CHAPTER 4
DISCUSSION

4.1 Physicochemical characteristics of liposome formulations

4.1.1 The physical appearances of liposome formulations

The neutral liposomes with and without tranexamic acid (TA) [7:2, 7:2:(5%TA), 7:2:(10%TA)] showed sedimentation after 24 hrs, whereas negative and positive liposomes without the drugs [7:2:1 (+), 7:2:1 (-)] and with the entrapped drug [7:2:1 (5%TA,+), 7:2:1 (10%TA,+), (5%TA,-), 7:2:1 (10%TA,-)] gave physical appearance of translucent white dispersion with no sedimentation (Table 3.1 and Figure 3.1). This appearance indicated the physical stability of the formulations. This may be due to the effects of charges on liposomes. The charges of liposomes also reduced and protected the aggregation following the formation of multilamellar liposomes (Betagen et. al., 1993).

4.1.2 pH measurement of liposome formulations

TA is a synthetic amino acid which has amino and carboxylic as functional groups. When TA dissolves in water, it will ionize in equilibrium as the following equations :

$$H_{15}NO_8 + H_2O \rightleftharpoons C_8H_{14}NO_8^- + H_3O^+ \quad (1)$$

$$C_8H_{15}NO_8 + H_2O \rightleftharpoons C_8H_{16}NO_8^+ + OH^- \quad (2)$$

In Table 3.2, when TA dissolved and ionized in DI water, the pH of the solution increased because of the more concentrations of hydroxyl ions than hydronium ions (Appendix A) demonstrating the increasing pH values. TA in DI water appeared to have weak positive charges

Stearylamine and dicetyl phosphate were amphiphile compounds. Stearylamine and dicetyl phosphate in liposomes and aqueous environments, they may ionize and give hydroxyl and hydronium ion as the following equations :

$$C_8H_{15}NO_8 + H_2O \rightleftharpoons C_8H_{16}NO_8^{+} + HO^{-} \quad (3)$$

$$C_{32}H_{67}O_4P + H_2O \rightleftharpoons C_{32}H_{66}O_4P^{-} + H_3O^{+} \quad (4)$$

In positively charged liposome dispersion with the entrapped TA, it gave the higher pH values than the positively charged liposome dispersion without the entrapped drug (Table 3.2). This is due to the entrapped TA which gave more hydroxyl ions to the liposome formulation dispersion. The negatively charged liposome dispersion with the entrapped TA gave the pH values higher than the blank negatively charged liposome dispersion (Table 3.2). Hydronium ion from dicetyl phosphate as in equation 4 in liposome formulation may be neutralized by hydroxyl ion from the ionization of TA (equation 2). The positively charged liposome dispersion received hydroxyl ion from stearylamine (equation 3), whereas the negatively charged liposome dispersion received hydronium ion from dicetyl phosphate (equation 4). Thus, all positively charged liposome dispersion gave higher pH values than all negatively charged liposome dispersion (Table 3.2).

4.1.3 Particle size and particle size distribution of liposome

The light scattering particle analyzer was used to determine the mean particle size and particle size distribution of liposome formulations after the 10-days preparation. The results were shown in Table 3.3 and Figures 3.2 to 3.13. The positively and negatively charged liposome formulation without the entrapped TA and the positively charged liposome with the entrapped 5% and 10% TA gave 10 times larger particle size than the negatively charged liposome with the entrapped 5% and 10% TA. All liposome formulations gave log – normal distribution of particle size. But, in The positively and negatively charged liposome formulation without the entrapped TA and the positively charged liposome with the entrapped 5% and 10% TA had two group of particlesize distribution whereas the negatively charged liposome with the entrapped 5% and 10% TA had only one group of particlesize

distribution. Stearylmine and dicetyl phosphate were incorporated in the liposomal bilayer membranes rendering the surface electrically like charges. This resulted in a repulsion and, hence an increase in the distance between the different bilayers was obtained (Benita, 1984). The similar positive charge of the lipid bilayer to the entrapped TA can increase the distance between the bilayers. On the other hand, the negative charge of the lipid bilayer which is opposite to charges from the entrapped TA will decrease this distance because charge neutralization was occurred.

4.1.4 The morphology of liposomes

All liposome formulations were prepared by a chloroform film with sonication method. Transmission electron microscope analysis showed that all liposome formulations were multilamellar vesicles. The transmission electron micrographs of 7:2:1 (+),7:2:1 (5%TA,+) and (5%TA,-) were shown in Figure 3.14 to 3.16 respectively. About 8 to 15 bilayers of the liposomes can be observed.

4.1.5 Thermal analysis of liposome formulations

Thermogravimetric analyzer was used to determined the glass transition temperature (T_g) of Emulmetik 950®, cholesterol, stearylamine, dicetyl phosphate, TA and the freeze dried liposome formulations [7:2:1(10%TA,+) and 7:2:1(10%TA,-)]. The result showed that the decomposition temperatures (glass transition temperatures, T_g) of Emulmetik 950®, cholesterol, stearylamine, dicetyl phosphate, TA, and the freeze dried liposome formulations [7:2:1(10%TA,+) and 7:2:1(10%TA,-)] were 257.77(Figure 3.17, No. 1 and Figure 3.18), 246.66 (Figure 3.17, No. 2 and Figure3.19), 162.22 (Figure 3.17, No. 3 and Figure 3.20), 218.47 (Figure 3.17, No. 4 and Figure 3.21), 223.63 (Figure 3.17, No. 5 and Figure 3.22), 222.22 (Figure 3.17, No. 6 and Figure 3.23), and 229.63 ^{0}C (Figure 3.17, No. 7 and Figure 3.24) respectively. Then, a scanning rate of 5 ^{0}C/min from 25 to 200 ^{0}C was used for the differential scanning calorimetry study since this range was the temperatures before reaching the decomposition of Emulmetik 950®, cholesterol, stearylamine, dicetyl phosphate, TA, and the freeze dried liposome formulations. Differential scanning calorimetry is an effective thermal method for studying the physicochemical interactions of two or more components. Differential effects associated with physical or chemical changes can be

recorded automatically as a function of temperature or time while the substance was heated at a uniform rate.

The DSC thermograms of Emulmetik 950®, cholesterol, stearylamine, and dicetyl phosphate showed an endothermic peak at 61.75 (Figure 3.25, No. 1),149.66 (Figure 3.25, No. 2), 57.08 (Figure 3.25, No. 3), and 79.33 ^{0}C (Figure 3.25, No. 4) respectively. For TA, it exhibited no transition temperature when scanned from 25 to 200 ^{0}C (Figure 3.25, No. 5). This indicated the high thermal stability of the drug.

The positively charged liposome without the entrapped drug (7:2:1,+) showed an endothermic peak with an average transition temperature ranging from 71.18 to 78.00 ^{0}C and the average maximum peak of transition temperature at 77.75 ^{0}C (Figure 3.26, No. 1) with the enthalpy of transition of 50.04 J/g. The positively charged liposome with the entrapped 5% TA (7:2:1 5%TA,+) showed an endothermic peak with an average transition temperature ranging from 70.37 to 76.50 ^{0}C and the average maximum peak of the transition temperature at 76.25 ^{0}C (Figure 3.26, No. 2) with the enthalpy of transition of 10.83 J/g. The positively charged liposome with the entrapped 10% TA (7:2:1,10%+) showed an endothermic peak with an average transition temperature ranging from 70.25 to 76.33 ^{0}C and the average maximum peak of the transition temperature at 77.08 ^{0}C (Figure 3.27, No. 2) with the enthalpy of transition of 9.31 J/g. The transition temperature of the positively charged liposome without the entrapped drug (7:2:1,+) did not differ from the positively charged liposome with the entrapped 5% and 10% drug [(7:2:1,5%+) and (7:2:1,10%TA,+)]. But, the enthalpy of transition of the blank positively charged liposome (7:2:1,+) was higher than the positively charged liposome with the entrapped drug [(7:2:1,5%TA,+) and (7:2:1,10%TA,+)]. These results indicated that TA was entrapped in an aqueous layer of the positive liposomes.

The negatively charged liposome without the entrapped drug (7:2:1,-) showed an endothermic peak with an average transition temperature ranging from 65.53 to 66.96 ^{0}C and the average maximum peak of the transition temperature at 70.00 ^{0}C (Figure 3.28, No. 1) with the enthalpy of transition of 45.36 J/g. Negatively charged liposome with the entrapped 5% drug (7:2:1,5% -) showed an endothermic peak with an average transition temperature ranging from 63.82 to 71.00 ^{0}C and the average maximum peak of transition temperature at 71.33 ^{0}C (Figure 3.28, No. 2) with the enthalpy of transition of 10.65 J/g. Negatively charged

liposome with the entrapped 10% drug (7:2:1,10%TA, -) showed an endothermic peak with an average transition temperature ranging from 63.66 to 70.50 ^{0}C and the average maximum peak of transition temperature at 70.83 ^{0}C (Figure 3.29, No. 2) with the enthalpy of transition 10.36 J/g similarly to the positive liposomes, the transition temperature of the blank positively charged liposome (7:2:1,-) did not differ from the negatively charged liposome with the entrapped 5% and 10% drug [(7:2:1,5%TA,-) and (7:2:1,10%TA,-)]. But, the enthalpy of transition of the blank negatively charged liposome (7:2:1,-) was higher than the negatively charged liposome with the entrapped 5% and 10% drug [(7:2:1,5%TA,-) and (7:2:1,10%TA,-)]. Similar to the positive liposomes, these results indicated that TA was entrapped in an aqueous layer of the liposomes.

Both positive and negative liposome formulations with the entrapped drug gave the enthalpy of transition values lower than their blank liposomes. TA may interfere with the lipid bilayer formation thereby reducing the enthalpy of transition of the liposome formulations. The higher the entrapped TA concentration, the more effect of this interferance can be observed (Table 3.5).

All liposome formulations with and without TA showed an endothermic peak with an average maximum peak of the transition temperature higher than Emulmetik 950®, because cholesterol incorporated in the liposomal membrane had its hydroxyl group oriented towards the aqueous layers with the aliphatic chain aligned parallel to the acyl chains in the center of the lipid bilayer. The presence of the rigid steriod nuclei along side the first ten or so of carbons of the phosphatidylcholine (Emulmetik 950®) chain has the effect of reducing the freedom of movement. So, this can result in an increase of the transition temperatures of liposome formulations (Guru V., 1993). The effects from the incorporated cholesterol also can reduce the leakage of TA from liposomes.

4.2 Quantitative analysis of tranexamic acid (TA) by spectrophotometric method

4.2.1 Standard curve of TA and the validation of TA assay by comparison between spectrophotometric and titrimetric methods

For the spectrophotometric method, the reaction between TA and 2,4,6 trinitrobenzosulfonic acid in an alkaline medium produced a yellow derivative as shown in

Figure 3.47. The optimum condition for the reaction was at pH 10, 25 ^{0}C for 30 min. The phosphate buffer (pH 4.5) was used to stop the reaction. The colour intensity was stable in the reaction medium for at least 3 hours when protected from light. The maximum absorption spectrum of the derivative was at 415 nm.

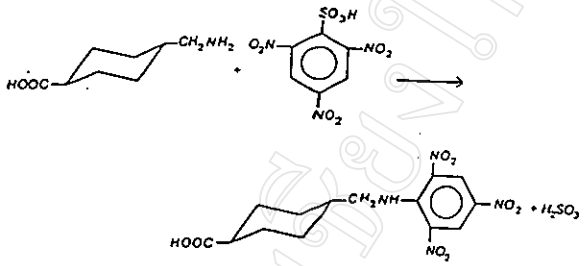

Figure 3.47 : Reaction of TA with 2,4,6 trinitrobenzosulfonic acid in alkaline medium (Atmaca,1989)

The absorbance of standard TA and the calibration curve of TA was showed in Table 3.22 and Figure 3.30 respectively. The calibration curve was fitted by a linear regression analysis program. The correlation coefficient was 0.9937. Thus, the equation was the following :

$$Y = 20.7903\,X - 0.1942$$

Where Y = the concentration of TA in solution (μg / ml)

X = the absorbance of TA

This spectrophotometric method was also used to assay TA in liposome formulations. The assay of TA in solution determind by this method was compared with that obtained by a nonaqueous titrimetric method as in Table 3.23. The mean values and the precisions of the two methods were compared statistically by the t - test and F – tests. At the 95% confidence level, there was no significant difference between them. The assay of TA in liposomes by this method was also validated as shown in Table 3.12. It has been indicated that the composition of liposome in all formulations did not absorb at the wave length of 415 nm and not react with the reagents of the assay.

4.3 The percentages of tranexamic acid (TA) entrapment in liposome formulations

The percentages of TA entrapped in liposome formulations were determined and calculated from the total amount of TA in the formulation (amount of TA in supernatant plus amount of TA in pellets) as the following equation :

$$\%TA\ encapsulated = TA_{Pel} \times 100 / (TA_{Pel} + TA_{Sup})$$

where TA_{Pel} = total amount of TA in pellets

TA_{Sup} = total amount of TA in supernatant

The percentages of TA entrapped in 7:2:1 (5%TA,+), 7:2:1 (10%TA,+), 7:2:1 (5%TA,-) and 7:2:1 (10%TA,-) were in the range of 13.20 ± 0.38 to 15.58 ± 0.80 (Table 3.27). The mean values and the precisions of TA entrapment in the four liposome formulations were compared by ANOVA at the 95% confidence level. There was no significant difference between them, which indicated that the charges as well as particle sizes had no effects on the percentages of TA entrapment in liposome formulations. The negatively charged liposome with the entrapped drug [7:2:1 (5%TA,-), 7:2:1 (10%TA,-)], which had different charge from the entrapped TA (positive charge) can improve the drug entrapment in liposomes by electrical charge. On the other hand, the positive charge liposomes [(5%TA,+), 7:2:1 (10%TA,+)] had larger particle size, and higher volume for the entrapped drug (Benita, 1984) than the negative liposomes. This may cause the balance of the entrapment of TA in both liposomes thereby almost the same magnitude of the percentages of entrapment was obtained.

In this study, the entrapment of TA in liposome formulations was presented by the percentages of loading of the drug (mg of TA / 100 mg of lipid). This value increased with the increase initial drug concentration. The increase of the initial TA concentration from 5% to 10% increased the percentages loading efficiency from 27.52 ± 1.68 (in positively charged liposomes with the entrapped 5% TA) to 62.70 ± 1.33 (in positively charged liposomes with the entrapped10% TA) and 29.04 ± 0.69 (in negatively charged liposomes with the entrapped 5% TA) to 54.73 ± 1.62 (in negatively charged liposomes with the

entrapped 10% TA) (Table 3.28) (Foldvari, 1993). This result indicated that positively and negatively charged liposomes with the entrapped10% TA had the amount of TA more than positively and negativey charged liposome with the entrapped 5% TA of about 2 times, although they had the similar aqueous entrapment volume.

4.4 Physical and chemical stability of tranexamic acid (TA) in liposome

4.4.1 Physical stability of TA in liposome

4.4.1.1 Physical appearance

After storage at 45 ± 1 ^{0}C for 90 days, the negatively charged liposome without the entrapped drug [7:2:1,(-)] produced sedimentation, but at 30 ± 1 and 4 ± 1 ^{0}C showed flocculation. Other liposome formulations [7:2:1 (+), 7:2:1 (5%TA,+), 7:2:1 (10%TA,+), (5%TA,-), 7:2:1 (10%TA,-)] appeared to be colloidal systems at all temperature (4 ± 1, 30 ± 1 and 45 ± 1 ^{0}C) (Tables 3.17 to 3.20 and Figures 3.31 to 3.33). These formulations may have stable electricaly double layers of the vesicles in the pH range of 6.89 to 7.91(Table 3.2). However, the negatively charged liposome without the entrapped drug [7:2:1 (-)] gave sedimentation. This may be due to the low pH that can cause the hydrolysis of phosphatidylcholine (Table 3.2). As known, phosphatidylcholine has four ester bonds. The two acyl ester bonds are most labile to hydrolysis while the glycerophosphate and the phosphocholine ester bonds are more stable. The major route of hydrolytic degradation of the lipid was depicted in Figure 3.48.

Interestingly, the 1-acyl-lysophosphatidylcholine isomer (1-acyl LPC) is the major lyso isomer found. However, detailed monitoring of hydrolysis reaction suggests that both 1-acyl LPC and 2-acyl LPC are formed at comparable rates, and that accumulation of mainly 1-acyl LPC occurs because of rapid conversion of 2-acyl LPC into 1-acyl LPC (acyl migration reaction (Figure 3.48). Following the pioneering work by Frokjaer (Frokjaer, S., 1984) phospholipid hydrolysis kinetics were extensively investigated by Grit and coworkers (Grit, M., 1993). Temperature, pH, bilayer rigid species influence can the hydrolysis rate. Optimum pH conditions were found around pH 6.5 (Figure 3.49) (Grit, 1993).

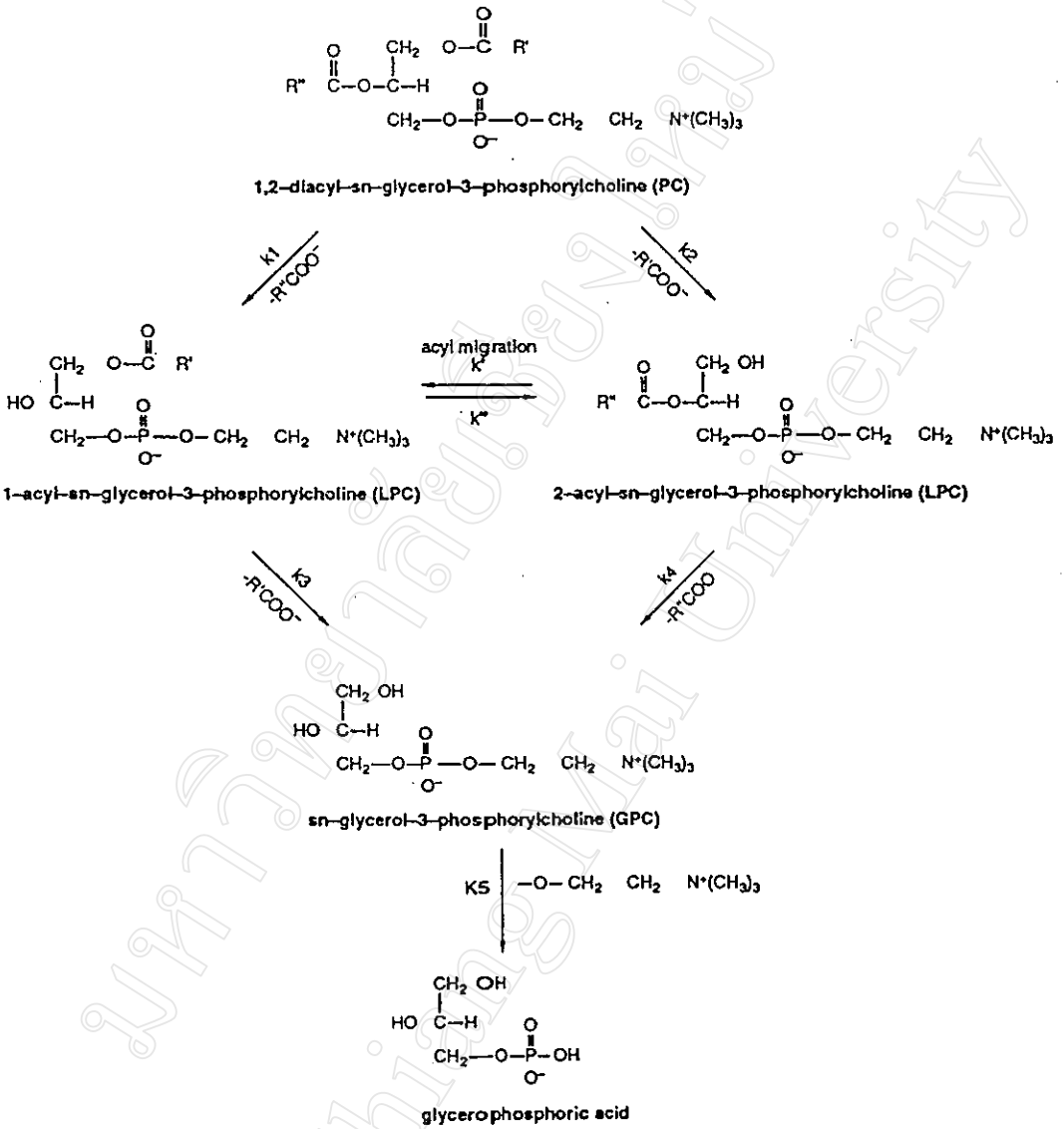

Figure 3.48 : Hydrolysis reactions of phosphatidylcholine in aqueous liposome dispersions. R' and R" are acyl chains. (Grit, 1993)

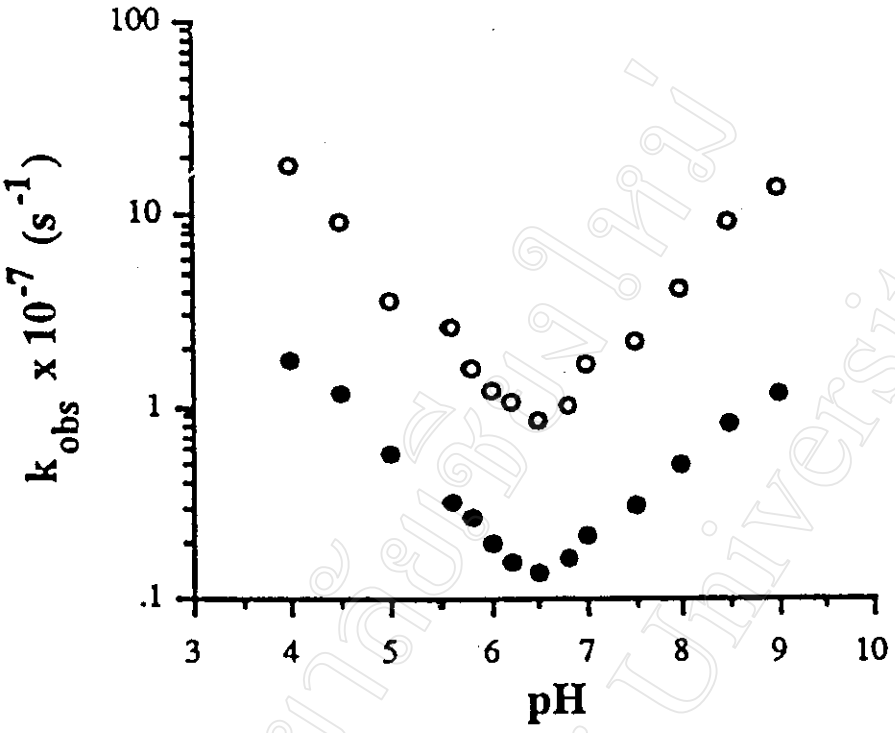

Figure 3.49 : The effect of pH on the hydrolysis of saturated soybean phosphatidylcholine each point represents the mean of at least two separate determinations(Grit, 1993). ● = 40 °C; O = 70 °C.

For the fluid crystalline state bilayers consisting of phosphatidylcholine, the temperature dependence of the hydrolysis rates is constant. In this study, the 7:2:1 (-) liposome that was kept at 45 °C was hydrolysed first and followed by 30 °C and 4 °C respectively. The results were in Tables 3.30 to 3.33. This can be adequately described by the Arrhenius equation (James K., 1994). A linear relationship was observed when plotting the log hydrolysis rate constant versus 1/T. For this study, the liposome formulations were examined to predict storage room temperature or refrigerated temperature stability on the basis of accelerated stability data. However, for bilayers with a phase transition in the experimental temperature range, a discontinuity in the Arrhenius plot was observed (Figure 3.37). The nature of this discontinuity depended on the pH. Hence, accelerated stability studies based on temperatures above the phase transition temperature were of little use in predicting shelf life stability under real-life conditions.

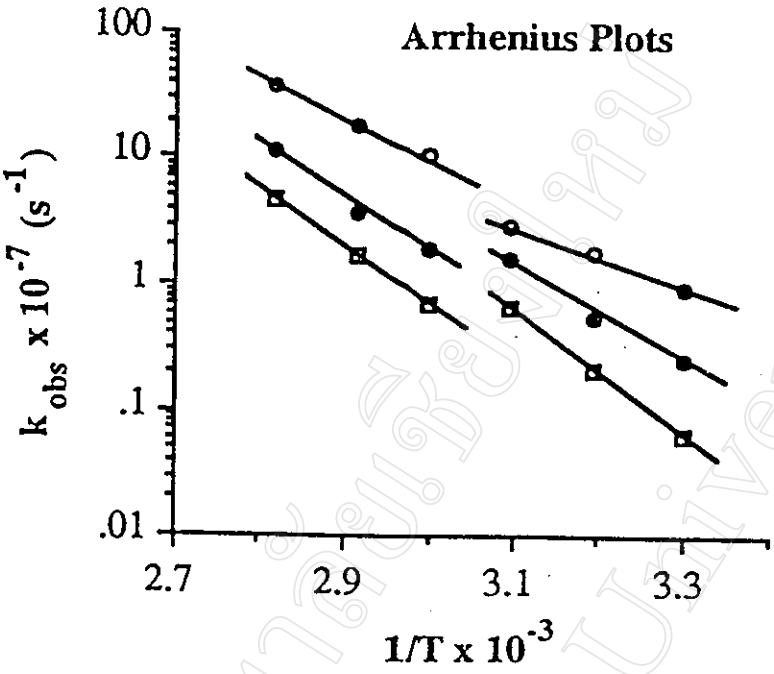

Figure 3.50 : The effect of temperature on the hydrolysis of saturated soybean phosphatidylcholine. The lines were calculated by linear regression analysis. Each point represents the mean of at least two separate determinations (Grit, M., 1993) . ● = pH 4.0; O = pH = 5.0; □ = pH 7.0.

Liposomal surface pH values can differ significantly from bulk pH values in the presence of charged lipid bilayer components. For negatively charged bilayers, the bulk pH values will be higher than the surface pH values. The difference between surface pH and bulk pH increases when the ionic strength in the aqueous phase is decreased or the surface charge density is raised (Grit, 1993). As the surface pH controls the hydrolysis kinetics of phospholipid, the bulk pH with maximum phospholipid stability will shift to higher pH values.

The hydrolysis of phospholipid (phosphatidylcholine, Emulmetik 950®) depending on pH and temperature were described above. The high temperature with low pH or high pH condition increased lipid hydrolysis, which can affect the stability of liposome formulation (Table 3.20).

4.4.1.2 The amount of the remaining tranexamic acid (TA) entrapped in liposomes of various liposome formulations

The chemical stability of liposomal TA preparation was investigated in terms of the remaining amount of TA content entrapped in liposome as a function of time after storage under various temperatures (4 ± 1 , 30 ± 1 and 45 ± 1 ^{0}C) up to three months. The remaining of TA entrapped in liposomes of various formulations (7:2:1 (5%TA,+), 7:2:1 (10%TA,+), (5%TA,-), 7:2:1 (10%TA,-) at 4 ± 1 , 30 ± 1 and 45 ± 1 ^{0}C for three months were shown in Tables 3.21 to 3.27 and Figures 3.34 to 3.36 respectively.

The shelf life of the prepared liposomal TA was calculated by fitting the data to the zero, the first order, and the Higuchi model (Table 3.28). The correlation cefficient from the first order showed higher relation than the zero and the Higuchi model. Thus, the leakage rate from the first order was used to predict the shelf life of the entrapped drug that remained in liposomes of more than 90%. The predicted shelf life values at 4 ± 1 , 30 ± 1 and 45 ± 1 ^{0}C was shown in Table 3.29.

In consideration of leakage rates (Table 3.28), the negatively charged liposome [(5%TA,-), 7:2:1 (10%TA,-)] were more stable than positively charged liposome [(5%TA,+), 7:2:1 (10%TA,+)]. The negatively charged liposome with the entrapped 10% drug [7:2:1 (10%TA,-)] was the most stable formulation. The leakage rates depend on temperatures. At high temperatures, the leakage rates was increased, whereas at low temperature the leakage rate was decreased (Table 3.28). It can be concluded that the shelf life (leakage) of the prepared liposomal TA is affected by charges and storage temperatures. From the experiment, a better formulation in term of leakage was found in negatively charged liposome because TA has positive charge properties, that can bind strongly with negative charge liposomal membrance, thereby reducing the leakage of the drug from liposomes (Benita, 1984). The best storage condition was 4 ± 1 ^{0}C because at higher temperature the lipid is more flexible and fluid (Sarbolouki, 1998). Then, the entrapped drug can leak from the lipid bilayers of liposome easily.

4.4.2 Chemical stability of tranexamic acid (TA) in liposomes

The chemical stability kinetic study of TA was investigated. The shelf life as well as the degradation rate of TA in liposome formulations were calculated by substituting in the equation of the zero order, first order, and Higuchi model. The equations of these models were demonstrated in the Appendix. The r square values from these equations were determined. From Table 3.37, the chemical kinetics of zero order, first order, and Higuchi model of all liposome formulations appeared to give no significant different r square value. The degradation rate of the drug appeared to be increased with increasing temperature. The amount of TA in all liposome formulations showed high stability of about more than 90% of the drug remaining at 4, 30 and 45 $^{\circ}C$ for 90 days. This indicated stability of the drug when entrapped in liposomes. This result appeared to agree with the previous study of the stability of TA in DI water (Manosroi, 2000).

4.5 The release study of tranexamic acid (TA) from solution and liposome of various liposome formulations

The release of TA from liposome formulations were studied. The freshly prepared TA liposomes were used to study in comparing with the TA in solution. The percent cumulative amount of TA released from liposome formulations [7:2:1 (5%TA,+), 7:2:1 (10%TA,+), 7:2:1 (5%TA,-), 7:2:1 (10%TA,-)] was shown in Table 3.46 and Figure 3.44. The percent cumulative amount of TA released from 5% solution and 10% solution was shown in Table 3.49 and Figure 3.45. The results showed that, the release of TA from 7:2:1 (5%TA,+) and 7:2:1 (5%TA,-) was slower than 7:2:1 (10%TA,+) and 7:2:1 (10%TA,-). This indicated that the release of TA from liposome formulations depended on liposomal surface charge, particle size, the initial TA concentration and the hydrophilicity of TA.

TA is a hydrophilic drug with slightly positive charge properties that can be incorporated in an aqueous layer of the multilamellar liposome. In positive charge liposome [(5%TA,+) and (10%TA,+)], TA took longer period to diffuse across the liposomal membrane. This result may suggest that positive liposomes had a larger particle size than the negative liposomes (Table 3.3). This also supported the result from particle size determination mentioned previously. In negatively charged liposomes [(5%TA,-) and

(10%TA,-)], TA may interact with the surface charge of liposome bilayer resulting in the difficulty of TA to be released from liposomes (Omaina, 1997 and Taylor, 1990). There was no significant difference in the release rate of the positively and negatively charged liposome formulations (Table3.52 and Figure 3.44).

The release of TA from all liposome formulations showed the release rate of about 6 to 8 % per $hr^{\frac{1}{2}}$. TA entrapped in all liposome formulations gave the slower release rate of about 3 times than in the solution at 37 ^{o}C, 24 hrs (Table 3.52 and Figure 3.46). This suggested that all liposomes can prolong and sustain the release of the drug comparing to the drug in solution (Sarrinen-Savolainen, 1997).