CHAPTER IV

RESULTS AND DISCUSSION

1. Saturation Solubility of Propylthiouracil at Ambient Temperature

The saturation solubility of propylthiouracil (PTU) was experimentally determined in water and phosphate buffer pH 7.4. The ambient temperature was monitored and found to be 27-28 °C during the time of study. The complete solubility data are shown in Table1.

Table 1 Solubility data of PTU in different aqueous media. Data are presented as mean±SD (n = 3)

Time (Days)	Solubility of PTU (mg/mL)	
	Water	Phosphate buffer
1	1.22±0.07	1.35±0.05
3	1.19±0.01	1.31±0.04
5	1.18±0.01	1.40±0.01
7	1.17±0.01	1.47±0.02
9	1.28±0.02	1.41±0.09
15	1.35±0.02	1.44±0.01
saturation solubility	1.23±0.07	1.40±0.04

PTU solution at ninety percent of saturation solubility in both aqueous media was used in subsequent studies.

2. Feasibility of Vesicle Formation by Sonication Method

In the feasibility study, the total lipid concentration was kept at 100 mg/mL. The lipid was hydrated with either 1.1 mg/mL PTU in water or 1.2 mg/mL PTU in phosphate buffer, pH 7.4. This concentration was at approximately 90% of PTU saturation solubility in both aqueous media. The groups of surfactant explored in this experiment were Tween® (20, 40, 60, 80 and 85), Span® (20, 40, 60, 80 and 83), Brij® (52, 72, 76, 93 and 97), sucrose laurate ester (L-595), glyceryl dilaurate (GDL) and glyceryl distearate (GDS). Surfactant to cholesterol (CHO) content was varied between 0-50% by weight with 10% increments. Table 2 shows all compositions of PTU vesicles that readily formed under the conditions used in this study. Tween® and glyceryl dilaurate did not form vesicles in the presence of PTU regardless of cholesterol content. The water soluble Tween® surfactants normally form micelles in water (Uchegbu and Vyas, 1998). However, there are many reports indicating that Tween® surfactants can form vesicles with both hydrophilic and lipophilic drugs in the presence of cholesterol. These include Tween® 40, 60, and 80, Tween® 20 and 80, Tween® 20, Tween® 60, and Tween® 85 (Udupa et al., 1993; Ruckmani et al., 2000; Carafa et al., 1998; Carafa et al., 2002; Pillai and Salim, 1999; Naresh et al., 1994). Glyceryl dilaurate can also form vesicles with hydrophilic and hydrophobic drugs, for example, cyclosporine-A (Waranuch et al, 1998), alpha-interferon (Niemiec et al., 1995), and glycolic acid (Ohta et al., 1996). In this present study, Span® (20, 40, and 60) and Brij® (52 and 72) formed vesicles only at certain cholesterol contents (Table 2). The ratios of surfactant to CHO that allowed complete vesicle formation were 70:30 for Span® 40 and Brij® 52, 60:40 for Span® 60 and Span® 20, and 50:50 for Brij® 76, respectively. These results are in contrast with many previous reports where vesicles can be formed with a wide range of Span® to cholesterol ratio (Yoshioka et al., 1994; Namdeo and Jain, 1999; Hao et al., 2002) and Brij® to cholesterol ratio (Niemiec et al., 1995; Arunothayanun et al., 1999). In all of those above reports, vesicles were prepared by film hydration method. The difference in the energy input might be responsible for the discrepancy seen here. The different methods used undoubtedly yield different input of the energy and thus the difference in ease of lipid dispersion in the aqueous medium.

Table 2 Compositions of lipid in formulations that formed complete vesicles

Water (pH 5.8-6.0)	Phosphate buffer pH 7.4
Span® 20:CHO 60:40	Span® 20:CHO 60:40
Span® 40:CHO 70:30	Span® 40:CHO 70:30
Span® 60:CHO 60:40	Span® 60:CHO 60:40
Brij® 52:CHO 70:30	Brij® 52:CHO 70:30
Brij® 76:CHO 50:50	Brij®76:CHO 50:50
L-595:PEG-8-L 50:50	L-595:PEG-8-L 70:30
GDS:CHO:Brij® 76 45:15:40	GDS:CHO:Brij® 76 45:15:40
Span® 20:CHO:Solulan® C24 57.5:37.5:5	Span® 20:CHO:Solulan® C24 57.5:37.5:5
Span® 40:CHO:Solulan® C24 67.5:27.5:5	Span® 40:CHO:Solulan® C24 67.5:27.5:5
Span® 60:CHO:Solulan® C24 57.5:37.5:5	Span® 60:CHO:Solulan® C24 57.5:37.5:5
Brij® 52: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	Brij® 76:CHO:Solulan® C24 47.5:47.5:5

The stabilizers used in this study were dicetyl phosphate (DCP), a negatively charged stabilizer, and Solulan® C24, a non-ionic stabilizer. When Solulan® C24 was added at 5% by weight, PTU vesicles formed from Span® (20, 40, and 60) and Brij® (52 and 76) retained their structures. On the contrary, when DCP was used at 5% by weight, none of the formulas tested could form vesicles even when PTU was absent. The systems with Brij® and Span® became viscous gel instead of milky suspensions and gave incomplete vesicle formation, implying that other structure rather than the vesicle might form. Similar results were observed when DCP was used at 1-4% by weight with 1% increments. Some researchers reported the successful use of DCP as a stabilizer for Span® (Reddy and Udupa, 1993; Yoshioka et al., 1994; Manconi et al., 2002). This may due to the difference in the method used. The successful use of DCP was reported with the hand shaking method, which employs complete dissolution of DCP in organic solvent. On the contrary, the method used in this study is devoid of organic solvent. Thus, the arrangement of lipid components upon hydration might differ. Stabilizers that embed themselves into the bilayer may change the elastic property of the bilayers. Thus,

neither Solulan® C24 nor DCP was included in the system with sucrose laurate (L-595) since this system was deliberately designed to display bilayer elasticity in this study.

Other previously reported compositions of lipid did not yield similar results in this study. The formulation consisting of L-595:CHO formed decent vesicles in this study only at the weight ratio of 60:40. This was different from a previous report where drug-free vesicles could be prepared at L-595:CHO molar ratio of 50:50 (van den Bergh, Vroom et al., 1999). The difference might be attributed to the presence of PTU in the system. PTU is a weak acid with a pKa of 8.3. At the pH of water (5.8-6.0), ratio of unionized to ionized form of PTU is about 200. Thus, PTU molecules could intercalate themselves into the bilayer, making the bilayer less accommodating for CHO molecules. However, the preparation was not stable after one week at ambient temperature. This result implies unfavorable interaction between PTU and L-595. On the contrary, stable vesicles formed when CHO was replaced with polyoxyethylene-8-laurate (PEG-8-L), a micelle forming surfactant, which is known to render elasticity to vesicles (Li et al., 2001; Honeywell-Nguyen, Frederik et al., 2002; Honeywell-Nguyen and Bouwstra, 2003). PEG-8-L has a laurate carbon chain. Strong interaction between PEG-8-L with L-595 may occur through the hydrophobic force between the carbon chains of the two esters.

Glyceryl distearate (GDS), another nonionic surfactant, formed complete vesicles with CHO and Brij® 76 at the weight ratio of 45:15:40. A similar system was report earlier with cyclosporine-A (Niemiec et al., 1995) and glycolic acid (Ohta et al., 1996). However, the ratio of GDS:CHO:Brij® 76 in that study was 57:15:28, and the method used was different in terms of the energy input. Thus, both the structure of the drug as well as the energy input into the system can strongly affect vesicle formation. This is of a particular importance with hydrophobic drugs where the drug molecules need to insert themselves into the bilayer.

When the medium was changed from water to phosphate buffer, pH 7.4, systems with Span® and Brij®, with or without Solulan® C24, as well as that with GDS still

formed complete vesicles at the same surfactant:CHO ratios. However, the properties of the aqueous medium largely influenced bilayer formation in L-595 systems. L-595:CHO did not form vesicles at all. In addition, the lipid ratio of PTU vesicles prepared from L-595:PEG-8-L changed from 50:50 to 70:30 when the medium was phosphate buffer instead of water. L-595 is a lipophilic surfactant with HLB value of 5.0 and composes of 30.3% monoester, 39.3% diester and 30.4% triester. It was dehydrated at elevated temperature (Kunida et al., 1993). Electrolytes induce dehydration of polar head groups of dialkyl polyoxyethylene ether and monoalkyl polyoxyethylene ether surfactants (Hofland et al., 1993; Harvey et al., 2005). The vesicles in the present study were prepared by sonication method at 70 °C. The salting out effect of electrolytes might be more pronounced at high temperature so that L-595:CHO could not form vesicles and the surfactant precipitated out. On the other hand, many researchers can prepare the vesicles from L-595 using buffer at various pH values by film hydration method (Li et al., 2001; Honeywell-Nguyen, Frederik et al., 2002; Honeywell-Nguyen, Arenja et al., 2003; Honeywell-Nguyen and Bouwstra, 2003; Honeywell-Nguyen, Groenink et al., 2003). It is likely that the higher pH and ionic strength might also affect the solubility or the critical micelle concentration of the drug and the lipid components. Besides, the two systems were prepared at the same PTU activity in the aqueous phase, since PTU had higher solubility in phosphate buffer, partition of the drug into the lipid bilayer might also differ from that of the system in water. This might be able to explain partly the molecular rearrangement of the bilayer components that was reflected in the change in ratio of the lipid components.

3. Characterization of PTU Vesicular Suspensions

The PTU vesicles were prepared from lipid compositions shown in Table 2 and were characterized regarding entrapment efficiency, size and size distribution, stability, elasticity, and phase transition.

3.1 PTU entrapment in vesicles

Table 3 displays PTU entrapment efficiencies (EE) in the vesicular pellets prepared from various compositions in water and phosphate buffer. Entrapment of PTU in vesicular pellets was approximately 4-8 times of its solubility in the aqueous phase. When water was used as the medium, PTU entrapment efficiency in the Span® 60 was the highest among all of Span® formulations. The entrapment efficiency follows the trend of C18 (Span® 60) > C16 (Span® 40) > C12 (Span® 20). Phase transition temperature of Span® 60, Span® 40, and Span® 20 from this present study was 54, 47, and 18 °C. So, the Span® having the highest phase transition temperature provided the highest entrapment. These results are in good agreement with previous reports where the entrapment efficiency of various drugs depended on phase transition temperature of the main lipid component (Yoshioka et al., 1994; Ruckmani et al., 2000; Hao et al., 2002) and lipophilicity of surfactants (Udupa et al., 1993). The same result was also seen in systems with Brij®.

Entrapment efficiency of PTU in phosphate buffer pH 7.4 did not depend on the phase transition temperature in both systems with Span® and Brij®. The entrapment efficiency follows the trend of C12 (Span® 20) > C18 (Span® 60) > C16 (Span® 40) and Brij® 52 > Brij® 76. These results are in accordance with the report of Shahiwala and Misra (2002) who found that entrapment efficiency of nimesulide vesicles in PBS, pH 7.4, prepared from Span® 20 was higher than that of Span® 60. In the Span® systems, PTU entrapment in phosphate buffer was higher than that in water, except for Span® 60. PTU is a weak acid and has a relatively low partition coefficient of 1.0 (Moffat et al., 2004). Thus, the drug can partly dissolve in the aqueous media. The solubility of PTU is

higher at pH 7.4 than at the lower pH value of water (1.4 versus 1.2 mg/mL). Thus, in phosphate buffer, there would be more PTU molecules in the aqueous core of vesicles than in water. The same result was seen in the GDS system. In the Span® 20 system, the entrapment efficiency was highest because of the increased vesicle size (see Table 4) and the high solubility of PTU in the buffer. On the contrary, PTU entrapment efficiencies of Span® 60, Brij® 52, and Brij® 76 systems were less than those in water. Harvey et al. (2005) investigated the effect of electrolytes on the morphology of vesicles composed of the dialkyl polyoxyethylene ether surfactants and concluded that electrolytes induced dehydration of polyoxyethylene head groups of surfactants and decreased bilayer thickness and increased lamellarity of vesicles. Hofland et al. (1993) found that non-ionic surfactant vesicles prepared from polyoxyethylene alkyl ether and cholesterol in PBS had a higher membrane stacking order than those prepared in water and the multilarmellar vesicles (MLVs) formed in PBS. These phenomena would reduce the aqueous compartment in the vesicles. PTU is a weak acid with a pKa of 8.3. At pH 7.4, fewer PTU molecules exist in the unionized form when compared with those at the pH of water (6.0). Thus, PTU would be associated with the bilayer in phosphate buffer less than in water. The size of Span® 60, Brij® 52, and Brij® 76 vesicles in buffer was smaller than that in water ($p<0.05$, Table 4). All of these factors simultaneously reflected in overall PTU entrapment in the vesicles. Hence, for a lyophobic drug like PTU, the vesicle size, the lipid components and the drug solubility in the membrane should all be taken into consideration to maximize its entrapment in vesicles.

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

Formulation	Entrapment efficiency (mg% by weight)		
	Water	Phosphate buffer	p-value
Span® 20:CHO	0.44±0.02	0.79±0.04	0.00
Span® 40:CHO	0.46±0.03	0.54±0.01	0.70
Span® 60:CHO	0.91±0.04	0.58±0.01	0.00
Brij® 52:CHO	0.92±0.00	0.83±0.03	0.55
Brij® 76:CHO	1.05±0.01	0.62±0.01	0.00
Span® 20:CHO:Solulan® C24	0.95±0.02	0.76±0.02	0.00
Span® 40:CHO:Solulan® C24	1.16±0.04	0.71±0.01	0.00
Span® 60:CHO:Solulan® C24	0.68±0.02	0.88±0.02	0.00
Brij® 52:CHO:Solulan® C24	0.71±0.01	0.55±0.02	0.00
Brij® 76:CHO:Solulan® C24	0.95±0.01	0.81±0.00	0.00
Sucrose laurate ester(L-595):PEG-8-L 50:50	0.53±0.01	N/A	-
Sucrose laurate ester(L-595):PEG-8-L 70:30	N/A	0.49±0.01	-
GDS:CHO:Brij® 76	0.71±0.03	1.00±0.05	0.00

N/A = not applicable

Table 4 Average size of PTU vesicles prepared from various formulations (Mean±SEM, n = 3)

Formulation	Size (μm)		
	Water	Phosphate buffer	p-value
Span® 20:CHO 60:40	4.02±0.37	6.46±0.15	0.00
Span® 40:CHO 70:30	8.57±0.08	2.34±0.02	0.00
Span® 60:CHO 60:40	7.85±0.02	2.12±0.03	0.00
Brij® 52:CHO 70:30	2.57±0.25	1.33±0.01	0.00
Brij® 76:CHO 50:50	5.29±0.02	3.93±0.00	0.00
Span® 20:CHO:Solulan® C24	2.58±0.00	3.60±0.08	0.04
Span® 40:CHO:Solulan® C24	4.76±0.08	3.20±0.04	0.00
Span® 60:CHO:Solulan® C24	4.12±0.01	3.23±0.01	0.00
Brij® 52:CHO:Solulan® C24	0.76±0.00	0.51±0.01	0.01
Brij® 76:CHO:Solulan® C24	5.01±0.00	3.47±0.00	0.00
Sucrose laurate ester(L-595):PEG-8-L 50:50	0.37±0.00	N/A	-
Sucrose laurate ester(L-595):PEG-8-L 70:30	N/A	0.51±0.00	-
GDS:CHO:Brij®76 45:15:40	3.20±0.01	3.42±0.02	0.99

N/A = not applicable

Figure 4 shows the effects of Solulan® C24 on PTU entrapment efficiency. Solulan® C24 is cholesteryl poly (24) oxythylene ether. It stabilizes vesicular suspension by steric hindrance, keeping vesicles far apart and preventing aggregation. When Solulan® C24 was added to the formulation, the PTU entrapment efficiency was decreased in the systems with Span® 60 and Brij® 52 in water ($p<0.05$) since the vesicle size was decreased ($p<0.05$, Table 4). PTU molecules in the bilayers could be reduced since the stabilizer molecules can interdigitate within the bilayers. On the other hand, EE of PTU was increased in the systems with Span® 20 and Span® 40. An explanation may be the change of vesicular structure when DCP was added to the bilayers. This has been the case with phosphates when surface charge density of 1-2 $\mu C/cm^2$ resulting in LUVs

instead of MLVs (Weiner et al., 1989). In addition, Namdeo and Jain (1996) found that the presence of surface charge by DCP increased the distance between bilayers of MLVs. The arguments for the observations in this present study should be the same as those for the effect of DCP. Instead of charge repulsion, however, intravesicular steric hindrance might play a key role to increase the internal aqueous volume. The increase EE can thus be attributed to PTU in the intravesicular aqueous phase when Solulan® C24 was added to the bilayer.

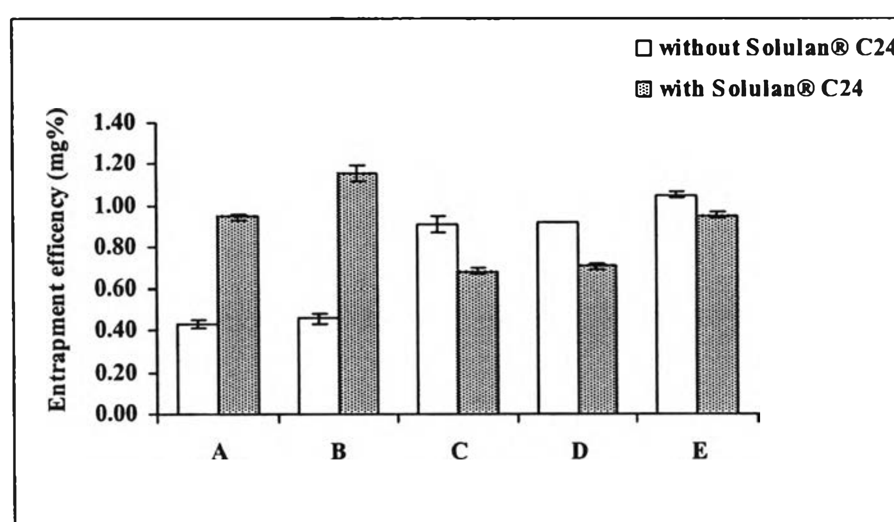

Figure 4 Effect of Solulan® C24 on PTU entrapment efficiency in niosomal systems in water (Mean$\pm$SEM, n = 3)
A = Span® 20:CHO, B = Span® 40:CHO, C = Span® 60:CHO,
D = Brij® 52:CHO, and E = Brij® 76:CHO

When phosphate buffer was used as the aqueous medium, the effects of Solulan® C24 on EE of PTU were different from that seen in water. EE of PTU was increased in the systems with Span® 40, Span® 60 and Brij® 76 but it was decreased in the systems with Brij® 52 ($p<0.05$, Figure 5). So the buffer affected the EE of PTU when Solulan® C24, which was hydrophilic stabilizer, was used in the formulations though ionic strength was not expected to affect the systems without charge.

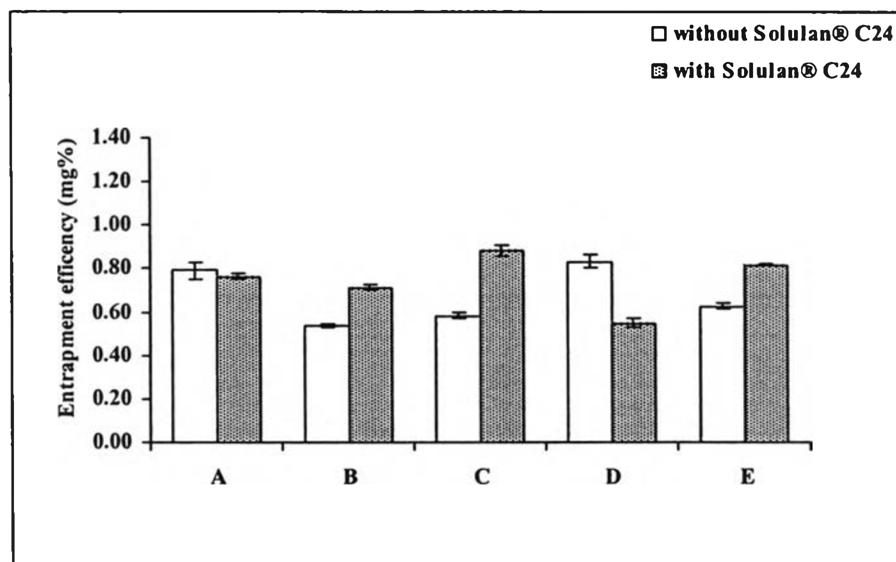

Figure 5 Effect of Solulan® C24 on PTU entrapment efficiency in niosomal systems in phosphate buffer, pH 7.4 (Mean±SEM, n = 3)
A = Span® 20:CHO, B = Span® 40:CHO, C = Span® 60:CHO,
D = Brij® 52:CHO, and E = Brij® 76:CHO

3.2 Size and size distribution

The size and size distribution of each vesicular system is shown in Table 4. The trend of the vesicles size prepared in phosphate buffer with or without Solulan® C24 was C12 (Span® 20) > C16 (Span® 40) > C18 (Span® 60). The vesicle size of Brij® 76 was greater than Brij® 52 in both aqueous media. There are many previous works reporting that the vesicle size of niosomes containing hydrophilic drugs such as 5-fluorouracil (Yoshioka et al., 1994) and cytarabine hydrochloride (Ruckmani et al., 2000) increases with increasing HLB of the system. On the contrary, in the water medium, the trend was C16 (Span® 40) > C18 (Span® 60) > C12 (Span® 20). This observation is consistent with many previous reports in which the vesicle size of niosomes containing various drugs did not depend on the HLB. These drugs were both hydrophilic and hydrophobic drugs including methotrexate (Udupa et al., 1993), 5-fluorouracil (Namdeo and Jain, 1999), enoxacin (Fang, Hong et al., 2001), and tretinoin (Manconi et al., 2002).

In this study, the vesicle sizes of all surfactant systems in phosphate buffer was smaller than those in water except the Span® 20 system ($p<0.05$, Table 4). These results contrasted with previous reports where the size of the non-ionic surfactant vesicles prepared in buffer was increased as compared to those prepared in water. Hofland et al. (1993) reported that the size of empty vesicles prepared from monoalkyl polypxyethylene ether surfactants in water was smaller than the size prepared in phosphate buffer saline. Harvey et al. (2005) also found similar results of the vesicle size of dialkyl polypxyethylene ether surfactants. The polar head groups of surfactants are dehydrated in buffer medium. The hydrophobicity of surfactants might be increased. Increasing hydrophobicity of surfactants decrease surface free energy, thereby the vesicle size was decreased (Yoshioka et al., 1994; Barlow et al., 2000).

When Solulan® C24 was used as stabilizer, the average size was either reduced or practically unchanged in most preparations (Figures 6 and 7). The exception was the system with Span® 40 and Span® 60 in phosphate buffer ($p<0.05$). In these formulations, the bimodal distribution seen in the systems without Solulan® C24 became almost monodispersed in the presence of the stabilizer (Figures 8-11). 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). The mechanism behind this is still unclear.

The average size does not give good correlation to PTU entrapment efficiency (Figures 12 and 13). Pearson correlation coefficients of the systems in water and in buffer were 0.085 and 0.388. This is in accordance with the fact that most of PTU molecules were unionized and embedded in the bilayer at the pH values of the aqueous media studied. In addition, different head groups of the surfactants could also play a role in accommodating foreign molecules into the bilayer. Thus, the size of the vesicle solely could not govern the entrapment of lipophilic molecules into the bilayer. On the other hand, the entrapment of water-soluble molecules generally increases as the vesicle size increases (Weiner et al., 1989; Ruckmani et al., 2000).

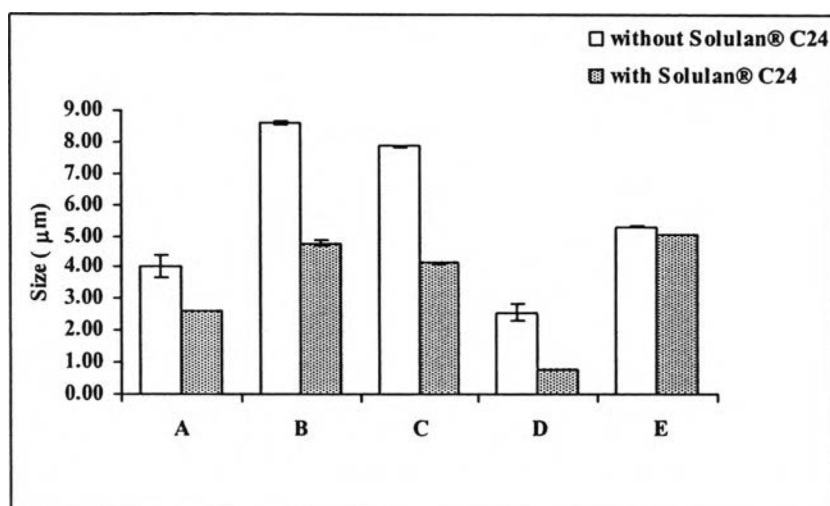

Figure 6 Effect of Solulan® C24 on the vesicle size of niosomes prepared in water (Mean±SEM, n = 3)
A = Span® 20:CHO, B = Span® 40:CHO, C = Span® 60:CHO,
D = Brij® 52:CHO, and E = Brij® 76:CHO

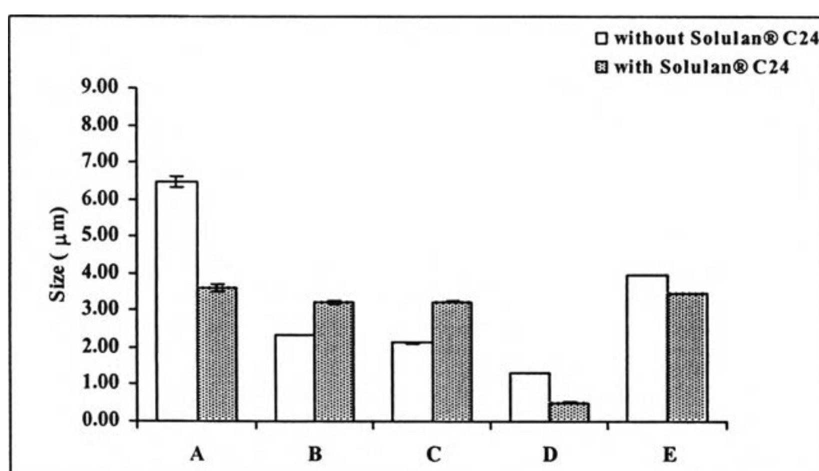

Figure 7 Effect of Solulan® C24 on the vesicle size of niosomes prepared in phosphate buffer, pH 7.4 (Mean±SEM, n = 3)
A = Span® 20:CHO, B = Span® 40:CHO, C = Span® 60:CHO,
D = Brij® 52:CHO, and E = Brij® 76:CHO

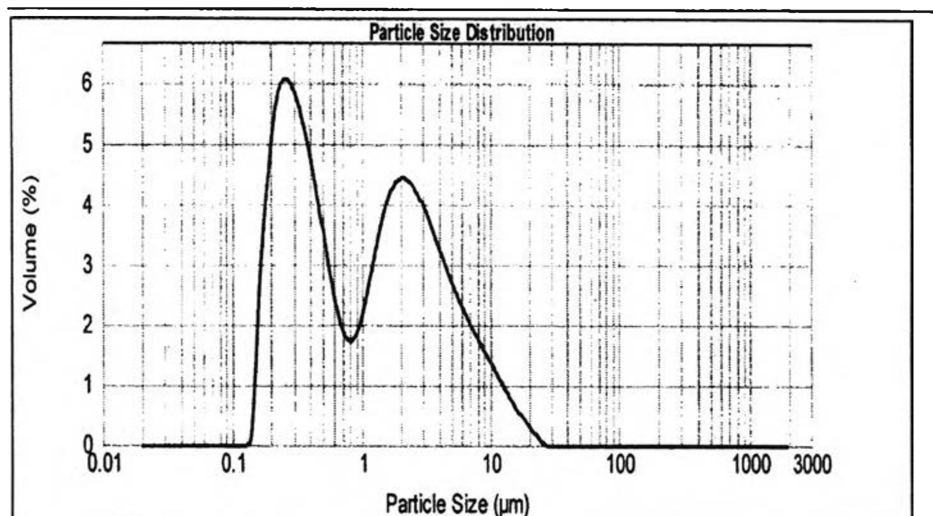

Figure 8 Size distribution of the vesicles prepared from Span® 40 without Solulan® C24 in phosphate buffer system

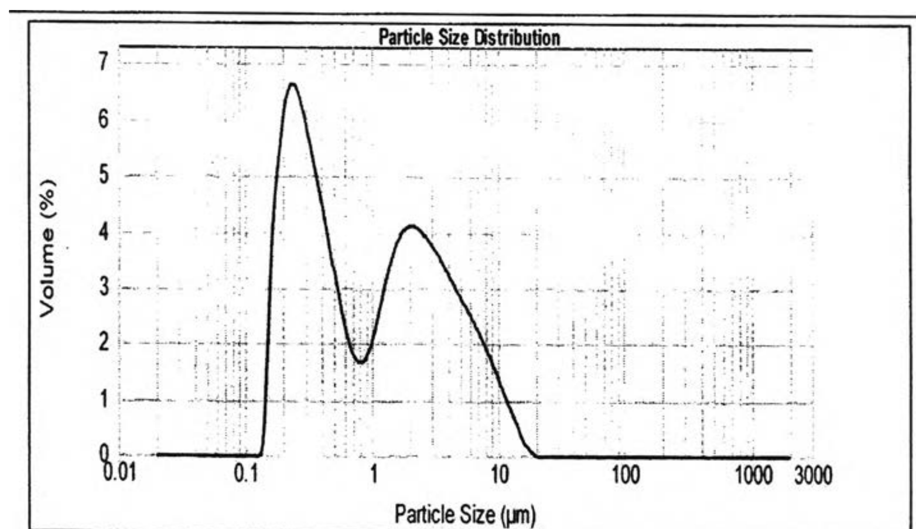

Figure 9 Size distribution of the vesicles prepared from Span® 60 without Solulan® C24 in phosphate buffer system

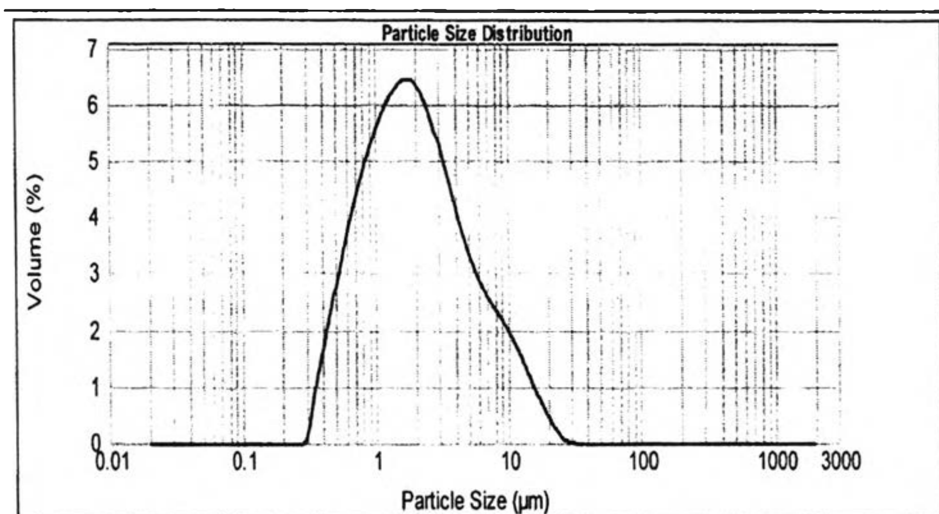

Figure 10 Size distribution of the vesicles prepared from Span® 40 with Solulan® C24 in phosphate buffer system

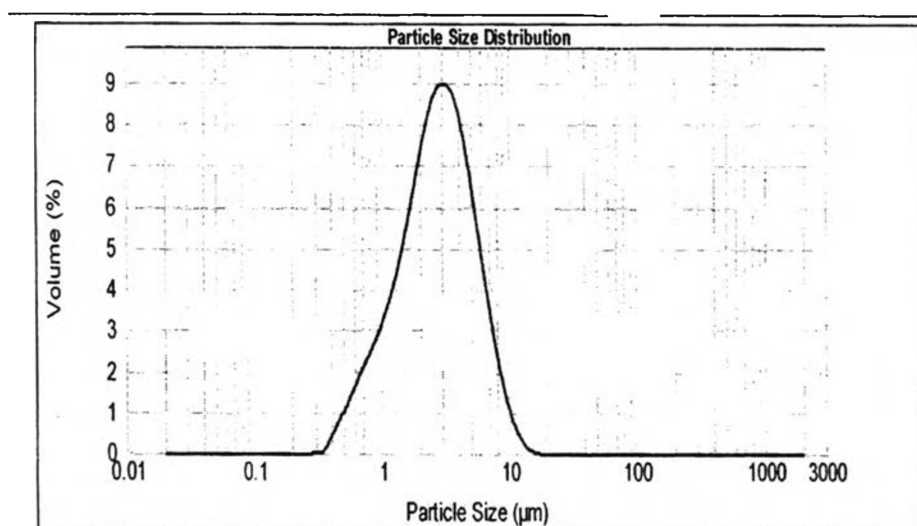

Figure 11 Size distribution of the vesicles prepared from Span® 60 with Solulan® C24 in phosphate buffer system

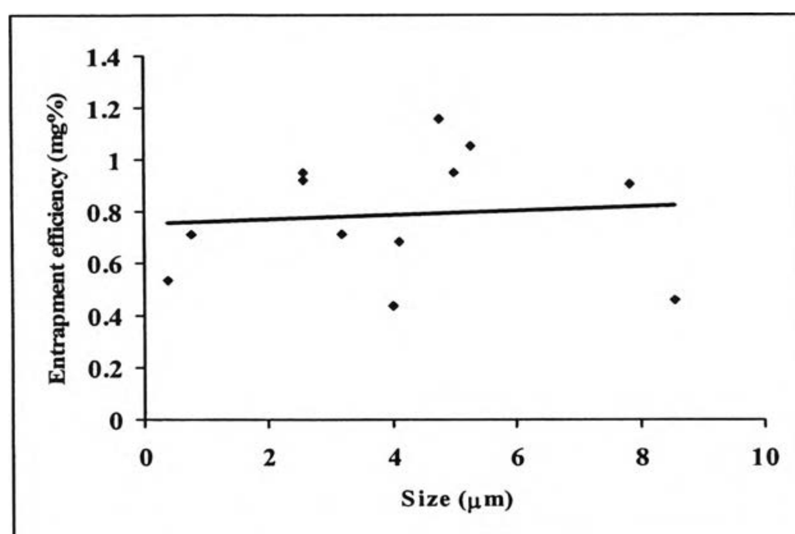

Figure 12 Relationship between entrapment efficiency and size of PTU niosomes in water

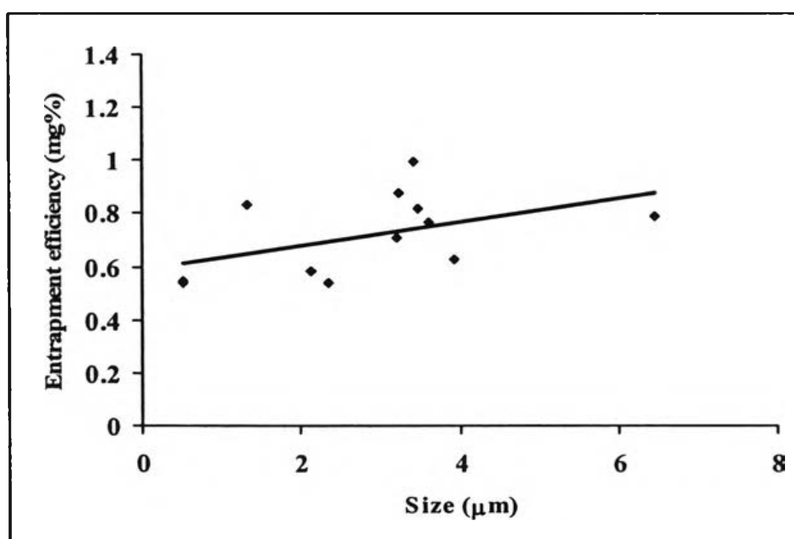

Figure 13 Relationship between entrapment efficiency and size of PTU niosomes in phosphate buffer, pH 7.4

3.3 Determination of phase transition temperature

Melting points from DSC scan of pure compounds and surfactant mixtures are shown in Table 5. These results of pure compounds were closed to those in previous reports (Niemiec et al., 1995; Kibbe, 2000; Moffat et al., 2004). Addition of cholesterol to surfactant shifted the melting point of the mixture. Cholesterol is known to abolish the gel to lipid phase transition of liposomal vesicles but the transition temperature is usually unaffected (Ladbrooke and Chapman, 1969; New, 1997; El Maghraby, Williams, and Barry, 2004).

The information from differential scanning calorimetric (DSC) method can be used for compound identification, compound interaction, or in an estimation of purity. The phase transition temperatures of the blank and PTU vesicles showed the same endothermic melting peak and no melting peak of PTU, CHO, and surfactants (Tables 6 and 7). This implies that the presence of drug crystals and lipid remnants was negligible. In the present study, Span® 40, Span® 60, and GDS vesicle systems clearly showed the transition peaks, which were above the room temperature. This means that they were the gel state vesicles (Ladbrooke and Chapman, 1969; Ganesan et al., 1984). The transition peak of Span® 60 vesicles was lower than that of the corresponding surfactant mixture. This observation may be due to the effect of water. The temperature at which the main endothermic transition occurs is lower when phosphatidylcholine is dispersed in water (Ladbrooke and Chapman, 1969). A similar result was observed in L-595 system. There was no detectable peak in Span® 20, Brij® 52, and Brij® 76 systems at the temperature range studied. It implies that they were in the liquid crystalline state (Ladbrooke and Chapman, 1969; Ganesan et al., 1984). Span® 20 mixture showed peak transition at a temperature lower than the ambient temperature. This system was in the liquid crystalline state (Table 5). The peak of Brij® system was a little lower than the ambient temperature. Thus, the effect of water in lowering the transition temperature of the system was clearly seen.

Table 5 Melting points of pure compounds and surfactant mixtures (Mean$\pm$SD, n = 3)

Compound	Melting point (°C)
Brij® 52	39.3$\pm$0.6
Brij® 76	37.4$\pm$0.2
Cholesterol (CHO)	147.9$\pm$0.3
Glyceryl distearate GDS)	54.4$\pm$0.4
Propylthiouracil	217.9$\pm$0.2
Polyoxyethylene-8-layrate (PEG-8-L)	7.1$\pm$0.2
Solulan® C24	47.8$\pm$0.5
Span® 20	18.3$\pm$0.4
Span® 40	47.1$\pm$0.5
Span® 60	54.1$\pm$0.2
Sucrose laurate ester (L-595)	135.8$\pm$1.1
Brij® 52:CHO 70:30	35.4$\pm$0.7
Brij® 76:CHO 50:50	34.6$\pm$0.6
Span® 20:CHO 60:40	1.5$\pm$0.8
Span® 40:CHO 70:30	42.7$\pm$1.6
Span® 60:CHO 60:40	45.6$\pm$0.8
Brij® 52:CHO:Solulan® C24 67.5:27.5:5	35.4$\pm$0.5
Brij® 76:CHO:Solulan® C24 47.5:47.5:5	35.0$\pm$0.7
Span® 20:CHO:Solulan® C24 57.5:37.5:5	5.1$\pm$1.7
Span® 40:CHO: Solulan® C24 57.5:37.5:5	42.4$\pm$0.8
Span® 60:CHO: Solulan® C24 57.5:37.5:5	41.8$\pm$2.3
GDS:CHO: Brij® 76 45:15:40	48.5$\pm$0.4
L-595:CHO 60:40	116.6$\pm$0.1
L-595:PEG-8-L 70:30	5.8$\pm$0.5
L-595:PEG-8-L 50:50	6.5$\pm$1.0

Table 6 Phase transition temperature of the blank and PTU vesicles in water (Mean±SD, n = 3)

Formulation	Peak temperature (°C)	
	Blank niosomes	PTU niosomes
Span® 20:CHO 60:40	-[a]	-[a]
Span® 40:CHO 70:30	42.2±0.2	42.1±0.2
Span® 60:CHO 60:40	36.4±0.2	36.8±0.2
Brij® 52:CHO 70:30	-[a]	-[a]
Brij® 76:CHO 50:50	-[a]	-[a]
L-595:PEG-8-L 50:50	-4.0±0.1, 6.1±0.3	-4.1±0.1, 6.2±0.2
GDS:CHO:Brij® 76 45:15:40	53.0±0.2	52.8±0.6
Span® 20:CHO:Solulan® C24 57.5:37.5:5	-[a]	-[a]
Span® 40:CHO:Solulan® C24 67.5:27.5:5	42.4±0.1	42.5±0.1
Span® 60:CHO:Solulan® C24 57.5:37.5:5	36.6±0.4	36.2±0.2
Brij® 52:CHO:Solulan® C24 67.5:27.5:5	-[a]	-[a]
Brij® 76:CHO:Solulan® C24 47.5:47.5:5	-[a]	-[a]

a = No transition peak was detected under the condition used.

Table 7 Phase transition temperature of the blank and PTU vesicles in phosphate buffer, pH 7.4 (Mean±SD, n = 3)

Formulation	Peak temperature (^{o}C)	
	Blank niosomes	PTU niosomes
Span® 20:CHO 60:40	-[a]	-[a]
Span® 40:CHO 70:30	41.8±0.2	41.9±0.1
Span® 60:CHO 60:40	40.1±3.4	37.6±0.4
Brij® 52:CHO 70:30	45.8±0.2	47.1±0.1
Brij® 76:CHO 50:50	-[a]	-[a]
L-595:PEG-8-L 70:30	-7.3±0.1, 6.6±0.2	-7.5±0.1, 6.5±0.1
GDS:CHO:Brij® 76 45:15:40	52.9±0.1	53.0±0.1
Span® 20:CHO:Solulan® C24 57.5:37.5:5	-[a]	-[a]
Span® 40:CHO:Solulan® C24 67.5:27.5:5	42.2±0.2	42.2±0.1
Span® 60:CHO:Solulan® C24 57.5:37.5:5	44.9±0.1	45.3±0.5
Brij® 52:CHO:Solulan® C24 67.5:27.5:5	47.2±0.1	47.1±0.1
Brij® 76:CHO:Solulan® C24 47.5:47.5:5	-[a]	-[a]

a = No transition peak was detected under the condition used.

The endothermic peaks in water and phosphate buffer systems were similar in all systems except the Brij® 52 systems where transition peaks were seen in phosphate buffer but not in water (Table 6 and 7). In the presence of salt solution lecithin and the salt compete for the free water and the phase transition is modified or disappears (Ladbrooke and Chapman, 1969). On the other hand, electrolytes induce dehydration of polyoxyethylene head group of dialkyl polyoxyethylene ether (Harvey et al., 2005) and monoalkyl polyoxyethylene ether (Hofland et al., 1993). Previous studies reported that electrolytes increase both layer thickness and interlamellar spacing of bilayer and promote the development of tighter membrane stacking. This could result in less bilayer fluidity. Brij® 52 (HLB = 5.3) is more hydrophobic than Brij® 76 (HLB = 12.4), thus the

dehydration effect might be predominant. Thus, the transition peaks appeared in the presence of salt.

3.4 Stability of PTU vesicular suspensions

In this study, there were no detectable changes in color and no gross precipitation occurred in any of the preparations after 2 months of storage at ambient temperature. No drug crystal was found in any preparations over the 2 months of study.

Tables 8 and 9 show the entrapment efficiencies of different types of PTU vesicles when they were stored at ambient temperature for 2 months. There was no statistical difference ($p>0.05$) between 1 and 60 days of storage in most formulations. During the storage time, PTU could partition among the aqueous phase outside the vesicles, the vesicular membrane, and the aqueous compartment of the vesicles until the system reached equilibrium. PTU entrapment efficiencies of some formulations increased a little after 2 months of storage, probably because of the effect of equilibrating time (Plookchit Chetratanont, 2002).

Tables 10 and 11 show average size of the vesicles at 1 and 60 days. The size at 60 days was the same as that at 1 day. This implies that all formulations were stable at ambient temperature for two months. The phase transition peaks of the vesicles are shown in Tables 12 and 13. All formulations showed the same peak during two months except the Brij® 52:CHO system. However, the peak appeared after 2 months of storage did not coincide with those of the surfactant or CHO. Thus, it was not likely that phase separation of the two components took place.

Table 8 Entrapment efficiencies of PTU vesicles in water after 2 months of storage at ambient temperature (Mean±SEM, n = 3)

Formulation	Entrapment efficiency (mg% by weight) Time (Days)		p-value
	1	60	
Span® 20:CHO 60:40	0.44±0.02	0.49±0.01	0.59
Span® 40:CHO 70:30	0.46±0.03	0.46±0.04	1.00
Span® 60:CHO 60:40	0.91±0.04	0.83±0.03	0.94
Brij® 52:CHO 70:30	0.92±0.00	1.02±0.00	0.00
Brij® 76:CHO 50:50	1.05±0.01	1.13±0.02	0.34
L-595:PEG-8-L 50:50	0.53±0.01	0.55±0.01	1.00
GDS:CHO:Brij® 76 45:15:40	0.71±0.03	0.61±0.03	0.73
Span® 20:CHO:Solulan® C24 57.5:37.5:5	0.95±0.02	1.19±0.02	0.01
Span® 40:CHO:Solulan® C24 67.5:27.5:5	1.16±0.04	1.24±0.02	0.19
Span® 60:CHO:Solulan® C24 57.5:37.5:5	0.68±0.02	0.81±0.01	0.01
Brij® 52:CHO:Solulan® C24 67.5:27.5:5	0.71±0.01	0.92±0.01	0.00
Brij® 76:CHO:Solulan® C24 47.5:47.5:5	0.95±0.01	1.06±0.04	0.04

Table 9 Entrapment efficiencies of PTU vesicles in phosphate buffer after 2 months of storage at ambient temperature (Mean±SEM, n = 3)

Formulation	Entrapment efficiency (mg% by weight) Time (Days)		p-value
	1	60	
Span® 20:CHO 60:40	0.79±0.04	1.05±0.04	0.00
Span® 40:CHO 70:30	0.54±0.01	0.66±0.03	0.38
Span® 60:CHO 60:40	0.58±0.01	0.64±0.03	1.00
Brij® 52:CHO 70:30	0.83±0.03	1.03±0.04	0.01
Brij® 76:CHO 50:50	0.62±0.01	0.93±0.06	0.00
L-595:PEG-8-L 70:30	0.49±0.01	0.54±0.01	1.00
GDS:CHO:Brij® 76 45:15:40	1.00±0.05	0.92±0.03	0.89
Span® 20:CHO:Solulan® C24 57.5:37.5:5	0.76±0.02	0.85±0.04	0.12
Span® 40:CHO:Solulan® C24 67.5:27.5:5	0.71±0.01	0.77±0.01	0.44
Span® 60:CHO:Solulan® C24 57.5:37.5:5	0.88±0.02	0.87±0.01	1.00
Brij® 52:CHO:Solulan® C24 67.5:27.5:5	0.55±0.02	0.71±0.01	0.00
Brij® 76:CHO:Solulan® C24 47.5:47.5:5	0.81±0.00	1.01±0.02	0.00

Table 10 Average sizes of PTU vesicles in water after 2 months of storage at ambient temperature (Mean±SEM, n = 3)

Formulation	Size (μm) Time (Days) 1	Size (μm) Time (Days) 60	p-value
Span® 20:CHO 60:40	4.02±0.37	3.27±0.20	0.07
Span® 40:CHO 70:30	8.57±0.08	8.98±0.22	0.81
Span® 60:CHO 60:40	7.85±0.02	7.06±0.13	0.05
Brij® 52:CHO 70:30	2.57±0.25	2.75±0.04	1.00
Brij® 76:CHO 50:50	5.29±0.02	6.10±0.02	0.03
L-595:PEG-8-L 50:50	0.37±0.00	0.36±0.00	1.00
GDS:CHO:Brij® 76 45:15:40	3.20±0.01	2.64±0.04	0.36
Span® 20:CHO:Solulan® C24 57.5:37.5:5	2.58±0.00	2.59±0.02	1.00
Span® 40:CHO:Solulan® C24 67.5:27.5:5	4.76±0.08	4.13±0.06	0.04
Span® 60:CHO:Solulan® C24 57.5:37.5:5	4.12±0.01	4.45±0.03	0.03
Brij® 52:CHO:Solulan® C24 67.5:27.5:5	0.76±0.00	2.37±0.16	0.00
Brij® 76:CHO:Solulan® C24 47.5:47.5:5	5.01±0.00	6.07±0.02	0.00

Table 11 Average sizes of PTU vesicles in phosphate buffer after 2 months of storage at ambient temperature (Mean±SEM, n = 3)

Formulation	Size (μm) Time (Days) 1	60	p-value
Span® 20:CHO 60:40	6.46±0.15	5.18±0.54	0.79
Span® 40:CHO 70:30	2.34±0.02	1.64±0.00	0.01
Span® 60:CHO 60:40	2.12±0.03	2.74±0.28	0.76
Brij® 52:CHO 70:30	1.33±0.01	1.56±0.00	0.00
Brij® 76:CHO 50:50	3.93±0.00	3.94±0.17	1.00
L-595:PEG-8-L 70:30	0.51±0.00	0.35±0.00	0.00
GDS:CHO:Brij® 76 45:15:40	3.42±0.02	2.54±0.06	0.02
Span® 20:CHO:Solulan® C24 57.5:37.5:5	3.60±0.08	3.31±0.09	0.06
Span® 40:CHO:Solulan® C24 67.5:27.5:5	3.20±0.04	3.06±0.02	0.81
Span® 60:CHO:Solulan® C24 57.5:37.5:5	3.23±0.01	3.16±0.00	1.00
Brij® 52:CHO:Solulan® C24 67.5:27.5:5	0.51±0.01	2.59±0.13	0.00
Brij® 76:CHO:Solulan® C24 47.5:47.5:5	3.47±0.00	3.29±0.05	0.55

Table 12 Phase transition temperatures of PTU vesicles in water after 2 months of storage at ambient temperature (Mean±SD, n = 3)

Formulation	Peak temperature (°C)	
	1 day	60 days
Span® 20:CHO 60:40	-[a]	-[a]
Span® 40:CHO 70:30	42.1±0.2	43.3±0.0
Span® 60:CHO 60:40	36.8±0.2	37.1±0.1
Brij® 52:CHO 70:30	-[a]	48.3±0.1
Brij® 76:CHO 50:50	-[a]	-[a]
L-595:PEG-8-L 50:50	-4.1±0.1, 6.2±0.2	-4.0±0.1, 6.4±0.2
GDS:CHO:Brij® 76 45:15:40	52.8±0.6	53.6±0.2
Span® 20:CHO:Solulan® C24 57.5:37.5:5	-[a]	-[a]
Span® 40:CHO:Solulan® C24 67.5:27.5:5	42.5±0.09	43.4±0.08
Span® 60:CHO:Solulan® C24 57.5:37.5:5	36.2±0.2	37.0±0.0
Brij® 52:CHO:Solulan® C24 67.5:27.5:5	-[a]	-[a]
Brij® 76:CHO:Solulan® C24 47.5:47.5:5	-[a]	-[a]

a = No transition peak was detected under the condition used.

Table 13 Phase transition temperatures of PTU vesicles in phosphate after 2 months of storage at ambient temperature (Mean±SD, n = 3)

Formulation	Peak temperature (°C)	
	1 day	60 days
Span® 20:CHO 60:40	-[a]	-[a]
Span® 40:CHO 70:30	41.9±0.1	43.0±0.0
Span® 60:CHO 60:40	37.6±0.4	39.8±3.5
Brij® 52:CHO 70:30	47.1±0.1	47.8±0.3
Brij® 76:CHO 50:50	-[a]	-[a]
L-595:PEG-8-L 50:50	-7.5±0.1, 6.5±0.1	-7.2±0.1, 7.8±0.3
GDS:CHO:Brij® 76 45:15:40	53.0±0.1	53.4±0.1
Span® 20:CHO:Solulan® C24 57.5:37.5:5	-[a]	-[a]
Span® 40:CHO:Solulan® C24 67.5:27.5:5	42.2±0.1	43.2±0.4
Span® 60:CHO:Solulan® C24 57.5:37.5:5	45.3±0.5	45.7±0.5
Brij® 52:CHO:Solulan® C24 67.5:27.5:5	47.1±0.1	47.4±0.4
Brij® 76:CHO:Solulan® C24 47.5:47.5:5	-[a]	-[a]

a = No transition peak was detected under the condition used.

3.5 Measurement of vesicle elasticity

Vesicles are considered to be elastic when it is possible to extrude the formulations through membranes with small pore sizes without significant changes in vesicular size. Rigid vesicles could not be extruded through membranes with small pore sizes (Honeywell-Nguyen, Frederik et al., 2002). The ultradeformable liposomes can pass through membranes with pore size smaller than their own size by a factor of approximately 3-4 (Cevc et al., 1995; Cevc et al., 1998; Cevc, 2004). To determine vesicle elasticity, the size of the L-595:PEG-8-L vesicle was measured before and after extrusion through polycarbonate membranes with a pore size of 50 nm 2 times using a hand-held extruder (LiposoFast™, Aveatin, Canada). The original size of L-595/PEG-8-

L vesicles was 270.63+3.00 nm. The size of the vesicles was 155.83+7.26 nm after extrusion through membranes with a pore size of 200 nm. After the first and the second extrusion through membranes with 50 nm pore size, the vesicle size was 158.50 nm+3.34 nm and 156.70+3.61 nm, respectively. Other formulations could not be extruded through membranes with 200 nm pore size. Thus, L-595:PEG-8-L vesicles were elastic vesicles. This conclusion was directly supported by the fact that the L-595:PEG-8-L vesicle size was nearly the same before and after the extrusion ($p>0.05$). Similar results were observed in many previous reports in which elastic vesicles contained various molar ratios of L-595:PEG-8-L (van den Bergh et al., 2001; Li et al., 2001; Honeywell-Nguyen, Frederik et al., 2002; Honeywell-Nguyen, Arenja et al., 2003; Honeywell-Nguyen and Bouwstra, 2003; Honeywell-Nguyen, Groenink et al., 2003). L-595:PEG-8-L vesicles are elastic vesicles consisting of the bilayer-forming surfactant L-595 and the micelle-forming surfactant PEG-8-L. Incorporation of a micelle-forming agent into a vesicle bilayer would result in solubilization of the bilayer and thereby increasing the elasticity of the vesicle. Therefore, a series of vesicles can be obtained, ranging from very rigid to very elastic by changing the ratio of the bilayer forming and the micelle forming agent (van den Bergh et al., 2001; Honeywell-Nguyen, Frederik et al., 2002). They found that elastic vesicles consisting of L-595, PEG-8-L and sodium sulfosuccinate in the molar ratio of 50:50:5 would give the most elastic and stable formulation with or without drugs. This formulation was not tested in this present study, however, since sodium sulfosuccinate was not commercially available. Thus, L-595:PEG-8-L vesicles were the only elastic formulation used in this study.

4. Drug Release Studies

In vitro drug release is generally used in evaluation of drug delivery from topical formulations. The result from such experiment can predict the behavior of drug release to the skin in vivo (Wester and Maibach, 1990).

In this study, four formulations of PTU vesicles: GDS:CHO:Brij® 76 45:15:40 w/w in phosphate buffer, pH 7.4; Span® 20:CHO:Solulan® C24 57.5:37.5:5 w/w in water;

L-595:PEG-8-L 50:50 w/w in water; and Span® 40:CHO:Solulan® C24 67.5:27.5:5 w/w in water were selected. The choices of formulations were based on thermodynamic state, size, and PTU entrapment of the vesicles. GDS:CHO:Brij® 76 in phosphate buffer was selected as vesicles in gel state. Span® 20:CHO:Solulan® C24 in water was a representative of vesicles in liquid crystalline state. L-595:PEG-8-L in water was in elastic state. The average sizes were approximately the same except for L-595:PEG-8-L (see Table 4). Span® 40:CHO:Solulan® C24 67.5:27.5:5 w/w in water was also selected since this formulation gave the highest entrapment of all noisome formulations in this study (see Table 3). The solutions of PTU in water and in phosphate buffer at 90% saturation were also tested as references. Ultrapure® water or phosphate buffer, pH 7.4, was used as a receptor fluid, corresponding to the composition of PTU vesicles being tested.

The profiles of PTU released from the vesicular suspensions and solutions are illustrated in Figure 14. All vesicular formulations gave slow PTU release when compared to the solutions. The diffusion of PTU from solution was nearly complete (>90%) within 6 hours. The vesicles gradually released associated PTU over 24 hours. The release of PTU was about 75-94% within 24 hours. Several researchers have reported similar release retardation of both hydrophilic and hydrophobic drugs from niosomes and liposomes. Examples of hydrophilic drugs were 5-fluorouracil (Namdeo and Jain, 1999), carboxyfluorescein (Yoshioka et al., 1994; Yoshioka and Florence, 1994), and cytarabine hydrochloride (Ruckmani et al., 2000) and those of hydrophobic drugs were enoxacin (Fang, Sung et al., 1999) and retinoic acid (Montenegro et al., 1996). The results from Figure 14 also clearly show that the release rate depended on entrapment efficiency of the formulation. The formulation with higher PTU entrapment released the drug more slowly than the formulation with lower entrapment. The similar negative relationship between drug entrapment and drug release was previously reported with mitoxantrone liposomes and acetazolamide niosomes (Law et al., 1994; Guinedi et al., 2005). This result indicates that PTU molecules associated with the vesicles were slowly released from the formulations. The small amount (approximately 10%) of PTU released at the early time point was practically the same for all formulations. This small

fraction should reflect the relatively rapid diffusion of free drug in the aqueous phase (outside the vesicles) of the formulations. The burst effect was unlikely because the formulations were in equilibrium at all times without any chances for PTU to migrate and accumulate at the surface of the vesicles. The more rapid diffusion of PTU at the early time point was seen with PTU solutions as expected.

The release of PTU from vesicles follows the first-order kinetics. This result agrees well with many previous reports (Margalit et al., 1992; Yoshioka and Florence, 1994; Lichtenstein and Margalit, 1995; Manconi et al., 2002). From the semi-logarithmic relationship of the percent drug remaining as a function time, values of release rate constant were obtained from the slope of the first order plot. Table 14 shows the release rate constants of these formulations. There were statistically significant differences in the release rate constants among the formulations tested ($p<0.05$). The results in Figure 14 and Table 14 agree well that the release rate depended on entrapment efficiency of the formulation with Pearson correlation coefficient of -0.731.

Table 14 Release rate constants of various formulations (Mean$\pm$SD, n = 4).

Lipid compositions	Release rate constant (Rate x 10 (hr^{-1}))	R^2
GDS:CHO:Brij® 76 45:15:40	1.24$\pm$0.11	0.9994
Span® 40:CHO:Solulan® C24 67.5:27.5:5	1.14$\pm$0.03	0.9975
Span® 20:CHO:Solulan® C24 57.5:37.5:5	1.40$\pm$0.15	0.9988
L-595:PEG-8-L 50:50	1.46$\pm$0.08	0.9994
p-value	0.003	-

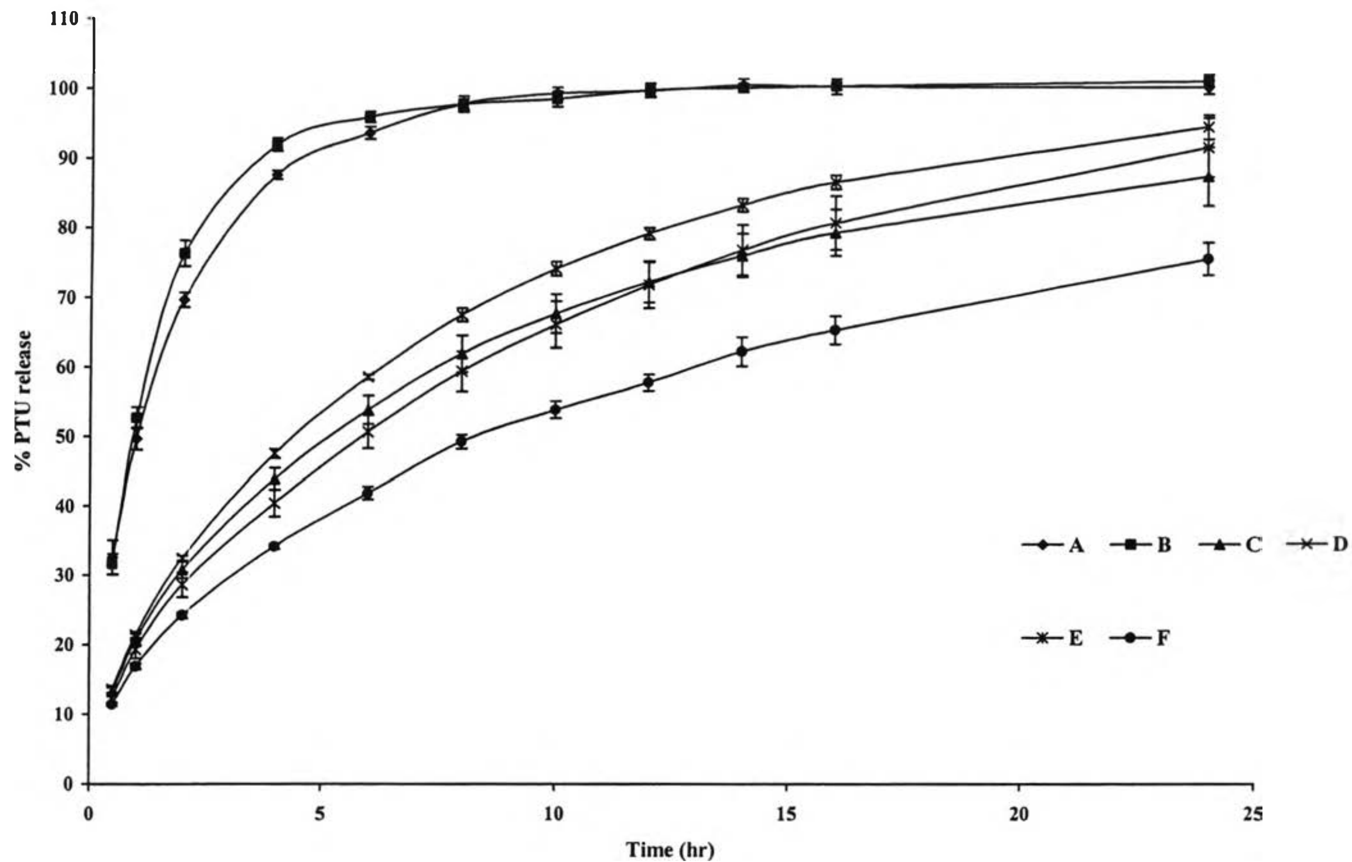

Figure 14 Release profiles of PTU vesicles and saturated solutions (Mean±SEM, n = 4)

A = solution in buffer (90% saturation), B = solution in water (90% saturation), C = Span® 20, D = L-595, E = GDS, and F = Span® 40

Besides the entrapment efficiency, the state of bilayer also influenced the release rate. There was no statistically significant difference between the release rates of the two vesicular formulations in the gel state (GDS and Span® 40 formulations) ($p>0.05$). Their release rates were significantly lower when compared to those of the other two formulations ($p<0.05$). The bilayer in the elastic state (L-595 formulation) seemed to be able to retain the drug better than what was expected solely from drug entrapment, which implied a relatively tight bilayer. Despite a much lower PTU entrapment (0.53 mg%, Table 3), its release was not significantly different from that of the formulation in the liquid crystalline state (Span® 20 formulation, entrapment: 0.95 mg %)($p>0.05$). Thus, the ease of drug release seemed to follow the trend of liquid crystalline state > elastic state > gel state. This trend was also seen within the same surfactant class. The release rate of PTU from Span® 20 formulation was significantly higher than that from Span® 40 formulation ($p<0.05$). The difference can be attributed to phase transition temperatures of the vesicular compositions. The temperature used in the release studies (32 °C in the donor compartment) was higher than the phase transition temperature of lipid mixture in Span®20 formulation (5.1 °C). The hydrocarbon chain of the vesicles was in the liquid crystalline state, which facilitated drug release. On the other hand, the lipid mixture used in Span®40 formulation had the phase transition temperature of 42.4 °C. The formulation was in the gel state, where the hydrocarbon chain were fully extended and closely packed. This would make the vesicle membrane more rigid and resisting to drug release. Other researchers have also reported faster release of drug from the vesicles in the liquid crystalline state than those in the gel state (Betageri and Parsons, 1992; Yoshioka et al., 1994; Saarinen-Savolainen et al., 1997; Fang, Sung et al., 1999; Namdeo and Jain, 1999; Ruckmani et al., 2000; Guinedi et al., 2005). The same explanation could also apply to the other two formulations with phase transition temperatures of 48.5 °C and 6.5 °C for GDS and L-595 systems, respectively.

5. In Vitro Permeation Study

The enhanced delivery through the stratum corneum of the vesicle-encapsulated drugs has been observed (Reddy and Udupa, 1993; Hofland et al., 1994; Lieb et al., 1994; Niemiec et al., 1995; Jayaraman et al, 1996; Waranuch et al., 1997; Agarwal et al., 2001; Fang, Hong et al., 2001; Carafa et al., 2002). Therefore, it remains to elucidate the mechanism of this delivery, especially as the stratum corneum is considered to be a particularly impermeable barrier (Junginger et al., 1991; Bouwstra and Honeywell-Nguyen, 2002; Bouwstra et al., 2003).

In comparison of the effect of one variable, the other variables should be fixed. For example, in comparison of the effect of thermodynamic state on permeation, the formulation should be composed of the same group of surfactants. However, the vesicles from the same group of surfactants did not form under the conditions used in this study. Therefore, four formulations of PTU vesicle: 1) GDS:CHO:Brij® 76 45:15:40 w/w in phosphate buffer, pH 7.4, 2) Span® 20:CHO:Solulan® C24 57.5:37.5:5 w/w in water, 3) L-595:PEG-8-L 50:50 w/w in water, 4) Span® 40:CHO:Solulan® C24 67.5:27.5:5 w/w in water were selected based on thermodynamic state, size, and PTU entrapment of the vesicles. GDS:CHO:Brij® 76 in phosphate buffer was selected as vesicles in gel state. Span®20:CHO:Solulan® C24 in water was a representative of vesicles in liquid crystalline state. L-595:PEG-8-L was in elastic state. The first two formulas have the approximately same size (see Table 4). Span® 40:CHO:Solulan® C24 in water was also selected since this formulation was in gel state and gave the highest entrapment among the formulations in this study (see Table 3).

6.Effects of Formulation Factors on Permeation of PTU from Vesicular Suspensions

There are several factors affecting drug permeation through the skin such as thermodynamic state (Bouwstra and Honeywell-Nguyen, 2002; Bouwstra et al., 2003), vesicle size (Verma et al., 2003), melting point of lipid composition (Waranuch et al., 1998), and the existence of vesicular structure (El Maghraby et al., 2000a; Fang, Hong

et al., 2001). In this present study, the effects of vesicle size were not examined because all formulations which their sizes were larger than 500 nm could not be extruded through Lipex™ Extruder at 70 °C. Glyceryl dilaurate:CHO:Brij® 76 at various ratios, which were expected to yield vesicles with different melting temperatures, did not form vesicles at all in this study. Consequently, the effects of melting point of lipid composition were not studied.

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

6.1 Thermodynamic state of vesicle bilayer

The present study confirmed that L-595:PEG-8-L vesicles were in elastic state since L-595:PEG-8-L vesicle size was nearly the same before and after extrusion through polycarbonate membranes with a pore size of 50 nm ($p>0.05$). This result well agrees with many previous reports (van den Bergh, Bouwstra et al., 1999; van den Bergh, Vroom et al., 1999; Li et al., 2001; van den Bergh et al., 2001; Honeywell-Nguyen, de Graaff et al., 2002; Honeywell-Nguyen, Frederik et al., 2002; Honeywell-Nguyen, Arenja et al., 2003; Honeywell-Nguyen and Bouwstra, 2003; Honeywell-Nguyen, Groenink et al., 2003).

Permeation of PTU from the three formulations which were in the different states: Span® 40:CHO:Solulan® C24 in water (gel state), Span® 20:CHO:Solulan® C24 in water (liquid crystalline state), and L-595:PEG-8-L in water (elastic state) were studied using modified Franz diffusion cells. Figure 15 shows permeation profiles of PTU from the three formulas. This result shows that liquid crystalline state vesicles were the most

effective in increasing PTU permeation across the pig skin among the three formulas tested in this study. Meanwhile, permeation of PTU from gel state vesicles was comparable to that of elastic vesicles.

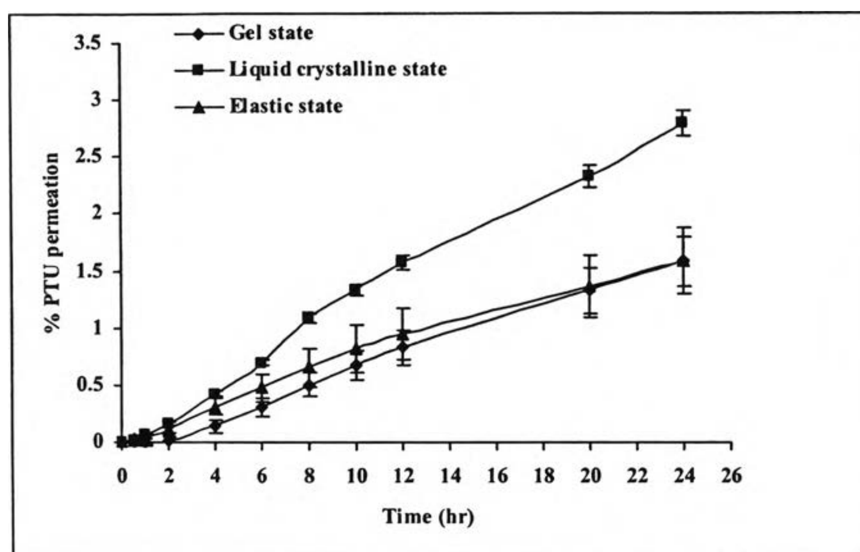

Figure 15 Permeation profiles of PTU from vesicles in different thermodynamic states (Mean±SEM, n = 4-6)

Table 15 shows permeation parameters of PTU release from the three formulations. All permeation parameters of liquid crystalline state vesicles were higher than those of others vesicles. As seen from RF, EF, and EF of $Q_{24;}$ the liquid crystalline state vesicles enhanced the flux, Ps, and Q_{24} of PTU by 7, 4, and 6-fold, respectively. However, all permeation parameters of the liquid crystalline state were not significantly different from those of the gel state (p>0.05) except Q_{24} and EF of Q_{24}. On the other hand, the differences of most permeation parameters between liquid crystalline state vesicles and elastic vesicles were statistically significant (p<0.05) except Ps and EF. Although gel state vesicles and elastic vesicles equally enhanced Ps (see EF) and Q_{24} (see EF of Q_{24}) the RF value of gel state vesicles was more than RF of elastic vesicles. However, no statistically difference was found between gel state and elastic vesicles (p>0.05).

Table 15 Effects of thermodynamic state on permeation parameters of PTU release from PTU vesicles (Mean±SEM, n = 4-6)

Permeation parameter	Span® 40:CHO:Solulan® C24 67.5:27.5:5	Span® 20:CHO:Solulan® C24 57.5:37.5:5	L-595:PEG-8-L 50:50	p-value
Flux x 10^2	10.68±1.16	16.48±2.38	7.66±1.34	0.02
Ps x 10^5	10.50±1.14	18.02±2.61	9.72±1.70	0.09
Q_s (%)	2.96±0.79	4.53±1.55	3.33±1.20	0.63
Q_{24} (%)	1.58±0.11	2.79±0.29	1.58±0.22	0.00
EF	4.10±0.45	7.03±1.02	3.79±0.66	0.09
EF of Q_s	0.12±0.03	0.18±0.06	0.13±0.05	0.63
EF of Q_{24}	2.43±0.17	4.29±0.44	2.43±0.34	0.00
RF	4.12±0.45	6.36±0.92	2.95±0.52	0.02
Recovery	82.65±2.24	86.90±2.45	93.41±2.07	-

It is generally believed 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 induced 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 induced ultrastructural changes in the deeper layers of the stratum corneum (Zellmer et al., 1995; van den Bergh et al., 1998; van den Bergh, Bouwstra et al., 1999). In addition, many studies examined vesicle-skin interaction using confocal laser scanning microscopic techniques. These in vitro studies indicate that the lipophilic fluorescent labels applied in liquid crystalline state vesicles penetrate deeper into the skin than when applied in gel state vesicles (van Kuijk, Junginger et al., 1998; Betz, Imboden et al., 2001). These results have been confirmed in vivo study (van Kuijk, Mougin et al., 1998). These above studies suggest that components of liquid crystalline state vesicles can enter the deeper layers of the stratum corneum and can modify the intercellular lipid lamellae. The superior mode of action of liquid crystalline state vesicles for skin interactions is the most possible explanation for their better effectiveness in enhancing drug transportation into and through the skin.

Several permeation studies in vitro were carried out to evaluate whether thermodynamic state of lipid composition of vesicles would affect skin penetration of both hydrophobic and hydrophilic compounds. Many hydrophobic compounds were studied such as triamcinolone acetonide (Yu and Liao, 1996), estradiol (Hofland et al., 1994), fluorescein (Perez et al., 2000), progesterone (Knepp et al., 1990). Furthermore, Ogiso et al. (1996) studied permeation of betahistine from gel state and liquid crystalline state liposomes in vivo using Wistar and hairless rats. These above studies reveal that liquid crystalline state vesicles are more effective than gel state vesicles in enhancing permeation of hydrophobic drug into and across the skin. El Maghraby et al. (2001) found that permeation of 5-fluorouracil from soybean phosphatidylcholine (PC) liposomes (liquid crystalline state vesicles) was higher than its permeation from dipalmitoyl phosphatidylcholine (DPPC) liposomes (gel state vesicles) within 12 hours, but permeation of 5-fluorouracil from both liposomes was not different within 36 hours. This result is in accordance with other studies of some hydrophilic drugs, for example,

low molecular weight sodium heparin (Betz, Nowbakht et al., 2001), methotrexate (Trotta et al., 2004), and sodium ascorbyl phosphate (Foco et al., 2005). From the above studies it seems that the effects of liquid crystalline state vesicles on drug permeation are very dominant with hydrophobic drugs. PTU is a lyophobic drug and has relatively low partition coefficient of 1.0. Partition of the drug from the bilayer would be relatively easy compared to that of hydrophobic drugs. Besides, some part of the drug was already in the solution form and ready to permeate the skin, even in the gel state system. Consequently, the fluidity of the vesicles did not largely influence PTU permeation. Therefore, PTU permeation from liquid crystalline state vesicles (Span® 20 formulation) was not significantly more than permeation from gel state vesicles (Span® 40 formulation).

From Figure 15 and Table 15, permeation of PTU from the L-595 vesicles was significantly lower when compared with Span® 20:CHO:Solulan® C24 and was comparable with that of Span® 40 formulation. In contrast, previous studies have demonstrated that elastic vesicles were better than the conventional rigid vesicles in the enhancement of drug transport across the skin. Honeywell-Nguyen, Frederik et al. (2002) investigated the effect of elastic (L-595:PEG-8-L 70:30 and 50:50 molar ratio in citrate buffer pH 5.0) and rigid (L-595:PEG-8-L 100:0 and 90:10 molar ratio in citrate buffer pH 5.0) vesicles using sodium sulfosuccinate as a stabilizer on the penetration of pergolide, which is a small lipophilic drug with pKa of 5-6 and log P of 2.3, across human skin. They found that elastic vesicles were superior to rigid vesicles. Honeywell-Nguyen, Arenja et al. (2003) studied permeation of rotigotine (pKa = 7.9) from L-595:PEG-8-L 50:50 elastic vesicles and L-595:PEG-8-L 100:0 rigid vesicles at pH 5.0 and 9.0. They demonstrated that elastic vesicles were more effective than rigid vesicles in the in vitro enhancement of rotigotine transport across human skin when using buffer solution with pH 9.0. In addition, they also found that elastic vesicles with high entrapment value at pH 9.0 gave rise to an enhancement effect of factor 80 as compared to the corresponding buffer solution, whereas vesicle suspensions at pH 5.0 with low entrapment efficiency did not significantly enhance the drug permeation as compared to its control. Therefore, it is essential that drug molecules are applied together with and

highly entrapped within the vesicles. This conclusion is in agreement with observations of El Maghraby et al. (1999) who studied the permeation of estradiol from deformable liposomes and Honeywell-Nguyen and Bouwstra (2003) who investigated in vitro transport of pergolide from elastic vesicles (L-595:PEG-8-L:sodium sulfosuccinate 50:50:5 molar ratio) across human skin. Furthermore, Honeywell-Nguyen and Bouwstra (2003) also suggested the proposed mechanism of action of L-595:PEG-8-L elastic vesicles with four main processes: 1) the association to the vesicle bilayers, 2) the partition of vesicles into the stratum corneum, 3) the drug release from the vesicles once in the stratum corneum, and 4) the diffusion of free drugs in the stratum corneum and partitioning into the viable skin tissue and subsequently into the systemic circulation. Therefore, from the above studies the explanations for the relatively low PTU permeation from L-595:PEG-8-L vesicles are: 1) this formula had low entrapment efficiency (see Table 2) and 2) PTU is lyophobic drug with partition coefficient of 1.0, PTU solubility in lipids of the stratum corneum might be low. Consequently, rate and amount of PTU released from elastic vesicles in the stratum corneum might be low.

6.2 Effects of surfactant type

To investigate the effects of surfactant type on permeation of PTU from vesicles, the permeation of PTU from GDS:CHO:Brij® 76 45:15:40 w/w in phosphate buffer, pH 7.4, and Span® 40:CHO:Solulan® C24 67.5:27.5:5 w/w in water were studied since both formulas had approximately the same entrapment and size . Figure 16 shows the permeation profiles of GDS:CHO:Brij® 76 and Span® 40:CHO:Solulan® C24 vesicles. The permeation parameters are summarized in Table 16. From the results in Figure 16 it is evident that the GDS vesicles were more effective for the transport of PTU across the skin than Span® 40 vesicles.

Table 16 Effects of surfactant type on permeation parameters of PTU from PTU niosomes (Mean±SEM, n = 6)

Permeation parameter	GDS:CHO:Brij® 76 45:15:40	Span® 40:CHO:Solulan® C24 67.5:27.5:5	p-value
Flux x 10^2	14.37±1.09	10.68±1.16	0.20
P_s x 10^5	17.29±1.31	10.50±1.14	0.02
Q_s (%)	2.27±0.73	2.96±0.79	0.98
Q_{24} (%)	2.22±0.15	1.58±0.11	0.04
EF	2.52±0.19	4.10±0.45	0.07
EF of Q_s	0.16±0.05	0.12±0.03	0.81
EF of Q_{24}	2.21±0.15	2.43±0.17	0.89
RF	2.12±0.16	4.12±0.45	0.03
Recovery	95.39±8.76	82.65±2.24	-

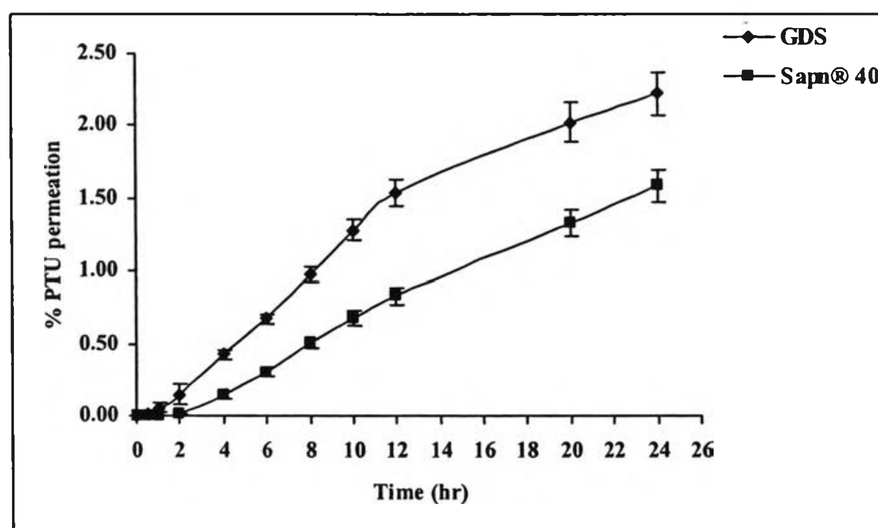

Figure16 Permeation profiles of PTU from GDS:CHO:Brij® 76 45:15:40 w/w and Span® 40:CHO:Solulan® C24 67.5:27.5:5 w/w vesicles (Mean±SEM, n = 6)

Permeation parameters of GDS formulation such as Jss, P_s, and Q_{24} were higher than parameters of Span® 40 especially P_s, and Q_{24}. On the other hand, permeation parameters which were compared with its control solution of GDS vesicles were less than the parameters of Span® 40 vesicles. However, they were not statistically significant except the RF value.

GDS vesicles were composed of glyceryl distearate 45 parts and Brij® 76 40 parts. Span® 40 vesicles contained Span® 40 67.5 parts. Glyceryl distearate is a diester and has an HLB value of 2.4. Span® 40 is sorbitan monopalmitate with an HLB of 6.7. Hydrophobic surfactants have a strong affinity to the skin (Dalvi and Zatz, 1981; Endo, et al., 1996). Therefore, GDS might partition into the stratum corneum better than Span® 40. Manconi et al. (2006) studied in vitro cutaneous delivery of tretinoin from the vesicles prepared from alkyl polyglucosides across newborn pig skin. They found that Oramix® NS10 vesicles (HLB = 11) gave lower permeation rate and higher skin accumulation than Brij® 30 (HLB = 9.7). Surfactants with high HLB are not able to penetrate into the strtum coeneum. However, when penetration occurs, it can strongly interact with skin lipids (Junginger et al., 1991). Moreover, in this present study, GDS vesicles were more potent penetration enhancer than Span® 40 (see Table 26). Therefore, the possible explanations about the better PTU transport across the skin from GDS vesicles than from Span® 40 vesicles are thus the better partition of GDS into the skin and its potent enhancing effect. However, the enhancement factors of all permeation parameters of GDS vesicles were less than those of Span® 40 vesicles. The only reason for this result is that the permeation parameters of saturated solution of PTU in phosphate buffer, pH 7.4, were higher than the permeation parameters of saturated solution of PTU in water though the differences were not statistically significant ($p>0.05$, Table 17). The reason for this result was not still unknown though it might not due to osmotic pressure effect.

Table 17 Permeation parameters of PTU from saturated solution in water and phosphate buffer pH 7.4 (Mean±SEM, n = 6)

Permeation parameters	solution in water (90% saturation)	solution in buffer (90% saturation)	p-value
Flux x 10^2	2.59±0.43	6.78±2.11	0.12
P_s x 10^5	2.56±0.43	6.85±2.14	0.12
Q_s (%)	25.07±4.51	13.99±7.05	0.20
Q_{24} (%)	0.65±0.12	1.01±0.27	0.23

6.3 Effects of the existence of vesicular structure

It was necessary to employ a solvent to solubilize the vesicular components so as to compare vesicular delivery with delivery from solution containing the same components as the corresponding vesicles. All the components used in the four vesicular formulations are soluble in isopropanol and ethanol. Although isopropanol and ethanol were a possibility, they were avoided because they could affect the skin which could possibly mask the enhancing effect of any additives (Megrab, Williams, and Barry, 1995; Yokomizo and Sagitani, 1996). Propylene glycol (PG) is widely used as an additive in pharmaceutical and cosmetic products and its enhancing effect on skin permeation arising from structural changes is marginal (Yamane, Williams, and Barry, 1995; Ross and Shah, 2000). Therefore, 90% v/v PG in water was selected as a solvent for the components of vesicles. However, only L-595:PEG-8-L 50:50 was soluble in this solvent. Solubility of PTU in 90% v/v PG in water at ambient temperature was 28.49±0.78 mg/mL. Ninety percent of PTU saturation in 90% v/v PG in water was 25.64 mg/mL.

Table 18 shows codes and compositions of formulations tested in this Section. The permeation of PTU from the L-595/PG, GDS/PG, Span® 20/PG and Span® 40/PG was studied according to the method described under Section 5 using CPG as control.

Similarly, PTU permeation from the four vesicular formulations: L-595, GDS, Span® 20, and Span® 40 were performed using CS as control.

Table 18 Codes and compositions of formulations tested

Form and code	Composition
Vesicles	
L-595	L-595:PEG-8-L 50:50
GDS	GDS:CHO:Brij® 76 45:15:40
Span® 20	Span® 20:CHO:Solulan® C24 57.5:37.5:5
Span® 40	Span® 40:CHO:Solulan® C24 67.5:27.5:5
Solution or physical mixture	
CS	90% PTU saturation in water (control for vesicles)
CPG	90% PTU saturation in 90% v/v PG in water (control for solution or physical mixture)
L-595/PG	Component of L-595 dissolved in CPG
GDS/PG	Component of GDS mixed in CPG
Span® 20/PG	Component of Span® 20 mixed in CPG
Span® 40/PG	Component of Span® 40 mixed in CPG

Ideally, drug delivery from saturated solutions in different solvents (equal thermodynamic activity) should provide the same flux unless the solvent has an effect on the skin. As propylene glycol only marginally changes skin structure, it might be expected that the flux of PTU from 90% saturated solution in 90% PG in water was close to the flux from aqueous solution. The fluxes (J_{ss} x 10^2) obtained from PG and aqueous solution were 3.41$\pm$0.33 and 2.5933$\pm$0.43 μg/cm^2 per hour and was not statistically difference ($p>0.05$). However, percent permeation of PTU from PG solution was lower than that from aqueous solution (Figure 17). Although propylene glycol acts as penetration enhancer at concentrations up to 50%, it is a humectant and induces skin dehydration at high concentrations (Ross and Shah, 2000). The skin dehydration and the high affinity of PTU for the solvent than for the skin (reduction in skin/vehicle partition

coefficient) may contribute to the reduced PTU permeation. Many researchers investigated the effects of propylene on permeation of various drugs such as p-aminoacetophenone (Flynn and Smith, 1972), lidocaine (Sarpotdar and Zatz, 1986), *N*, *N*-diethyl-*m*-tolbuamide (Ross and Shah, 2000), and flurbiprofen (Fang, Hwang, and Leu, 2003). They found the same results as in the present study. However, Yamane et al. (1995) reported that propylene glycol did not affect the permeation of a hydrophilic drug, 5-fluorouracil, since the solubility of this drug in water and in propylene glycol is comparatively insignificant.

The permeation profiles of PTU from surfactants in vesicles and solution or physical mixtures are presented in Figures 18-21. Percent PTU permeation of all vesicles was found to be superior to solution and physical mixtures of corresponding surfactants in propylene glycol. Tables 19-22 show permeation parameters of the vesicles and solution or physical mixtures in propylene glycol.

Table 19 reveals that all permeation parameters of Span® 20 vesicles (Span® 20) were significantly higher than parameters of Span® 20 in PG ($p<0.05$) except flux ($p>0.05$). On the other hand, Q_s and EF of Q_s values of vesicles were less than those of Span® 20 in PG. The Span® 40 vesicles significantly increased all permeation parameters ($p<0.05$, Table 20) compared with physical mixture in PG (Span® 40/PG) while no statistically difference was observed for Q_s and EF of Q_s values ($p>0.05$). Thus, the Span® 20 and Span® 40 vesicles were better than their physical mixtures in PG since most enhancement factors from the vesicles were higher than those from the physical mixtures in PG.

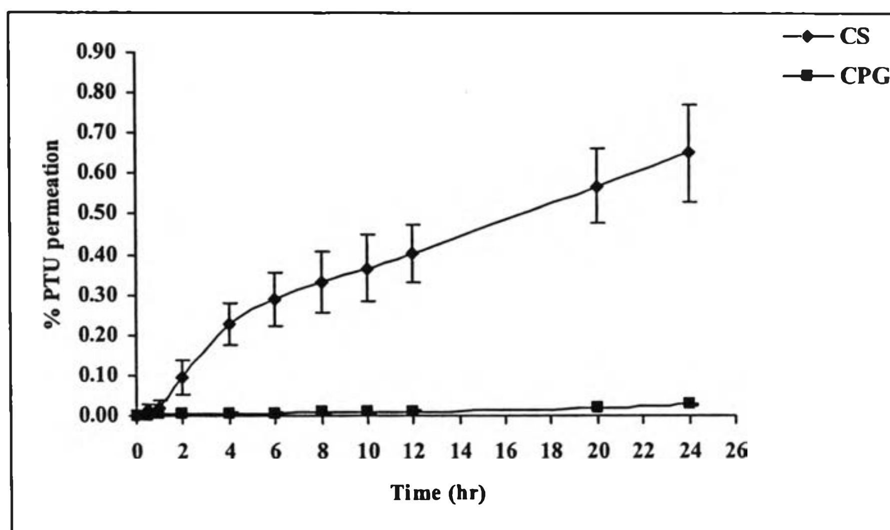

Figure 17 Permeation profiles of PTU from aqueous solution (CS) and propylene glycol solution (CPG) at 90% saturation (Mean±SEM, n = 5-6)

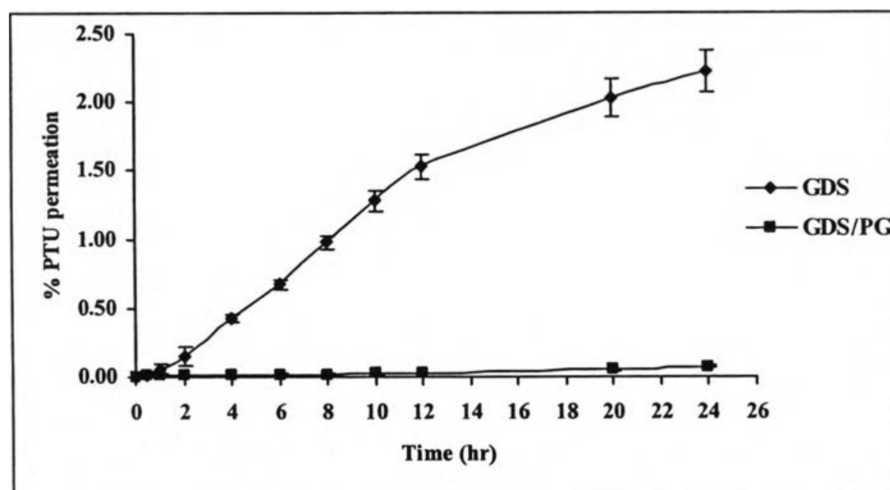

Figure 18 Permeation profiles of PTU from GDS vesicles and GDS/PG physical mixtures (Mean±SEM, n = 6)

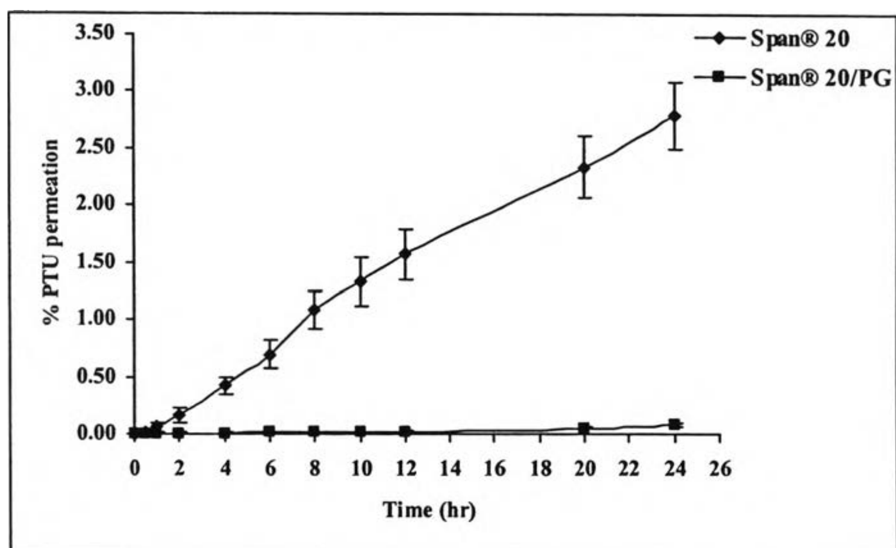

Figure 19 Permeation profiles of PTU from Span® 20 vesicles and Span® 20/PG physical mixtures (Mean±SEM, n = 6)

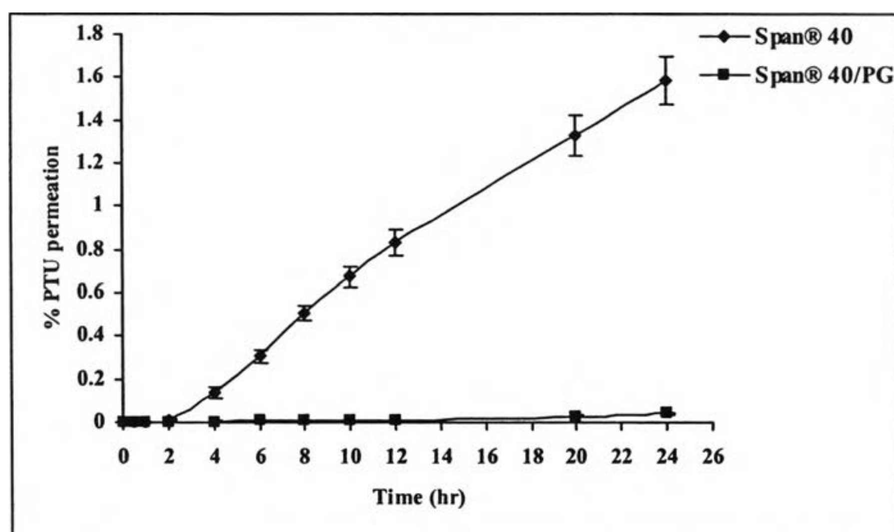

Figure 20 Permeation profiles of PTU from Span® 40 vesicles and Span® 40/PG physical mixture (Mean±SEM, n = 6)

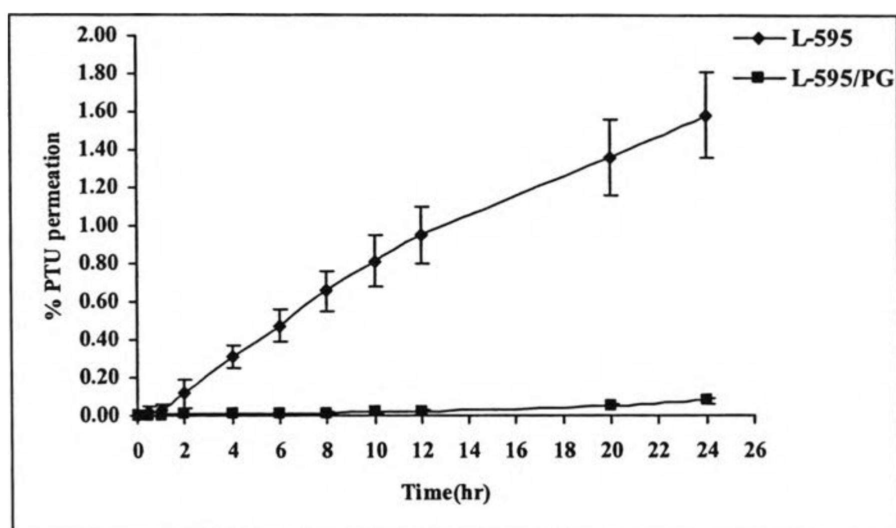

Figure 21 Permeation profiles of PTU from L-595 vesicles and L-595/PG solution (Mean±SEM, n = 4-6)

Table 19 Permeation parameters of PTU from Span® 20:CHO:Solulan® C24 vesicles and physical mixtures of Span® 20:CHO:Solulan® C24 in PG (Mean±SEM, n = 6)

Permeation parameter	Span® 20:CHO:Solulan® C24 in 90% v/v PG in water	Span® 20:CHO:Solulan® C24 vesicles	p-value
Flux x 10^2	10.29±1.6	16.48±2.38	0.05
Ps x 10^6	5.09±0.77	180.18±26.03	0.00
Q_s (%)	10.62±4.46	4.53±1.55	0.24
Q_{24} (%)	0.08±0.01	2.79±0.29	0.00
EF	3.63±0.55	7.03±1.02	0.02
EF of Q_s	1.70±0.71	0.18±0.06	0.09
EF of Q_{24}	3.10±0.46	4.29±0.44	0.04
RF	3.01±0.45	6.36±0.92	0.01
Recovery	87.27±7.01	86.90±2.45	-

Table 20 Permeation parameters of PTU from Span® 40:CHO:Solulan® C24 vesicles and physical mixtures of Span® 40:CHO:Solulan® C24 in PG (Mean±SEM, n = 6)

Permeation parameter	Span® 40:CHO:Solulan® C24 in 90% v/v PG in water	Span® 40:CHO:Solulan® C24 vesicles	p-value
Flux x 10^2	4.66±0.56	10.68±1.16	0.00
Ps x 10^6	2.55±0.34	105.01±11.41	0.00
Q_s (%)	2.98±1.68	2.96±0.79	0.99
Q_{24} (%)	0.04±0.00	1.58±0.11	0.00
EF	1.82±0.22	4.10±0.45	0.00
EF of Q_s	0.48±0.27	0.12±0.03	0.21
EF of Q_{24}	1.54±0.17	2.43±0.17	0.00
RF	1.37±0.16	4.12±0.45	0.00
Recovery	79.97±0.81	82.65±2.24	-

The similar results were observed in the studies of El Maghraby et al. (2000a), Fang, Hong et al. (2001), and Carafa et al. (2002). El Maghraby et al. (2000a) investigated the importance of liposome structure in permeation of oestradiol across the human skin. They studied four formulas of liposomes: PC, PC and sodium cholate, PC and Span® 80, and PC and oleic acid compared with lipid solutions in 90% w/w propylene glycol in water. They found that the vesicular forms of all four formulas tested were superior compared with lipid solutions when comparing relative fluxes. Fang, Hong et al. (2001) studied the transport of enoxacin across the nude mouse skin from Soybean PC liposomes and Span® 60 niosomes. They reported that Soybean PC liposome gave insignificantly higher permeation than Soybean PC physical mixture and that Span® 60 niosomes had a significantly higher permeation than Span® 60 physical mixture. In the study of Carafa et al. (2002), it was evident that permeation rates of lidocaine and lidocaine hydrochloride from drug dispersion in the presence of surfactant (Tween® 20:CHO) or phosphatidylcholine in micellar form are quite lower than that from the same vesicles. These results of the present study thus suggest that the existence of vesicular structure promoted in vitro skin delivery of PTU for Span® 20 and Span® 40 systems.

The J_{ss}, P_s, and Q_{24} values of GDS vesicles were significantly higher than the values of GDS/PG ($p<0.05$, Table 21). Relative flux (RF), enhancement factor of P_s (EF), and Q_{24} (EF of Q_{24}) of GDS vesicles was not significantly different from that of physical mixture in PG ($p>0.05$). Table 22 shows that all permeation parameters of L-595 vesicles were insignificantly different from those of L-595 in PG solution ($p>0.05$) except P_s, and Q_{24}, which were significantly higher than those of L-595/PG ($p<0.05$). Q_s and EF of Q_s of both GDS/PG and L-595/PG were higher than those of their vesicles. Therefore, the vesicular form of the GDS and L-595 system was not clearly superior to the solution or physical mixture form. Therefore, the existence of vesicular structure might not be clearly important in GDS and L-595 systems

Table 21 Permeation parameters of PTU from GDS:CHO:Brij® 76 vesicles and physical mixtures of GDS:CHO:Brij® 76 in PG (Mean±SEM, n = 6)

Permeation parameter	GDS:CHO:Brij® 76 in 90% v/v PG in water	GDS:CHO:Brij® 76 vesicles	p-value
Flux x 10^2	6.37±0.55	14.37±1.09	0.00
Ps x 10^6	3.81±0.36	172.89±13.12	0.00
Q_s (%)	5.64±1.61	2.27±0.73	0.10
Q_{24} (%)	0.07±0.01	2.22±0.15	0.00
EF	2.72±0.24	2.52±0.19	0.53
EF of Q_s	0.90±0.26	0.16±0.05	0.03
EF of Q_{24}	2.68±0.30	2.21±0.15	0.18
RF	1.87±0.16	2.12±0.16	0.29
Recovery	88.93±2.96	95.39±8.76	-

Table 22 Permeation parameters of PTU from L-595:PEG-8-L vesicles and physical mixtures of L-595:PEG-8-L in PG (Mean±SEM, n = 4-6)

Permeation parameter	L-595:PEG-8-L in 90% v/v PG in water	L-595/PEG-8-L vesicles	p-value
Flux x 10^2	10.08±1.22	7.66±1.34	0.23
Ps x 10^6	4.62±0.61	97.19±16.97	0.01
Q_s (%)	7.25±1.74	3.33±1.20	0.14
Q_{24} (%)	0.08±0.01	1.58±0.22	0.01
EF	3.30±0.40	3.79±0.66	0.51
EF of Q_s	1.16±0.28	0.13±0.05	0.01
EF of Q_{24}	2.86±0.39	2.43±0.34	0.43
RF	2.95±0.36	2.95±0.52	1.00
Recovery	85.25±7.73	93.41±2.07	-

The critical micelle concentration (CMC) of surfactants in 90% v/v propylene glycol is higher than the CMC in water (Sarpotdar and Zatz, 1986). Since L-595 system was clearly more soluble in 90% v/v propylene glycol, CMC should be accordingly higher. L-595 and PEG-8-L molecules could be more in the monomer form which acts as a penetration enhancer. Consequently, permeation of PTU from L-595 solution in PG might be higher. Thus, enhancement factors of J_{ss} (RF), P_s (EF), and Q_{24} (EF of Q_{24}) of L-595 solution were not significantly different when compared with L-595 vesicles. Solubility of GDS system seemed to be higher in 90% v/v propylene glycol in water. The same phenomenon might occur in the GDS system but the effect was not as clearly as that in the L-595 system. In addition, Okuyama et al. (1999) studied the effect of Brij® on the permeation of piroxicam from cataplasm in vivo using guinea-pig and reported that formulation containing Brij® 76 and 20 % propylene glycol significantly increased the piroxicam plasma concentration compared to the formula without propylene glycol. The more monomer form of the lipid components of the GDS and L-595 systems in PG might be the explanation of the high permeation enhancement of GDS/PG and L-595/PG systems.

The proposed mechanism of action as penetration enhancer of propylene glycol is to partition into the stratum corneum and increase drug solubility in the stratum corneum and solvent drag may carry drug into the skin (Williams and Barry, 2004). El Maghraby et al. (2000a) reported that permeation of oestradiol from PG solution was higher than from aqueous solution. Bowen and Heard (2006) found that the permeation of ketoprofen from gel formulations was inversely proportional to the content of PG, whereas the permeation of PG was directly proportional. PTU accumulated in the skin of all systems in PG was higher than that of the vesicles. However, Yamane et al. (1995) reported that propylene glycol did not affect the permeation of hydrophilic drug 5-fluorouracil. Therefore, the effect of PG on permeation of PTU was not remarkable since PTU is a lyophobic drug with low partition coefficient and it can partly dissolve in water. However, this effect of PG might be the reason for the increased Q_s and EF of Q_s values of all systems in PG.

From the overall results in this part, the effect of vesicular structure on PTU permeation was clearly important in Span® 20 and Span® 40 systems while in GDS and L-595 systems it was not remarkable. However, percent of PTU permeation from the vesicle system of all formulas was much higher than that from solution and physical mixture.

7. Elucidation of the Dominating Mechanisms of PTU Permeation from Vesicular Suspensions

There are many ways to maximize permeation of drugs into and across the skin, for example, to modify vehicle-drug interaction, to use of the vesicle and particulate systems, to modify the stratum corneum, to bypass or remove the stratum corneum, and to use the electricity (Barry, 2001). One of the most controversial methods is the use of the vesicle and particulate systems. There are a number of papers in which liposomes have increased permeation of both hydrophilic and hydrophobic drugs (Lieb et al., 1994; Niemiec et al., 1995; Waranuch et al., 1997; Betz, Nowbakht et al., 2001; El Maghraby et al., 2000b; Agarwal et al., 2001; Fang, Hong et al., 2001; Carafa et al., 2002). Similar

to liposomes, niosomes improve transport of many drugs (Reddy and Udupa, 1993; Hofland et al., 1994; Lieb et al., 1994; Niemiec et al., 1995; Ohta et al., 1996; Jayaraman et al., 1996; Waranuch et al., 1997; Agarwal et al., 2001; Fang, Hong et al., 2001; Carafa et al., 2002). Although it has been accepted that the vesicles increase skin drug delivery, mechanism of action is not clear. There are many studies to elucidate mechanism of action of vesicles and many dominating mechanisms of action of the vesicles have been proposed. The "free drug" mechanism is stated that the drug is released from the vesicles and then freely permeates the skin. This mechanism is tested by comparing drug release profile from the vesicles with drug permeation (Ganesan et al., 1984; El Maghraby et al., 1999). The mechanism is that vesicles may increase thermodynamic activity of drug which is tested by comparing the different entrapment efficiencies of formulations with drug permeation (El Maghraby et al., 1999). The vesicles may act as penetration enhancers. This mechanism is studied by skin pretreatment with empty vesicles and compared permeation between direct application of drug containing vesicles and pretreatment (Hofland et al., 1994; Fang, Hong et al., 2001; Honeywell-Nguyen, Arenja et al., 2003). Some intact vesicles can permeate across the skin, so it can be proved by comparing permeation from small vesicles (200-300 nm) with that from larger vesicles (Cevc et al., 1995; El Maghraby et al., 1999). The vesicles may improve drug uptake by adsorption, fusion, and mixing with skin lipid. This mechanism is tested by dipping stratum corneum into the different vesicles and determining drug uptake (El Maghraby et al., 1999). Waranuch et al. (1998) suggested that the transport of cycloscoporin-A from liposomal formulations (GDL:CHO:Brij® 76) occurs as a result of dehydration of the liposomes followed by melting of the lipid components on the skin and permeation from liposomes is the same as that from lipid melts. In addition, Honeywell-Nguyen and Bouwstra (2003) studied permeation of pergolide from sucrose laurate ester elastic vesicles and found that the possible mechanisms were penetration enhancement of the vesicles and the mechanism involving transepidermal osmotic gradient.

Therefore, the dominating mechanisms of the permeation of PTU from the vesicles were: 1) increased drug thermodynamic activity, 2) the "free drug" mechanism,

3) penetration enhancement of the vesicles, and 4) the effect of transepidermal osmotic gradient

7.1 Increased drug thermodynamic activity

The rank order of the entrapment efficiency of PTU vesicles was Span® 40 > GDS > Span® 20 > L-595 (Table 2), whereas for permeation parameters; J_{ss}, P_s and Q_{24}; it was Span® 20 > GDS > Span® 40 > L-595. For EF, EF of Q_{24} and RF the rank order was Span® 20 > Span® 40 > L-595> GDS (Tables 15 and 16). No correlations were observed between entrapment efficiency and all permeation parameters (Table 23).

Table 23 Pearson correlation coefficients between entrapment efficiency and permeation parameters

Permeation parameter	Pearson correlation coefficient
Flux (J_{ss})	0.553
Permeability coefficient (P_s)	0.489
PTU in skin (Q_s)	-0.167
PTU permeated at 24 hr (Q_{24})	0.379
Enhancement Factor of P_s (EF)	0.219
Enhancement Factor of Q_s (EF of Q_s)	-0.060
Enhancement Factor of Q_{24} (EF of Q_{24})	0.227
Relative flux (RF)	0.367

In comparison between the system with highest entrapment efficiency (Span® 40 system) and the system with lowest entrapment efficiency (L-595 system), PTU permeation of both system was not significantly different (Table 24). Thus, variation in entrapment efficiency was not a factor responsible for the difference in PTU delivery of the vesicles. Similar results were observed by others researchers. Hofland et

al. (1999) studied permeation of eatradiol from niosomes prepared from polyoxyethylene monoalkyl ether-type surfactants and found that drug permeation across human stratum corneum did not depend on entrapment efficiency but on thermodynamic state of the vesicles. Similar results were also noted by El Maghraby et al. (1999) who investigated skin delivery of estradiol from deformable and traditional liposomes using human stratum corneum. In addition, Fang, Hong et al. (2001) also reported that transport parameters of enoxacin from both niosomes and liposomes across nude mouse skin did not directly relate with drug encapsulation. Sinico et al. (2005) reported that permeation of tretinoin liposomes across newborn pig skin depended on phase transition temperature of the main liposomal component.

Table 24 Permeation parameters of PTU from Span® 40:CHO:Solulan® C24 and L-595:PEG-8-L vesicles (Mean±SEM, n = 4-6)

Permeation parameter	Span® 40:CHO:Solulan® C24 67.5:27.5:5	L-595:PEG-8-L 50:50	p-value
Flux x 10^2	10.68±1.16	7.66±1.34	0.32
Ps x 10^5	10.50±1.14	9.72±1.70	0.97
Q_s (%)	2.96±0.79	3.33±1.20	0.99
Q_{24} (%)	1.58±0.11	1.58±0.22	1.00
EF	4.10±0.45	3.79±0.66	0.96
EF of Q_s	0.12±0.03	0.13±0.05	0.99
EF of Q_{24}	2.43±0.17	2.43±0.34	1.00
RF	4.12±0.45	2.95±0.52	0.32

The vesicles fuse at the stratum corneum surface and form stacks of bilayers. However, no differences in PTU permeation were expected since all formulations were at equal thermodynamic activity. The difference in permeation found may be explained by differences in skin-vesicle interactions, i.e., not mere adsorption of bilayers, but also the ultrastructural changes in the case of liquid crystalline and elastic vesicles. There are a number of papers on vesicles-skin interaction using fluorescent

probes of liposomes (Zellmer et al., 1995; Kirjavainen et al., 1996; van den Bergh et al., 1998; Cevc et al., 2002) and niosomes prepared from L-595 system (van den Bergh, Bouwstra et al., 1999; van den Bergh, Vroom et al., 1999; Honeywell-Nguyen, de Graaff et al., 2002). The results were similar that liquid crystalline and elastic vesicles cause ultrastructural changes of the deeper layer of stratum corneum, while gel state vesicles only adsorb and fuse on the stratum corneum surface. These mechanisms are consistent with the results of PTU niosoms.

7.2 The "free drug" mechanism

If the mechanism of action of the vesicles is solely the free drug mechanism whereby the drug is released from the vesicles and then freely permeates the skin, the formulation with a faster release rate would permeate the skin faster. Thus, the rate limiting step of skin transport is the release rate. To investigate the free drug mechanism, the correlation between release rate constants and permeation parameters of corresponding formula was determined. No correlation was observed in all formulations in this study (Table 25). PTU permeation from Span® 20 and L-595 formulations which had equal release rates was significantly different (Tables 13 and 15). The same result was observed with Span® 40 and GDS systems, i.e., GDS significantly increased PTU permeation compared to Span® 40 while their release rates were not significantly different. On the contrary, Span® 20 formulation with faster release rate Span® 40 did not significantly increased PTU permeation when compared with Span® 40. This strongly suggests that the free drug mechanism did not operate on any of the formulations. This result agrees well with the reported mechanism for some lipophilic drugs in liposomes and niosomes such as hydrocortisone and progesterone (Ganesan et al., 1984; Ho et al., 1985), retinoic acid (Montenegro et al., (1996), estradiol (El Maghraby et al., 1999), enoxacin (Fang, Hong et al., 2001), and lidocaine (Carafa et al., (2002).

Ganesan et al. (1984) and Ho et al. (1985) studied the mechanism of action of dipalmitoyl phosphatidylcholine liposomes containing glucose, hydrocortisone and progesterone using hairless mouse skin. Their results suggest 3 probable mechanisms: 1)

release of associated drug and permeation of free drug, 2) release of entrapped solute coupled with skin permeation of free drug and also direct liposome-skin transfer, and 3) skin permeation involving liposome-skin transfer. The first mechanism is applied to hydrophilic drugs like glucose entrapped in the aqueous phase of the vesicles. When glucose is released from the vesicles it can permeate across the skin freely. The third one is appropriate for progesterone, a lipophilic drug associated in the lipid bilayers. Permeation of progesterone from vesicles is higher than that from solution but the release rate is immeasurably slow. For hydrocortisone which is less hydrophobic than progesterone, it is released from liposomes slowly but it permeates as fast as progesterone. Thus, the second mechanism, both the free drug and liposome-skin transfer mechanism operate on permeation of hydrocortisone. PTU is a lyophobic drug which can locate both in the bilayer and in the aqueous phase of the vesicles. Therefore, mechanism of action for PTU could not solely be the free drug mechanism, but the other mechanisms should also apply.

Table 25 Pearson correlation coefficients between release rate constants and permeation parameters

Permeation parameter	Pearson correlation coefficient
Flux (J_{ss})	0.019
Permeability coefficient (P_s)	0.132
PTU in skin (Q_s)	0.048
PTU permeated at 24 hr (Q_{24})	0.281
Enhancement Factor of P_s (EF)	0.384
Enhancement Factor of Q_s (EF of Q_s)	0.034
Enhancement Factor of Q_{24} (EF of Q_{24})	0.432
Relative flux (RF)	0.232

7.3 Penetration enhancement of the vesicles

It is well known that nonionic surfactants can enhance the skin penetration of several drugs such as piroxicam (Okuyama et al., 1999; Shin, Cho, and Oh, 2001), ibuprofen (Park et al., 2000), sumatriptan succinate (Femenia-Font et al., 2005), 5-fluorouracil, antipyrine, 2-phenyl ethanol, and 4-phenyl butanol (Lopez et al., 2000). One of the possible mechanisms of action of the vesicles was the facilitation of drug permeation by penetration enhancing effect of the vesicles or vesicular components. In order to explore this mechanism, the effect of skin pretreatment with empty vesicles on PTU permeation from aqueous solution at 90 % saturation was performed. The main component of the vesicles tested in the present study is non-ionic surfactants. The results are summarized in Table 26

The rank order of some permeation parameters such as J_{ss}, P_s, and Q_{24} shown in Table 26 was GDS > Span® 40 > Span® 20 > L-595. GDS empty vesicles were the most enhancing while L-595 empty vesicles were the least ($p<0.05$). On the other hand, most enhancement factors of all formulations were not statistically significantly different ($p>0.05$). The exception was seen with the EF of Q_{24} value ($p<0.05$). Pretreatment with Span® 20 was not statistically significantly different as compared with Span® 40 ($p>0.05$). Table 27 shows p-value from Dunnett test. The results in Tables 26 and 27 imply that GDS empty vesicles had a remarkable penetration enhancing effect. Enhancing effect of Span® 40 and Span® 20 empty vesicles was moderate, but the L-595 had a marginal enhancing effect on PTU permeation.

Table 26 Permeation parameters of PTU from PTU solution at 90% saturation after pretreatment with empty vesicles (Mean+SEM, n = 4)

Permeation parameter	Span® 40:CHO:Solulan® C24 67.5:27.5:5	Span® 20:CHO:Solulan® C24 57.5:37.5:5	L-595:PEG-8-L 50:50	GDS:CHO:Brij® 76 45:15:40	p-value
Flux x 10^2	3.19±0.37	3.26±0.26	2.88±0.43	4.48±0.24	0.02
Ps x 10^5	3.11±0.37	2.88±0.23	2.54±0.38	4.10±0.22	0.02
Q_s (%)	4.85±0.64	8.95±2.10	7.04±2.89	4.68±2.40	0.49
Q_{24} (%)	0.99±0.16	0.68±0.05	0.59±0.10	1.49±0.23	0.00
EF	2.35±0.28	2.17±0.17	1.91±0.29	2.48±0.14	0.37
EF of Q_s	1.36±0.18	2.52±0.59	1.98±0.81	0.35±0.18	0.06
EF of Q_{24}	1.63±0.25	1.12±0.09	0.98±0.16	2.21±0.34	0.01
RF	2.12±0.25	2.17±0.17	1.91±0.29	2.36±0.13	0.56
Recovery	88.49±0.82	81.23±3.30	80.23±2.64	93.34±2.75	-

Table 27 The p-values from Dunnett test of permeation parameters against control (n = 4)

Parameter	p-value			
	Span® 40	Span® 20	GDS	L-595
Flux x 10^2	0.02	0.03	0.00	0.08
Ps x 10^5	0.02	0.01	0.00	0.08
Q_s (%)	0.95	0.13	0.19	0.44
Q_{24} (%)	0.13	0.18	0.01	1.00
EF	0.01	0.02	0.00	0.08
EF of Q_s	0.96	0.13	0.19	0.44
EF of Q_{24}	0.14	0.98	0.01	1.00
RF	0.03	0.02	0.00	0.08

GDS system was composed of Brij® 76 40 parts and GDS 45 parts. Brij® 76 is a good enhancer for many drugs such as cyclosporine-A (Dowton et al., 1993; Waranuch et al., 1997; Waranuch et al., 1998), alpha-interferon (Niemeic et al., 1995), growth hormone releasing peptide (Fleisher et al., 1995), piroxicam (Okuyama et al., 1999), and ibuprofen (Park et al., 2000). In addition, Sentjurc et al (1999) studied permeation of hydrophilic spin probes from GDS:CHO:lipoamino salt LAS (45:45:10 w/w) using electron paramagnetic resonance imaging method. They found that the vesicles increase spin probes permeation. Thus, GDS system increased PTU permeation from aqueous solution since it contained both GDS and Brij® 76 which are good penetration enhancers.

Lopez et al. (2000) investigated the effects of Span® 20, Tween® 20, and Azone® on the penetration of compounds with different lipophilicities. They pretreated epidermal membrane of Wistar rat overnight with 1 or 5 % w/v of the enhancers in ethanol and found that Span® 20 can be considered as suitable as Azone® in enhancing permeation of compounds with intermeadiate lipophilicities like 2-phenyl ethanol (log P = 1.34). Femenia-Font et al. (2005) studied sumatripan succinate permeation across porcine skin after pretreatment the skin with 5 % w/w of various enhancers in ethanol for

12 hours. They concluded that Span® 20 have shown a moderate enhancing activity. The reason that enhancement activity of Span® 20 empty vesicles was not clearly shown in this present study might be due to the difference in the dosage form and pretreatment time. In the present study, the skin was pretreatment with dispersion of empty vesicles in aqueous for only 4 hours while in the previous reports the skin was pretreated with ethanolic solution for 12 hours.

Sucrose laurate ester (l-595) system was not superior to the aqueous medium in the present study. This result agrees well with the study of many researchers. Ayala-Bravo et al. (2003) performed the enhancement effect of sucrose laurate ester on penetration of 4-hydroxybenzonitrile from 2 % and 10 % w/v solutions in water across human skin in vivo after pretreatment for 1 hour. They found that L-595 solution did not increase drug permeation compared with its aqueous solution. Honeywell-Nguyen, Arenja et al. (2003) studied permeation of rotigotine solution after pretreatment human skin with empty vesicles of L-595:PEG-8-L (50:50 molar ratio) for 1 hour and concluded that pretreatment clearly had no effect on drug permeation as compared to the control. The same results were observed by Honeywell-Nguyen and Bouwstra (2003) who studied the permeation of pergolide using system contained L-595:PEG-8-L (50:50 by mole).

L595 empty vesicles did not act as penetration enhancer. This may be due to the lower concentration of total lipid used. Thus, to prove this idea, L-595:PEG-8-L (50:50 w/w) empty vesicles were prepared using total lipid at 200 mg/mL. PTU permeation from solution at 90 % saturation in water was carried out with the same of pretreatment test. Table 28 shows permeation parameters after pretreatment with L-595:PEG-8-L at 100 and 200 mg/mL. All parameters from the system with lipid concentration of 200 mg/mL were higher than those from the system with 100 mg/mL total lipid. Thus, with increased lipid concentration, penetration enhancement effect of L-595 empty vesicles was clearly increased. Although increased lipid concentration gave more potent enhancement, PTU permeation from L-595:PEG-8-L (200 mg/mL) vesicles was lower, though not statistically significant, than that of L-595:PEG-8-L (100 mg/mL)

vesicles (Table 29, Figure 22). L-595:PEG-8-L (200 mg/mL) vesicles was as viscous as gel. Higher viscosity of L-595:PEG-8-L (200 mg/mL) vesicles might cause slower release of PTU and thus decreased skin permeation. Therefore, penetration enhancing effect of L-595 (100 mg/mL) vesicles was not the mechanism of this system since PTU permeation after pretreatment with empty vesicles was not significantly different as compared with aqueous solution.

Table 28 Permeation parameters of PTU solution (at 90% saturation) after pretreatment with L-595:PEG-8-L empty vesicles at 100 and 200 mg/mL (Mean±SEM, n = 4)

Permeation parameter	L-595:PEG-8-L at 200 mg/mL	L-595:PEG-8-L at 100 mg/mL	p-value
Flux x 10^2	5.11±0.45	2.88±0.43	0.01
Ps x 10^5	4.50±0.39	2.54±0.38	0.01
Q_s (%)	3.58±0.94	7.04±2.89	0.58
Q_{24} (%)	1.09±0.12	0.59±0.10	0.04
EF	3.39±0.30	1.91±0.29	0.01
EF of Q_s	1.01±0.26	1.98±0.81	0.58
EF of Q_{24}	1.80±0.20	0.98±0.16	0.04
RF	3.39±0.30	1.91±0.29	0.00
Recovery	82.75±3.27	80.23±2.64	-

Table 29 Permeation parameters of PTU from L-595:PEG-8-L vesicles at 100 and 200 mg/mL (Mean±SEM, n = 4)

Permeation parameter	L-595:PEG-8-L at 200 mg/mL	L-595:PEG-8-L at 100 mg/mL	p-value
Flux x 10^2	4.66±0.87	7.66±1.34	0.11
Ps x 10^5	6.06±1.13	9.72±1.70	0.12
Q_s (%)	1.21±0.13	3.33±1.20	0.18
Q_{24} (%)	1.14±0.24	1.58±0.22	0.22
EF	2.37±0.44	3.79±0.66	0.12
EF of Q_s	0.05±0.01	0.13±0.05	0.18
EF of Q_{24}	1.74±0.37	2.43±0.34	0.22
RF	1.80±0.34	2.95±0.52	0.11
Recovery	86.48±3.98	93.41±2.07	-

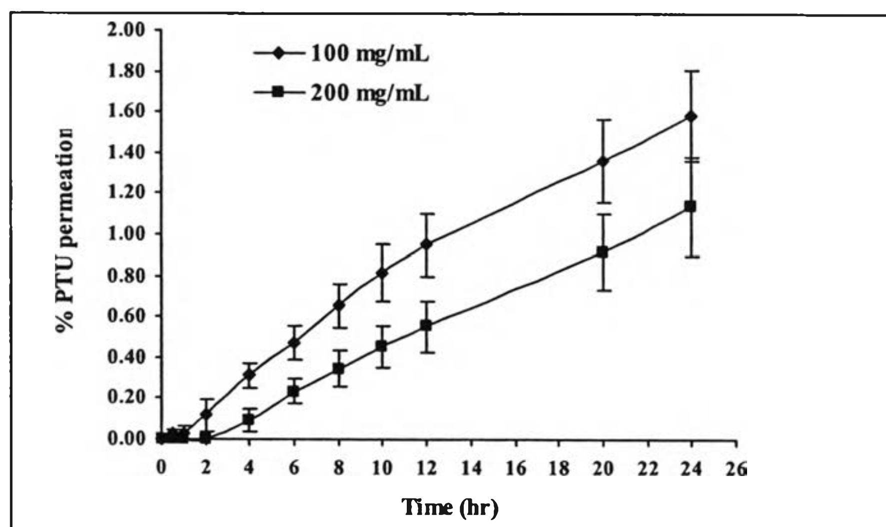

Figure 22 Permeation profiles of PTU from L-595:PEG-8-L at 100 and 200 mg/mL (Mean±SEM, n = 4)

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Comparison of permeation parameters of PTU from solution after pretreatment with empty vesicles with those from corresponding vesicles was carried out. The application of Span® 20, Span® 40 and L-595 systems in the form of vesicles was superior to pretreatment (Tables 30-32). On the other hand, GDS was not remarkably superior to pretreatment (Table 33). There were many researchers who investigated drug permeation from the vesicles compared with penetration after pretreatment with empty vesicles. For example, Hofland et al. (1994) reported that direct contact between estradiol niosomes and the skin was imperative to exert the highest effect on drug transport. El Maghraby et al. (1999) revealed that permeation of estradiol from traditional and deformable liposomes was superior to pretreatment with empty vesicles. Similary, Honeywell-Nguyen, Arenja et al. (2003) and Honeywell-Nguyen and Bouwstra (2003) used L595:PEG-8-L elastic vesicles for enhancing permeation of rotigotine and pergolide, respectively. They concluded that application of drug containing L595:PEG-8-L vesicles enhanced drug permeation more than pretreatment with empty vesicles.

Table 30 Permeation parameters of PTU from PTU containing vesicles and pretreatment of Span® 20 system (Mean±SEM, n = 4-6)

Permeation parameter	Pretreatment with empty vesicles	Span® 20:CHO:Solulan® C24 niosomes	p-value
Flux x 10^2	3.26±0.26	16.48±2.38	0.00
Ps x 10^5	2.88±0.23	18.02±2.61	0.00
Q_s (%)	8.95±2.10	4.53±1.55	0.12
Q_{24} (%)	0.68±0.05	2.79±0.29	0.00
EF	2.17±0.17	7.03±1.02	0.01
EF of Q_s	2.52±0.59	0.18±0.06	0.03
EF of Q_{24}	1.12±0.09	4.29±0.44	0.00
RF	2.17±0.17	6.36±0.92	0.01
Recovery	81.23±3.30	86.90±2.45	-

Table 31 Permeation parameters of PTU from PTU containing vesicles and pretreatment of Span® 40 system (Mean±SEM, n = 4-6)

Permeation parameter	Pretreatment with empty vesicles	Span® 40:CHO:Solulan® C24 niosomes	p-value
Flux x 10^2	3.19±0.37	10.68±1.16	0.00
Ps x 10^5	3.11±0.37	10.50±1.14	0.00
Q_s (%)	4.85±0.64	2.96±0.79	0.13
Q_{24} (%)	0.99±0.16	1.58±0.11	0.01
EF	2.35±0.28	4.10±0.45	0.02
EF of Q_s	1.36±0.18	0.12±0.03	0.01
EF of Q_{24}	1.63±0.25	2.43±0.17	0.02
RF	2.12±0.25	4.12±0.45	0.01
Recovery	88.49±0.82	82.65±2.24	-

Table 32 Permeation parameters of PTU from PTU containing vesicles and pretreatment of L-595 system (Mean±SEM, n = 4)

Permeation parameter	Pretreatment with empty vesicles	L595:PEG-8-L niosomes	p-value
Flux x 10^2	2.88±0.43	7.66±1.34	0.03
Ps x 10^5	2.54±0.38	9.72±1.70	0.02
Q_s (%)	7.04±2.89	3.33±1.20	0.22
Q_{24} (%)	0.59±0.10	1.58±0.22	0.01
EF	1.91±0.29	3.79±0.66	0.04
EF of Q_s	1.98±0.81	0.13±0.05	0.11
EF of Q_{24}	0.98±0.16	2.43±0.34	0.02
RF	1.91±0.29	2.95±0.52	0.13
Recovery	80.23±2.64	93.41±2.07	-

Table 33 Permeation parameters of PTU from PTU containing vesicles and pretreatment of GDS system (Mean$\pm$SEM, n = 4-6)

Permeation parameter	Pretreatment with empty vesicles	GDS:CHO:Brij® 76 niosomes	p-value
Flux x 10^2	4.48$\pm$0.24	14.37$\pm$1.09	0.00
Ps x 10^5	4.10$\pm$0.22	17.29$\pm$1.31	0.00
Q_s (%)	4.68$\pm$2.40	2.27$\pm$0.73	0.27
Q_{24} (%)	1.49$\pm$0.23	2.22$\pm$0.15	0.02
EF	2.48$\pm$0.14	2.52$\pm$0.19	0.87
EF of Q_s	0.35$\pm$0.18	0.16$\pm$0.05	0.27
EF of Q_{24}	2.21$\pm$0.34	2.21$\pm$0.15	0.99
RF	2.36$\pm$0.13	2.12$\pm$0.16	0.31
Recovery	93.34$\pm$2.75	95.39$\pm$8.76	-

Since GDS is a good penetration enhancer, application in the form of vesicles increased PTU permeation as high as that of pretreatment with empty vesicles. From these results, it is no doubt that drug entrapment in vesicles was essential in order to achieve PTU transport enhancement for Span® 20, Span® 40, and L-595 systems but not essential for the GDS system. Although the difference of the GDS system was not statistically significant, all parameters of PTU containing vesicles were higher than those of pretreatment.

Since GDS, Span® 20 and Span® 40 empty vesicles showed most and moderate enhancing activity, respectively, a penetration enhancing mechanism was possible for these systems particularly for the GDS system. It was not the only mechanism accounting for increased skin delivery of PTU vesicles since direct application of all vesicles gave higher PTU permeation than pretreatment. On the other hand, enhancement effect of L-595 vesicles on PTU permeation across newborn pig skin was not operated by penetration enhancing effect of the components.

7.4 Transepidermal osmotic gradient

According to Cevc and Bloom (1992), the water gradient is an important driving force for drug diffusion. They have shown that transdermal lipid transport may occur spontaneously provided that the special vesicles, Transfersomes®, are exposed to a dehydration force resulting from an osmotic gradient between the skin surface and the deeper skin tissue. Therefore, it has been suggested that elastic vesicles are most efficient under non-occlusive condition. To verify this mechanism, PTU permeation from L-595 (elastic) and Span® 40 (gel state) vesicles under non-occlusive and occlusive conditions was studied.

Table 34 and Figure 23 show permeation parameters and profiles from L-595 vesicles under non-occlusive and occlusive conditions. Occlusive application in this study gave the higher values of Flux, P_s, Q_s, and Q_{24} than non-occlusive application, but the difference were not statistically significantly. These results show that application of L-595 elastic vesicles under non-occlusive increased PTU permeation as highly as under occlusive condition. The higher values of some parameters during occlusion were simply caused by the enhancement effect of water. This indicates that water was an excellent penetration enhancer for PTU (see Figure 24). However, most parameters (as compared with aqueous solution) under non-occlusive condition were significantly higher (about 2-4 times) than those under occlusive condition ($p<0.05$). This means that the action of vesicles themselves was decreased under occluded condition. Similar results were observed in Span® 40 vesicles (Table 35 and Figure 25). This is in agreement with Cevc and Bloom (1992) who suggested that occlusion would eliminate the osmotic gradient thereby preventing the partitioning of vesicles into the skin.

Table 34 Permeation parameters from L-595 vesicles under non-occlusive and occlusive conditions (mean±SEM, n = 4)

Permeation parameter	L595:PEG-8-L (non-occlusive condition)	L595:PEG-8-L (occlusive condition)	p-value
Flux x 10^2	7.66±1.34	9.06±1.12	0.45
Ps x 10^5	9.72±1.70	11.50±1.43	0.45
Q_s (%)	3.33±1.20	2.84±1.67	0.82
Q_{24} (%)	1.58±0.22	2.18±0.26	0.13
EF	3.79±0.66	1.51±0.19	0.04
EF of Q_s	0.13±0.05	0.31±0.18	0.38
EF of Q_{24}	2.43±0.34	1.51±0.18	0.05
RF	2.95±0.52	1.06±0.13	0.03
Recovery	93.41±2.07	91.77±7.39	-

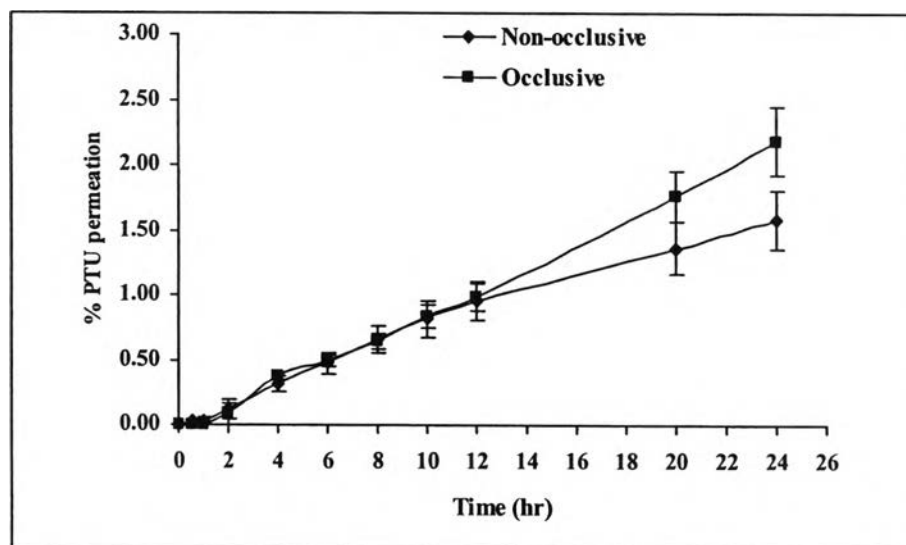

Figure 23 Permeation profiles of PTU from L-595:PEG-8-L vesicles under non-occlusive and occlusive conditions

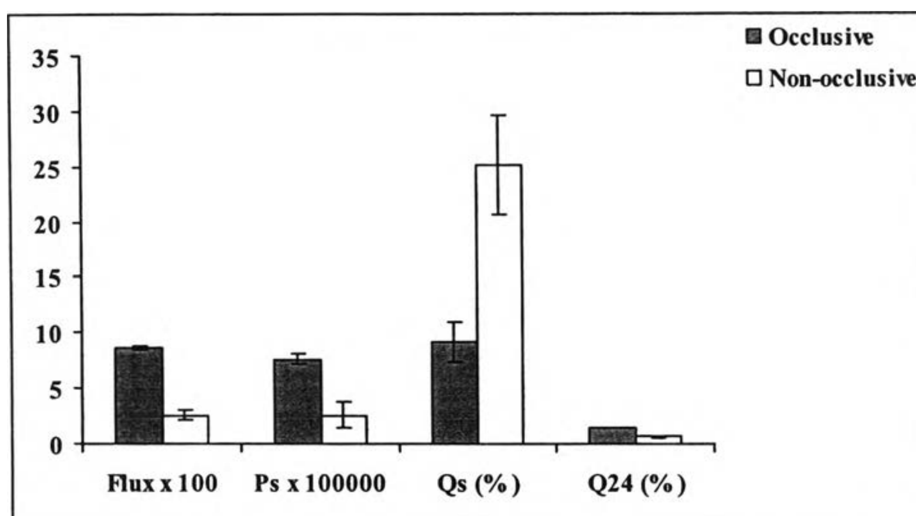

Figure 24 Permeation parameters of PTU from solution (90 % saturation) under non-occlusive and occlusive conditions (mean±SEM, n = 6)

Table 35 Permeation parameters from Span® 40 vesicles under non-occlusive and occlusive conditions (mean±SEM, n = 6)

Permeation parameter	Span® 40:CHO:Solulan® C24 (non-occlusive condition)	Span® 40:CHO:Solulan® C24 (occlusive condition)	p-value
Flux x 10^2	10.68±1.16	8.51±0.96	0.18
Ps x 10^5	10.50±1.14	9.00±1.01	0.35
Q_s (%)	2.96±0.79	3.76±1.98	0.72
Q_{24} (%)	1.58±0.11	1.61±0.18	0.89
EF	4.10±0.45	1.18±0.13	0.00
EF of Q_s	0.12±0.03	0.41±0.22	0.21
EF of Q_{24}	2.43±0.17	1.11±0.12	0.00
RF	4.12±0.45	1.00±0.11	0.00
Recovery	82.65±2.24	85.13±3.64	-

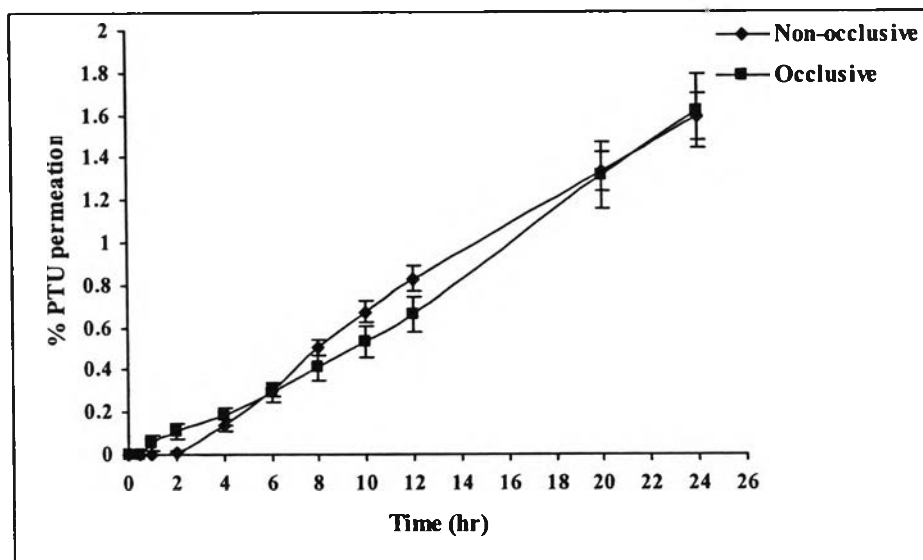

Figure 25 Permeation profiles of PTU from Span® 40 vesicles under non-occlusive and occlusive conditions (mean±SEM, n = 6)

L-595:PEG-8-L is an elastic vesicle system with high elasticity. It can penetrate through membrane with pore size of 30-50 nm. Several studies have demonstrated that these elastic vesicles are better than the conventional rigid vesicles in the enhancement of drug transport across the skin. These studies were performed using some lipophilic drugs: pergolide, rotigotine, and lidocaine. The exact mechanisms of action of this system remain a subject of discussion. Furthermore, it is not clear under which conditions elastic vesicles provide the best enhancement of drug transport. van den Bergh, Bouwstra et al. (1999) studied the interaction of L-595:PEG-8-L elastic and rigid vesicles with hairless mouse skin both in vivo and in vitro and examined skin ultrastructure by TEM and histology. They found lamellar stacks in the intercellular lipid spaces after treatment with elastic vesicles, a feature not observed after treatment with rigid vesicles. Van den Bergh, Vroom et al. (1999) performed vesicle-skin interaction using TEM, FFEM and two-photon excitation microscopy. They showed that after treatment of human skin with L-595:PEG-8-L vesicles, the transport of lipophilic fluorescent marker was via a fine meshwork of thread-like channels with increased permeation of the marker. On the contrary, there was a homogeneous intercellular

penetration of the marker after treatment with Wasag-7 rigid vesicles or with PEG-8-L micelles. In contrast to the study of Cevc and Bloom (1992), both studies did not show any evidence that elastic vesicles could penetrate through the stratum corneum. Honeywell-Nguyen, de Graaff et al. (2002) investigated both in vitro and in vivo interaction of L595:PEG-8-L elastic vesicles with human skin using tape stripping and freeze fracture electron microscopy. They found a fast penetration of intact elastic vesicles into the deeper layer of stratum corneum, where these vesicles accumulated in channel-like regions. No ultrastructural change was found in skin treated with rigid vesicles. Treating with micelle resulted in rough, irregular fracture planes. Also, there was no evidence to suggest that elastic material could penetrate beyond the stratum corneum in to the viable epidermis in large quantity.

From these above studies, the condition used was non-occlusion. It implies that elastic vesicles improve skin drug delivery or induce ultrastructural changes of skin under non-occlusive condition. Cevc and Bloom (1992) reported that Transfersome® is an ultradeformable vesicle that can penetrate though intact skin only when applied under non-occlusive condition. Phospholipids have a tendency to avoid any dry surroundings. Thus, it will partially dehydrate by evaporation when the phospholipids-based liposomes is applied on the skin under non-occlusive condition. For vesicles to remain maximally swollen under such condition, they follow the local transepidermal hydration gradient between skin surface and deeper skin layer. Vesicles can only do this if they are ultradeformable vesicles like Transfersome®. In this condition, traditional liposomes will adsorp, completely dehydrate, and fuse on skin surface. Accordingly, occlusion is believed to abolish the natural hydration gradient in the skin and should inhibit the action of deformable vesicles. There are some reports on the studies using occlusive condition compared with non-occlusive condition to elucidate which condition would be suitable for elastic vesicles. El Maghraby et al. (2001) investigated human skin delivery of estradiol from ultradeformable vesicles containing soybean PC and edge activators such as sodium cholate, Tween® 80, and Span® 80 and different state traditional liposomes and compared between occlusive and non-occlusive conditions. They reported that under non-occlusive condition both ultradeformable and traditional liposomes improved

estradiol skin delivery but ultradeformable liposomes were superior. Occlusive condition reduced skin delivery of both vesicles. Honeywell-Nguyen and Bouwstra (2003) compared pergolide skin penetration from L-595:PEG-8-L elastic vesicles under occlusive and non-occlusive conditions across human skin. They concluded that non-occlusive condition improved the skin delivery of pergolide compared to buffer control. Occlusion increased drug transport from both vesicles as well as buffer solution due to the fact that water is an excellent penetration enhancer. However, the action of elastic vesicles themselves was diminished under occlusion because it showed a lower flux compared with buffer control. In addition, Honeywell-Nguyen, Groenink et al. (2003) investigated the in vivo interaction of L-595:PEG-8-L elastic vesicles with human skin using the tape stripping in combination with freeze-fracture electron microscopy method. They found a fast penetration of intact elastic vesicles into the stratum corneum via channel-like regions after non-occlusive treatment. Although micrographs showed very few intact vesicles in the deeper layers of the stratum corneum, the presence of lipid plaques was frequently observed after occlusion condition. The results from the present study agree with the other previous studies. From the results of the previous and present studies, non-occlusion should be a suitable condition for the elastic vesicles. These results support the hypothesis for L-595:PEG-8-L elastic vesicles that the vesicles improve skin drug delivery by penetrating through the stratum corneum under non-occlusive condition due to the transepidermal osmotic gradient.

Occlusion has been reported to increase percutaneous absorption of various other topically applied compounds. On the contrary, it is essential that liposomes undergo significant dehydration under non-occlusive condition so as to be effective. The bulk aqueous medium in the vesicular suspension in this present study was about 90 % of the formulation because the lipid concentration was kept at 100 mg/mL. Thus, without a high degree of dehydration, no advantages over a simple aqueous solution could be obtained from vesicular systems. Two factors that control the extent of dehydration of a vesicular suspension are phase transition temperature and the presence of humectants (Touitou et al., 1994; Dowton et al., 1993). Ohta et al. (1996) investigated influence of formulation type on the deposition of glycolic acid and glycerol in hairless mouse skin in vivo. They

concluded that glycerol affected skin deposition of glycolic acid by slowing down dehydration of the formulation containing GDL:CHO:Brij® 76 (57:15:28 by weight). Therefore, PTU permeation from Span® 40 under non-occlusive condition was higher than that under occlusive condition when the aqueous control was brought into account.

From all the studies to elucidate the dominating mechanism of PTU vesicles to improve PTU permeation, increased drug thermodynamic activity and the "free drug" mechanism did not operate on PTU permeation because there was no correlation between PTU entrapment and release rate constant and all permeation parameters. The mechanism of action of PTU vesicles depended on the component of vesicles.

For GDS system, penetration enhancement effect of the component might play an important role because pretreatment with GDS empty vesicles clearly increased PTU permeation from solution in buffer. However, % PTU permeation from GDS vesicles was much higher than that of the physical mixture in 90% propylene glycol in water. Thus, another mechanism for GDS system might involve vesicle-skin transfer because direct skin contact with GDS vesicles was essential.

The dominating mechanism of action for Span® 20 and Span® 40 vesicles might include penetration enhancing process and vesicle-skin transfer for two reasons:

1) PTU permeation was a little increased after skin pretreatment with empty vesicles. Thus, the improved PTU penetration by Span® 20 and Span® 40 vesicles might not be caused solely by the penetration enhancing effect of the main surfactant components.

2) PTU permeation from PTU vesicles was significantly higher than that from pretreatment. Therefore, the optimal drug transport could be achieved only when drug molecules were applied together with the vesicles. This also suggests that the penetration enhancing mechanism was not the most predominant mechanism responsible for the increased PTU transport from Span® 20 and Span® 40 vesicles. PTU vesicles clearly increased % PTU permeation compared with the physical mixtures in 90% propylene glycol in water. From these results, another mechanism might be vesicle-skin

transfer because it was essential to entrap PTU into the vesicles so as to improve PTU permeation.

The results of the present study are also suggestive of a mechanism for L-595 system as a drug carrier system for these reasons:

1) Pretreatment showed no difference from aqueous control, whereas PTU niosomes clearly enhanced PTU permeation. Thus, the improved PTU permeation by the elastic vesicles was not solely caused by the penetration enhancing properties of the surfactant components.

2) PTU vesicles significantly enhanced PTU permeation. PTU permeation from L-595 vesicles was higher than that from solution in 90% propylene glycol in water. Therefore, the most predominant mechanism responsible for the increased PTU transport was vesicle-skin transfer.

3) PTU vesicles significantly enhanced PTU permeation under non-occlusive condition compared to occlusive condition. Thus, it implies that L-595:PEG-8-L elastic vesicles penetrated through the stratum corneum using transepidermal osmotic gradient.