CHAPTER IV

RESULTS AND DISCUSSION

1. Solubility of PTU

The solubilities of PTU in three different buffers: citrate buffer pH 5.5, HEPES buffer pH 7.4, and borate buffer pH 9.0 were 0.8 mg/ml, 1.1 mg/ml, and 1.8 mg/ml, respectively. The complete solubility data are shown in Table 1. These saturation concentrations were used in subsequent studies.

Table 1. The solubility data of PTU in three different buffers.

day	Solubility of PTU (mg/ml)		
	pH 5.5	pH 7.4	pH 9.0
3	0.80	1.08	1.95
6	0.82	1.05	1.88
9	0.85	1.22	1.82
12	0.80	1.12	1.86

2. Effects of composition and process variables on PTU entrapment

2.1 Effects of preparation method, lipid concentration, and equilibrating time

Three methods of preparation were compared to select the most appropriate method for PTU liposomal preparation. Since PTU does not have high solubility in lipid, hydration of the lipid phase with a large amount of water resulted in partitioning of the drug into the aqueous phase due to the partial solubility of the drug in water. To reduce partitioning of the drug into the aqueous phase, hydration was performed with a saturated solution of PTU (Supawadee,

1998). The organic PTU method represented the situation where PTU would be allowed to incorporate into the bilayer before partitioning took place. The encapsulation efficiency of PTU liposomes prepared by the aqueous PTU method, in which PC without PTU was dissolved in chloroform and PTU was dissolved in HEPES buffer pH 7.4 before adding to the organic phase, was compared to the first method to investigate whether PTU could partition from aqueous phase to lipid phase as it does from the lipid phase to the aqueous phase. Results in Tables 2 and 3 show that the concentration of lipid in system affected the entrapment. Interactions are seen with concentration, equilibrating time, and the method used.

In an attempt to enhance the entrapment of the drug, the organic-aqueous PTU method was performed. The method was expected to yield the highest PTU entrapment due to its saturation in both lipid and aqueous phases. The encapsulation efficiency and the statistical analysis are shown in Tables 4 and 5, respectively. The complete data of these experiments are in Appendix D.

When the PTU used was not at its saturation in both phases, entrapment of PTU in the liposomal systems depended largely on the lipid concentration ($p < 0.05$). Preparation method or equilibrating time by itself did not have any significant effect on PTU entrapment. Interaction effects between these two factors might play some roles. However, when PTU encapsulation is to be predicted, interaction effects among lipid concentration, preparation method, and equilibrating time must be the prime consideration.

High entrapment was observed immediately after preparation by organic PTU method. Some part of the drug in the bilayer, during storage, was likely to partition into the aqueous phase (Figure 8a). This caused a reduction in PTU entrapment after one week of storage. On the other hand, preparation of liposomes by aqueous PTU method gave a low entrapment after preparation (Figure 8b). This probably due to unsaturation of the drug in the bilayer. Upon allowing partitioning to take place, an increase in entrapment occurred. This finding is the result of further partitioning of PTU from aqueous phase into bilayer.

Table 2: Effects of preparation method, lipid concentration, and equilibrating time on encapsulation efficiency of PTU liposomes. Data are shown as mean ± SD.

PC conc. (mg/ml)	Encapsulation efficiency (mmol PTU/mol PC)			
	Organic PTU method		Aqueous PTU method	
	0 wk	1 wk	0 wk	1wk
10	78.64±11.06	67.40±7.61	60.95±8.62	80.47±1.00
100	37.36±5.09	39.85±5.70	40.82±1.16	45.11±3.56

Organic PTU method : Total PTU in the system was 1.1 mg with PC 10 and 100 mg/ml

Aqueous PTU method : Total PTU in the system was 1.1 mg with PC 10 and 100 mg/ml

Table 3: Interactions of preparation method, lipid concentration, and equilibrating time on encapsulation efficiency of PTU liposomes.

Source of variation	Sum of squares	DF	Mean square	F-Ratio	P-value
Error	656.335	16	41.021		
Concentration	5795.488	1	5795.488	141.281	.000
Time	85.089	1	85.089	2.074	.169
Method	6.314	1	6.314	.154	.700
Concentration-time	.840	1	.840	.020	.888
Concentration-method	66.500	1	66.500	1.621	.221
Time-method	397.639	1	397.639	9.694	.007
Concentration-time-method	314.433	1	314.433	7.665	.014
Model	6666.303	7	952.329	23.216	.000
Total	7322.638	23			

R-squared = 0.910

Adjusted R-Squared = 0.871

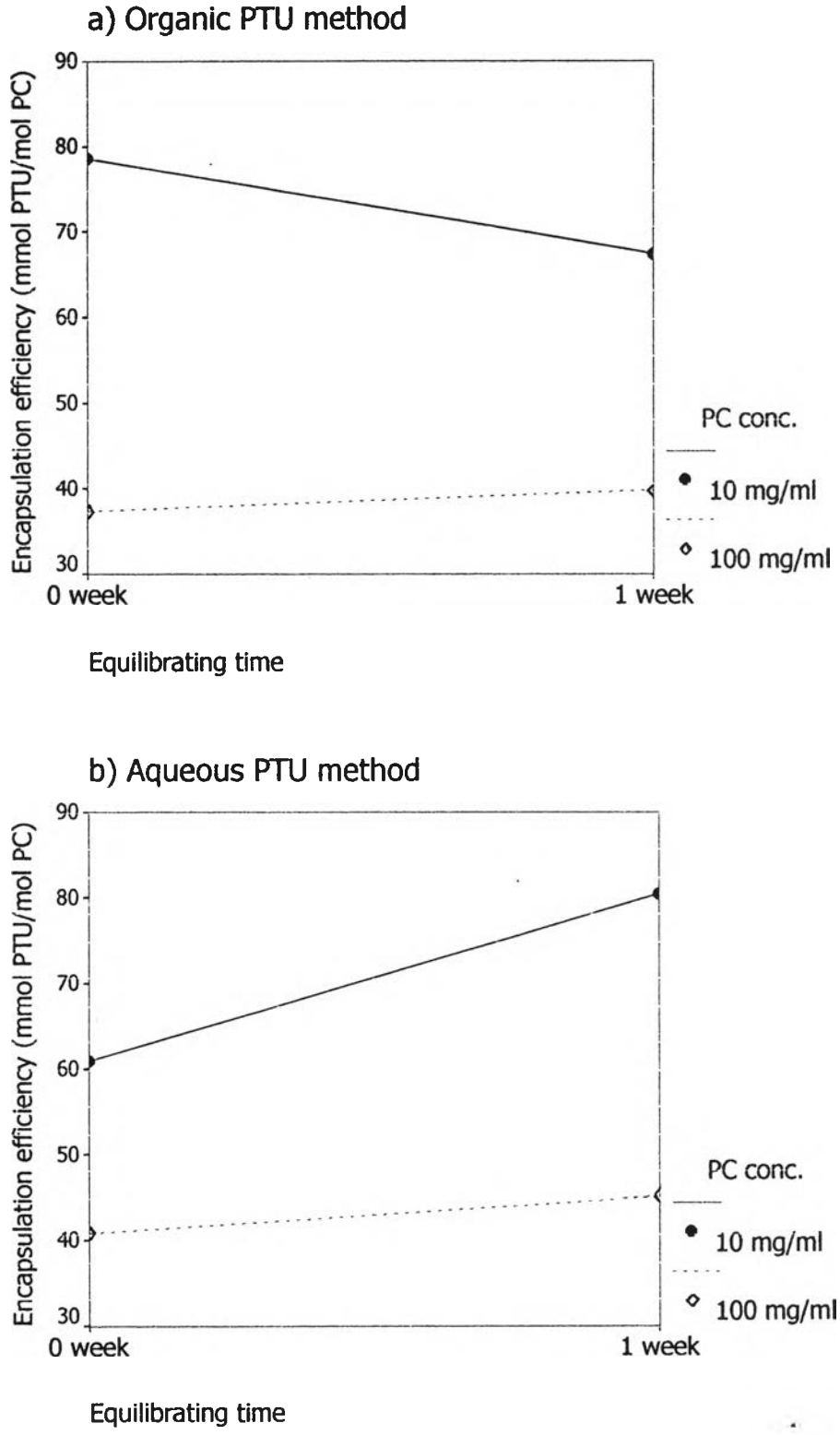


Figure 8: Interactions of preparation method, lipid concentration, and equilibrating time on encapsulation efficiency of PTU liposomes.

Table 4: Encapsulation efficiency of PTU liposomes prepared by organic-aqueous PTU method. Data are shown as mean ± SD.

PC conc. (mg/ml)	Encapsulation efficiency (mmol PTU/mol PC)	
	Organic-Aqueous PTU method	
	0 wk	1wk
10	70.82±4.18	88.37±7.75
100	85.55±8.62	89.18±7.39

Organic-Aqueous PTU method : Total PTU in the system was 1.5 (0.4 mg in organic phase and 1.1 mg in aqueous phase) or 2.5 mg (1.4 mg in organic phase and 1.1 mg in aqueous phase) when using PC 10 and 100 mg/ml, respectively

Table 5: Interactions of preparation method, lipid concentration, and equilibrating time on encapsulation efficiency of PTU liposomes prepared by organic-aqueous PTU method.

Source of variation	Sum of squares	DF	Mean square	F-Ratio	P-value
Error	412.918	8			
Concentration	180.808	1	180.808	3.503	.098
Time	336.233	1	336.233	6.514	.034
Concentration-time	145.325	1	145.325	2.816	.132
Model	662.365	3	220.788	4.278	.044
Total	1075.284	11			

The effect of equilibrating time was not as dramatic when the lipid concentration was ten-fold increased. However, the encapsulation efficiency was much reduced (see Figures 8a and 8b). This indicates that the aqueous core of the vesicle did not proportionately increase as the lipid quantity was increased. The REV method used in this study is expected to give large unilamellar liposomes. However, excess lipid is known to produce oligolamellar or multilamellar vesicles when the REV method is used (New, 1990). These vesicles have much smaller aqueous cores than do large unilamellar vesicles (Weiner, Martin, and Riaz, 1989). Thus, the reduction in PTU entrapment could be the result of changes in liposomal structure.

When the organic-aqueous PTU method was used, the amount of PTU was adjusted for the different amount of lipid used. When PC concentration was 10 mg/ml, the maximum PTU dissolved in organic phase was 0.4 mg/ml of the final preparation. When the PC concentration was 100 mg/ml, the maximum PTU dissolved in organic phase was 1.4 mg/ml of the final preparation. These concentrations were experimentally determined as described under 2.1 in Materials and Methods. Thus, the total amounts of PTU in the systems prepared by organic-aqueous PTU method with 10 mg/ml and 100 mg/ml PC were 1.5 and 2.5 mg/ml of the final preparation, respectively.

In contrast to the systems with unsaturation of PTU, lipid concentration did not have any significant effects on encapsulation efficiency of the drug. The equilibrating time might have some effects on PTU entrapment, especially when low lipid concentration was used (see Table 4). This would not expected if saturation took place in both phases. However, the temperature used in liposomal preparation was higher than the storage temperature (35 °C versus 4-8 °C). At this higher temperature, solubility of PTU in the aqueous phase should increase, allowing some partitioning to take place. This was more obvious at low lipid concentration where higher fraction of the drug was in the aqueous phase. The organic-aqueous PTU method also gave the highest PTU encapsulation efficiency as expected. Therefore, this method was selected for further studies. Although lipid concentration did not influence the encapsulation efficiency of liposomes prepared by the organic-aqueous PTU method, PC concentration of 100 mg/ml was selected to minimize time and organic solvent required for preparation of liposomes.

2.2 Effects of liposomal surface charge, pH, and cholesterol content

Fifty four experiments (18 treatments x 3 runs) were carried out according to the factorial design in order to describe the encapsulation of PTU in REV liposomes under different conditions of surface charge, pH, and cholesterol content. All liposomal formulations in this study were prepared with PTU

saturating in both the organic phase and the aqueous phase. The saturated concentration in the aqueous phase varied upon pH (see above). The total lipid amount was considered similar in terms of thermodynamic activity. Thus, it was not treated as another variable. Table 6 presents the average values (mean $\pm$ SD) of encapsulation efficiency expressed as mmol of PTU/mol of total lipid. (Raw data are shown in Appendix E.) Encapsulation efficiencies obtained range from 26.94 to 98.40 mmol PTU/mol lipid. The highest value was achieved with negatively charged liposomes at pH 7.4. Contrarily, the lowest values correspond to liposomes with cholesterol at pH 5.5, regardless of charge.

From the contingency table of the ANOVA analysis (Table 7), pH and cholesterol content had some effects on PTU entrapment. Both two-way and three-way interactions between all factors studied are seen. These interactions are clearly shown in Figure 9.

The entrapment of PTU in neutral liposomes, composed of PC alone, did not show any difference among the three different pH values. While the negatively charged liposomes, composed of PC/DCP, gave a higher encapsulation at pH 7.4 than at pH 5.5 and at pH 9.0. Based upon the pK_a of PTU which is equal to 8.3 at 20 °C (Reynolds, 1993), it can be deduced that PTU is presented mostly in the unionized molecular form at pH 5.5, while at pH 7.4 the percentage of ionized form of the drug may increase. Neutral or negatively charged bilayers are not likely to interact with unionized PTU at pH 5.5 via any ionic interaction. Incorporation of PTU into the bilayer was limited to a certain extent according to the optimized hydrophobic interaction between the drug and the lipid molecules. However, increased encapsulation was seen at pH 7.4 since the concentration of the drug in the aqueous phase was higher at that pH than at pH 5.5. The increased encapsulation was the result of increased amount of drug entrapped in the aqueous core of the vesicles rather than association of the drug to the bilayer.

On the contrary, when negatively charged liposomes were hydrated with borate buffer pH 9.0, PTU encapsulated in liposomes decreased. It is very likely

that most of PTU at this pH was present in the ionized form with a negative charge. Although aqueous solubility of PTU at pH 9.0 is increased, the encapsulation of PTU in negatively charged liposomes was significantly lower at pH 9.0 than at pH 7.4. This indicates that the fraction of PTU associated with the bilayer was much reduced according to the lower fraction of unionized PTU at pH 9.0. The same scenario might also apply to neutral liposomes. The neutral liposomal bilayer would not assume any net charge at pH 9.0. However, the smaller fraction of PTU molecules present in the unionized form resulted in fewer PTU molecules within the bilayer. Thus, the entrapment at pH 9.0 remained almost unchanged, despite the increased concentration of the drug in the aqueous core.

The different degree of electrostatic interactions may explain the difference in encapsulation seen with liposomes formed by positively charged lipid (PC/SA). Stearylamine, with a pK_a of 10.6 (Klang et al., 1994), has a net positive charge in the range of pH studied. The increased extent of the ionized form of the drug with pH resulted in increased electrostatic interactions with the positively charged lipid bilayer. Such attractive interaction allowed more association of the drug molecules with the bilayer. Thus, the highest encapsulation of PTU in positively charged liposomes was obtained at pH 9.0. The encapsulation at this pH was the result of both the interaction of the ionized drug with the opposite charges on the bilayer and the increase in PTU concentration in the aqueous core. The reason that the encapsulation at pH 7.4 (89.3% unionized) did not increase from pH 5.5 (99.8% unionized) remains unclear. Some increase in encapsulation was expected, though the change would not have been as dramatic as what was seen from pH 7.4 to pH 9.0 (16.6% unionized). However, other factors may also play some roles in drug entrapment in liposomes. Besides pH of the medium and degree of ionization of the drug, vesicle size may play an important role (Sharma, 1997). However, this experiment did not allow evaluation of liposomal size. Some polydispersity of liposomal vesicles was also evident from the results of morphology study below (Figure 10).

Table 6: Effects of liposomal charge, pH, and cholesterol content on encapsulation efficiency of PTU liposomes. Data are shown in mean ± SD.

Composition	Encapsulation Efficiency (mmolPTU/mol total lipid)					
	With Cholesterol			Without Cholesterol		
pH	5.5	7.4	9.0	5.5	7.4	9.0
Neutral (PC)	28.51±2.23	28.60±4.69	47.63±4.16	77.07±5.33	85.55±8.62	75.05±7.73
Positive (PC/SA)	32.43±1.73	46.19±2.95	61.76±2.25	59.74±2.18	60.41±20.51	97.08±7.62
Negative (PC/DCP)	26.94±4.28	33.21±4.16	42.24±5.64	64.66±12.64	98.40±14.08	71.07±2.90

Table 7: Interactions of liposomal charge, pH, and cholesterol content on encapsulation efficiency of PTU liposomes.

Source of variation	Sum of squares	DF	Mean square	F-Ratio	P-value
Error	2282.854	36	63.413		
Charge	118.263	2	59.132	.932	.403
Cholesterol	19438.148	1	19438.148	306.534	.000
PH	2817.059	2	1408.530	22.212	.000
Charge-cholesterol	1026.995	2	513.497	8.098	.001
Charge-pH	2297.275	4	574.319	9.057	.000
Cholesterol-pH	501.417	2	250.708	3.954	.028
Charge-cholesterol-pH	1611.536	4	402.884	6.353	.001
Model	27810.692	17	1635.923	25.798	.000
Total	30093.546	53			

R-squared = 0.924

Adjusted R-Squared = 0.888

Incorporation of cholesterol into lipid bilayers resulted in reduction of PTU encapsulated in liposomes (see Table 6 and Figure 9). The results from the experiment 2.1 indicates that PTU was in equilibrium in both hydrophilic (the aqueous core) and hydrophobic (the lipid bilayer) parts of liposomes. Cholesterol molecules fill in the hydrophobic region of bilayer (Weiner, Martin and Riaz, 1989). The capacity to entrap unionized form of the drug in this region of bilayer is likely to decrease in the presence of cholesterol molecules. Similar results have

been reported with procaine hydrochloride (Hatfield and Fung, 1999) and anthracycline derivatives (Frezard and Garnier-Suillerot, 1998). In contrast, cholesterol strongly increases the entrapment of hydrophilic spin probe (ASL) in liposomes (Vrhovnik et al., 1998), where cholesterol prevents leakage of the probe from the aqueous core.

From the encapsulation efficiency studies, four formulations of PTU liposomes: PC, pH 7.4, PC/DCP, pH 7.4, PC/CH/DCP, pH 7.4, and PC/SA, pH 9.0 were selected for further studies on the release behavior, the water evaporation, and the stability. The selected formulations possessed three different charges with the apparently highest encapsulation efficiency of each surface charge. In addition, to investigate the influence of cholesterol, a formulation with cholesterol (PC/CH/DCP, pH 7.4) was also included.

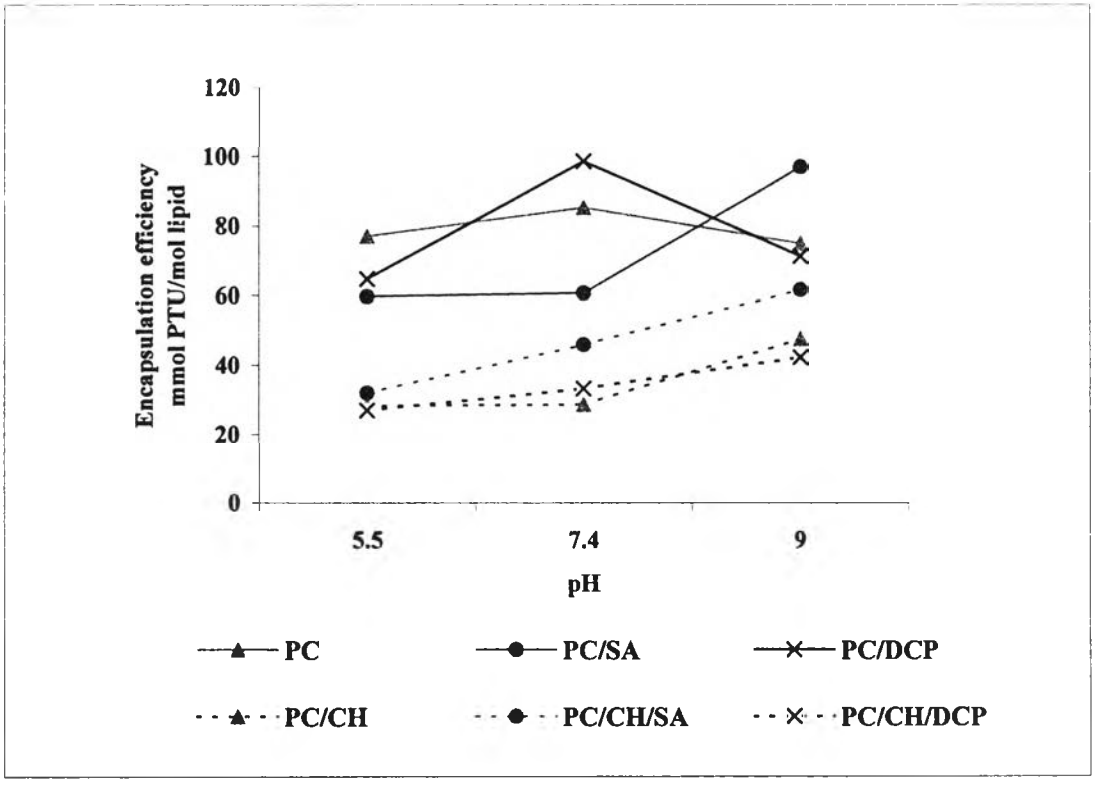


Figure 9: Interactions of liposomal charge, pH, and cholesterol content on encapsulation efficiency of PTU liposomes.

3. Morphology of PTU liposomes

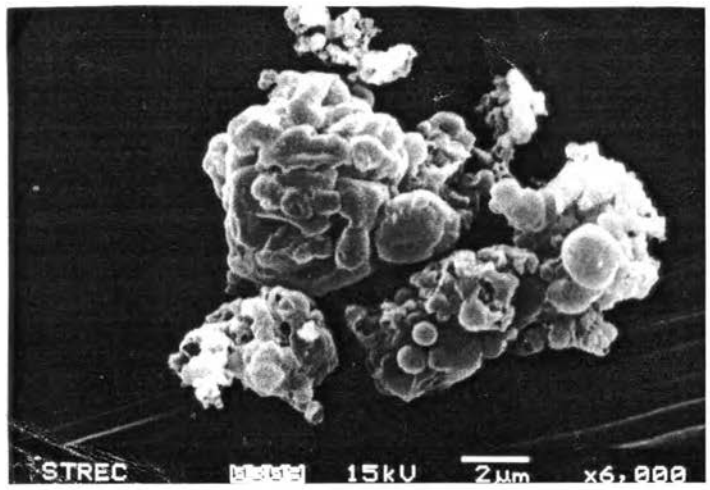
The morphology of four liposomal preparations, selected from the experiment 2.2, was studied using scanning electron microscopy. Electron micrographs show that most liposomes were spherical in shape (Figure 10). Though the particle size of liposomes observed from scanning electron micrographs was mostly smaller than 1 μm, all liposomal preparations were not homogeneous in size. Neutral vesicles aggregated (see figure 10a), while charged liposomes did not suffer such severe aggregation. Electrostatic charges in the bilayer prevent close contact of liposomal vesicles and, hence, aggregation did not take place (Betageri, Jenkins, and Parsons, 1993).

4. Drug Release studies

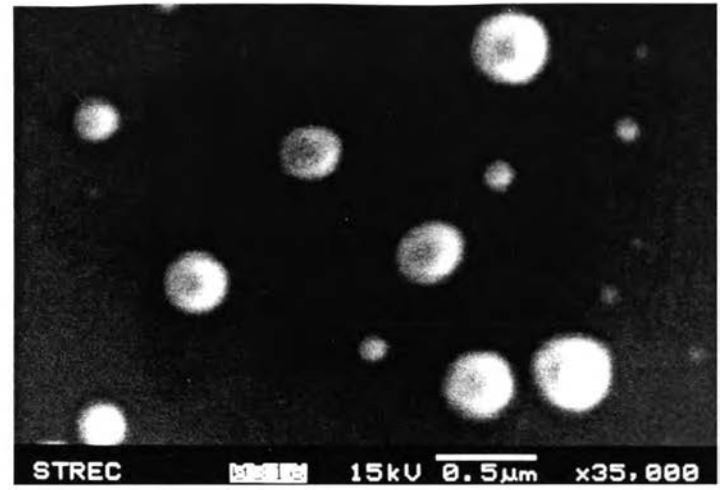
In vitro drug release is generally used in evaluation of drug delivery from topical formulations. The result from such experiment can predict the behavior of drug release to the skin in vivo (Wester and Maibach, 1990).

In this study, four formulations of PTU liposomes: PC, pH 7.4, PC/DCP, pH 7.4, PC/CH/DCP, pH 7.4, and PC/SA, pH 9.0 were selected. The saturated aqueous solution of PTU in borate buffer pH 9.0 was also studied for comparison. The release of PTU was studied using systems saturated with PTU in order to ascertain equivalent thermodynamic activity in all formulations. The data of standard calibration curve prepared for each analysis of PTU are shown in Appendix F. A blank system of similar liposomal composition but without incorporation of PTU was similarly processed. The UV spectra, shown in Appendix F, indicate that there was no interference for the analysis of PTU released from each carrier. HEPES buffer pH 7.4 or borate buffer pH 9.0 was used as a receptor fluid, according to the composition of PTU liposomes being tested.

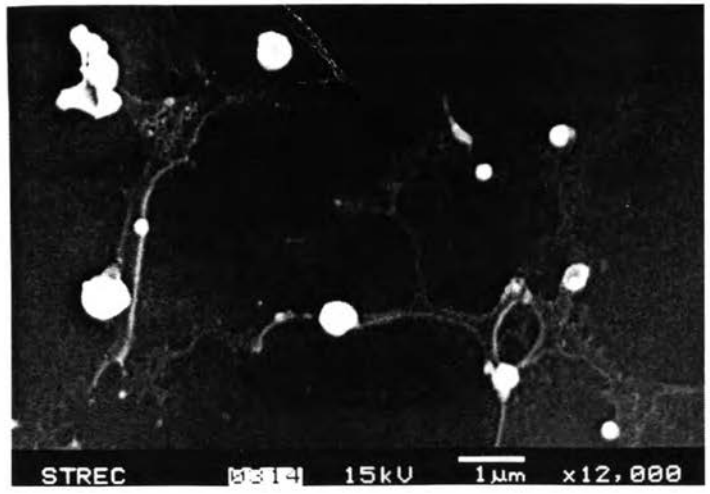
The release profiles of PTU from liposomal systems and from solution are illustrated in Figure 11. The complete release data are in Appendix F. The release of PTU from solution was nearly complete (> 90%) within 5 hours. Liposomes gradually released encapsulated PTU during 24 hours. The cumulative amount of PTU released at 48 hours



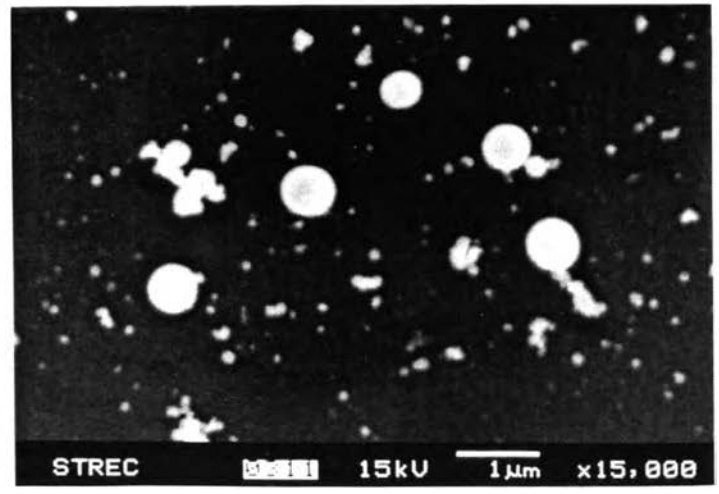
(a) PC



(b) PC/DCP



(c) PC/CH/DCP



(d) PC/SA

Figure 10: Morphology of PTU liposomes determined by scanning electron microscopy at 1-2 months after preparation.

was also determined to monitor whether PTU was completely released from liposomes. The release of PTU was about 70% of the drug content from all liposomal systems.

After the experiment was terminated at 48 hours, the physical appearance of liposomal samples in the donor compartment was observed. A long duration of experiment that the liposomes were exposed to a high temperature of diffusion cells may cause some changes in liposomal structure, influencing PTU release from the lipid vesicles. In this study, the liposomal pellets from centrifugation of 1.5 gram of liposomal suspension was added onto the donor side of the diffusion cells. This amount of liposomal pellets was more than that required to cover the surface area of membrane. Supawadee (1998) suggested that during the release experiment the composition of the system had been changed due to the water from the receptor compartment diffusing through the membrane. During this experiment, some changes in the physical appearance of the sample were also detected. The surface of the liposomal pellet in the donor, which was far from the membrane, seemed to deform. Some part of the liposomal mass changed from white to yellow, especially at the surface. Only the drug in the liposomes which was still properly hydrated could diffuse through the membrane to the receptor fluid. This surface dehydration might cause incomplete release of the drug. Additional explanation for the incomplete release of drugs from their vehicles was proposed by Gummer (1989). Gummer stated that with diffusion cells, it might be difficult to maintain equal pressure around the whole junction between the donor and the receptor. In this case, erroneous data may be generated when the applied dose is allowed to contact the cell joint. In this study such possibility could not be ruled out since the liposomal pellet was relatively fluidized, especially when the receptor fluid had come in contact.

The present study showed the slower PTU release from liposomal formulations than that from aqueous solution. Similar findings have been reported by Thibault and Poelman (1992) who attributed the slower retinoic acid release from liposomes to the fact that the drug did not diffuse freely, but the entrapped drug in the vesicles must diffuse through several barriers, including the membrane of adjacent vesicles. Besides, the release rate of the drug from the vehicle is inversely related to the vehicle viscosity (Bonina, et al., 1995; Kriwet and Muller-Goymann, 1995). This consideration could account for the

different drug release profiles observed from low viscosity vehicles such as aqueous solution and higher viscosity vehicles such as liposomal pellets.

Drug transport across the stratum corneum from aqueous solution or from vehicles with high effective diffusion coefficients is controlled by the stratum corneum since the diffusion resistance in the stratum corneum becomes greater than that inside the vehicle (Kriwet and Muller-Goymann, 1995). In contrast, for vehicle with a low drug release rate, such as PTU liposomes in this study, the release rate from the delivery system is the rate limiting step in drug permeation.

No significant difference in cumulative percent of PTU released after 48 hours was observed with the different liposomal formulations tested. However, the highest PTU entrapment formulation was the most appropriate formulation in terms of cost-effectiveness. At the end of the experiment, about 70% of drug content was released from all liposomal formulations. The highest cumulative PTU amount was thus received with the formulation with highest entrapment.

4. Water evaporation study

The evaporation profiles from different preparations of PTU liposomes as well as from the saturated solution of PTU are displayed in Figures 12-13. Figure 12 shows the weight loss from PTU liposomes composed of three different charges: neutral (PC), positive (PC/SA), and negative (PC/DCP) in comparison with saturated solution of PTU. Figure 13 shows water loss from PTU liposomes in the presence and absence of cholesterol. The numerical results of this study are presented in Appendix G. The saturated solution of PTU showed the highest evaporation rate due to loss of bulk water. The lower evaporation rate shown by all formulations of PTU liposomes means that water can be retained in the preparation for a longer period of time. This property is desirable for most percutaneous preparations since drug delivery will stop as soon as there is no water left to dissolve the drug (Touitou et al., 1994a).

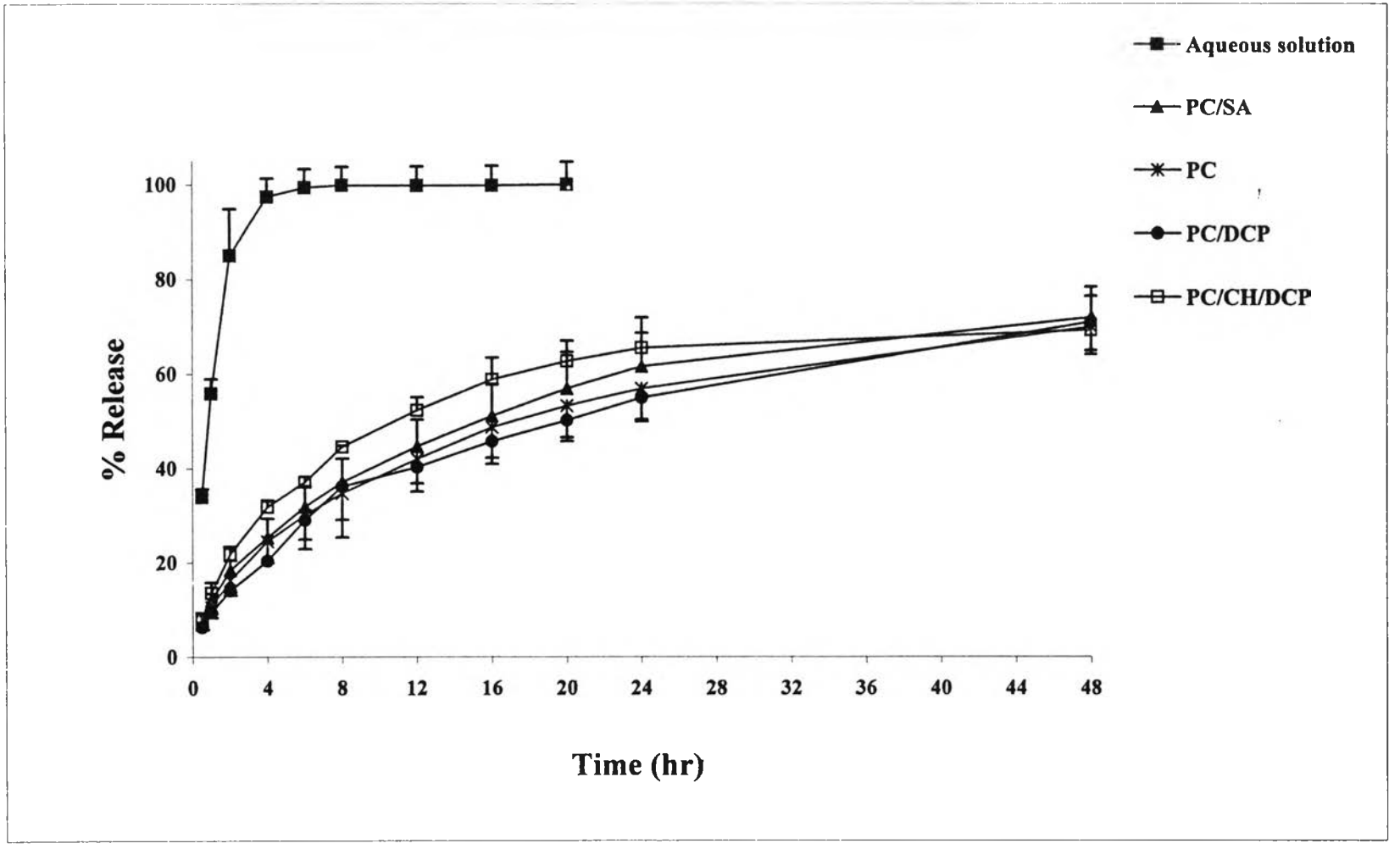


Figure 11: Release profiles of PTU liposomes and saturated PTU solution (Mean ± SEM).

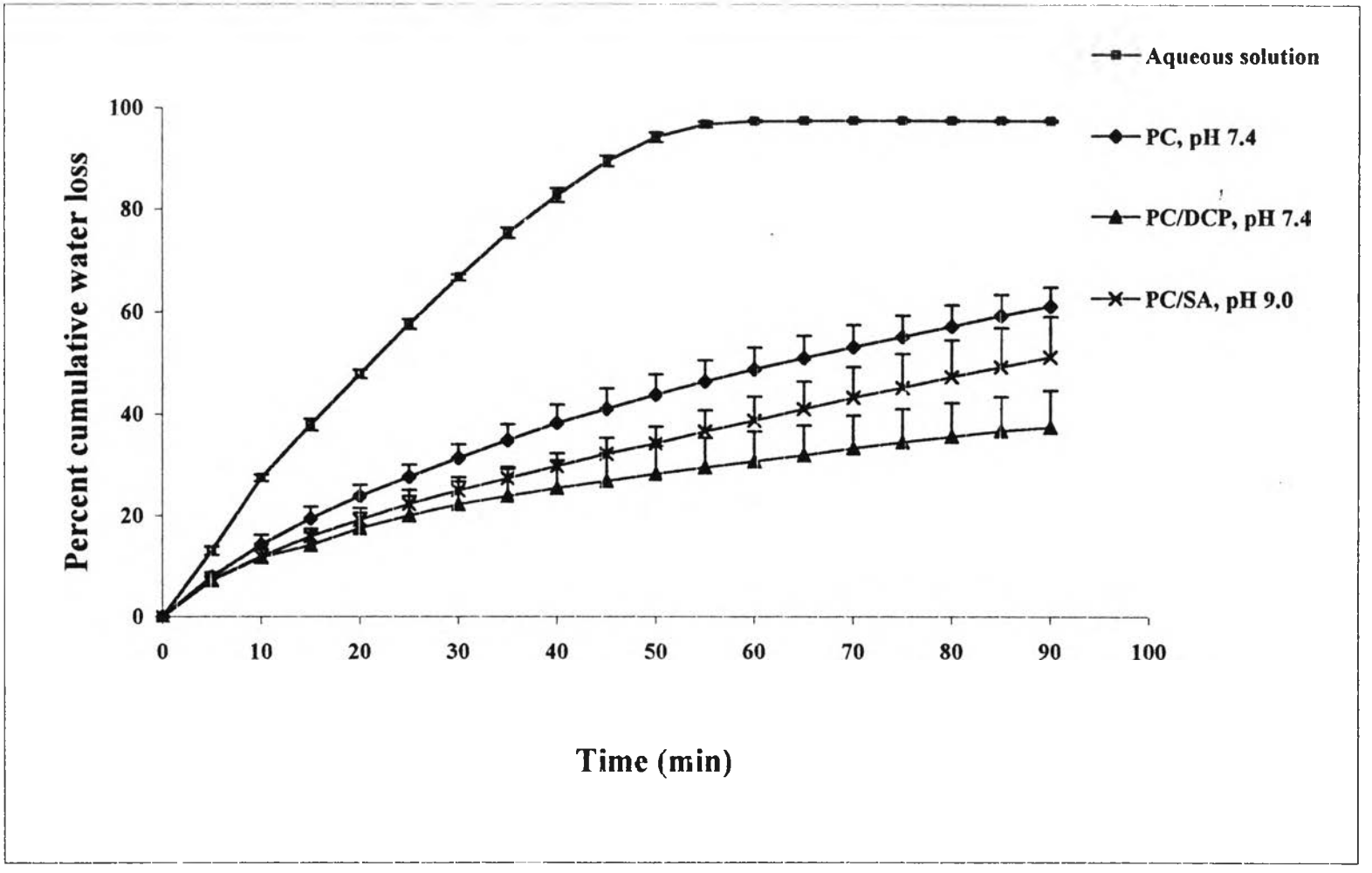


Figure 12: Water evaporation profiles of PTU liposomes and PTU saturated solution (Mean ± SEM).

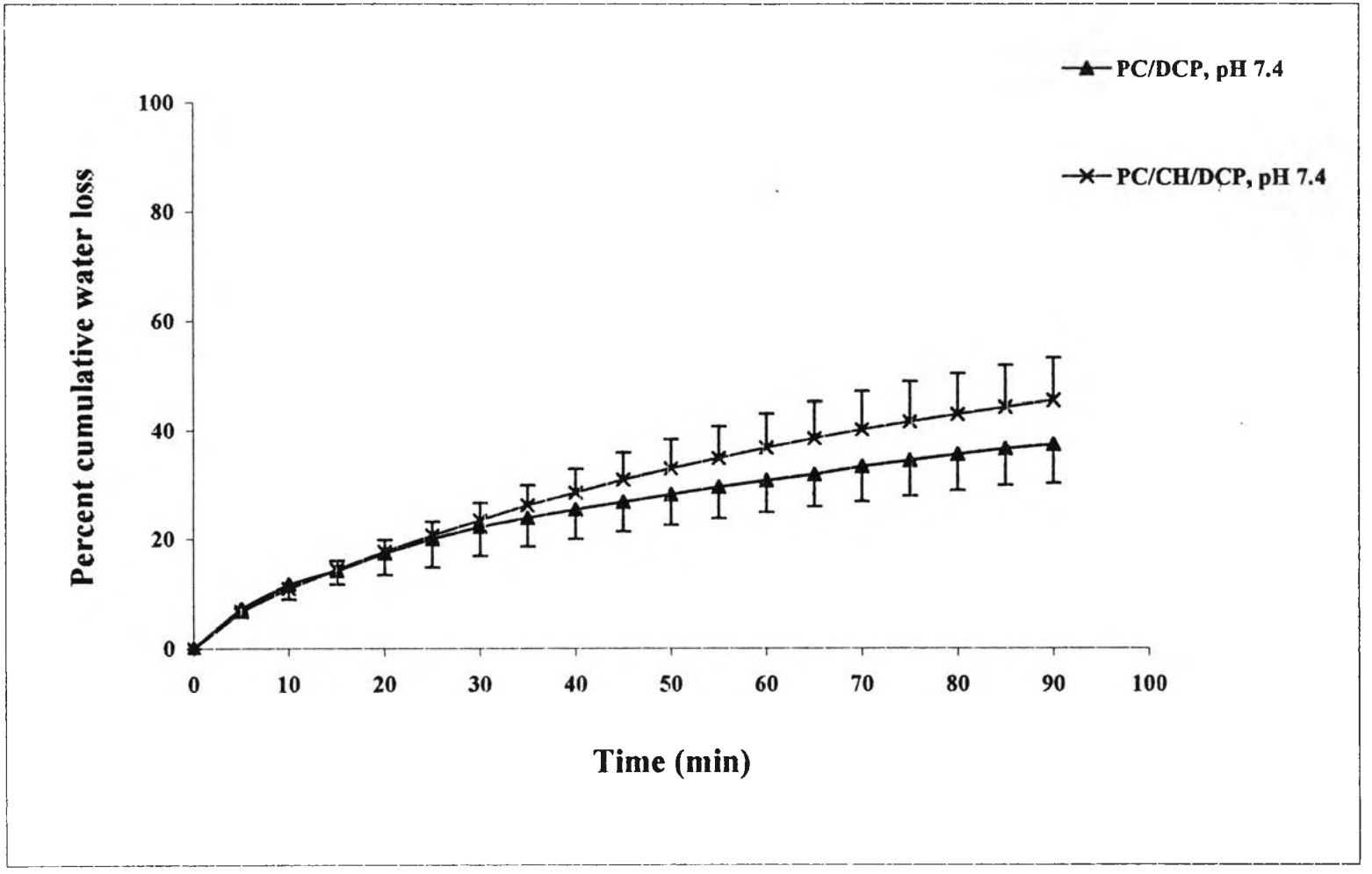


Figure 13: Water evaporation profiles of PTU liposomes in the presence and absence of cholesterol (Mean ± SEM).

When compared among differently charged liposomes, there is no statistical difference in water loss. The trend, however, is that neutral liposomes gave a higher evaporation rate than both positively and negatively charged liposomes. The presence of cholesterol in the formulation (PC/CH/DCP) showed a trend of faster evaporation rate than the formulation without cholesterol (PC/DCP) as presented in Figure 13, though there is no statistical significance. Incorporation of cholesterol into liposomal membrane known to modify the physical properties of liposomes. Fluidity of the bilayer in liquid crystalline phase is reduced when cholesterol is incorporated into liposomal membranes above their T_c (New, 1990). The bilayer in gel phase takes up less water than does the bilayer in liquid crystalline phase (Cevc, 1993). Thus, less water might be bound to the bilayer with cholesterol. The difference can not be clearly shown by these data due to the high standard deviation. Such high variation was seen because the experiment was performed with three different batches of liposomes. In addition, the experiment set-up used in this study might not allow detection of the difference caused by the composition of the vesicles. The aluminium crucibles used in this experiment provided a thick sample layer of 1.6 mm. Thus, the sample might not be thin enough to let all bulk water leave and the effect of bound water be seen in a relatively short period of time (90 min). Friberg and Kayali (1989) used similar approach to study water evaporation from stratum corneum lipid models with 0.5 mm thickness of the sample layer.

5. Biological activities of PTU liposomes

The fact that psoriasis is a chronic skin disease characterized by scaling and inflammation has led researchers to explore new approaches to control the abnormal cell proliferation. The conspicuously increased turnover of epidermis in psoriasis has diverted scientists' attention away from several hints of abnormality occurring in the dermis. Fibroblasts resident in the dermis can regulate epidermal proliferation, and fibroblasts obtained from psoriatic dermis are functionally and biochemically different from normal fibroblasts (Krueger, 1987). One of the altered properties is that psoriatic fibroblasts have an increased proliferation rate. Priestley (1983) and Priestley and Adams (1983) found that fibroblasts from psoriatic skin were hyperactive in vitro. Touitou et al. (1991) reported that theophylline markedly inhibited both normal and psoriatic dermal fibroblast

proliferation in cell cultures. Moreover, xanthine derivatives; caffeine, theophylline, and dyphylline; were shown to inhibit proliferation of normal and human psoriatic fibroblasts as well as 3T3 mouse fibroblasts (Schaffer and Touitou, 1991; Touitou et al., 1994b). Therefore, 3T3 fibroblasts appear to be an appropriate experimental system that do not require animal and human subjects for the screening of fibroblast antiproliferative drugs.

In this study, BALB/c mouse 3T3 fibroblasts were chosen to be a model system to assess the antiproliferative effect of PTU. From the reported method of study (Schaffer and Touitou, 1991; Touitou et al., 1991; Touitou et al., 1994b), cells were incubated with antiproliferative compounds for 3 days. Since phospholipids are prone to decomposition especially at high temperature, in the presence of light and oxygen (Cevc, 1993). The shorter incubation time over which the antiproliferation effect can be seen may be a better approach to minimize decomposition of liposomes. Therefore, two incubation periods, 1 day and 3 days, were compared. The results are shown in figure 14. There was no significant difference between these two incubation periods at every concentration of PTU tested ($p < 0.05$). The following experiments, therefore, were performed with the incubation time of one day.

In order to validate the method of study with previous reports (Schaffer and Touitou, 1991; Touitou et al., 1994b), comparison between the antiproliferative effect of caffeine and that of PTU was performed. Figure 15 shows that both PTU and caffeine at the same concentration of 1.1 mg/ml displayed an inhibitory effect on BALB/c 3T3 fibroblast proliferation ($p < 0.05$). It can be concluded that both PTU and caffeine effectively inhibit fibroblast proliferation.

Most cell lines grow well at pH 7.4 (Freshney, 1994). Therefore, the liposomal formulation that gave highest PTU encapsulation and was hydrated with HEPES buffer pH 7.4, PC/DCP, was selected to study the effect of PTU liposomes on proliferation of BALB/c mouse 3T3 cells. The presence of cholesterol in otherwise the same composition of anionic PTU liposomes (PC/CH/DCP) was also studied. Liposomes are known to lose their ability to retain entrapped substances when incubated with blood or plasma (Weiner, Martin and Riaz, 1989). In addition, the presence of serum in the medium can cause

alteration of the rate, extent, and mode of interaction of liposomes and cells. Thus, one would prefer using a serum-free medium in cell culture to evaluate liposomes for topical or transdermal applications. The antiproliferative effects of PTU solution (1.1 mg/ml to 1.1 µg/ml) in the presence and absence of CS in DMEM+ were determined to ensure that there was no unacceptable difference between the two media.

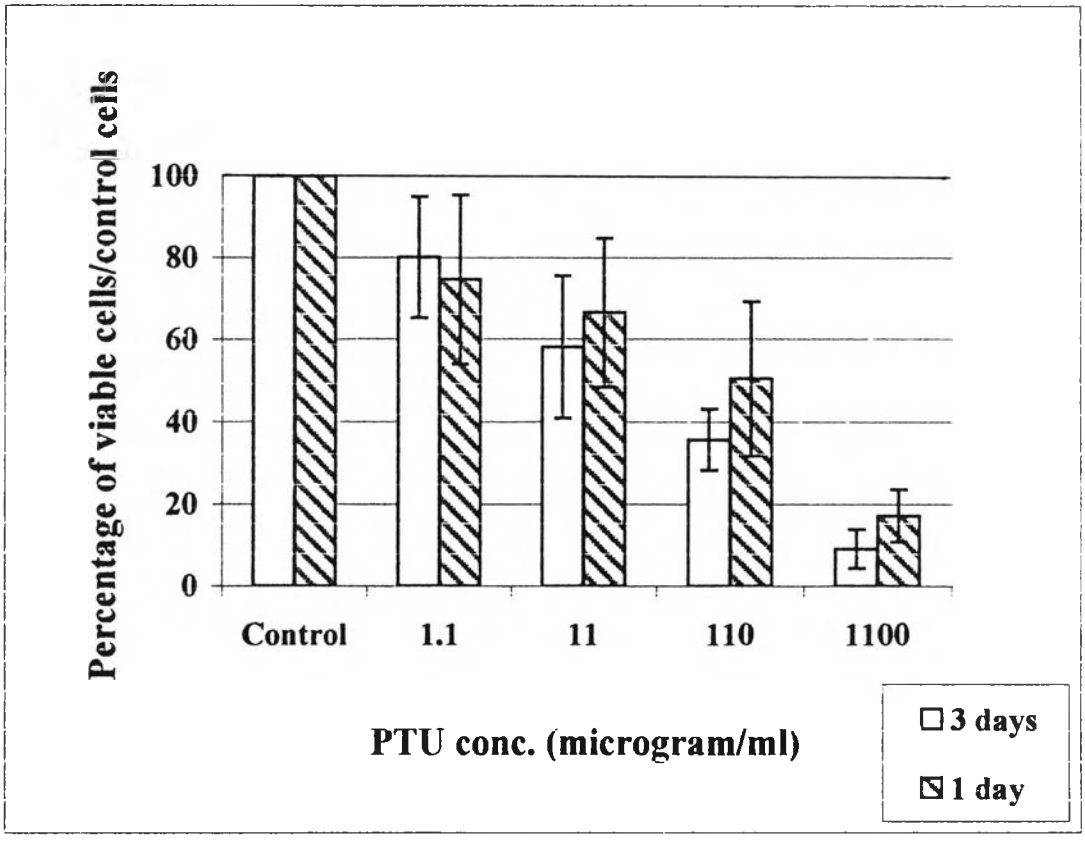


Figure 14: Effect of the incubation time on proliferation of BALB/c 3T3 fibroblasts. Data are shown as mean values (Error bar = SEM; Control = DMEM+).

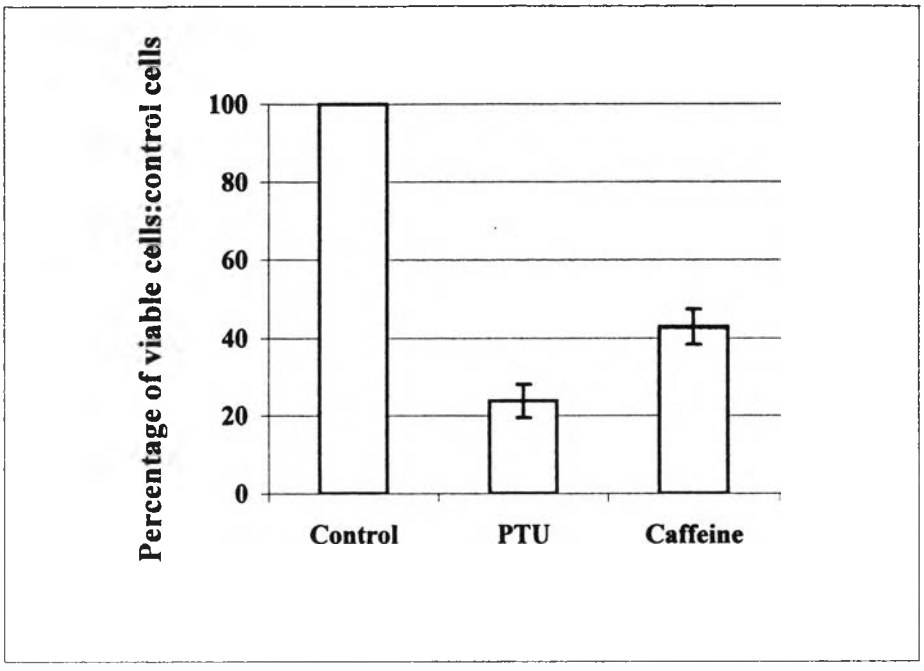


Figure 15: Effect of PTU and caffeine (1.1 mg/ml) on BALB/c 3T3 proliferation. Data are shown as mean values (Error bar = SEM; Control = DMEM+; Incubation time = 1 day).

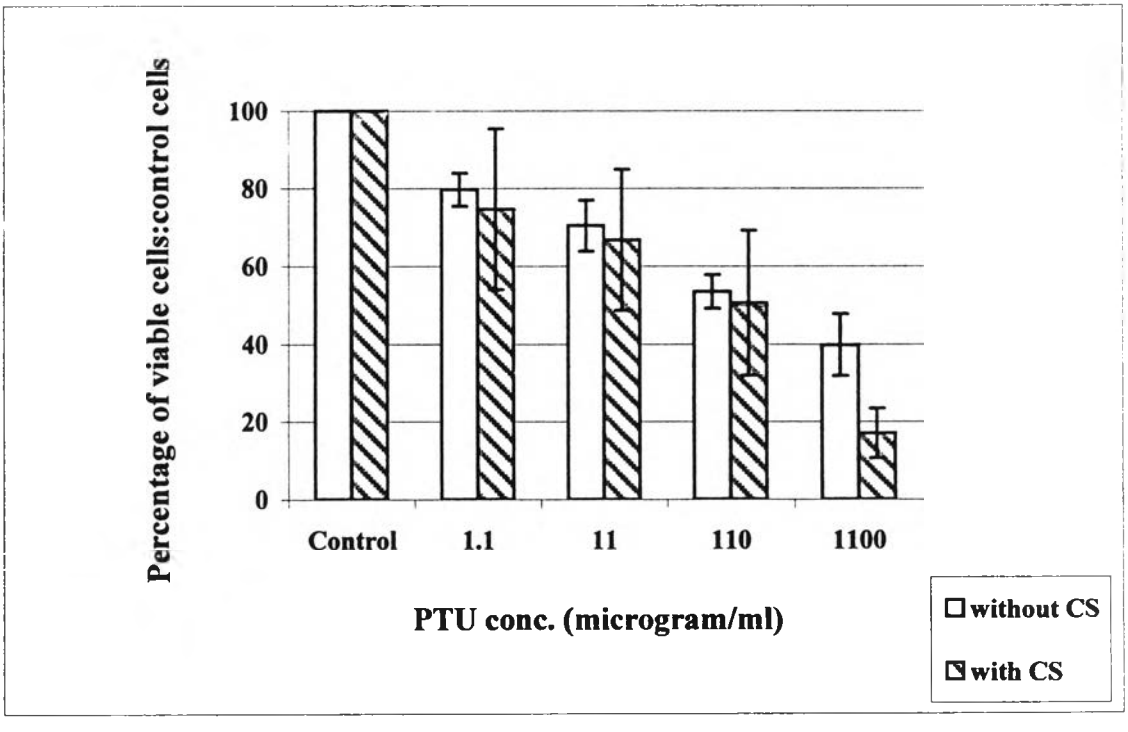


Figure 16: Effect of PTU solution in the presence and absence of CS in DMEM+ on BALB/c 3T3 proliferation. Data are shown as mean values (Error bar = SEM; Incubation time = 1 day).

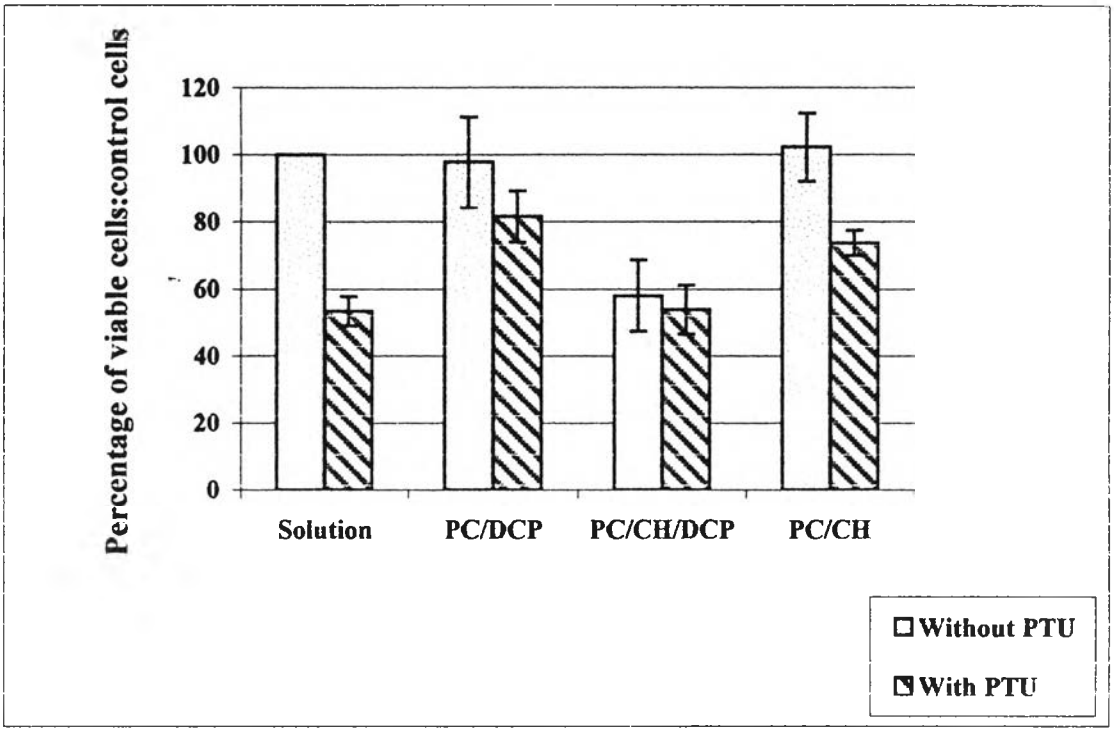


Figure 17: Effect of PTU liposomes on BALB/c 3T3 proliferation. Data are shown as mean values (Error bar = SEM; Control = DMEM+ without CS; Incubation time = 1 day).

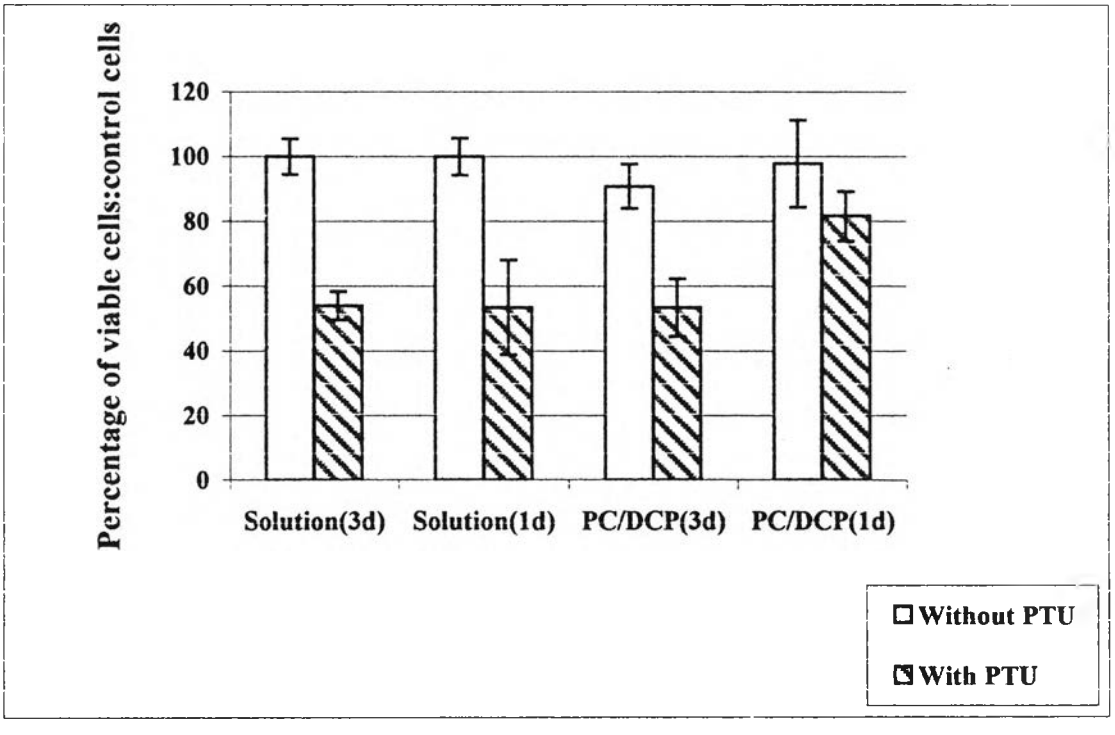


Figure 18: Effect of incubation time of 1 day and 3 days on antiproliferative effect of PTU liposomes on BALB/c 3T3 proliferation. Data are shown as mean values (Error bar = SEM; Control = DMEM+ without CS).

As illustrated in figure 16, a progressive decrease in BALB/c 3T3 cell proliferation was evident with increasing PTU concentrations, both in the presence and the absence of CS in the medium. No significant difference regarding the use of CS was seen at each PTU concentration tested. The PTU concentration of 0.11 mg/ml in the absence of CS in DMEM+ was then chosen to assess the effect of PTU liposomes on proliferation of fibroblasts. This concentration was expected to be moderate that would inhibit cell proliferation without decreasing cell counts to the point where accuracy was compromized.

Poste and Papahadjopoulos (1976) stated that vesicle surface charge and lipid fluidity, rather than vesicle size, are more important in determining the pathway of uptake into BALB/c mouse 3T3 fibroblast cells. On the other hand, Monkkonen et al. (1994) reported that a decrease in the size of liposomes by extrusion did not affect the delivery of studied compounds to RAW 264 macrophages, but drastically increased their potency for L929 fibroblasts. In this study, to eliminate the variation in vesicle size, extrusion of all liposomal formulations through 0.1 μm polycarbonate membranes was performed.

The antiproliferative effects of PTU encapsulated in liposomes and free PTU on BALB/c 3T3 cells are presented in figure 17. Percent of viable cells seen with PTU as free drug in solution was 53.70% which was significantly different from control. This inhibitory effect of free PTU was more potent than that of PC/DCP/PTU liposomes (81.63% of control). Empty PC/DCP liposomes had no effect on the proliferation of BALB/c 3T3 cells when used at the same amount of lipid as PC/DCP/PTU liposomes. Thus, the inhibition caused by PC/DCP/PTU liposomes was due to PTU, not the lipid. The number of PC/CH/DCP/PTU liposomes treated cells was decreased from control (53.98% of control). This was almost the same as what observed from PTU solution. However, a relevant intrinsic inhibitory effect of empty liposomes on cell cultured was clearly observed (see Figure 17). Therefore, it was not possible to evaluate the inhibitory effect of these PTU-encapsulating liposomes. In order to indicate the role of DCP in those liposomes on fibroblast proliferation, PC/CH/PTU liposomes and PC/CH liposomes were tested with the cells. From figure 17, PC/CH liposomes had no effect, while PC/CH/PTU liposomes inhibited cell proliferation to a similar degree as PC/DCP/PTU liposomes.

In the present study, neither PC/CH nor PC/DCP empty liposomes inhibited BALB/c 3T3 cell proliferation, but this effect was observed only in the presence of both CH and DCP in the same liposomes. This result might be explained partly by a previous study by Bajoria, Sooranna and Contractor (1997). They found that anionic liposomes composed of PC/CH/DCP (7:7:1) were internalized by human trophoblast cells in culture more avidly than neutral and cationic ones. This observation agrees with the findings of others using fibroblast and macrophage cell lines (Molnar et al., 1977; Monkkonen et al., 1993). Thus, the present study indicated direct toxicity of PC/CH/DCP liposomes on BALB/c 3T3 cells which might be the result of better liposomal uptake by the cells.

It is generally expected that the presence of cholesterol in liposomal formulations will not adversely affect their safety (Gregoriadis, 1993). However, incorporation of cholesterol into PC bilayers which results in reduction of bilayer fluidity might increase liposomal uptake to the cells. However, Li and Mitra (1996) found that charge-inducing agents, i.e., SA and DCP, can cause apparent disruption of rat pulmonary epithelial cells. Similarly, DCP was seemed to produce tissue necrosis in mouse (Gregoriadis, 1993). Hence, cholesterol seemed to enhance the antiproliferative effect of DCP-containing liposomes, probably by increasing their uptake.

Alternatively, negative charges on liposomal bilayers can also promote intracellular uptake of liposomes by target cells (Sharma, 1997). Thus, the intrinsic inhibitory effect of empty PC/CH/DCP liposomes observed in this study may be caused by cholesterol in the liposomes. This idea is supported by Mende et al. (1992) who indicated that exogenous cholesterol may be detrimental to endothelial cells. In addition, the proliferation rate of human keratinocytes in culture markedly decreased when treated with CH/Phospholipid (5:10) liposomes (Pitto et al., 1999).

However, it is noteworthy that impurities from the preparation process present in PC/CH/DCP liposomes could not be ruled out since only one batch was used. No matter what the true reason was, PC/CH/DCP formulation was considered not acceptable for further development in this study.

Earlier studies (Bajoria, Sooranna and Contractor, 1997; Fresta et al., 1995) have shown that liposomes are able to deliver many drugs into various types of cells in a greater amount than the free drug. However, in the present study free PTU is more effective than PTU liposomes in the inhibition of fibroblast proliferation when the same amount of PTU was used with the incubation period of one day. This finding is consistent with a previous study with free caffeine and caffeine liposomes, where free caffeine was more effective than liposomal caffeine (Touitou et al., 1994a). In this study, PC/DCP/PTU liposomes and PC/CH/PTU liposomes tended to be effective in inhibiting fibroblast proliferation. The lower extent in antiproliferative effect of PTU liposomes was probably associated with a sustained release characteristic of liposomes, as seen in the release study. To clarify this, PC/DCP/PTU liposomes were incubated with BALB/c 3T3 cells for a longer period of time (3 days) to allow a complete release of PTU from liposomes. As illustrated in figure 18, PTU solution showed a similar antiproliferative effect with both incubation time of 1 day and 3 days. Hence, one day was enough for PTU solution to present its complete effect but not for PTU liposomes. When incubation time was increased to 3 days, PTU liposomes, however, inhibited proliferation of fibroblasts to a similar degree as PTU solution. This result may indicate that the inhibitory effect of PC/DCP/PTU liposomes was probably caused by free PTU released from PTU-encapsulating liposomes, and not by cellular uptake. In vivo, however, conventional liposomes are not expected to come in contact with fibroblasts in the dermis; they should serve only as a method of prolonged delivery as a reservoir of drugs (Ganesan et al., 1984).

6. Physical stability of PTU liposomes

In this study, the influence of formulation factors on physical properties of liposomes, including the ability of liposomes to retain the entrapped PTU after 8 weeks of storage in a refrigerator was determined. There was no detectable changes in color of all preparations. Eventhough there was no gross precipitation of all liposomal formulations observed, neutral liposomes aggregated when inspected under an optical microscope even at 4 weeks of storage. Scanning electron micrographs (see Figure 10) also show severe aggregation of neutral liposomes. Inclusion of charged components into a particular

liposomal formulation can inhibit close approach of the vesicles and prevent aggregation (Weiner, Martin and Riaz, 1989). No drug crystal was found in all preparations along 8 weeks of study.

Figure 19 shows the encapsulation efficiency of different types of PTU liposomes when they were stored in a refrigerator for 4 and 8 weeks. There was no statistical difference, using Kruskal-Wallis test, among different intervals of storage in all liposomal formulations. This implies that there was no severe destruction in liposomal structure during the storage interval.

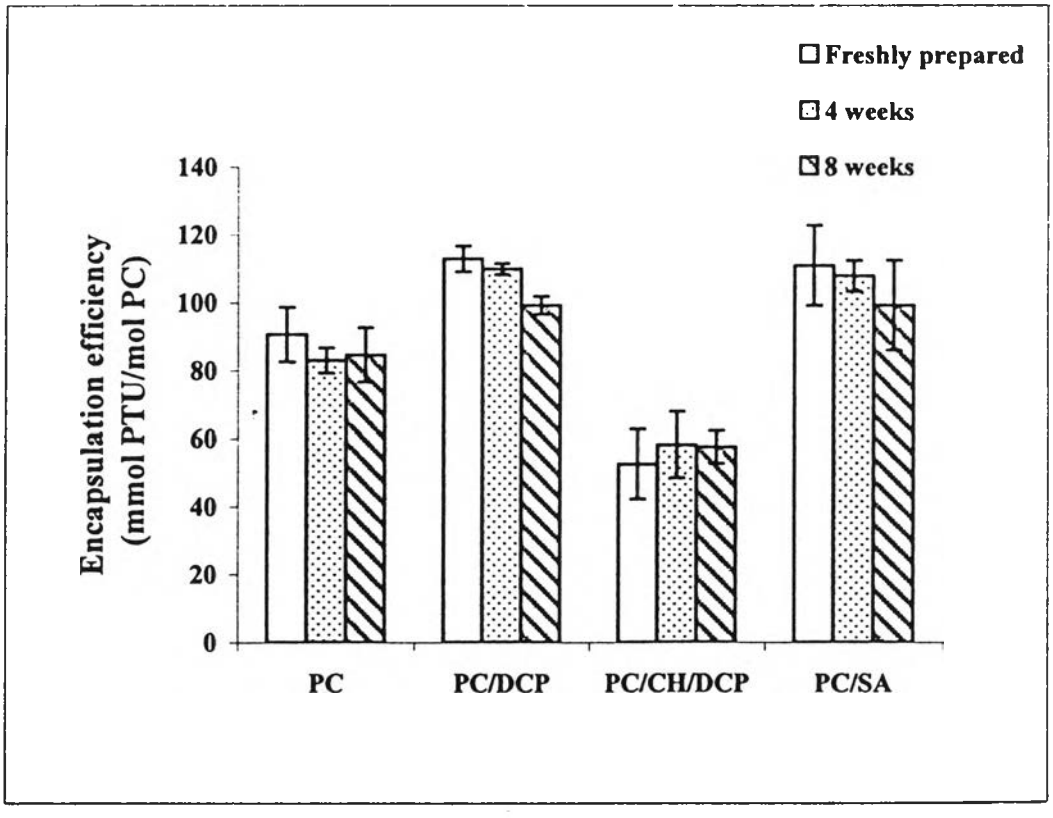


Figure 19: Encapsulation efficiency of PTU liposomes after 8 weeks of storage in a refrigerator. Data are shown as mean values (error bar = SEM).

It is not feasible to prepare the liposomes immediately prior to use. This experiment intended to show the difference between liposomal compositions and the tendency to be a practically useful product. However, stability of these liposomes seemed to be sufficient for the purpose of clinical trials. In clinical trials, patients should be monitored regularly, probably no more than 8 weeks apart. If the results of clinical trials are encouraging, then a more stable product should be aimed at. The stability of liposomal products should preferably meet the standards of conventional pharmaceutical products, where a shelf life of one year is considered to be an absolute minimum (Cevc, 1993).