CHAPTER IV

RESULTS AND DISCUSSION

1. Quantitative analytical method of ibuprofen in liposomal preparations

1.1 Preliminary assay of ibuprofen in liposomal preparations

In the preliminary study, there were many problems in HPLC Assay. The extraction of ibuprofen from liposomal preparation by sodium hydroxide or phosphate buffer saline pH 7.4 was not complete because it preferred to dissolve in the mixture of organic solvents of chloroform and isopropyl alcohol, which were used to lyse the ibuprofen liposomes. Additionally, all lipid components of liposomes also dissolved very well in isopropyl alcohol, an intermediate solvent. Thus, they were extracted into the extraction solvent, sodium hydroxide or phosphate buffer saline pH 7.4. Experimentally, the complete extraction of ibuprofen from lipid components of liposomes could not be achieved.

On further attempts, ibuprofen liposomes were dissolved in methanol and then directly injected into HPLC column. These led to an obstruction of the HPLC column by lipid components due to the difficulty in the removal of lipids from the column.

1.2 UV spectrophotometric assay

As mentioned above, the complete extraction of ibuprofen from other liposomal components in HPLC method could not be experimentally achieved. Additionally, in spite of an attempt was carried out by directly injection of liposomal sample solution into the column, it led to contaminate the column. It was difficult to remove lipids completely from the column and might cause the column damages. Thus, UV spectrophotometric assay was investigated for the analysis of ibuprofen in ibuprofen liposomal preparations.

1.2.1 Selection of blank solvent

Blank solvents for UV spectrophotometric assay were investigated. Isopropyl alcohol was firstly selected in the study at the point that it could dissolve ibuprofen and all lipid components used in liposomal preparations. The other solvent selected in the investigation was phosphate buffer saline pH 7.4 (Sivakumar et al, 1994). The latter solvent could also dissolve the drug but not for the lipid components.

The problems were found in UV spectrophotometric assay using isopropyl alcohol as a blank solvent. Since it could dissolve both ibuprofen and lipid components, the absorbance of ibuprofen was interfered with one of soybean lecithin at wavelength 264.2 nm which was the maximum absorption wavelength of ibuprofen in isopropyl alcohol (Appendix II). However, it could subtract the absorbance of soybean lecithin from absorbance of ibuprofen liposomes because the absorbance of soybean lecithin was not greater than 0.4 which was the maximum value of blank that permitted in the British Pharmacopoeia 1993.

The major problem of UV spectrophotometric analysis was the absorbance of (±)-α-tocopherol in isopropyl alcohol which interfered one of ibuprofen in liposomal preparations (Appendix II). This caused the higher concentration of ibuprofen after analysis which was greater than the actual value. Thus, isopropyl alcohol was not selected as a blank solvent in the UV spectrophotometric analysis.

Phosphate buffer saline pH 7.4 was chosen to be the blank solvent since it could dissolve ibuprofen of concentration used in the study. As reported by Sivakumar et al (1994), phosphate buffer pH 7.4 was used as a blank solvent in the release study of ibuprofen liposomes.

1.2.2 Study of maximum absorption wavelength of ibuprofen

UV spectrophotometer model UV 160A was used to analyze the absorbance of ibuprofen. The maximum absorption wavelength of ibuprofen in phosphate buffer saline pH 7.4 was found at wavelength 222.6 nm (Appendix II). The representative calibration curve data of ibuprofen in phosphate buffer saline pH 7.4 and Beer's law plot of various concentrations of ibuprofen and their absorbances at wavelength 222.6 nm were freshly prepared in each analysis.

The calibration curve data were fitted into a straight line equation using a linear regression analysis program. The coefficient of determination (r^2) was found to be nearly 1 in all calibration curve data (Appendix II).

2. Method of preparation of the empty liposomes

The method for preparation of ibuprofen liposomes was selected depending on the solubility of ibuprofen. Ibuprofen is a hydrophobic drug which can be incorporated in bilayer membranes of liposomes. It exists as a white crystalline powder or colorless crystals (Appendix II). By this point, the mechanical dispersion method is the appropriate method using for preparation of ibuprofen liposomes. In this method, both hydrophobic drug and lipids were dissolved in organic solvent and deposited on the wall of round bottom flask after the removal of organic solvent. The resulting final product was found as multilamellar liposomes in which containing hydrophobic drug in their multibilayer membranes. The entrapment efficiency was found that it could arise up to 100% (New, 1990a).

Since the formation of an optimal thin film of lipid with larger surface area is desirable to facilitate the efficient hydration of the bilayer, appropriate method of preparation of empty liposomes by mechanical dispersion method was investigated.

In this study, round bottom flasks were selected for preparation of liposomes even though use of pear-shaped flasks were reported to offer larger surface area. It was found that 1,000 ml round bottom flask gave more appropriate thin film deposited than other sizes.

From the investigation of optimal amount of chloroform, evaporating temperature, time for removal of chloroform, amount of soybean lecithin, temperature and time for hydration of the thin film, the empty liposomes were prepared using preparation methods as follows.

Optimal amount of soybean lecithin, 90.9 μmol/ml or 70.4 mg/ml, was dissolved in suitable amount of chloroform of 20 ml in a 1,000 ml round bottom flask. Then, the lipid solution was evaporated at 35°C using rotary evaporator under reduced pressure for 3 hours until the thin film deposited on the wall of the round bottom flask and chloroform odor no longer existed. After that, 3 ml of sterile water for injection was added to hydrate the film at 35°C for 2 hours. Then, the preparation was mixed occasionally by vortex mixer and the final product was found as a milky suspension.

3. Preparation of ibuprofen liposomes

The initial ibuprofen liposomes were prepared under the above conditions in section 2. The process initiated with the dissolving of ibuprofen and 90.9 μmol/ml of soybean lecithin in chloroform 20 ml. Then, the mixture was evaporated by rotary evaporator under reduced pressure for 3 hours. After that, 3 ml of sterile water was added to hydrate at 35°C for 2 hours. Then, the preparation was mixed occasionally by vortex mixer and the final product was found as a milky suspension.

4. Analysis of ibuprofen encapsulated in ibuprofen liposomal preparations

Free or unencapsulated ibuprofen was separated from the liposomal preparation by using ultracentrifuge at 4°C, 50,000 rpm for 5 hours. Then, the supernatant liquid was collected into a 25 ml volumetric flask and adjusted final volume with phosphate buffer saline pH 7.4. The solution was analyzed by UV spectrophotometer at wavelength 222.6 nm. The average absorbance of supernatant

liquid of corresponding empty liposomes in each formulation was used to subtract from the absorbance of supernatant liquid of each ibuprofen liposomal preparations. After that, the concentration of ibuprofen was calculated from the regression equation of calibration curve which were freshly prepared for each analysis. Encapsulated ibuprofen in liposomal preparation was calculated by the equation as described in experimental methodology in section 8.1.2 in term of percentage ibuprofen entrapment.

5. Effects of the soybean lecithin to drug molar ratios on the preparation of ibuprofen liposomes

Ibuprofen liposomes with various amounts of ibuprofen (formula 1-5) of 0.9, 1.8, 2.7, 3.2 and 3.6 mg/ml using 90.9 μmol/ml of constant soybean lecithin concentration, that were 1:0.047, 1:0.096, 1:0.144, 1:0.170 and 1:0.191 molar ratio of soybean lecithin to drug, respectively, were prepared as shown in Table 3. It was

Table 3 Effects of the soybean lecithin to drug molar ratios on the preparation of ibuprofen liposomes prepared with 90.9 μmol/ml of constant soybean lecithin concentration

Formula	soybean lecithin to drug molar ratio	amount of ibuprofen (mg/ml)	appearance of ibuprofen liposomes	crystal of ibuprofen
1	1:0.047	0.9	milky suspension	not found
2	1:0.096	1.8	milky suspension	not found
3	1:0.144	2.7	milky suspension	not found
4	1:0.170	3.2	milky suspension	not found
5	1:0.191	3.6	milky suspension	found

found that ibuprofen liposomes containing with all molar ratios of soybean lecithin to drug, except of 1:0.191 molar ratio, using the appropriate preparation conditions in section 2 could be obtained which ibuprofen crystals could not be found by examination under optical microscopy. While drug crystals were found in the final product containing 1:0.191 molar ratio of soybean lecithin to drug. The drug crystallization may be explained on the basis that the unencapsulated drug remaining in the aqueous phase of preparation exceeds the drug solubility (1: 1,900) in water (Lund, 1994).

Although ibuprofen liposomes containing 1:0.170 molar ratio of soybean lecithin to drug (formula 4) could be prepared without drug crystallization in the final product, the 1:0.144 molar ratio (formula 3; lower drug concentration than 1:0.170) was selected to prepare in the further study. The reason was that from the preliminary study of the formulae with other lipid components and higher drug concentration, the drug crystallization occurred.

The two batches of empty liposomes containing the similar composition as in formula 3 were prepared. The average absorbance of supernatant liquid of these empty liposomes after ultracentrifugation at 4°C, 50,000 rpm was used to subtract from the absorbance of supernatant liquid of formula 3 for calculation of the percentage drug entrapment of formula 3.

The percentage drug entrapment was investigated based on the analysis of four batches of liposomal preparations as shown in Table 4. The average percentage drug entrapment was 99.08 ± 0.03% which showed very interestingly high drug encapsulated. This resulted from the hydrophobic characteristic of the drug that can directly interact with the liposomal membranes. From the result, it was shown that in

Table 4 The percentage drug entrapment of ibuprofen liposomes (formula 3) prepared with 90.9 μmol/ml of constant soybean lecithin concentration and 2.7 mg/ml of ibuprofen (1 :0.144 molar ratio of soybean lecithin to drug)

Batch no.	% drug entrapment (SD)	conc. of ibuprofen entrapped (μmol/ml) (SD)
1	99.09	13.10
2	99.08	13.10
3	99.11	13.10
4	99.04	13.10
average (SD)	99.08 (0.03)	13.10 (0.00)

spite of different batches prepared, the standard deviation of percentage drug entrapment was quite low.

Particle size analysis of liposomal preparations (formula 3) was determined by particle size laser scattering analysis of three batches of samples. The measurement was based on three different times of observation as shown in Table 5. It

Table 5 Particle size of ibuprofen liposomes (formula 3) prepared with 90.9 μmol/ml of constant soybean lecithin concentration and 2.7 mg/ml of ibuprofen (1:0.144 molar ratio of soybean lecithin to drug)

Batch no.	size (μm)			average size (μm) (SD)
	1	2	3	
1	7.11	7.06	7.05	7.07 (0.03)
2	5.41	5.38	5.30	5.36 (0.06)
3	4.90	4.89	4.91	4.90 (0.01)
average size (μm) (SD)				5.78 (0.99)

was found that the average particle size was 5.78 ± 0.99 μm. The results as shown in Table 5 revealed that different batches of preparation resulted in different particle sizes. It was found that they had unimodal particle size distributions (Appendix II).

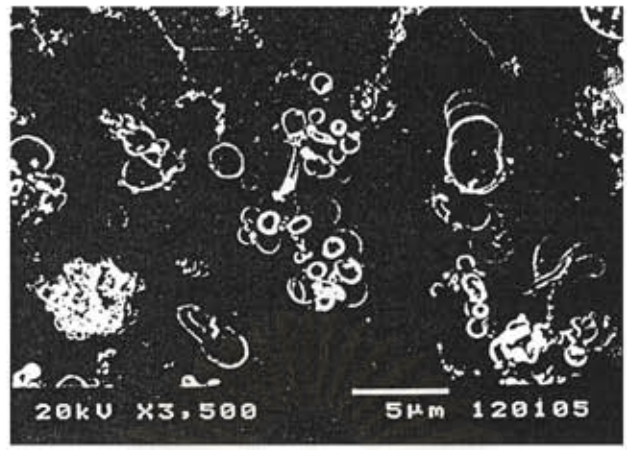
The scanning electron photomicrographs of the liposomal preparation (formula 3) were shown in Figure 8. The liposomal vesicles appeared to be spherical shaped.

From the transmission electron microscopy as shown in Figure 9, the lamellar structures of the liposomal vesicles appeared. It thus can be concluded that the ibuprofen liposomes can be classified to be multilamellar vesicles (MLV) which have several bilayers which capable directly interact with hydrophobic drug molecules.

Multilamellar vesicles vary in the number of lipid bilayers and have a low aqueous encapsulation volume. Therefore, they are suitable for the encapsulation of bilayer-interacting hydrophobic drugs and less appropriate for hydrophilic drugs (New, 1990a; Kulkarni, Betageri and Singh, 1995).

6. Effects of the soybean lecithin to cholesterol molar ratios on the preparation of ibuprofen liposomes

From the previous study, formula 3, liposomes containing 2.7 mg/ml of ibuprofen and 90.9 μmol/ml of constant soybean lecithin concentration was selected to this study. On the efforts to prepare ibuprofen liposomes with various molar ratios of soybean lecithin to cholesterol of 9:1, 8:2, 7:3, 6:4 and 5:5 whereas the whole lipid



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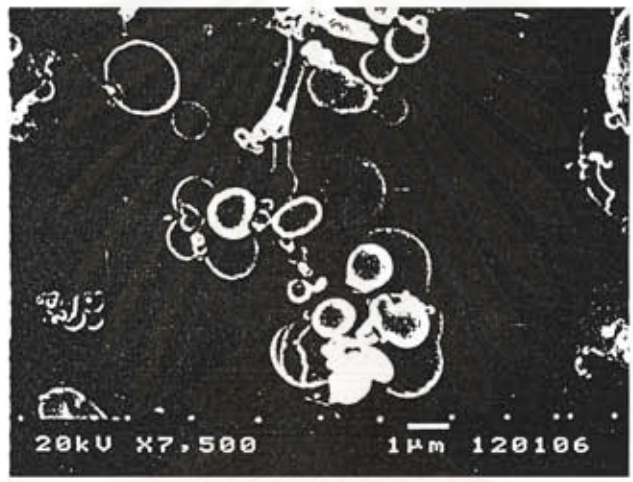

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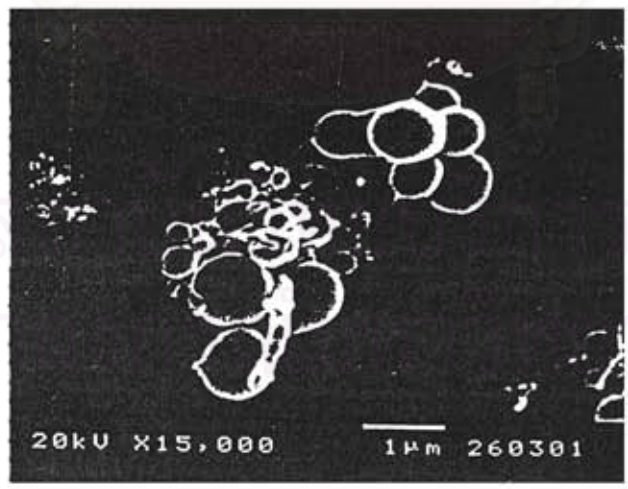

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Figure 8 Scanning electron photomicrographs of ibuprofen liposomes (formula 3) prepared with 90.9 µmol/ml of constant soybean lecithin concentration and 2.7 mg/ml of ibuprofen (1:0.144 molar ratio of soybean lecithin to drug) at t_0 (freshly prepared)

A , magnification : 3,500 x ; B , magnification : 7,500 x ; C , magnification : 15,000 x

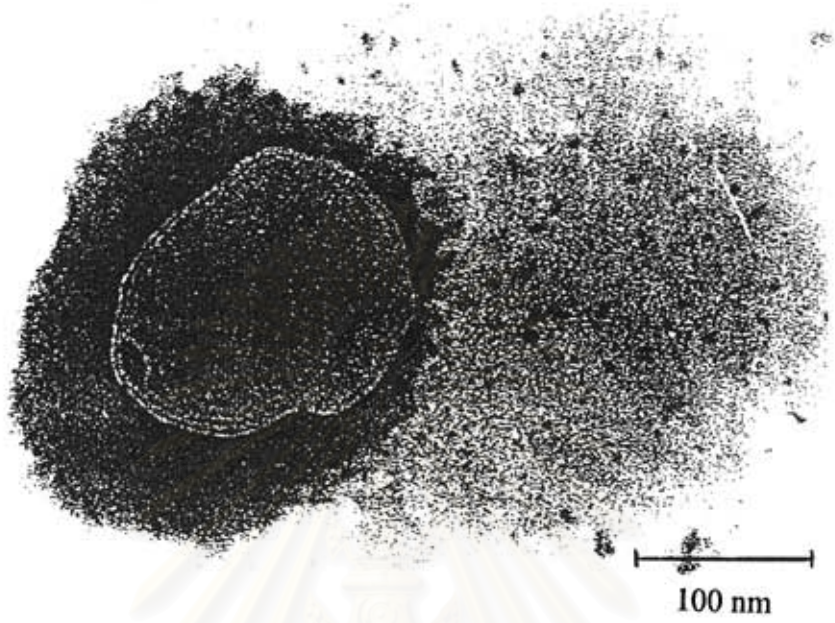


Figure 9 Transmission electron photomicrograph of ibuprofen liposomes (formala 3) prepared with 90.9 μmol/ml of constant soybean lecithin concentration and 2.7 mg/ml of ibuprofen (1:0.144 molar ratio of soybean lecithin to drug) at t_0 (freshly prepared), magnification : 230,000 x

was kept constant at 90.9 μmol/ml, formula 6-10, were obtained as shown in Table 6. It was found that only one ibuprofen liposomal preparation (formula 6) composed of 9:1 molar ratio of soybean lecithin to cholesterol could be obtained as milky suspension and showed no drug crystal. The remainders were found as the viscous gel-like suspension.

The two batches of empty liposomes containing the similar compositions as in formula 6 were prepared. The average absorbance of supernatant liquid of these empty liposomes after ultracentrifugation at 4°C, 50,000 rpm was used to subtract from the absorbance of supernatant liquid of formula 6 for calculation the percentage

Table 6 Effects of the soybean lecithin to cholesterol molar ratios on the preparation of ibuprofen liposomes prepared with 90.9 μmol/ml of constant whole lipid and 2.7 mg/ml of ibuprofen

Formula	soybean lecithin to drug molar ratio	appearance of ibuprofen liposomes	crystal of ibuprofen
6	9:1	milky suspension	not found
7	8:2	viscous suspension	not found
8	7:3	viscous suspension	not found
9	6:4	viscous suspension	not found
10	5:5	viscous suspension	not found

drug entrapment of formula 6.

The percentage drug entrapment based on the analysis of four batches of liposomal preparations which had 9:1 molar ratio of soybean lecithin to cholesterol was shown in Table 7. The average percentage drug entrapment was 98.65 ± 0.12%.

Table 7 The percentage drug entrapment of ibuprofen liposomes (formula 6) prepared with 9:1 molar ratio of soybean lecithin to cholesterol, 90.9 μmol/ml of constant whole lipid and 2.7 mg/ml of ibuprofen

Batch no.	% drug entrapment (SD)	conc. of ibuprofen entrapped (μmol/ml) (SD)
1	98.82	13.06
2	98.57	13.03
3	98.57	13.03
4	98.64	13.04
average (SD)	98.65 (0.12)	13.04 (0.01)

That showed a quite high drug encapsulation. However, it was found that the percentage drug entrapment was less than that of the liposomal preparation containing without cholesterol.

.Cholesterol is incorporated into lipid bilayers to impart bilayer rigidity and makes it more hydrophobic. It revealed in this study that there was an appropriate amount of cholesterol that could be used to obtain liposomal preparation.

The average size of liposomal vesicles based on size analysis of three different batches and at three different times of observation was shown in Table 8. It

Table 8 Particle size of ibuprofen liposomes (formula 6) prepared with 9:1 molar ratio of soybean lecithin to cholesterol, 90.9 μmol/ml of constant whole lipid and 2.7 mg/ml of ibuprofen

Batch no.	size (μm)			average size
	1	2	3	(μm) (SD)
1	5.26	5.23	5.25	5.25 (0.01)
2	4.90	4.86	4.86	4.87 (0.02)
3	4.45	4.45	4.45	4.45 (0)
average size (μm) (SD)				4.86 (0.34)

was shown to be 4.86 ± 0.34 μm that was smaller than that of liposomal preparation (formula 3) without cholesterol. It was found that they had unimodal particle size distributions (data not shown).

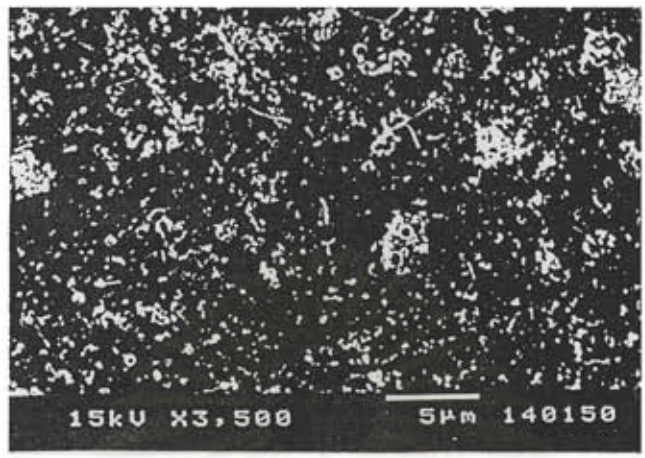
The scanning electron photomicrographs of the formula 6 were shown in Figure 10, Similarly, it demonstrated the spherical shaped vesicles of liposomes.

7. Effects of the amount of stearylamine on the preparation of ibuprofen liposomes

Stearylamine was added to formula 6 in various amounts as described in Table 9. It was found that ibuprofen liposomes (formula 11-13) prepared with 20.00, 15.00 and 10.00 mole% of stearylamine and 90.9 μmol/ml of constant whole lipid could not be obtained as milky suspension. They appeared to be viscous gel. Thus, an attempt was carried out on less amounts of stearylamine of 5.00, 2.50 and 1.25 mole% added to the formulation. Similarly, at 5.00 mole% of stearylamine (formula14), a milky suspension also could not be obtained. Whereas there was aggregated lipid in ibuprofen liposomes containing 1.25 mole% of stearylamine (formula 16). It was demonstrated that the appropriate amount of stearylamine was 2.50 mole% (formula 15) because a milky suspension without drug crystal could be achieved. Therefore, formula 15 was selected for the further study.

The two batches of empty liposomes containing the similar compositions as in formula 15 were prepared. The average absorbance of supernatant liquid of these empty liposomes after ultracentrifugation at 4°C, 50,000 rpm was used to subtract from the absorbance of supernatant liquid of formula 15 for calculation the percentage drug entrapment of formula 15.

The average percentage drug entrapment of liposomal preparations (formula 15) was investigated based on analysis of four different batches of liposomal preparations which had 9:1 molar ratio of soybean lecithin to cholesterol and 2.50


15kV X3,500
5µm 140150


15kV X7,500
1µm 140151

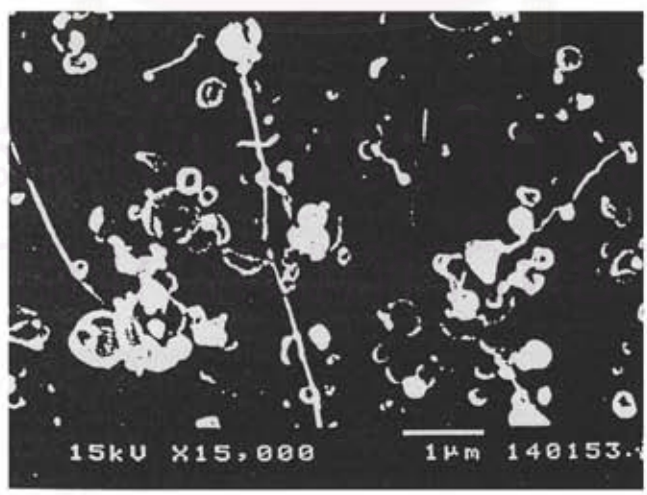

15kV X15,000
1µm 140153

Table 9 Effects of the amount of stearylamine on the preparation of ibuprofen liposomes prepared with 9:1 molar ratio of soybean lecithin to cholesterol, 90.9 μmol/ml of constant whole lipid and 2.7 mg/ml of ibuprofen

Formula	amount of stearylamine (mole%)	appearance of ibuprofen liposomes	crystal of ibuprofen
11	20.00	viscous gel	not found
12	15.00	viscous gel	not found
13	10.00	viscous gel	not found
14	5.00	viscous gel	not found
15	2.50	milky suspension	not found
16	1.25	aggregation	not found

mole% of stearylamine. It was determined to be 98.05 ± 0.03% drug entrapped as shown in Table 10. The entrapment was shown a little smaller than the formula containing only soybean lecithin.

Table 10 The percentage drug entrapment of ibuprofen liposomes (formula 15) prepared with 9:1 molar ratio of soybean lecithin to cholesterol, 2.50 mole% of stearylamine, 90.9 μmol/ml of constant whole lipid and 2.7 mg/ml of ibuprofen

Batch no.	% drug entrapment (SD)	conc. of ibuprofen entrapped (μmol/ml) (SD)
1	98.07	12.96
2	98.09	12.97
3	98.03	12.96
4	98.02	12.96
average (SD)	98.05 (0.03)	12.96 (0)

The particle size of three different batches of formula 15 were averaged to be 4.72 ± 0.25 μm as shown in Table 11. It seemed that the size was smaller than that

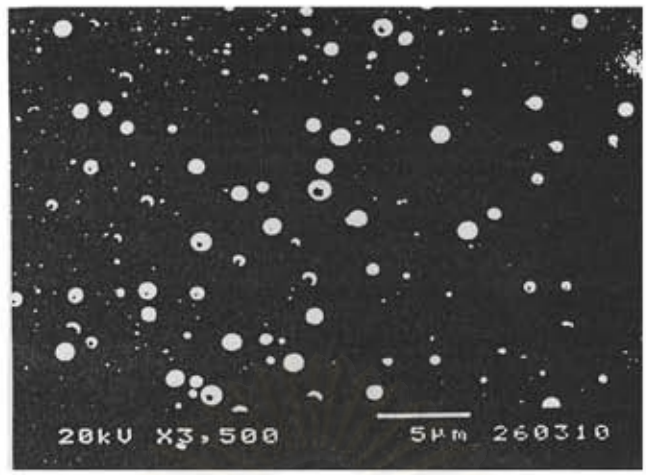
Table 11 Particle size of ibuprofen liposomes (formula 15) prepared with 9:1 molar ratio of soybean lecithin to cholesterol, 2.50 mole% of stearylamine, 90.9 μmol/ml of constant whole lipid and 2.7 mg/ml of ibuprofen

Batch no.	size (μm)			average size (μm) (SD)
	1	2	3	
1	5.05	5.06	5.04	5.05 (0.01)
2	4.61	4.55	4.50	4.55 (0.05)
3	4.59	4.58	4.48	4.55 (0.06)
average size (μm) (SD)				4.72 (0.25)

of the liposomal formulation containing only soybean lecithin as lipid content. It was found that they had unimodal particle size distributions (data not shown).

From the scanning electron photomicrographs, similarly, they showed the spherical shaped vesicles as shown in Figure 11. Furthermore, it was noticed that liposomal vesicles distinctly separated from each other.

Incorporation of stearylamine, a positively charged component, can induce charges on the bilayers. In such type of liposomes, due to the presence of a charged interface, there is an electrostatic repulsion between adjacent bilayers, causing an increase in the distance between the bilayers; this leads to a rise in the volume of the internal aqueous component of multilamellar liposomes. The presence of charge may also prevent aggregation of liposomes (Weiner, Martin, and Riaz, 1989; New, 1990g;



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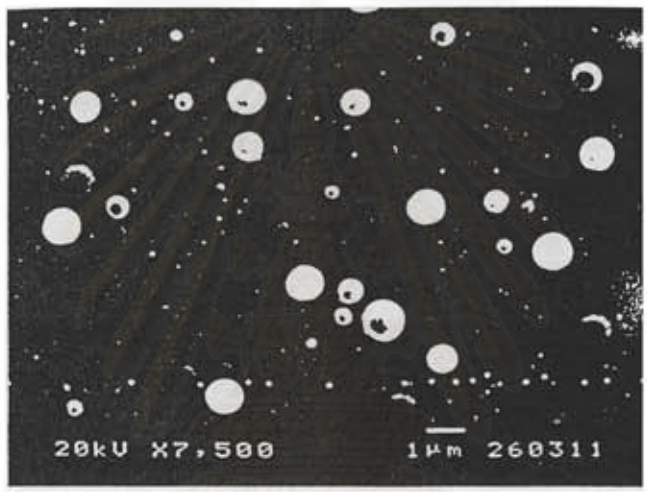

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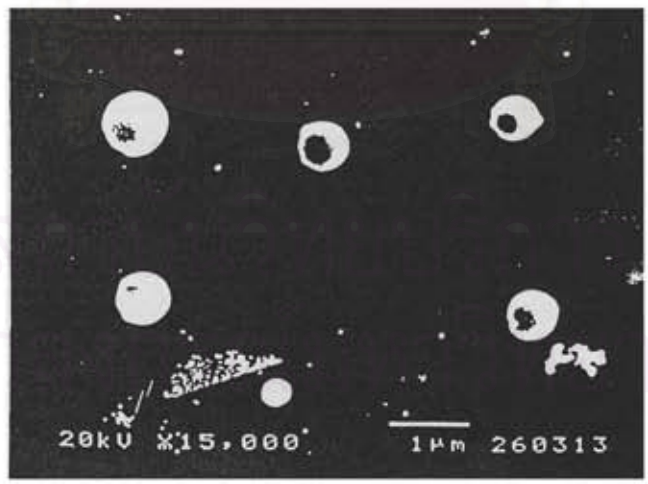

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Figure 11 Scanning electron photomicrographs of ibuprofen liposomes (formula 15) prepared with 9:1 molar ratio of soybean lecithin to cholesterol, 2.50 mole% of stearylamine, 90.9 µmol/ml of constant whole lipid and 2.7 mg/ml of ibuprofen at t_0 (freshly prepared)

A , magnification : 3,500 x ; B , magnification : 7,500 x ; C , magnification : 15,000 x

Kalkarni, Betageri, and Singh, 1995). Thus, for the further study , the formula 15 was selected.

8. Effects of the amount of (±)-α-tocopherol on the preparation of ibuprofen liposomes

In the addition of various amount of (±)-α-tocopherol of, 1.00, 0.50 and 0.10% (formulae 17-19) as shown in Table 12, it was found that the liposomal

Table 12 Effects of the amount of (±)- α-tocopherol on the preparation of ibuprofen liposomes (formula 17-24) prepared with 9:1 molar ratio of soybean lecithin to cholesterol, 2.50 mole% of stearylamine, 90.9 μmol/ml of constant whole lipid and 2.7 mg/ml of ibuprofen

Formula	amount of (±)-α-tocopherol (%)	appearance of ibuprofen liposomes	crystal of ibuprofen
17	1.00	viscous gel	not found
18	0.50	viscous gel	not found
19	0.10	viscous gel	not found
20	0.05	viscous gel	not found
21	0.025	milky suspension	not found
22	0.0125	milky suspension	not found
23	0.00625	milky suspension	not found
24	0.001	milky suspension	not found

preparations could not be obtained as milky suspension. Instead, the viscous gel were produced. Thus, the additional efforts to prepare liposomal formulations with less amount of (±)-α-tocopherol, 0.050% (formula 20), was carried out. A milky

suspension could not be also obtained, it was still viscous gel. So, the lesser amounts of (±)-α-tocopherol, 0.025, 0.0125, 0.00625 and 0.001% (formulae 21-24, respectively), were carried out. Liposomal preparations as milky suspension were achieved from all formulae.

However, the empty liposomes containing the similar component as in formula 21-24 could not be obtained. It was found that they appeared to be viscous gel and it was difficult to remove from the round bottom flask. Thus, there were no absorbance values of supernatant liquid of corresponding empty liposomes after separation used to subtract from the absorbanes of supernatant liquid of ibuprofen liposomal preparations of formula 21-24.

The average percentage drug entrapment of liposomal preparations (formula 21-24) that can be obtained as milky suspension was determined based on drug analysis of four different batches as shown in Table 13. The entrapment of these

Table 13 The percentage drug entrapment of ibuprofen liposomes (formula 21-24) prepared with 0.025, 0.0125, 0.00625 and 0.001% of (±)-α-tocopherol, 9:1 molar ratio of soybean lecithin to cholesterol, 2.50 mole% of stearylamine, 90.9 μmol/ml of constant whole lipid and 2.7 mg/ml of ibuprofen (n = 4)

Formula	amount of (±)-α-tocopherol (%)	% drug entrapment				average % drug entrapment (SD)	average conc. of ibuprofen entrapped (μmol/ml) (SD)
		n_1	n_2	n_3	n_4		
21	0.025	98.33	98.44	98.19	98.37	98.33 (0.10)	13.00 (0.01)
22	0.0125	98.29	98.26	97.82	98.63	98.25 (0.33)	12.99 (0.04)
23	0.00625	98.38	98.33	97.98	98.44	98.29 (0.21)	12.99 (0.03)
24	0.001	97.89	98.15	98.42	98.59	98.26 (0.31)	12.99 (0.04)

liposomal preparations appeared to be very closed in the range 98.25-98.33%.

The average particle size of four liposomal preparations (formula 21-24) were investigated from three different batches as shown in Table 14. They ranged

Table 14 Particle size of ibuprofen liposomes (formula 21-24) prepared with 0.025, 0.0125, 0.00625 and 0.001% of (±)-α-tocopherol, 9:1 molar ratio of soybean lecithin to cholesterol, 2.50 mole% of stearylamine, 90.9 μmol/ml of constant whole lipid and 2.7 mg/ml of ibuprofen

Formula	Batch no.	size (μm)			average size (μm) (SD)
		1	2	3	
21	1	5.51	5.51	5.51	
	2	5.53	5.40	5.36	
	3	5.20	5.20	4.92	5.35 (0.21)
22	1	5.22	5.22	5.30	
	2	5.55	5.48	5.44	
	3	5.20	5.14	5.12	5.30 (0.16)
23	1	4.91	5.12	5.13	
	2	4.66	4.57	4.53	
	3	4.23	4.28	4.27	4.63 (0.35)
24	1	4.89	4.90	4.84	
	2	5.52	5.35	5.24	
	3	4.35	4.30	4.36	4.86 (0.45)

between 4.63-5.35 μm, that seemed to be smaller than those of liposomal preparations containing only soybean lecithin. It was found that they had unimodal particle size distributions (data not shown).

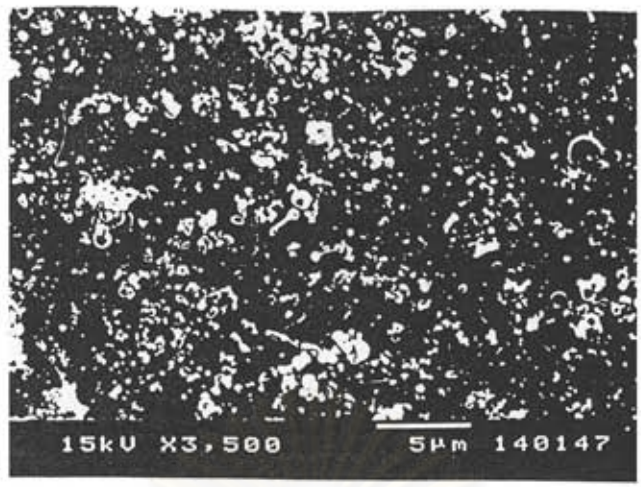
The scanning electron photomicrographs of four liposomal preparations containing 0.025, 0.0125, 0.00625 and 0.001% of (±)-α- tocopherol were shown in Figure 12, 13, 14, and 15, respectively. They similarly demonstrated spherical shaped vesicles. However, it was noticed that there were liposomal vesicles attached closely, like aggregated, much more than the liposomal preparation containing 2.50 mole% of stearylamine without (±)-α-tocopherol (formula 15).

9. Effects of compounds used for preparing ibuprofen liposomes on the percentage drug entrapment, size, and the morphology of ibuprofen liposomes

Important compounds used for preparing ibuprofen liposomes including drug, ibuprofen, were lipophilic substances. They preferred to attach and interact with the bilayer membranes. It was found that the percentage drug entrapment of ibuprofen liposomes using only 90.9 μmol/ml of soybean lecithin and 2.7 mg/ml of ibuprofen (formula 3) was significantly highest among formulae ($P < 0.05$) as shown in Table 15 and 16. Beside this, particle size of this formula was also significantly highest ($P < 0.05$) as shown in Table 17 and 18.

When cholesterol was added at 9:1 molar ratio of soybean lecithin to cholesterol (formula 6), the percentage drug entrapment and size of ibuprofen liposomes significantly decreased ($P < 0.05$) as shown in Table 15-16 and 17-18, respectively.

The incorporation of cholesterol caused the decrease of ibuprofen liposomes sizes when compared with those containing no cholesterol. Generally, the



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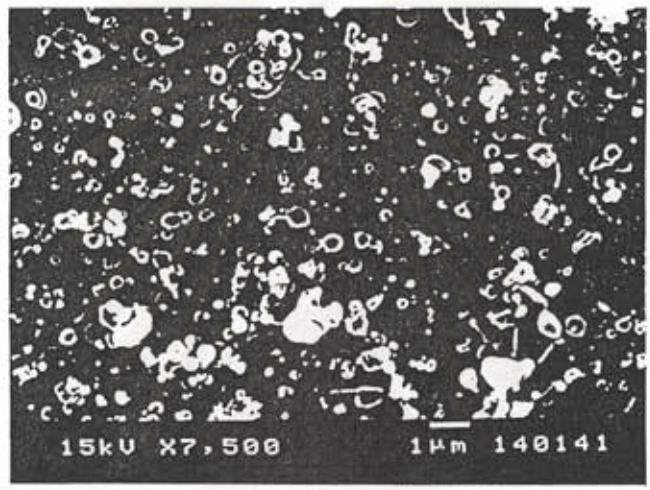

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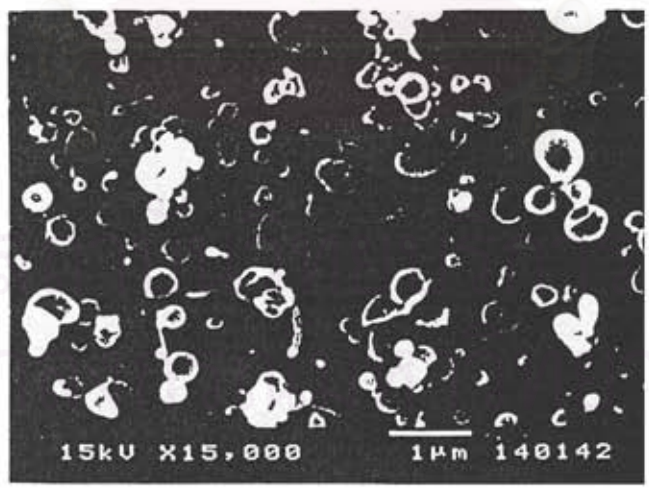
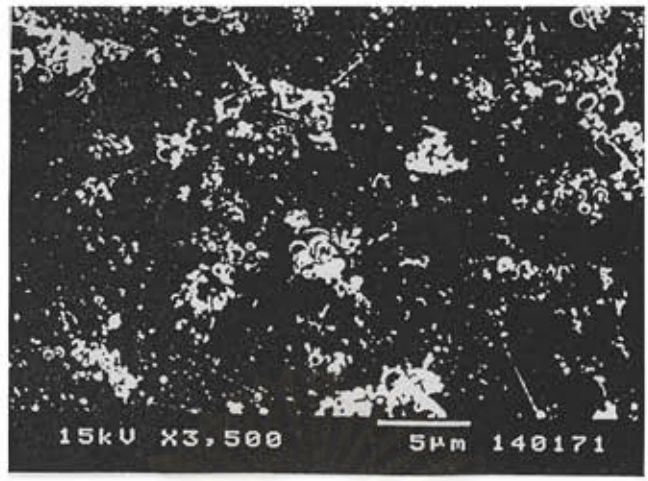


C

Figure 12 Scanning electron photomicrographs of ibuprofen liposomes (formula 21) prepared with 9:1 molar ratio of soybean lecithin to cholesterol, 2.50 mole% of stearylamine, 0.025% of (±)-α-tocopherol, 90.9 µmol/ml of constant whole lipid and 2.7 mg/ml of ibuprofen at t_0 (freshly prepared)

A , magnification : 3,500 x ; B , magnification : 7,500 x ; C , magnification : 15,000 x



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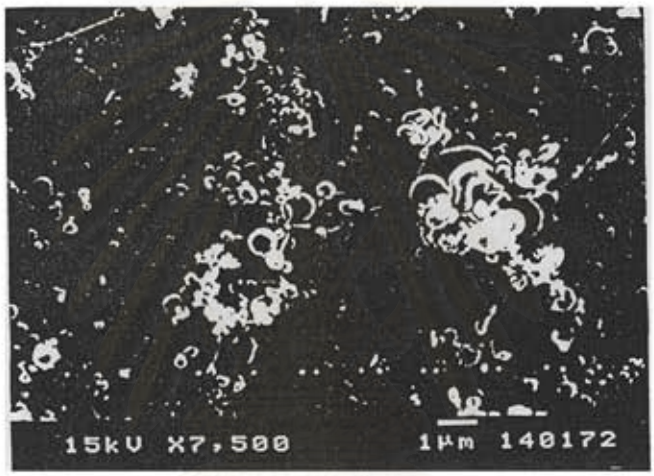

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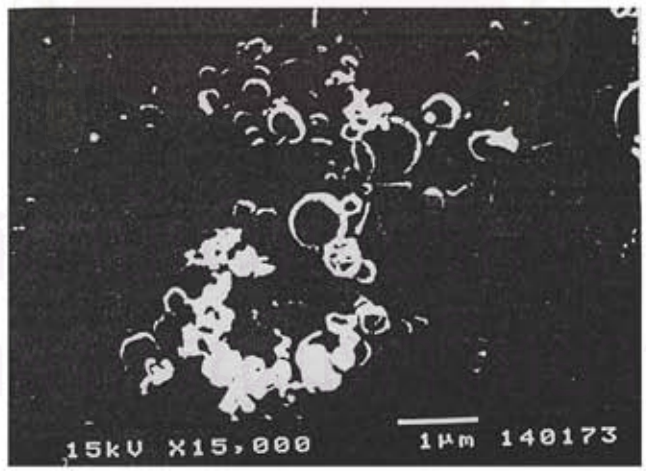

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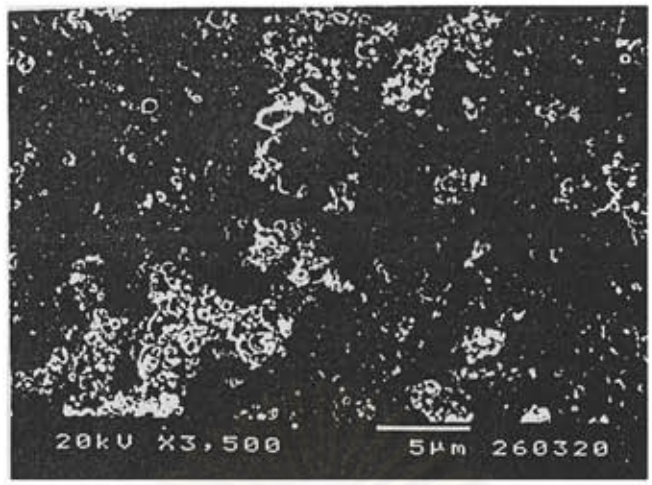
Figure 13 Scanning electron photomicrographs of ibuprofen liposomes (formula 22) prepared with 9:1 molar ratio of soybean lecithin to cholesterol, 2.50 mole% of stearylamine, 0.0125% of (±)-α-tocopherol, 90.9 µmol/ml of constant whole lipid and 2.7 mg/ml of ibuprofen at t_0 (freshly prepared)

A , magnification : 3,500 x ; B , magnification : 7,500 x ; C , magnification : 15,000 x



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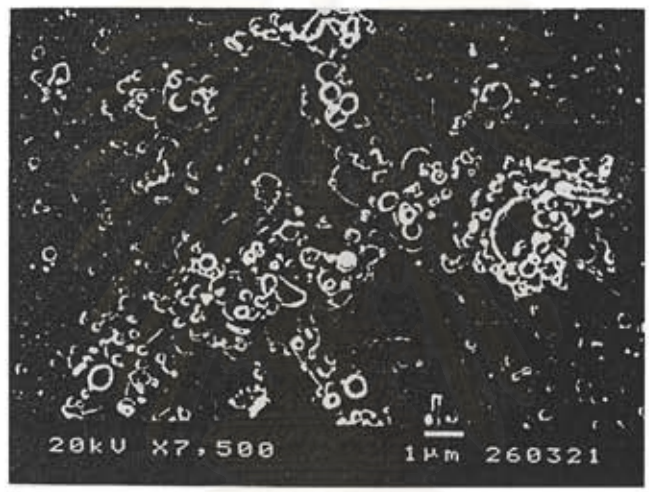

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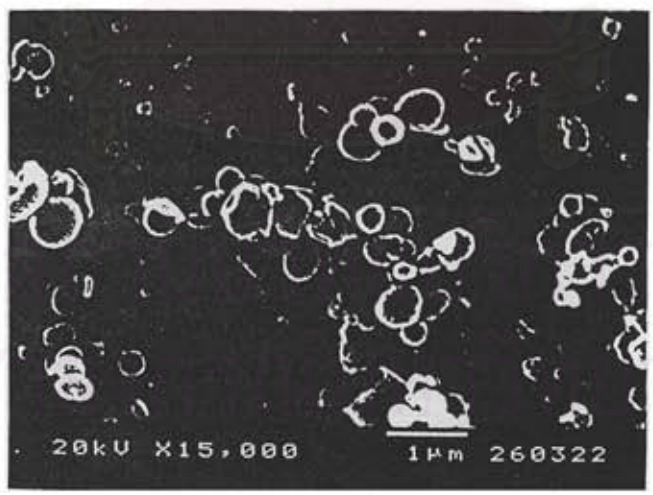
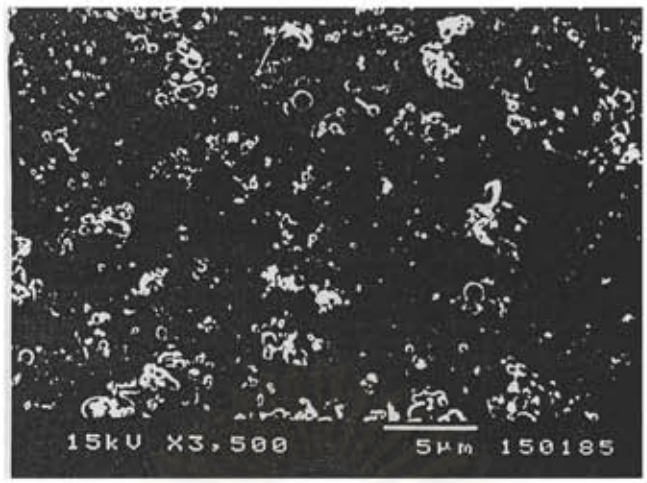


C

Figure 14 Scanning electron photomicrographs of ibuprofen liposomes (formula 23) prepared with 9:1 molar ratio of soybean lecithin to cholesterol, 2.50 mole% of stearylamine, 0.00625% of (±)-α-tocopherol, 90.9 µmol/ml of constant whole lipid and 2.7 mg/ml of ibuprofen at t_0 (freshly prepared)

A , magnification : 3,500 x ; B , magnification : 7,500 x ; C , magnification : 15,000 x



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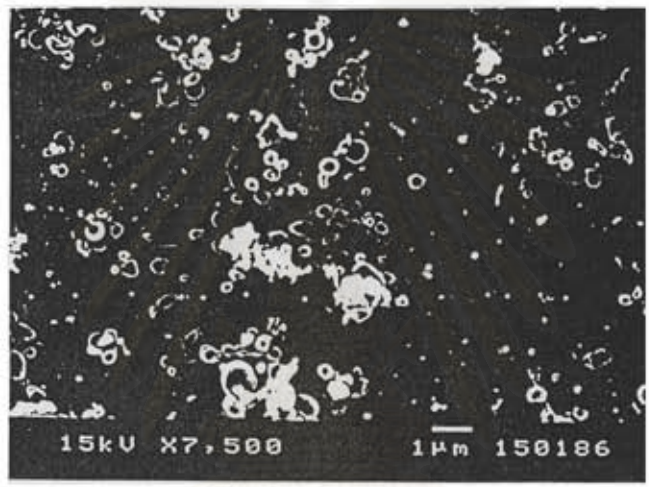

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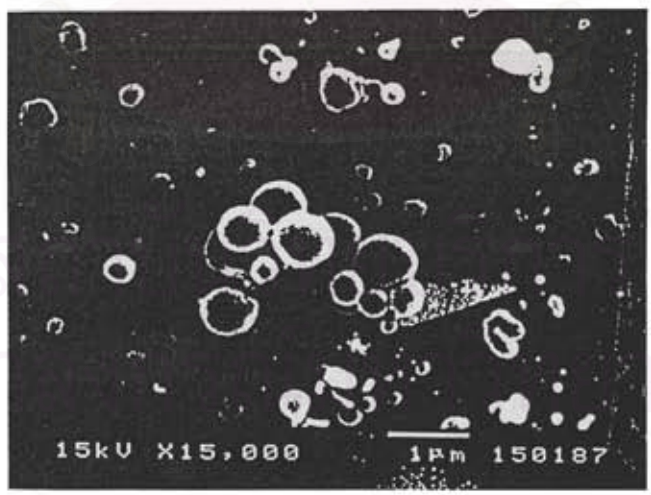


C

Figure 15 Scanning electron photomicrographs of ibuprofen liposomes (formula 24) prepared with 9:1 molar ratio of soybean lecithin to cholesterol, 2.50 mole% of stearylamine, 0.001% of (±)-α-tocopherol, 90.9 µmol/ml of constant whole lipid and 2.7 mg/ml of ibuprofen at t_0 (freshly prepared)

A , magnification : 3,500 x ; B , magnification : 7,500 x ; C , magnification : 15,000 x

Table 15 One way ANOVA data of average percentage drug entrapment of selected ibuprofen liposomes formulae at t_0 (freshly prepared).

Source	df	SS	MS	F	F-critical
Among Groups	6	2.81	0.47	11.96	2.57
Within Groups	21	0.82	0.04		
Total	27	3.63			

Level of significance = 0.05

df = Degree of freedom MS = Mean square

SS = Sum of square F = Variance ratio

Table 16 Duncan's new multiple range test of average percentage drug entrapment of selected ibuprofen liposomes formulae at t_0 (freshly prepared).

formula	15	22	24	23	21	6	3
average percentage of drug entrapment	98.05	98.25	98.26	98.29	98.33	98.65	99.08

p	2	3	4	5	6	7
SSR (df 21)	2.95	3.10	3.18	3.25	3.30	3.34
LSR	0.29	0.31	0.32	0.32	0.33	0.33

p = number of mean paired associated with the same number of steps

LSR = Least significant range

SSR = Significant studentized range

formula	3	6	15	21	22	23	24
3		×	×	×	×	×	×
6			×	×	×	×	×
15				-	-	-	-
21					-	-	-
22						-	-
23							-
24							

× = Statistical difference ($p < 0.05$)

\- = Not statistical difference ($p > 0.05$)

Table 17 One way ANOVA data of average particle size of selected ibuprofen liposomes formulae at t_0 (freshly prepared).

Source	df	SS	MS	F	F-critical
Among Groups	6	9.32	1.55	6.95	2.26
Within Groups	56	12.51	0.22		
Total	62	21.83			

Level of significance = 0.05

df = Degree of freedom MS = Mean square

SS = Sum of square F = Variance ratio

Table 18 Duncan's new multiple range test of average particle size of selected ibuprofen liposomes formulae at t_0 (freshly prepared).

formula	23	15	6, 24	22	21	3
average particle size (μm)	4.63	4.72	4.86	5.30	5.35	5.78

p	2	3	4	5	6
SSR (df 56)	2.83	2.98	3.08	3.14	3.20
LSR	0.45	0.47	0.49	0.49	0.50

p = number of mean paired associated with the same number of steps

LSR = Least significant range

SSR = Significant studentized range

formula	3	6	15	21	22	23	24
3		×	×	-	×	×	×
6			-	×	-	-	-
15				×	×	-	-
21					-	×	×
22						×	-
23							-
24							

× = Statistical difference ($p < 0.05$)

\- = Not statistical difference ($p > 0.05$)

addition of cholesterol caused in the increase of liposomal vesicle sizes. It was found that the increasing amount of cholesterol caused in the increasing of surface area per phospholipid molecule. This caused from the molecules of cholesterol which were put into the bilayers, the heads of the phospholipid molecules, that could obtain their water of hydration by overlapping the cholesterol, and their hydrocarbon chain straighten out behind, thereby increasing the membrane thickness which caused in the increasing in size of vesicles. However, it was found that when the proportion of cholesterol added was low, the decrease in membrane thickness of liposomal size occurred. This caused from the tilting the rest of the cholesterol molecules sideways (Johnson, 1973). As a result of this, it caused in the decrease of liposomal sizes. That was the reason why the sizes of ibuprofen liposomes containing 9:1 molar ratio of soybean lecithin to cholesterol decreased.

The decrease of percentage drug entrapment of ibuprofen liposomes in this study was consistent with Stamp and Juljano (1979) who found that the addition of cholesterol reduced the encapsulation of hydrophobic drugs. This caused from the decrease of ibuprofen liposomal sizes. Ibuprofen is a hydrophobic drug which prefers to attach with the bilayer membranes. When the membrane thickness of liposomal sizes decreased, the sizes decreased, and the areas for the attachment of ibuprofen also decreased. This caused in the decrease of percentage drug entrapment.

It was distinctive that addition of 2.50 mole% of stearylamine, the positively charged compound, in formula 15 led to the decrease of both percentage drug entrapment and particle size. It might attribute to the presence of charged interface induced by stearylamine, there was an electrostatic repulsion between adjacent bilayer and vesicles ; this led to a decrease in the number of bilayes in liposomal vesicles. This might result in the decrease in the drug entrapment (Weiner,

Martin, and Riaz, 1989; New, 1990g; Kalkarni, Betageri, and Singh, 1995). Moreover, it might attribute to the competitive interaction between stearylamine, amphiphilic molecules, and ibuprofen drug molecules to the bilayer membranes, that resulted in the slight decreasing of drug entrapment.

Although there is approximately 0.1% of α-tocopherol in soybean lecithin (Phospholipon ®) which was used in this study but it was found that the antioxidant efficiency of α-tocopherol in liposomal membranes was lower than in homogeneous solution (Niki, Takahashi, and Komuro, 1986). Thus, the amount of α-tocopherol in Phospholipon ® may be not enough for the inhibition of oxidation in liposomal membranes. The addition of α-tocopherol might increase antioxidant activity and act as an antioxidant in the ibuprofen liposomal preparations. Therefore, additional amounts of 0.001-0.025% of (±)-α-tocopherol were incorporated and their effects to the percentage drug entrapment and particle size including the antioxidant activity in the protection of ibuprofen liposomal preparations were studied.

From Table 17-18, (±)-α-tocopherol at only two concentration, 0.025 and 0.0125% (formulae 21 and 22, respectively), led to significantly increase in particle sizes of liposomal preparations ($P < 0.05$) when compared with formula 15, the formula containing stearylamine without (±)-α-tocopherol. Whereas from Table 15-16, there was no significant change in the percentage drug entrapment of the formulae containing all concentrations of (±)-α-tocopherol (formulae 21-24) and the formula containing stearylamine without (±)-α-tocopherol (formula 15) ($P > 0.05$). It was possible that the increase in size of ibuprofen liposomes when (±)-α-tocopherol was added caused from the insertion of (±)-α-tocopherol molecules in the bilayer membranes. This might cause in the reorientation of lipophilic molecules in the bilayer membranes. As a result of this, it caused in the increase of ibuprofen

liposomes sizes but not of percentage drug entrapment. However, there was the optimum amount of (±)-α-tocopherol to do this. Thus, it was found that 0.00625% and 0.001% of (±)-α-tocopherol added in formulae 23 and 24, respectively, did not affect to size of ibuprofen liposomes when compared with formula 15.

The characteristics from the scanning electron photomicrographs of all different ibuprofen liposomal preparation were quite the same. The spherical shape vesicles were demonstrated. However, it was obvious that the liposomal preparation containing 2.50 mole% of stearylamine (formula 15) had different appearances that liposomal vesicles clearly separated from each other.

However, it was noticed that particle sizes measured by particle size laser scattering analyzer were larger than ones observed by scanning electron microscope. These might cause from the drawback of particle size laser scattering analyzer. This method can not distinguish between a large particle and a flocculated mass of smaller particles (Weiner, Martin, and Riaz, 1989). If aggregated or overlapped liposomal vesicles passed through the laser channel beam, the instrument measured them as larger apparent sizes. These resulted in the different particle sizes measured by particle size laser scattering analyzer and SEM.

Ibuprofen liposomes selected from the most appropriate formula in section 5, 6, 7, and 8 were further studied in stability of liposomal preparations by terms of percentage drug entrapment, size and size distribution, and the morphology of ibuprofen liposomes. The percentage drug entrapment and particle size of all formulae selected was summarized in Table 19.

Table 19 The percentage drug entrapment and particle size of selected ibuprofen liposomes at t_0 (freshly prepared)

Formula	average percentage drug entrapment (SD)	average particle size (μm) (SD)
3	99.08 (0.03)	5.78 (0.99)
6	98.65 (0.12)	4.86 (0.34)
15	98.05 (0.03)	4.72 (1.25)
21	98.33 (0.10)	5.35 (0.21)
22	98.25 (0.33)	5.30 (0.16)
23	98.29 (0.21)	4.63 (0.35)
24	98.26 (0.31)	4.86 (0.45)

However, four batches of corresponding ibuprofen liposomal preparations selected were prepared for stability study in term of the percentage drug entrapment because whole ibuprofen liposomal preparation prepared was used to analyze for the percentage drug entrapment.

10. Comparison of the stability of ibuprofen liposomes between freshly prepared and after one month storage at 4°C

The stability of ibuprofen liposomal preparations selected from the most appropriate formula in each section was investigated after one month storage at refrigerated temperature, 4°C. The percentage drug entrapment and size of formulae selected after storage were shown in Table 20 and Appendix II. The statistic used for data analysis was one way ANOVA following Duncan's new multiple range test.

Table 20 The percentage drug entrapment and particle size of selected ibuprofen liposomes at t_1 (after storage at 4°C for 1 month)

Formula	average percentage drug entrapment (SD)	average particle size (μm) (SD)
3	99.16 (0.10)	6.73 (0.77)
6	99.13 (0.03)	5.71 (0.11)
15	98.20 (0.26)	5.01 (0.42)
21	98.39 (0.04)	9.46 (2.12)
22	98.46 (0.06)	5.45 (0.27)
23	98.09 (0.06)	6.33 (0.31)
24	98.26 (0.26)	6.01 (0.53)

The results showed that there were only two formulations which showed no significant changes in percentage drug entrapment and sizes after storage ($P < 0.05$) as shown in Table 21-22 and 23-24, respectively. One was the formulation containing 9:1 molar ratio of soybean lecithin to cholesterol and 2.50 mole% of stearylamine (formula 15). The other was the liposomal preparation containing additional 0.0125% of (±)-α-tocopherol (formula 22).

In addition, physical appearance of the two formulae were still achieved as stable milky suspension. By the meaning of these, it can be said that the above two formulations had good stability. They could be described that 2.50 mole% of stearylamine could stabilize liposomes from the aggregation and fusion.

Obviously, an only one appropriate amount of (±)-α-tocopherol added in the formulation acted as an antioxidant. It demonstrated that using more amounts of (±)-α-tocopherol led to the aggregation of liposomal vesicles. Possibly, it might

Table 21 One way ANOVA data of average percentage drug entrapment of selected ibuprofen liposomes formulae compared at t_0 (freshly prepared) and t_1 (after storage at 4°C for 1 month).

Source	df	SS	MS	F	F-critical
Among Groups	13	7.63	0.59	19.07	1.96
Within Groups	42	1.29	0.03		
Total	55	8.93			

Level of significance = 0.05

df = Degree of freedom MS = Mean square

SS = Sum of square F = Variance ratio

Table 22 Duncan's new multiple range test of average percentage drug entrapment of selected ibuprofen liposomes formulae compared at t_0 (freshly prepared) and t_1 (after storage at 4°Cfor 1 month).

formula	$15(t_0)$	$23(t_1)$	$15(t_1)$	$22(t_0)$	$24(t_0)$	$24(t_1)$	$23(t_0)$	$21(t_0)$	$21(t_1)$	$22(t_1)$	$6(t_0)$	$3(t_0)$	$6(t_1)$	$3(t_1)$
average percentage of drug entrapment	98.05	98.09	98.20	98.25	98.26	98.26	98.29	98.33	98.39	98.46	98.65	99.08	99.13	99.16

p	2	3	4	5	6	7	8	9	10	11	12	13
SSR (df 42)	2.86	3.01	3.10	3.17	3.22	3.27	3.30	3.33	3.35	3.39	3.42	3.42
LSR	0.25	0.26	0.27	0.28	0.28	0.29	0.29	0.29	0.29	0.30	0.30	0.30

p = number of mean paired associated with the same number of steps

LSR = Least significant range

SSR = Significant studentized range

formula	$3(t_1)$	$6(t_1)$	$15(t_1)$	$21(t_1)$	$22(t_1)$	$23(t_1)$	$24(t_1)$
$3(t_0)$	-						
$6(t_0)$		×					
$15(t_0)$			-				
$21(t_0)$				-			
$22(t_0)$					-		
$23(t_0)$						-	
$24(t_0)$							-

× = Statistical difference ($p < 0.05$)

\- = Not statistical difference ($p > 0.05$)

Table 23 One way ANOVA data of average particle size of selected ibuprofen liposomes formulae compared at t_0 (freshly prepared) and t_1 (after storage at 4°C for 1 month).

Source	df	SS	MS	F	F-critical
Among Groups	13	180.57	13.89	26.65	1.81
Within Groups	112	58.36	0.52		
Total	125	238.93			

Level of significance = 0.05

df = Degree of freedom MS = Mean square

SS = Sum of square F = Variance ratio

Table 24 Duncan's new multiple range test of average particle size of selected ibuprofen liposomes formulae compared at t_0 (freshly prepared) and t_1 (after storage at 4°C for 1 month).

formula	$23(t_0)$	$15(t_0)$	$6(t_0)$	$24(t_0)$	$15(t_1)$	$22(t_0)$	$21(t_0)$	$22(t_1)$	$6(t_1)$	$3(t_0)$	$24(t_1)$	$23(t_1)$	$3(t_1)$	$21(t_1)$
average particle size (μm)	4.63	4.72	4.86	4.86	5.01	5.30	5.35	5.45	5.71	5.78	6.01	6.33	6.73	9.46

p	2	3	4	5	6	7	8	9	10	11	12	13
SSR (df 42)	2.77	2.92	3.02	3.09	3.15	3.19	3.23	3.26	3.29	3.34	3.38	3.38
LSR	0.67	0.70	0.73	0.74	0.76	0.77	0.78	0.78	0.79	0.80	0.81	0.81

p = number of mean paired associated with the same number of steps

LSR = Least significant range

SSR = Significant studentized range

formula	$3(t_1)$	$6(t_1)$	$15(t_1)$	$21(t_1)$	$22(t_1)$	$23(t_1)$	$24(t_1)$
$3(t_0)$	×						
$6(t_0)$		×					
$15(t_0)$			-				
$21(t_0)$				×			
$22(t_0)$					-		
$23(t_0)$						×	
$24(t_0)$							×

× = Statistical difference ($p < 0.05$)

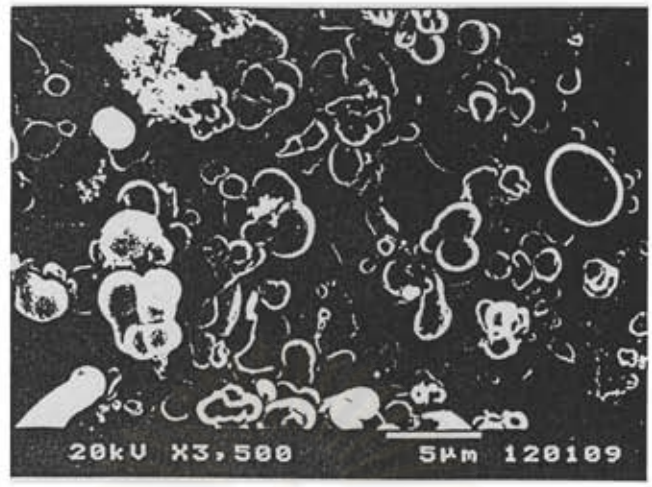
\- = Not statistical difference ($p > 0.05$)

interfere the electrostatic activity of stearylamine in the protection of aggregation of vesicles.

The increase in sizes of formula 3, ibuprofen liposomes containing only soybean lecithin, might be caused from the hydrolysis of soybean lecithin which resulted in the presence of lyso-PC. Lyso-PC in the micellar form could interact with liposomes and caused in the fusion with the liposomal membranes and led to the increase of the liposomal vesicle sizes (Zuidam et al, 1995).

Generally, the incorporation of cholesterol could stabilize liposomal vesicles by decreasing the permeability of liposome membranes. It reduced the chain mobility and consequently decreased the permeability of the structural membranes (De Gier, Mandersloot, and Van Deenen, 1968; New, 1990d). Consequently, inclusion of cholesterol into unsaturated membranes was often essential in order to achieve sufficient stability. However, it was not consistent with this study. It was found that the sizes of formula 6, ibuprofen liposomes containing 9:1 molar ratio of soybean lecithin to cholesterol, increased after storage at $4^{\circ}C$ for 1 month. The increase of liposomal sizes was designated instability of ibuprofen liposomes. This referred to the formation of lyso-PC as mentioned above. The condensing effect of cholesterol might be interfered with the penetration of lyso-PC molecules or fusion of lyso-PC micelles which caused in the increasing of liposomal vesicle sizes (Inoue and Kitagawa, 1974).

The electron micrographic characteristics of all ibuprofen liposomal preparations selected after storage at $4^{\circ}C$ from the observation by SEM were shown in Figure 16-22. It was found that they certain changed from the initial time as shown in Figure 23. However, it was noticed that vesicular aggregations occurred in the



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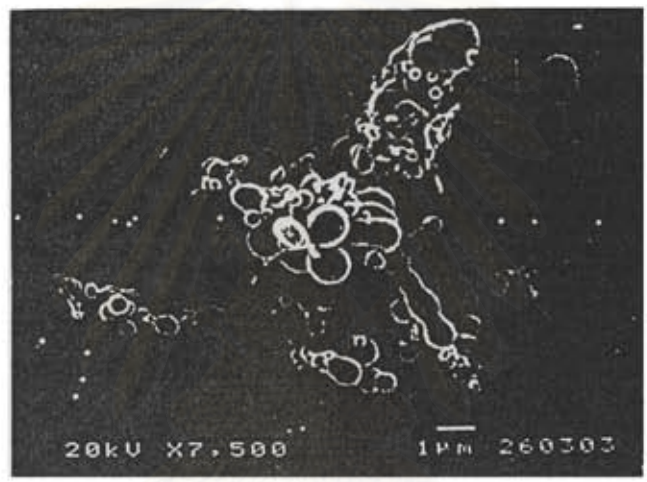

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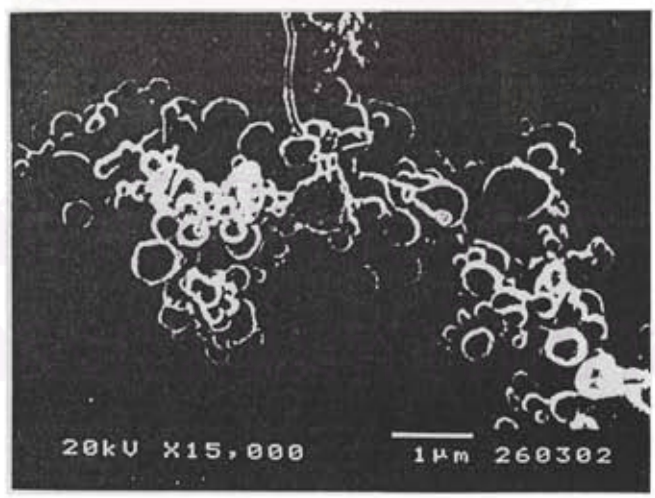

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Figure 16 Scanning electron photomicrographs of ibuprofen liposomes (formula 3) prepared with 90.9 µmol/ml of soybean lecithin concentration and 2.7 mg/ml of ibuprofen at t_1 (after storage at 4°C for 1 month)

A , magnification : 3,500 x ; B , magnification : 7,500 x ; C , magnification : 15,000 x

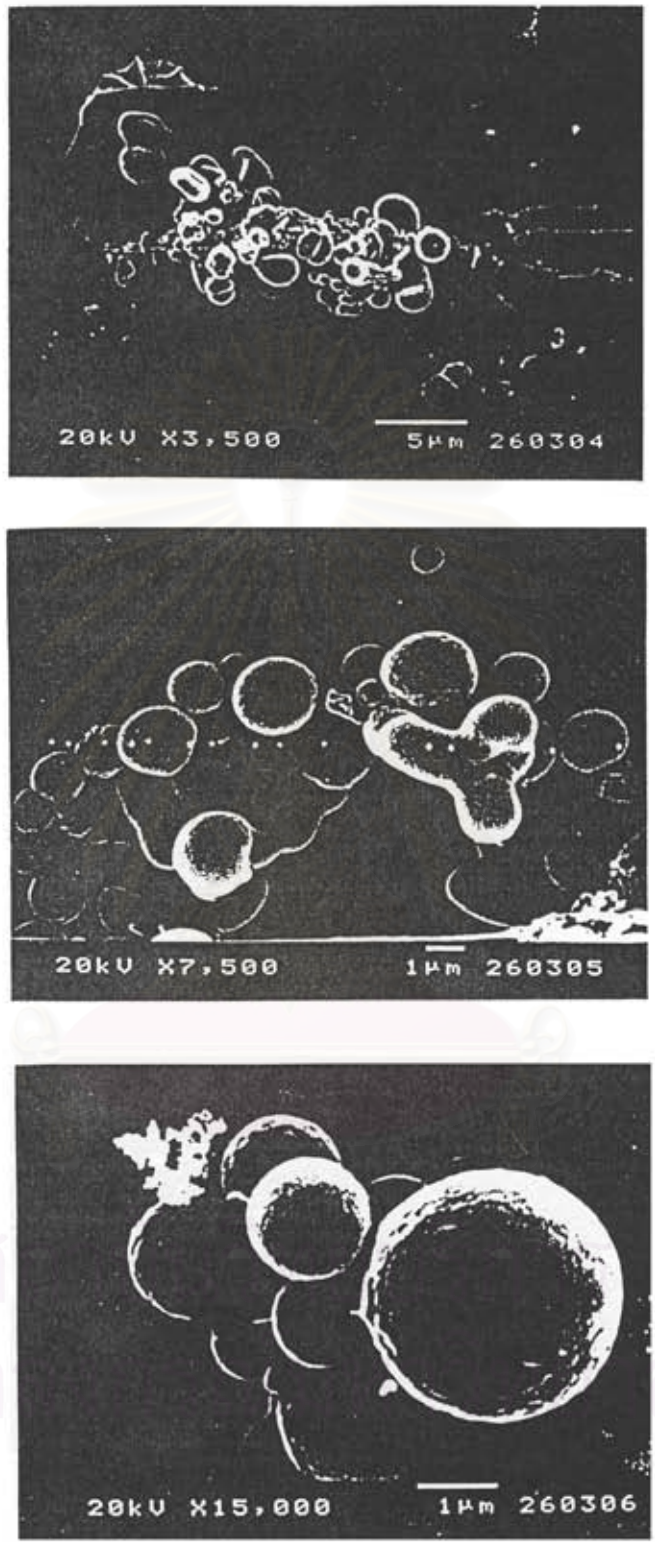
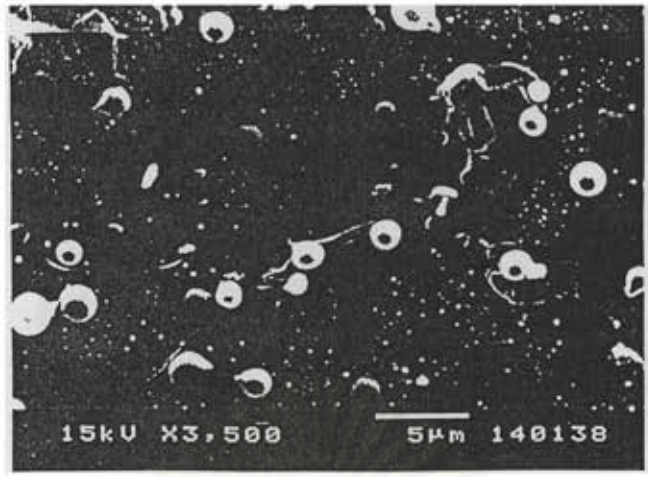
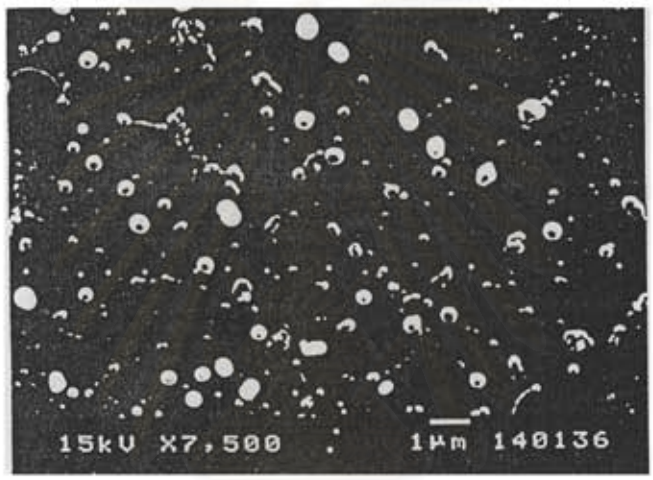


Figure 17 Scanning electron photomicrographs of ibuprofen liposomes (formula 6) prepared with 9:1 molar ratio of soybean lecithin to cholesterol, 90.9 µmol/ml of constant whole lipid and 2.7 mg/ml of ibuprofen at t_1 (after storage at 4°C for 1 month)

A , magnification : 3,500 x ; B , magnification : 7,500 x ; C , magnification : 15,000 x



A



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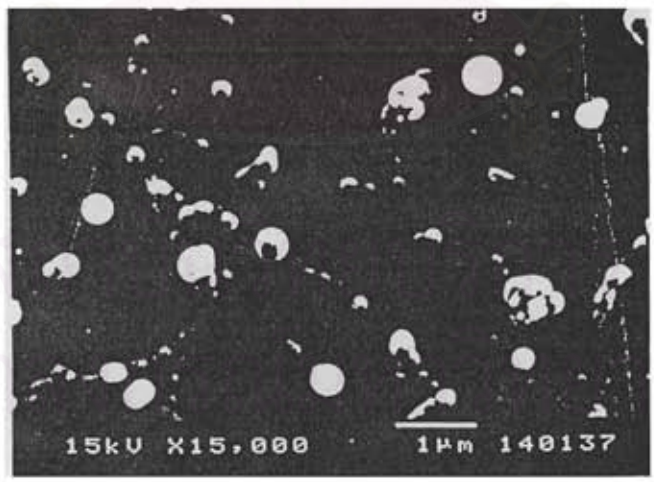
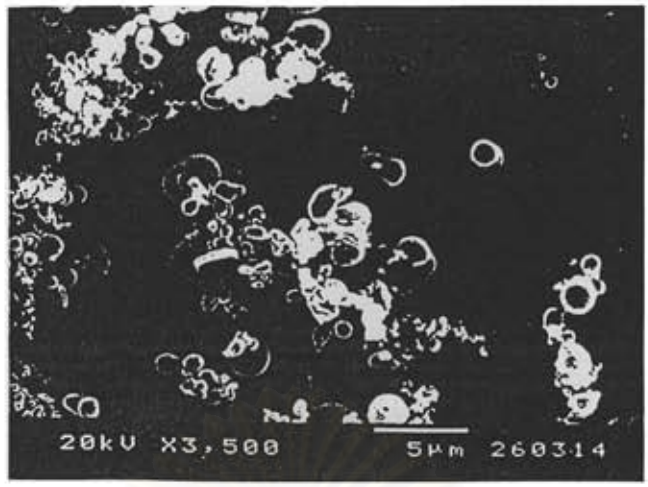


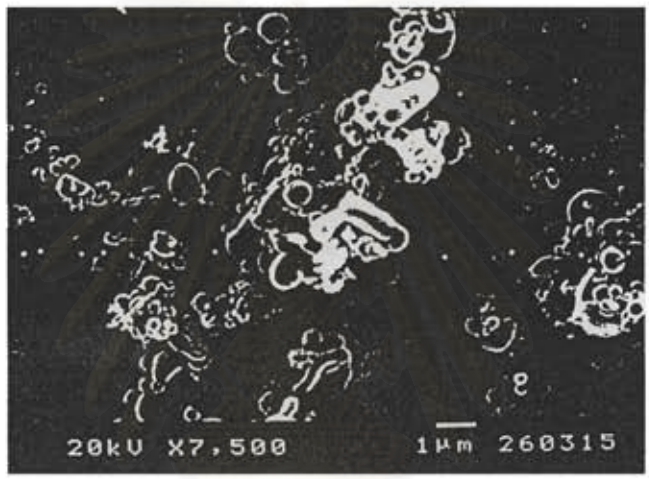
C

Figure 18 Scanning electron photomicrographs of ibuprofen liposomes (formula 15) prepared with 9:1 molar ratio of soybean lecithin to cholesterol, 2.50 mole% of stearylamine, 90.9 µmol/ml of constant whole lipid and 2.7 mg/ml of ibuprofen at t_1 (after storage at 4°C for 1 month)

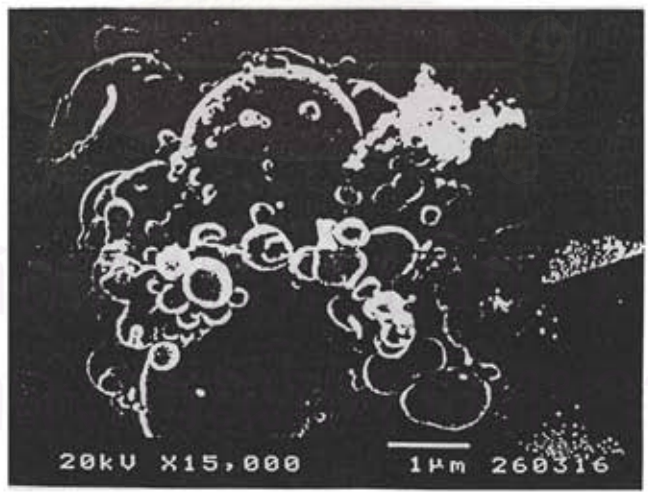
A , magnification : 3,500 x ; B , magnification : 7,500 x ; C , magnification : 15,000 x



A



B



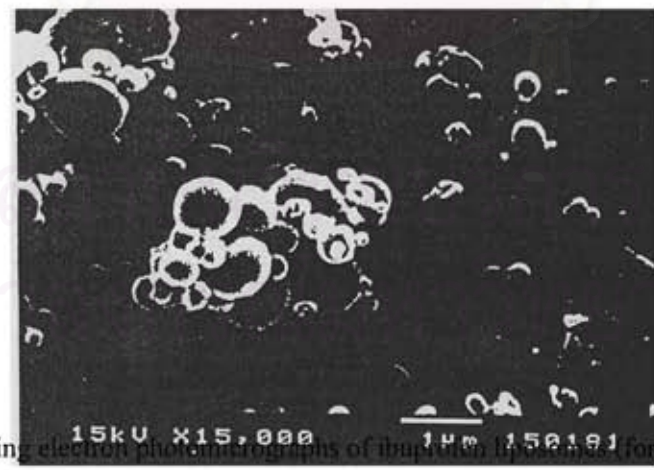
C

Figure 19 Scanning electron photomicrographs of ibuprofen liposomes (formula 21) prepared with 9:1 molar ratio of soybean lecithin to cholesterol, 2.50 mole% of stearylamine, 0.025% of (±)-α-tocopherol, 90.9 µmol/ml of constant whole lipid and 2.7 mg/ml of ibuprofen at t_i (after storage at 4°C for 1 month)

A , magnification : 3,500 x ; B , magnification : 7,500 x ; C , magnification : 15,000 x

A

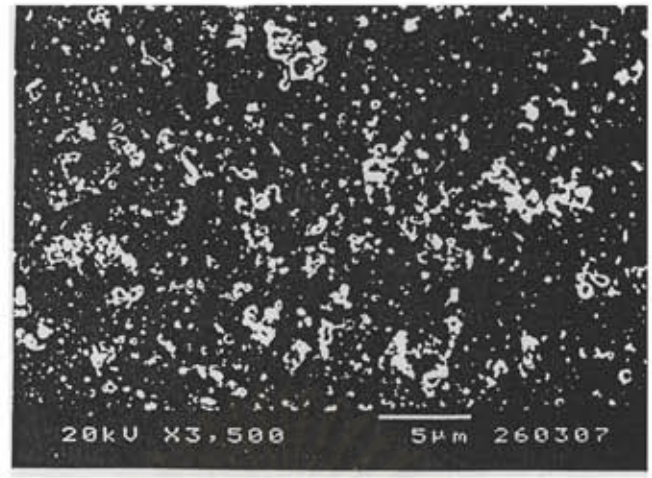
B



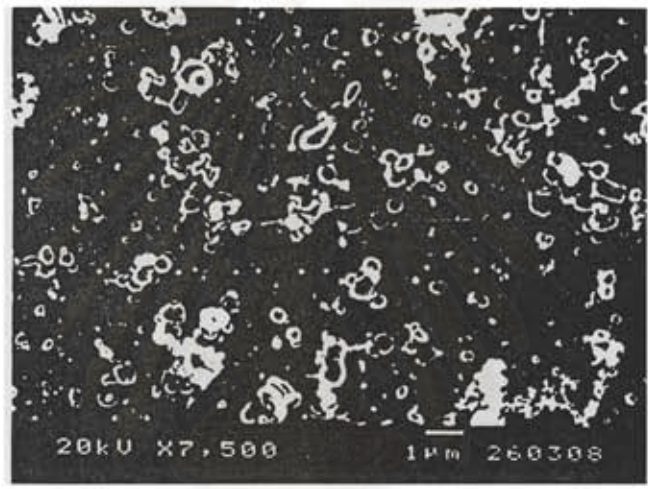
C

Figure 20 Scanning electron photomicrographs of ibuprofen liposomes (formula 22) prepared with 9:1 molar ratio of soybean lecithin to cholesterol, 2.50 mole% of stearylamine, 0.0125% of (±)-α-tocopherol, 90.9 µmol/ml of constant whole lipid and 2.7 mg/ml of ibuprofen at t_1 (after storage at 4°C for 1 month)

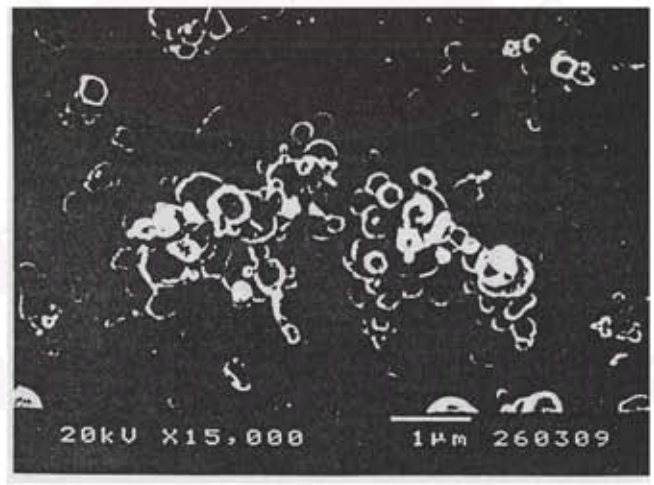
A , magnification : 3,500 x ; B , magnification : 7,500 x ; C , magnification : 15,000 x



A



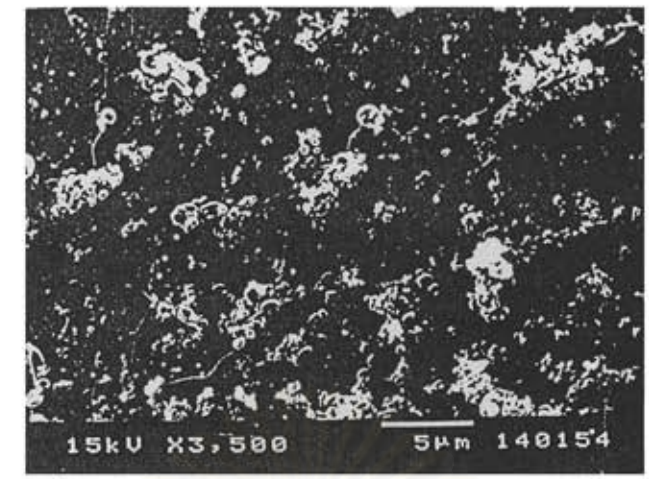
B



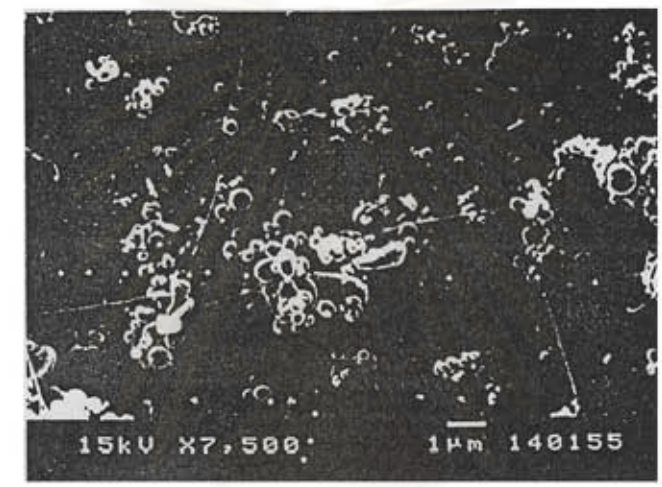
C

Figure 21 Scanning electron photomicrographs of ibuprofen liposomes (formula 23) prepared with 9:1 molar ratio of soybean lecithin to cholesterol, 2.50 mole% of stearylamine, 0.00625% of (±)-α-tocopherol, 90.9 µmol/ml of constant whole lipid and 2.7 mg/ml of ibuprofen at t_1 (after storage at 4°C for 1 month)

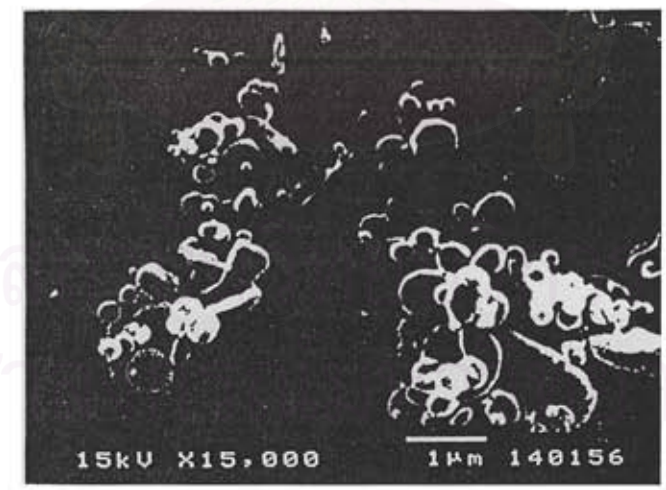
A , magnification : 3,500 x ; B , magnification : 7,500 x ; C , magnification : 15,000 x



A



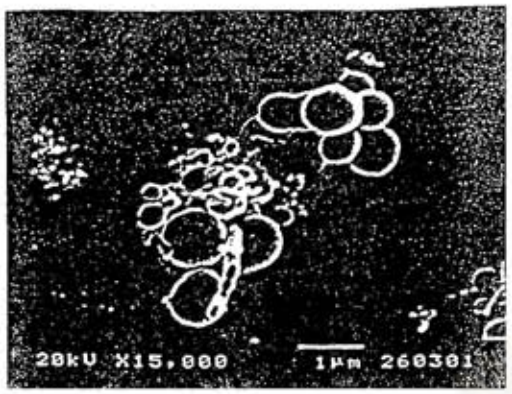
B

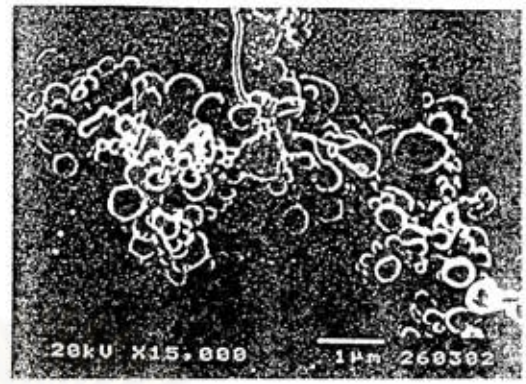


C

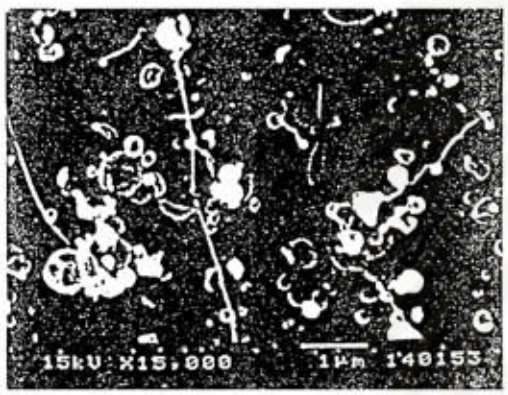
Figure 22 Scanning electron photomicrographs of ibuprofen liposomes (formula 24) prepared with 9:1 molar ratio of soybean lecithin to cholesterol, 2.50 mole% of stearylamine, 0.001% of (±)-α-tocopherol, 90.9 µmol/ml of constant whole lipid and 2.7 mg/ml of ibuprofen at t_1 (after storage at 4°C for 1 month)

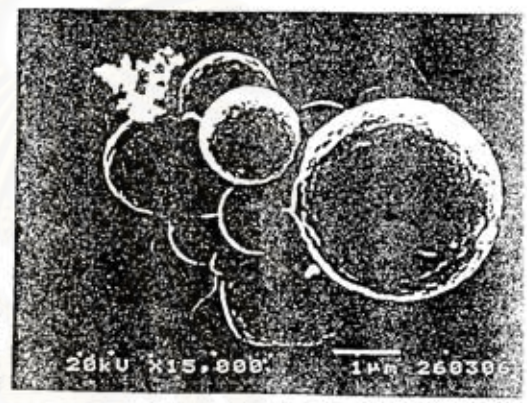
A , magnification : 3,500 x ; B , magnification : 7,500 x ; C , magnification : 15,000 x



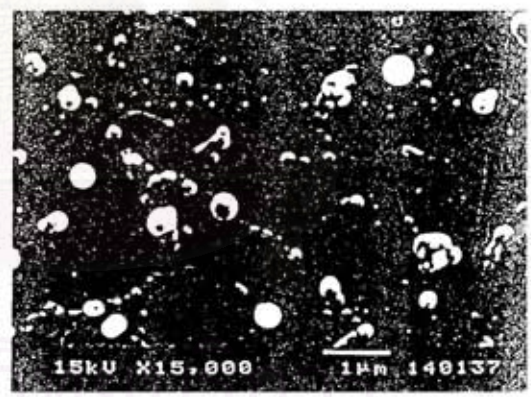


formula 3

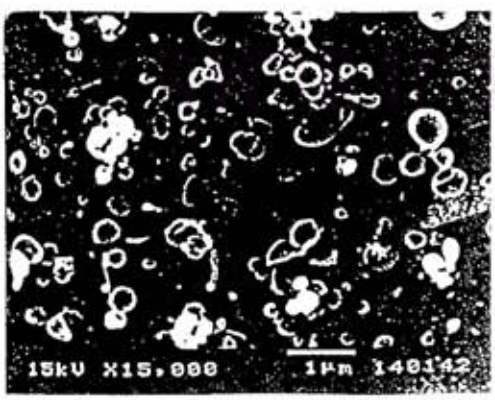


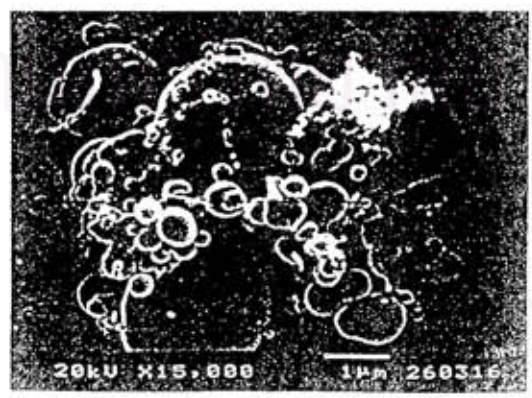


formula 6



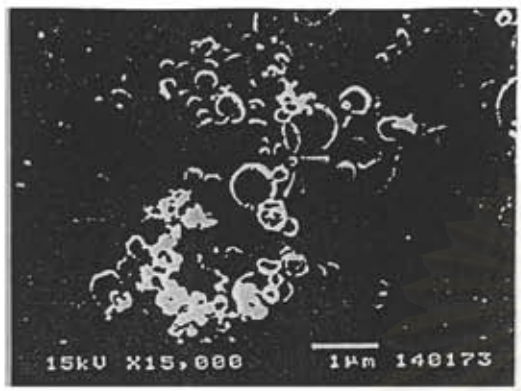
formula 15

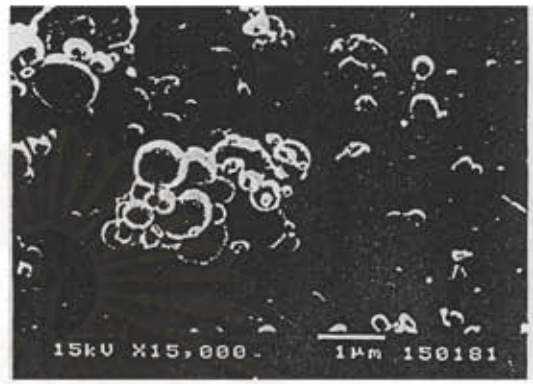




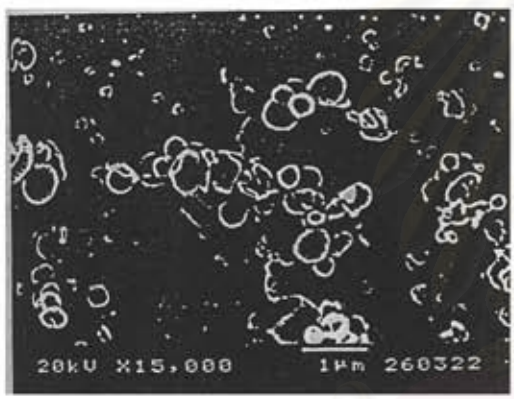
formula 21

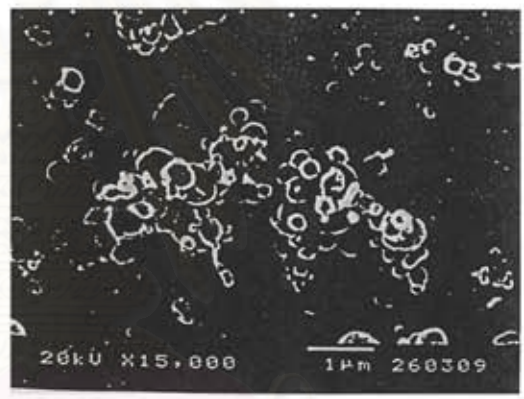
Figure 23 Scanning electron photomicrographs of selected ibuprofen liposomes compared at t_0 (left) and t_1 (right)



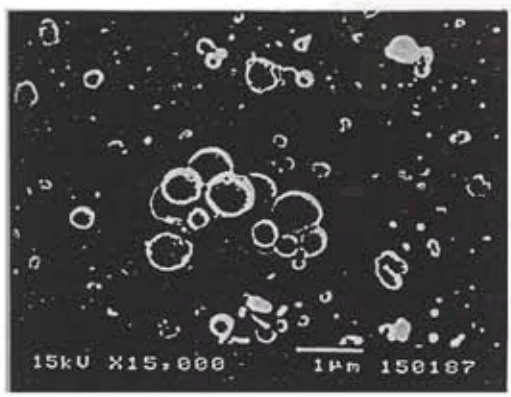


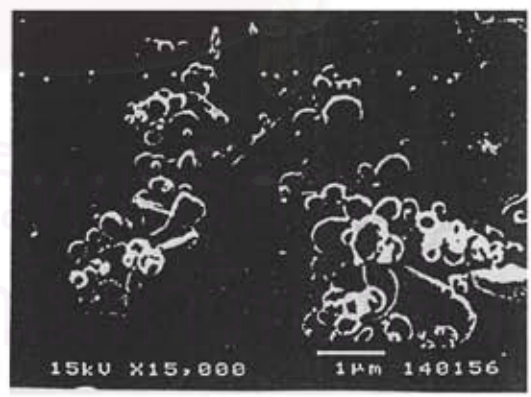
formula 22





formula 23





formula 24

Figure 23 (continued) Scanning electron photomicrographs of selected ibuprofen liposomes compared at t_0 (left) and t_1 (right)

formulations except the formula containing 2.50 mole% of stearylamine without (±)-α-tocopherol and the formula containing with 0.0125% of (±)-α-tocopherol.

The explanation from the size distribution diagrams (data not shown), it was found that the only one preparation containing 0.025% of (±)-α-tocopherol (formula 21) had bimodal size distributions (Appendix II). This promoted the resulting data from the size and physical appearance of this formula.

Suggestions for further researches

For further investigations, several aspects of ibuprofen liposomes could be carried out.

1. Scaling up of laboratory scale of ibuprofen liposomes is required for the future need of the final and stable preparation.

2. The release kinetic of stable ibuprofen liposomes may be studied in order to realize the efficiency of ibuprofen releasing from liposomes to skin.

3. The incorporation of ibuprofen liposomes in pharmaceutical dosage forms, such as gel and cream is required to formulate the actual topical preparation including the study of the stability of ibuprofen liposomes in formulation and release kinetic of ibuprofen from the base formulation.