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คณะเภสัชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2551

EFFECTS OF ENTRAPPED COMPOUNDS AND CHEMICAL STRUCTURE AND CONCENTRATION OF BRIJ® ON FORMATION AND CHARACTERISTICS OF LIPOSOMES

Miss Amaraporn Roopdee

A Thesis Submitted in Partial Fulfillment of the Requirements

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Thesis Title	**EFFECTS OF ENTRAPPED COMPOUNDS AND CHEMICAL STRUCTURE AND CONCENTRATION OF BRIJ® ON FORMATION AND CHARACTERISTICS OF LIPOSOMES**
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การศึกษานี้มีวัตถุประสงค์เพื่อศึกษาอิทธิพลของโครงสร้างทางเคมี และความเข้มข้นของบริจ® ต่อการเกิดลิโพโซมและเพื่อศึกษาผลของสารที่ถูกกักเก็บต่อการเกิด และลักษณะของลิโพโซม จากผลการทดลอง แสดงว่า บริจ® 30 บริจ® 35 และ บริจ® 72 สามารถถูกใช้ในการเตรียมลิโพโซมโดยวิธีธินฟิล์มไฮเดรชันได้ ลักษณะของลิโพโซมเปล่า (พีซีลิโพโซม) และลิโพโซมที่ประกอบด้วยบริจ® (พีซี-บริจ® ลิโพโซม) ถูกประเมินตัวชี้วัดต่าง ๆ ประกอบด้วย รูปร่าง และสัณฐาน, ขนาด และการกระจายขนาด ลักษณะของลิโพโซมที่เตรียมได้ เป็นแบบมัลติลาเมลลาที่มีพื้นผิวเรียบ ขนาดของอนุภาคที่แตกต่างกันของลิโพโซม อธิบายได้ว่าเกิดจากความแตกต่างกันในคุณสมบัติทางเคมีฟิสิกส์ของบริจ® ลิโพโซมที่ประกอบด้วยบริจ® 30 ที่ความเข้มข้น 20 % โดยมวลของปริมาณไขมันทั้งหมด (L_30/20) และพีซี-บริจ® ลิโพโซมที่ประกอบด้วยบริจ® 72 ที่ความเข้มข้น 15 % โดยมวลของปริมาณไขมันทั้งหมด (L_72/15) ถูกเลือกสำหรับใช้ในการศึกษาต่อไป พีซีลิโพโซม และพีซี-บริจ®ลิโพโซมที่บรรจุกริซิโอฟูลวิน มีขนาดอนุภาคที่เล็กกว่าอนุภาคของพีซีลิโพโซม และพีซี-บริจ® ลิโพโซมเปล่า เปอร์เซ็นต์ประสิทธิภาพในการกักเก็บกริซิโอฟูลวินสอดคล้องกับขนาดอนุภาค ความไม่ชอบน้ำของกริซิโอฟูลวินทำให้คาดว่ามีผลกระทบโดยตรงกับลักษณะเฉพาะของไบแลร์ กริซิโอฟูลวินสามารถถูกกักเก็บในอนุภาคได้ดี เนื่องจากความไม่ชอบน้ำของกริซิโอฟูลวินทำให้มีประมาณ 20 % ของกริซิโอฟูลวินที่รั่วออกมาหลังจากเก็บไว้ที่ 4 องศาเซลเซียส นาน 1 เดือน พีซีลิโพโซม และพีซี-บริจ® ลิโพโซมที่บรรจุคาร์บอกซีฟลูออเรสซีนซึ่งชอบน้ำมีขนาดอนุภาคที่ใกล้เคียงกับขนาดอนุภาคของพีซีลิโพโซม และพีซี-บริจ® ลิโพโซมเปล่า เปอร์เซ็นต์ประสิทธิภาพในการกักเก็บคาร์บอกซีฟลูออเรสซีนก็เป็นสัดส่วนโดยตรงกับขนาดอนุภาคเช่นเดียวกัน การรั่วของคาร์บอกซีฟลูออเรสซีนจากทุกตำรับอาจเป็นไปได้ว่าเกิดจากการแตก และการรั่วของอนุภาค

ภาควิชา.....เภสัชกรรม.....ลายมือชื่อนิสิต.....อมราภรณ์ รูปดี.....

สาขาวิชา.....เภสัชกรรม.....ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

ปีการศึกษา.....2551.....ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม ร.ต.ท.หญิง ม่วงศิริ

4876621133: MAJOR PHARMACEUTICS

KEY WORD: BRIJ® / EDGE ACTIVATOR / LIPOSOMES / CHEMICAL STRUCTURE /GRISEOFULVIN / CARBOXYFLUORESCEIN.

AMARAPORN ROOPDEE: EFFECTS OF ENTRAPPED COMPOUNDS AND CHEMICAL STRUCTURE AND CONCENTRATION OF BRIJ® ON FORMATION AND CHARACTERISTICS OF LIPOSOMES. THESIS PRINCIPAL ADVISOR: ASST. PROF. PORNPEN WERAWATGANONE, Ph.D., THESIS CO-ADVISOR: ASST. PROF. Pol. Lt. WALAISIRI MUANGSIRI, Ph.D., 74pp.

The purposes of this study were to study the influence of chemical structure and concentration of Brij® on liposome formation and to investigate effect of entrapped compounds on formation and characteristic of liposomes. The results showed that, Brij® 30, Brij® 35 and Brij® 72 could be employed in preparation of liposomes using thin film hydration method. Blank liposomes (PC liposomes) and liposomes containing Brij® (PC-Brij® liposomes) were characterized for various parameters including vesicle shape and morphology, size and size distribution. The obtained PC-Brij® liposomes were characterized as multilamellar vesicles (MLVs) with smooth surface. Size differences of PC-Brij® liposomes were discussed on difference in physicochemical properties of Brij®. PC-Brij® liposomes composed of Brij® 30 at 20 % w/w of total lipid content (L_30/20) and PC-Brij® liposomes composed of Brij® 72 at 15 % w/w of total lipid content (L_72/15) were chosen for further study. Vesicle size reduction was observed from PC and PC-Brij® liposomes loaded with griseofulvin when compared to the corresponding blank PC and PC-Brij® liposomes. Percent griseofulvin entrapment efficiencies were related to vesicle size. Hydrophobicity of griseofulvin was expected to directly affect the bilayer characteristic. Griseofulvin was well retained inside the vesicles due to its hydrophobicity nature with approximately 20 % leakage after storage at 4 °C for 1 month. Sizes of PC and PC-Brij® liposomes loaded with hydrophilic carboxyfluorescein were comparable to the corresponding blank PC and PC-Brij® liposomes. % Carboxyfluorescein entrapment efficiencies were proportional to vesicle size. Leakage of carboxyfluorecein from all formulations was observed probably due to lyze and leak of vesicles.

Department:.....Pharmacy.....Student's Signature.....Amaraporn R.....

Field of Study:.....Pharmaceutics...Principal Advisor's Signature.....Pornpen Werawatganone.....

Academic Year:.....2008.....Co-Advisor's Signature.....W. Muangsiri.....

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LIST OF ABBREVIATIONS

%RSD	=	percentage of relative standard deviation
%v/v	=	percentage of volume by volume
%w/v	=	percentage of weight of volume
°C	=	degree Celsius
µM	=	micromolar
µm	=	micrometer
ANOVA	=	analysis of variance
AR	=	analytical reagent
CF	=	carboxyfluorescein
L	=	phosphatidylcholine liposomes
DPPC	=	dipalmitoylphosphatidylcholine
EE	=	entrapment efficiency
et al.	=	*et alii*, ‘and others’
FFTEM	=	freeze fracture transmission electron microscope
FRVs	=	freeze-dried rehydration vesicles
GF	=	griseofulvin
h	=	hour
HPLC	=	high performance liquid chromatography
kPa	=	kilopascal
LUVs	=	large unilamellar vesicles

LV	=	large unilamellar
mg	=	milligram
min	=	minute
ml	=	milliliter
MLVs	=	multilamellar vesicles
mM	=	millimolar
MPas	=	megapascal
mPas	=	millipascal
MW	=	molecular weight
nm	=	nanometer
nM	=	nanomolar
no.	=	number
NSEM	=	negative staining electron microscope
PC	=	phosphatidylcholine
R^2	=	coefficient of determination
rpm	=	revolution per minute
SEC	=	size exclusion chromatography
SEM	=	scanning electron microscope
SUVs	=	small unilamellar vesicles
T	=	temperature
T_C	=	phase transition temperature

TEM	=	transmission electron microscope

CHAPTER I

INTRODUCTION

Pharmaceutical and cosmetic industries have been developed a wide variety of vesicular systems with different physicochemical characteristics. Colloidal vesicular drug delivery systems, such as liposomes, niosomes and ethosomes, possess distinct advantages over conventional dosage forms. These carriers can act as drug reservoirs and drug release rate and/or drug targeting is controlled by modification of their compositions or vesicular surface (Alsarra et al., 2005; Honeywell-Nguyen, Groenink and Bouwstra, 2006). Phosphatidylcholine liposomes (PC liposomes) cannot effectively deliver active substances; therefore, compounds are incorporated into the lipid bilayer in order to improve physicochemical characteristics of the vesicles. Those compounds include cholesterol (Kerby, Clake and Papahadjopoulos, 1980), polyethylene glycol (PEG) (Allen et al., 1991), alcohol (Takeuchi et al., 2000), sugar (Wolkers et al., 2004) and surfactant (Mishra et al., 2007). Generally, PC liposomes are not stable because of electrostatic repulsion. Cholesterol is added to overcome the limitation of low stability and increasing permeability (Cócera et al., 2003). Cholesterol improves the structural and dynamic properties resulting in a progressive reduction of membrane fluidity. α-Tocopherol is used as an antioxidant for lipid to increase liposome stability. Small alcohol molecules, such as ethanol, propanol and butanol (Westh and Trandum, 1999) interact with lipid bilayer and increase water adsorption at the bilayer surface. Sugars, for example trehalose and sucrose, are often used as cryoprotectants. The interaction between sugar and phospholipid head groups reduces leakage of the entrapped compound through the bilayer. Non-ionic surfactant, Tween® 80 (Kronberg et al., 1990; Cevc, 1996; Jorg, 2001), is added the liposomes to modify the surface of vesicle bilayer.

In this study, liposomes were prepared from soybean phosphatidylcholine (PC) and Brij® (Polyoxyethylene alkyl ethers), non-ionic PC and non-ionic surfactant, respectively. The use of non-ionic compound was taken into considerations in order to reduce chance of incompatability. Brij® is a large group of non-ionic surfactants and often uses as pharmaceutical excipients. However, the effect of Brij® structure on

formation and properties of phosphatidylcholine- Brij® liposomes (PC-Brij® liposomes) has never been reported. This study was undertaken to evaluate the feasibility of Brij® as an additive in liposomal preparation, to determine effects of Brij® structures on vesicle formation, vesicle shape and morphology, size and size distribution of liposomes containing Brij® (PC-Brij® liposomes). The PC-Brij® liposomes loaded with model compounds, polar and non-polar compound, were prepared and consequently evaluated their entrapment efficiency and leakage.

Objectives :

1. To study effect of type of Brij®, on preparation of liposomes.
2. To find the relationship between chemical structure and concentrations of Brij® on characteristic of PC-Brij® liposomes.
3. To investigate effect of entrapped compounds on formation and characteristic of PC-Brij® liposomes.

CHAPTER II

LITERATURE REVIEWS

Liposomes

Liposomes are usually composed of phospholipids (PL) such as phosphatidylcholine (PC) and dipalmitoyl phosphatidylcholine (DPPC) (Monroig et al., 2007) and cholesterol (Were et al., 2003). The liposomes consist of two parts, which are hydrophilic core and lipophilic wall of vesicles. Generally, the lipid wall contains phospholipids (Figure 1). Phospholipids, such as soybean phosphatidylcholine (SPC) (Cevc et al., 1998; Cevc and Blume, 2003; El Maghraby, Williams and Barry, 2000; Yang et al., 2002; Lopes et al., 2004; Mishra et al., 2007) and egg phosphatidylcholine (EPC) (Fang et al., 2006; Hiruta et al., 2006; Sabín et al., 2006) are often used.

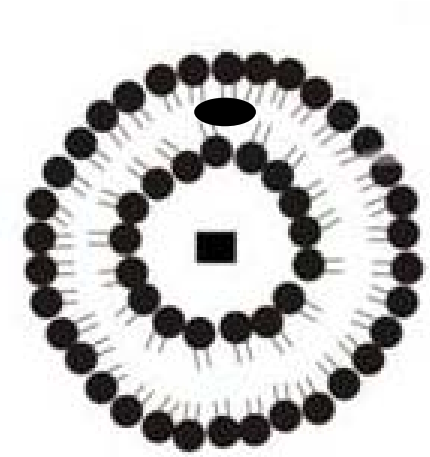


Figure 1. Structure of liposomes (; phosphatidylcholine, ; hydrophobic compounds and ■; hydrophilic compounds)

However, the limited stability of liposomes during storage and administration restricts their application and development. Many attempts have been made to enhance the stability of liposomes. The initial research showed the improved stability of liposomes composed of cholesterol and neutral long chain saturated phospholipids (Kerby, Clake and Papahadjopoulos, 1980). Two classic examples are the inclusion of cholesterol to reduce passive drug leakage (Ipsen and Mouritsen, 1988) and an addition of polymeric lipids to increase the blood circulation time (Woodle and Lasic, 1992).

1. Materials used in the preparation of liposomes

The basic compositions of the liposomes are composed of the phospholipids and the additives. Examples of phospholipids are phosphatidylcholine and dipalmitoyl phosphatidylcholine (DPPC). Additive substances; such as a biocompatible surfactant, are added in a bilayer softener.

1). Phospholipids

Phospholipids, the major components of biological membranes, are the main constituent of liposome wall. Phospholipids molecule consists of a hydrophilic polar head group and two hydrophobic tails (Figure.2). The polar head group contains one or more phosphate groups. Most phospholipids head groups are phosphoglycerides, which contain glycerol joining the head and the tail. Examples of phosphoglycerides include phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine, and phosphatidylinositol. The fatty acyl chain in biomembranes often contains even number of carbon atoms. They may be saturated or unsaturated. Regarding ionic state, phospholipids can be classified into three groups; neutral phospholipids such as phosphatidylcholine, negatively charged phospholipids such as phosphatidylserine and positively charged phospholipids such as DOTAP (Kim et al., 2004).

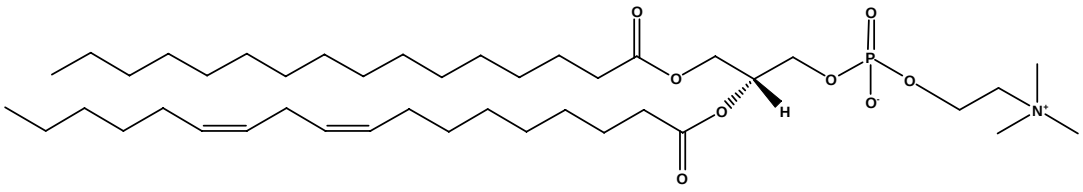
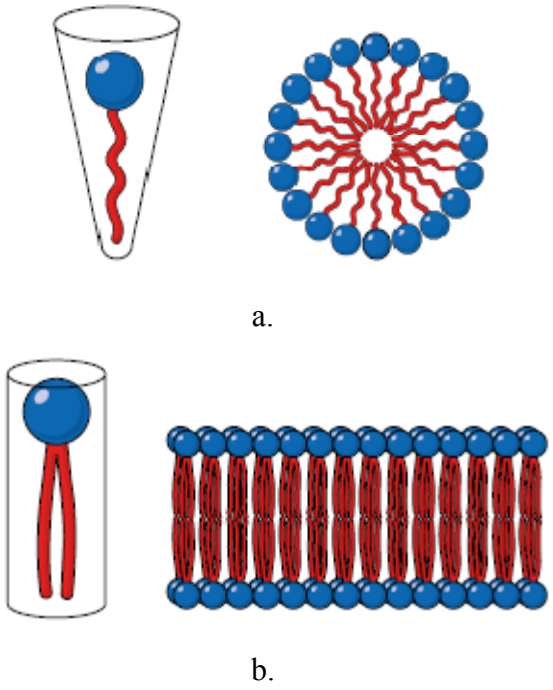


Figure 2. Structure of Phosphatidylcholine (Soybean)

The most commonly used in liposome preparations phospholipid is phosphatidylcholine (PC) (New, 1990). PC is an amphipathic molecule in which a glycerol bridge links a pair of hydrophobic acyl hydrocarbon chains with a hydrophilic polar head group (Figure 2). In aqueous media, PC molecules align themselves closely in planner bilayer sheets in order to minimize the unfavorable interactions between the bulk aqueous phase and the long hydrocarbon fatty acid chains. Such interactions are completely eliminated when the sheets fold on themselves to form close sealed vesicles. PC molecules contrast with other amphipathic molecules, such as detergent and lysolecithin, in that bilayer sheets are formed in preference to micellar structures. The reason is that the double fatty acid chain gives the molecule an overall tubular shape, more suitable for aggregation in planar sheets compared with detergent with a polar head and a single hydrocarbon chain. The detergent usually forms conical shape and fits into a spherical micellar structure (Figure 3).



a.

b.

Figure 3. Molecular shapes of surfactant (a) and phospholipids (b)

PC, also known as lecithin, can be derived from both natural and synthetic sources. They are readily extracted from egg yolk and soybean but less readily from bovine heart and spinal cord. They are often used as the principal phospholipids in liposome for a wide range of applications because of their low cost relative to other phospholipids, their neutral charge, and their chemical inertness. Lecithin from natural sources is, in fact, a mixture of PC with different chain lengths and various degrees of unsaturation. Lecithin from plant sources has a high level of polyunsaturation in the fatty acyl chains, while that from mammalian sources contains a higher proportion of fully saturated chains.

2). Additive for modifying of liposomes

Many studies have been studied the modification of bilayer composition to improve stability of liposomes. Several substances, such as non-ionic surfactant; Tween® 80, PEG, PVA and sucrose, are also used to improve liposomal physical stability.

Several researches successfully reported modification of liposomal bilayer with several substances such as polyethylene glycol (PEG) (Klibanov Zhang et al., 2008; Leal et al., 2008), polyvinyl alcohol (PVA) (Takeuchi et al., 2000; Xiaomei and Zhensheng, 2006), polyacrylic acid (PAA) (Takeuchi et al., 2001), oleyl alcohol (Sudimack et al., 2002), polysorbate 80 (Tween® 80) (Jorg, 2001; Sudimack et al., 2002), carboxymethyl chitin (Dong and Rogers, 1991), chitosan (Guo et al., 2003; Rengel and Barisic, 2002), cyclodextrin (Puskás and Csempesz, 2007), dextran derivatives (Elferink, Wit and Veld, 1992) and sugar (Maitani et al., 2007; Christensen et al., 2008).

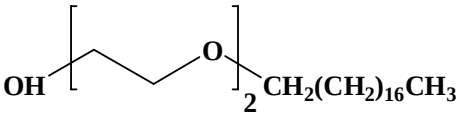
PEG liposomes have shown to prolong blood circulation time and to possess affinity to specific peptides on tumor cells. Thus, PEG liposomes are reported to improve therapeutic activity over non-targeted formulations (Torchilin et al., 1994; Terada et al., 2007; Rigacci et al., 2007; Zhang et al., 2008).

Disaccharides, trehalose and sucrose, are well-known compounds to protect destruction of lipid bilayer structures upon water removal during freeze-drying process (Christensen et al., 2007; Christensen et al., 2008). The protective effect of disaccharides is based on an extent of sugar-lipid interaction and high glass transition temperature of the carbohydrate. The interaction between the sugar and the phospholipid head groups is pivotal to prevent leakage through the bilayer (Crowe et al., 1997).

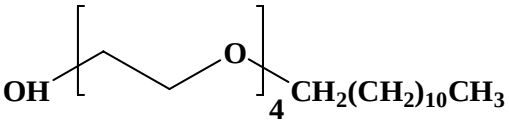
The influence of alcohols, such as ethanol, propanol and butanol, on the properties of lipid bilayers has been extensively investigated. Interactions of small alcohols with lipid bilayer membranes are water adsorption at the membrane-water interface (Westh and Trandum, 1999).

- **Brij®** (Rowe, 2003)

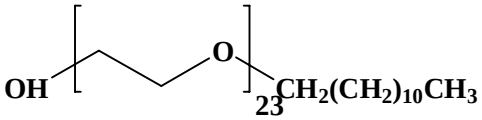
Brij®, polyoxyethylene alkyl ethers, is a non-ionic surfactant. Brij® varies considerably in their physical appearance from liquids, to pastes, to solid waxy substances (Figure 4). For example, Brij® 35 and Brij® 72 are off-white solid while Brij® 30 is clear liquid. They are colorless, white or cream-colored materials with a slight odor. They are nonionic surfactants and widely used in topical pharmaceutical formulations and cosmetics primarily as emulsifying agents for water-in-oil and oil-in-water emulsions. Each of polyoxyethylene alkyl ethers tend to be a polymer mixture of slightly varying molecular weights and the specified numbers describing polymer lengths are average values. Polyoxyethylene alkyl ethers are chemically stable even in strongly acidic or alkaline conditions.



polyoxyethylene (2) stearyl ether (Brij 72)



polyoxyethylene (4) lauryl ether (Brij 30)



polyoxyethylene (23) lauryl ether (Brij 35)

Figure 4. Structures of Brij® 72, Brij® 30 and Brij® 35

3). Entrapped substances

A wide variety of drugs have been incorporated in or associated with liposomes, one of the colloidal drug carrier systems for pharmaceutical and bio-medical applications. Liposomal drug delivery systems have the distinct advantage of being both non-toxic and biodegradable because they are mainly composed of phospholipids and phospholipid derivatives (Schiffelers, Storm and Bakker-Woudenberg, 2001). Moreover, liposomes have the capability of incorporating both hydrophilic and lipophilic drugs. Liposomes are phospholipid bilayer vesicles that have been studied extensively as potential drug carriers.

The liposomes can accommodate various of substances: lipophilic substances such as amphotericinB (Moribe, Maruyama and Iwatsuru, 1999), dexamethasone (Jain et al., 2003), ibuprofen (Mohammed et al., 2004), tamoxifen (Engelke et al., 2001) and triamcinolone-acetonide (Cevc and Blume, 2003) are incorporated into lipid bilayer; hydrophilic compounds such as clindamycin hydrochloride (Škalko, Čajkovac and Jalšenjak, 1992), 5-fluorouracil (El Maghraby, Williams and Barry, 2001; Glavas-Dodov et al., 2005), ketorolac tromethamine (Ruozi et al., 2005), indocyanine (Lopes et al., 2004), ofloxacin (Furneri et al., 2000), ketotifen fumarate (Elsayed et al., 2006) and propranolol hydrochloride (Mishra et al., 2007) are incorporated in the sheltered aqueous core; amphiphatic substances such as amitriptyline (Nii and Ishii, 2005) and tetracycline (Kohno, Tomono and Maesaki, 1998) typically adsorb to the lipid-water interface.

Liposomes improve release and biological activity in vivo. The liposomes increase local drug concentration when applied on the skin under nonocclusive conditions (Cevc and Blume, 1992). After application on the skin, water soluble drugs release from the vesicles according to concentration gradient. Such gradient is pronounced in the water-rich environment like in the living skin.

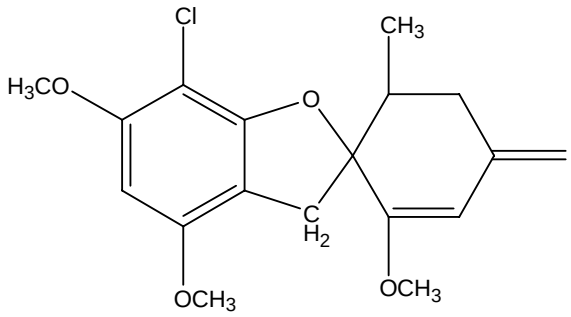
In general, release of fatty drugs from liposomes is much slower than the release of water soluble substances. Liposomes can prolong action of lipophilic drug. Fat soluble drugs are strongly anchored in liposome lipid bilayer. Such drugs

therefore do not leak from, but rather leach out off the vesicles. The extent of drug leaching out off liposomes depends on proximity of liposomes and cell membrane or receptors. The drug leaching is a function of local drug concentration and lipophilicity of the drug molecule. Amphiphatic molecules combine the features of water and fat soluble compounds. Such molecules consequently have intermediate and prolong release characteristics.

Griseofulvin and carboxyfluorescein are examples of hydrophobic and hydrophilic compounds, respectively, and could be loaded in vesicles (Fatouros and Antimisiaris, 2002; Barbet et al., 1984).

a). Griseofulvin (Florey, 1979)

The chemical name of griseofulvin is (2S-trans)-7-Chloro-2′,4,6-trimethoxy-6′-methylspiro[benxofuran-2 (3H), 1′-(2) cyclohexene] 3, 4′-dione or 7-Chloro-4,6-dimethoxycoumaran-3-one-2-spiro-1′-(2′-methoxy-6′-methylcyclohex-2′-en-4′-one) (Figure 5). Molecular weight equals to 352.77 ($C_{17}H_{17}ClO_6$). Griseofulvin is a white, odorless, crystalline powder.



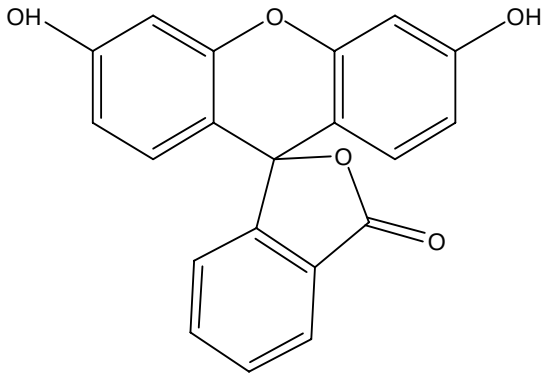
Griseofulvin

Figure 5. Structure of griseofulvin

Griseofulvin exhibits both fluorescence and luminescence. The solubilities of griseofulvin at 25°C are 30 g/L in acetone, 0.4 g/L in methanol; 0.2 g/L in Span® 80 and 0.2 g/L in water. Griseofulvin is a stable drug substance. After 12 years storage at room temperature no decomposition was detected. Griseofulvin is converted to griseofulvic acid under acidic conditions. There is no photo degradation under reasonable conditions of light exposure.

b). Carboxyfluorescein

Carboxyfluorescein, molecular weight equals to 376.32. Its structure is shown in Figure 6. Carboxyfluorescein is a fluorescent dye with an excitation and emission wavelength of 492 and 517 nm, respectively. Carboxyfluorescein can be dissolved in water pH > 5. It is commonly used as a tracer agent. The dye is membrane-impermeant and can be loaded into cells by microinjection or scrape loading. It can be incorporated into liposome, and allow for the tracking of liposome as they pass through the body. In addition, carboxyfluorescein has been used to track division of cells (Barbet et al., 1984).



6-Carboxyfluorescein

Figure 6. Structure of 6-carboxyfluorescein

2. Preparation methods

The preparation methods have been classified according to the three basic categories; physical dispersion, two-phase separation and detergent solubilization (New, 1990). Different types of liposomes are obtained as a consequence of preparation method selection. Some general methods are described herein.

1). Physical dispersion

Generally, thin lipid film is formed and rehydrated with appropriate medium. The mixture is shaked until milky dispersion is formed. The physical dispersion technique gives rise to multilamellar vesicles. This technique is also sub-classified as:

a). Hand-shaken multilamellar vesicles

In order to produce liposomes, lipids are dissolved in an appropriate solvent, dried by a rotary evaporator at a temperature above the phase transition temperature of the lipid, hydrated with the appropriate aqueous solution and agitated by hand until the milky dispersion is formed. In order to increase the surface-to-volume ratio of the dried lipid film, addition of glass beads to the lipid aqueous dispersion is optional. Generally, this technique is provided heterogeneous multilamellar vesicles. The most traditional method of liposome formulation is the thin film hydration method (Ferreira et al., 2005; Pavelić, Škalko-Basnet and Schubert, 2001; Sabín et al., 2006), which is similar to this technique in the concept. Many studies are chosen the thin film hydration method because it is an easy and simple method.

b). Sonicated vesicles

In order to reduce the vesicle size as small as possible, high energy input using either a sonicator probe or a sonicator bath is necessary.

- **Probe sonication**

Probe sonication is still probably the most widely used method for the preparation of small unilamellar vesicles (SUVs) on a small scale. Probe sonication generates heat leading to lipid degradation and introduces titanium ion, which is released from the probe, to the dispersion as a contaminant (Tasi, Liu and Chen, 2003).

- **Bath sonication**

This method is much milder than the probe sonication technique. The temperature awareness is usually unnecessary since the low generated heat is easily absorbed by the bath. However, vesicle size obtained by this technique is larger than the vesicle size obtained by the probe sonication technique.

c). Freeze-dried rehydration vesicles (FRVs)

The liposomal dispersion is lyophilized and rehydrated with aqueous solution (Torchilin and Weissig, 2003; Glavas-Dodov et al., 2005). This technique provides high entrapment efficiency, even for macromolecule.

2). Solvent dispersion

In this method, the lipids are dissolved in appropriate organic solvent followed by an addition of aqueous solution containing active compounds. After removing the organic solvent, the lipids align themselves into monolayer and form liposomes. Ethanol injection (Betz et al., 2005) and water in organic phase techniques are described below.

a). Ethanol injection

Lipid in ethanolic solution is rapidly injected in aqueous solution. The mixture is vigorously stirred using a magnetic stirrer. Liposomes are formed while ethanol is evaporated. This method gives low percent encapsulation of hydrophilic compound and leaves ethanol as contaminant in the phospholipid membranes.

b). Water in organic phase

Water in organic phase technique involves two steps; formation of the inner half and the outer half. The most popular technique under this category is the reverse phase evaporation technique (Sulkowski et al., 2005). The procedure includes preparation of w/o emulsion and reversion to o/w emulsion. After the w/o emulsion is formed, the solvent is removed from the emulsion by a rotary evaporator under reduced pressure. The emulsion is dried to a semi solid gel. In order to bring about the gel to collapse, the gel is shaken vigorously with a vortex mixer until large unilamellar vesicles (LUVs) are formed. The major disadvantage is the direct exposure of the entrapped compounds to organic solvent, which may introduce the instability of compound (New, 1990).

3). Detergent solubilization

In this technique, the lipids are brought into contact with detergent forming mixed micelles. The detergent is then removed from the mixed micelles, whereupon unilamellar vesicles are spontaneously formed. The most common detergents employed are bile salts and Triton-X (Monroig et al., 2007; New, 1990; Pavelić, Škalko-Basnet and Schubert, 2001).

3. Liposome characterization

Many methods have been used for characterizations of both PC liposomes and PC-Brij® liposomes. Some general characteristics are:

1) Vesicle shape and type

Vesicle shape is a fundamental characteristic of liposomes. Methods determining the morphology of liposomes vary in requirement and complexity. Some microscopic techniques, which are used in the morphological examination of liposomes and other vesicular carriers, are explained below (Talsma et al., 1987; New, 1990).

- Light microscopy is used to determine the gross view and rough size of the vesicles. The optical microscopy with phase contrast technique is a powerful technique for investigation of large unilamellar (LV) and multilamellar vesicles (MLVs). Samples are smeared on a glass slide and visualized under the microscope. Large MLVs gives very bright satellite feature under the phase contrast light microscope. The artifacts of this method are rather few. The sample thickness is critical because too thick sample interfere visualization of multilamellarity of the liposomes.

- Electron microscope gives precise information about size, configuration of lipid vesicles and their stability. Scanning electron microscope (SEM) is often used in many researches for reporting the shape and surface of the liposomes. Negative staining electron microscope (NSEM) freeze fracture electron microscope (Furneri et al., 2000) and transmission electron microscope (TEM) (Cho et al., 2006; Han et al., 2008; Ruozi et al., 2005; Sulkowski et al., 2005) are suitable techniques in observing lamellarity of the MLVs, large unilamellar vesicles (LUVs) and small unilamellar vesicles (SUVs). Both SEM and TEM are often used for investigation of vesicles, but drawbacks of TEM include several sample preparation steps and high cost.

2) Vesicle size and size distribution

The size distribution of liposomes is often difficult to estimate because vesicle sizes are often spread over a very wide range. This is particularly true for multilamellar liposomes (MLVs), which usually exhibit broad size distributions (Betageri, 1993). Methods in determination of size and size distribution are photon correlation spectroscopy (PCS) (Betz et al., 2005; Furneri et al., 2000; Lindner et al., 2008; Zhu et al., 2005), dynamic light scattering (DLS) (Lopes et al., 2004; Nagami, Matsumoto and Ueoka, 2006; Sezgin, Yüksel and Baykara, 2006; Were et al., 2003), laser diffraction method (Bendas and Tadros, 2007; Glavas-Dodov et al., 2005; Liu, Sun and Wang, 2008) and coulter counter (Han et al., 2008).

- DLS is useful technology in measurement of size of small vesicles and powerful technique for vesicle size ranging from 1 nm to 1 μm (Malvern). The artifacts of this method are the afterpulsing effect of photomultiplier, Doppler Effect, and the medium effect.

- Laser diffraction is used for vesicle size ranging from < 1 to 1000 μm (Malvern).

3) Lamellarity

The lamellarity determination is often accomplished by ^{31}P NMR. In this technique, the addition of Mn^{2+} quenches the ^{31}P NMR signal of phospholipids on the exterior face of the liposomes. The degree of lamellarity is determined from the signal ratio before and after the Mn^{2+} addition. While frequently used, this technique has recently been found to be quite sensitive to the Mn^{2+} and buffer concentration and the types of liposomes under analysis (Edward and Baeumner, 2006). Another method for determining this characteristic is freeze fracture transmission electron microscope (FFTEM).

4) Entrapment efficiency

It is clearly essential to measure the quantity of entrapped substance inside liposomes. The entrapment efficiency is determined after separation of the unentrapped drug. The amount of entrapped drug in the vesicles is then determined by disrupting the vesicles by using Triton X-100 (Ono et al., 2002) or ethanol. The lyzing solvent depends on solubility of active substance, PC, and other stabilizers. The centrifugation method (Elsayed et al., 2006; Fang et al., 2006; Glavas-Dodov et al., 2005), size exclusion chromatography (SEC) (Ioele et al., 2005; Li, He and Li, 1996; Lopes et al., 2004; Pavelić, Škalko-Basnet and Jalšenlak, 2004; Ruysschaert et al., 2005; Stensrud et al., 2000; Were et al., 2003) and dialysis (Muñoz et al., 1998) are techniques to separate vesicles from dispersion.

- Ultracentrifugation is fast and simple technique with less sample preparation and low cost. Thus, centrifugation is proven to be a fast and easy technique for separating large liposomes from non-encapsulated (Torchilin and Weissig, 2003).

- SEC separates vesicles based on size using a stationary phase making from crosslinked polysaccharide beads such as Sephadex G-50 (Li, He and Li, 1996; Essa, Bonner and Barry, 2002), Sephadex G-75 or Sepadex G-100. The stationary phase is packed in a column. Sample is applied on the column and eluted with mobile phase. Gel packing technique is critical since air bubble in the column is prohibited (Torchilin and Weissig, 2003). Moreover, this technique is time consuming.

5) Stability of liposomes

Stability of liposomes includes physical stability and chemical stability. Physical stability is concerned the interaction forces between liposomes resulting in liposomes aggregation. The forces between membranes include Van der Waals forces and hydration force. Furthermore, leakage is another parameter measuring vesicle stability (Hashizaki et al., 2006; Carafa et al., 2006; Ji et al., 2006) and aggregation and fusion of vesicles (New, 1990). Method determining leak of substance is similar to entrapment efficiency technique. In addition, chemical stability is related to oxidation and hydrolysis reactions.

CHAPTER III

MATERIALS AND METHODS

MATERIALS

1. Brij® 30 (East Asiatic Company, Thailand)
2. Brij® 35 (East Asiatic Company, Thailand, Lot no. 57078)
3. Brij® 72 (East Asiatic Company, Thailand, Lot no. 57487)
4. Carboxyfluorescein (Approx. ≥95%, Sigma, USA, Lot no. 1284701)
5. Chloroform, AR grade (Lab-Scan Co., Ltd Ireland)
6. Ethanol, AR grade (Merck, Germany)
7. Griseofulvin (Approx. ≥95%, Lot no. 362385, Sigma, USA)
8. Hydrochloric acid solution
9. Methanol, HPLC grade (Lab-Scan Co., Ltd Ireland)
10. Phosphatidylcholine (from soybean, Phospholipon 90) (Rhodia, Germany)
11. Sodium hydroxide, AR grade (Merck, Germany, Lot no. 630)
12. Standard buffer solution pH 10 (Merck, Germany)
13. Standard buffer solution pH 4 (Merck, Germany)
14. Standard buffer solution pH 7 (Merck, Germany)
15. Triton X-100 (Sigma-Aldrich, USA, Lot no. 065K0122)

APPARATUS

1. Analytical balance (AG 285, Mettler Toledo, Switzerland)
2. Analytical balance (PG 403-S, Mettler Toledo, Switzerland)
3. Cellulose acetate membrane filter 25 mm, 10μm (Millipore, USA)
4. Cellulose acetate membrane filter 25 mm, 2 μm (Millipore, USA)
5. Centrifuge bottles, polycarbonate (10.4ml) (Beckman Instruments, USA)
6. Extruder (Lipex ™ Nothern Lipids, Canada)
7. Light Microscope (Nikon Eclipse E200, Japan)

8. Micropipette (Biohit, Finland)
9. Particle size analyzer (Mastersizer 2000 Ver 5.1, Malvern Instruments, UK)
10. pH meter (Model 420A, Orion, USA)
11. Refrigerated Incubator (FOC 225i, VELP Scientifica, Italy)
12. Rotary Evaporator (Rotavapor R-215, Buchi, Switzerland)
13. Round bottom-flask 1000 ml (Schott Duran, Germany)
14. Scanning electron microscope (Model JSM-5410LV, JEOL®, Japan)
15. Spectrofluorometer (Jasco, Japan)
16. Ultracentrifuge (L80, Beckman, USA)
17. Ultrasonicator (Transsonic Digital S T900/H, Elma, Germany)
18. UV-visible spectrophotometer (UV-1601, Shimadzu, Japan)
19. Vortex mixer (Vortex Genies-2, Scientific Industries, Inc., USA)

METHODS

1. Quantification of griseofulvin and carboxyfluorescein by spectrofluorometry

1.1. Preparation of stock solution

1.1.1. Preparation of griseofulvin stock solution

The stock solution was prepared by completely dissolving 3.53 mg of griseofulvin in 50 % v/v methanol in chloroform in a 100-ml volumetric flask. The solution was adjusted to the volume, giving the final concentration of 0.10 mM.

1.1.2. Preparation of carboxyfluorescein stock solution

The stock solution was prepared by dissolving 5.64 mg of carboxyfluorescein in 100-ml ultrapure water and adjusting pH to 8.0 with 2 N sodium hydroxide. The solution was adjusted to the volume, giving the final concentration of 0.17 mM.

1.2. Determination of excitation and emission wavelengths by spectrofluorometry

1.2.1. Griseofulvin

The baseline was prepared using 50 % v/v methanol in chloroform. Excitation and emission spectra were scanned from 200 to 400 nm and from 300 to 500 nm, respectively. The excitation and emission wavelengths were determined at wavelengths giving highest absorption and emission intensities.

1.2.2. Carboxyfluorescein

The baseline was settled with ultrapure water, adjusted to pH 8.0 using 2 N sodium hydroxide. The setting excitation and emission spectra were scanned from 400 to 510 nm and from 300 to 600 nm, respectively. The excitation and emission wavelengths were determined at wavelengths giving highest absorption and emission intensities.

1.3. Validation of spectrofluorometry

Fluorescence spectroscopic assay of both griseofulvin and carboxyfluorescein were validated. The essential parameters to ensure the acceptability of the performance of the analytical method are specificity, linearity, precision and accuracy (ICH Q2R1).

1.3.1. Specificity

The specificity of the method was examined by comparing emission spectrum of model compounds in the present of other components in the blank liposomal formulations. The experiment was aimed to confirm that the emission peak of the interest was free from interference by other components in the sample.

1.3.2. Linearity

Linearity of griseofulvin in a concentration range of 1.00 to 7.00 μM and carboxyfluorescein in a concentration range of 6.94 to 24.5 nM were studied. Five concentrations of standard mixture solutions in the aforementioned ranges and

three replicates of each concentration were prepared and analyzed. The relationship between logarithms of fluorescence intensity versus their logarithm concentrations was evaluated with the least square linear regression. The linearity was determined from the coefficient of determination (R^2).

Acceptance criteria (ICH Q2R1)

The coefficient of determination (R^2) should greater than 0.999.

1.3.3. Accuracy

The accuracy of an analytical method describes the closeness of the mean test results obtained by the method to the true value (concentration) of the analyte. Three concentrations and five determinations per concentration were prepared and analyzed. The accuracy of the method was shown as the percentage of recovery.

Acceptance criteria (ICH Q2R1)

The percentage of recovery should be within 98-102 %.

1.3.4. Precision

a) Within run precision

Within run precision was performed by measuring five replicates of each three standard concentrations (low, medium, high) of standard mixture solutions in the same day. The estimated concentrations were obtained and the percentage of relative standard deviation (% RSD) of each concentration was determined.

b) Between run precision

Between run precision was determined by analyzing three concentrations (low, medium, high) of standard mixture solutions on five different days. The estimated concentrations were compared and the percentage of relative standard deviation (% RSD) of each concentration was determined.

Acceptance criteria (ICH Q2R1)

The percentage of relative standard deviation (% RSD) for both within run and between run precision should less than 2 %.

2. Preparations and evaluations of blank PC and PC-Brij® liposomes (New, 1990; Torchilin and Weissig, 2003)

2.1. Preparation of blank PC and PC-Brij® liposomes

2.1.1. Optimization of liposomal preparation procedure

Effects of pressure during evaporation step, pressure reduction rate and % lipid content on film formation and film appearance were evaluated. To determine optimum operating conditions of the rotary evaporator, PC liposomes were prepared under following conditions, using thin film hydration method. Pressure during evaporation step was varied between 200 and 300 mbar, pressure reduction rate was varied between 5 and 10 mbar/min and % lipid content was varied between 2 and 5 % w/v.

2.1.2. Blank PC liposomes

Four milliliters of 5 % w/v PC stock solution in chloroform was transferred into a 1000-ml round bottom-flask. Then, 6 ml chloroform was added into the flask to get the final lipid concentration at 2 %w/v. The flask was attached to a rotary evaporator, immersed in a 40 °C water bath and rotated under vacuum at a speed of 100 rpm under pressure of 200 mbar until the dried lipid film was formed. The deposited lipid film was hydrated with 10 ml of ultrapure water by rotating the round bottom-flask at 100 rpm for 1 h at 40 °C. Liposomal dispersions were further sonicated in an ultrasonic bath at 40 °C for 30 min, resulting in a coarse liposomal dispersion. The formation of vesicles was visualized under cross polarized lightmicroscope.

2.1.3. Blank PC-Brij® liposomes

Each PC-Brij® liposomal preparation was composed of PC and Brij® series; Brij® 30, Brij® 35 or Brij® 72. The ratio of Brij® to PC was varied from 10 to 20 % w/w of total lipid content as shown in Table 1. These liposomes were prepared in the same manner as PC liposomes in 2.1.2. The various amounts of Brij® were added in PC solution. Finally, the formation of PC-Brij® liposomes was evaluated under cross polarized lightmicroscope.

Table 1. Composition of PC liposomes and PC-Brij® liposomes using various ratios of PC* to Brij®

Formulation Code	Brij® type	PC: Brij® (% w/w)
L	-	100 : 0
L_30/10	Brij® 30	90 : 10
L_30/15	Brij® 30	85 : 15
L_30/20	Brij® 30	80 : 20
L_35/10	Brij® 35	90 : 10
L_35/15	Brij® 35	85 : 15
L_35/20	Brij® 35	80 : 20
L_72/10	Brij® 72	90 : 10
L_72/15	Brij® 72	85 : 15
L_72/20	Brij® 72	80 : 20

* PC concentration was kept constant at 2 % w/v in liposomal dispersion.

2.2. Evaluations of blank PC and PC-Brij® liposomes

The characteristics of each formulation such as microscopic appearance, size and size distribution were investigated.

2.2.1. Microscopic appearance

The PC and PC-Brij® liposomes were visualized using a cross polarized light microscope. The appearances were perceived in vesicle formation, shape, size and aggregation.

2.2.2. Vesicle size and size distribution

The vesicle size and size distribution were determined by laser diffraction method (Mastersizer 2000 Ver 5.1, Malvern Instruments equipped with a computerized inspection system) at 25 °C. The sample was diluted using deionized water to an appropriate concentration, the obscuration value more than 10 %. All the measurements were conducted in triplicate. Data were analyzed using a one-way analysis of variance (ANOVA) along with Tukey's multiple comparisons (SPSS version 16.0). The PC liposomes and two PC-Brij® liposomal formulations were selected for further drug loading study.

3. Preparations and evaluations of PC and PC-Brij® liposomes loaded with griseofulvin and carboxyfluorescein

Griseofulvin (GF) and carboxyfluorescein (CF) were loaded into PC liposomes and selected formulations of PC-Brij® liposomes.

3.1. Preparations of PC and PC-Brij® liposomes loaded with griseofulvin and carboxyfluorescein

3.1.1. PC liposomes loaded with griseofulvin and carboxyfluorescein

The maximum concentration of griseofulvin (GF) that could be loaded in liposomes was determined by titrating process. Various concentrations of GF were added in PC solution. During thin film formation step, GF was expected to precipitate and form crystal in the dried film if the GF concentration was above the solubility limitation. The drug crystal was observed under a cross polarized lightmicroscope. The selected GF concentration was the first highest concentration that no drug crystal was observed.

a) Griseofulvin loaded in PC liposomes

GF stock solution was prepared at concentration of 0.10 mM in chloroform. Ten milliliters of 0.10 mM GF stock solution was mixed with 4 ml of 5 % w/v PC stock solution in chloroform. Then, the solution was transferred to a 1000-ml round bottom-flask with a ground glass-neck. The conditions and method were

similar to the preparation process of blank PC liposomes (section 2.1.2.). The deposited lipid film was subsequently hydrated with 10 ml of ultrapure water. Vesicle formation was visualized under a cross polarized light microscope.

b) Carboxyfluorescein loaded in PC liposomes

CF solution was prepared at concentration of 0.17 mM in ultrapure water adjusted to pH 8.0 using 2 N sodium hydroxide. Four milliliters of 5 % w/v PC stock solution was transferred into a 1000-ml round bottom-flask with a ground glass-neck and 10 ml of chloroform was added into the flask. Using the same method as mentioned in 2.1.2., PC thin film was obtained and the deposited lipid film was hydrated with 10 ml of 0.17 mM CF solution. Vesicle formation was visualized under a cross polarized light microscope.

3.1.2. PC-Brij® liposomes loaded with griseofulvin and carboxyfluorescein

a) Griseofulvin loaded in PC-Brij® liposomes

Ten milliliters of 0.10 mM griseofulvin was added with selected amounts of Brij® and four milliliters of 5 % w/v PC stock solution in chloroform. Then, solution was added in the 1000-ml round bottom-flask with a ground glass-neck. The conditions and method were the same process as blank PC-Brij® liposomal preparation (section 2.1.3.).

b) Carboxyfluorescein loaded in PC-Brij® liposomes

Four milliliters of 5 % w/v PC stock solution was transferred to a 1000-ml round bottom-flask with a ground glass-neck and added with a selected ratio of Brij®. Then, ten milliliters chloroform was added into the flask. PC thin film was prepared in the same manner as mentioned in 2.1.3. The deposited lipid film was disrupted by hydration with 10 ml of 0.17 mM carboxyfluorescein in ultrapure water adjusted to pH 8.0 using 2 N sodium hydroxide.

3.2. Evaluations of PC and PC-Brij® liposomes loaded with griseofulvin and carboxyfluorescein

The characteristics of drug loaded liposomes were evaluated using the same methods as mentioned in 2.2. The entrapment efficiency and leakage were also investigated in drug loaded liposomes.

3.2.1. Microscopic appearances

Morphology of drug loaded the liposomes was investigated using both cross polarized light microscope and scanning electron microscope (SEM). For SEM, samples were adhered on the glass slide using a specific fixation technique. Samples were dehydrated with a graded series of ethanol, made critical point dryer, mounted and coated with gold. Finally, SEM micrographs of the treated sample were taken under SEM.

3.2.2. Vesicle size and size distribution

The vesicle size and size distribution were determined by laser diffraction method and performed as described in 2.2.2.

3.2.3. Entrapment efficiency (EE)

The drug loaded liposomal dispersions were ultracentrifuged at 60,000 rpm for 6 h at 4 C° using a cooling ultracentrifuge. The clear supernatant was carefully decanted and determined for griseofulvin and carboxyfluorescein by spectrofluoroscopy technique. To determine the entrapped compounds, GF liposomes were lyzed by 50 % v/v methanol in chloroform while CF liposomes were dissolved in 1 % w/v Triton X-100. Then, amounts of entrapped GF and CF were analyzed using spectrofluorometry technique. The fluorescence intensity of griseofulvin was recorded at excited wavelength of 294 nm and emission wavelength of 412.5 nm. The fluorescence intensity of carboxyfluorescein was recorded at excitation and emission wavelengths of 495 and 515 nm, respectively. The entrapment efficiency and % recovery were calculated using Equation 1 and 2, respectively (Dubey et al., 2006; Peltonen et al., 2004). The experiments were performed in triplicate.

$$\% \text{ Entrapment efficiency} = \frac{\text{Amount of entrapped in liposomes}}{\text{Total amount loaded compound in sample}} \times 100 \qquad \text{Equation 1}$$

$$\% \text{ Recovery} = \% \text{ entrapped in vesicles} + \% \text{ non entrapped in supernatant} \qquad \text{Equation 2}$$

3.2.4. Determination of leakage

The leakage of entrapped compound from the PC-Brij® liposomes was tested and compared to that of PC liposomes. In this study, six formulations of PC and PC-Brij® liposomes loaded with GF and CF were separately prepared as previously described. Each formulation was prepared 5 times and pooled together. Then, each of liposomal dispersions was divided into 5 10-ml portions. Each portion was stored in a sealed glass vial at 4 °C. At appropriate time, samples were taken to analyze for concentration of GF or CF in supernatant and vesicles as mentioned in 3.2.4. In case of liposomes loaded with GF, the samples were taken at 0, 1, 2, 3 and 4 weeks. For liposomes loaded with CF, the samples were taken at 0, 6, 12, 18 and 24 hours. Amounts of GF or CF in the supernatant and in the vesicles were determined using spectrofluorometry and presented as extent of leakage (Alam et al., 2008) (Equation 3) and % drug retained (Alam et al., 2008) (Equation 4) during the storage time.

$$\begin{aligned}\text{Extent of leakage} &= \text{Initial entrapment efficiency of sample } (\%) \\ &\quad - \% \text{ Retained after storage}\end{aligned} \qquad \text{Equation 3}$$

$$\% \text{ Drug retained} = \frac{\text{Amount of entrapped compound in vesicles at time t}}{\text{Amount of entrapped compound at initial time}} \times 100 \qquad \text{Equation 4}$$

4. Data analysis

Data analysis was done with the software package SPSS version 16.0. Statistical significance was checked with ANOVA and considered to be granted at $P < 0.05$. All results give the mean of all measured value ± standard deviation (SD).

CHAPTER IV

RESULTS AND DISCUSSION

1. Quantification of griseofulvin and carboxyfluorescein by spectrofluorometry

1.1. Determination of excitation and emission wavelengths by spectrofluorometry

1.1.1. Griseofulvin

Figure 7 illustrates that excitation and emission wavelengths of griseofulvin in 50 % v/v methanol in chloroform were 294.0 and 412.5 nm, respectively.

1.1.2. Carboxyfluorescein

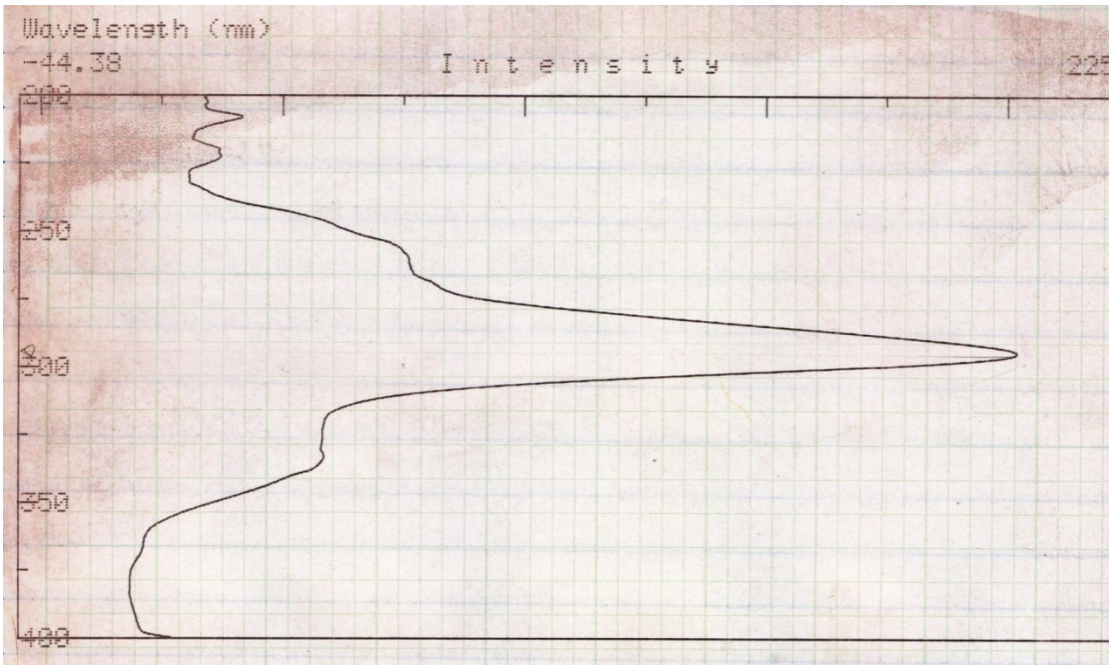
Figure 8 illustrates that excitation and emission wavelengths of carboxyfluorescein in ultrapure water adjusted to pH 8.0 using 2 N sodium hydroxide were 495.0 and 515.0 nm, respectively.

1.2. Validation of spectrofluorometry

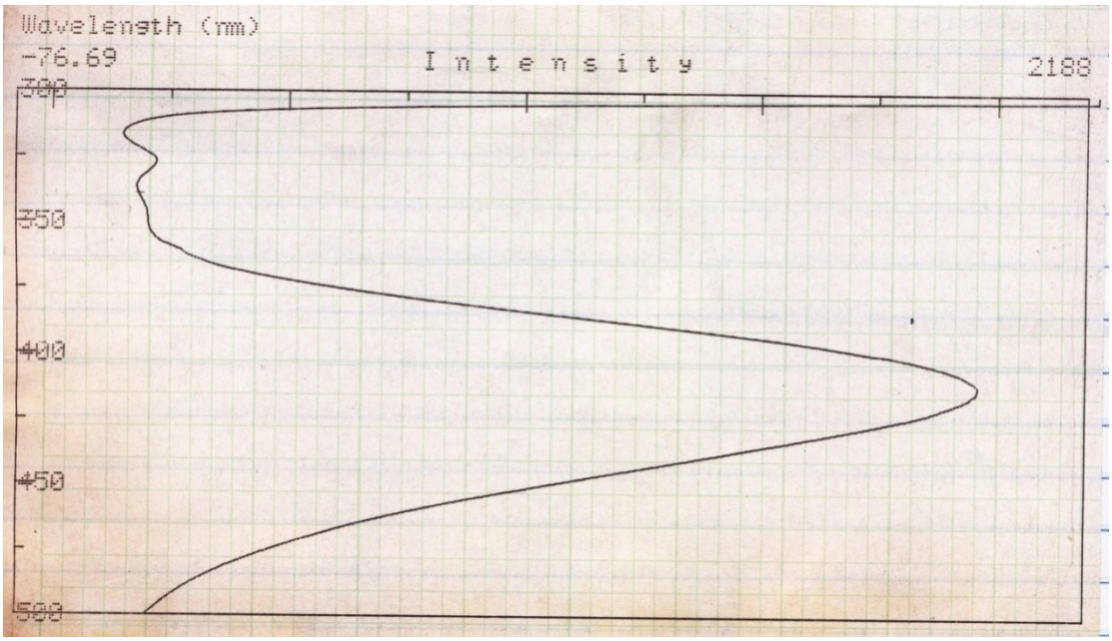
1.2.1. Specificity

a) Griseofulvin

Spectrums of griseofulvin in 50 % v/v methanol in chloroform gave a maximum excitation wavelength of 294.0 nm and a maximum emission wavelength of 412.5 nm. Using an excitation wavelength of 294.0 nm, mixtures of phosphatidylcholine (PC) and Brij® 72, Brij® 35 or Brij® 30, gave a maximum emission wavelength of 346.5, 346.5 and 398 nm, respectively. Although, maximum emission wavelength of placebo was in proximity of the emission wavelength of GF, the fluorescence intensity at 412.5 nm of placebo mixtures was less than 7.0 %. In other words, GF concentration was overestimated by 7.0 % in the presence of Brij®.

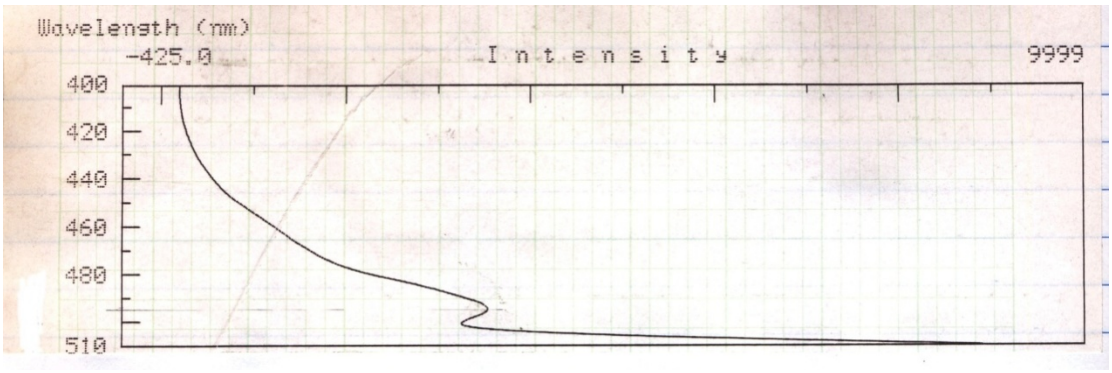


a)



b)

Figure 7. Excitation (a) and emission (b) spectrum of GF in 50 % v/v methanol in chloroform



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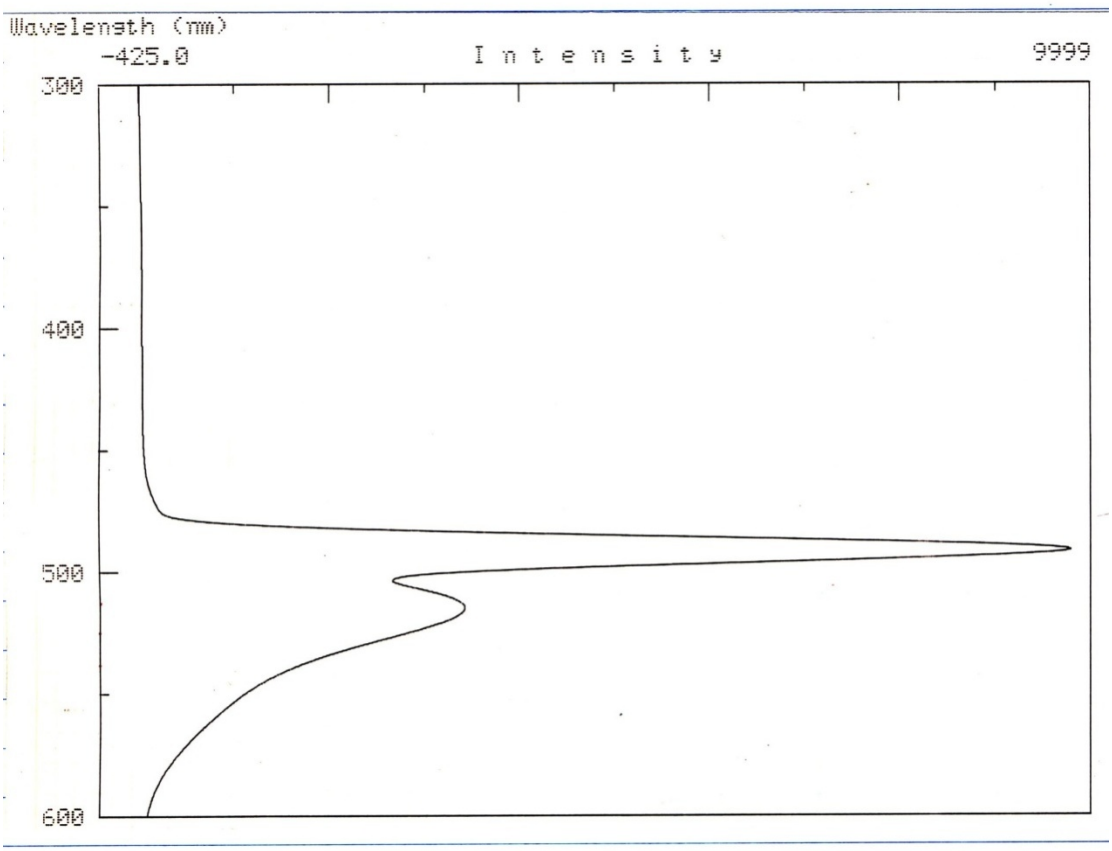


b)

Figure 8. Excitation (a) and emission (b) spectrum of CF in ultrapure water adjusted to pH 8.0 using 2 N sodium hydroxide

b) Carboxyfluorescein

Solution of carboxyfluorescein (CF) in 1 %w/v Triton X-100 in ultrapure water adjusted to pH 8.0 using 2 N sodium hydroxide gave a maximum excitation wavelength of 495.0 nm and a maximum emission wavelength of 512.0 nm. Using an excitation wavelength 495 nm, the solution of PC and each of Brij® 72, Brij® 35 or Brij® 30, in 1 %w/v Triton X-100, gave an emission spectrum at maximum wavelength of 515.0 nm due to Brij®. Although, maximum emission wavelength of placebo was in proximity of the emission wavelength of CF, the fluorescence intensity at 515.0 nm of placebo mixtures was less than 0.9 %. In other words, CF concentration was overestimated by 0.9 % in the presence of Brij®.

1.2.2. Linearity

a) Griseofulvin

Typical calibration curve of griseofulvin shows a coefficient of determination (R^2) of 0.9998 (Figure 9). The approximated slope value of 1.0 indicates that the spectrofluorometer was in good conditions. The acceptable concentration range of griseofulvin was 1.00 to 7.00 µM.

b) Carboxyfluorescein

Typical calibration curve of carboxyfluorescein shows a coefficient of determination (R^2) of 0.9997 (Figure 10). The slope value close to 1.0 indicates that the spectrofluorometer was in good conditions. The acceptable concentration range of carboxyfluorescein was 6.94 to 24.98 nM.

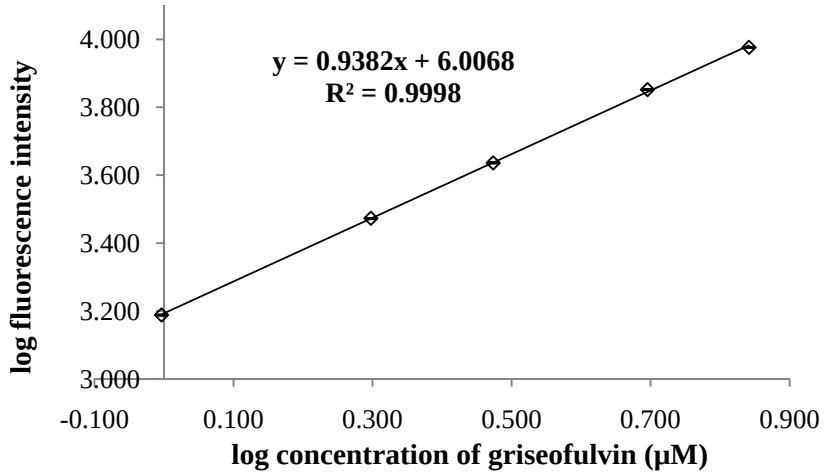


Figure 9. The logarithmic transformation relationship between fluorescence intensities and griseofulvin concentrations (values represented as mean diameter ± *SD*, n=3).

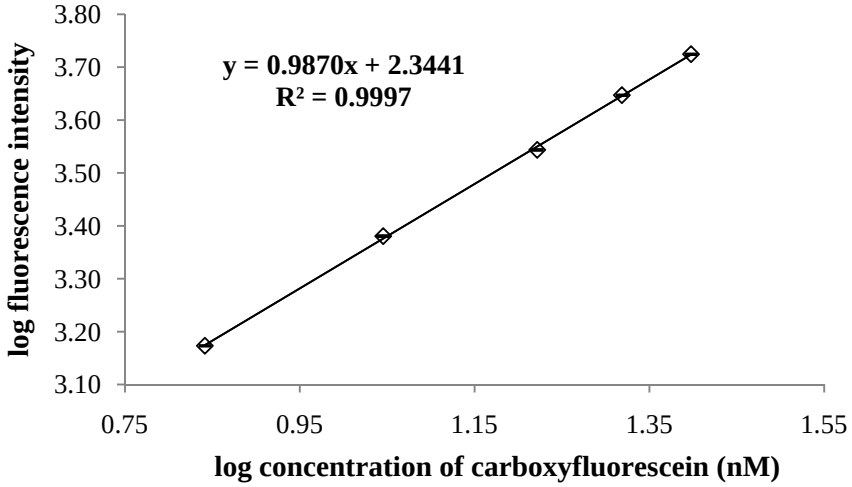


Figure 10. The logarithmic transformation relationship between fluorescence intensities and carboxyfluorescein concentrations (values represented as mean diameter ± *SD*, n=3).

1.2.3. Accuracy

a) Griseofulvin

- **Analysis of GF in solution**

Theoretical concentrations of GF standard solutions were 2.00, 2.80 and 5.00 µM. The estimated concentrations were established to be 2.000 ± 0.012, 2.812 ± 0.006 and 5.033 ± 0.013 µM. Percent analytical recovery was calculated to be 99.50 ± 0.44, 100.22 ± 0.16 and 100.48 ± 0.22 %, respectively. (Data as shown in APPENDIX, page 61, Table 11)

- **Analysis of GF in PC-Brij® liposomes dispersion**

Theoretical concentrations of GF standard solutions were 2.00, 2.80 and 5.00 µM. The estimated concentrations were established to be 2.044 ± 0.004, 2.853 ± 0.003 and 5.083 ± 0.016 µM. Percent analytical recovery was calculated to be 101.71 ± 0.22, 101.61 ± 0.21 and 101.36 ± 0.18%, respectively. (Data as shown in APPENDIX, page 62, Table 14)

These experiments were conducted to verify that the methods used for GF analysis in the supernatant and in the PC-Brij® liposomes were sufficiently accurate in the analytical range. The mean of percent analytical recovery was sufficiently high (close to 100 %) with a low % RSD, which indicates that spectrofluorometry method was accurate for quantitative analysis of GF in solution and GF in PC-Brij® liposomal dispersion. The values of percent analytical recovery were in the range of 98-102 %; therefore, this analytical procedure shows accuracy in the analytical range.

b) Carboxyfluorescein (CF)

- **Analysis of CF in solution**

Theorectical concentrations of CF standard solutions were 8.32, 13.87 and 22.19 nM. The estimated concentrations were established to be 8.34 ± 0.03, 11.14 ± 0.03 and 22.48 ± 0.05 nM. Percent analytical recovery was calculated to be 100.29 ± 0.47, 100.39 ± 0.26 and 101.29 ± 0.24 %, respectively (Data as shown in APPENDIX, page 66, Table 23).

- **Analysis of CF in PC-Brij® liposomes dispersion**

Theorectical concentrations of CF standard solutions were 8.32, 13.87, and 22.19 nM. The estimated concentrations were established to be 8.42 ± 0.04, 11.25 ± 0.05 and 22.56 ± 0.02 nM. Percent analytical recovery was calculated to be 101.19 ± 0.54, 101.44 ± 0.46 and 101.67 ± 0.12 %, respectively (Data as shown in APPENDIX, page 67, Table 26).

The results indicate that the satisfactory quantitation of CF in solution and in PC-Brij® liposomal dispersion were achieved using spectrofluorometry. The average of percent analytical recovery was sufficiently high (close to 100%) with a low % RSD. The values of percent analytical recovery were in the range of 98-102 %; therefore, this analytical procedure shows accuracy in the analytical range.

1.2.4. Precision

Data indicate that the spectrofluorometry method was sufficiently precise for quantitation of GF and CF. Better precision would be obtained when the analyte concentration was less than 5.00 µM and 22.19 nM, respectively. Thus, the samples were diluted accordingly before being subjected to the spectrofluorometry method (Data as shown in APPENDIX, page 63-64, 68-69).

a) Griseofulvin

The precision of griseofulvin quantification was determined as shown in Table 2. The % RSD values from both within and between run were less than 2 %.

b) Carboxyfluorescein

The precision of carboxyfluorescein quantification was determined as shown in Table 3. The % RSD values from both within and between run were less than 2 %.

Table 2. Within and between run precision of griseofulvin

Concentration (µM)	% RSD	
	within run	between run
2.00	0.39	0.34
2.80	0.12	0.11
5.00	0.12	0.09

Table 3. Within and between run precision of carboxyfluorescein

Concentration (nM)	% RSD	
	within run	between run
8.32	0.39	0.31
13.87	0.28	0.41
22.19	0.21	0.11

2. Preparation and evaluation of blank PC and PC-Brij® liposomes (New, 1990)

2.1. Preparation of blank PC and PC-Brij® liposomes

During condition optimization process, PC liposomes were employed as a model preparation. Film formation is an important step in thin film hydration method. Smooth, even and homogeneous film with suitable thickness is preferred. Factors affecting film formation include temperature, pressure reduction rate, reduced pressure during evaporation step, evaporation rate and % lipid content. In this study, the temperature was set at 40 C°. The flask rotation rate was fixed at 100 rpm. In process to form lipid film, the reduced pressure was varied at 200 and 300 mbar. Moreover, the pressure reduction rate was varied at 5 and 10 mbar/min. At reduced pressure of 300 mbar and pressure reduction rate of 10 mbar/ min, the film rupture took place resulting in rough film surface. At reduced pressure of 200 mbar and pressure reduction rate of 5 mbar/ min, smooth and even film was formed. Thus, the reduced pressure and pressure reduction rate were chosen to be 200 mbar and 5 mbar/ min.

Effect of lipid content on liposome formation was evaluated after the film formation conditions were established. % Lipid content was varied between 2 and 5 % w/v. At 2 % lipid content, presence of lipid fragment and aggregation was less than those observed at 5 % w/v total lipid content; so, the PC concentration was chosen to be 2 % w/v total lipid content.

In summary, film formation conditions were set as followed. % Lipid content was 2 % w/v. The temperature of water bath was set at 40 C°. The flask rotation speed was 100 rpm. The pressure reduction rate was set at 5 mbar/ min until reduced pressure of 200 mbar was obtained. The evaporation period took about 1.50 h. The vesicles were formed when adding water to hydrate film. Finally, the liposomal dispersion was sonicated at 40 °C, 30 min in an ultrasonic bath.

The preparation conditions of liposomes and % lipid content from this step were used in preparation of PC-Brij® liposomes by the addition of Brij® 30, Brij® 35 or Brij® 72 in the formulation. The result showed that vesicles were formed after each type and concentration of Brij® was incorporated in the preparation process.

2.2. Evaluations of blank PC and PC-Brij® liposomes

2.2.1. Microscopic appearance

Milky colloidal dispersions were obtained from both PC and PC-Brij® liposomal formulations. Under a cross polarized light microscope, all formulations contributed spherical lamellar vesicles with diameter larger than 1 μm. No obvious difference in appearance between PC and PC-Brij® liposomes was observed. The observation indicated that Brij® 30, Brij® 35 and Brij® 72 could be incorporated into the lipid bilayer. Micrographs show that both PC and PC-Brij® liposomes are smooth spherical vesicles with diameter more than 1.0 μm (data not shown). In addition, the vesicles fluoresced and showed the satellite feature when they were visualized under a cross polarized light microscope corresponding to lamellarity of vesicles. Furthermore, the obtained vesicles were expected to be multilamellar vesicles according to the preparation method, thin film hydration technique (New, 1990).

2.2.2. Vesicle size and size distribution

From laser diffraction studies, the particle size distribution of all formulations showed a typical normal distribution pattern. ANOVA statistic analysis indicated that the mean size diameters of all formulations were significantly different from each other ($P < 0.05$). As shown in Table 4, the mean diameters of the PC and PC-Brij® liposomes were 7.79 ± 0.03 μm and ranged from 7.94 ± 0.15 to 12.67 ± 0.72 μm, respectively. Tukey's multiple comparisons showed that vesicle size of L_30/20, L_35/10 and PC liposomes were insignificantly different.

For PC-Brij® liposomes containing Brij® 35, the average mean diameter increased as the surfactant concentration increased from 10 to 20 % w/w of total lipid content (Table 4). Brij® 35 possessed a short hydrophobic tail with a large hydrophilic head (Figure 11). Therefore, the hydrophilic head of Brij® 35 can be drawn water molecules to hydrate the lipid wall. As a consequence, the mean diameter was increased when Brij® concentration was increased.

Table 4. Composition and mean particle size of PC and PC-Brij® liposomal formulations

Formulation Code of vesicle	**Composition PC: Brij® (%w/w)**	**Particle size (µm)**
L	-	7.79 ± 0.03
L_30/10	90 : 10	11.02 ± 0.05*
L_30/15	85 : 15	7.94 ± 0.15
L_30/20	80 : 20	12.03 ± 0.06*
L_35/10	90 : 10	7.95 ± 0.02
L_35/15	85 : 15	11.38 ± 0.03*
L_35/20	80 : 20	12.67 ± 0.72*
L_72/10	90 : 10	11.04 ± 0.03*
L_72/15	85 : 15	9.92 ± 0.03*
L_72/20	80 : 20	12.42 ± 0.06*

Values represented as mean diameter ± *SD* (n=3).

* Significant at 95 % confident interval comparing with L (PC liposomes).

Effect of Brij® 30 and Brij® 72 on vesicle size was similar to each other (Table 4). Brij® 30 and Brij® 72 are similar in their hydrophilic head size. They incorporated into lipid bilayer better than Brij® 35. In the presence of 10 % w/w Brij® 30 and Brij® 72 of total lipid content, the mean size diameter of vesicles was bigger than that of in the absence of Brij®. The observation implied that the incorporation of Brij® 30 and Brij® 72 resulted in swelling of vesicles. When Brij® 30 and Brij® 72 concentrations were increased from 10 to 15 % w/w of total lipid content, mean diameter was decreased. With further increasing in Brij® 30 and Brij® 72 concentration from 15 to 20 % w/w of total lipid content, particle size mean of vesicle increases. Unpredictable change of vesicle size according to Brij® 30 and Brij® 72

concentration was unexplainable. However, this size reduction was consistented with the effect of Span® on size of liposomes (Mishra et al., 2007).

Among 3 Brij®, Brij® 72 has the longest hydrophobic chain with the lowest HLB value of 4.9 (Figure 11), so Brij® 72 can well incorporate in lipid bilayer. Brij® 30 has a short hydrophobic chain and a small hydrophilic head group (Figure 11) with a HLB value of 9.7. Thus, Brij® 30 can be partially dissolved in the lipid bilayer. On the contrary, Brij® 35 has the largest hydrophilic head group with HLB value of 16.9. So, Brij® 35 prefers hydrophilic environment to the lipid bilayer. Due to the large hydrophilic part of Brij® 35, loose pack of bilayer is expected resulting in instability of vesicles. Therefore, Brij® with medium to high hydrophilic properties from medium to low HLB values should be selected to incorporate in liposomes. HLB values of surfactant also play an important role on formation of niosomes. Surfactants with HLB value in a range of 1.8 to 8.6 is recommended in niosome preparation (Agarwal et al., 2004). Formulations containing Brij® 35 were dropped off. Thus, two formulations containing 20 % w/w Brij® 30 and 15 % w/w Brij® 72 were selected for further studies.

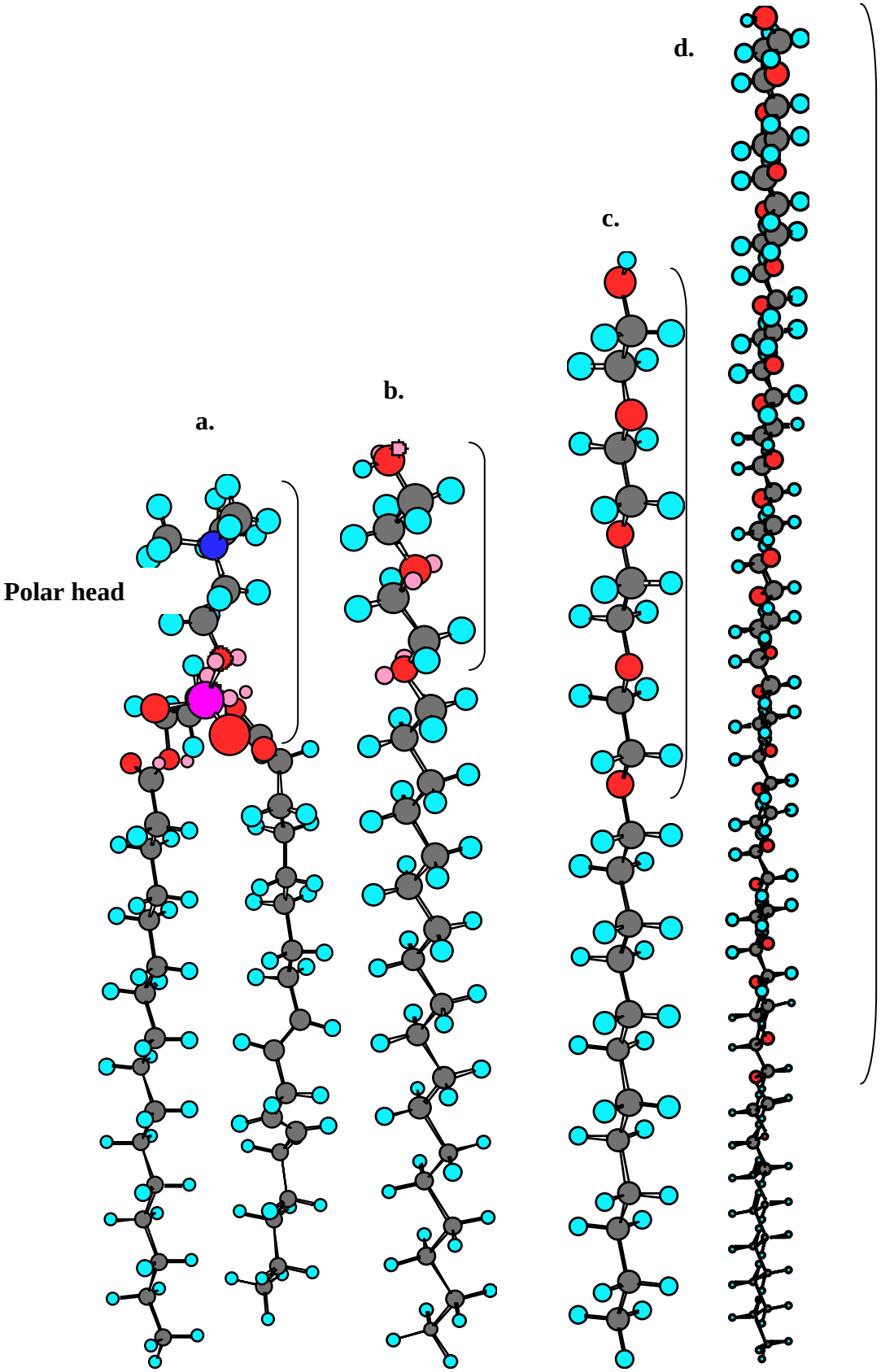


Figure 11. 3D Structures of PC (a), Brij® 72 (b), Brij® 30 (c) and Brij® 35 (d)

3. Preparations and evaluations of PC and PC-Brij® liposomes loaded with griseofulvin and carboxyfluorescein

3.1. Preparations of PC and PC-Brij® liposomes loaded with griseofulvin and carboxyfluorescein

In this study, PC liposomes, L_30/20 and L_72/15 loaded with griseofulvin and carboxyfluorescein were prepared and characterized. Both of PC and PC-Brij® liposomes loaded with griseofulvin and carboxyfluorescein could be prepared by thin film hydration technique using optimized conditions as previously described.

3.1.1. PC and PC-Brij® liposomes loaded with griseofulvin

According to griseofulvin titration process, the maximum concentration of griseofulvin that could be incorporated into the liposomal formulations was found to be 0.01 mM.

3.1.2. PC and PC-Brij® liposomes loaded with carboxyfluorescein

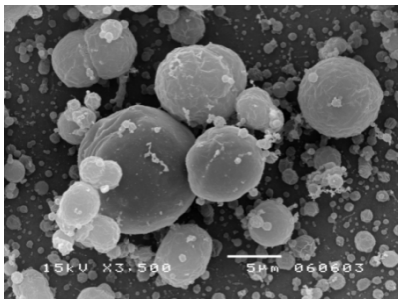
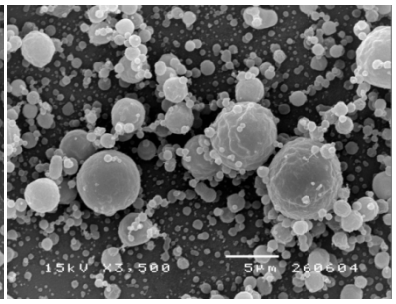
To prepare liposomes loaded carboxyfluorescein, the dried lipid film was hydrated by carboxyfluorescein solution in the dark. Both PC and PC-Brij® liposomes loaded with carboxyfluorescein were obtained and characterized later on. The concentration of incorporated carboxyfluorescein into the liposomal formulations was 0.17 mM.

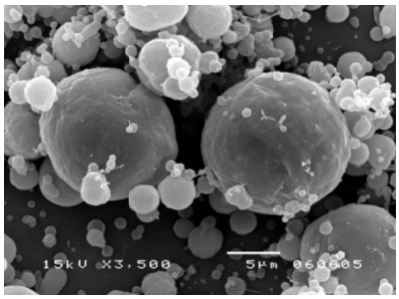
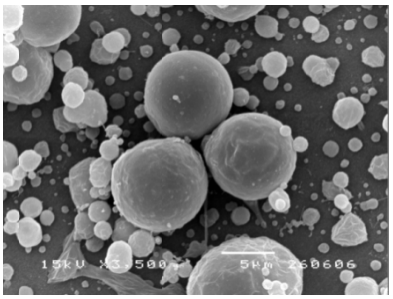
3.2. Evaluations of PC and PC-Brij® liposomes loaded with griseofulvin and carboxyfluorescein

The effects of entrapped compounds, lipophilic (griseofulvin) and hydrophilic (carboxyfluorescein) substances, on the formation and characteristics of PC and PC-Brij® liposomes were investigated through determination of the microscopic appearances, vesicle size and size distribution, entrapment efficiency and leakage.

3.2.1. Microscopic appearances

Both PC and PC-Brij® liposomes loaded with griseofulvin and carboxyfluorescein were examined by a cross polarized light microscope. All formulations showed lamellarity with diameter larger than 1 µm, which confirmed with visualization under the microscope. Multilamellar vesicles were expected to obtain from all formulations since the thin film hydration technique was employed. Moreover, The SEM micrographs confirmed that the vesicles were spherical shape with diameter more than 5 µm (Figure 12).

a)  b) 

c)  d) 

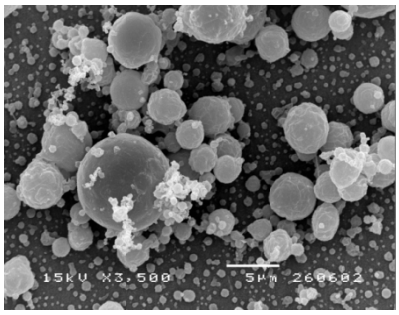
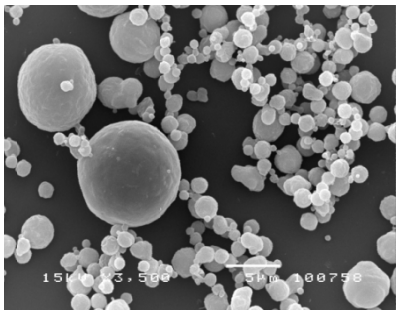
e)  f) 

Figure 12. Scanning electron micrographs before and after extrusion of GF_L (a and b), GF_L_30/20 (c and d) and GF_L_72/15 (e and f), respectively

3.2.2. Vesicle size and size distribution

a) PC and PC-Brij® liposomes loaded with griseofulvin

As shown in Table 5, vesicle size of PC liposomes loaded with griseofulvin was comparable to that of blank PC liposomes while vesicle size of PC-Brij® liposomes loaded with griseofulvin was reduced when compared to those of blank PC-Brij® liposomes. Griseofulvin is a lipophilic drug; thus, it is incorporated inside the lipid bilayer. Generally, PC possessed high hydrophobicity while Brij® contains both hydrophilic and hydrophobic parts. Thus, hydrophilic part of Brij® may impede hydrophobic interaction between PC and Brij® resulting formation of loose bilayer. In other words, the lipid bilayer may loosely align due to difference in hydrophobicity and hydrophilicity of PC and Brij®. Griseofulvin, an amphoteric compound, can be interacted with both PC and Brij®. As a result, the lipid bilayer may pack together in the presence of griseofulvin. In the presence of griseofulvin, hydrophobicity of the lipid bilayer was increased. As a result, less water molecules were drawn to hydrate the bilayer. Thus, extent of membrane swelling and vesicle size were reduced. Effect of membrane hydrophilicity on membrane swelling and increase in particle size were obviously seen in the previous studies. In the absence of Brij®, size of blank PC liposomes was 7.79 ± 0.03 µm. In the presence of Brij® mean sizes diameter of blank PC-Brij® liposomes were about 11.00 µm for both L_30/10 and L_72/10.

Table 5. The mean particle size of liposomes loaded with griseofulvin and carboxyfluorescein formulations

Formulation	**Composition**	**Particle size (µm)**		
Code of vesicle	**PC : Brij® (%w/w)**	**Blank**	**GF**	**CF**
L	-	7.79 ± 0.03	8.07 ± 0.02	7.78 ± 0.01
L_30/20	80:20	12.03 ± 0.06	7.77 ± 0.01	11.91 ± 0.01
L_72/15	85:15	9.92 ± 0.03	7.54 ± 0.02	8.95 ± 0.01

Values represented as mean diameter ± *SD* (n=3).

b) PC and PC- Brij® liposomes loaded with carboxyfluorescein

Carboxyfluorescein was entrapped in the aqueous core; thus, carboxyfluorescein showed negligible effect on the lipid bilayer. As a consequence, size of vesicle loaded with carboxyfluorescein was comparable to those of blank vesicles.

3.2.3. Entrapment efficiency

Entrapment efficiency is an important factor in evaluation of PC-Brij® liposomes and PC liposomes. The incorporation efficiency may be altered by several factors such as the physicochemical properties of drug and lipid bilayer composition.

a) PC and PC-Brij® liposomes loaded with griseofulvin

Entrapment efficiency is a percentage of the drug incorporated into the liposomes to total drug concentration. PC liposomes showed the highest griseofulvin entrapment efficiency (Table 6). This result was proportional to the vesicle size. Griseofulvin could be incorporated into bilayer. Large vesicle size housed more drug molecules in the bilayer. Moreover, the lipid bilayer of L_30/20 and L_72/15 contained molecules of Brij® leaving less space for griseofulvin to reside. Therefore, griseofulvin entrapment efficiency in PC-Brij® liposomes was less than that of in PC liposomes. The longer hydrocarbon chain of Brij®, Brij® 72, the less griseofulvin entrapped.

Table 6. Entrapment efficiency of PC and PC-Brij® loaded with griseofulvin and carboxyfluorescein

Formulation Code of vesicle	Composition PC : Brij® (%w/w)	% Entrapment efficiency	
		GF	CF
L	-	89.85 ± 4.17	5.59 ± 0.35
L_30/20	80: 20	85.03 ± 4.92	7.14 ± 0.28
L_72/15	85: 15	76.45 ± 1.71	6.45 ± 0.16

Values represented as mean diameter ± *SD* (n=3).

b) PC and PC-Brij® liposomes loaded with carboxyfluorescein

Table 7 shows low percent entrapment efficiency of CF since thin film hydration method was utilized in liposomal preparation. Generally, hydrophilic drugs give rise to low percent entrapment efficiency in liposomes. Furthermore, thin film hydration method is also reported to give low entrapment efficiency for hydrophilic molecules. Thus, the observed entrapment efficiency of carboxyfluorescein in both PC and PC-Brij® liposomes was very low. However, the increasing in carboxyfluorescein percent entrapment efficiency was consistent with the mean diameter of vesicles. In other words, the large vesicles enclose large volume of aqueous core resulting in improvement of percent entrapment efficiency of hydrophilic drugs.

3.2.4. Leakage

In terms of stability of all liposomes, leakage is a feature to show entrapment efficiency of formulation.

a) PC and PC-Brij® liposomes loaded with griseofulvin

Figure 13 shows that profiles of % drug remaining in the vesicles. % GF remaining during storage time did not show the difference among the formulations. Approximately 20 % of griseofulvin leaches from the vesicles in the first week and griseofulvin in the supernatant was analyzed and % recovery was calculated using Equation 4. The percent analytical recovery was in the range of 99.38-101.5 % in all formulations. Thus, the loss of griseofulvin from the vesicles was due to drug immigration into water but not due to degradation. Griseofulvin is reported to be a stable compound which undergoes degradation in acidic solution (Florey, 1979). The reason for griseofulvin leaching from the particle could be due to supersaturated condition of griseofulvin in bilayer during the vesicle preparation process. Griseofulvin could partition in and out the vesicles and reached equilibrium in a week. In addition, concentration of griseofulvin in the supernatant was determined to be 0.08-0.10 mM which was lower than the solubility of griseofulvin in ultrapure water at 4 °C (0.46 mM, preliminary data).

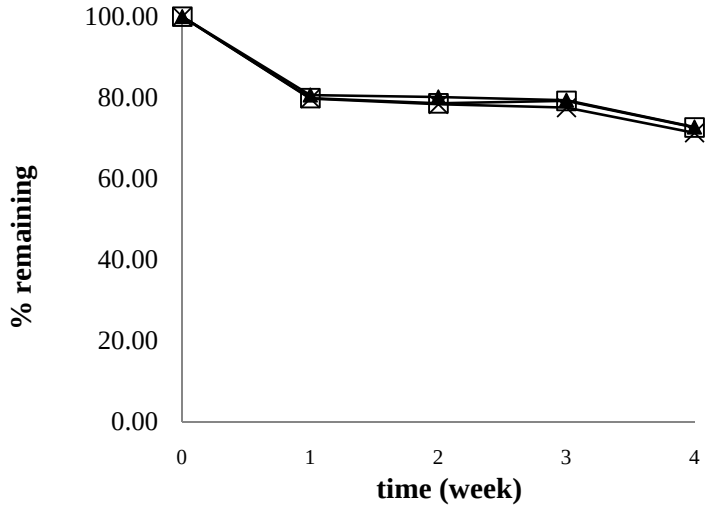


Figure 13. The percentage of griseofulvin remaining in vesicles of L (×); L_30/20 (▫); L_72/15 (▲) after the formulations was storage at 4 °C.

b) PC and PC-Brij® liposomes loaded with carboxyfluorescein

Figure 14 illustrates that % carboxyfluorescein remaining inside the vesicles were decreased during storage. % Carboxyfluorescein entrapment efficiency was in an order of L_30/20 > L_72/15 > L. Carboxyfluorescein in the supernatant was also determined. % Total analytical recovery of carboxyfluorescein in the dispersions at various times was in a range of 98.34 to 101.97 %. The carboxyfluorescein leakage depended on the composition of bilayer. Bilayer containing Brij® could retain carboxyfluorescein, a hydrophilic compound, better than bilayer with absence of Brij®. Brij® may increase the affinity of vesicles to hydrophilic molecule. Thus, entrapment efficiency enhancement was observed and consequently, longer retention of carboxyfluorescein was found in PC-Brij® liposomes. Brij® 30 possess a bigger hydrophilic part than Brij® 72; therefore, % remaining in liposome containing Brij® 30 was more than that in liposome containing Brij® 72.

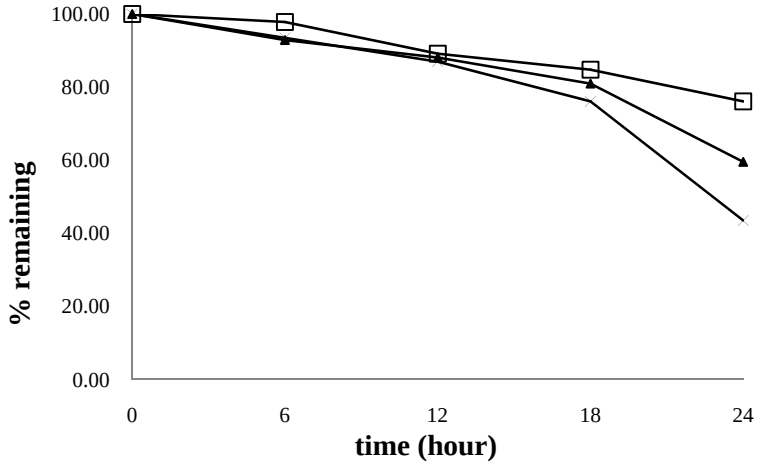


Figure 14. The percentage of carboxyfluorescein remaining in vesicles of CL (×); L_30/20 (▫); L_72/15 (▲) after the formulations was storage 4 °C.

CHAPTER V

CONCLUSIONS

The present study was to study the effect of type of Brij® or in preparation of liposomes. The outcomes were concluded as followed.

1. Brij® 30, Brij® 35 and Brij® 72 could be incorporated in the preparation of liposomes.

2. Both of PC and PC-Brij® liposomes were multilamellar vesicles (MLVs). PC-Brij® liposomes trends to provide bigger vesicle size than that of PC liposomes. This could be due to the hydrophilic part structure of PC-Brij® molecule drawing water to the vesicles. The change of PC-Brij® liposome size also depends on the concentration and structure of Brij®.

3. Entrapment efficiency of the vesicles depends on type of the loaded drugs. Hydrophobic griseofulvin could be loaded in both PC and PC-Brij® liposomes greater than hydrophilic carboxyfluorescein. Moreover, entrapment efficiency correlated to the size of vesicles.

4. % Griseofulvin remaining values in PC and PC-Brij® liposomes were not obviously different after 1 month storage and they showed approximately 80 % remaining. On the contrary, % Carboxyfluorescein remaining values were affected by vesicle formulations. It revealed that Brij® 30 in the vesicles retained hydrophilic carboxyfluorescein better than Brij® 72 providing greater % carboxyfluorescein remaining.

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APPENDIX

Linearity of the spectrofluorometry method for griseofulvin (GF)

Table 7. Fluorescence intensities of 1.0-7.0 μM griseofulvin (GF) standard solution in 50 % v/v methanol in chloroform

Concentration of GF (μM)	Fluorescence intensity				SD	% RSD
	run1	run2	run3	mean		
1.00	1538	1544	1540	1541.67	3.05	0.20
2.00	2966	2971	2968	2968.33	2.52	0.08
3.00	4324	4322	4328	4325.67	3.05	0.07
5.00	7111	7113	7109	7111.00	2.00	0.03
7.00	9469	9471	9468	9469.33	1.53	0.02

Table 8. Logarithm of fluorescence intensities of GF standard solution

Log concentration of GF (μM)	Log fluorescence intensity				SD	% RSD
	run1	run2	run3	mean		
0.00	3.187	3.189	3.188	3.1880	0.0008	0.0269
0.30	3.472	3.473	3.472	3.4733	0.0004	0.0114
0.48	3.636	3.636	3.636	3.6360	0.0003	0.0082
0.70	3.852	3.852	3.852	3.8520	0.0002	0.0034
0.85	3.976	3.976	3.976	3.9760	0.0004	0.0019

Accuracy of the spectrofluorometry method for griseofulvin (GF)

Table 9. The fluorescence intensities of GF standard solution in 50 % v/v methanol in chloroform

Concentration of GF (µM)	Fluorescence intensity					Mean ± SD
	run1	run2	run3	run4	run5	
2.00	2989	2999	3010	3007	3018	3004.60 ± 11.06
2.80	4143	4138	4140	4148	4136	4141.00 ± 4.69
5.00	7138	7129	7120	7130	7140	7131.40 ± 7.99

Table 10. The estimated concentrations of GF standard solution in 50 % v/v methanol in chloroform

Concentration of GF (µM)	Estimated concentration from standard curve (µM)					Mean ± SD
	run1	run2	run3	run4	run5	
2.00	1.998	1.999	2.000	2.000	2.000	2.000 ± 0.012
2.80	2.812	2.812	2.812	2.821	2.812	2.812 ± 0.006
5.00	5.034	5.031	5.029	5.032	5.032	5.033 ± 0.013

Table 11. The percentage of analytical recovery of GF standard solution in 50 % v/v methanol in chloroform

Concentration of GF (µM)	Percentage of analytical recovery					Mean ± SD
	run1	run2	run3	run4	run5	
2.00	99.06	99.14	99.81	99.42	100.13	99.50 ± 0.44
2.80	100.39	99.98	100.31	100.23	100.21	100.22 ± 0.16
5.00	100.69	100.27	100.42	100.29	100.72	100.48 ± 0.22

Accuracy of the spectrofluorometry griseofulvin in L_30

Table 12. The fluorescence intensities of GF standard solution in the presence of L_30/20 physical mixture

Concentration of GF (µM)	Fluorescence intensity					Mean ± SD
	run1	run2	run3	run4	run5	
2.00	3030	3041	3037	3034	3045	3037.40 ± 5.86
2.80	4158	4163	4167	4157	4160	4161.00 ± 4.06
5.00	7144	7153	7148	7155	7160	7152.00 ± 6.20

Table 13. The estimated concentrations of GF standard solution in the presence of L_30/20 physical mixture

Concentration of GF (µM)	Estimated concentration from standard curve (µM)					Mean ± SD
	run1	run2	run3	run4	run5	
2.00	2.038	2.042	2.042	2.042	2.043	2.044 ± 0.004
2.80	2.851	2.851	2.858	2.852	2.854	2.853 ± 0.003
5.00	5.074	5.082	5.083	5.084	5.088	5.083 ± 0.016

Table 14. The percentage of analytical recovery of GF standard solution in the presence of L_30/20 physical mixture

Concentration of GF (µM)	Percentage of analytical recovery					Mean ± SD
	run1	run2	run3	run4	run5	
2.00	101.33	101.73	101.87	101.76	101.87	101.71 ± 0.22
2.80	101.42	101.55	101.94	101.68	101.47	101.61 ± 0.21
5.00	101.12	101.26	101.47	101.58	101.36	101.36 ± 0.18

Precision of the spectrofluorometry method for griseofulvin (GF)

Within run precision of GF standard solution

Table 15. The fluorescence intensities of GF standard solution within run precision

Concentration of GF (μM)	Fluorescence intensity					Mean ± SD
	run1	run2	run3	run4	run5	
2.00	2989	2999	3010	3007	3018	3004.60 ± 11.06
2.80	4143	4138	4140	4148	4136	4141.00 ± 4.69
5.00	7138	7129	7120	7130	7140	7131.40 ± 7.99

Table 16. The estimated concentrations of GF standard solution in within run precision

Concentration of GF (μM)	Estimated concentration from standard curve (μM)					Mean ± SD	% RSD
	run1	run2	run3	run4	run5		
2.00	1.98	1.99	2.00	2.00	2.00	1.99 ± 0.01	0.45
2.80	2.81	2.81	2.81	2.82	2.81	2.81 ± 0.00	0.16
5.00	5.04	5.03	5.02	5.03	5.04	5.03 ± 0.01	0.17

Between run precision of GF standard solution

Table 17. The fluorescence intensities of GF standard solution between run precision

Concentration of GF (μM)	Fluorescence intensity					Mean ± SD
	run1	run2	run3	run4	run5	
2.00	2990	2997	3003	2980	3001	2994.20 ± 9.36
2.80	4143	4140	4148	4142	4150	4144.60 ± 4.22
5.00	7136	7139	7143	7148	7150	7143.20 ± 5.89

Table 18. The estimated concentrations of GF standard solution in between run precision

Concentration of GF (μM)	Estimated concentration from standard curve (μM)					Mean ± SD	% RSD
	run1	run2	run3	run4	run5		
2.00	1.98	1.99	1.99	1.98	1.99	1.99 ± 0.01	0.28
2.80	2.81	2.81	2.82	2.81	2.82	2.81 ± 0.01	0.19
5.00	5.04	5.04	5.04	5.04	5.05	5.04 ± 0.00	0.09

Linearity of the spectrofluorometry method for carboxyfluorescein (CF)

Table 19. Fluorescence intensities of carboxyfluorescein (CF) standard solution in ultrapure water adjusted to pH 8.0

Concentration of CF (µM)	Fluorescence intensity				SD	% RSD
	run1	run2	run3	mean		
6.94	1488	1490	1493	1490.33	2.52	0.17
11.10	2403	2396	2408	2402.33	6.03	0.25
16.66	3499	3488	3503	3496.67	7.77	0.22
20.82	4433	4442	4438	4437.67	4.51	0.10
24.98	5305	5298	5309	5304.00	5.57	0.10

Table 20. Logarithm of fluorescence intensities of CF standard solution

Log concentration of CF (µM)	Log fluorescence intensity				SD	% RSD
	run1	run2	run3	mean		
0.84	3.173	3.173	3.174	3.173	0.001	0.023
1.05	3.381	3.379	3.382	3.381	0.001	0.032
1.22	3.544	3.543	3.544	3.544	0.001	0.027
1.32	3.647	3.648	3.647	3.647	0.001	0.012
1.40	3.725	3.724	3.725	3.725	0.001	0.012

Accuracy of the spectrofluorometry method for carboxyfluorescein (CF)

Table 21. The fluorescence intensity of carboxyfluorescein (CF) standard solution in ultrapure water adjusted to pH 8.0

Concentration of GF (μM)	Fluorescence intensity					Mean ± SD
	run1	run2	run3	run4	run5	
8.32	1787	1794	1801	1785	1798	1793.00 ± 6.89
13.87	2388	2376	2392	2385	2379	2384.00 ± 6.52
22.19	4777	4765	4754	4762	4780	4767.60 ± 10.78

Table 22. The estimated concentrations of carboxyfluorescein (CF) standard solution in ultrapure water adjusted to pH 8.0

Concentration of GF (μM)	Estimated concentration from standard curve (μM)					Mean ± SD
	run1	run2	run3	run4	run5	
8.32	8.32	8.35	8.38	8.31	8.37	8.35 ± 0.03
13.87	11.16	11.10	11.18	11.14	11.11	11.14 ± 0.03
22.19	22.52	22.52	22.47	22.41	22.45	22.48 ± 0.05

Table 23. The percentage analytical recovery of carboxyfluorescein (CF) standard solution in ultrapure water adjusted to pH 8.0

Concentration of GF (μM)	Percentage of analytical recovery					Mean ± SD
	run1	run2	run3	run4	run5	
8.32	99.86	100.41	100.81	99.75	100.64	100.29 ± 0.47
13.87	100.47	100.11	100.79	100.34	100.24	100.39 ± 0.26
22.19	101.41	101.56	101.31	100.91	101.24	101.29 ± 0.24

Accuracy of the spectrofluorometry method for carboxyfluorescein (CF) in L_30

Table 24. The fluorescence intensities of GF in physical mixture of L_30/20

Concentration of GF (µM)	Fluorescence intensity					Mean ± SD
	run1	run2	run3	run4	run5	
8.32	1800	1811	1820	1814	1799	1808.80 ± 9.09
13.87	2395	2402	2411	2415	2420	2408.60 ± 10.07
22.19	4780	4788	4790	4785	4791	4786.80 ± 4.44

Table 25. The estimated concentrations of GF in physical mixture of L_30/20

Concentration of GF (µM)	Estimated concentration from standard curve (µM)					Mean ± SD
	run1	run2	run3	run4	run5	
8.32	8.38	8.43	8.47	8.44	8.37	8.42 ± 0.04
13.87	11.19	11.22	11.27	11.28	11.31	11.25 ± 0.05
22.19	22.54	22.54	22.58	22.59	22.56	22.56 ± 0.02

Table 26. The percentage analytical recovery of GF in physical mixture of L_30/20

Concentration of GF (µM)	Percentage of analytical recovery					Mean ± SD
	run1	run2	run3	run4	run5	
8.32	100.60	101.37	101.88	101.39	100.69	101.19 ± 0.54
13.87	100.76	101.22	101.60	101.62	101.99	101.44 ± 0.46
22.19	101.47	101.63	101.80	101.69	101.74	101.67 ± 0.12

Precision of the spectrofluorometry method for carboxyfluorescein (CF)

Within run precision

Table 27. The fluorescence intensities of CF standard solution

Concentration of GF (µM)	Fluorescence intensity					Mean ± SD
	run1	run2	run3	run4	run5	
8.32	1787	1794	1801	1785	1798	1793.00 ± 6.89
13.87	2388	2376	2392	2385	2379	2384.00 ± 6.52
22.19	4777	4765	4754	4762	4780	4767.60 ± 10.78

Table 28. The estimated concentrations of CF standard solution

Concentration of GF (µM)	Estimated concentration from standard curve (µM)					Mean ± SD	% RSD
	run1	run2	run3	run4	run5		
8.32	8.38	8.41	8.44	8.37	8.43	8.40 ± 0.03	0.39
13.87	11.24	11.18	11.25	11.22	11.19	11.22 ± 0.03	0.28
22.19	22.68	22.62	22.57	22.61	22.70	22.64 ± 0.05	0.23

Between run precision

Table 29. The fluorescence intensities of CF standard solution

Concentration of GF (µM)	Fluorescence intensity					Mean ± SD
	run1	run2	run3	run4	run5	
8.32	1777	1787	1784	1779	1790	1783.40 ± 5.413
13.87	2344	2354	2361	2368	2365	2358.40 ± 9.607
22.19	4744	4738	4740	4751	4747	4744.00 ± 5.244

Table 30. The estimated concentrations of CF standard solution

Concentration of GF (µM)	Estimated concentration from standard curve (µM)					Mean ± SD	% RSD
	run1	run2	run3	run4	run5		
8.32	8.27	8.32	8.30	8.28	8.33	8.30 ± 0.03	0.02
13.87	10.95	11.00	11.03	11.06	11.05	11.02 ± 0.04	0.41
22.19	22.37	22.37	22.34	22.35	22.40	22.36 ± 0.02	0.11

Table 31. Particle size of liposomal formulation (before and after passed the extrusion)

Formulation Code	size	
	Before passed	**After passed**
L_30/10	11.08	3.16
	11.00	3.21
	10.99	3.18
L_30/15	7.78	4.01
	7.97	3.87
	8.07	4.12
L_30/20	12.09	4.46
	11.97	4.42
	12.04	4.42
L_35/10	7.97	3.09
	7.94	3.41
	7.94	3.71
L_35/15	11.41	3.40
	11.35	3.63
	11.38	3.63
L_35/20	12.78	3.87
	12.72	3.89
	12.52	3.91
L_72/10	11.02	3.74
	11.02	4.30
	11.07	4.04
L_72/15	9.92	4.25
	9.95	4.82
	9.90	4.19
L_72/20	12.36	3.63
	12.47	4.19
	12.42	3.98
L	7.80	3.90
	7.81	3.87
	7.75	3.82

Table 32. Size of PC and PC-Brij® liposomes loaded with griseofulvin

Formulation Code.	Particle size	
	Before passed	After passed
L	8.04	6.67
	8.08	6.71
	8.07	6.74
L_30/20	7.78	5.86
	7.77	5.85
	7.77	5.83
L_72/15	7.53	5.62
	7.52	5.62
	7.56	5.63

Table 33. Size of PC and PC-Brij® liposomes loaded with carboxyfluorescein (Before and after passed)

Formulation Code.	Particle size	
	Before passed	After passed
L	7.79	5.32
	7.78	5.48
	7.79	5.49
L_30/20	7.79	6.53
	7.77	6.49
	7.78	6.52
L_72/15	7.69	5.59
	7.67	5.58
	7.68	5.59

Table 34. Analysis the particle size of blank PC and PC-Brij®liposomal formulations by one-way ANOVA ($P < 0.05$)

Source of Variation	df	Sum of Squares	Mean Square	F	Significant level
Between Groups	9	82.030	9.114	166.691	S*
Within Groups	20	1.094	0.055		
Total	29	83.123			

* S: Significant level at α = 0.05

Table 35. Tukey HSD test of the mean particle size of blank PC and PC-Brij® liposomal formulations ($P < 0.05$)

Formulation	Formulation	Significant level
L	L_30/10	S*
L	L_30/15	NS**
L	L_30/20	S*
L	L_35/10	NS**
L	L_35/15	S*
L	L_35/20	S*
L	L_72/10	S*
L	L_72/15	S*
L	L_72/20	S*

* S: Significant level at α = 0.05

** NS: Insignificant level at α = 0.05

Table 36. % Entrapped, % non-entrapped and % recovery of griseofulvin (week 0-4)

Formulation code	% Drug	Time (week)				
		0	**1**	**2**	**3**	**4**
L	% entrapped	94.36	75.26	73.94	73.15	67.27
	% non-entrapped	-	-	27.72	28.31	30.89
	% recovery	-	-	101.74	101.58	98.16
L_30/20	% entrapped	84.83	67.77	66.68	67.81	61.60
	% non-entrapped	-	-	31.67	31.90	37.81
	% recovery	-	-	98.35	99.09	99.40
L_72/15	% entrapped	79.64	64.62	64.62	63.23	57.91
	% non-entrapped	-	-	33.81	36.15	41.16
	% recovery	-	-	98.44	99.38	99.08

Table 37. % Entrapped, % non-entrapped and % recovery of carboxyfluorescein (hour 0-24)

Formulation code	% Drug	Time (hour)				
		0	**6**	**12**	**18**	**24**
L	% entrapped	7.01	6.51	6.05	5.43	3.09
	% non-entrapped	93.64	94.67	94.67	95.48	97.64
	% recovery	100.64	100.76	100.75	100.92	100.78
L_30/20	% entrapped	7.11	6.94	6.30	6.01	5.31
	% non-entrapped	92.97	94.22	94.22	95.74	95.90
	% recovery	99.41	100.53	100.59	101.80	101.23
L_72/15	% entrapped	6.37	5.96	5.71	5.27	3.77
	% non-entrapped	95.43	95.43	96.26	96.59	97.56
	% recovery	99.99	101.45	101.95	101.90	101.32

VITA

Miss Amaraporn Roopdee was born on September 26, 1982 in Ubon Rachathani, Thailand. She received her Bachelor's degree in Pharmaceutic from the Faculty of Pharmaceutical Sciences, Khon Kaen University, Khon Kaen, Thailand in 2005. Then, she entered the Master's degree program in Pharmacy at Chulalongkorn University in the same year.