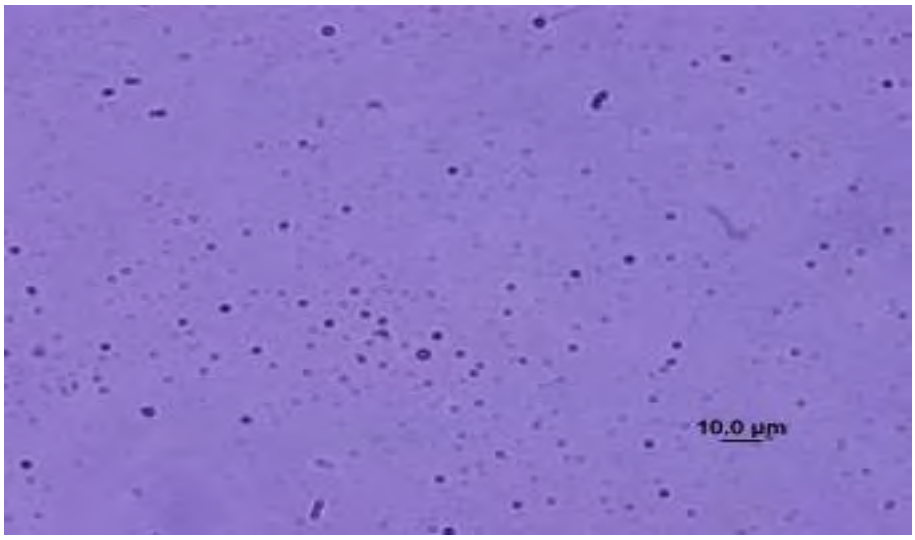


Figure D13 Photograph of Span®60:CHO:Solulan®C24 57.5:37.5:5 (400X)

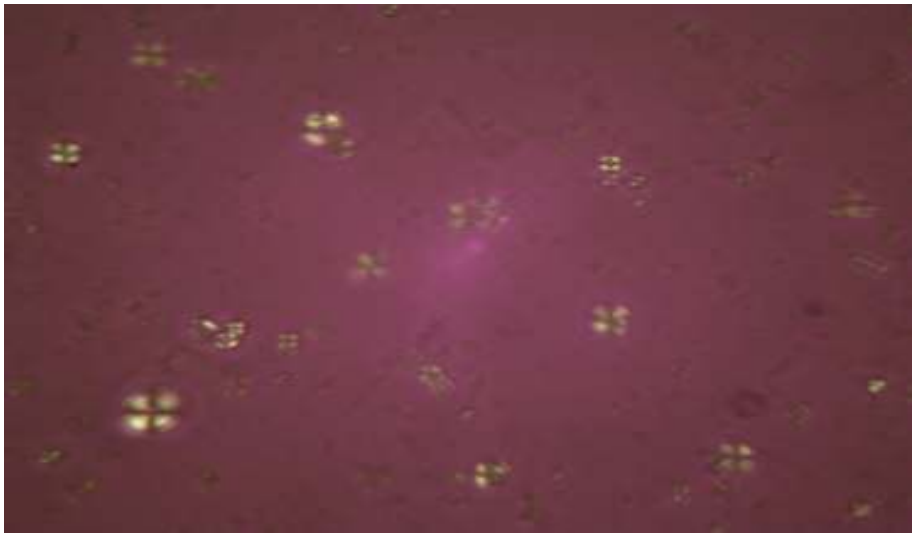


Figure D14 Polarized light microscopic image of Span®60:CHO: Solulan®C24 57.5:37.5:5 (1000X)

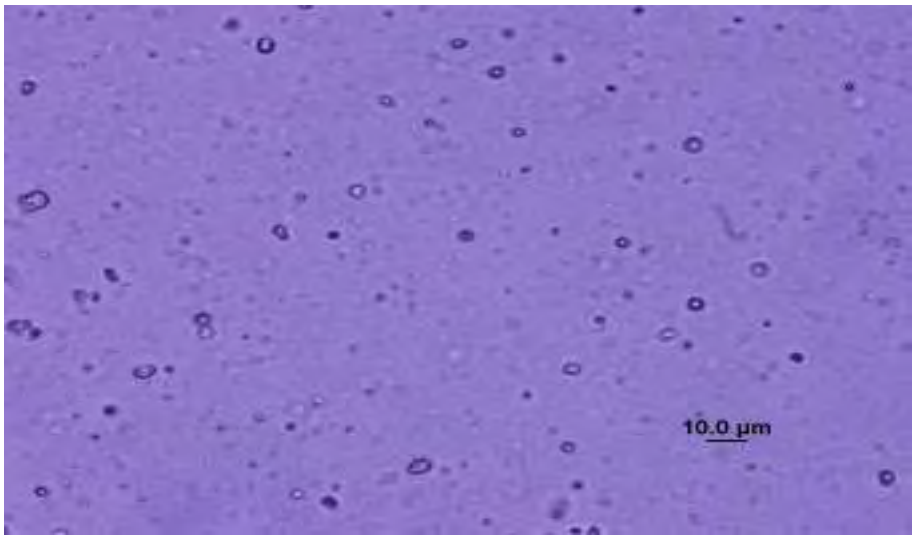


Figure D15 Photograph of GDS:CHO:Brij®76 45:15:40 (400X)

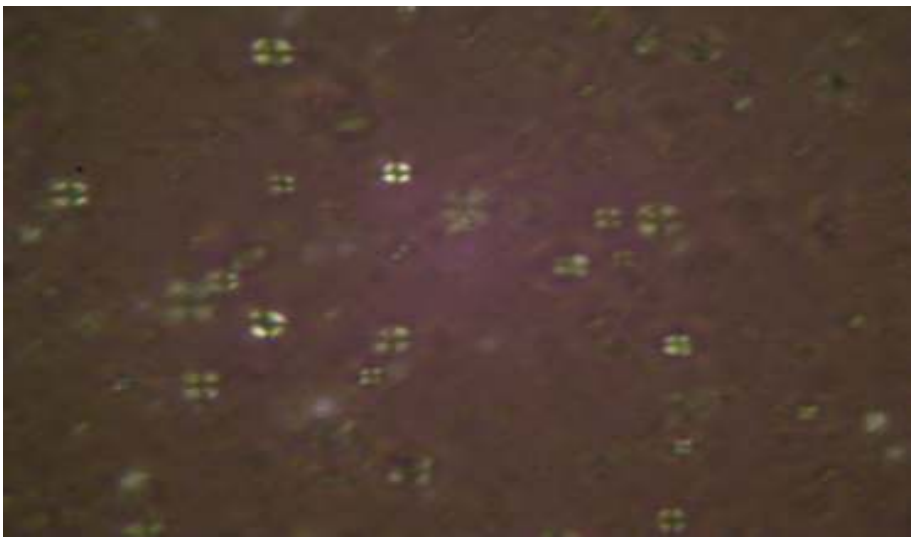


Figure D16 Polarized light microscopic image of GDS:CHO:Brij®76 45:15:40 (1000X)

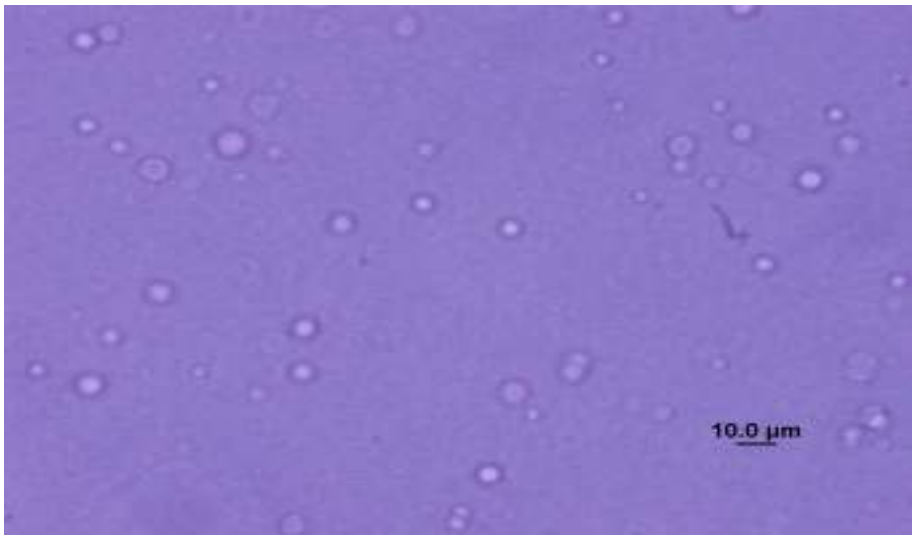


Figure D17 Photograph of L-595:PEG-8-L 50:50 (400X)

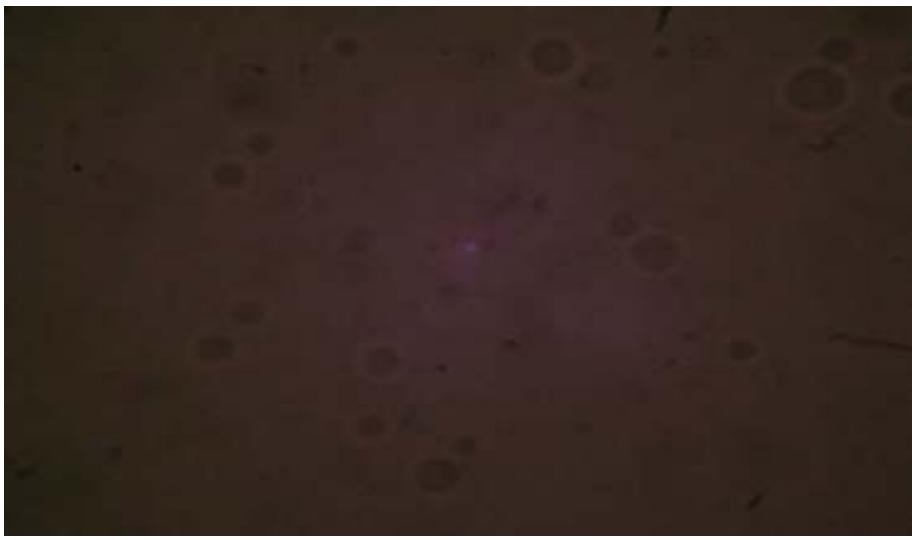


Figure D18 Polarized light microscopic image of L-595:PEG-8-L 50:50 (1000X)

APPENDIX E

Size distribution of glycolic acid niosomes

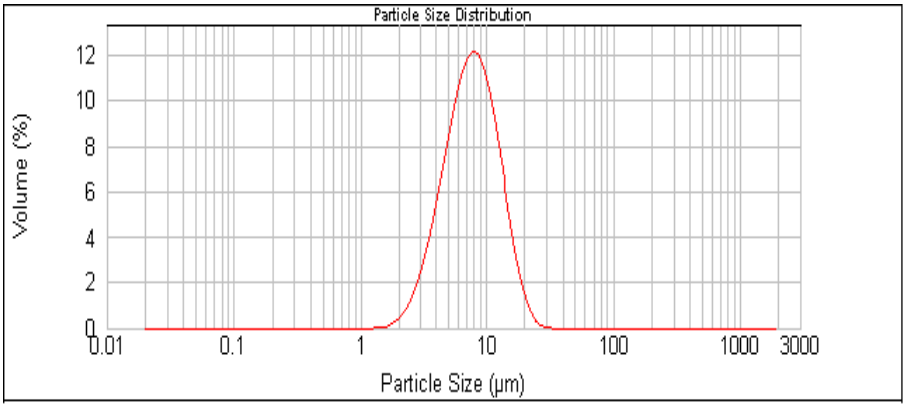


Figure E1 Size distribution of Brij®76:CHO (50:50)

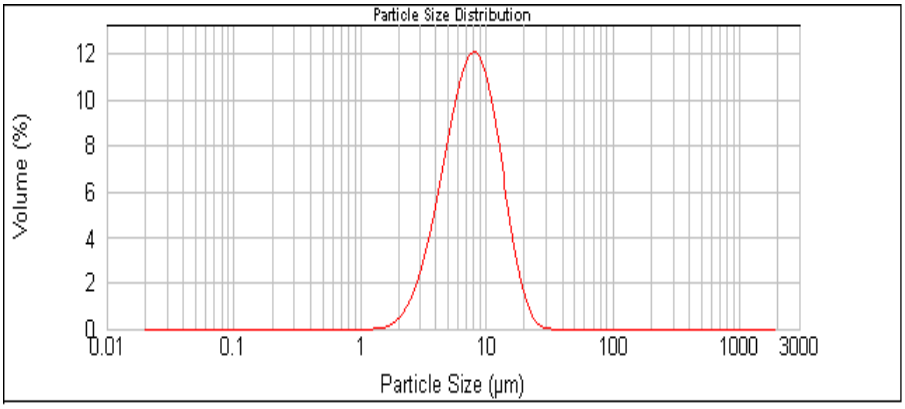


Figure E2 Size distribution of Brij®76:CHO:Solulan®C24 (47.5:47.5:5)

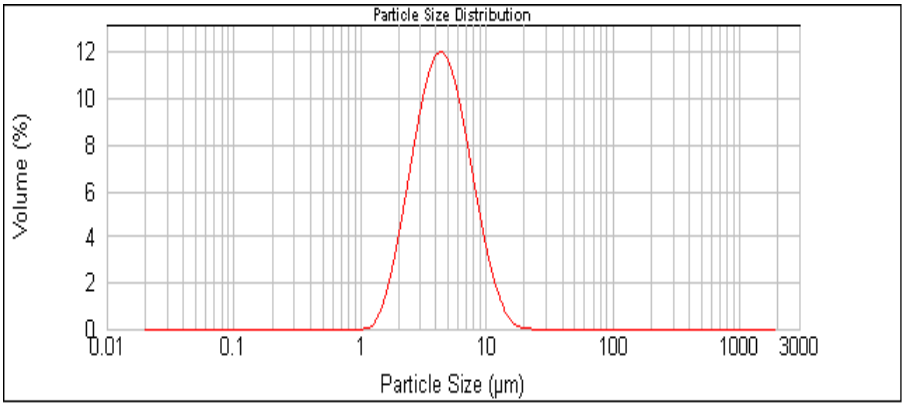


Figure E3 Size distribution of Span®20:CHO:Solulan®C24 (57.5:37.5:5)

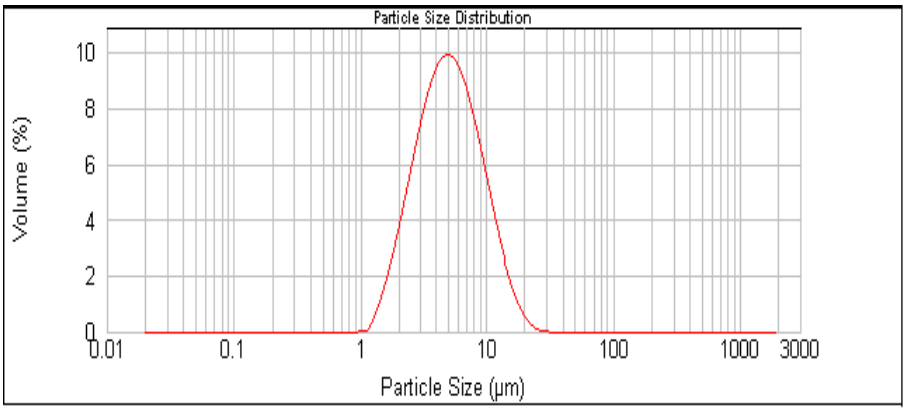


Figure E4 Size distribution of Span®40:CHO:Solulan®C24 (67.5:27.5:5)

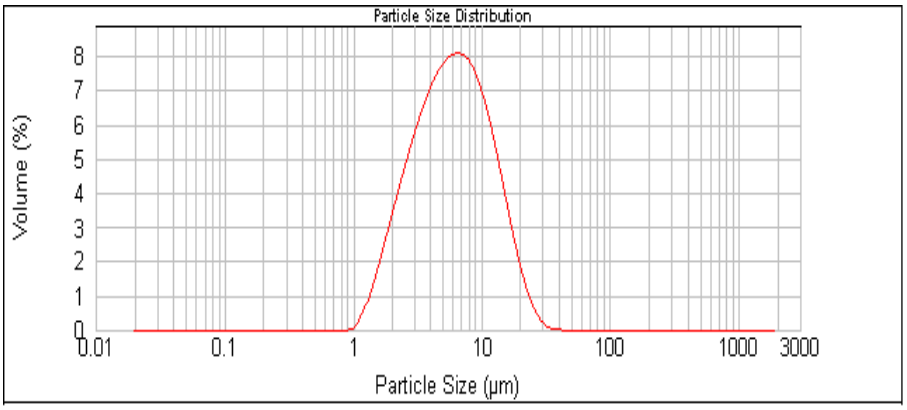


Figure E5 Size distribution of Span®60:CHO:Solulan®C24 (57.5:37.5:5)

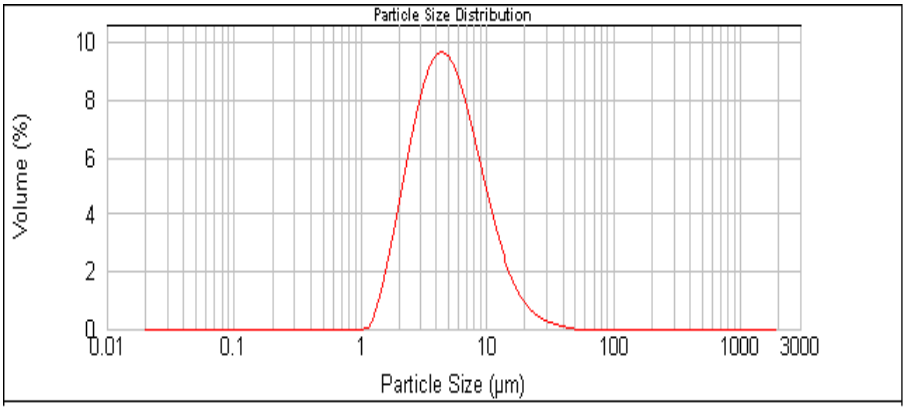


Figure E6 Size distribution of GDS:CHO:Brij®76 (45:15:40)

APPENDIX F

Release study

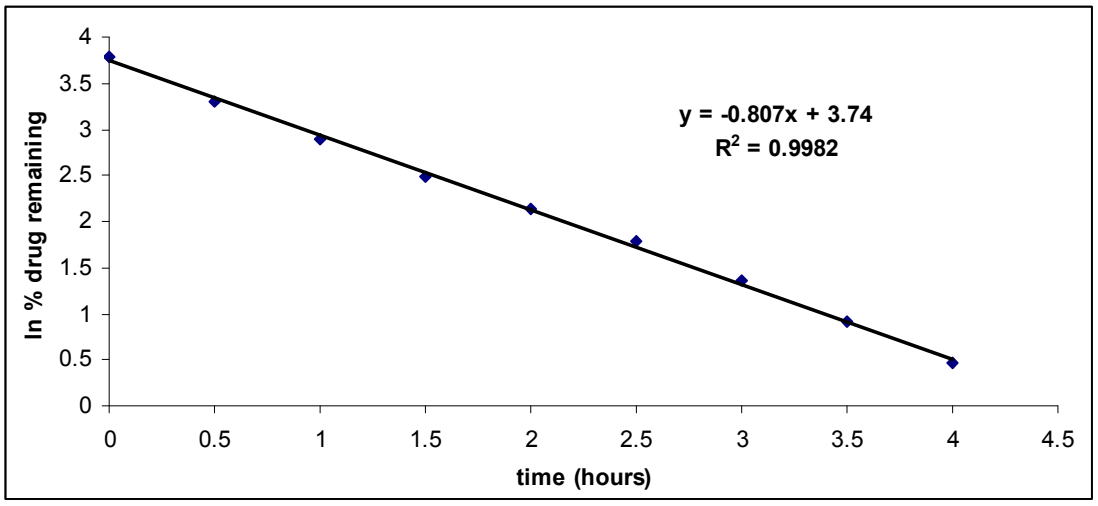


Figure F1 First order plot of GA solution

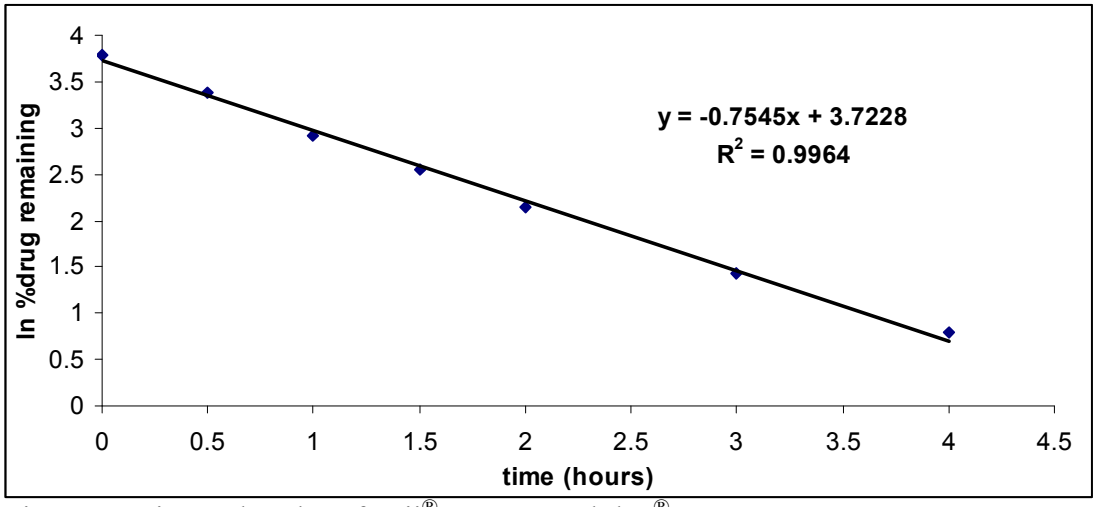


Figure F2 First order plot of Brij®76:CHO:Solulan®C24

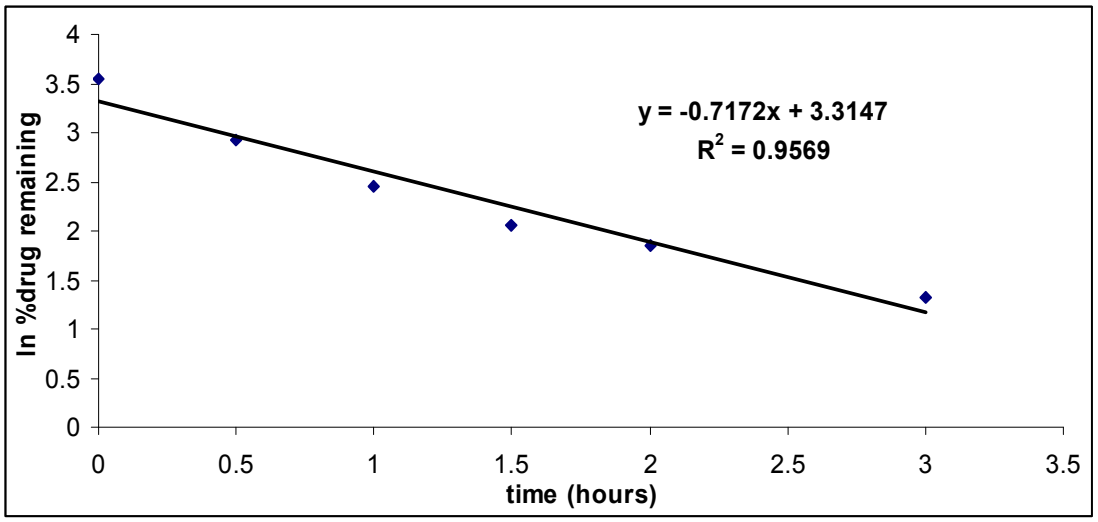


Figure F3 First order plot of Span®20:CHO:Solulan®C24

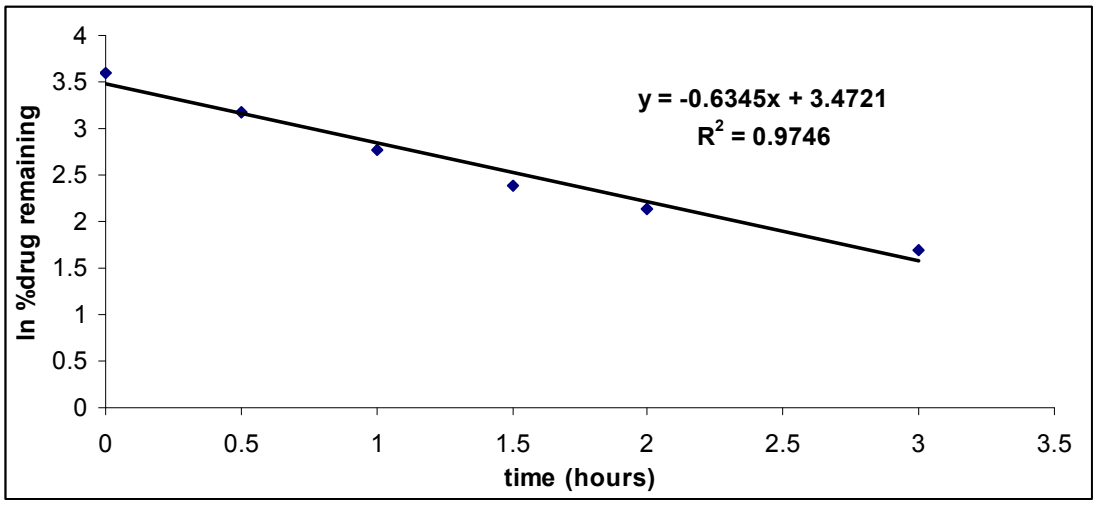


Figure F4 First order plot Span®40:CHO:Solulan®C24

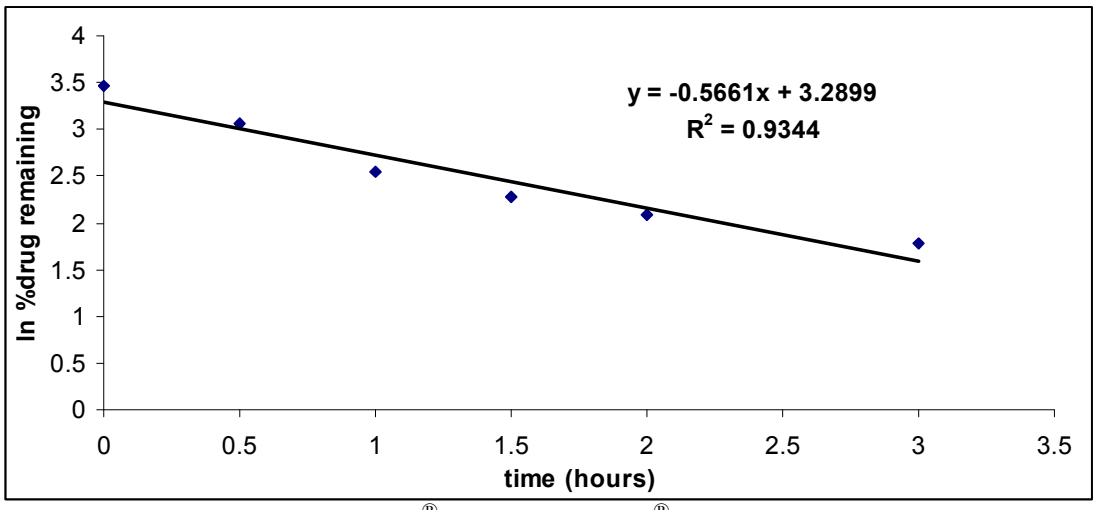


Figure F5 First order plot of Brij®52:CHO:Solulan®C24

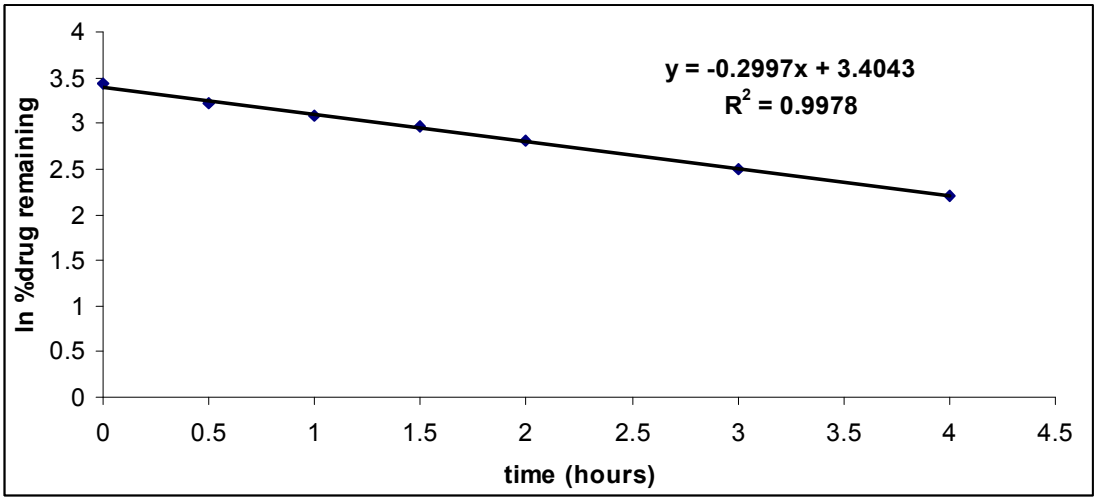


Figure F6 First order plot of GDS:CHO:Brij®76

VITA

Miss Sasiwimol Klinhom was born on January 12, 1981 in Ratchaburi, Thailand. She received the Bachelor's degree of Pharmaceutical Sciences, Chulalongkorn University, Bangkok in 2004. Since graduation, she has worked as a pharmacist at Department of Medicinal Sciences, Ministry of Public Health, Nonthaburi. She entered the Master's degree program in Pharmaceutics at Chulalongkorn University in 2006.