CHAPTER IV

RESULTS AND DISCUSSION

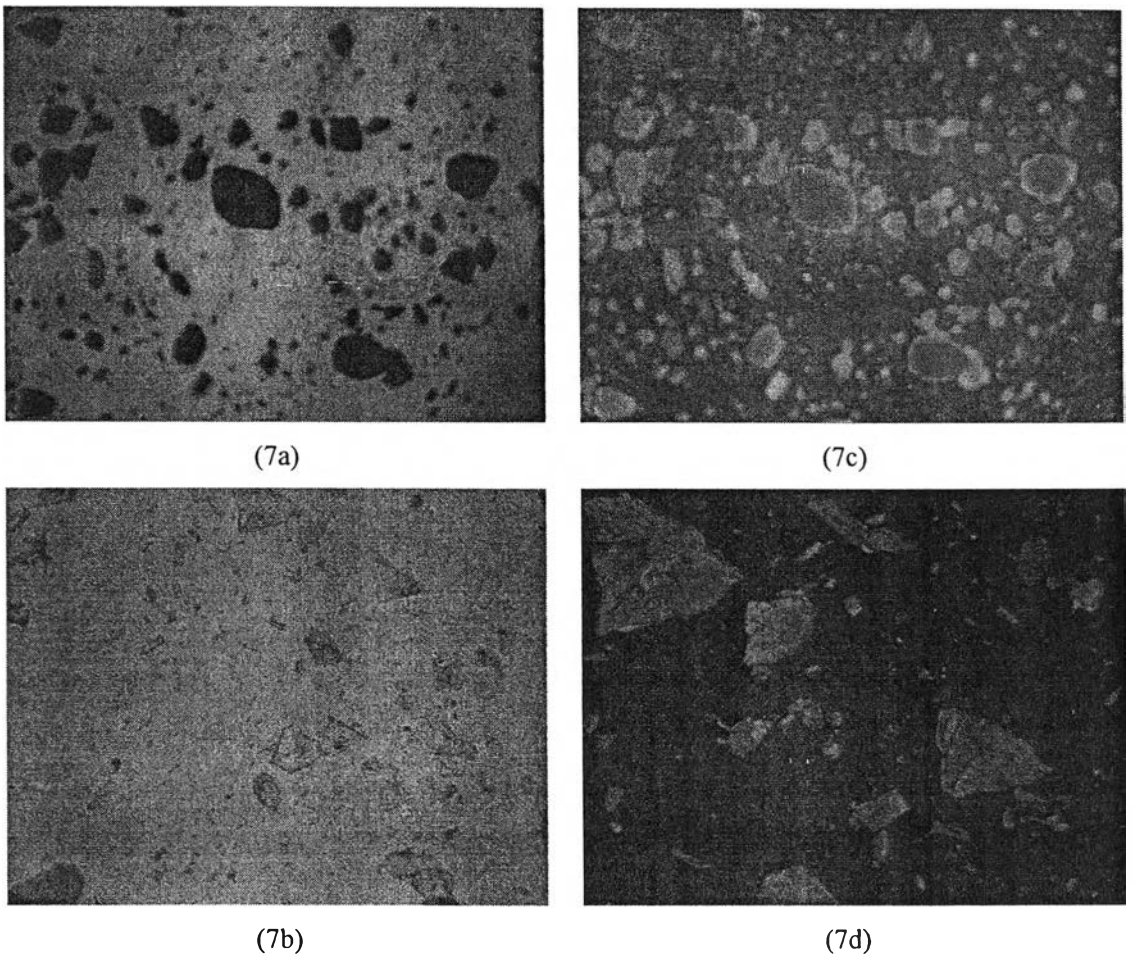
1. Characterization of saquinavir mesylate (SQV) and recrystallized SQV (R-SQV)

Morphological study

Saquinavir mesylate powder (SQV) and recrystallized SQV (R-SQV) were investigated for their morphology. The morphology of SQV and R-SQV was observed by an optical microscope **(Figures 7a-7d)** and a scanning electron microscope **(Figures 9a-9d).** When observed under polarlized light, the birefringence property of SQV and R-SQV was shown as presented in **Figure 7c** and **Figure 7d**, indicating that both SQV and R-SQV were crystals. The birefringence property is found only in crystal while amorphous form would not reflect under polarlized light.

The crystallinity of R-SQV was also detected by a hot stage microscope in the temperature range of 30-260°C. When the temperature was increased from 95°C to the end of experiment, the solvent bubbles which may be alcohol or water existed from the R-SQV crystal **(Figure 8).** It would be the effect of solvent, 95% ethanol, and the simulated conditions of drug dissolving and solvent evaporation during niosome and proniosome preparation. R-SQV could be solvate, hydrate or mixed molecular adduct. The solvate or mixed molecular adduct form of SQV may possess lower solubility compared to SQV solubility in aqueous media.

Size and shape of SQV and R-SQV were observed using a scanning electron microscope. The morphology of both substances is presented in **Figure 9.** SQV and R-SQV were in plate-like shape. The figures showed that the particle size of R-SQV **(Figure 9b and Figure 9d)** was markedly larger than that of SQV **(Figure 9a and Figure 9c).** The larger size of R-SQV resulted from the recrystallization process which may allowed slow crystal growth rate which provided larger size of crystal.



(7a) (7c)

(7b) (7d)

Figure 7. The photomicrographs from a light microscope in the 2.5×10 magnification:

(7a) SQV (saquinavir mesylate)

(7b) R-SQV (recrystallized saquinavir mesylate)

(7c) SQV (saquinavir mesylate) observed under polarized light

(7d) R-SQV (recrystallized saquinavir mesylate) observed under polarized light

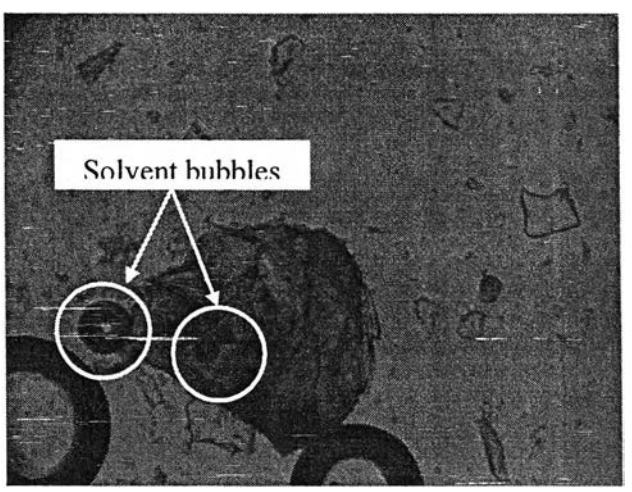


Figure 8. Recrystallized saquinavir mesylate (R-SQV) under hot stage microscope in the 2.5×4 magnification

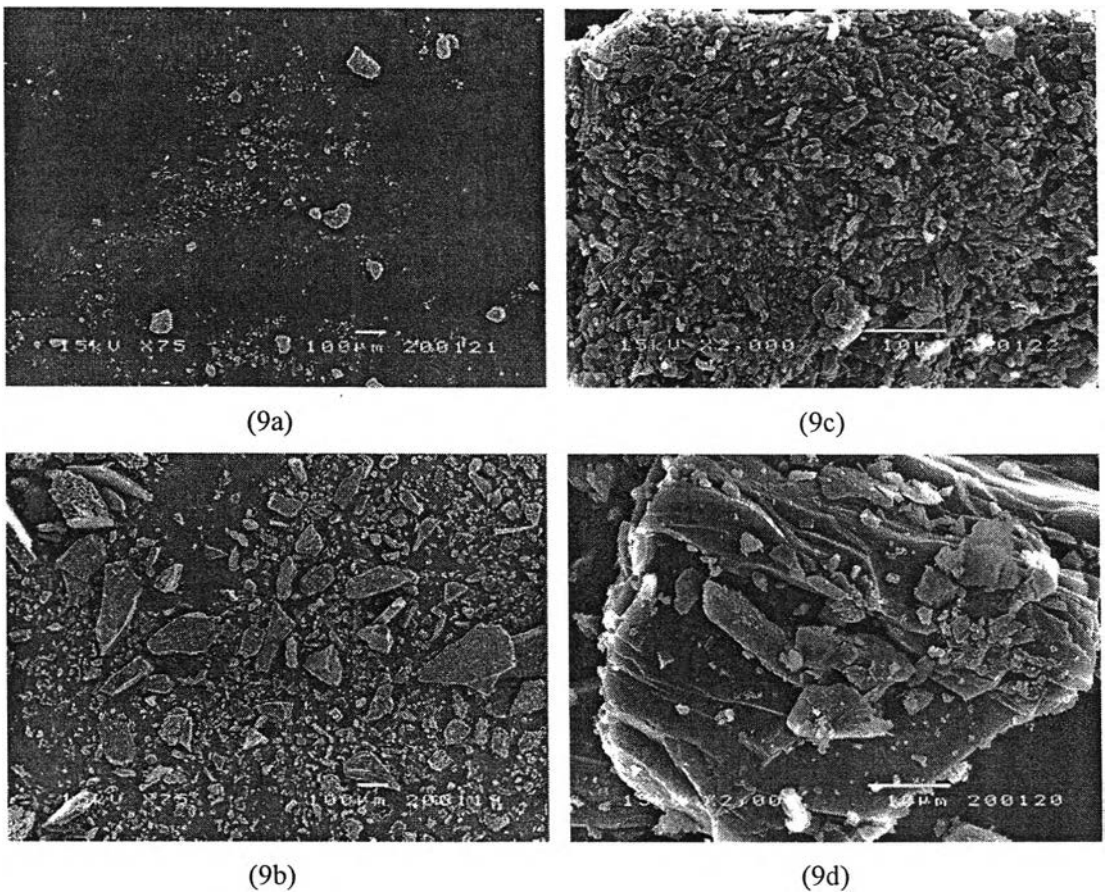


Figure 9. The morphology of SQV (saquinavir mesylate) and R-SQV (recrystallized saquinavir mesylate) (9a) SQV (×75), (9b) R-SQV (×75), (9c) SQV (×2000) and (9d) R-SQV (×2000) observed under a scanning electron microscope in the ×75 and ×2000 magnification

Particle size and size distribution

Particle size and size distribution of SQV was measured using a laser diffraction particle size analyzer. The result showed that the mean diameter of SQV was 109.02 ± 2.80 μm.

Flowability

SQV did not flow through the glass funnel, indicating poor flowability. The plate-like shape of the particles, as presented in **Figure 9a and Figure 9c**, may be the main cause of their poor flowability, whereas the negligible moisture content (0.1 %) may not be attributed to poor flowability. In addition, although in this study the particle size of SQV powder was more than 100 μm, it was observed that SQV powder could perform particle aggregation by static electricity.

Identification

The infrared spectroscopy was used to identify the functional groups of SQV and R-SQV as presented in **Figure 10.** The main peaks of SQV and R-SQV spectra were found in the same regions spanning, i.e. 1025-1140 cm^{-1} (C-O stretching), 1168-1195 cm^{-1} (S=O stretching), 2700-2900 cm^{-1} (C-H stretching) and 3300-3400 cm^{-1} (O-H stretching) (พิมพ์จิต ดามพวรรณ และ วัชรินทร์ รุกขไชยศิริกุล, 2542).

The N-H bending and C=O stretching of amide groups were represented around 1550 cm^{-1} and 1670 cm^{-1}, respectively. There was a difference in peak intensity of the region spanning 3300-3400 cm^{-1} due to the O-H stretching mode. Around this wave number, SQV expressed broad spectrum with small peaks which be due to noises or impurities, whereas R-SQV displayed broader spectrum without those small peaks. The difference in IR spectra of O-H stretching was possibly influenced by O-H groups in drug molecules or solvent (95% ethanol) used in the recrystallization process. This evidence indicated that after recrystallization, R-SQV obtained did not transform into new material.

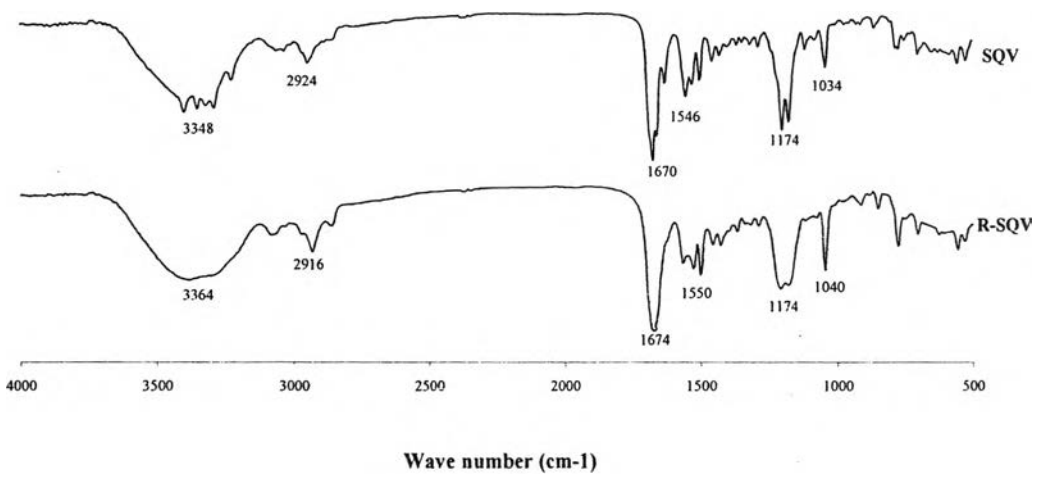
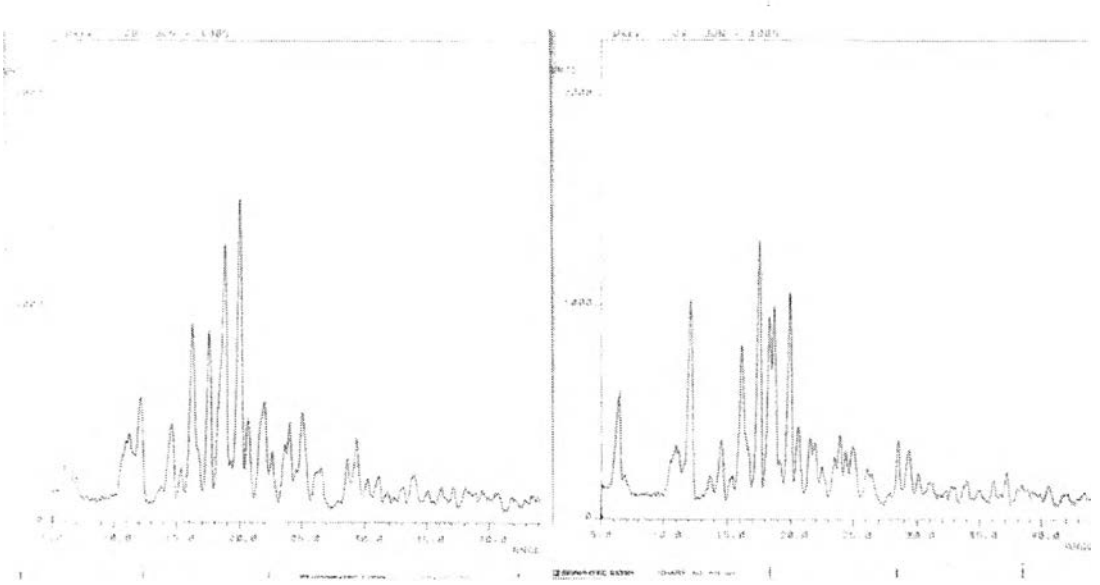


Figure 10. IR spectra of Saquinavir mesylate (SQV) and recrystallized saquinavir mesylate (R-SQV)

Solid state morphology

The X-ray diffraction technique which is useful for characterizing polymorphs was utilized to examine the solid state morphology of SQV and R-SQV **(Figure 11).** The fingerprint patterns of SQV **(Figure 11a)** and R-SQV **(Figure 11b)** revealed that both SQV and R-SQV were in crystal forms. However, their peaks were not identical, implying that these substances are not in the same form. Recrystallization of SQV with the condition used herein can therefore, to some extent, produce another form of SQV. It might also be due to the heat or solvent used in recrystallization entrapped in SQV crystal structure and affected the change in d-spacing, the spacing between the atomic planes lattice unit of drug molecules.



(11a) (11b)

Figure 11. X-ray patterns of (11a) saquinavir mesylate and (11b) recrystallized saquinavir mesylate

Thermal property

Differential scanning calorimetry is generally used to establish the melting points of polymorphic substances. The well-defined melting points of both SQV (251.7°C) and R-SQV (237.5°C) are shown in **Figure 12.** R-SQV is speculated to contain some residual solvent, ethanol, used in the recrystallization process of SQV. DSC thermogram of R-SQV showed the desolvation of solvent entrapped in drug

molecules around 80°C while smooth thermogram was observed in this temperature region of SQV. The drug molecules rearranged in recrystallization process when the temperature was raised to around 200°C, following with drug melting at 237.5°C. These results confirmed that R-SQV was a different form from SQV which showed the sharp endothermic peak of melting point at 251.7°C.

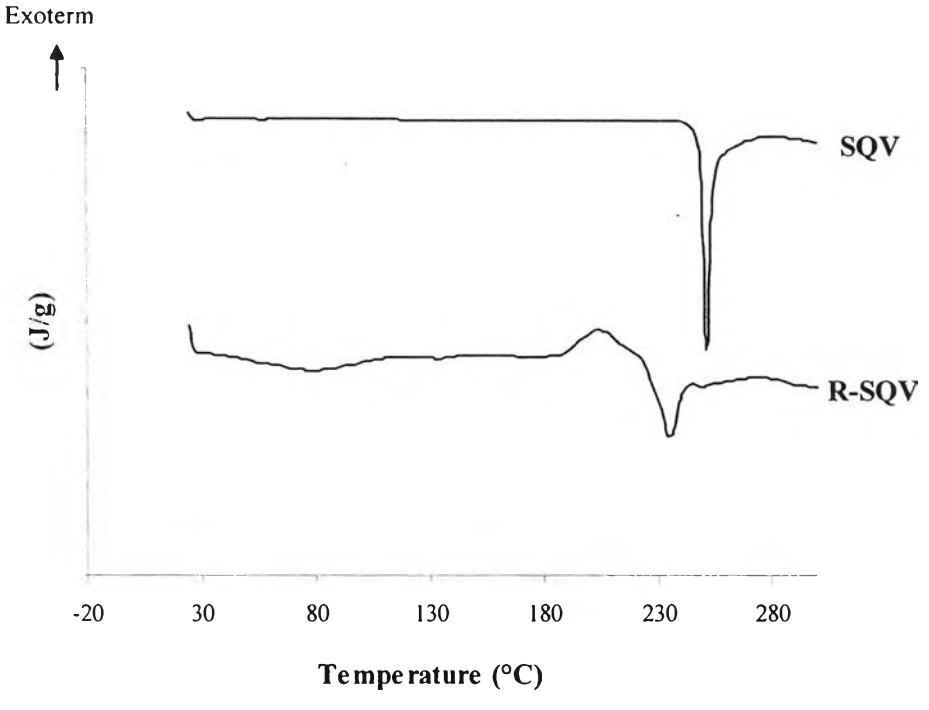


Figure 12. DSC thermograms of saquinavir mesylate (SQV) and recrystallized saquinavir mesylate (R-SQV)

According to the evidence from infrared spectroscopy, SQV and R-SQV were the same materials because they expressed the same functional group. Both SQV and R-SQV exhibited birefringence under a polarizing optical microscope; therefore these substances were crystalline solids. The result from X-ray diffraction showed that SQV and R-SQV were of different crystal forms. Thus, the recrystallization process affected the crytallinity and might allow SQV to be solvate, hydrate or mixed molecular adduct.

Solubility

The solubility of SQV in water, 0.1N hydrochloric acid (0.1N HCl) and phosphate buffer pH 6.8 (PBS pH 6.8) were studied at 37°C over 48 h (**Figure 13**). **Figure 13** illustrates that solubility of SQV was highest in water. The SQV solubility was found to be 2.45 ± 0.02 mg/ml, 0.07±0.01 mg/ml and 0.06±0.00 mg/ml in water, 0.1N HCl and PBS pH 6.8, respectively.

This effect resulted from the difference in pH of media. As pKa of SQV is 7.01 (McEvoy, 2004), at the pH lower than pKa, SQV would ionize hence possess the high solubility. Therefore, SQV was ionized and soluble in water (pH 5.80) in a higher amount than that in PBS pH 6.8. In addition, according to SQV structure which possesses amide groups and quinoline nitrogen (Tan et al., 2003), SQV might be sensitive to hydrolysis and oxidation in acidic environment. Therefore, SQV solubility in 0.1N HCl was low even though SQV was ionized form in 0.1N HCl (pH 1.2).

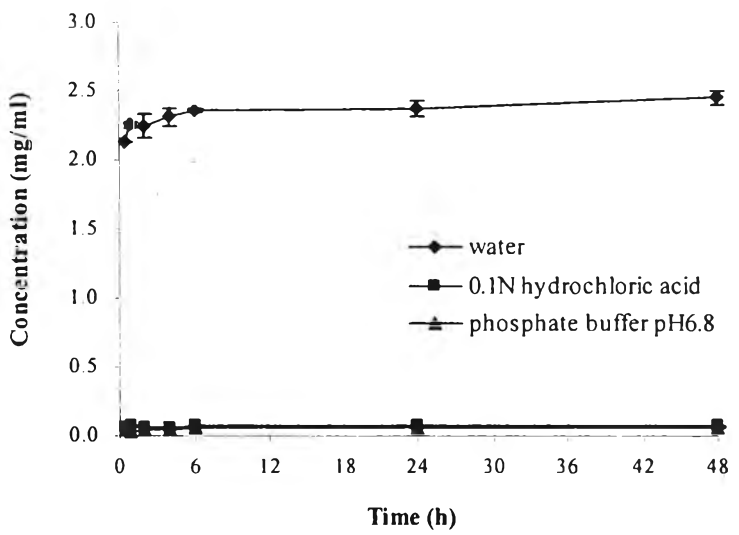


Figure 13. Saquinavir mesylate solubility profiles studied at 37°C, for 48 h, in three different media (♦) water, (▪) 0.1N hydrochloric acid, (▲) phosphate buffer pH 6.8

2. Preparation and characterization of niosomes

As the chemical reaction resistant property and inert to concentrated acidic/alkaline conditions of polyoxyethylene alkyl ether surfactants (Rieger, 1988), the ability to form niosomes of this surfactant group was investigated. The series of polyoxyethylene alkyl ether surfactants, i.e. Brij®30 ($C_{12}EO_4$), Brij®52 ($C_{16}EO_2$), Brij®72 ($C_{18}EO_2$) and Brij®98 ($C_{18}EO_{20}$), were used to study the effects of hydrophilic head groups and hydrophobic side chains of surfactants on niosome formation.

Phase transition temperatures (Tc) of Brij®30, Brij®52, Brij®72 and Brij®98 as studied by running DSC were 5°C, 42°C, 49°C and 41°C, respectively **(Figure 14).** The preliminary study showed that all nonionic surfactants studied together with cholesterol and Simulsol®M52 could form niosomes in water at 70°C, i.e. above their phase transition temperature. The selected mole ratio of nonionic surfactant: cholesterol: Simulsol®M52, 45:45:10, was used to investigate the ability to form niosomes at the body temperature, 37°C, in three different media, i.e. water, 0.1N hydrochloric acid and phosphate buffer pH 6.8. The result of stability study for these formulations would identify the appropriate nonionic surfactant used to form niosomes encapsulating SQV.

2.1 Characterization of niosome prepared in water at 70°C

Morphological study

The morphology of niosomes prepared in water at 70°C was observed using a light microscope. It was found that saturated alkyl surfactants, i.e. Brij®30, Brij®52 and Brij®72, could form niosomes with mean size well below 10 μm as presented in **Table 7.** The unsaturated surfactant, Brij®98 did not form niosomes, possibly due to its improper geometrical structure. The critical packing parameter (CPP) (Israelachvili, 1992) has been used to explain the aggregated forms of surfactant molecules. Surfactant molecules with CPP equal to 1 would aggregate to lamellar phase, while those with CPP less than 1 may form hexagonal and micellar phase. Comparing to the other surfactants, Brij®98 molecule having a large hydrophilic head group area in the structure, i.e. CPP < 1, may promote the formation of micelles rather than vesicles. In addition, a hydrophilic lipophilic balance (HLB) is a good indicator for the ability of amphiphiles to form vesicles. As reported earlier,

surfactants with HLB number between 14-17 could not form niosomes (Shahiwala and Misra, 2002). Therefore, Brij®98 having high hydrophilicity with high HLB value of 15 are preferable to form micelle in aqueous media.

Table 7. Mean size of niosomes prepared with various lipid/ surfactant compositions in water at 70°C (n=3)

Nonionic surfactant			mole ratio of SF: Chol: SM 52	Mean size (SD) (μm)
Type	Chemical structure	HLB		
Brij®30	$C_{12}EO_4$	9.7	60:30:10	5.70 (0.52)
			45:45:10	5.94 (0.61)
			30:60:10	9.18 (0.92)
Brij®52	$C_{16}EO_2$	5.3	60:30:10	8.31 (1.16)
			45:45:10	8.62 (0.79)
			30:60:10	10.04 (0.64)
Brij®72	$C_{18}EO_2$	4.9	60:30:10	8.81 (0.63)
			45:45:10	8.92 (0.76)
			30:60:10	9.91 (1.51)
Brij®98	$C_{18}EO_{20}$	15.3	60:30:10	NA
			45:45:10	NA
			30:60:10	NA

C_xEO_y where x =number of C atom in alkyl chain and y = number of ethylene oxide group in hydrophilic chain

HLB = hydrophilic lipophilic balance (Walters et al, 1981)

SF = nonionic surfactant; Chol = cholesterol; SM52 = Simulsol®M52 and NA = not applicable

Particle size and size distribution

Laser diffraction particle size analyzer was used to determine the particle size in a size range of 0.2-2000 μm. Brij®30, Brij®52 and Brij®72 were found to form niosomes with different sizes in a range of 5.70-10.04 μm. The mean size of these niosomes was affected by the amount of cholesterol added (**Table 7**). The niosome size was found to increase with increased amount of cholesterol. As previously reported, the influence of cholesterol when intercalated in the bilayer membranes

could result in the increase of vesicle size (Yoshioka et al., 1994). The mean size of niosomes also depends on the alkyl chain length of the surfactants (Manosroi et al, 2003). In this study, the result showed that the alkyl chain of Brij®30 (C_{12}) surfactant which is shorter than that of Brij®52 (C_{16}) and Brij®72 (C_{18}) could form the smaller size of niosomes than Brij®52 niosomes and Brij®72 niosomes, respectively **(Table 7)**.

It could be concluded herein that Brij®30, Brij®52 and Brij®72 could form niosomes with various ratios, i.e. 60:30:10, 45:45:10 and 30:60:10 mole ratio of nonionic surfactant: cholesterol: Simulsol®M52. As there was no marked difference in the ability of niosome formation for different mole ratios of lipid/ surfactants, the incorporation of nonionic surfactant and cholesterol in a ratio of 1:1 in bilayer membranes which has been proved to possess physical stability (Uchegbu and Vyas, 1998) was chosen for further study.

2.2 Characterization of niosome prepared in different media at 37°C

In this study, the proposed property of niosomal oral delivery is the ability of nonionic surfactant molecules to form vesicles at body temperature, 37°C. The formation of niosomes comprising nonionic surfactant: cholesterol: Simulsol®M52 45:45:10 mole ratio, where 1:1 nonionic surfactant to cholesterol was incorporated in bilayer membranes in aqueous media at 37°C was investigated.

The results showed that niosomes of Brij®30, Brij®52, Brij®72 which could form niosomes in water at 70°C could also be prepared in three different media, i.e. water, 0.1N hydrochloric acid and phosphate buffer pH 6.8 at 37°C. As the results, it showed that Brij®52 niosomes and Brij®72 niosomes could form at the hydrating temperature lower than their phase transition temperatures, although some crystals-like structures were also found which may imply the uncompleted formation of niosomes **(Figure 17)**. It did not possible to prepare niosomes with Brij®98 at 37°C which could be explained by the critical packing parameter (CPP) as previously described describe again here. It is worth mentioned that nonionic surfactants with certain CPPs and HLB values could form vesicles despite of the varied pH.

The phase transition temperature (Tc) for niosomes containing equimolar amounts of surfactant and cholesterol in water was studied by differential scanning calorimetry (DSC). The DSC thermograms showed that Tc of each surfactant in all niosome formulations could not be detected as depicted in **Figure 14.** Only two main endothermic peaks of water at around 0°C and 100°C most likely belonged to freezing and boiling points of water. This maybe due to the fact that cholesterol abolishes the gel to liquid phase transition of niosomal membranes as previously reported (Cable, 1989). The high relative amount of water in niosomal dispersion may also be the reason for undetectable peak of Tc. Total lipid/ surfactant in niosomal dispersions were low (0.3067-0.3138 mg), being 6.13-6.30% w/w of surfactant sample.

Morphological study

The niosomes were observed under an optical microscope **(Figures 15-17).** The morphology of Brij®30 niosomes, Brij®52 niosomes and Brij®72 niosomes dispersed in various media freshly prepared and after stored at room temperature, 4°C and 45°C for 2 weeks are shown in **Figures 15a-15f, Figures 16a-16f and Figures 17a-17f, respectively.** It was found that these systems exhibited the stability problems like aggregation and fusion, pointed out in the figures, as commonly found in vesicular systems.

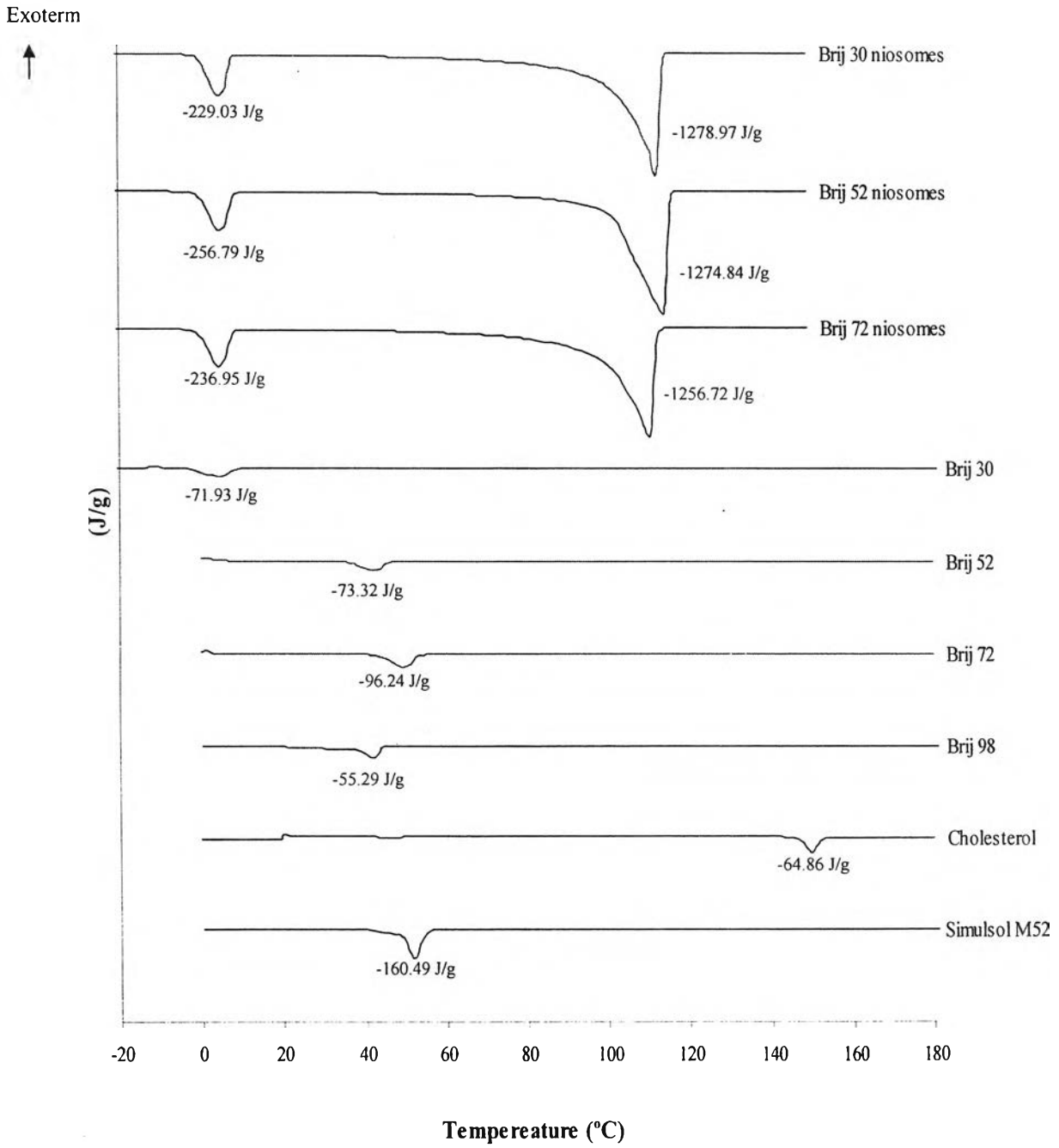


Figure 14. DSC thermograms of niosomal dispersions prepared with various nonionic surfactants, cholesterol and Simulsol®M52 (45:45:10 mole ratio) in water at 37°C, Brij®surfactants, cholesterol and Simulsol®M52

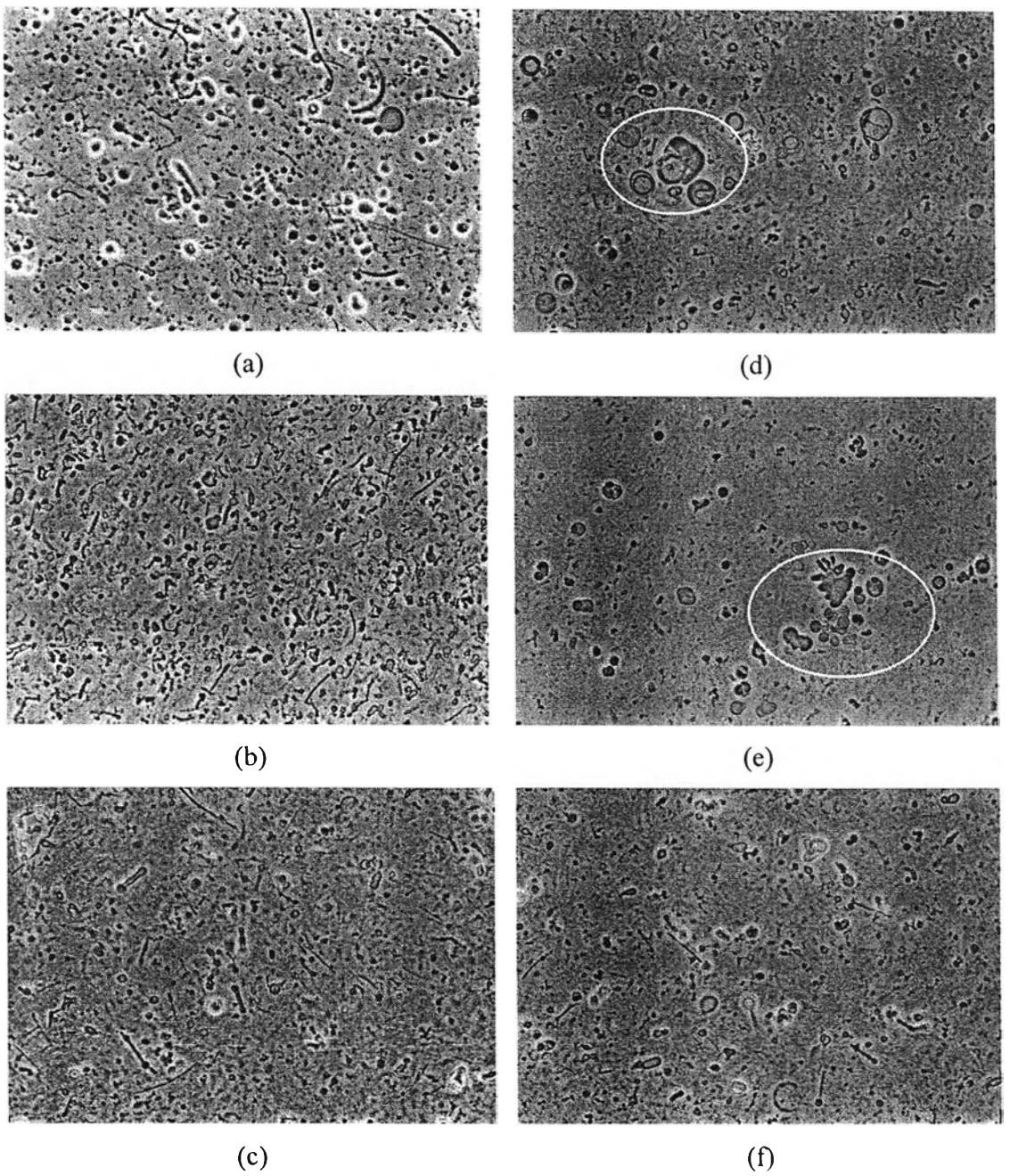


Figure 15 Photomicrographs of Brij®30 niosomes (45:45:10 Brij®30: cholesterol: Simulsol®M52) prepared in various media at 37°C in magnification of x 40

(a) Freshy prepared niosomes dispersed in water

(b) Freshy prepared niosomes dispersed in 0.1N HCl

(c) Freshy prepared niosomes dispersed in PBS pH 6.8

(d) Niosomes dispersed in water after stored at 4°C for 2 weeks

(e) Niosomes dispersed in 0.1N HCl after stored at 4°C for 2 weeks

(f) Niosomes dispersed in PBS pH 6.8 after stored at 4°C for 2 weeks

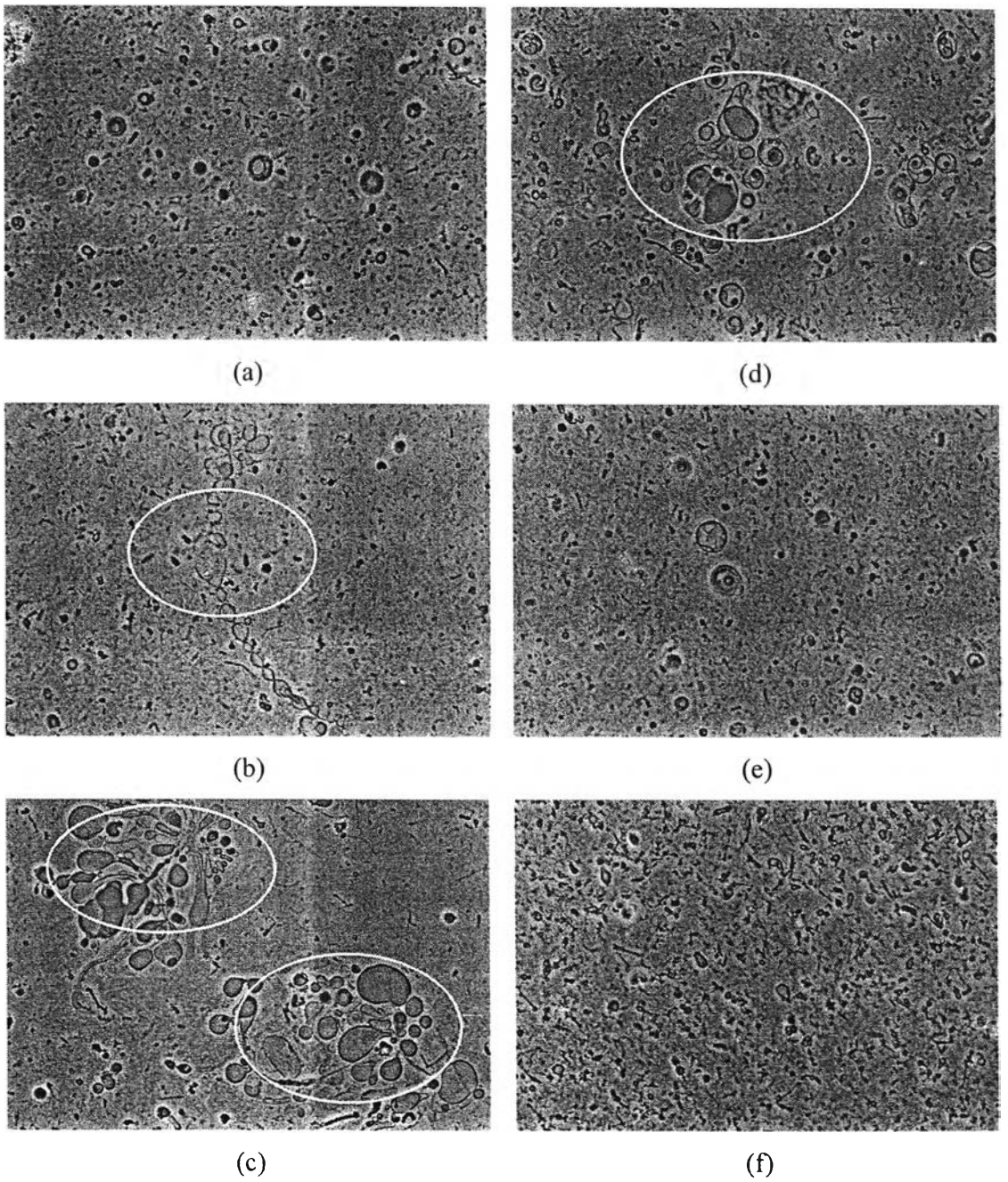


Figure 15 (continue) Photomicrographs of Brij®30 niosomes (45:45:10 Brij®30: cholesterol: Simulsol®M52) prepared in various media at 37°C in magnification of x 40

(a) Niosomes dispersed in water after stored at room temperature for 2 weeks

(b) Niosomes dispersed in 0.1N HCl after stored at room temperature for 2 weeks

(c) Niosomes dispersed in PBS pH6.8 after stored at room temperature for 2 weeks

(d) Niosomes dispersed in water after stored at 45°C for 2 weeks

(e) Niosomes dispersed in 0.1N HCl after stored at 45°C for 2 weeks

(f) Niosomes dispersed in PBS pH 6.8 after stored at 45°C for 2 weeks

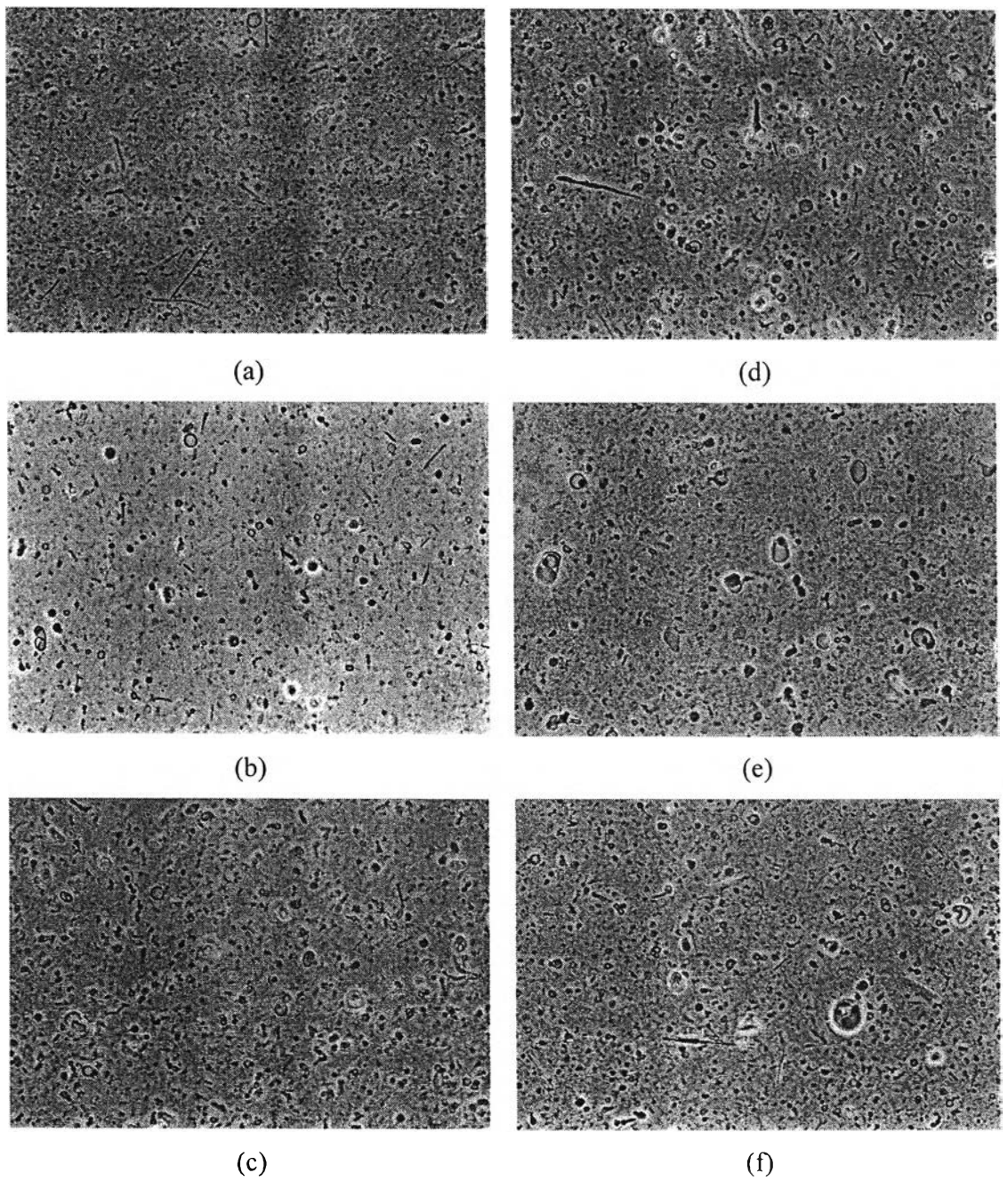


Figure 16 Photomicrographs of Brij®52 niosomes (45:45:10 Brij®52: cholesterol: Simulsol®M52) prepared in various media at 37°C in magnification of x 40

(a) Freshy prepared niosomes dispersed in water

(b) Freshy prepared niosomes dispersed in 0.1N HCl

(c) Freshy prepared niosomes dispersed in PBS pH 6.8

(d) Niosomes dispersed in water after stored at 4°C for 2 weeks

(e) Niosomes dispersed in 0.1N HCl after stored at 4°C for 2 weeks

(f) Niosomes dispersed in PBS pH 6.8 after stored at 4°C for 2 weeks

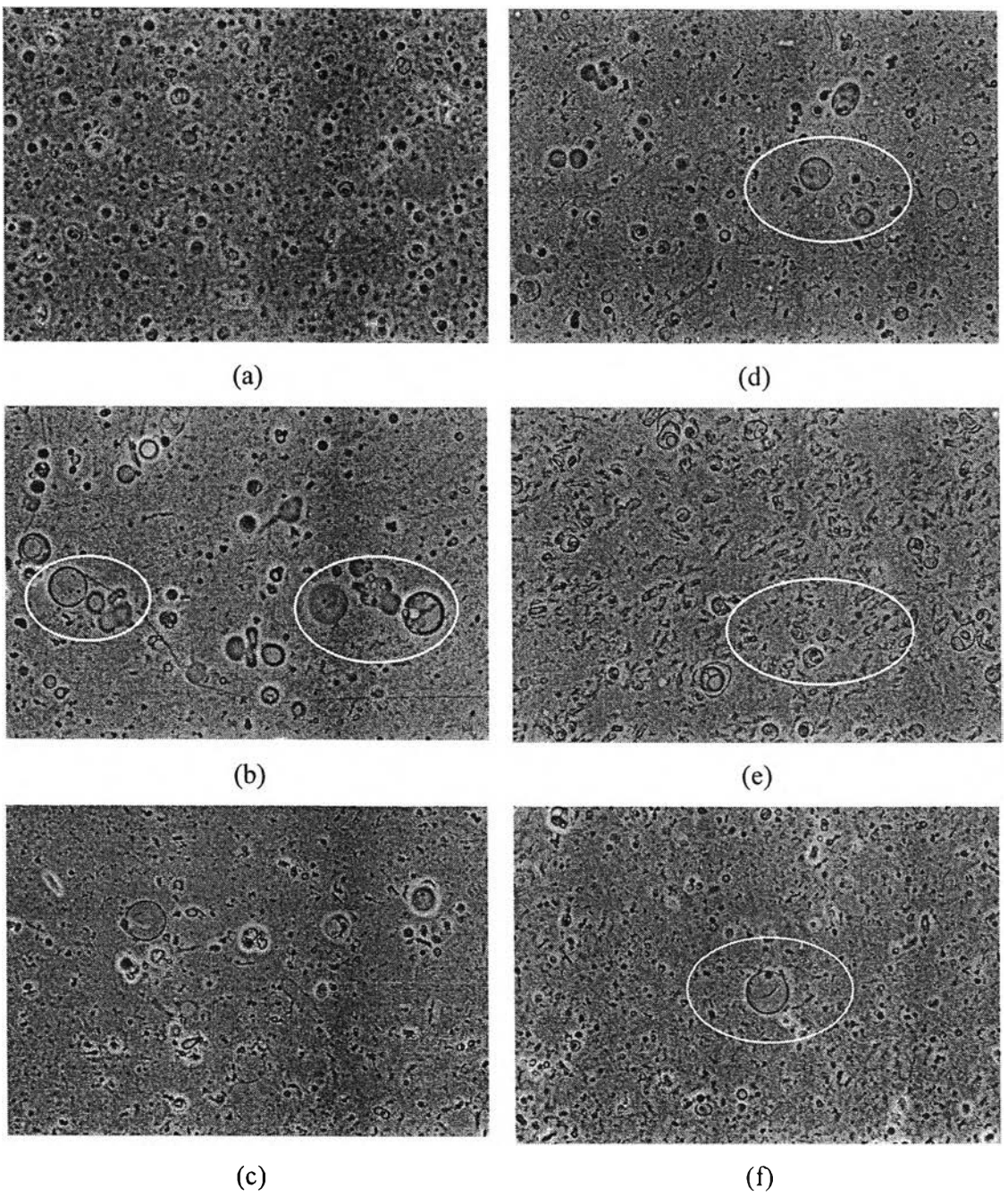


Figure 16 (continue) Photomicrographs of Brij®52 niosomes (45:45:10 Brij®52: cholesterol: Simulsol®M52) prepared in various media at 37°C in magnification of x 40

(a) Niosomes dispersed in water after stored at room temperature for 2 weeks

(b) Niosomes dispersed in 0.1N HCl after stored at room temperature for 2 weeks

(c) Niosomes dispersed in PBS pH 6.8 after stored at room temperature for 2 weeks

(d) Niosomes dispersed in water after stored at 45°C for 2 weeks

(e) Niosomes dispersed in 0.1N HCl after stored at 45°C for 2 weeks

(f) Niosomes dispersed in PBS pH 6.8 after stored at 45°C for 2 weeks

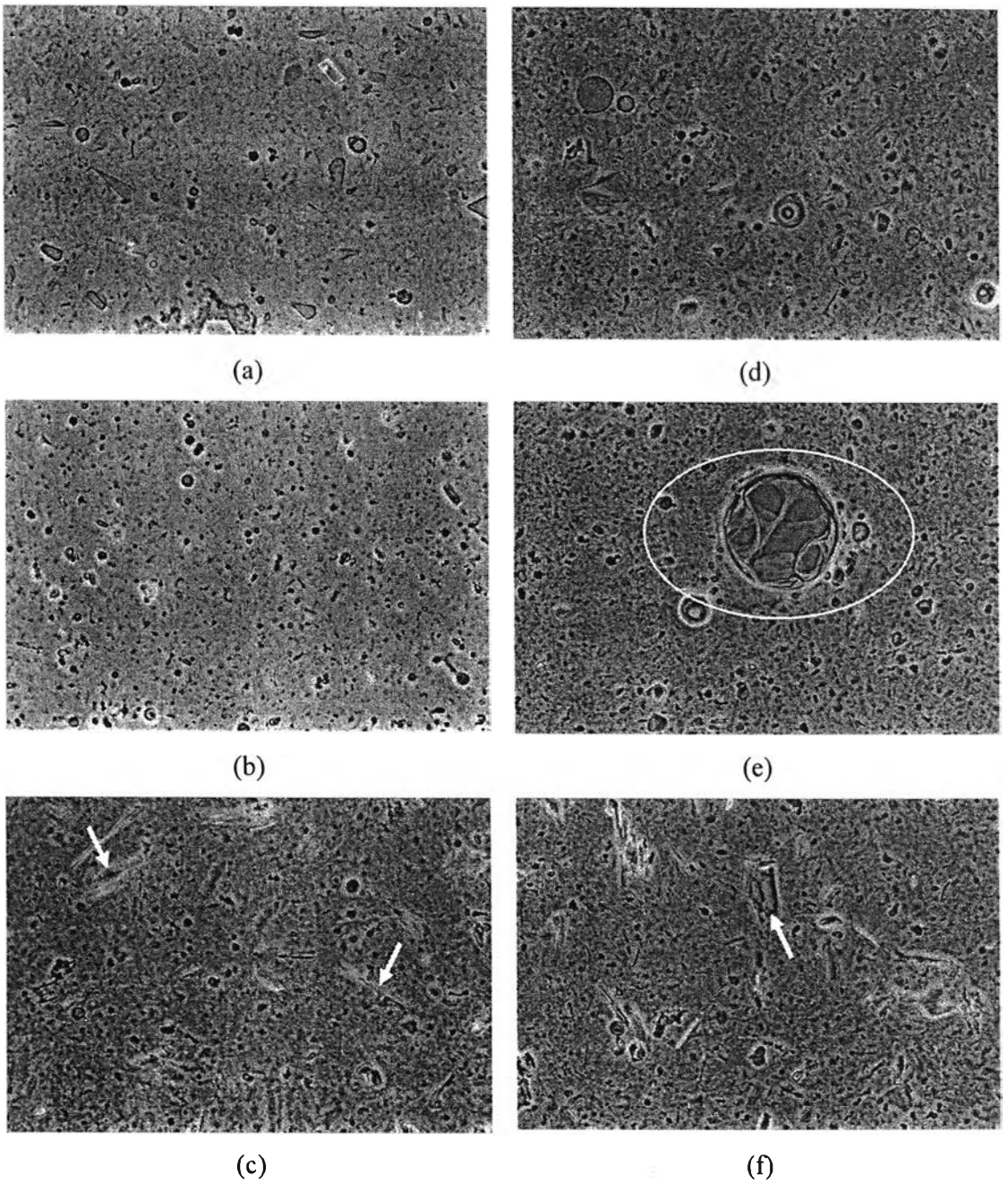


Figure 17 Photomicrographs of Brij®72 niosomes (45:45:10 Brij®72: cholesterol: Simulsol®M52) prepared in various media at 37°C in magnification of x 40

(a) Freshy prepared niosomes dispersed in water

(b) Freshy prepared niosomes dispersed in 0.1N HCl

(c) Freshy prepared niosomes dispersed in PBS pH 6.8

(d) Niosomes dispersed in water after stored at 4°C for 2 weeks

(e) Niosomes dispersed in 0.1N HCl after stored at 4°C for 2 weeks

(f) Niosomes dispersed in PBS pH 6.8 after stored at 4°C for 2 weeks

Arrow point the crystal-like structure found accompanying with vesicles

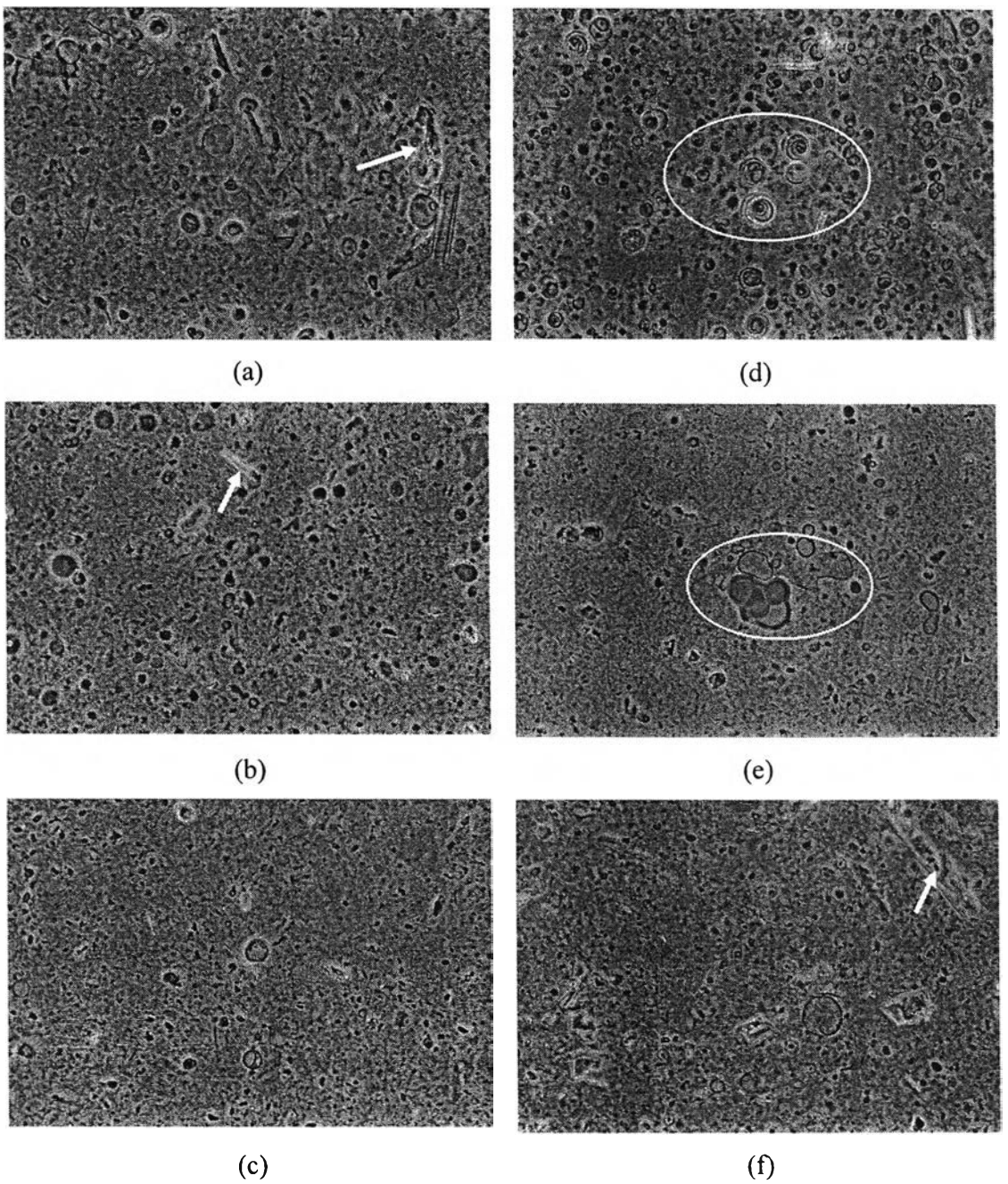


Figure 17 (continue) Photomicrographs of Brij®72 niosomes (45:45:10 Brij®72: cholesterol: Simulsol®M52) prepared in various media at 37°C in magnification of x 40

(a) Niosomes dispersed in water after stored at room temperature for 2 weeks

(b) Niosomes dispersed in 0.1N HCl after stored at room temperature for 2 weeks

(c) Niosomes dispersed in PBS pH6.8 after stored at room temperature for 2 weeks

(d) Niosomes dispersed in water after stored at 45°C for 2 weeks

(e) Niosomes dispersed in PBS pH6.8 after stored at 45°C for 2 weeks

(f) Niosomes dispersed in PBS pH 6.8 after stored at 45°C for 2 weeks

Arrow point the crystal-like structure found accompanying with vesicles

The particle size and size distribution

Size of Brij®30, Brij®52 and Brij®72 niosomal dispersions freshly prepared (day0) and stored at room temperature, 4°C and 45°C for 1 and 2 weeks are shown in **Table 8, 9 and 10**, respectively. As presented in **Figures 15-17**, aggregation and fusion, usually found in niosomal dispersion systems, were also the problems found in this study. The noticeable effect was easily observed for niosomes stored at accelerated temperature, 45°C. It was found that at 45°C storage temperature, size of niosomes dispersed in water and phosphate buffer pH 6.8 was likely to noticeably decrease, while that of in 0.1N hydrochloric acid tended to markedly increase **(Table 10)**.

Table 8. Size of various types of niosomes (45:45:10) prepared in different media at 37°C and stored at room temperature for 2 weeks (n=3)

Formulation	Medium	Mean size (SD) (μm)		
		Day 0	1 week	2 weeks
Brij®30:Chol:SM52	water	9.88 (0.19)	10.19 (0.34)	9.92 (0.25)
	0.1 N HCl	7.52 (0.15)	7.69 (0.25)	11.53 (0.75)
	PBS pH 6.8	7.53 (0.44)	7.22 (0.05)	7.53 (0.43)
Brij®52:Chol:SM52	water	11.46 (1.40)	13.09 (0.29)	13.47 (0.30)
	0.1 N HCl	11.29 (1.44)	13.08 (0.73)	13.27 (0.26)
	PBS pH 6.8	10.43 (0.64)	8.93 (0.12)	9.80 (1.08)
Brij®72:Chol:SM52	water	40.15 (1.13)	38.80 (1.26)	37.97 (2.44)
	0.1 N HCl	24.39 (1.48)	22.44 (1.34)	20.56 (1.56)
	PBS pH 6.8	13.29 (1.61)	10.69 (2.68)	10.52 (2.43)

Chol = cholesterol and SM52 = Simulsol®M52

Table 9. Size of various types of niosomes (45:45:10) prepared in different media at 37°C and stored at 4°C for 2 weeks (n=3)

Formulation	Medium	Mean size (SD), (μm)		
		Day 0	1 week	2 weeks
Brij®30:Chol:SM52	water	9.88 (0.19)	10.19 (0.29)	10.56 (0.65)
	0.1 N HCl	7.52 (0.15)	9.27 (0.36)	8.01 (0.86)
	PBS pH 6.8	7.53 (0.44)	7.50 (0.41)	7.51 (0.44)
Brij®52:Chol:SM52	water	11.46 (1.40)	12.40 (0.64)	12.39 (0.47)
	0.1 N HCl	11.29 (1.44)	11.64 (0.90)	10.98 (1.29)
	PBS pH 6.8	10.43 (0.64)	10.30 (0.74)	9.98 (0.46)
Brij®72:Chol:SM52	water	40.15 (1.13)	36.671.83()	37.84 (1.13)
	0.1 N HCl	24.39 (1.48)	26.82 (1.84)	26.95 (1.91)
	PBS pH 6.8	13.29 (1.61)	11.33 (0.59)	11.50 (0.66)

Chol = cholesterol and SM52 = Simulsol®M52

Table 10. Size of various types of niosomes (45:45:10) prepared in different media at 37°C and stored at 45°C for 2 weeks (n=3)

Formulation	Medium	Mean size (SD), (μm)		
		Day 0	1 week	2 weeks
Brij®30:Chol:SM52	water	9.88 (0.19)	10.90 (0.63)	8.19 (0.98)
	0.1 N HCl	7.52 (0.15)	12.72 (0.72)	13.74 (0.99)
	PBS pH 6.8	7.53 (0.44)	7.04 (0.22)	7.18 (0.43)
Brij®52:Chol:SM52	water	11.46 (1.40)	10.90 (0.30)	10.38 (0.36)
	0.1 N HCl	11.29 (1.44)	13.35 (0.36)	13.06 (0.48)
	PBS pH 6.8	10.43 (0.64)	8.22 (0.24)	7.83 (0.21)
Brij®72:Chol:SM52	water	40.15 (1.13)	13.83 (1.05)	10.68 (1.38)
	0.1 N HCl	24.39 (1.48)	15.10 (1.48)	13.25 (1.65)
	PBS pH 6.8	13.29 (1.61)	12.03 (1.67)	10.64 (2.43)

Chol = cholesterol and SM52 = Simulsol®M52

As the results, Brij®30 surfactant could form niosomes in different media at body temperature, 37°C with the smallest mean size of less than 10 μm. Although on storages all formulations had the stability problems, Brij®30 niosomes was likely to be the most interesting system because their size only increased to about 10 μm. For particulate oral delivery, the size of delivery system is considered as one of the most important factors for the gastrointestinal uptake. Particle of a size below 10μm were found to be taken up mainly via M cells of Peyer's patches (Eldridge et al., 1990). Therefore, Brij®30 surfactant was chosen to prepare SQV encapsulated niosomal dispersion.

2.3 Preparation and characterization of SQV niosomes

According to the appropriate size of Brij®30 niosomes, as observed in section 2.2, with 45:45:10 mole ratio of Brij®30: cholesterol: Simulsol®M52 in water, 0.1N hydrochloric acid and phosphate buffer pH 6.8 were chosen for incorporation of SQV. The effects of lipid/ surfactants mole ratio and lipid/ surfactants concentration on SQV solubility enhancement were studied for niosomes prepared in water. The niosome formulation with appropriate lipid/ surfactants mole ratio and lipid/ surfactants concentration was then selected to investigate the effect of hydrating medium on niosome formation and drug release.

Niosomes prepared from the self-assembly of the nonionic amphiphiles in aqueous media resulting in closed bilayer structure which are capable to dissolve both hydrophobic and hydrophilic substances by incorporating soluble substances into aqueous compartments and incorporating insoluble substances into bilayer membranes. Niosomal system composes of nonionic surfactant vesicles (niosomes) and micelles. Thus, the solubility of SQV was increased by incorporating drug into bilayer membranes of niosomes and solubilzing drug in micelles.

2.3.1 Effects of lipid/ surfactants mole ratio on SQV solubility enhancement

The effect of molar ratio of lipid/ surfactants on solubility enhancement was examined by keeping the total lipid/ surfactants concentration constant at 60 mM. The amount of SQV solubilized in 60 mM of niosomes formed from Brij®30: cholesterol: Simulsol®M52 with various molar ratios, i.e. 90:0:10, 80:0:20 and

70:0:30 and 45:45:10 was investigated. The solubilized SQV is defined here as both SQV encapsulated in niosomal pellets and free SQV dissolved in clear supernatants following ultracentrifugation.

Both entrapped drug and free drug for each formulation are described in terms of amount of SQV (mg/ml) as illustrates in **Figure 18**. The amount of SQV encapsulated in niosomal pellets was markedly lower than those found in clear supernatants. However, there were significant differences in amount of encapsulated SQV between niosomes containing cholesterol (45:45:10 mole ratio) and non-cholesterol niosomes (90:0:10, 80:0:20 and 70:0:30) ($p<0.05$). Brij®30: cholesterol: Simulsol®M52 (45:45:10 mole ratio) encapsulated SQV 1.7-2.3 folds more than other non-cholesterol niosome formulations. It is due to the fact that cholesterol improves the membrane rigidity. Rogerson et al. (1987) found that the efflux of entrapped adriamycin, a hydrophobic drug was decreased by inclusion of cholesterol into the niosomes. Thus, niosomes containing cholesterol were therefore capable to entrap higher amount of SQV.

The amount of solubilized SQV in the niosomal dispersions were also compared among niosomal suspensions formed with various molar ratio of Brij®30: cholesterol: Simulsol®M52 (90:0:10, 80:0:20 and 70:0:30). The highest amount of solubilized SQV was found in niosomal suspension with 30%mole of Simulsol®M52, 70:0:30 mole ratio, being 0.57±0.01 mg/ml in niosomal pellets and 3.93±0.33 mg/ml in clear supernatants. The micelle formation of Simulsol®M52, PEG-40-stearate, may also increase solubilization of drug as amount of SQV in clear supernatants was increased with increased amount of Simulsol®M52. The amount of free SQV in clear supernatants was increased 1.4 fold when Simulsol®M52 increased from 10% to 30% in formulation.

The SQV entrapment efficiency, presented in terms of mole entrapped SQV per mole lipid/ surfactants used for niosome preparation composed of Brij®30: cholesterol: Simulsol®M52 with various molar ratios (90:0:10, 80:0:20 and 70:0:30) was investigated following ultracentrifugation, being 0.0092, 0.0115, and 0.0133, respectively. It was possible due to that the increased amount of Brij®30 readily soluble in the interior of surfactant aggregates, but poorly soluble in the aqueous

solution, resulted in an effective increase in the hydrophobic volume, and therefore also in an increase in the CPP. The resulting structure would therefore alter from lamellar to reverse hexagonal or reversed micelles (Malmsten, 2002). This would be a reason for reduction of encapsulated drug when the proportion of Brij®30 was increased.

In order to investigate the effect of cholesterol on drug entrapment, the amount of entrapped SQV in non-cholesterol niosomes composed of molar ratio of Brij®30: cholesterol: Simulsol®M52 (80:0:20) was compared to that in niosomes containing cholesterol (45:45:10 mole ratio) which included Brij®30 and Simulsol®M52 about 81.12%mole and 18.18%mole, respectively. It was found that niosomes containing cholesterol could entrap SQV 1.7 fold more than non-cholesterol niosomes. The increase in entrapped SQV was attributed to the ability of cholesterol to cement the leaking space in the bilayer membrane, which in turn allowed an increased enhanced SQV level in niosomes (Yoshioka et al.,1994).

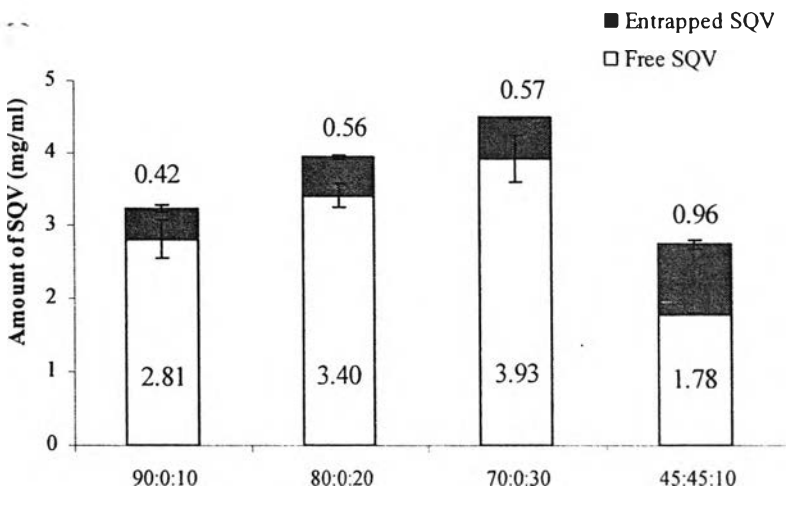


Figure 18. The amount of free saquinavir mesylate (Free SQV) dissolved in clear supernatants and entrapped in niosomal pellets (Entrapped SQV) following ultracentrifugation of 60mM niosomes of Brij®30: cholesterol: Simulsol®M52 (n=3)

The mean size of niosomes prepared from Brij®30: cholesterol: Simulsol®M52 in the mole ratios 70:0:30, 80:0:20 and 90:0:10 determined by photon

correlation spectroscopy was significantly different ($p<0.05$), being 219.5±18.5 nm, 253.3±12.7 nm and 524.2±18.4 nm, respectively. The increase in amount of Simulsol®M52 was found to affect the size of niosomes by allowing the higher curvature of bilayer membrane and produced the smaller size of vesicle. This might be a reason for niosomes forming smaller size with increased Brij®30 content.

In this study, SQV niosomes were developed as a model for an oral drug delivery system via gastrointestinal tract. SQV, a model drug, was found to be entrapped in Brij®30 niosomes in very small amount. This system may therefore be useful only for potent drug delivery such as luteinizing-hormone releasing hormone (Arunothayanun et al., 1999a), doxorubicin (Rogerson et al., 1988) and methotrexate (Azmin et al., 1984). However, niosomal dispersions contain 2 forms of aggregated surfactant systems, i.e. niosomes and micelles. Micelles are formed mostly from Simulsol®M52 and could also be formed from a mixture of Brij®30 and Simulsol®M52. Simulsol®M52, a hydrophilic surfactant, used as a steric stabilizer in niosome formulation was also absorbed in gastrointestinal tract (Francis et al., 2004). It was speculated that niosomal dispersions would enhance the absorption of insoluble drugs utilizing both niosomes and micelles to solubilize the drugs. Although the highest amount of entrapped SQV was found in niosomes formed with cholesterol (45:45:10 mole ratio), the total amount of solubilized SQV in the dispersion (niosomes and micelles) niosomes containing cholesterol (45:45:10 mole ratio) was only 2.74 mg/ml. The total SQV solubilized in the dispersion of 70:0:30 formulation was highest, being 4.50 mg/ml. Thus, this ratio was selected for further studies to improve SQV solubilization.

2.3.2 Effects of lipid/ surfactants concentration on SQV solubility enhancement

Niosomes prepared with 60 mM of 70:0:30 molar ratio of Brij®30: cholesterol: Simulsol®M52 was proved to be the most appropriate molar ratio solubilizing the greatest quantity of SQV in previous study. In order to investigate the effect of lipid/ surfactants concentration on solubility enhancement, varied concentrations of lipid/ surfactants, i.e. 60, 120, 180 and 300 mM were used to prepared niosomes in water.

Figure 19 illustrates the total amount of SQV (mg) solubilized in niosomal dispersion 15 ml with varied lipid/ surfactants concentrations of niosomes. Significant differences ($p<0.05$) in the amount of SQV solubilized in clear supernatants of niosomal dispersions with lipid/ surfactants concentrations of 60, 120, 180 and 300 mM following ultracentrifugation was observed. The increase in the amount of solubilized SQV in the clear supernatants was clearly dependent on the amount of total lipid/ surfactants concentrations. It is known that the solubility of hydrophobic drugs is directly related to the number of micelles (Malmsten, 2002). Besides, SQV might prefer to partition into micelles than vesicle bilayer. The logarithm of the octanol-water partition coefficient (log P) of SQV, being 4.1, indicating SQV is lipophilic drug (log $P>0$) would be able to partition through lipid bilayer. However, there was no significant difference in the amount of SQV entrapped in niosomal pellets ($p>0.05$) when increased lipid/ surfactants concentration from 60 mM to 300 mM. Increasing the lipid/ surfactants concentration did not improve the amount of SQV encapsulation in niosomes, but could increase the amount of SQV solubilized in clear supernatants.

As a result of the amount of entrapped SQV in Brij®30 niosomes (mole ratio 70:0:30), the concentration of 60 mM, was appeared to require the minimum amount of nonionic surfactant used. The weight ratio of encapsulated to free SQV of 60, 120, 180 and 300 mM of niosome suspensions were 1:6.88, 1:16.35, 1:19.44 and 1:25.62, respectively. The concentration of 60 mM was the minimum amount of lipid/surfactants required to give the greatest ratio of encapsulated to free SQV in the system. This concentration was therefore further studied for the effect of hydrating medium on niosome formation.

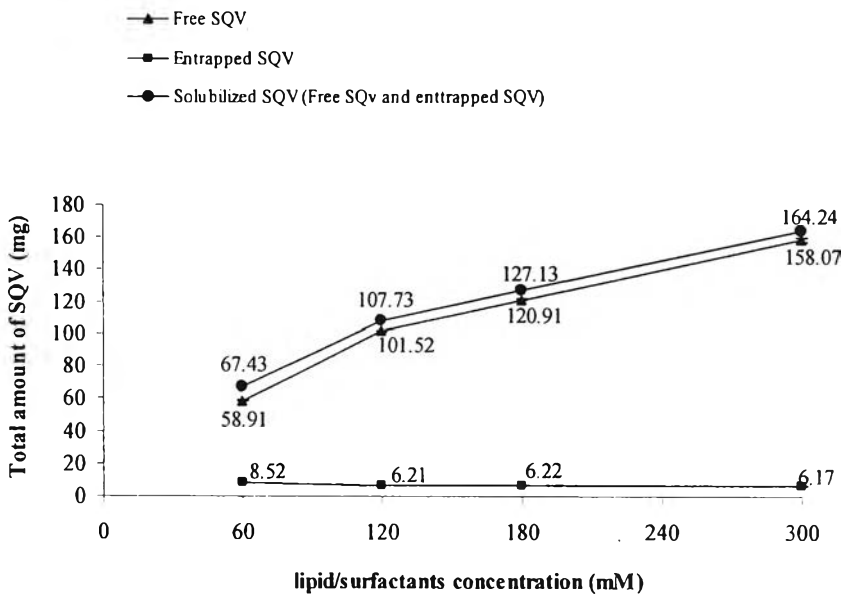


Figure 19. The amount of saquinavir mesylate (SQV) solubilized in niosomal dispersion 15 ml, both in niosomal pellets and dissolved as free SQV in supernatants, in various concentrations of niosomes prepared with Brij®30, cholesterol and Simulsol®M52 (mole ratio 70:0:30) in water (n=3)

2.3.3 Effects of hydrating medium on formation of niosomes

In order to study the possibility of using niosomes for oral delivery, SQV niosomes formed from 60 mM 70:0:30 mole ratio of Brij®30, cholesterol and Simulsol®M52 were prepared in different media, mimicking gastrointestinal fluid. The morphology and phase transition temperature of Brij®30 niosomes dispersed in water, as a representative of niosomes dispersed in aqueous media were studied. Further, the size and size distribution, entrapment efficiency and drug release of niosomes dispersed in water, 0.1N hydrochloric acid and phosphate buffer pH 6.8 were investigated.

The size of SQV niosomes dispersed in aqueous media was too small to be observed by a light microscope. However, it was proved by a transmission microscope that oligolamellar niosomes could form upon hydration dry film of SQV, Brij®30, cholesterol and Simulsol®M52 (70:0:30 molar ratio) with water at 37°C. Niosomes form with approximate size of 180 nm **(Figure 20)**.

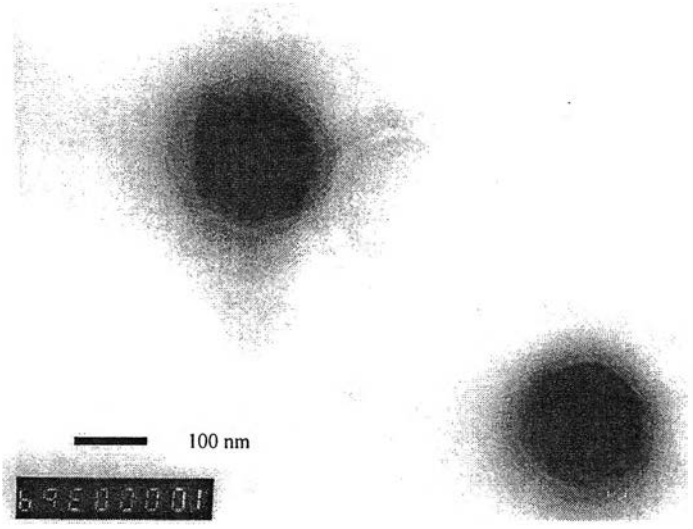


Figure 20. Transmission electron micrograph of SQV niosomes prepared in water at 37°C

Phase transition temperature of niosomes in aqueous media was studied using differential scanning calorimetry (DSC). The DSC thermogram showed that the gel to liquid phase transition temperature of SQV entrapped in Brij®30 niosomes did not appear **(Figure 21)**. There was only the endothermic peak around 100°C which was known as the boiling point of water. Although this formulation was not added cholesterol, there was no DSC peak at 5°C which was the gel to liquid phase transition temperature of Brij®30 surfactant. This might because the amount of Brij®30 surfactant used in the formulation was only 1.43%, while water was the main component which was about 94.65%.

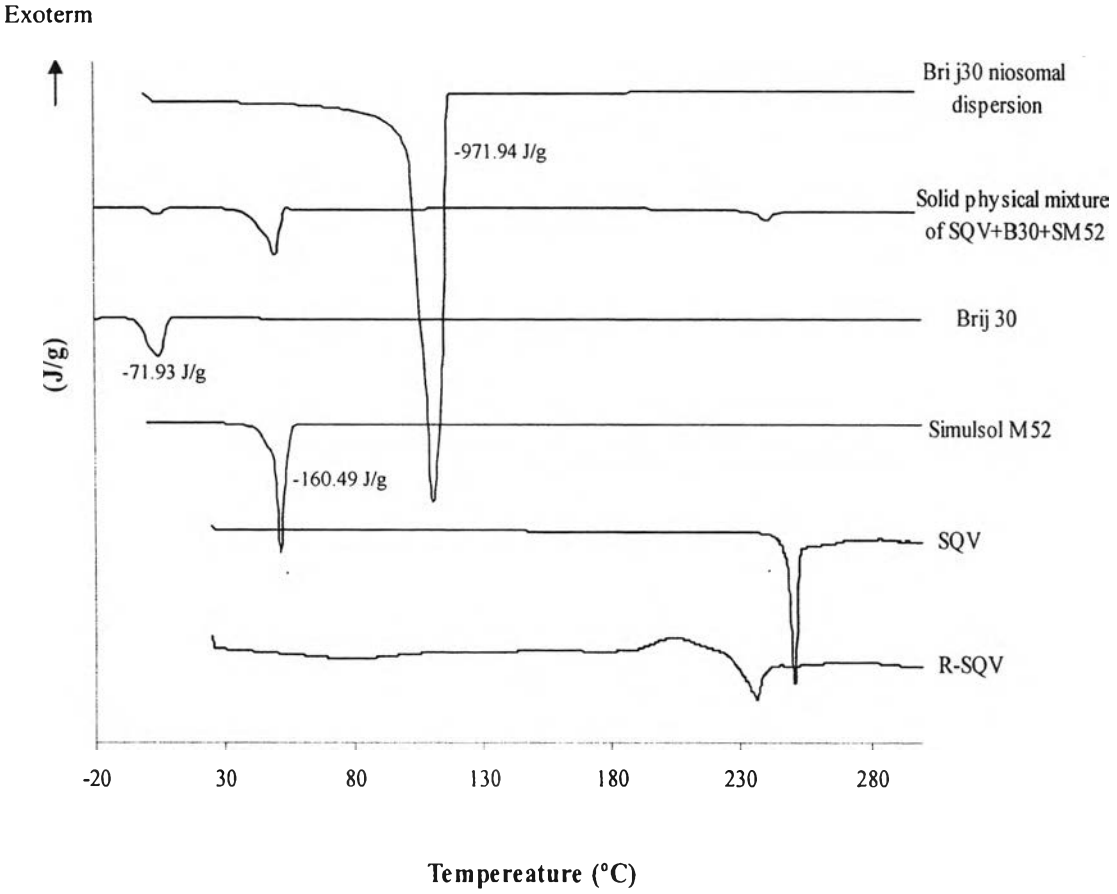


Figure 21. DSC thermograms of SQV niosomes (70:0:30 mole ratio of Brij®30, cholesterol and Simulsol®M52) with total lipid/ surfactants concentration of 4.92 %w/w in dispersion, SQV, R-SQV, Brij®30 and Simulsol®M52

Particle size and particle size distribution

Particle size analysis of 60 mM Brij®30: cholesterol: Simulsol®M52 (70:0:30) niosomes using laser diffraction particle size analyzer was limited to since the lower limit at 0.2 μm. Photon correlation spectroscopy therefore was utilized to measure particle size and size distributions of niosomes in various media. The mean size of niosomes in water, 0.1N hydrochloric acid and phosphate buffer pH 6.8 was comparable, being 206.1±12.2 nm, 206.8±10.0 nm and 244.3±51.8 nm, respectively.

SQV entrapment efficiency

SQV niosomes were analyzed for the quantity of entrapped SQV in terms of entrapment efficiency following ultracentrifugation. The entrapment efficiency of SQV in 60 mM niosome dispersion prepared from Brij®30: cholesterol: Simulsol®M52 in water, 0.1N hydrochloric acid (0.1N HCl) and phosphate buffer pH 6.8 (PBS pH6.8) were neligible, being 0.0030, 0.0003 and 0.0066 (n=3), respectively. SQV was able to be entrapped in niosomes dispersed in PBS pH 6.8 more than those in water and 0.1N HCl. This may due to pKa of SQV (7.01) (McEvoy, 2004) and SQV was less ionized in PBS pH 6.8. As the Handerson-Hasselbach equation, it was found that ratios of ionized form to unionized form of SQV in water, 0.1N HCl and PBS pH6.8 were 16.22, 6.46×10^5 and 1.62, respectively. It could be used to explain that SQV ionized less in phosphate buffer pH 6.8 medium than water pH 5.80 and 0.1N hydrochloric acid (pH 1.2).

Stability of SQV niosomes

Niosomes prepared from Brij®30: cholesterol: Simulsol®M52 with the mole ratio of 70: 0: 30 in water, 0.1N hydrochloric acid (0.1N HCl) and phosphate buffer pH 6.8 (PBS pH 6.8) were pipetted into separated test tubes and incubated at 37±0.5°C with a shaking speed of 120 rpm. The samples were collected at each time intervals, then ultracentrifuged at 4°C to separate the clear supernatants from niosomal pellets. At this temperature niosomes was expected to have minimal thermodynamic energy and hence reduction of SQV partitioning during pellet separation. However, it was also possible that decrease in temperature from 37°C to 4°C might induce SQV precipitation and cause reduction of solubilized drug in clear supernatants. The amount of SQV released was analyzed from clear supernatants and calculated in terms of the percentage of SQV released. Percentage released SQV in each time interval was compared with entrapped SQV at initial time point (t_0).

The SQV released from niosomes (n=3) in three different media is depicted in **Figure 22** showing that SQV released slower in PBS pH 6.8 than in water. It is speculated that SQV (pKa 7.01) may ionize in water (pH 5.80) more than in PBS pH 6.8. Ionized SQV would freely partition from bilayer membrane to aqueous media. Thus, SQV which had more ionized form in water would release from

niosomes more than that in PBS pH 6.8. The amount of SQV released in water increased dramatically and reached the plateau within 2 h, being 80.59±6.57 %. In PBS pH 6.8, the release of SQV was gradual and peaked at 4 h at 62.88±3.03 %. The release in both media was then controlled by niosomes. On the other hand, the release of SQV in 0.1N HCl did not appear in this study. The amount of SQV in clear supernatants which was investigated as released SQV was found to reduce from the initial time point (t_0) in acidic medium. Surprisingly, at 12h, the encapsulated SQV was 49.09±3.59 μg/ml, which was higher than the initially encapsulated amount (16.08±2.85 μg/ml). The fact that the slight amount of SQV was encapsulated in niosomes at the initial time (entrapment efficiency 0.0003) may cause high concentration gradient between bulk solution and the bilayer membrane at the initial time. The concentration gradient would be a driving force for drug molecule to partition into the membrane where the concentration of SQV was lower until it obtained the steady state.

In this study, there was high variation of percentage of SQV released from niosomes because released SQV was analyzed from niosomal dispersions which was collected from separated tubes of each time interval. This result is still useful to explain the stability of SQV niosomes in various media. Stable vesicular dispersion would be expected to remain the initial level of entrapped drug or insignificant amount of drug partitioning into or out of the membrane throughout the time of study. According to the results (**Figure 22**), Brij®30 niosomes were unstable in water, 0.1N HCl and PBS pH6.8.

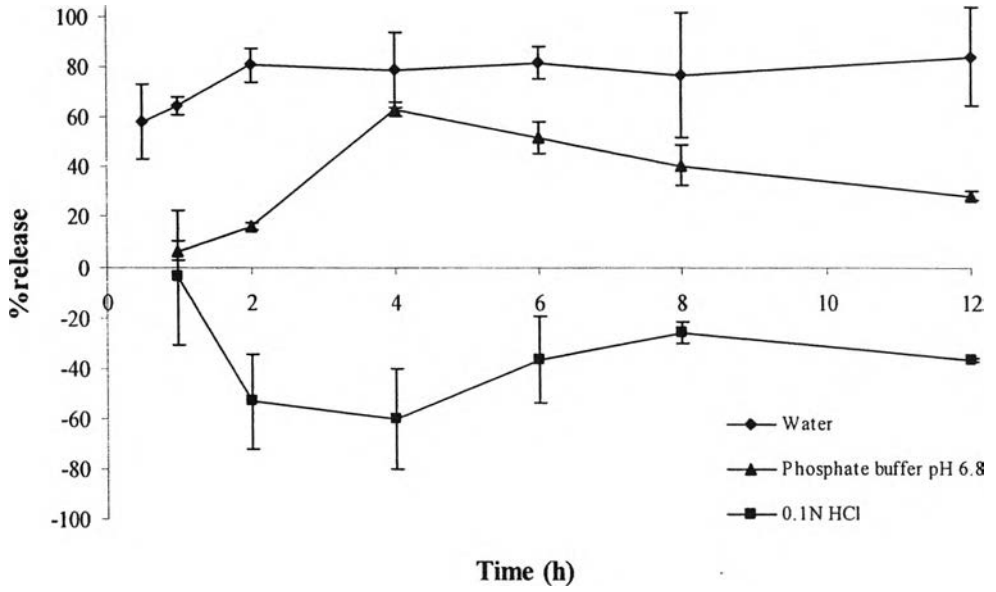


Figure 22. The saquinavir mesylate released from Brij®30 niosomes dispersed in different media at 37°C

3. Preparation and characterization of SQV proniosomes

3.1 Investigation of the possibility to form proniosomal granules

Plain proniosomes were prepared from a mixture of 60 mM Brij®30 niosomes (Brij®30: cholesterol: Simulsol®M52 70:0:30 molar ratio) and lactose. The dried mass was accepted for further study if the moisture content was less than 2%. The amount of lactose required in the formulation was the lowest amount providing oven-dried proniosomal granules within 24 h **(Table 3)**. The amount of niosomal dispersion was kept constant in all formulations, whereas the amount of lactose used was varied from 15 to 35 folds of the total amount of SQV dissolved in niosomal dispersion as determined in section 2.3.1.

Plain proniosomal granules were examined for the flowability **(Table 11)** and moisture content **(Table 12)**. Except for formulation NL15 and NL20, all formulations could be oven-dried within 24 h at 70°C. However these dried granules did not flow. Formulation NL25 was found to require the minimum amount of lactose (112.5 mg, 65.57 % w/w of proniosomal granules) to achieve dried mass

which contained the greatest proportion of drug. The appearance of proniosome formulation NL25 prepared using mass ratio of SQV to lactose being 1:25 is shown in **Figure 23.**

It is worth mentioning that dextrose and sorbitol were also studied as soluble carriers for proniosomes with the weight ratio of SQV and carrier being 1:25. However, all formulations using either dextrose or sorbitol as carriers could not be oven-dried to obtain dried proniosome mass within 24 h at 70°C. This may due to the higher solubility in water of dextrose (1 mg/ml) and sorbitol (2 mg/ml) when compared to lactose (0.22 mg/ ml) (Kibbe, 2000). Dextrose and sorbitol may be therefore considered poor carriers when proniosomes were prepared with the methods stated herein.

Table 11. Flowability of proniosomes prepared from a mixture of plain niosomal dispersion and lactose and subsequent oven drying

Code	Weight ratio of SQV : Lactose	Amount (mg) of solubilized SQV : Lactose	Flowability (g/sec)
NL15	1:15	4.5 : 67.5	NA
NL20	1:20	4.5 : 90.0	Not flow through glass funnel
NL25	1:25	4.5 : 112.5	Not flow through glass funnel
NL30	1:30	4.5 : 135.0	Not flow through glass funnel
NL35	1:35	4.5 : 157.5	Not flow through glass funnel
Lactose	-	-	Not flow through glass funnel

NA = Not applicable

SQV = Saquinavir mesylate

Table 12. Moisture content of proniosomes prepared from a mixture of plain niosomal dispersion and lactose and subsequent oven drying

Code	Weight ratio of SQV : Lactose	Amount (mg) of solubilized SQV : Lactose	% Loss on drying Mean (SD)
NL15	1:15	4.5 : 67.5	NA
NL20	1:20	4.5 : 90.0	2.33 (0.07)
NL25	1:25	4.5 : 112.5	1.67 (0.03)
NL30	1:30	4.5 : 135.0	1.32 (0.01)
NL35	1:35	4.5 : 157.5	1.15 (0.01)
Lactose	-	-	0.12 (0.01)

NA = Not applicable

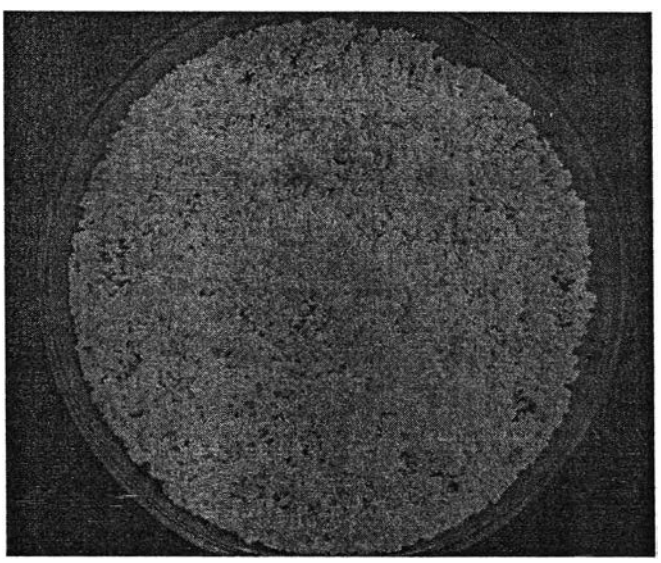


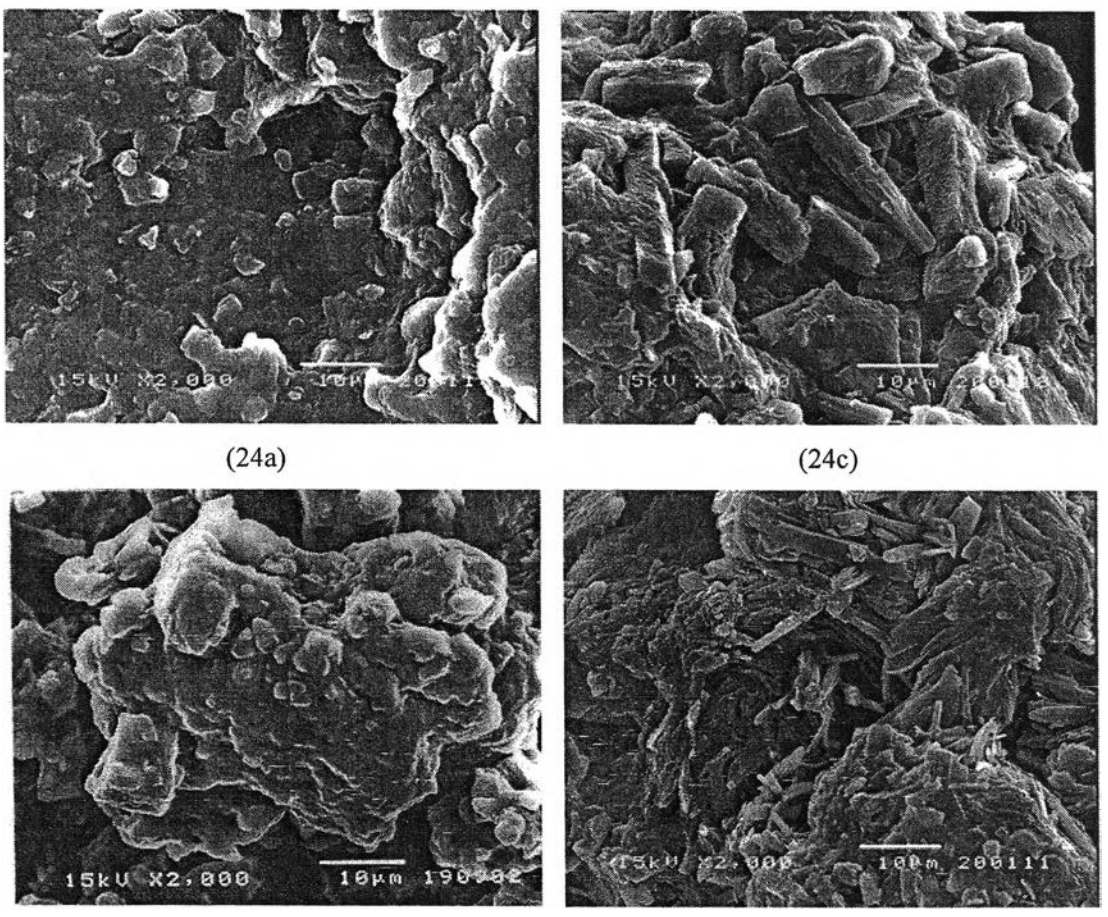
Figure 23. The appearance of proniosome formulation NL25 prepared from a mixture of 60 mM plain niosomes (Brij®30: cholesterol: Simulsol®M52 70:0:30 molar ratio) and lactose (weight ratio1:25) and subsequent oven drying

3.2 Preparation and characterization of SQV proniosomes

Two methods were applied to prepare proniosomes: (i) oven dried mixture of SQV niosomal dispersion and lactose, and (ii) oven-dried mixture of alcoholic solution of SQV, lipid/surfactants and lactose. Dried proniosomal granules could be produced from the mixtures of lactose with both niosomal dispersions and alcoholic solution of lipid/ surfactants.

Morphological study

The sieved dried SQV granules were also observed for the morphology under a scanning electron microscope **(Figure 24).** SQV crystals were found in SQV proniosomal granules, SQV-NL **(Figure 24c)** and SQV-AL **(Figure 24d).** The morphology of SQV proniosomal granules prepared from different methods was different. Proniosome formulation SQV-NL **(Figure 24c)** clearly showed larger crystals which may be resulted from the slower drying of granules in water, which was hydrating medium of niosomal dispersion.



(24a) (24c)

(24b) (24d)

Figure 24. Scanning electron micrograph (×2000) (24a) NL (plain proniosomes prepared from a mixture of plain niosomes and lactose), (24b) AL (plain proniosomes prepared from a mixture of lipid/ surfactants alcoholic solution and lactose), (24c) SQV-NL (SQV proniosomes prepared from a mixture of saquinavir mesylate entrapped niosomes and lactose) and (24d) SQV-AL (SQV proniosomes prepared from a mixture of saquinavir mesylate and lipid/ surfactants alcoholic solution and lactose)

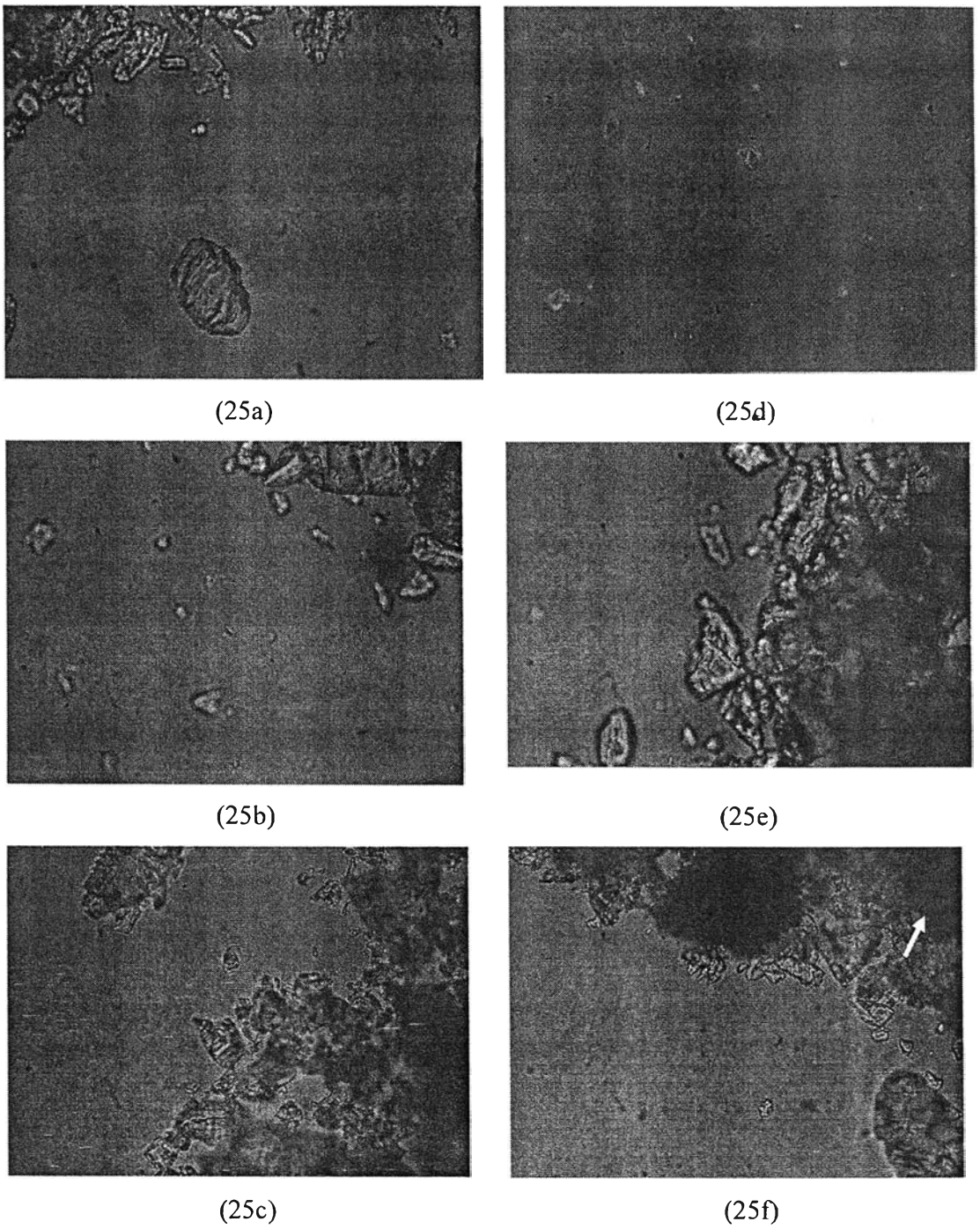
SQV proniosomes prepared from 2 different methods, SQV proniosomes prepared from a mixture of saquinavir mesylate entrapped niosomes and lactose (SQV-NL) and SQV proniosomes prepared from a mixture of saquinavir mesylate and lipid/ surfactants alcoholic solution and lactose (SQV-AL), were investigated for the ability of transformation from proniosomal granules to niosomes under a light microscope **(Figure 25-26)**. SQV proniosomes, formulation SQV-NL and SQV-AL were rehydrated with water, 0.1N hydrochloric acid (0.1N HCl) and phosphate buffer pH 6.8 (PBS pH 6.8). The transformation to niosomes after rehydrating plain proniosomes, formulation NL (plain proniosomes from a mixture of plain niosomes and lactose) and AL (plain proniosomes from a mixture of lipid/ surfactants alcoholic solution and lactose) were also observed for their transformation after rehydrating with these media. The photomicrographs of these proniosomes were recorded 30 seconds after rehydration as shown **in Figure 25 and Figure 26**.

It was found that niosome-derived from proniosome formulation NL and SQV-NL could not be observed upon rehydrating with water, 0.1N HCl and PBS pH 6.8 under a light microscope. **Figure 25** shows the appearance of proniosome formulation NL and SQV-NL with a drop of water **(Figures 25a-25b)**, 0.1N HCl **(Figures 25c-25d)** and PBS pH 6.8 **(Figures 25e-25f)** after rehydrating with these media which no niosomes was observed. It was possible that proniosome formulation NL and SQV-NL transformed to niosomes with size less than that of niosomes prior to prepare proniosomes, being about 200 nm **(Figure 20)**. Hence niosome-derived from proniosomes could not be observed under a light microscope. This assumption agreed with previous study by Hu and Rhodes (1999). They found that size of span®60 niosomes prepared by hand-shaking method was larger and slightly more heterogenous than those derived from proniosomes.

On the other hand, the transformation of proniosomes to niosomes in formulation AL and SQV-AL could be observed **(Figure 26)**. Niosomes spontaneously formed upon rehydrating proniosomal granules with a drop of water **(Figures 26a-26b)**, 0.1N HCl **(Figures 26c-26d)** and PBS pH 6.8 **(Figures 26e-26f)**.

In addition, an amount of 312 mg of proniosome formulation NL, AL, SQV-NL and SQV-AL was added with water, acidic or basic media to 1000 ml and observed under a light microscope. It was found that SQV niosome-derived from proniosome formulation SQV-AL still presented in diluted dispersions (**Figure 27**). Again, after rehydrating proniosome formulation SQV-NL with various media of 1000 ml, none of niosomes could be observed. Niosomes larger than 0.5μm are able to be seen by light microscope (Uchecbu and Vayas, 1998). It was possible that SQV proniosomes prepared from a mixture of entrapped SQV niosome and lactose transformed to niosomes with size less than 200 nm which required a transmission microscope in order to detect niosomes.

The morphology of proniosome-derive niosomes in these diluted media from proniosome formulation NL and SQV-NL (**Figure 28**) and proniosome formulation AL and SQV-AL (**Figure 29**) were also observed under a transmission electron microscope. It showed that Brij®30 niosomes were capable to present in diluted dispersions. Proniosomal granules using Brij®30 and Simulsol®M52 as vesicle forming agents prepared by both methods could therefore transform from proniosomes to niosomes in water, acidic and basic media.



(25a) (25d)

(25b) (25e)

(25c) (25f)

Figure 25. The transformation from proniosomal granules to niosomes of formulation NL (plain proniosomes prepared from a mixture of plain niosomes and lactose), and SQV-NL (SQV proniosomes prepared from a mixture of saquinavir mesylate entrapped niosomes and lactose) observed under a light microscope in 2.5 × 40 magnification (25a) NL rehydrated with water, (25b) NL rehydrated with 0.1N hydrochloric acid, (25c) NL rehydrated with phosphate buffer pH 6.8, (25d) SQV-NL rehydrated with water, (25e) SQV-NL rehydreated with 0.1Nhydrochloric acid and (25f) SQV-NL rehydrated with phosphate buffer pH 6.8

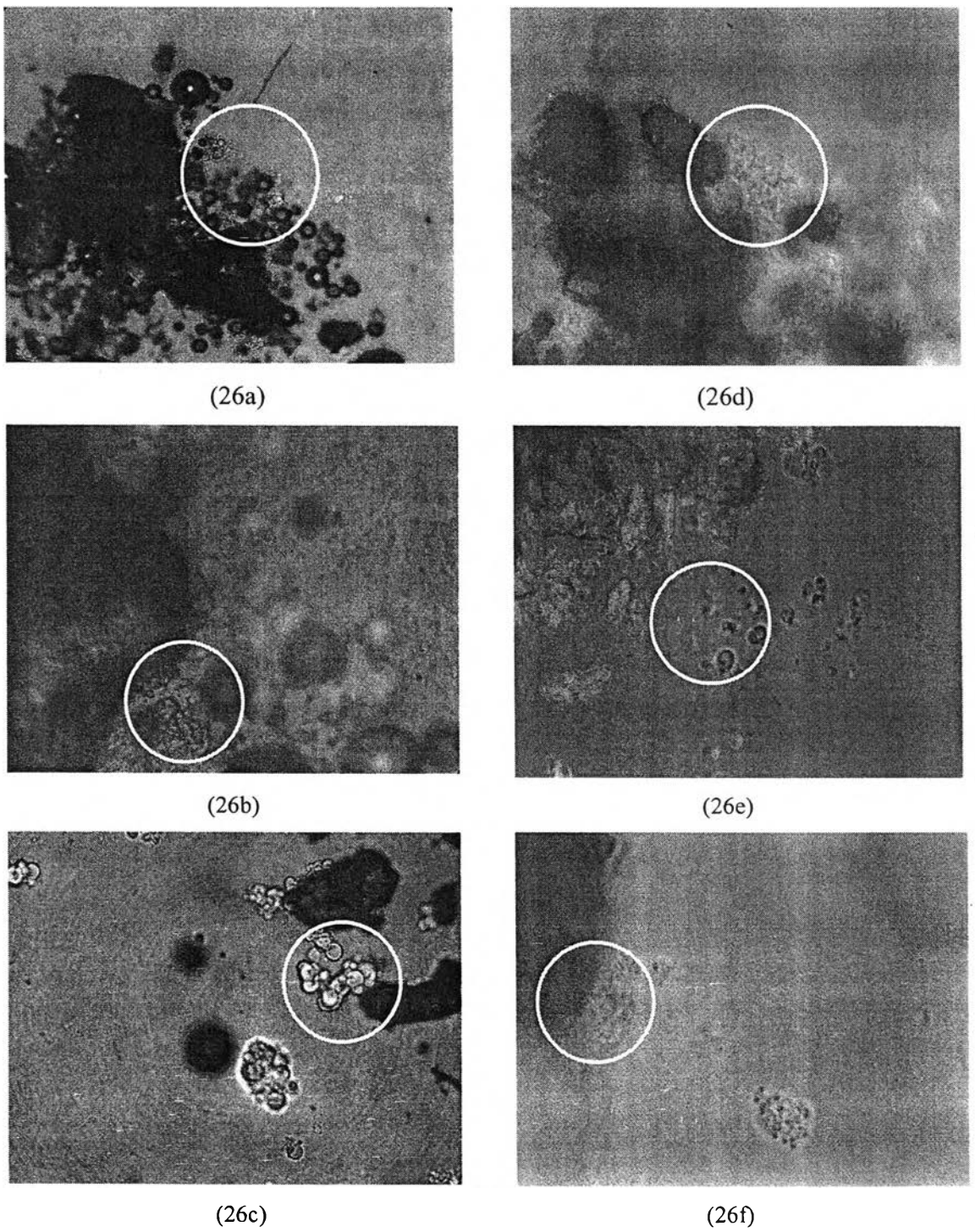
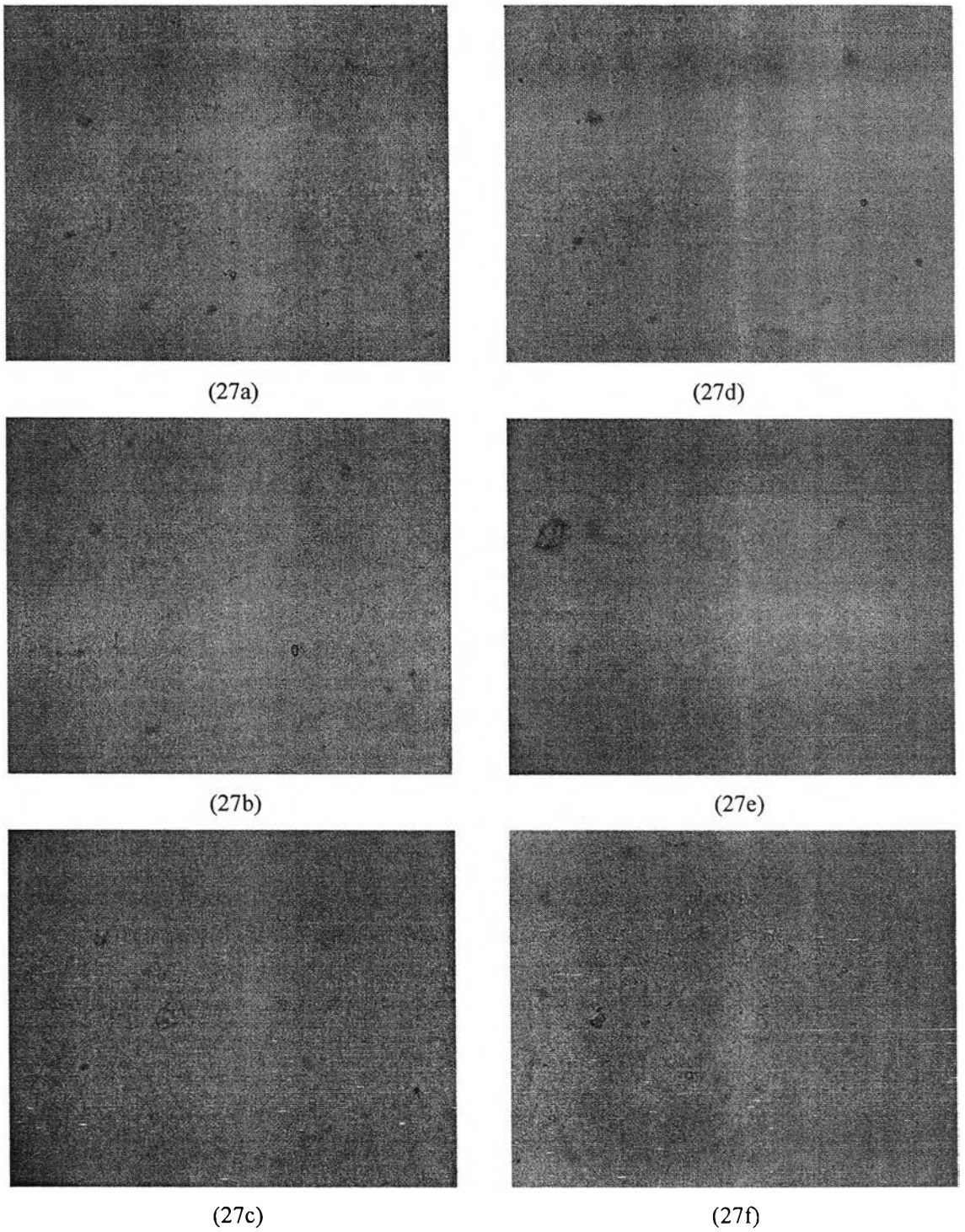


Figure 26. The transformation from proniosomal granules to niosomes (circled) of formulation AL (plain proniosomes prepared from a mixture of lipid/ surfactants alcoholic solution and lactose), and SQV-AL (SQV proniosomes prepared from a mixture of saquinavir mesylate and lipid/ surfactants alcoholic solution and lactose) observed under a light microscope in 2.5 × 40 magnification (26a) AL rehydrated with water, (26b) AL rehydrated with 0.1N hydrochloric acid, (26c) AL rehydrated with phosphate buffer pH 6.8, (26d) SQV-AL rehydrated with water, (26e) SQV-AL rehydreated with 0.1Nhydrochloric acid and (26f) SQV-AL rehydrated with phosphate buffer pH 6.8

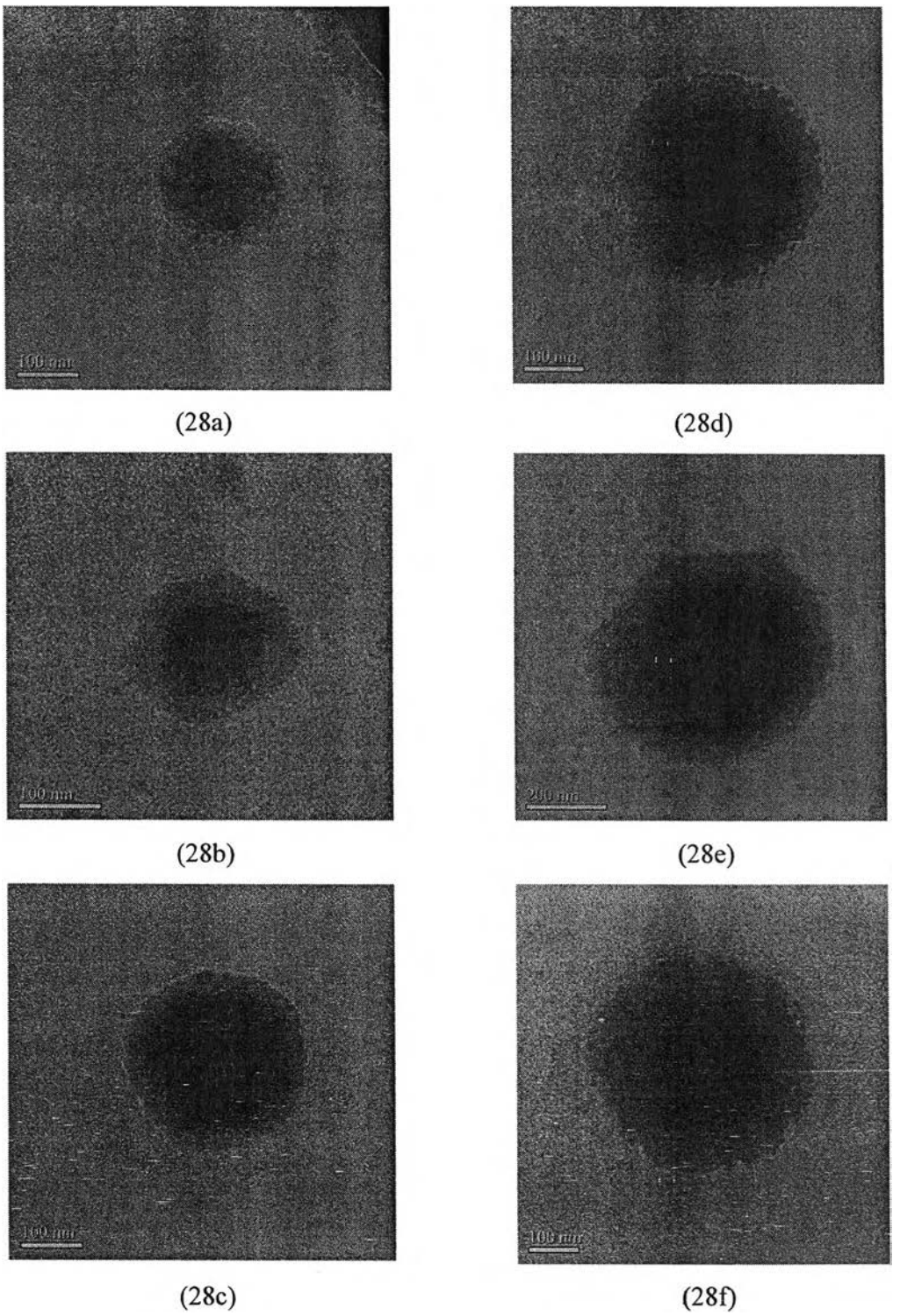


(27a) (27d)

(27b) (27e)

(27c) (27f)

Figure 27. The micrographs of proniosome-derive niosomes in 1000 ml of various media from formulation AL (plain proniosomes prepared from a mixture of lipid/ surfactants alcoholic solution and lactose), and SQV-AL (SQV proniosomes prepared from a mixture of saquinavir mesylate and lipid/ surfactants alcoholic solution and lactose) observed under a light microscope in 2.5 × 40 magnification (27a) AL rehydrated with water, (27b) AL rehydrated with 0.1N hydrochloric acid, (27c) AL rehydrated with phosphate buffer pH 6.8, (27d) SQV-AL rehydrated with water, (27e) SQV-AL rehydreated with 0.1Nhydrochloric acid and (27f) SQV-AL rehydrated with phosphate buffer pH 6.8



(28a) (28d)

(28b) (28e)

(28c) (28f)

Figure 28. The transmission electron micrographs of proniosome-derive niosomes in 1000 ml of various media from formulation NL (plain proniosomes prepared from a mixture of plain niosomes and lactose), and SQV-NL (SQV proniosomes prepared from a mixture of saquinavir mesylate entrapped niosomes and lactose) (28a) NL rehydrated with water, (28b) NL rehydrated with 0.1N hydrochloric acid, (28c) NL rehydrated with phosphate buffer pH 6.8, (28d) SQV-NL rehydrated with water, (28e) SQV-NL rehydreated with 0.1Nhydrochloric acid and (28f) SQV-NL rehydrated with phosphate buffer pH 6.8

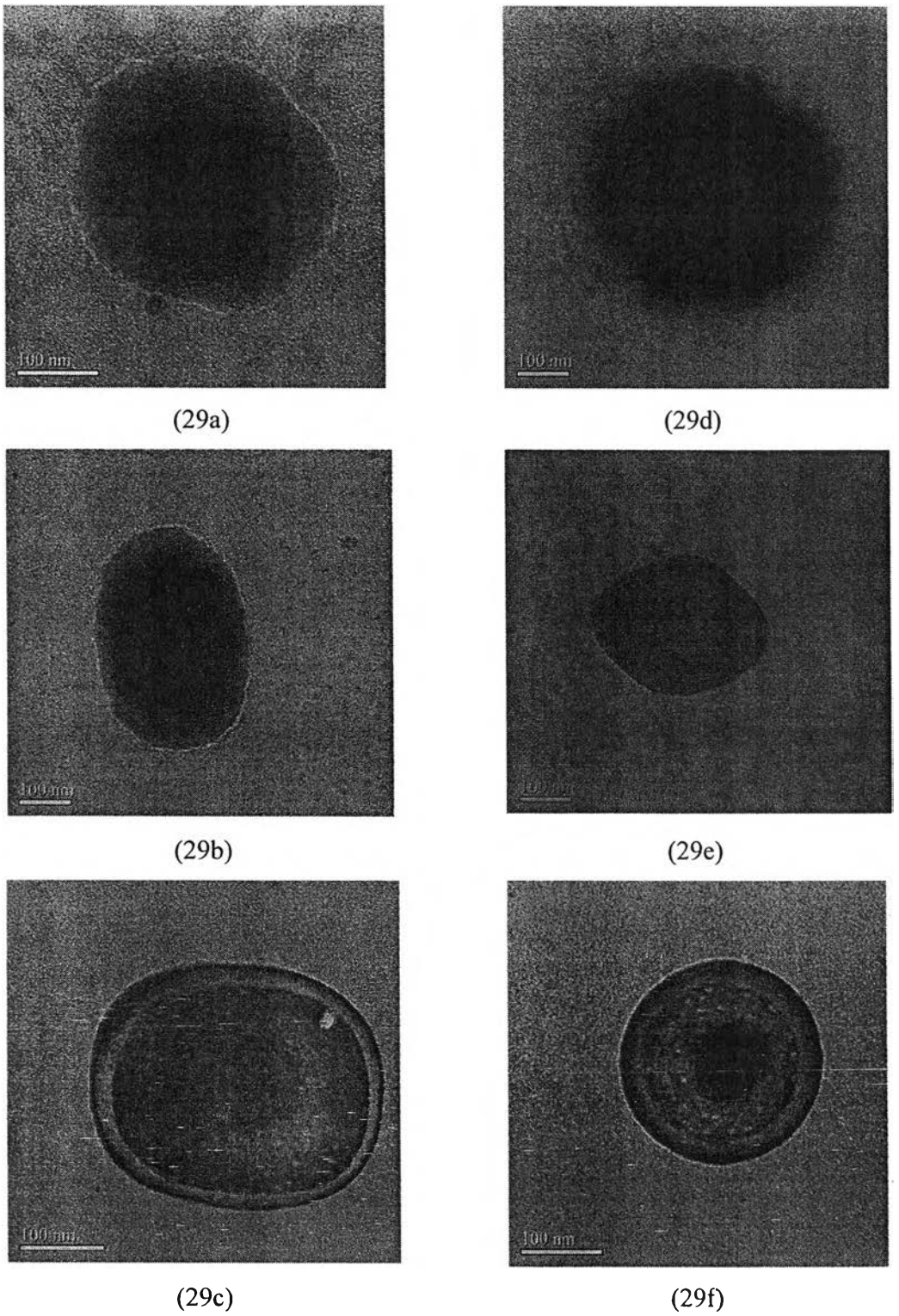


Figure 29. The transmission electron micrographs of proniosome-derive niosomes in 1000 ml of various media from formulation AL (plain proniosomes prepared from a mixture of lipid/ surfactants alcoholic solution and lactose), and SQV-AL (SQV proniosomes prepared from a mixture of saquinavir mesylate and lipid/ surfactants alcoholic solution and lactose) (29a) AL rehydrated with water, (29b) AL rehydrated with 0.1N hydrochloric acid, (29c) AL rehydrated with phosphate buffer pH 6.8, (29d) SQV-AL rehydrated with water, (29e) SQV-AL rehydreated with 0.1Nhydrochloric acid and (29f) SQV-AL rehydrated with phosphate buffer pH 6.8

Particle size and size distribution

The particle size and size distribution of proniosomes formulation NL (plain proniosomes prepared from a mixture of plain niosomes and lactose), AL (plain proniosomes prepared from a mixture of lipid/ surfactants alcoholic solution and lactose), SQV-NL (SQV proniosomes prepared from a mixture of saquinavir mesylate entrapped niosomes and lactose) and SQV-AL (SQV proniosomes prepared from a mixture of saquinavir mesylate and lipid/ surfactants alcoholic solution and lactose) was measured by sieve analysis as shown in **Figure30**. All formulations were to give normal size distribution with the mean size around 0.595-1.00 μm. The result showed that the different methods had no effect on size and size distribution of proniosomal granules. It was a result of controlling particle size of proniosomal granules by sieving the granules containing the same amount of the main components, lipid/ surfactants and lactose. The normal size distribution was worthy for reducing surface area variation of the granules in dissolution studies.

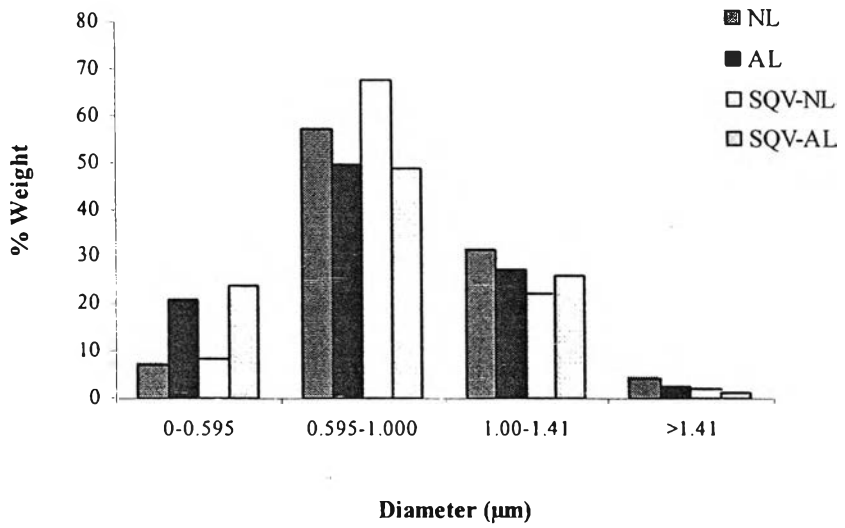


Figure 30. Particle size and size distribution of proniosomes formulation NL (plain proniosomes prepared from a mixture of plain niosomes and lipid/ surfactants solution and lactose), AL (plain proniosomes prepared from a mixture of lipid/ surfactants alcoholic solution and lactose), SQV-NL (SQV proniosomes prepared from a mixture of saquinavir mesylate entrapped niosomes and lactose) and SQV-AL (SQV proniosomes prepared from a mixture of saquinavir mesylate and lipid/ surfactants alcoholic solution and lactose)

Loss on drying

All proniosome formulations, i.e. NL, AL, SQV-NL and SQV-AL, were measured for their moisture content in terms of %loss on drying. It was found that proniosomal granules prepared form a mixture of niosomal dispersion and lactose had slightly higher moisture content than proniosomes prepared from a mixture of lipid/ surfactants alcoholic solution and lactose. The percent loss on drying of proniosome formulation NL and SQV-NL were 1.67% and 1.52%, respectively. Proniosome formulation AL and SQV-AL, which prepared using alcohol, showed percent loss on drying of 1.26% and 1.14%, respectively. Lactose is a water soluble substance therefore it is hardly dried in aqueous media compared with alcoholic media. The rate of evaporation of aqueous media was lower than alcoholic solution in the equal drying time. This caused remained moisture content higher in proniosome formulation NL and SQV-NL.

Flowability

The proniosomal granules formulated with varied amount of lactose did not flow through glass funnel, indicating their poor flowability.

Bulk density and tapped density and compressibility

Table 13 shows bulk density and tapped density of proniosomes. The Carr's compressibility of all proniosome formulations was in a range of 23%-33%, indicating these proniosomal granules had poor flowability property. The poor flowability would be due to the moisture content and irregular shape or roughness of granules. As the results, proniosomal granule formulations did not improve the flowability of SQV.

Table 13. Bulk density and tapped density of proniosomes (n=3)

Formulation	Bulk density (g/cm^3)	Tapped density (g/cm^3)	Carr's compressibility (%)
NL	0.43	0.58	25.00
	0.43	0.57	23.91
	0.44	0.57	23.08
Mean (SD)	0.44 (0.00)	0.57 (0.00)	24.00 (0.96)
AL	0.43	0.56	23.40
	0.43	0.56	23.66
	0.43	0.56	24.47
Mean (SD)	0.43 (0.00)	0.56 (0.00)	23.84 (0.56)
SQV-NL	0.42	0.56	24.21
	0.42	0.56	24.21
	0.43	0.56	25.26
Mean (SD)	0.42 (0.00)	0.59 (0.00)	24.05 (0.32)
SQV-AL	0.42	0.56	24.21
	0.42	0.55	22.34
	0.42	0.54	22.92
Mean (SD)	0.42 (0.00)	0.55 (0.00)	23.43 (0.68)

NL = Plain proniosomes prepared from a mixture of plain niosomes and lactose

PL-AL = Plain proniosomes prepared from a mixture of saquinavir mesylate and lipid/ surfactants solution and lactose

SQV-NL = SQV proniosomes prepared from a mixture of saquinavir mesylate entrapped niosomes and lactose

SQV-AL = SQV proniosomes prepared from a mixture of saquinavir mesylate and lipid/ surfactants solution and lactose

Identification

SQV in proniosomal granules was identified using IR spectroscopy. The IR spectra of SQV-NL (SQV proniosomes prepared from a mixture of SQV entrapped niosomes and lactose) and SQV-AL (SQV proniosomes prepared from a mixture of SQV and lipid/ surfactants alcoholic solution and lactose) were compared to IR spectra of NL (plain proniosomes prepared from a mixture of plain niosomes and lactose), SQV and R-SQV (recrystallized SQV) as presented in **Figure 31.**

Overall, the peak characteristics of SQV-AL and SQV-NL were similar to plain proniosomes (NL), except for the peak of C=O bending of amide group. Both SQV-AL and SQV-NL exhibited C=O stretching in the same region. IR spectrum of SQV-AL presented C=O stretching at 1640 cm^{-1} and 1732 cm^{-1}. While IR spectrum of SQV-NL presented C=O stretching at 1648 $cm^{-1}$and 1729 cm^{-1}. However, it was found that IR spectra of SQV and R-SQV presented C=O stretching of amide group around 1670 cm^{-1} and 1674 cm^{-1} which were around peak positions of C=O stretching of amide group in SQV-AL (1640 cm^{-1}) and SQV-NL (1648 cm^{-1}). Another peak positions of C=O stretching in SQV-AL (1732 cm^{-1}) and SQV-NL (1729 cm^{-1}), reflecting ester group may be affected by ester group in Simulsol®M52 structure. This peak position was also appeared in IR spectrum of plain proniosomes (NL). The C=O stretching of plain proniosomes only presented at wave number 1733 cm^{-1}, indicating the peak of C=O stretching around 1729-1733 cm^{-1} was affected by Simulsol®M52.

SQV-AL (SQV proniosomes prepared from a mixture of SQV and lipid/ surfactants solution and lactose), SQV-NL (SQV proniosomes prepared from a mixture of SQV entrapped niosomes and lactose) and NL (plain proniosomes prepared from a mixture of plain niosomes and lactose) possess broad spectrum in the region spanning 3300-3400 cm^{-1} as well as SQV and R-SQV. The noise pattern shown for SQV was not observed for IR-spectra of SQV-AL, SQV-NL and plain proniosomes (NL). It would be the result of O-H stretching mode from O-H group of the drug and the residual moisture or solvent (95% ethanol) left after the drying process. In addition, IR spectra of SQV-AL and SQV-NL and NL expressed the peak of C-H out of plane bending in region spanning 800-860 cm^{-1} as IR spectra of lactose, whereas IR spectra of SQV and R-SQV did not.

IR spectra of SQV-AL and SQV-NL as presented in **Figure 29** did not clearly show the peaks of C-O stretching and S=O stretching in regions spanning 1025-1140 cm^{-1} and 1168-1195 cm^{-1}, respectively which found in both IR spectra of SQV and R-SQV. Moreover, the peak of N-H bending which presented around 1550 cm^{-1} in IR spectra of SQV and R-SQV was not found in IR spectra of SQV-NL and SQV-AL.

As the result of this study there might be the interaction of the components in proniosomes because both peak position and intensity of the main peaks were noticeably changed. The possible interaction may be between SQV and lactose as the peak of N-H bending disappeared in IR-spectra of SQV-NL and SQV-AL. The Maillard reaction is a chemical reaction between amino groups of the drug and carbonyl group of reducing sugar (lactose), usually requiring the addition of heat (van Boekel, 1998).

Solid state morphology

The interaction of the components in proniosomes was examined by using an X-ray diffractrometer. The dissimilar X-ray patterns of plain proniosomes and SQV proniosomes are observed in **Figure 32,** however, it was difficult to identify the crystal form compared with X-ray patterns of SQV and R-SQV (recrystallized SQV) **(Figure 33)**. This is due to the intensity of X-ray diffraction pattern of lactose and Simulsol®M52 **(Figure 34)** which presented in large proportion, being 66.57% and 21.77% of the formulation. Therefore these excipients would dominate the X-ray patterns of proniosomes, which was contained in much lesser amount, and caused the unclear X-ray pattern of SQV in proniosomes.

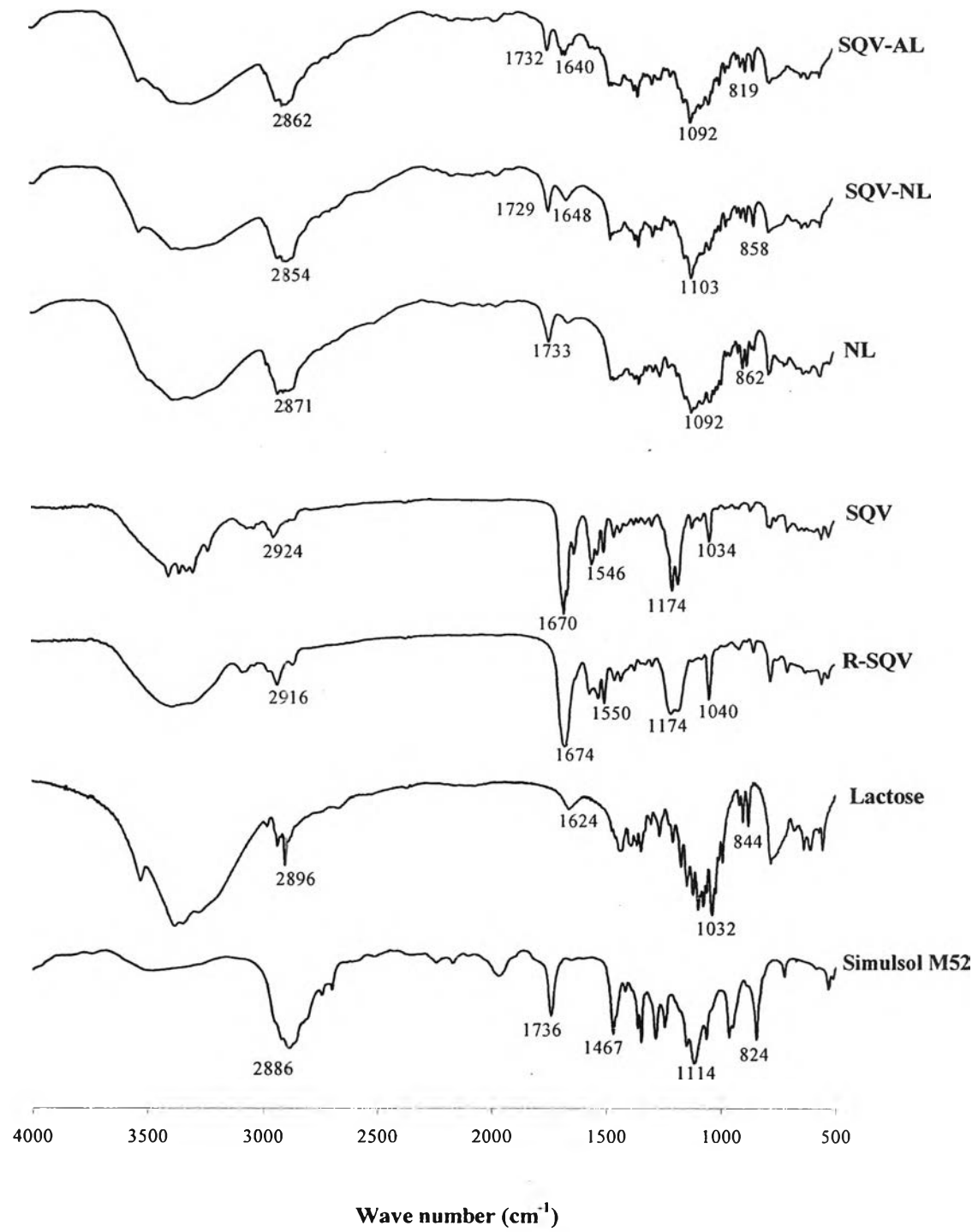
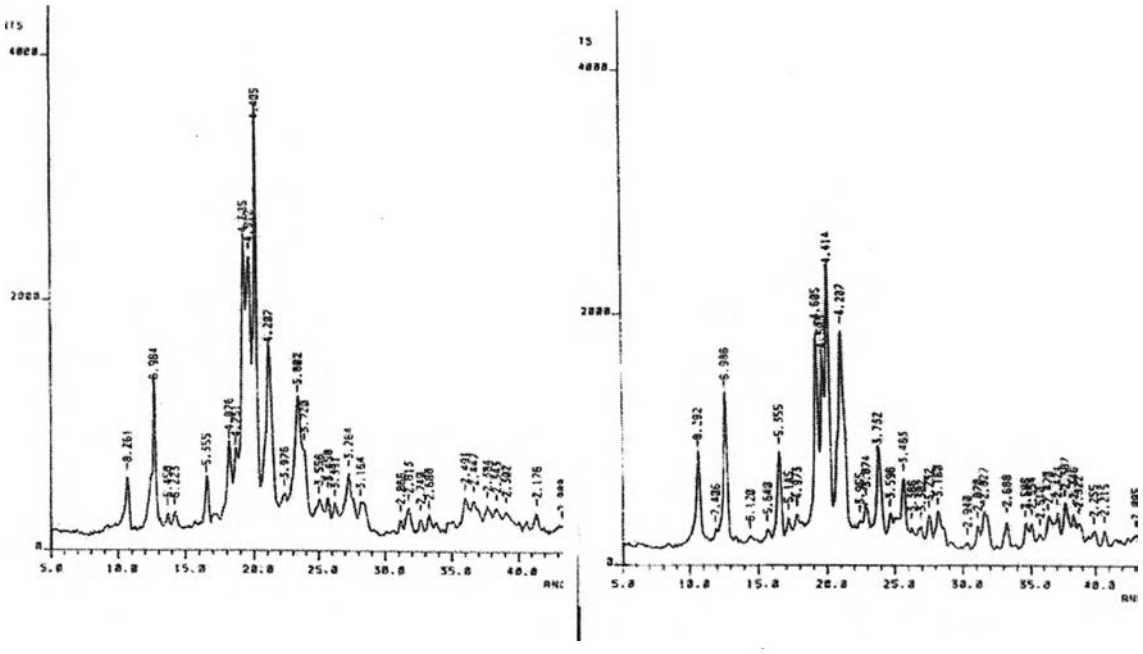
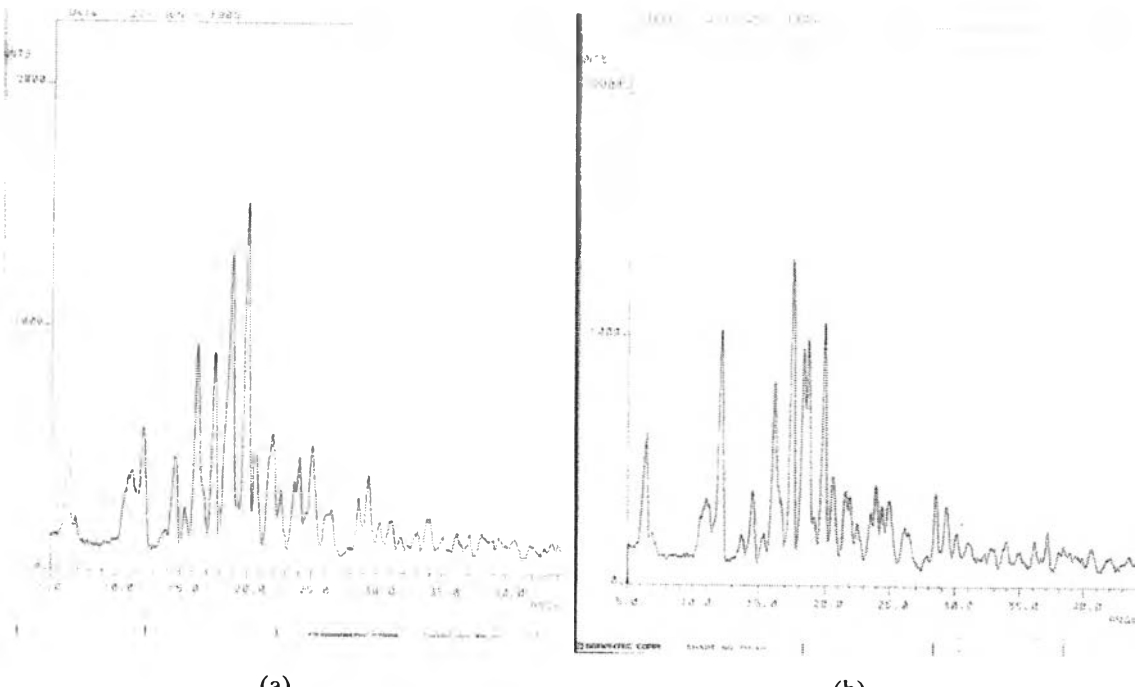


Figure 31. IR spectra of SQV (saquinavir mesylate), R-SQV (recrystallized SQV), proniosomal granules formulation SQV-AL (SQV proniosomes prepared from a mixture of saquinavir mesylate and lipid/ surfactants alcoholic solution and lactose), SQV-NL (SQV proniosomes prepared from a mixture of saquinavir mesylate entrapped niosomes and lactose) and plain proniosomal granules



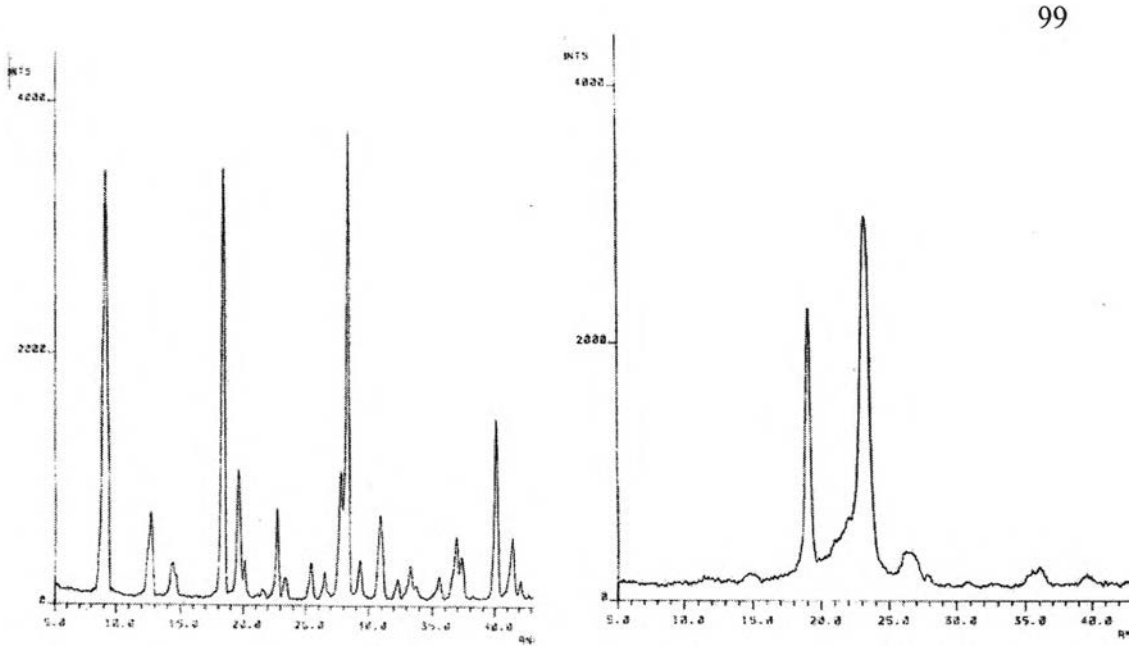
(a) (b)

Figure 32. The X-ray patterns of (a) plain proniosomes and (b) SQV proniosomes



(a) (b)

Figure 33. The X-ray patterns of (a) saquinavir mesylate and (b) recrystallized saquinavir mesylate



(a) (b)

Figure 34. The X-ray patterns of (a) lactose and (b) Simulsol®M52

Thermal property

Thermal property of proniosome formulation SQV-NL (SQV proniosomes prepared from a mixture of SQV entrapped niosomes and lactose) and SQV-AL (SQV proniosomes prepared from a mixture of SQV and lipid/ surfactants alcoholic solution and lactose) was examined and compared to that of a physical mixture of SQV and lipid/ surfactant, plain proniosomes formulation AL and NL, SQV, R-SQV and lactose **(Figure 35)**. DSC thermograms were presented in the temperature range of 25°C to 300°C. It appeared from the thermograms that all proniosome formulations presented the peak of Simulsol®M52 around 50°C and the peak of lactose around 215°C. However, the peaks of SQV (251.7°C) or R-SQV (235.7°C) were not clearly shown in the DSC thermograms of proniosome formulation SQV-NL and SQV-AL. As the low proportion of SQV presented in the proniosomes, thus the peak response of SQV was extremely low comparing with peak of lactose.

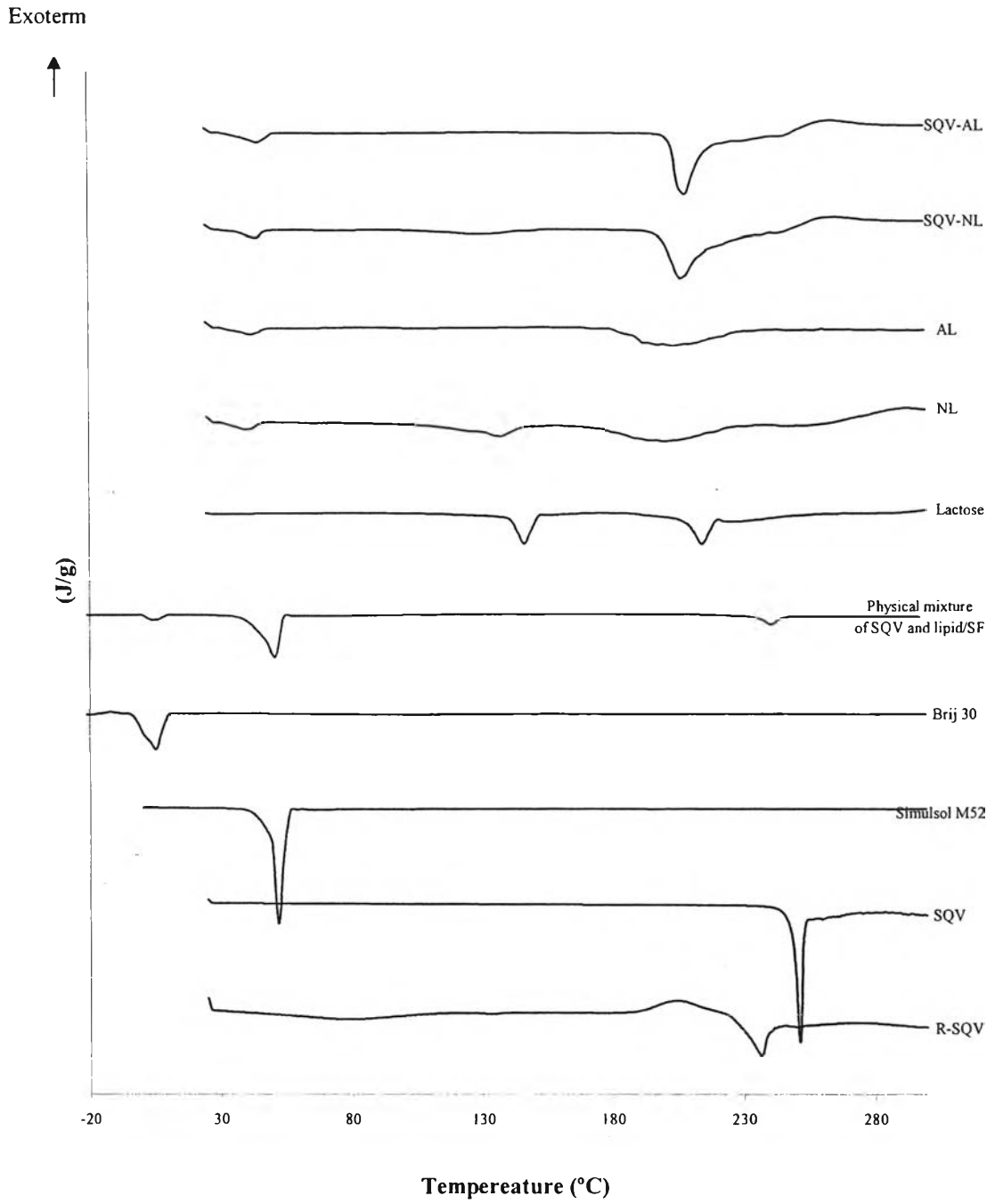


Figure 35. DSC thermograms of SQV-NL (SQV proniosomes prepared from a mixture of saquinavir mesylate entrapped niosomes and lactose) and SQV-AL (SQV proniosomes prepared from a mixture of saquinavir mesylate and lipid/ surfactants alcoholic solution and lactose), NL (plain proniosomes prepared from a mixture of plain niosomes and lactose), a mixture of SQV+Brij®30+Simulsol®M52, lactose, Brij®30, Simulsol®M52, SQV and R-SQV (recrystallized SQV)

Drug content

Proniosomes formulation SQV-NL was filled into capsules, so-called capsule SQV-C, and the SQV content in the capsule was analyzed by HPLC method. The results showed that average SQV content was 8.73±0.03 mg per capsule, less than theoretically loaded amount of SQV which was equivalent to 12 mg per capsule. The drug loss might be involved with SQV degradation. The chemical structure of SQV expressed functional groups of amide, quinoline, amino group and chromophore. These functional groups could be induced to chemical reactions which will be discussed further in the stability study.

SQV release from proniosomal granules

The sink conditions are approximated if saturation volume is 5-10 times to the test volume (Hanson, 1991). In this study, the dissolution study was performed in volume 1000 ml of media, i.e. water, 0.1N hydrochloric acid (0.1N HCl) and phosphate buffer pH 6.8 solution (PBS pH 6.8) to obtain sink condition. The cumulative percentage of drug release was plotted against dissolution time. The dissolution of capsule A (capsule filled with a physical mixture of SQV and lactose), B (capsule filled with a physical mixture of R-SQV and lactose), SQV-C (capsule filled with SQV-NL) and SQV-D (capsule filled with SQV-AL) in water and PBS pH 6.8 was studied for 12 h. The dissolution of these capsules in 0.1N HCl was studied only for 4 h to mimic the period of time when the dosage form could stay in the stomach before emptying to small intestine.

The release profiles of SQV from capsule A, B, SQV-C and SQV-D were compared in water **(Figure 36)**, 0.1N HCl **(Figure 37)** and PBS pH 6.8 **(Figure 38).** In order to compare the SQV release from each formulation, the amount of drug release was presented in terms of AUC (area under the curve) of drug release profiles in various media **(Table 14).** It was found that in the first 4 hour-period, the AUC of drug release profile for capsule A in 0.1N HCl or PBS pH 6.8 was significantly different from that of SQV-C and SQV-D ($p<0.05$), so did capsule B. Therefore, the amount of drug released from capsule A and B was significantly lower than that of SQV-C and SQV-D both in acidic and basic media.

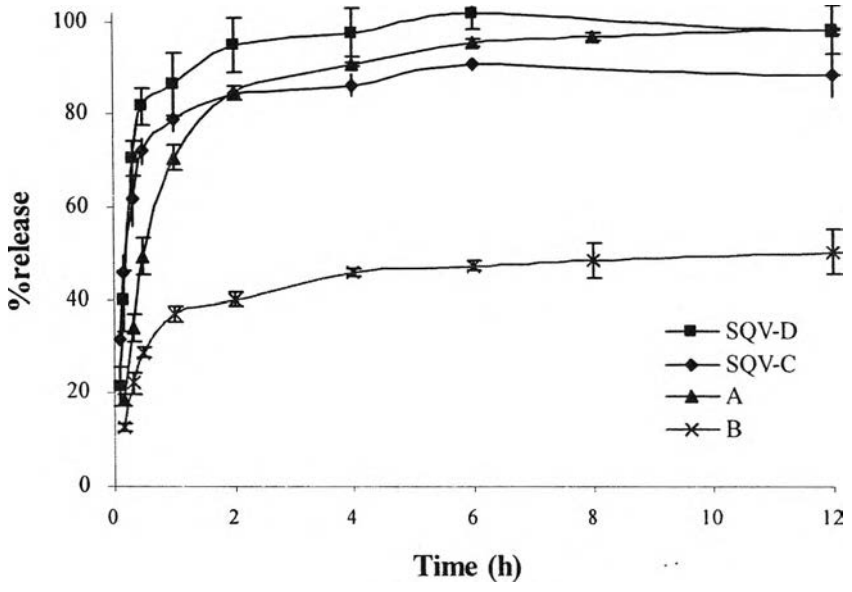


Figure 36. Saquinavir mesylate (SQV) proniosomes released from capsule A (capsule filled with a physical mixture of SQV and lactose), B (capsule filled with a physical mixture of R-SQV and lactose), SQV-C (capsule filled with SQV-NL) and SQV-D (capsule filled with SQV-AL) in water at 37°C

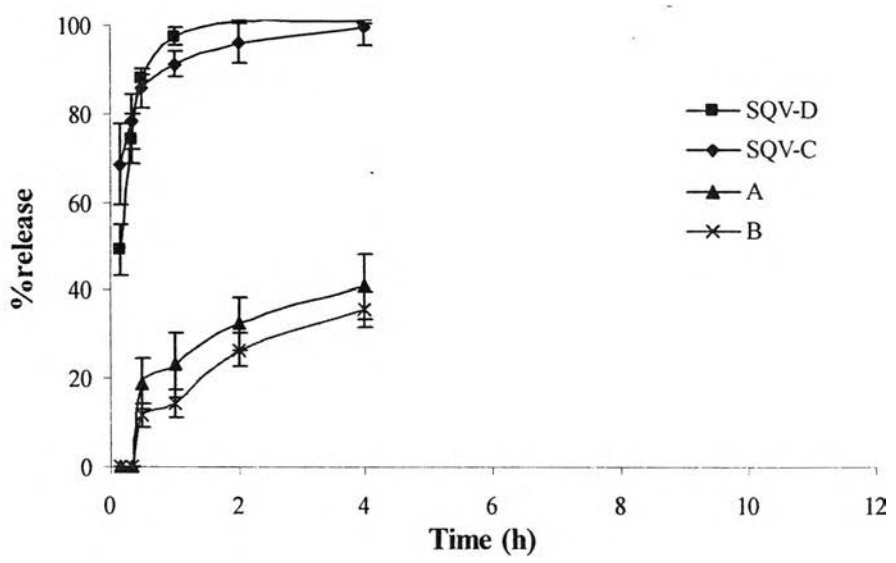


Figure 37. Saquinavir mesylate (SQV) proniosomes released from capsule A (capsule filled with a physical mixture of SQV and lactose), B (capsule filled with a physical mixture of R-SQV and lactose), SQV-C (capsule filled with SQV-NL) and SQV-D (capsule filled with SQV-AL) in 0.1N hydrochloric acid at 37°C

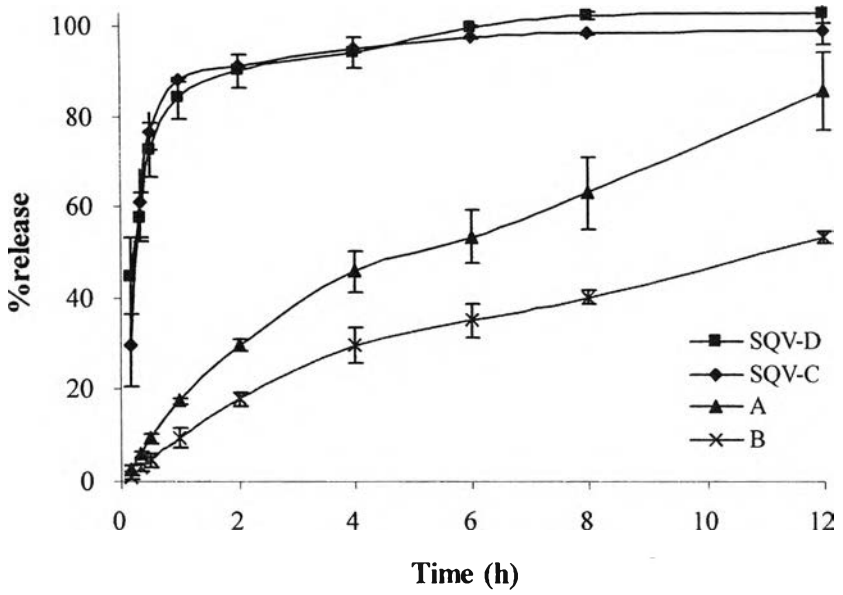


Figure 38. Saquinavir mesylate (SQV) proniosomes released from capsule A (capsule filled with a physical mixture of SQV and lactose), B (capsule filled with a physical mixture of R-SQV and lactose), SQV-C (capsule filled with SQV-NL) and SQV-D (capsule filled with SQV-AL) in phosphate buffer pH 6.8 at 37°C

On the other hand, there was no significant difference between the AUC of drug release profile for capsule SQV-C and SQV-D in all media up to 4 hour-period ($p>0.05$). In addition, the drug released from these capsules up to 12 hour-period in water and PBS pH 6.8 was also comparable. The increase in the amount of drug dissolved from capsule SQV-C and SQV-D, compared to capsule A and capsule B, may be contributed by the lipid/surfactants that are capable of forming both niosomes and micelles.

The release profiles of the drug for niosomal systems in all media depicted the fast drug release of more than 80% in 30 min **(Figures 36-38).** Then, the release remained constant over the period of time. The fast drug release in the first period could be a result of the drug solubilization by niosomes and micelles-forming surfactants. The results agreed with the previous study by Alkan-Onyuksel and Son (1992). They showed that adding lipid/ surfactants would increase drug solubility in micelles. On the other hand, the slow drug release was the effect of controlled drug release by niosomes. It was previously reported by Hao et al. (2002) that Span®60

Table 14. Area under the curves of dissolution profiles of saquinavir mesylate released from capsule A, B, SQV-C and SQV-D in water, 0.1N hydrochloric acid and phosphate buffer pH 6.8 up to 4, 12 and 24 h (n=3), Mean (SD)

Formulation	AUC (%h) up to 4 h			AUC (%h) up to 12h			AUC (%h) up to 24 h		
	Water	0.1N HCl	PBS pH 6.8	Water	0.1N HCl	PBS pH 6.8	Water	0.1N HCl	PBS pH 6.8
A	295.84 (3.63)	113.29 (23.75)	110.68 (5.63)	1061.46 (4.17)	NA	615.33 (60.63)	2223.19 (7.14)	NA	1631.76 (156.76)
B	148.80 (4.23)	90.01 (13.38)	71.46 (6.18)	533.79 (25.98)	NA	415.51 (28.44)	1159.23(60.77)	NA	1082.57 (38.87)
SQV-C	314.50 (5.59)	365.09 (16.51)	337.85 (4.19)	1028.34 (15.91)	NA	1119.77 (10.58)	NA	NA	NA
SQV-D	350.48 (17.66)	374.99 (3.35)	333.34 (16.28)	1148.05 (45.18)	NA	1137.64 (25.85)	NA	NA	NA

AUC = Area under the curve, NA = not applicable, A= capsule filled with a physical mixture of SQV and lactose, B = capsule filled with a physical mixture of R-SQV and lactose, SQV-C = capsule filled with SQV-NL and SQV-D = capsule filled with SQV-AL

niosomes exhibited a prolonged release of colchicine in 100 ml simulated gastric fluid (or simulated intestinal fluid) at 37°C for 24 h compared to non-surfactants formulation. The increase in the amount of solubilized colchicine was influenced by increasing of Span®60 concentration.

Although SQV was not stable in acidic environment probably due to hydrolysis and oxidation (Tan et al., 2003) in acidic media, the amount of released SQV in the niosomal systems was stable over the period of dissolution study. This may be the effect of surfactant, in proniosome-derived niosomes that increased the amount of solubilized drug. Moreover, Brij®30 surfactant, vesicle-forming agent, was chemically stable in strongly acidic or alkaline condition (Kibbe, 2000). Therefore this system also protected encapsulated drug.

The pH of media was found to influence the amount of SQV released from the capsules which did not contain lipid/surfactants. The drug released from capsule A (capsule filled with a physical mixture of SQV and lactose) and capsule B (capsule filled with a physical mixture of R-SQV and lactose) in water was significantly different from that in 0.1N HCl and PBS pH 6.8 ($p<0.05$) over 4 h. It was found that the amount of SQV dissolved from capsule A and capsule B was greatest in water. SQV, basic drug, has pKa 7.01 (McEvoy, 2004) would be ionized more in water (pH 5.80) than in PBS pH 6.8. However, capsule A and capsule B exhibited low amount of drug released in 0.1N HCl since the chemical instability of SQV in acidic media as described earlier. **Figure 37** shows drug released from capsule A and capsule B in 0.1N HCl was low and not significantly different ($p>0.05$). The amount of drug released from both formulations in acidic media was less than 40%. The result agreed with the solubility of SQV in these media.

However, it was found that there was a significant difference between the amount of the drug released from capsule A and capsule B in water **(Figure 36)** and PBS pH 6.8 **(Figure 38)** over 12 h ($p<0.05$). This might be the effect of the physical morphology of drug as observed under a microscope **(Figure 7)** and a scanning electron microscope **(Figure 9)**. SQV was smaller and had more surface area than R-SQV. Hence the amount of drug dissolved from capsule A was higher than that from

capsule B in water and PBS pH 6.8. In addition, as R-SQV was solvate, it was more difficult to dissolve in aqueous media. As presented in **Figures 36-38**, the amount of drug released from capsule B (capsule filled with a physical mixture of R-SQV and lactose) was lower than that of capsule A (capsule filled with a physical mixture of SQV and lactose) in all media studied. However, there was no significant difference in the amount of drug released from these capsules in 0.1N HCl up to 4 h **(Figure 37)** ($p>0.05$). Therefore, the recrystallization process of SQV was influent to drug release in water and PBS pH 6.8 by physical morphology and solvate. Besides, drug dissolution was limited by the chemical instability in 0.1N HCl.

In order to evaluate the capability of proniosomes in SQV release enhancement, the AUC of release profiles for capsule A (capsule filled with a physical mixture of SQV and lactose), SQV-C (capsule filled with SQV-NL) and SQV-D (capsule filled with SQV-AL) in water and PBS pH 6.8 media was compared up to 12 h, while the AUC of released profiles of capsule A, capsule SQV-C and capsule SQV-D in 0.1N HCl was compared up to 4 h. It was found that proniosome capsule SQV-C and capsule SQV-D dramatically increased drug release in PBS pH 6.8 compared to capsule A **(Figure 38)**, where as the amount of SQV released from these capsules in water was comparable **(Figure 36)**. In addition, proniosome capsule SQV-C and capsule SQV-D markedly increased drug release in acidic media **(Figure 37)**. **Table 14** shows that the amount of drug released from proniosome capsules (SQV-C and SQV-D) was more than that of capsules containing SQV and lactose (capsule A), being about 3 folds in 0.1N HCl up to 4 h and being 2 folds in PBS pH 6.8 up to 12 h, respectively.

Figures 39-42 are obtained by redrawing the previous data to describe the effect of dissolution media, i.e. water, 0.1N hydrochloric acid (0.1N HCl) and phosphate buffer pH 6.8 (PBS pH 6.8) on SQV release. The amount of released SQV was calculated in terms of AUC up to 4 h of the dissolution time. There was a significant difference in the amount of drug released from SQV-C (capsule filled with SQV-NL) in each medium over a 4 hour-period ($p<0.05$), it was found that the extent of SQV released from this capsule was highest in 0.1N HCl **(Figure 41)**. The amount of SQV released from capsule SQV-D (capsule filled with SQV-AL) was also markediy higher in 0.1N HCl than that in water and PBS pH 6.8 **(Figure 42)**.

However, the amount of the amount of SQV released might be affected by the presence of lipid/ surfactant. **Figure 41** and **Figure 42** show the increase in the amount of drug released from capsule SQV-C (capsules filled with SQV-NL) and capsule SQV-D (capsule filled with SQV-AL) in both acidic and basic media compared to capsule A and B. The released SQV increased rapidly in 30 min and was then controlled by niosomes. The fast released of SQV in the first period was most likely due to the effect of surfactants on solubility enhancement and burst effect.

The patterns of drug released from capsule A (capsule filled with a physical mixture of SQV and lactose) and capsule B (capsule filled with a physical mixture of R-SQV and lactose) were similar to the profiles of SQV solubility **(Figure 13)**. The SQV solubility was found to be 2.45 ± 0.02 mg/ml, 0.07±0.01 mg/ml and 0.06±0.00 mg/ml in water, 0.1N HCl and PBS pH 6.8 solution, respectively. As presented in **Figure 39 and Figure 40**, the amount of drug released from capsule A and B was greatest in water, while those in 0.1N HCl and PBS pH 6.8 were low. The amount of released SQV of both capsule A **(Figure 39)** and capsule B **(Figure 40)** in water was significantly different from those in 0.1N HCl and PBS pH 6.8 ($p<0.05$). It was possible that these formulations did not contain surfactants, thus the release of SQV was mainly affected by pH of the media. SQV (pKa 7.01) had a larger unionized proportion in water (pH 5.80) than in PBS pH 6.8, therefore SQV was more soluble in water. There was lag time period in the first 20 minutes of the dissolution profile before the drug was released from capsule A **(Figure 39)** and capsule B **(Figure 40)** in acidic media, after that drug released slowly over a 4 h period. This was probably due to poor wettability of the drug particle in aqueous media. In addition, a lack of lipid/surfactants to form niosomes hence increase SQV solubility and protect encapsulated SQV from acidic environment might allow SQV to undergo hydrolysis easier in the acidic condition.

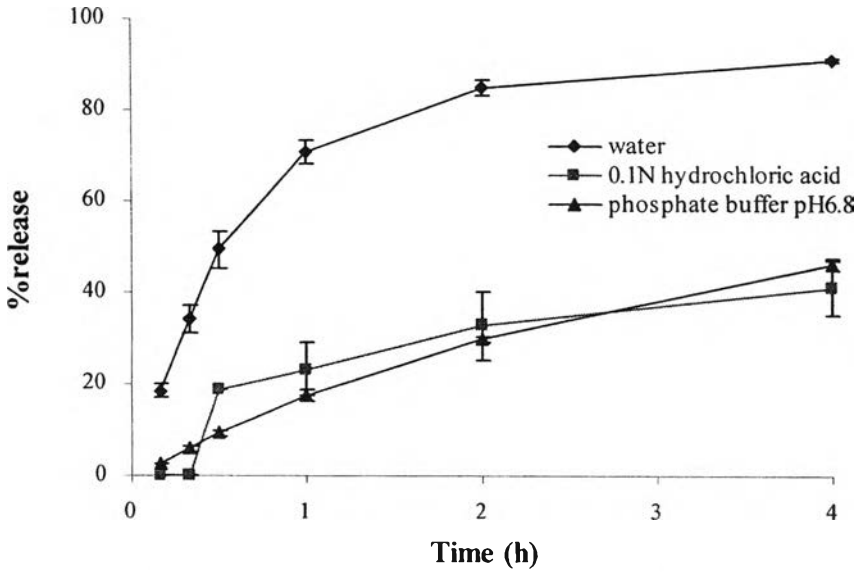


Figure 39. Saquinavir mesylate (SQV) released from capsule A (capsule filled with a physical mixture of SQV and lactose) in water, 0.1N hydrochloric acid and phosphate buffer pH 6.8 at 37°C

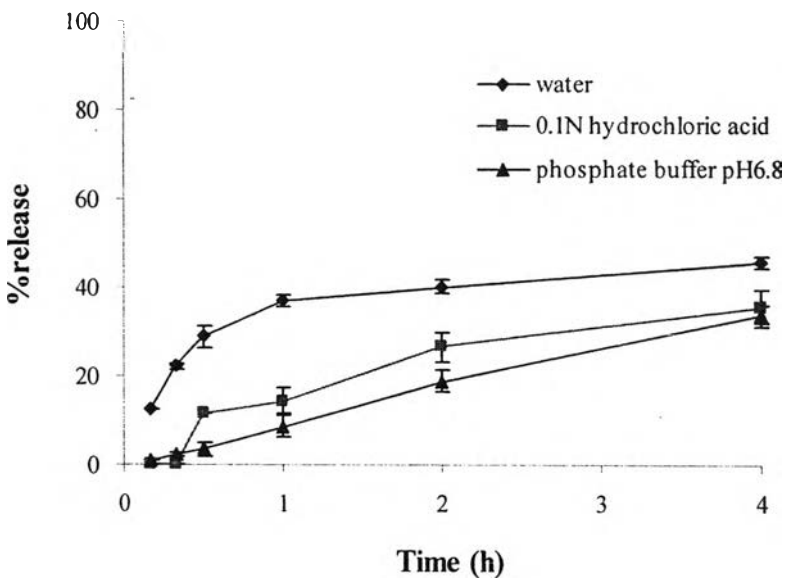


Figure 40. Saquinavir mesylate (SQV) released from capsule B (capsule filled with a physical mixture of R-SQV and lactose) in water, 0.1N hydrochloric acid and phosphate buffer pH 6.8 at 37°C

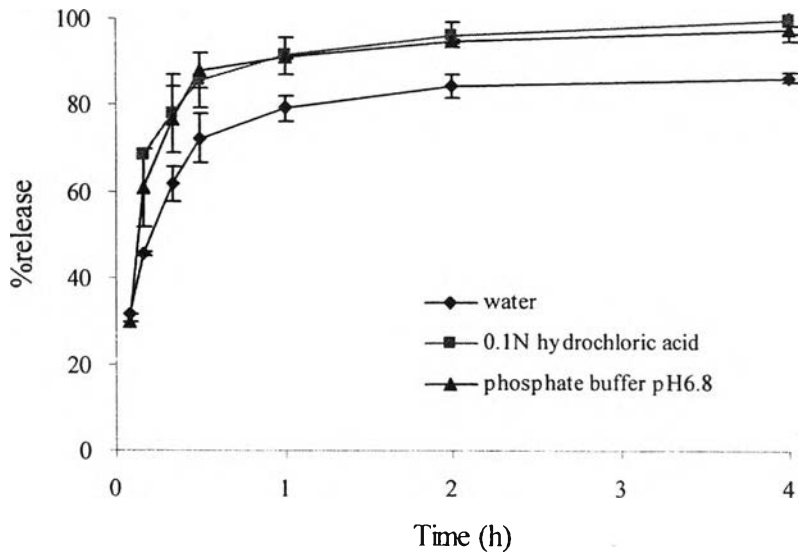


Figure 41. Saquinavir mesylate (SQV) proniosomes released from capsule SQV-C (capsule filled with SQV-NL) in water, 0.1N hydrochloric acid and phosphate buffer pH 6.8 at 37°C

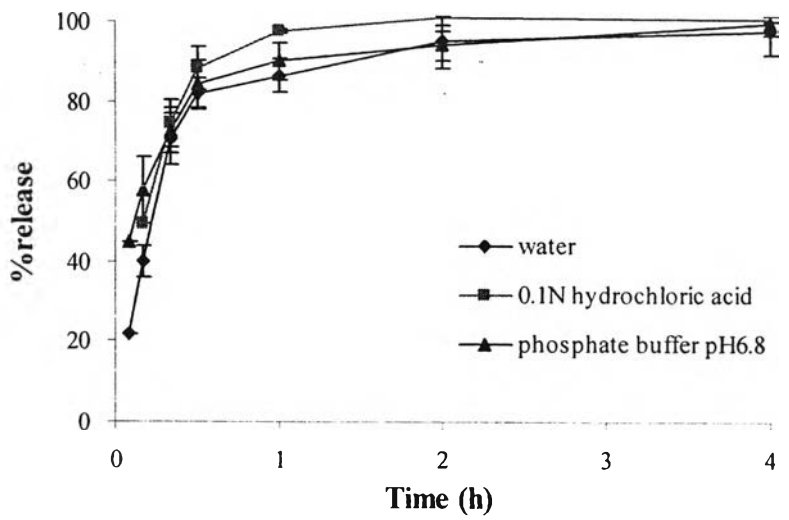


Figure 42. Saquinavir mesylate (SQV) proniosomes released from capsule SQV-D (capsule filled with SQV-AL) in water, 0.1N hydrochloric acid and phosphate buffer pH 6.8 at 37°C

The drug released slowly from capsule A (capsule filled with a physical mixture of SQV and lactose) and capsule B (capsule filled with a physical mixture of R-SQV and lactose) in 0.1N HCl and PBS pH 6.8 compared to capsule SQV-C (capsule filled with SQV-NL) and SQV-D (capsule filled with SQV-AL), associated with a lack of surfactants. This indicated that proniosome capsules were efficient in improving amount of drug release. Therefore, it could be concluded that proniosome capsules prepared by two different methods were successfully improve SQV release in 0.1N HCl and PBS pH 6.8.

Data analysis

Nonlinear regression analysis of release data fitted to three different kinetic models, zero-order, first-order and Higuchi model, are presented in **Table 15, Table 16 and Table 17**, respectively. The release kinetic constants were calculated using release data from the initial time to the first time point after the amount of drug release reached the plateau. The release profiles of all formulations in different media were found to fit either first-order model (equation 7), or Higuchi model (equation 9). It was found that the release profiles of all capsule formulations, capsule A (capsule filled with a physical mixture of SQV and lactose), capsule B (capsule filled with a physical mixture of R-SQV and lactose), capsule SQV-C (capsule filled with SQV-NL) and capsule SQV-D (capsule filled with SQV-AL) fitted to either first-order or Higuchi release kinetics. It was found that SQV released from these capsules in acidic and basic media fitted to both first order model and Higuchi model **(Table 16 and Table 17)**. The coefficient of determination, r^2, of SQV released in 0.1N hydrochloric acid and phosphate buffer pH 6.8 were in the range of 0.8501-0.9958 and 0.8073-0.9849, respectively. Therefore, the release of the drug in these media was dependent on the remained amount of SQV in granules and also could be related to square root of dissolution time. While SQV release in water was found to fit to Higuchi model **(Table 17)**, the r^2 value was in the range of 0.7266-0.9017. Therefore, the release of the drug in water was dependent on the square root of dissolution time.

Table 15. The saquinavir mesylate released from capsules in different media fitted to zero-order model (Mean (SD), n=3)

Formulation	Dissolution medium					
	water		0.1N HCl		PBS pH 6.8	
	r^2	K ($\% t^{-1}$)	r^2	K ($\% t^{-1}$)	r^2	K ($\% t^{-1}$)
A	0.6914 (0.0193)	6.8740 (0.8226)	0.7969 (0.0517)	10.5517 (1.7707)	0.9406 (0.0230)	19.9530 (0.3089)
B	0.6365 (0.0739)	4.6939 (0.3164)	0.8988 (0.0261)	9.3856 (1.1091)	0.9063 (0.0573)	9.0723 (0.0728)
SQV-C	0.4521 (0.0213)	14.7483 (0.1593)	0.7626 (0.0545)	159.9100 (8.1198)	0.5923 (0.0636)	37.7600 (2.9308)
SQV-D	0.4684 (0.0469)	18.2703 (1.6352)	0.9260 (0.0408)	173.5667 (0.7257)	0.5982 (0.0546)	34.6427 (1.2117)

A = capsule filled with a physical mixture of SQV and lactose, B = capsule filled with a physical mixture of R-SQV and lactose filled capsule, SQV-C = capsule filled with SQV-NL and SQV-D = capsule filled with SQV-AL, r^2 = coefficient of determination and k = release rate constant

Table 16. The saquinavir mesylate released from capsules in different media fitted to first-order model (Mean (SD), n=3)

Formulation	Dissolution medium					
	water		0.1N HCl		PBS pH 6.8	
	r^2	K ($\log\% t^{-1}$)	r^2	K ($\log\% t^{-1}$)	r^2	K ($\log\% t^{-1}$)
A	0.8936 (0.0291)	-0.2568 (0.0017)	0.8501 (0.0372)	-0.0597 (0.0137)	0.9674 (0.0053)	-0.0260 (0.0731)
B	0.7095 (0.0259)	-0.0556 (0.0011)	0.9308 (0.0173)	-0.0507 (0.0075)	0.9412 (0.0544)	-0.0285 (0.0020)
SQV-C	0.6647 (0.0130)	-0.1852 (0.0079)	0.9039 (0.0448)	-1.6403 (0.2817)	0.8073 (0.0489)	-0.5077 (0.0326)
SQV-D	0.7650 (0.0494)	-0.4037 (0.1446)	0.9958 (0.0036)	-1.8417 (0.0627)	0.8661 (0.0087)	-0.4710 (0.0754)

A = capsule filled with a physical mixture of SQV and lactose, B = capsule filled with a physical mixture of R-SQV and lactose filled capsule, SQV-C = capsule filled with SQV-NL and SQV-D = capsule filled with SQV-AL, r^2 = coefficient of determination and k = release rate constant

Table 17. The saquinavir mesylate released from capsules in different media fitted to Higuchi model (Mean (SD), n=3)

Formulation	Dissolution medium					
	water		0.1N HCl		PBS pH 6.8	
	r^2	K ($\% t^{-1/2}$)	r^2	K ($\% t^{-1/2}$)	r^2	K ($\% t^{-1/2}$)
A	0.9017 (0.0147)	48.7987 (0.7101)	0.8791 (0.0260)	23.7770 (4.3456)	0.9849 (0.0047)	24.8983 (2.8407)
B	0.8744 (0.0728)	22.7737 (0.3276)	0.9207 (0.0050)	20.4097 (2.5623)	0.9650 (0.0257)	17.1690 (1.3261)
SQV-C	0.7266 (0.0212)	39.4370 (0.2271)	0.9447 (0.0273)	124.9167 (7.1169)	0.8437 (0.0292)	68.2650 (2.8358)
SQV-D	0.7305 (0.0350)	48.1223 (3.2638)	0.9935 (0.0033)	126.1233 (3.2208)	0.8592 (0.0309)	63.3673 (1.9429)

A = capsule filled with a physical mixture of SQV and lactose, B = capsule filled with a physical mixture of R-SQV and lactose filled capsule, SQV-C = capsule filled with SQV-NL and SQV-D = capsule filled with SQV-AL, r^2 = coefficient of determination and k = release rate constant

Stability study

The stability of proniosome capsule SQV-C (capsule filled with SQV-NL) stored at room temperature and 45°C for 4 months was studied. **Table 18** shows the amount of SQV content of capsules after stored at room temperature and 45°C for 4 months. The initial SQV content was 8.73 mg as analyzed by HPLC method. The content of SQV gradually decreased to 8.46 mg (96.91%), 7.87 mg (90.15%) and 5.77 mg (66.09%) after stored at room temperature for 2, 3 and 4 months, respectively. In the accelerated condition, SQV content in capsules stored at 45°C for 4 months markedly decreased to 8.60 mg (98.51%), 7.49 mg (85.80%), 7.54 mg (86.37%) and 5.76 mg (65.98%) when stored for 1, 2, 3 and 4 months, respectively.

Table 18. The amount of saquinavir mesylate (SQV) content in proniosome capsules (formulation SQV-C) after stored at room temperature and 45°C for 4 months

Time (month)	Room temperature		45°C	
	mg per capsule (mean (SD))	%SQV remained	mg per capsule (mean (SD))	%SQV remained
0	8.73 (0.03)	100.00	8.73 (0.03)	100.00
1	8.82 (0.17)	101.03	8.60 (0.10)	98.51
2	8.46 (0.17)	96.91	7.49 (0.08)	85.80
3	7.87 (0.19)	90.15	7.54 (0.18)	86.37
4	5.77 (0.13)	66.09	5.76 (0.14)	65.98

Proniosome capsule SQV-C (capsule filled with SQV-NL) and SQV-D (capsule filled with SQV-AL) stored at 45°C for 4 months could remain the ability to form niosomes after rehydrated with water, 0.1N hydrochloric acid and phosphate buffer pH 6.8 in a proportion in 1000 ml medium as equivalent to dissolution medium. The photomicrographs of niosome-derived from proniosomes, capsule SQV-D, observed under a light microscope are presented in **Figure 45**. The morphology of niosomes-derived from both proniosome capsule SQV-C **(Figure 46)** and SQV-D **(Figure 47)** in various media were also observed under a transmission electron microscope. The figures showed that Brij®30 niosomes could form in diluted media after the storage.

The reduction of SQV content was associated with the chemical instability of drug. SQV degradation may be due to the humidity, i.e. hydrolysis corresponding to amide groups. Oxidation could also be involved in the SQV degradation triggered by atmospheric oxygen on quinoline nitrogen (Tan et al., 2003). In addition, SQV may undergo the Maillard reaction, non-enzymatic browning reaction, with lactose which was the main component of proniosomes. Maillard reaction occurred between a reducing sugar and amine, which produced water and brown color to product (Zhihu, 2005). However, the alteration of color could also be the result of oxidation on chromophores. **Figure 43** shows the appearances of proniosomes formulation SQV-NL (SQV proniosomes prepared from a mixture of saquinavir mesylate entrapped niosomes and lactose) **(Figure 43a)** and NL (plain proniosomes prepared from a mixture of plain niosomes and lactose) **(Figure 43b)** stored at 45°C for 4 months. A slight change in color to yellow-brown was observed for SQV proniosomes (SQV-

NL). The appearance of proniosome formulation SQV-AL (SQV proniosomes prepared from a mixture of saquinavir mesylate and lipid/ surfactants solution and lactose) and SQV proniosomes after stored at 45°C for 4 months were presented in **Figure 44a** and **Figure 44b**, respectively. It was found that proniosome formulation SQV-AL also changed in color as SQV-NL did, while AL still appeared white color. The change in color of SQV proniosomes prepared by different methods might be the results of the oxidation on chromophores and Maillard reaction corresponding to amine in the SQV structure. The agglomeration of SQV proniosomes indicated an increase in moisture content as found that %loss on drying being 2.84±0.04%. The humidity therefore could interact with SQV by hydrolysis and oxidation. Although proniosome granules prepared from Brij®30, Simulsol®M52 and lactose were efficient to improve the SQV solubility. Hydrolysis, oxidation and Maillard reaction were the problem of SQV instability found in proniosomes using lactose as a carrier.

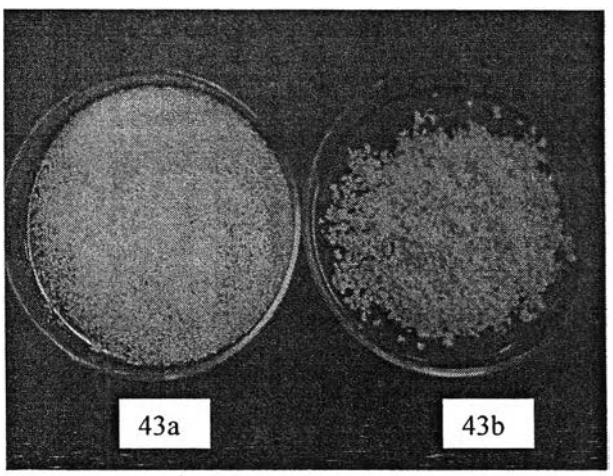


Figure 43. The appearances of (43a) plain proniosomes NL and (43b) SQV proniosomes NL after stored at 45°C for 4 months

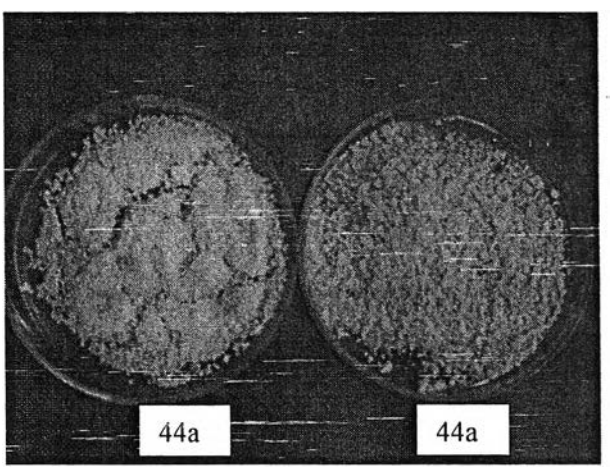
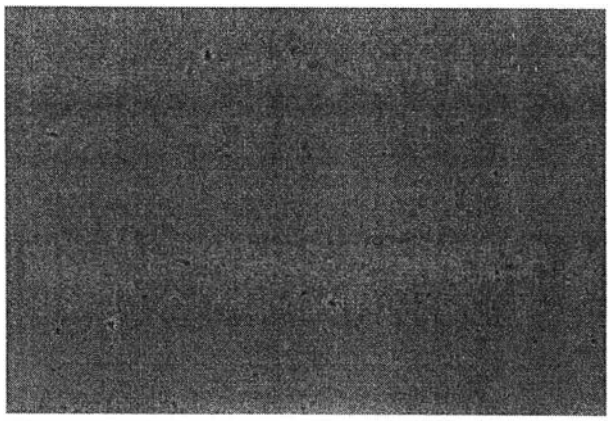
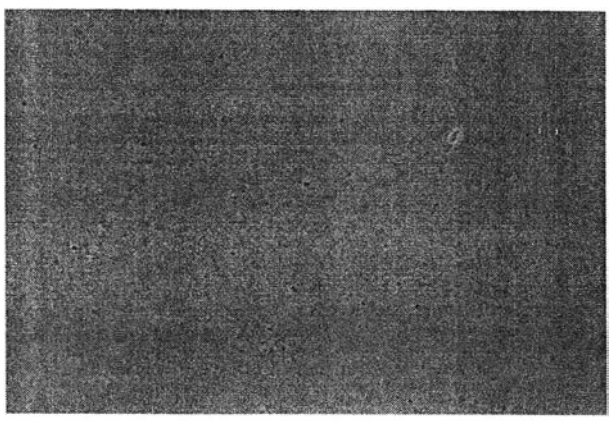


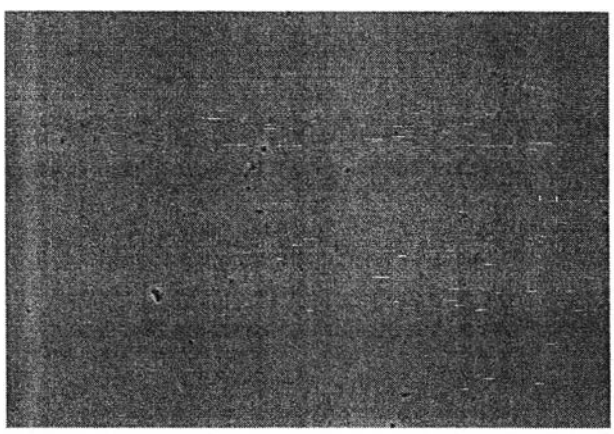
Figure 44. The appearances of (44a) plain proniosomes AL and (44b) SQV proniosomes AL after stored at 45°C for 4 months



(a)



(b)



(c)

Figure 45. Niosome-derived from 312 mg SQV-AL (SQV proniosomes prepared from a mixture of saquinavir mesylate and lipid/ surfactants solution and lactose) after storage at 45°C for 4 months dissolved in 1000 ml water (a), 0.1N hydrochloric acid (b) and phosphate buffer pH 6.8 (c)

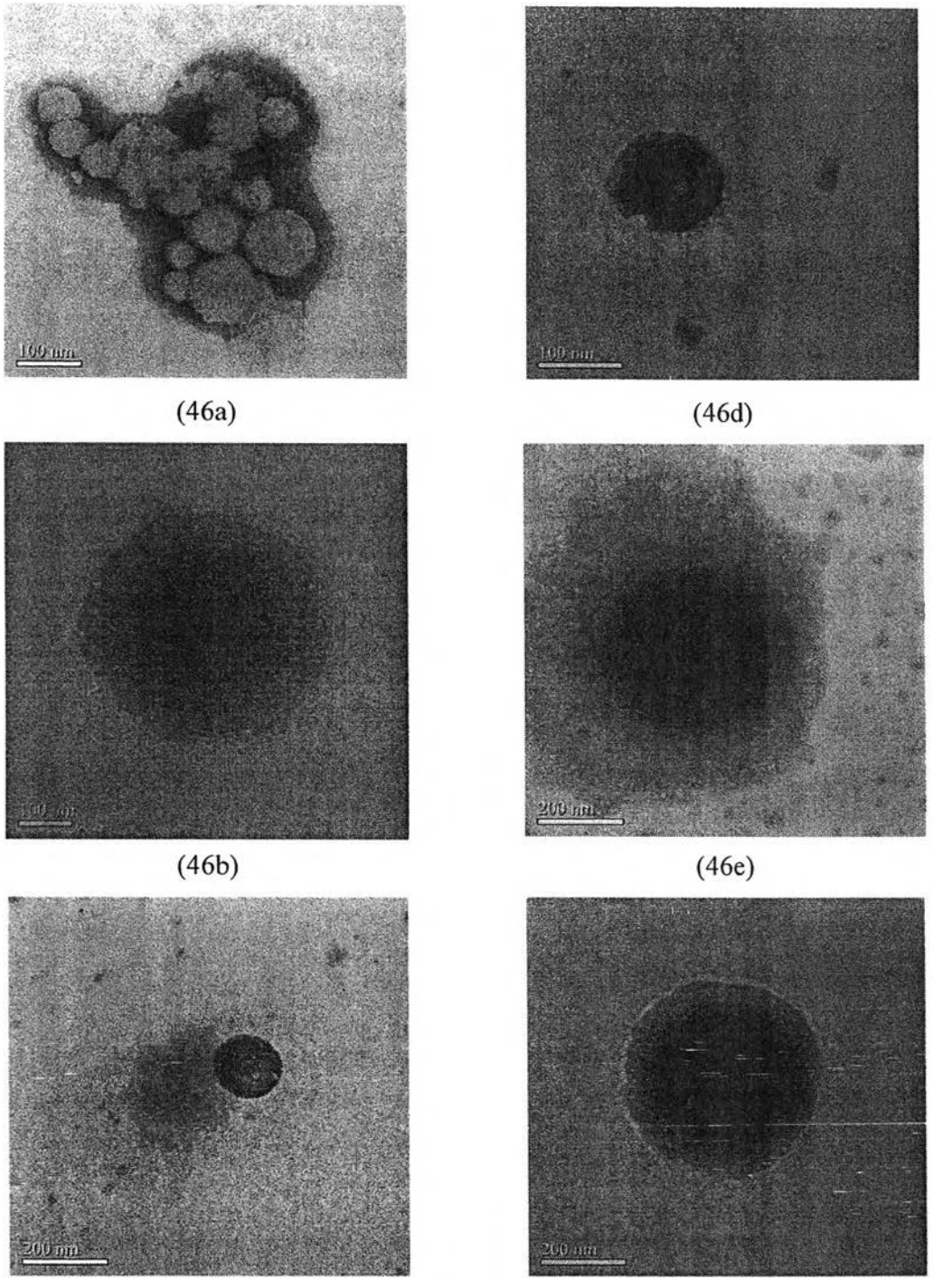


Figure 46. The transmission electron micrographs of proniosome-derive niosomes in 1000 ml of various media from plain capsule (capsule filled with plain proniosomes prepared from a mixture of plain niosomes and lactose) and capsule SQV-C (capsule filled with SQV-NL) stored at 45°C for 4 months (46a) plain capsule rehydrated with water, (46b) plain capsule rehydrated with 0.1N hydrochloric acid, (46c) plain capsule rehydrated with phosphate buffer pH 6.8, (46d) SQV-C rehydrated with water, (46e) SQV-C rehydreated with 0.1N hydrochloric acid and (46f) SQV-C rehydrated with phosphate buffer pH 6.8

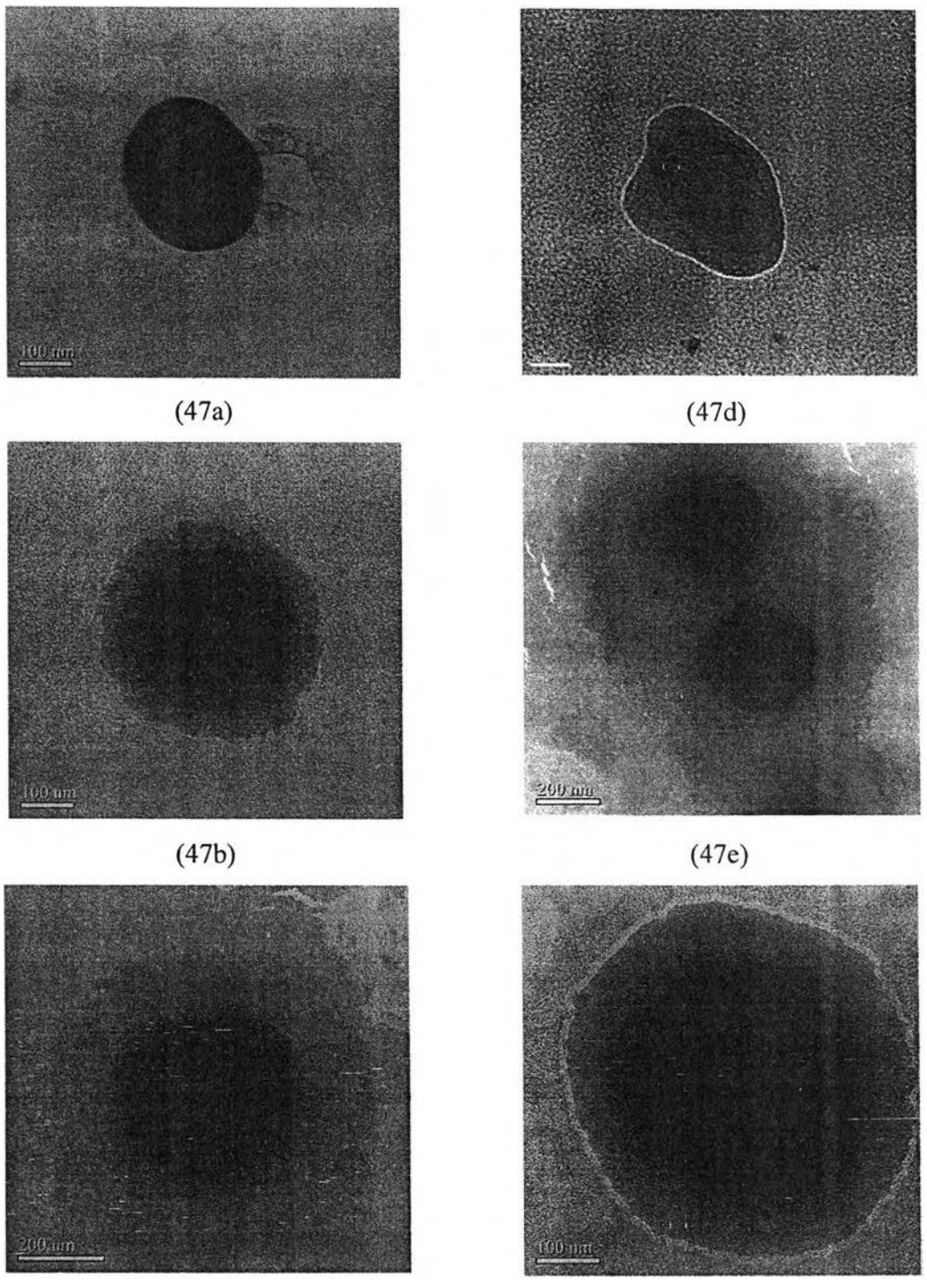


Figure 47. The transmission electron micrographs of proniosome-derive niosomes in 1000 ml of various media from plain capsule (capsule filled with plain proniosomes prepared from a mixture of lipid/ surfactants alcoholic solutin and lactose) and capsule SQV-D (capsule filled with SQV-AL) stored at 45°C for 4 months (47a) plain capsule rehydrated with water, (47b) plain capsule rehydrated with 0.1N hydrochloric acid, (47c) plain capsule rehydrated with phosphate buffer pH 6.8, (47d) SQV-D rehydrated with water, (47e) SQV-D rehydreated with 0.1N hydrochloric acid and (47f) SQV-D rehydrated with phosphate buffer pH6.8