CHAPTER 3

RESULTS AND DISCUSSION

3.1 Preparation of cationic lipids

3.1.1 Synthesis of cholest-5-en-3ol (3β) (trimethylammonio) acetate chloride(CTA) from cholesterol

CTA was successfully synthesized by the DCC/DMAP coupling method with Steglich esterification. The reaction was completed at 48 hours. The percentage yield of the product was approximately 32.22% at 1 to 1 molar ratio of cholesterol to betaine hydrochloride. The appearance of the CTA product was white powder (Figure 3.1). The procedure of the synthesis was presented in figure 3.2.

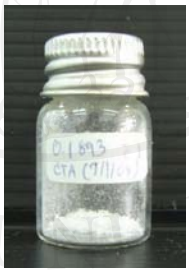


Figure 3.1 Appearance of CTA

3.1.2 Purification of CTA

The crude CTA product was purified by column chromatography. CTA was detected with TLC in fractions 8-16. The crude pooled from this fractions were recrystallized with methanol to give the purified which only one spot on the TLC was observed (Figure 3.3). The melting point of this product was in the range of 138-140 ^{o}C in comparing to the melting point of the reactant cholesterol which gave in the renge of 146-147 ^{o}C.

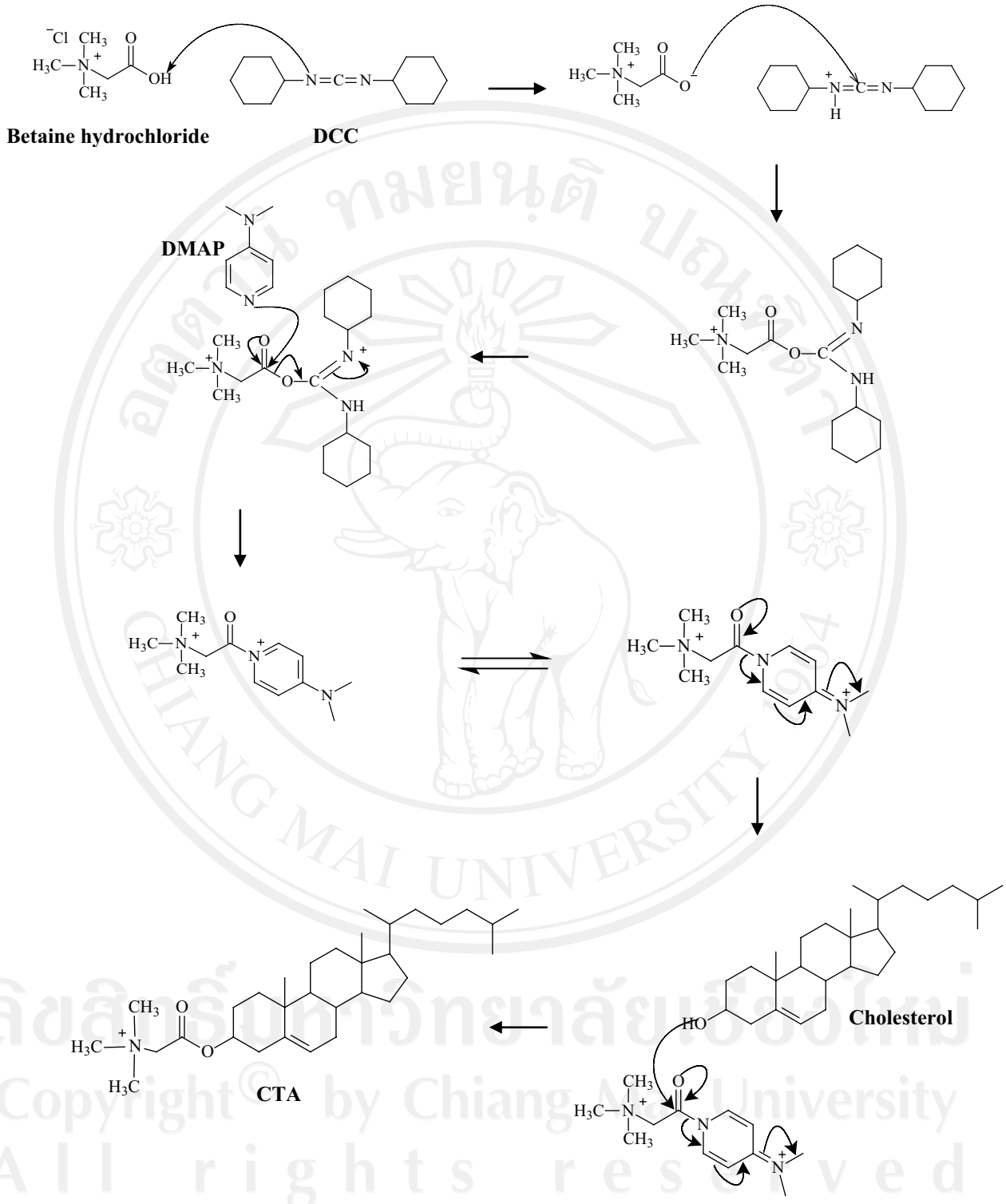
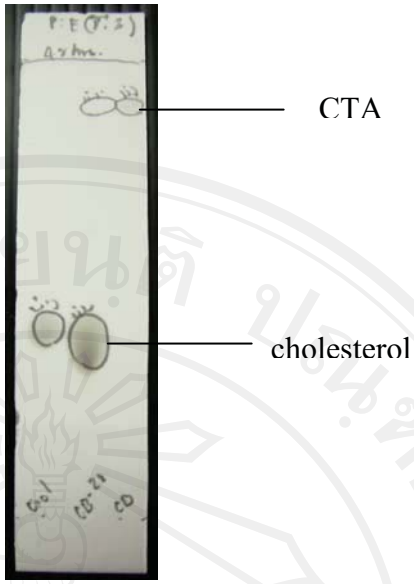


Figure 3.2 Synthesis steps of CTA from cholesterol and betaine hydrochloride by the method of Steglich esterification



Figures 3.3 TLC of the synthesized product comparing to cholesterol (reactant)

3.1.3 Identification of the synthesized products

Figure 3.4-3.6 showed the chromatograms of the purified CTA identifed by IR, ^{1}H NMR and LC-MS. The data of the chromatograms was shown in Table 3.1.

A. Infrared spectroscopy (IR)

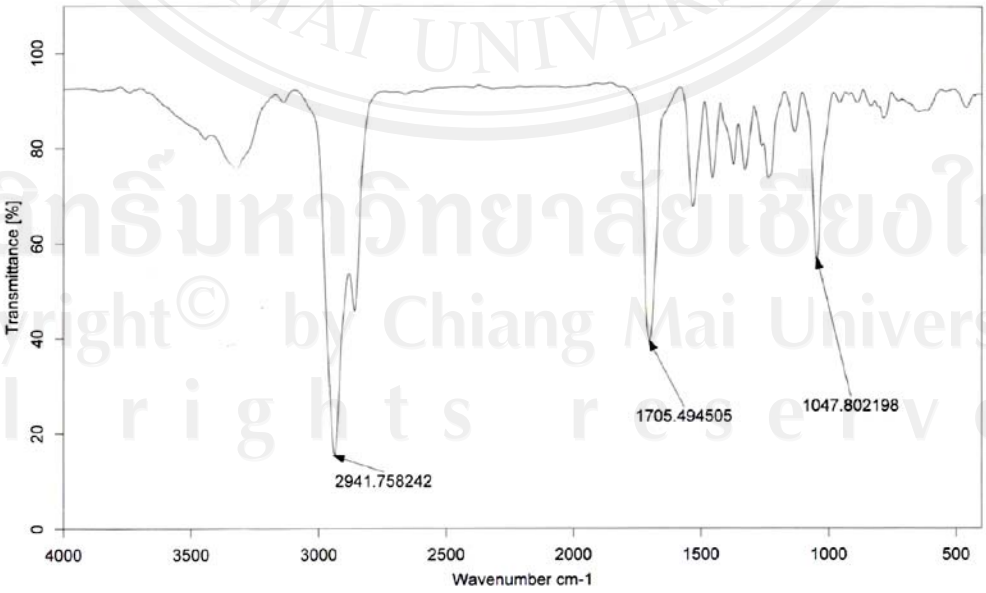


Figure 3.4 The IR spectrum of the purified CTA

B. Nuclear magnetic resonance chromatography (NMR)

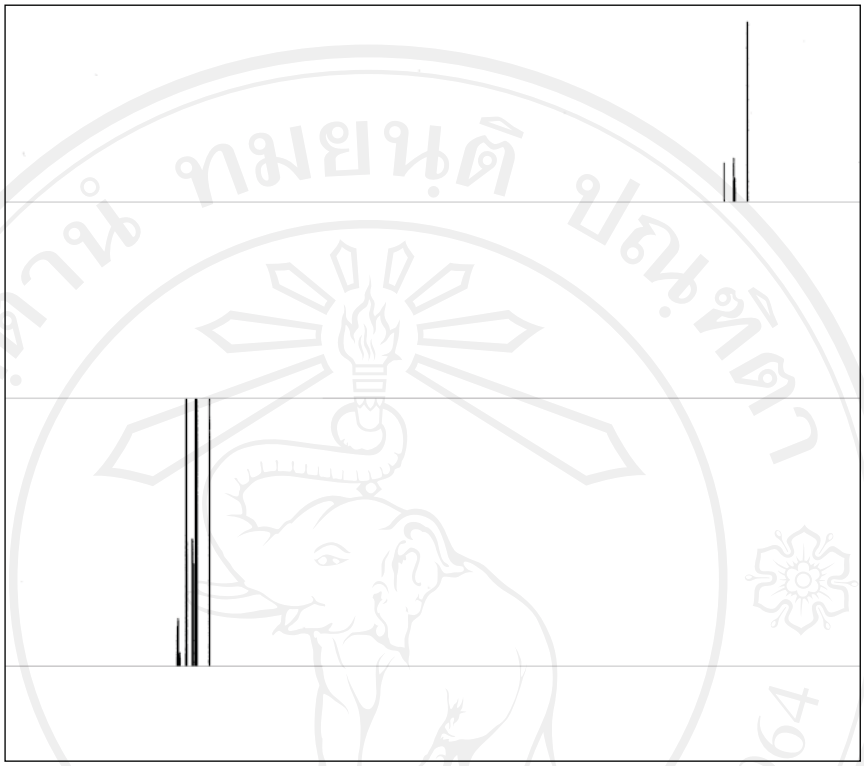


Figure 3.5 The ^{1}H NMR spectrum of the purified CTA

C. Liquid chromatography/ mass spectrometry (LC/MS)

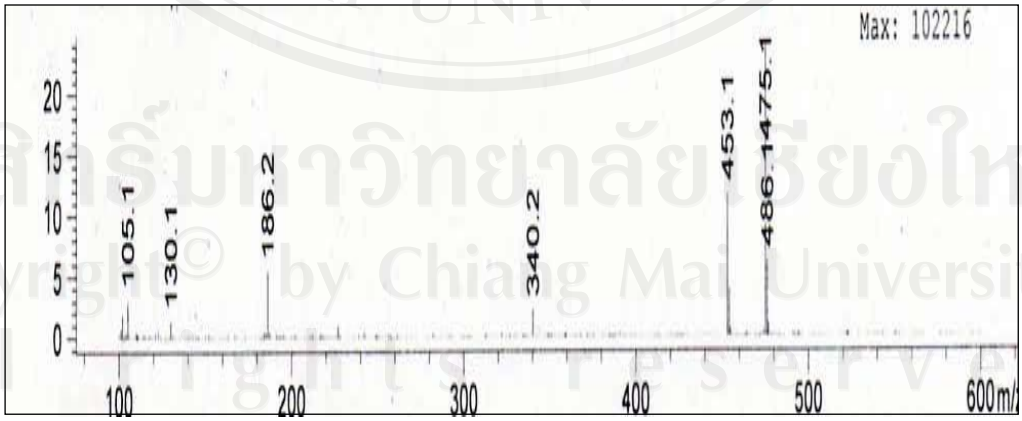


Figure 3.6 The MS spectrum of the purified CTA

Table 3.1 Summary of the CTA product identification information

Physical properties	
white powder, melting point (m.p.) 138-140 ^{o} C	
IR spectroscopy	
ν_{max} (cm^{-1})	Type of vibration
1047	C-O stretching
1602	C=C stretching
1705	C=O stretching of ester
2941	C-H stretching of CH_2, CH_3
NMR spectroscopy	
Chemical shift (δ, ppm)	Type of proton
0.68	s, 3H (CH_3)
0.86	d, 3H (CH_3)
0.89	d, 3H (CH_3)
1.01	s, 3H (CH_3)
1.024	s, 9H ($3CH_3$)
1.03-1.61	m, 28H (cholesteryl)
2.30	m, 2H ($OCHCH_2C=CH$)
4.48	s, 2H(OCH_2N^+)
5.37	m, 1H (C=CH)
Mass spectrometry(ESI-MS)	
Molecular wight	m/z
Calc.for $C_{32}H_{56}NO_2$	486.1475 $(M+H)^+$

CTA was characterized by ^{1}H-NMR techniques (Figure 3.5 and Table 3.1), 1H of C=CH (cholesteryl) appeared as δ at 5.37 ppm, 2H of OCH_2N^+ (betainate) appeared as δ at 4.48 ppm, 9H of $3CH_3$ (betainate) appeared as δ at 1.024 ppm with high field chemical shift because the effect from N^+ atom and the proton of CH_3(cholesteryl, betainate),CH_2 (cholesteryl) and CH (cholesteryl) which appeared at high field chemical shift.

3.1.4 Quantitative analysis of the CTA product by HPLC

The CTA contents in the synthesized purified product were analyzed by HPLC. In comparing the chromatograms, cholesterol (reactant) gave the retention time at 6.985 mins (Figure 3.7), whereas CTA showed at 6.003 mins (Figucre 3.8). The content of CTA was determined from the peak area. The percentage of content of purified CTA was 91.30 %.

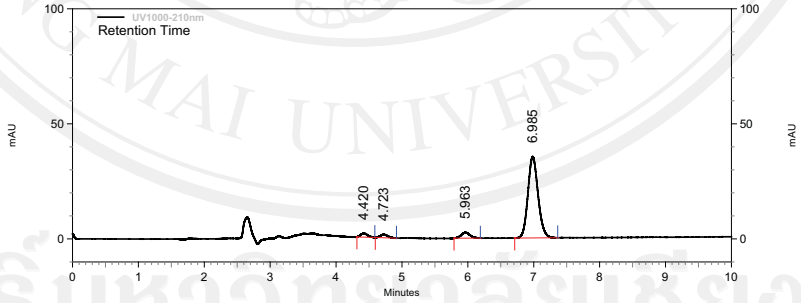


Figure 3.7 HPLC chromatogram of cholesterol

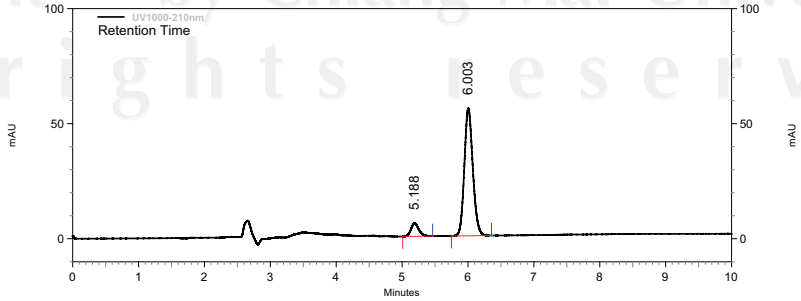


Figure 3.8 HPLC chromatogram of the purified CTA product

3.1.5 Stability of CTA

The synthesized product was kept in glass vials and stored at 4±2°C, room temperature (30±2°C) and 45±2°C for 3 months. The amount of CTA was determined by HPLC at different time intervals (0, 1, 2, and 3 months). The percentages of CTA remaining was compared to that at initial. The chemical stability sharing the CTA content remaining as difference temperature was shown in Figure 3.9. After 3 months, the percentages of CTA remaining at 4±2°C, room temperature (30±2°C) and 45±2°C were 87.5, 84.7 and 83.7 respectively. CTA seemed to be chemically stable when exposed to light and high temperature.

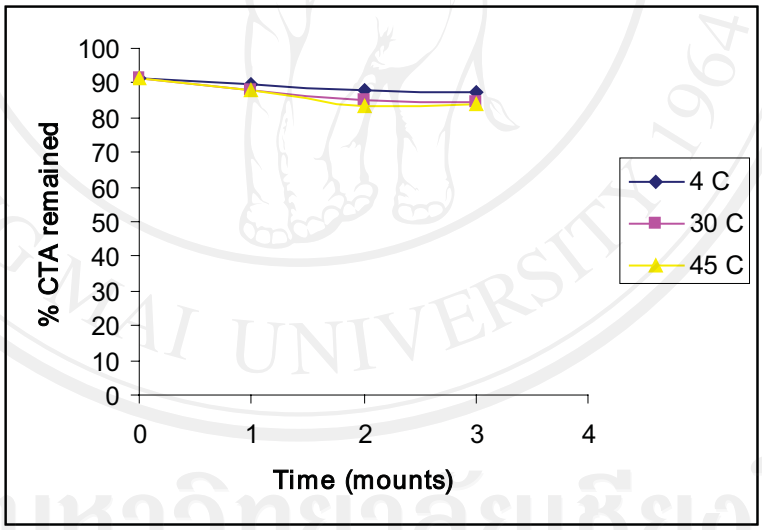
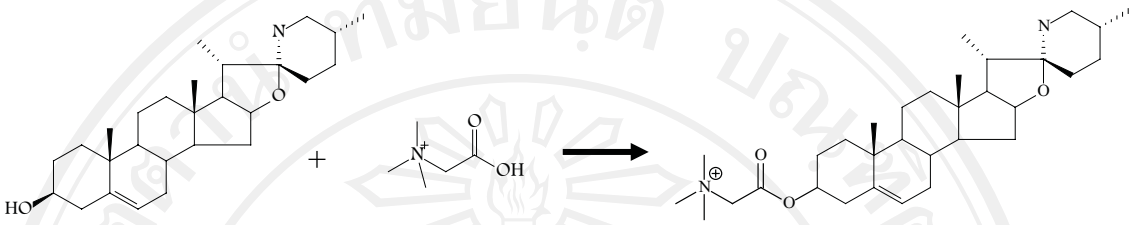


Figure 3.9 The percentages of CTA remaining at different temperatures for 3 months

3.1.6 Preparation of a cationic lipid from solasodine

A purified cationic lipid can not be obtained from the reaction of solasodine and betaine hydrochloride by the DCC/DMAP coupling method with Steglich esterification (Ficgure 3.10) because the reaction gave many by-products from the

nitrogen and oxygen functional group in the solasodine structure which, can react with betaine hydrochloride, DCC and DMAP. These side products were indicated in the TLC chromatogram (Figure 3.11).



solasodine trimethyl glycine

Figure 3.10 Synthesis reaction of a cationic lipid from solasodine

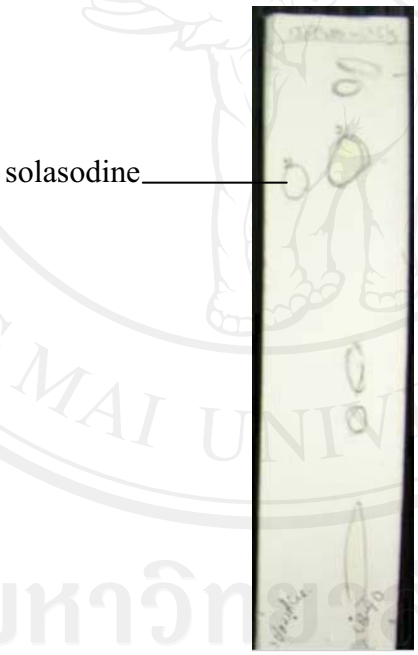


Figure 3.11 TLC chromatogram of the synthesized product from solasodine (reactant)

3.1.7 Preparation of cholestranol from cholesterol

Cholestranol was synthesized from cholesterol and hydrogen (gas) using Pd/C as a catalyst. The percentage yields of the synthesized product was 55.05 %. This

product was identified by TLC and melting point. The TLC chromatogram of the product was shown in Figure 3.12. Its melting point was in the range of 100-104 ^{0}C in comparing to 146-147 ^{0}C of cholesterol.

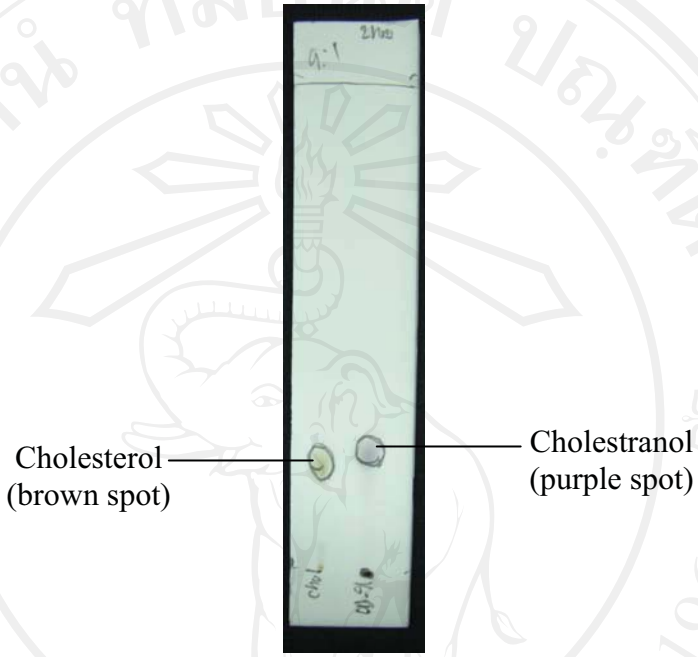


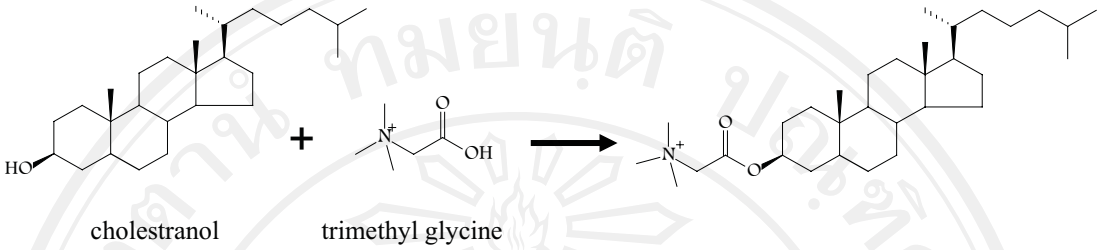
Figure 3.12 TLC chromatogram of cholestranol synthesized from cholesterol (reactant)

3.1.8 Preparation of a cationic lipid from cholestranol

The cationic lipid, cholestranol(3β) (trimethylammonio)acetate(HCTA) was synthesized from cholestranol and trimethyl glycine by an esterification reaction(Figure 3.13). The percentage yield of the product was 15.05 %.

This product was identified by TLC, melting point, IR and NMR. The TLC chromatogram of the product was shown in Figure 3.14. Its melting point was in the range of 128-132 ^{0}C in comparing to 100-104 ^{0}C of cholesthanol, which was used as a reactant.

Figure 3.15-3.16 showed the chromatograms of HCTA identified by IR, and ^{1}H NMR. The summary of HCTA identification information was presented in Table 3.2.



cholestranol trimethyl glycine

Figure 3.13 Synthesis of a cationic lipid from cholestranol

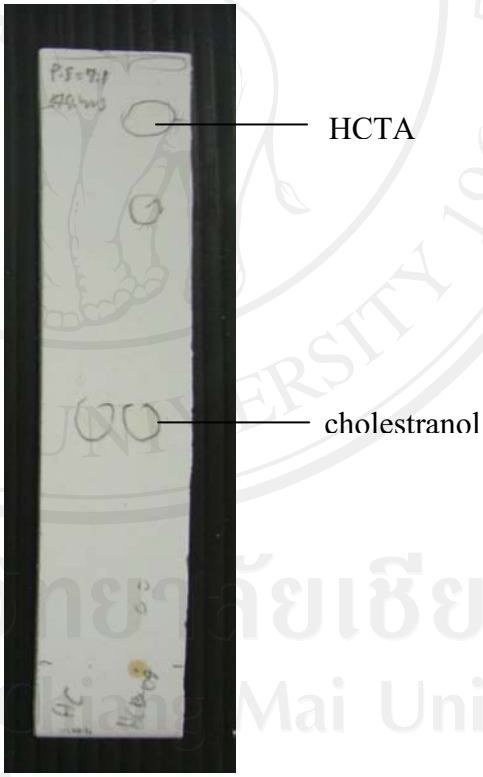


Figure 3.14 TLC chromatogram of the synthesized product in comparing to cholesterol (reactant)

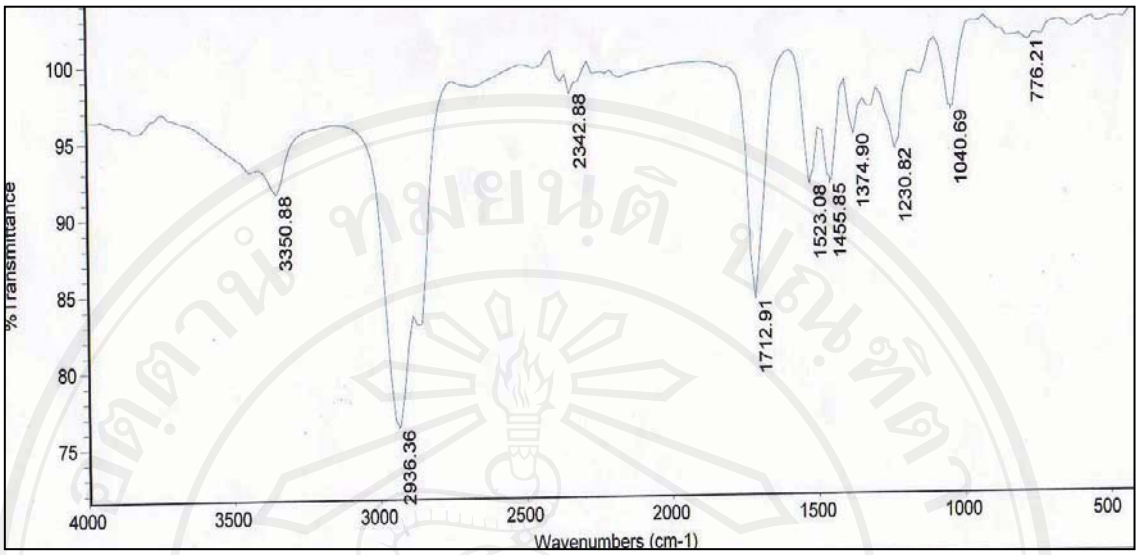


Figure 3.15 The IR spectrum of the synthesized product (HCTA)

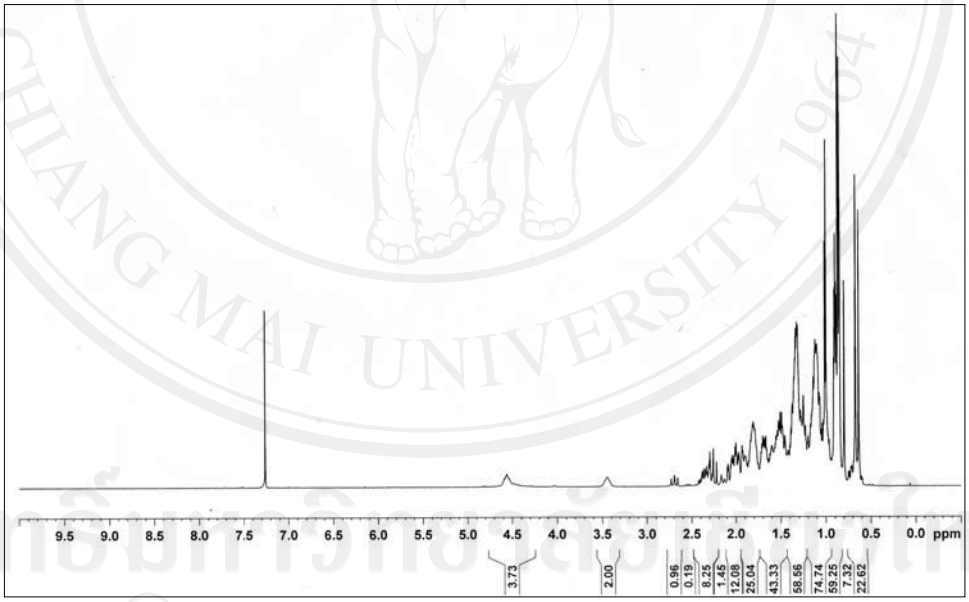


Figure 3.16 The H^1NMR spectrum of the synthesized product (HCTA)

Table 3.2 Summary of HCTA identification information

Physical properties	
white powder melting point (m.p.) 128-132 ^{0}C	
IR spectroscopy	
ν_{max} (cm^{-1})	Type of vibration
1040	C-O stretching
1712	C=O stretching of ester
2936	C-H stretching of CH_2, CH_3
NMR spectroscopy	
Chemical shift (δ, ppm)	Type of proton
0.67	s, 3H (CH_3)
0.86	d, 3H (CH_3)
0.89	d, 3H (CH_3)
1.01	s, 3H (CH_3)
1.024	s, 9H ($3CH_3$)
1.03-1.61	m, 28H (cholesteryl)
2.30	m, 2H ($OCHCH_2C$=CH)
3.46	s, 2H(OCH_2N^+)
4.57	m, 1H(OCOCH)

HCTA was characterized by ^{1}H-NMR techniques (Figure 3.16 and Table 3.2), 2H of OCH_2N^+ (betainate) appeared as δ at 3.46 ppm, 1H of OCOCH (cholesteryl) appeared as δ at 4.57 ppm and the proton of CH_3(cholesteryl, betainate),CH_2 (cholesteryl), CH (cholesteryl) appeared at high field chemical shift. Mass spectrometry of HCTA will be studied in the future because this study was concern in CTA only.

3.2 Preparations of liposomes from the synthesized cationic lipid (CTA)

A. Formulations of blank liposome

Seven liposomal formulations were prepared by a chloroform film method with sonication with the total concentrations of the lipids mixture of 20 mM (Table 3.3). Liposomes composed of DPPC/ Chol/ charged lipids (anionic or cationic) were prepared in the molar ratio of 7:2:1 since the cationic liposomes composed of DPPC/ Chol/cationic charged lipid (stearylamine) at the molar ratio of 7:2:1 gave high physical stability in our previous study and showed deep penetration of amphotericin B into the skin (Manosroi et al., 2004). In this study, five liposomal formulations prepared from DPPC/Chol at 7:3, DPPC/Chol/ DDAB at7:2:1, DPPC/Chol/ CTA at 7:2:1, DPPC/CTA/DDAB at 7:2:1, and DPPC/ CTA at 7:3 molar ratios gave no sedimentation or layer separation (Figure 3.17). These liposomal formulations were selected for further determination of morphology, particle size and zeta potential.

Table 3.3 Descriptions of seven liposomals formulations prepared by chloroform film method with sonication

Type	Composition	Molar ratio
Neutral	DPPC/ Chol	7:3
Cationic	DPPC/Chol/ DDAB	7:2:1
	DPPC/Chol/CTA	7:2:1
	DPPC/ CTA/DDAB	7:2:1
	DPPC/CTA	7:3 1:1 1:2

(A) (B) (C) (D) (E) (F) (G)

Figure 3.17 Physical appearances of seven liposomal formulations composing of (A) DPPC/Chol at 7:3, (B)DPPC/Chol/ DDAB at7:2:1, (C) DPPC/Chol/ CTA at 7:2:1 , (D) DPPC/CTA/DDAB at 7:2:1 , (E) DPPC/ CTA at 7:3 molar ratio, (F) DPPC/ CTA at 1:1 and (G) DPPC/ CTA at 1:2 molar ratio

B. Morphology, particle size and zeta potential determination

Figures 3.18 and 3.19 showed the optical microscopic and TEM images of the blank liposome. The morphology of the blank liposomal formulations indicated spherical shape.

The zeta potential and vasicular sizes of five liposomal formulations, which gave no sedimentation of layer separation, were demonstrated in Table 3.4. The zeta potential of liposomes contained no cholesterol was higher (more positive) than those with cholesterol. When CTA was added in the composition, more zeta potential was observed. Also, CTA appeared to be able to decrease the negative charge of cholesterol, since the zeta potential of DPPC/CTA/DDAB (7:2:1) was higher than DPPC/Chol/DDAB (7:2:1). The more physical stability of the liposomes which had more zeta potential values from adding CTA, especially the DPPC/CTA / DDAB liposomal formulation was observed. This formulation is one of the 3 formulations which showed good physical stability due to the zeta potential of at least $\pm$30 mV

which is normally required to achieve a reasonably stable dispersion(Sentein et al., 2009).

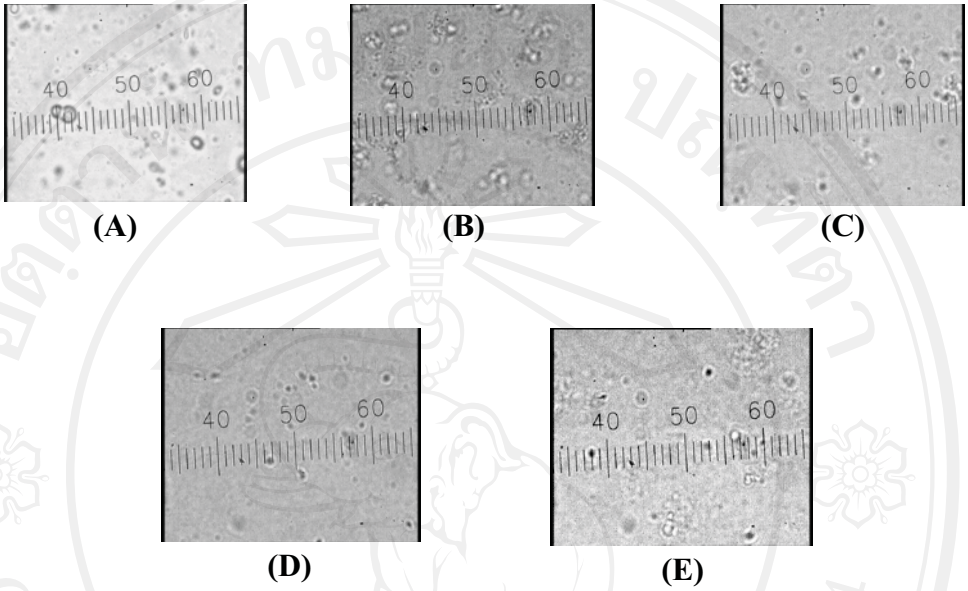
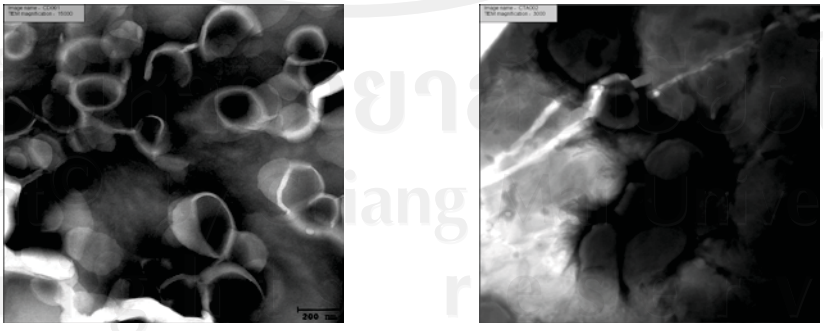


Figure 3.18 Optical microscopic images of five blank liposomal formulations (A) DPPC/Chol at 7:3 molar ratio, (B) DPPC/Chol/CTA at 7:2:1 molar ratio, (C) DPPC/Chol/DDAB at 7:2:1 molar ratio, (D) DPPC/CTA/ DDAB at 7:2:1 molar ratio and (E) DPPC/CTA at 7:3 molar ratio



(A) (B)

Figure 3.19 The negative staining TEM of the blank liposomes prepared by the chloroform film method (A) DPPC/Chol/DDAB at 7:2:1 molar ratio and (B) DPPC/Chol/CTA at 7:2:1 molar ratio

Table 3.4 The zeta potential values and vasicular sizes of five liposomal formulations and their ingredients determined by dynamic light scattering (DLS)

Formulations	Zeta potential average (mV)	Average physical size (nm)
DPPC/Chol (7:3)	-18.9±0.87	242.9±4.80
DPPC/Chol/DDAB (7:2:1)	+68.82±2.14	137.2±0.22
DPPC/Chol/CTA (7:2:1)	-8.99±1.28	201.6±1.30
DPPC/CTA/DDAB(7:2:1)	+75.74±0.62	166.4±2.99
DPPC/CTA(7:3)	+1.20±0.93	242.9±4.80
DPPC	-3.92±0.70	94.49±0.83
Chol	-33.6±0.72	331.8±29.59
DDAB	+75.3±9.94	93.3±2.28
CTA	+11.7±0.60	1624±361.9

C. Physical stability of blank liposomes

The freshly prepared five liposomal formulations were transferred to transparent vials and kept at different temperatures (4 ± 2 ^{o}C, room temperatue (30 ± 2 ^{o}C) and 45 ± 2 ^{o}C) for 3 months. Physical stability of the formulations was observed visually (color, odor, clarity and precipitation).

Table 3.5 showed physical appearances of five blank liposomal formulation kept at different temperatures for 3 months. At all temperatures, the physical characteristics of the liposomal formulations of DPPC/Chol/ DDAB at7:2:1 and DPPC/CTA/DDAB at 7:2:1 molar ratio did not change after 3 months but the liposomal formulation of DPPC/ CTA at 7:3 molar ratio was precipitated after 1

month. At 45 ± 2 ^{o}C, the color of the liposomal formulation of DPPC/ CTA at 7:3 molar ratio was changed from white to yellow after 2 months.

Table 3.5 Physical appearances of the five liposomal formulations in distilled water (pH =5.6) kept at different temperatures for 3 months

Formulations (molar ratio)	**Time (months)**	**Physical appearances**		
		4 ± 2 ^{o}C	**30 ± 2 ^{o}C**	**45 ± 2 ^{o}C**
DPPC/Chol (7:3)	0	translucent, white dispersion, no sedimentation		
	1	translucent, white dispersion, sedimentation, +1	translucent, white dispersion, sedimentation, +1	translucent, white dispersion, sedimentation, +1
	2	translucent, white dispersion, sedimentation, +1	translucent, white dispersion, sedimentation, +1	translucent, white dispersion, sedimentation, +1
	3	translucent, white dispersion, sedimentation, +2	translucent, white dispersion, sedimentation, +1	translucent, white dispersion, sedimentation, +1

Note: +1 to +5 = degree of sedimentation from low to high

Table 3.5 Physical appearances of the five liposomal formulations in distilled water (pH =5.6) kept at different temperatures for 3 months (continued)

Formulations (molar ratio)	**Time (months)**	**Physical appearances**		
		4 ± 2 °C	**30 ± 2 °C**	**45 ± 2 °C**
DPPC/Chol/DDAB (7:2:1)	0	translucent, white dispersion, no sedimentation		
	1	translucent, white dispersion, no sedimentation		
	2	translucent, white dispersion, no sedimentation		
	3	translucent, white dispersion, no sedimentation		
DPPC/Chol/CTA (7:2:1)	0	translucent, white dispersion, no sedimentation		
	1	translucent, white dispersion, sedimentation, +1	translucent, white dispersion, sedimentation, +1	translucent, white dispersion, sedimentation, +1
	2	translucent, white dispersion, sedimentation, +1	translucent, white dispersion, sedimentation, +1	translucent, white dispersion, sedimentation, +1
	3	translucent, white dispersion,	translucent, white dispersion,	translucent, white dispersion,

Note: +1 to +5 = degree of sedimentation from low to high

Table 3.5 Physical appearances of the five liposomal formulations in distilled water (pH =5.6) kept at different temperatures for 3 months (continued)

Formulations (molar ratio)	**Time (months)**	**Physical appearances**		
		4 ± 2 °C	**30 ± 2 °C**	**45 ± 2 °C**
DPPC/Chol/CTA (7:2:1)	3	sedimentation, +1	sedimentation, +1	sedimentation, +1
DPPC/CTA/DDAB (7:2:1)	0	translucent, white dispersion, no sedimentation		
	1	translucent, white dispersion, no sedimentation		
	2	translucent, white dispersion, no sedimentation		
	3	translucent, white dispersion, no sedimentation		
DPPC/CTA (7:3)	0	translucent, white dispersion, no sedimentation		
	1	translucent, white dispersion, sedimentation, +4	translucent, white dispersion, sedimentation, +4	translucent, white dispersion, sedimentation, +4
	2	translucent, white dispersion, sedimentation, +4	translucent, white dispersion, sedimentation, +4	translucent, yellow dispersion, sedimentation, +4

Note: +1 to +5 = degree of sedimentation from low to high

Table 3.5 Physical appearances of the five liposomal formulations in distilled water (pH =5.6) kept at different temperatures for 3 months (continued)

Formulations (molar ratio)	Time (months)	Physical appearances		
		4 ± 2 °C	**30 ± 2 °C**	**45 ± 2 °C**
DPPC/CTA (7:3)	3	translucent, white dispersion, sedimentation, +5	translucent, white dispersion, sedimentation, +5	translucent, yellow dispersion, sedimentation, +5

Note: +1 to +5 = degree of sedimentation from low to high

3.3 Entrapment of human insulin in the selected CTA liposomal formulation

A. Preparations of liposomes entrapped with human insulin

From the physical stability study of blank liposomes, the four liposomes which exhibited good physical stability, composed of DPPC/Chol at 7:3, DPPC/Chol/DDAB at 7:2:1, DPPC/Chol/CTA at 7:2:1 and DPPC/CTA/DDAB at 7:2:1 molar ratios were selected to entrap with human insulin. These liposomal formulations were prepared by freeze dried empty liposomes (FDEL) method.

B. Determination of entrapment efficiency of human insulin entrapped in the selected liposomal formulations

The entrapped human insulin in the selected liposomes were separated from the not entrapped insulin by gel filtration. The fractions containing the entrapped human

insulin in liposomes and the fractions containing the not entrapped human insulin were detected in the range of 9-24 and 27-50 fractions, respectively (Figure 3.20).

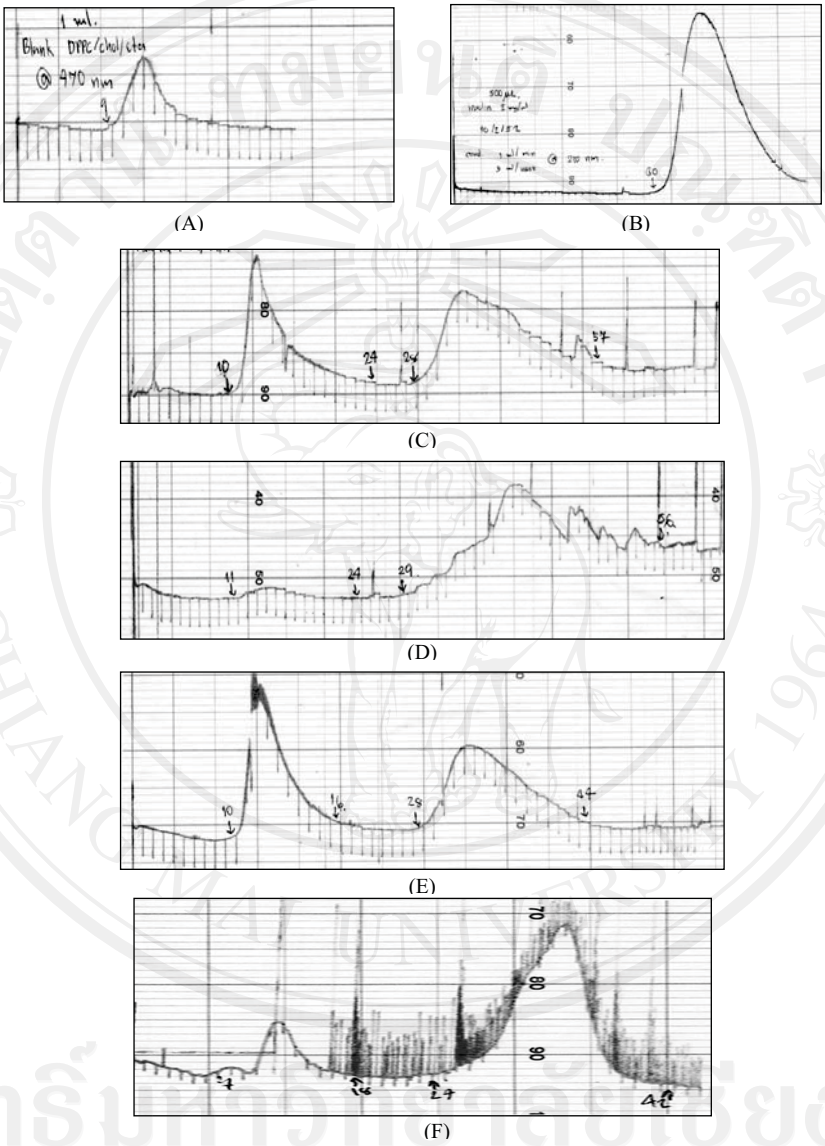


Figure 3.20 Chromatograms from fractional collector of (A) blank liposome (B) free human insulin and various liposomal formulations entrapped with human insulin at the molar ratio of insulin/total liposomal lipid of 1:200 (C) DPPC/Chol at 7:3, (D) DPPC/Chol/DDAB at 7:2:1, (E) DPPC/Chol/CTA at 7:2:1, (F) DPPC/CTA/DDAB at 7:2:1 molar ratio

The molar ratio of insulin to total lipid was prepared at 1:200 (Appendix B).

Human insulin was entrapped in liposomal formulations at 0.45 mg/ml with the molar ratio of human insulin to total lipid was 1:200 (Appendix B). The standard curve of the standard human insulin was determined by HPLC shown in Figure 3.21. The linear relationship (r^2) between the concentrations and the peak area was 0.9999. Formulation of proteins and peptide is more challenging than formulation of small molecules because of protein conformation and the potential for chemical degradation during preparation. In this study found that the percentages of the entrapment efficiency of human insulin in the liposomal formulations DPPC/Chol at 7:3, DPPC/Chol/ DDAB at 7:2:1, DPPC/Chol/CTA at 7:2:1, and DPPC/CTA/DDAB at 7:2:1 molar ratio were at 44.68%, 59.16%, 55.41% and 62.72%, respectively shown in Table 3.6. The percentages of the entrapment efficiency of human insulin in neutral liposomal (DPPC/Chol at 7:3 molar ratio) was lower than other liposomal formulation because of charge interaction between negatively charge of liposome and negatively charge of human insulin. However, it was reported that, the entrapment efficiency of human insulin in neutral liposomal (DPPC/Chol at 7:3 molar ratio) using membrane destabilizing method was as high as 52 % but it was easily aggregated (Huang and Wang., 2006). Usually, the water soluble compounds can be entrapped in the bilayer vesicles such as liposomes of not more than 10-20% (Gulati et al.,1997 and Peltonen et al., 2002). In this study, insulin which is water soluble gave the entrapment efficiency higher than this amount. This was not only due to the entrapment of the drug inside the aqeous phase of the vesicles, but also the adsorbbance of the drug on the surface of the vesiclular by electrostatic interaction, as well.

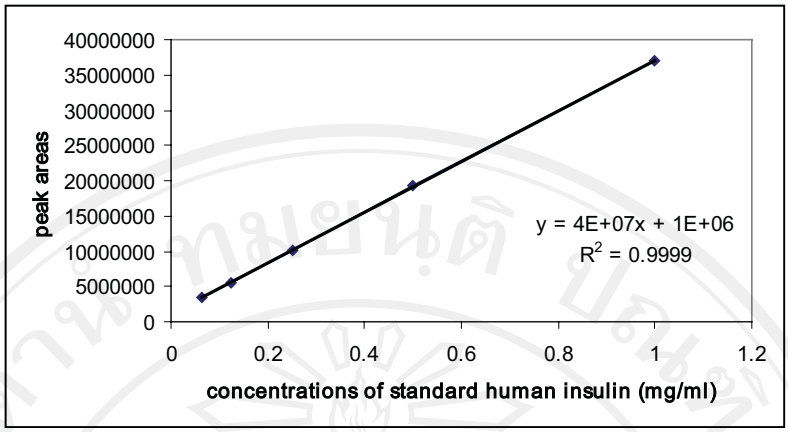


Figure 3.21 The standard curve of the standard human insulin. The linear relationship (r^2) between the concentrations and peak areas was 0.9999

Table 3.6 Percentages of entrapment efficiency of human insulin in four liposomal formulations determined by gel filtration and HPLC

Liposomal formulations	**Molar ratio of insulin/total liposomal lipid**	**Entrapment efficiency (%)**
DPPC/chol 7:3	1:200	48.68
DPPC/chol/DDAB 7:2:1	1:200	59.16
DPPC/chol/CTA 7:2:1	1:200	55.41
DPPC/CTA/DDAB 7:2:1	1:200	62.72

C. Determination of morphology, particle size and zeta potential of human insulin entrapped in the selected liposomal formulations

Figure 3.22 showed the morphology of the liposomal formulations entrapped with human insulin at 1:200 molar ratio of insulin/total liposomal lipid. All showed the existing of the vesicles by an optical microscopy. Figure 3.23 showed the negative staining TEM images of the liposome composed of DPPC/CTA/DDAB at 7:2:1 molar ratio entrapped with human insulin. All vesicles appeared to be large unilamellar vesicles. Table 3.7 showed the vesicular size and the values zeta potential of form liposomal formulations entrapped with human insulin. The liposome DPPC/Chol (at 7:3 molar ratio) gave the smallest particle size of 1.03 μm, while DPPC/CTA/DDAB (at 7:2:1 molar ratio) showed the largest size of 2.26μm. Other cationic liposomes showed the sizes in the range of 1.4-1.8 μm. The zeta potential values of DPPC/Chol/DDAB and DPPC/CTA/DDAB were more positive values (41.8$\pm$1.93 and 47.7$\pm$1.44 mV) than DPPC/Chol and DPPC/Chol/CTA which gave the negative values of -8.69$\pm$1.19 and -6.09$\pm$0.49 mV, respectively. These results have indicated that when cholesterol was increased, the zeta potential values were more negative. However, when CTA was used instead of cholesterol, the zeta potential value was more positive. Thus, the synthesized CTA can be used as a cationic charge increasing lipid for liposome preparation.

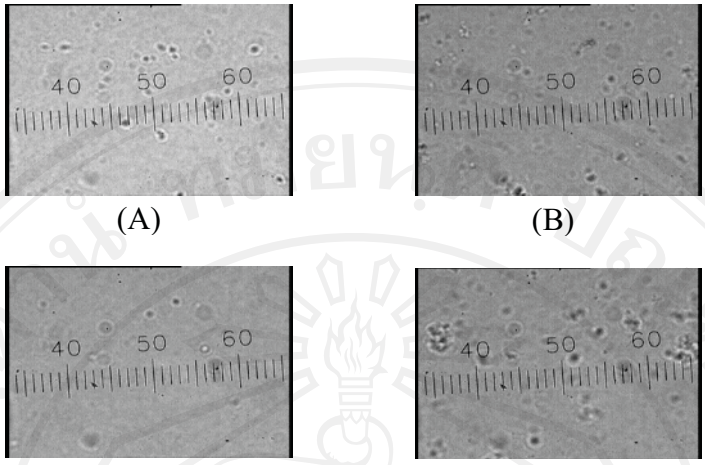


Figure 3.22 Images under optical microscope (1000x) of four liposomal formulations entrapped with human insulin at the molar ratio of insulin/total liposomal lipid of 1:200 (A) DPPC/Chol at 7:3, (B) DPPC/Chol/DDAB at 7:2:1, (C) DPPC/Chol/CTA at 7:2:1, (D) DPPC/CTA/DDAB at 7:2:1 molar ratio

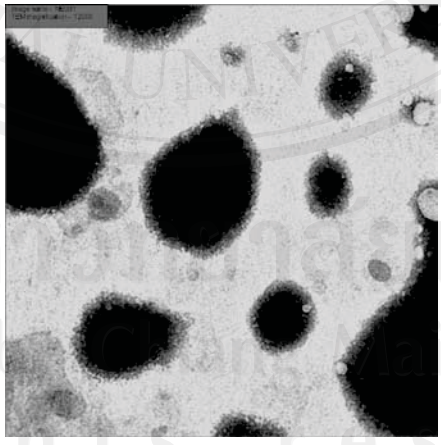


Figure 3.23 The negative staining TEM of the liposome composed of DPPC/CTA/DDAB at 7:2:1 molar ratio entrapped with human insulin at the molar ratio of insulin/total liposomal lipid of 1:200

Table 3.7 The zeta potential values and vesicular sizes of human insulin entrapped in four liposomal formulations

Liposomal formulation	Molar ratio of insulin/total liposomal lipid	Average zeta potential (mV)	Average vesicular size
DPPC/chol (7:3)	1:200	-8.69±1.19	1.03±0.43 μm
DPPC/chol/DDAB (7:2:1)	1:200	41.8±1.93	1.69±0.11 μm
DPPC/chol/CTA (7:2:1)	1:200	-6.09±0.49	1.40±0.19 μm
DPPC/CTA/DDAB (7:2:1)	1:200	47.7±1.44	2.26±0.87 μm

D. Stability study of the selected liposomes entrapped with human insulin

The physical stability of the selected form liposomal formulations entrapped with human insulin at 1:200 molar ratio of insulin/total lipid in phosphate buffer (pH 7) at 4 ^{0}C, room temperatue (30 ± 2 ^{0}C) and 45 ^{0}C) for 4 months was demonstated in Table 3.8.

Table 3.9 and figure 3.24 demonstrated the percentage remaining of human insulin entrapped in liposome compose of DPPC/CTA/DDAB at 7:2:1 molar ratio in compare with human insulin solution at initial and stored at 4 ^{0}C, room temperatue (30 ± 2 ^{0}C) and 45 ^{0}C for 4 months.

Table 3.8 Physical appearances of the four selected liposomes entrapped with human insulin stored at different temperatures for 4 months

Formulations (molar ratio)	**Time**	**Physical appearances at various temperatures**		
		4 ^{o}C	**30 ± 2 ^{o}C**	**45 ^{o}C**
DPPC/Chol (7:3)	0	translucent, white dispersion, no sedimentation		
	1 weeks	translucent, white dispersion, sedimentation, +4	translucent, white dispersion, sedimentation, +4	translucent, white dispersion, sedimentation, +4
DPPC/Chol (7:3)	2 weeks	translucent, white dispersion, sedimentation, +5	translucent, white dispersion, sedimentation, +5	translucent, yellow dispersion, sedimentation, +5
	3 weeks	translucent, white dispersion, sedimentation, +5	translucent, white dispersion, sedimentation, +5	translucent, yellow dispersion, sedimentation, +5

Note: +1 to +5 = degree of sedimentation from low to high

Table 3.8 Physical appearances of the four selected liposomes entrapped with human insulin stored at different temperatures for 4 months (continued)

Formulations (molar ratio)	**Time**	**Physical appearances at various temperatures**		
		4 °C	**30 ± 2 °C**	**45 °C**
DPPC/Chol (7:3)	1 month	translucent, white dispersion, sedimentation, +5	translucent, white dispersion, sedimentation, +5	translucent, yellow dispersion, sedimentation, +5
	2 months	translucent, white dispersion, sedimentation, +5	translucent, white dispersion, sedimentation, +5	translucent, yellow dispersion, sedimentation, +5
	3 months	translucent, white dispersion, sedimentation, +5	translucent, yellow dispersion, sedimentation, +5	translucent, yellow dispersion, sedimentation, +5

Note: +1 to +5 = degree of sedimentation from low to high

Table 3.8 Physical appearances of the four selected liposomes entrapped with human insulin stored at different temperatures for 4 months (continued)

Formulations (molar ratio)	**Time**	**Physical appearances at various temperatures**		
		4 ^{o}C	**30 ± 2 ^{o}C**	**45 ^{o}C**
DPPC/Chol (7:3)	4 months	translucent, white dispersion, sedimentation, +5	translucent, yellow dispersion, sedimentation, +5	translucent, yellow dispersion, sedimentation, +5
DPPC/Chol/DDAB (7:2:1)	0	translucent, white dispersion, no sedimentation		
	1 weeks	translucent, white dispersion, no sedimentation		
	2 weeks	translucent, white dispersion, sedimentation, +2	translucent, white dispersion, sedimentation, +2	translucent, white dispersion, sedimentation, +2
	3 weeks	translucent, white dispersion, sedimentation, +2	translucent, white dispersion, sedimentation, +2	translucent, yellow dispersion, sedimentation, +2

Note: +1 to +5 = degree of sedimentation from low to high

Table 3.8 Physical appearances of the four selected liposomes entrapped with human insulin stored at different temperatures for 4 months (continued)

Formulations (molar ratio)	**Time**	**Physical appearances at various temperatures**		
		4 °C	**30 ± 2 °C**	**45 °C**
DPPC/Chol/DDAB (7:2:1)	1 month	translucent, white dispersion, sedimentation, +2	translucent, white dispersion, sedimentation, +4	translucent, yellow dispersion, sedimentation, +3
	2 months	translucent, white dispersion, sedimentation, +3	translucent, white dispersion, sedimentation, +5	translucent, yellow dispersion, sedimentation, +5
	3 months	translucent, white dispersion, sedimentation, +3	translucent, white dispersion, sedimentation, +5	translucent, yellow dispersion, sedimentation, +5

Note: +1 to +5 = degree of sedimentation from low to high

Table 3.8 Physical appearances of the four selected liposomes entrapped with human insulin stored at different temperatures for 4 months (continued)

Formulations (molar ratio)	**Time**	**Physical appearances at various temperatures**		
		4 °C	**30 ± 2 °C**	**45 °C**
DPPC/Chol/DDAB (7:2:1)	4 months	translucent, white dispersion, sedimentation, +3	translucent, white dispersion, sedimentation, +5	translucent, yellow dispersion, sedimentation, +5
DPPC/Chol/CTA (7:2:1)	0	translucent, white dispersion, no sedimentation		
	1 week	translucent, white dispersion, sedimentation, +3	translucent, white dispersion, sedimentation, +3	translucent, white dispersion, sedimentation, +1
	2 weeks	translucent, white dispersion, sedimentation, +4	translucent, white dispersion, sedimentation, +4	translucent, white dispersion, sedimentation, +1

Note: +1 to +5 = degree of sedimentation from low to high

Table 3.8 Physical appearances of the four selected liposomes entrapped with human insulin stored at different temperatures for 4 months (continued)

Formulations (molar ratio)	**Time**	**Physical appearances at various temperatures**		
		4 ^{o}C	**30 ± 2 ^{o}C**	**45 ^{o}C**
DPPC/Chol/CTA (7:2:1)	3 weeks	translucent, white dispersion, sedimentation, +5	translucent, white dispersion, sedimentation, +5	translucent, yellow dispersion, sedimentation, +1
	1 month	translucent, white dispersion, sedimentation, +5	translucent, white dispersion, sedimentation, +5	translucent, yellow dispersion, sedimentation, +4
	2 months	translucent, white dispersion, sedimentation, +5	translucent, white dispersion, sedimentation, +5	translucent, yellow dispersion, sedimentation, +5

Note: +1 to +5 = degree of sedimentation from low to high

Table 3.8 Physical appearances of the four selected liposomes entrapped with human insulin stored at different temperatures for 4 months (continued)

Formulations (molar ratio)	**Time**	**Physical appearances at various temperatures**		
		4 ^{o}C	**30 ± 2 ^{o}C**	**45 ^{o}C**
DPPC/Chol/CTA (7:2:1)	3 months	translucent, white dispersion, sedimentation, +5	translucent, white dispersion, sedimentation, +5	translucent, yellow dispersion, sedimentation, +5
	4 months	translucent, white dispersion, sedimentation, +5	translucent, yellow dispersion, sedimentation, +5	translucent, yellow dispersion, sedimentation, +5
DPPC/CTA/DDAB (7:2:1)	0	translucent, white dispersion, no sedimentation		
	1 week	translucent, white dispersion, no sedimentation		
	2 weeks	translucent, white dispersion, no sedimentation		
	3 weeks	translucent, white dispersion, no sedimentation		
	1 month	translucent, white dispersion, no sedimentation		

Note: +1 to +5 = degree of sedimentation from low to high

Table 3.8 Physical appearances of the four selected liposomes entrapped with human insulin stored at different temperatures for 4 months (continued)

Formulations (molar ratio)	**Time**	**Physical appearances at various temperatures**		
		4 ^{o}C	**30 ± 2 ^{o}C**	**45 ^{o}C**
DPPC/CTA/DDAB (7:2:1)	2 months	translucent, white dispersion, no sedimentation,	translucent, white dispersion, sedimentation, +1	translucent, white dispersion, sedimentation, +1
	3 months	translucent, white dispersion, no sedimentation,	translucent, white dispersion, sedimentation, +3	translucent, white dispersion, sedimentation, +2
	4 months	translucent, white dispersion, no sedimentation,	translucent, white dispersion, sedimentation, +3	translucent, white dispersion, sedimentation, +2

Note: +1 to +5 = degree of sedimentation from low to high

Table 3.8 showed the physical appearances of liposomal formulations composed of DPPC/chol(at 7:3 molar ratio), DPPC/Chol/DDAB (at 7:2:1 molar ratio), DPPC/ Chol/CTA (at 7:2:1molar ratio), and DPPC/CTA/DDAB (at 7:2:1 molar ratio)

entrapped with human insulin at different temperatures for 4 months. The best liposomal formulation which was selected for the entrapment of human insulin was DPPC/CTA/DDAB because it exhibited the best physical stability than other formulation during 1 month. Other liposomal formulations were precipitated and the color was changed from white in yellow.

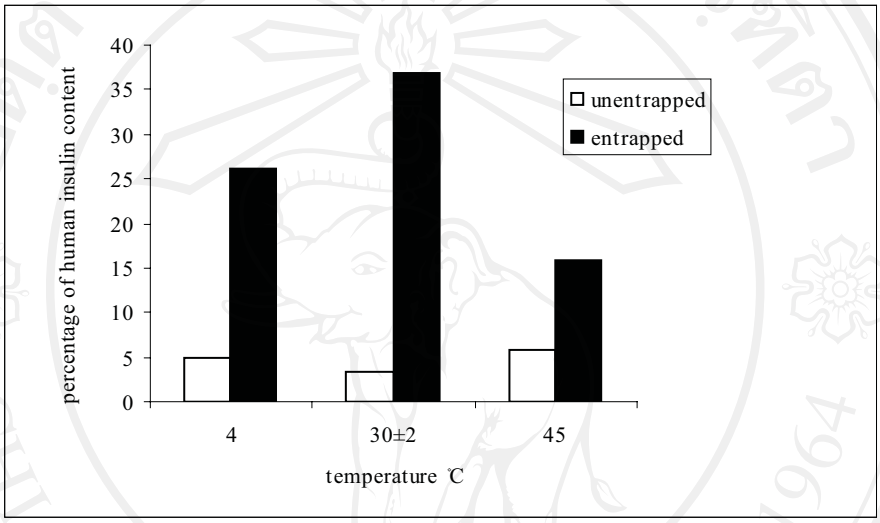


Figure 3.24 Percentages remaining of human insulin entrapped and not entrapped in liposome (DPPC/CTA/DDAB at 7:2:1 molar ratio) stored at different temperatures for 4 months

Table 3.9 Percentage remaining of human insulin entrapped and not entrapped in liposome (DPPC/CTA/DDAB at 7:2:1 molar ratio) at initial and stored at different temperatures for 4 months

Temperature	**The percentage remaining of human insulin**			
	At initial		**After 4 month**	
	Liposome	**Solution**	**Liposome**	**Solution**
4 ^{0}C	86.10%	88.74%	26.21%	4.86%
30 $\pm$ 2 ^{0}C			36.86%	3.26%
45 ^{0}C			15.75%	5.82%

The percentages remaining of human insulin entrapped in DPPC/CTA/DDAB (at 7:2:1 molar ratio) was higher than the unentrapped human insulin solution after 4 months at all temperatures. The highest percentages of human insulin remaining was human insulin entrapped in the DPPC/CTA/DDAB (at 7:2:1 molar ratio) liposome at 30 $\pm$ 2 ^{0}C of 36.86% . At higher temperature, the structure of the lipid membrane becames more irregular and looser, and the permeability of the lipid and as a result, the encapsulation efficiency was higher (Huang an wang., 2006) Thus, more insulin can be promoted by entrapping in liposome thereby increase the entrapment efficiency after 4 months than other temperature (Figure 3.25). At 4 ^{o}C the liposomal membrane may be more rigid than at 30 ^{o}C.

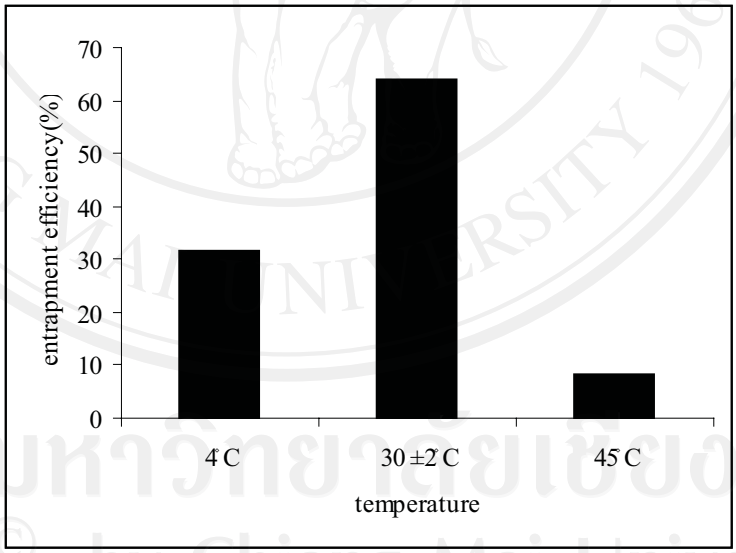


Figure 3.25 Percentage of entrapment efficiency of human insulin entrapped in liposome composing DPPC/CTA/DDAB at 7:2:1 molar ratio at different temperatures for 4 months

However, the percentages remaining of human insulin entrapped in the liposome at 45 ^{0}C was the lowest because this temperature was higher than the human

physiological temperature (37 ^{0}C), which may degrade the human insulin (Shriniwas et al., 1981).

Our previous study has demonstrated that the entrapped plasmid in the vesicles was more stable than the plasmid not entrapped due to the vesicular protection of the plasmid against high temperature (Manosroi et al., 2008). This study has suggested that human insulin entrapped in the developed liposomal formulations gave high entrapment efficiency and was more stable than the human insulin in an aqueous solution when exposured to light and different temperatures for 4 months.