CHAPTER IV

RESULTS AND DISCUSSION

1. Test for methods of quantitation of lactic acid in liposomes and phospholipid analysis

1.1 Validation of analytical method for quantitative determination of lactic acid in liposomes by HPLC

1.1.1 Accuracy

These experiments were conducted to verify that the methods used for lactic acid analysis in the supernatant and the pellet for determination of drug entrapment in liposomes were sufficiently accurate and precise. Since the studies also involved seperation and extraction procedures, accuracy and precision of the seperation and extraction procedures were also verified.

a) Analysis of lactic acid in solution

Table 3 shows percent analytical recovery at each lactic acid concentration . The mean percent analytical recovery was sufficiently high (close to 100%) with a low %CV, which indicates that HPLC method was accurate for quantitative analysis of lactic acid in solution.

b) Analysis of lactic acid in liposome suspension

Table 4 shows percent analytical recovery of each concentration of lactic acid liposome suspension. The results show that the recovery was

sufficiently high (close to 100%). However, %CV of the recovery is higher than that obtained from the solution due to the many steps of seperation and extraction of lactic acid from liposome suspension. These results indicates that satisfactory quantitation of lactic acid in liposome suspension was acheived by using HPLC.

Table 3. Accuracy data of lactic acid solution analysis

Actual Concentration (mcg/ml)	Analytical Concentration (mcg/ml)	%Recovery
100.00	95.10	95.10
	100.87	100.87
	102.24	102.24
300.00	290.98	96.99
	304.99	101.66
	298.12	99.37
500.00	482.73	96.55
	513.23	102.65
	505.81	101.16
		Mean=99.62, SD=2.76 %CV=2.77

Table 4. Data for accuracy of analysis of lactic acid in liposome suspension

Actual Concentration (mcg/ml)	Analytical Concentration (mcg/ml)	%Recovery
10.02	9.79	97.75
10.02	9.71	96.88
10.02	10.88	108.59
20.08	21.18	105.50
20.30	19.16	94.39
20.45	19.86	97.11
60.80	62.16	102.24
60.40	60.36	99.93
60.00	54.97	91.61
		Mean=99.33, SD=5.36 %CV=5.40

c) Separation of lactic acid in liposomes from the free drug in supernatant

Table 5 shows amounts of entrapped lactic acid which obtained from seperation and extraction of lactic acid for 3 times from the same liposome suspension. The %CV of amount of entrapped lactic acid is in the acceptable range. Since the precision of the separation and extraction procedures was very high, no replication was performed for each batch of liposomes in determination of encapsulation efficiency. Instead, three batches were used to account for any deviation that might have occurred during the process of liposome production.

Table 5. Accuracy and precision data of separation of lactic acid from liposomes

Lactic acid concentration (mg/ml)	Amount of lactic acid(mg)		Total (mg)	%Recovery
	free drug	pellet		
10.50	8.77	1.65	10.42	99.24
	8.71	1.64	10.35	98.57
	8.77	1.73	10.50	100.00
Mean ± SD	8.75 ± 0.03	1.67 ± 0.05	10.42 ± 0.08	99.27 ± 0.72
%CV	0.39	2.95	0.72	0.73

1.1.2 Precision

Table 6 and 7 show %CV for each lactic acid concentration in the range of 0.57-7.46. This indicates that the HPLC method are sufficiently precise for quantitation of lactic acid solution and that better precision would be obtained when the concentrations were in the range of 100-500 mcg/ml. Thus, the samples were diluted accordingly before being subjected to the HPLC analysis.

Table 6. Within run precision data

Concentration (mcg/ml)	Peak Area Ratio			Mean	SD	%CV
	n1	n2	n3			
20	0.062	0.067	0.072	0.067	0.005	7.46
50	0.162	0.186	0.178	0.175	0.012	6.86
100	0.339	0.360	0.365	0.355	0.014	3.94
200	0.707	0.736	0.733	0.725	0.016	2.21
300	1.052	1.103	1.078	1.078	0.026	2.41
500	1.750	1.861	1.834	1.815	0.058	3.20

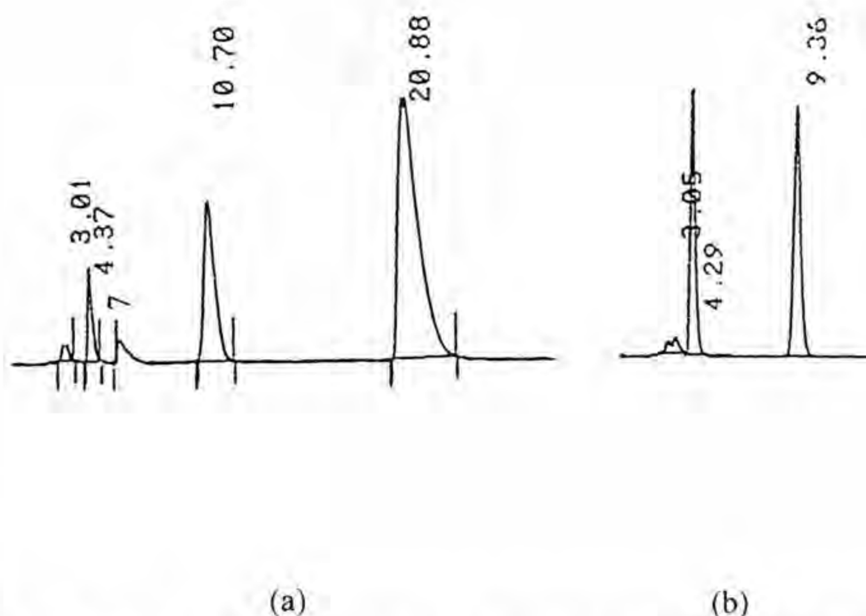
Table 7. Between run precision data

Concentration (mcg/ml)	Peak Area Ratio			Mean	SD	%CV
	Day1	Day2	Day3			
20	0.067	0.064	0.059	0.063	0.004	6.42
50	0.175	0.176	0.174	0.175	0.001	0.57
100	0.355	0.370	0.358	0.361	0.008	2.19
200	0.725	0.750	0.726	0.734	0.014	1.93
300	1.078	1.121	1.081	1.093	0.024	2.20
500	1.815	1.867	1.801	1.828	0.034	1.90

1.1.3 Specificity

Figure 10 shows typical chromatograms of lactic acid in the pellets and in the supernatant. Lactic acid in the supernatant eluted as a distinct peak with a retention time of 4.37 min. Lactic acid in the pellets, after being extracted from lipids , gave a single peak with a retention time of 4.29 min. These peaks were not interfered by the two peaks of lipids which had retention times of 7 and 20 min.

In summary, no interference peaks were observed in the samples of both the free drug in the supernatant and the entrapped drug in the pellet after extraction.



(a) (b)

Figure 10. The HPLC chromatograms of lactic acid in the pellet (a) and in the supernatant (b)

The retention time of lactic acid is 4.37 min in the pellet.

The retention time of free lactic acid in the supernatant is at 4.29 min.

The retention time of tyrosine (the internal standard) is 10.70 and 9.86 min in the pellet and in the free drug, respectively.

The retention times of lipids are at 7 and 20.08 min.

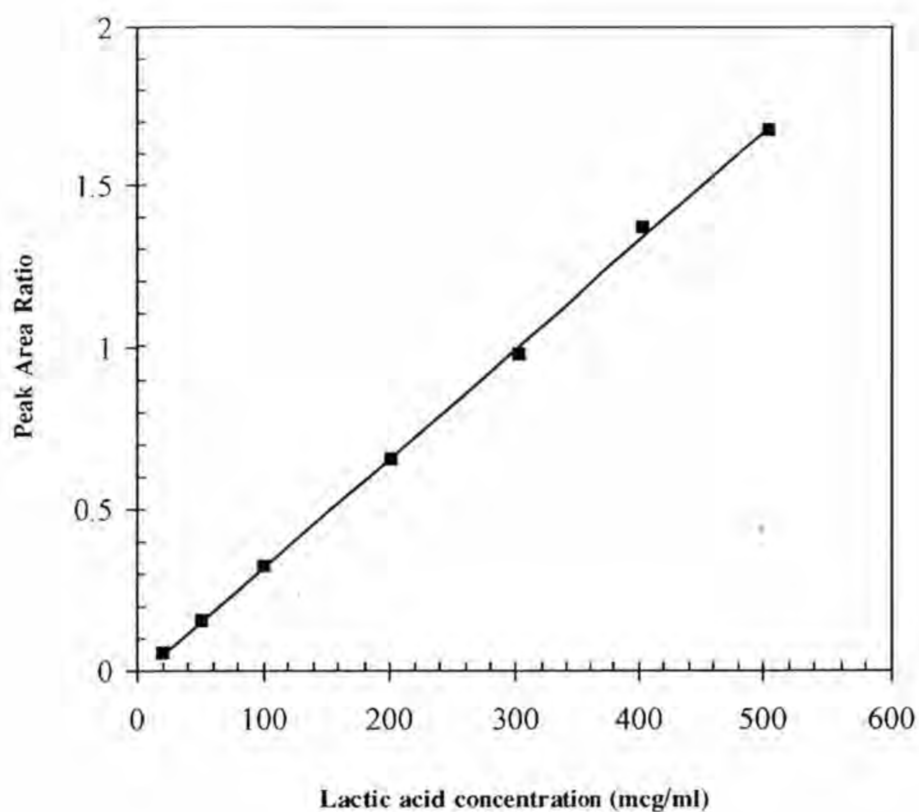
1.1.4 Linearity

Linear regression analysis of the peak area ratio was performed with a correlation coefficient (r) of 0.9997 and a coeffcient of determination (R^2) of 0.9994. Data for the calibration curve of lactic acid is presented in Table 8. The calibration curve and the HPLC chromatograms of the standard solutions are shown

in Figure 11 and 12, respectively. Figure 11 shows that the relationship between peak area ratio and lactic acid concentration is linear. These results indicate that the HPLC method was acceptable for quantitative analysis of lactic acid solutions.

Table 8. Data for a calibration curve of standard solutions of lactic acid.

Actual concentration (mcg/ml)	Peak Area Ratio	Analytical concentration (mcg/ml)	%Recovery
20.16	0.054	20.53	101.83
50.40	0.158	51.12	101.42
100.80	0.327	100.82	100.02
201.60	0.657	197.88	98.16
302.40	0.978	292.29	96.66
403.20	1.372	408.18	101.23
504.00	1.675	497.29	98.67



$$y = 0.0034x - 0.0158$$

$$R^2 = 0.9994$$

where y = Peak Area Ratio

x = Lactic acid concentration (mcg/ml)

Figure 11. A representation of calibration curve of standard solutions of lactic acid.

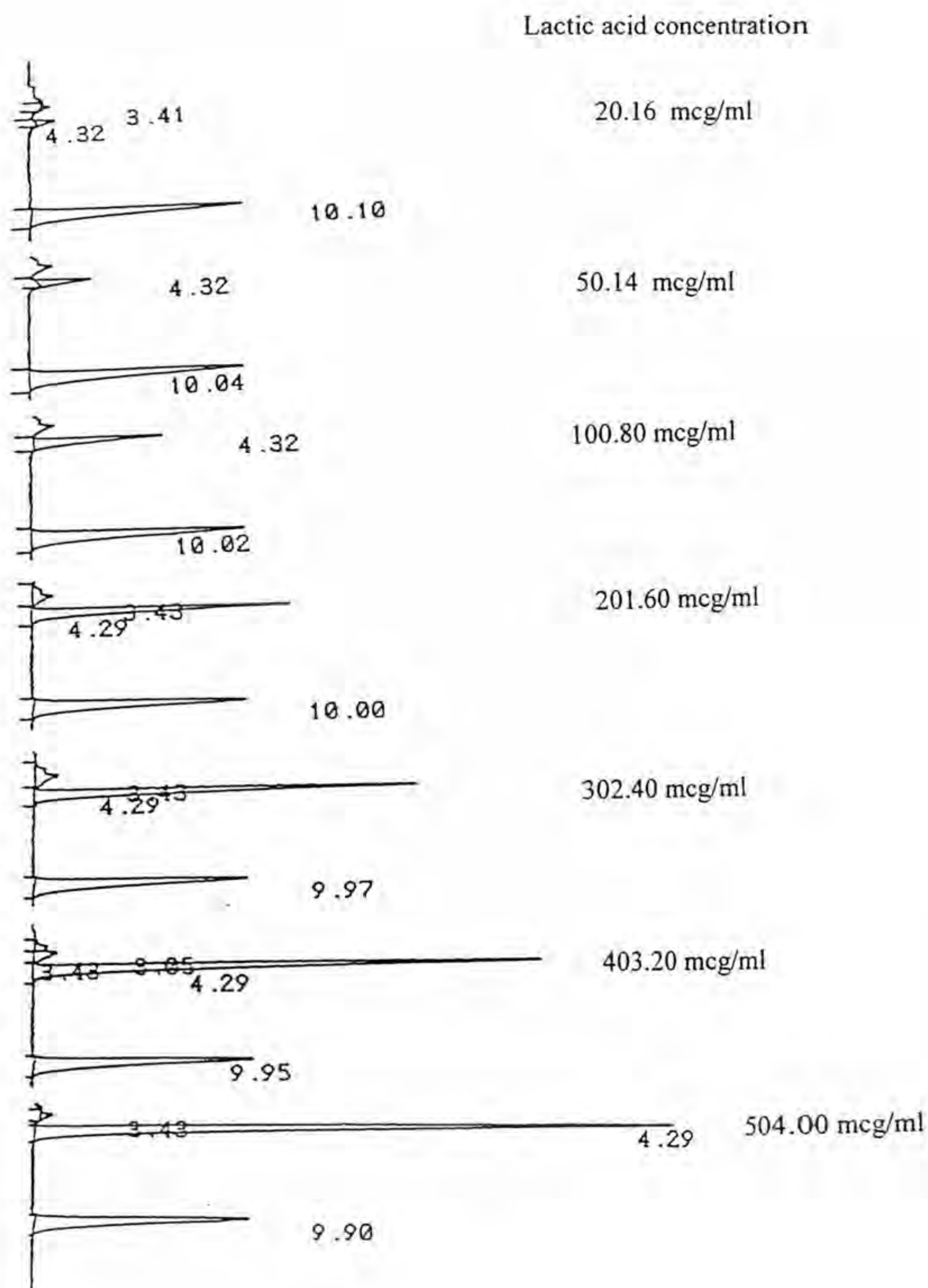


Figure 12. The HPLC chromatograms of the standard solutions (concentration=20-500 mcg/ml) of lactic acid (retention time=4.29-4.32 min) and the internal standard (retention time=9.90-10.10 min).

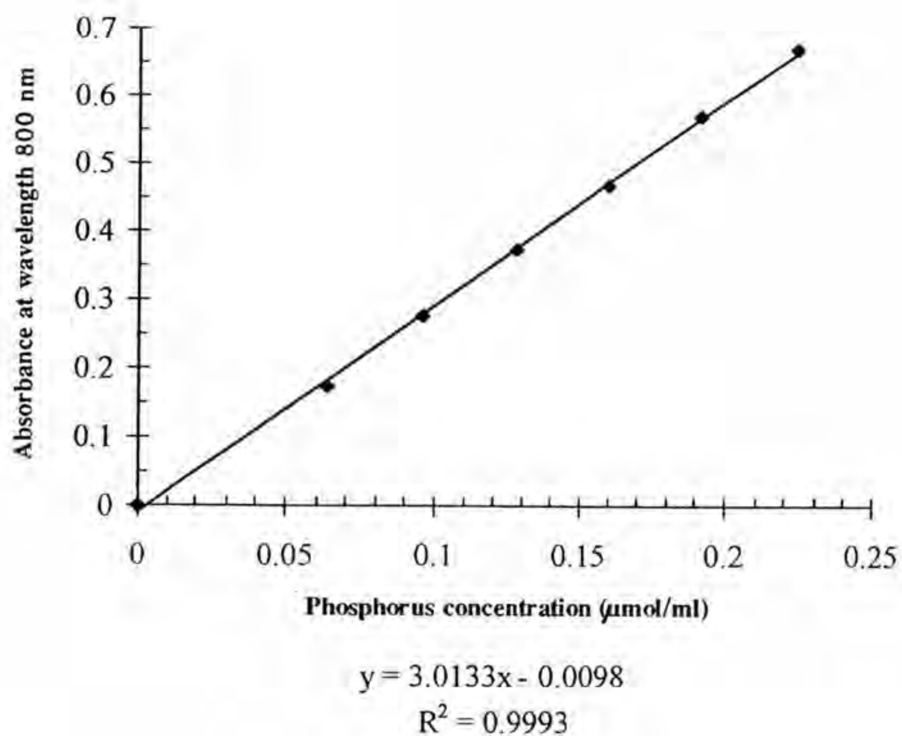
In summary, the results obtained from the validation indicate that the HPLC method for the quantitative determination of lactic acid in liposomes was specific, precise, and accurate. The lack of interference by other liposomal components means that quantitative analysis of lactic acid entrapped in liposomes using HPLC was appropriate.

1.2. Quantitative determination of phospholipid in prepared liposomes

Table 9 and Figure 13 show the linearity of phosphorus calibration curve assayed in the concentration range of 0.064-0.22 µmol/ml. The relationship between absorbance at 800 nm and phosphorus concentration was linear ($r = 0.9997$) and the coefficient of determination was high ($R^2 = 0.9993$) This indicates that the Bartlett method of phosphorus analysis was acceptable for quantitation of phosphorus in this concentration range.

Table 9. Calibration curve data of standard phosphorus solutions; absorbances were measured at 800 nm

Anhydrous potassium dihydrogen phosphate concentration (µmol/ml)	Total Absorbance	Total Absorbance - Blank Absorbance
0.000	0.301	0.000
0.064	0.476	0.175
0.096	0.577	0.276
0.128	0.674	0.373
0.160	0.770	0.469
0.192	0.871	0.570
0.224	0.973	0.672



$$y = 3.0133x - 0.0098$$
$$R^2 = 0.9993$$

where y = Absorbance

x = Concentration (μmol phosphorus/ml)

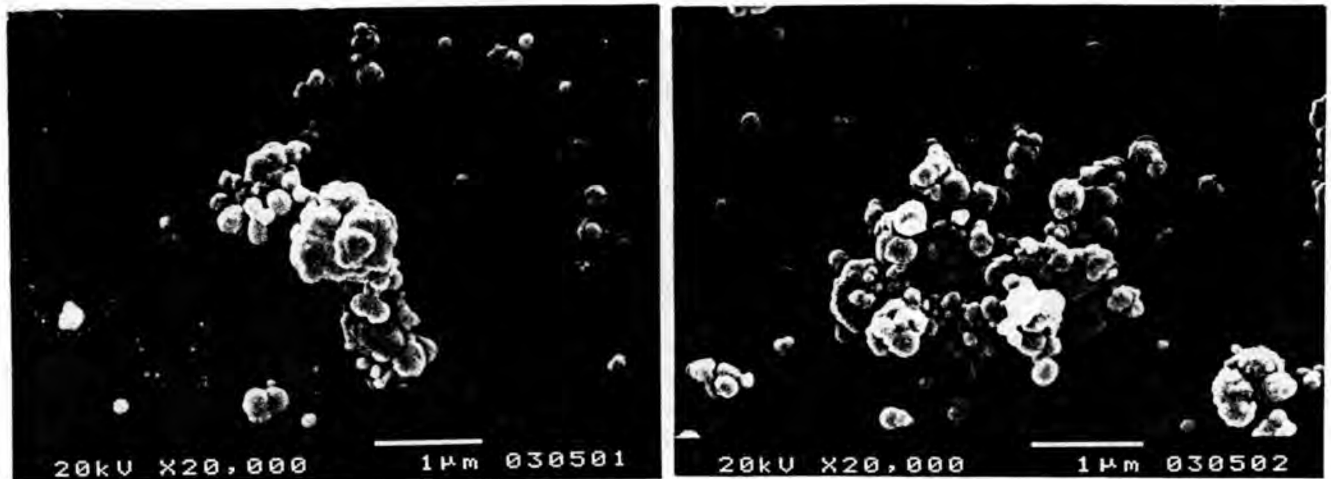
Figure 13. A representation of calibration curve of standard phosphorus solutions using the Bartlett assay; absorbances were measured at 800 nm.

2. Preparation of lactic acid liposomes by reverse phase evaporation technique

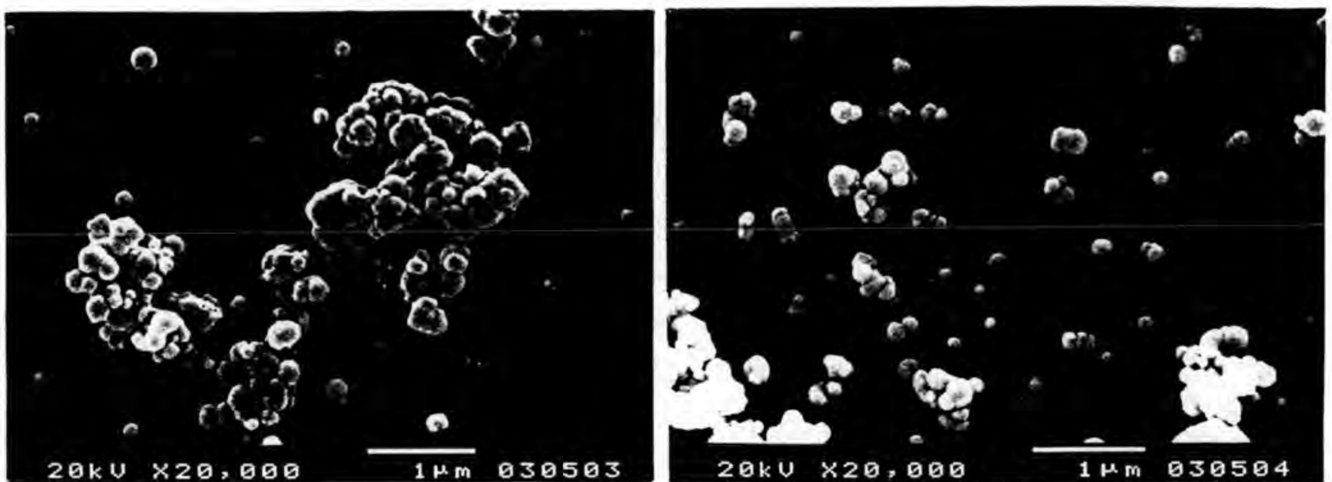
In this study, lactic acid liposomes could be prepared by reverse phase evaporation method. This method was modified from the method of Szoka and Papahadjopoulos (Szoka and Papahadjopoulos, 1978). Instead of diethyl ether, chloroform:methanol (1:1) was used for dissolving lipids. The ratio of organic solvent to water, the sonication temperature, the sonication time, and the temperature for evaporation of organic solvent were adjusted to obtain suitable conditions of preparation of lactic acid liposomes as follows. The ratio of chloroform:methanol (1:1) to water was 6:1 by volume, the sonication time was 5 min at room temperature, and the organic solvent was evaporated using a rotary evaporator at 35°C. These conditions were used for all preparations of lactic acid liposomes. By the method of reverse phase evaporation, large unilamellar and oligolamellar vesicles are formed, usually consisting of phospholipid bilayers of not more than about 10 lamellae (Hamaguchi et al., 1989). Thus, it was probable to obtain LUVs in the preparation of lactic acid liposomes by this technique, especially when the lipid concentration was low and the volume of water was sufficient (New, 1990).

3. Characteristics of lactic acid liposomes prepared by reverse phase evaporation procedure

Visual examination by light microscopy of prepared vesicles revealed some vesicles with diameter larger than 1µm, while elctron micrographs indicated the presence of vesicles smaller than 1µm. Approximate size and organization of the reverse phase evaporation vesicles prepared from egg yolk lecithin are illustrated in Figure 14 and Figure 15. Scanning electron microscopy showed that the liposomes were spherical vesicles (Figure14), whereas transmission electron microscopy showed some deformed structures (Figure 15), apparently due to osmotic pressure changes during the staining process. Though Figure 15 shows some lamellar patterns on liposomes, it is usually difficult to report for multilamellar vesicles from

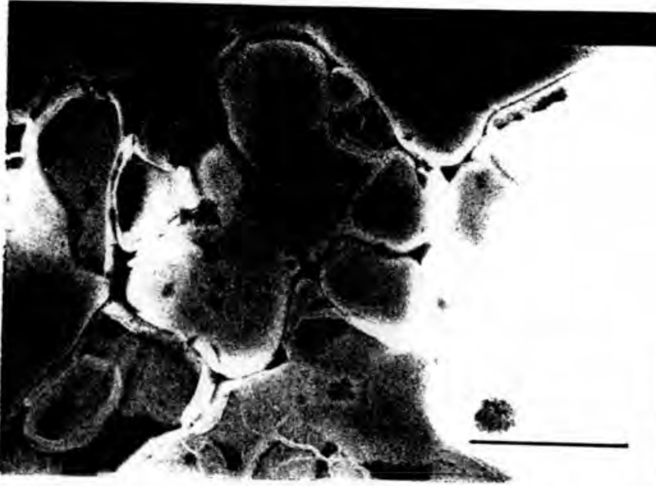


(A) (B)



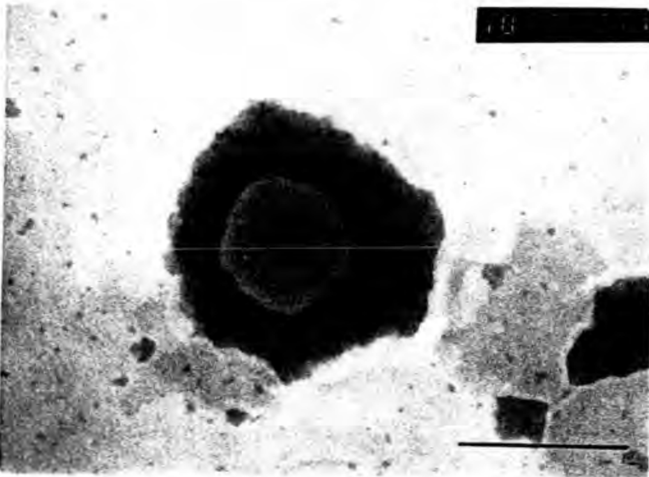
(C) (D)

Figure 14. Scanning electron photomicrographs of EPC lactic acid liposomes (20,000 x). Some individual liposomes are shown and are spherical in shape.

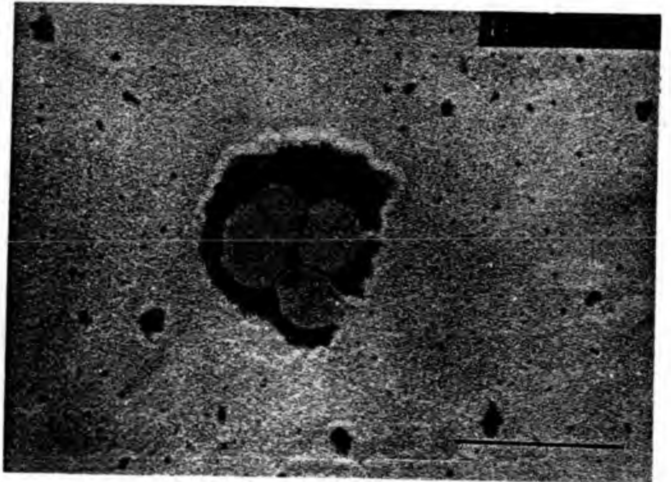


(A)

(B)



(C)



(D)

Figure 15. Transmission electron photomicrographs of EPC lactic acid liposomes.

Key: A magnification: 37,000 x ; Bar = 0.5 μm

B, C, D magnification: 100,000 x ; Bar = 0.2 μm

transmission microscopy. It has been suggested that the apparent multilamellarity may be an artifact of negative stainning and that multilamellations that are seen when liposomes are partially overlapping thus do not reflect the actual internal structure (New, 1990).

Apart from method of preparation, it is evident that the lactic acid liposomes prepared in these experiments can be unilamellar from the values of the captured volume. Table 10 shows the captured volumes of neutral liposomes prepared at different EPC concentrations which are in range of 15.01-15.73 μl/mg lipid. As described previously in Chapter II (Table 1), the capture volume of liposomes (μl/mg lipid) may be used to identify type of prepared vesicles. If this value is higher than 9, the vesicles are identified as LUVs. Therefore, the prepared lactic acid liposomes should be LUVs.

Table 10. The captured volume of EPC liposomes prepared at different EPC concentrations.

EPC concentration (mg/ml)	captured volume (μl/mg lipid)*
10.00	15.37±1.44
20.00	15.01±1.56
30.00	15.73±2.04
40.00	15.37±0.36

* Mean ± SD

Due to aggregation of prepared vesicles, the particle size of these vesicles could not be accuratedly measured by laser light scattering, which is the most convenient method for particle size analysis. However, from electron micrographs, size range of the prepared lactic acid vesicles in this sample (Figure14 and 15) was in the range of 100-500 nm. This size range, however, might represent only the lower end of overall particle size distribution owing to the intrinsic nature of the technique. Estimation of particle size distribution by electron microscopy requires unbiased

sampling, and an average of at least 400-500 vesicles is usually needed in order to achieve a satisfactory accuracy (Hauser, 1993). Estimatation of particle size distribution by electron microscopy, however, would not give an accurate result for these liposomes because liposomal structures in the micrographs were distorted (Figure 15) after negative staining. On the other hand, scanning electron microscopy also requires partical coating, which caused severe aggregation of the vesicles (Figure 14), making accurate estimation of particle size range not possible. As expected the upper end of the distribution (particles larger than 1 μm seen under light microscope) was not shown by electron microscopy.

4. Factors affecting encapsulation efficiency of lactic acid in liposomes

4.1 Effect of total concentration of lipid on lactic acid entrapment

The effect of phospholipid concentration on lactic acid entrapment at constant lactic acid concentration of 10 mg/ml is illustrated in Table11 and Figure16. As phospholipid concentration increased from 10 mg/ml to 40 mg/ml, the percentage of lactic acid entrapped was enhanced from 13.59 % to 71.97 % due to more liposome vesicles formed. An increase in the number of liposomes resulted in more aqueous compartments available for lactic acid entrapment in liposomes; consequently, the percentage of entrapment was higher. However, when comparing the encapsulation efficiency of lactic acid in liposomes at constant phospholipid concentration by calculation of the amount of lactic acid entrapped per mole of lipid, one found no significant difference in the encapsulation efficiency with altered phospholipid concentrations (Table 12 and Figure 17). Thus, in terms of cost-effectiveness, the encapsulation efficiency of lactic acid in liposomes did not depend on the phospholipid concentration used for preparing liposomes. To acheive optimal efficacy for encapsulation capacity, it is necessary to encapsulate the maximum possible quantity of lactic acid by using least amount of phospholipid. Since the

Table 11. Effect of phospholipid concentration on lactic acid entrapment.

Phospholipid concentration (mg/ml)	% Lactic acid entrapment*
10.00	13.59 ± 0.95
20.00	26.69 ± 1.49
30.00	48.22 ± 7.27
40.00	71.97 ± 5.54

* Mean ± SD, n=3

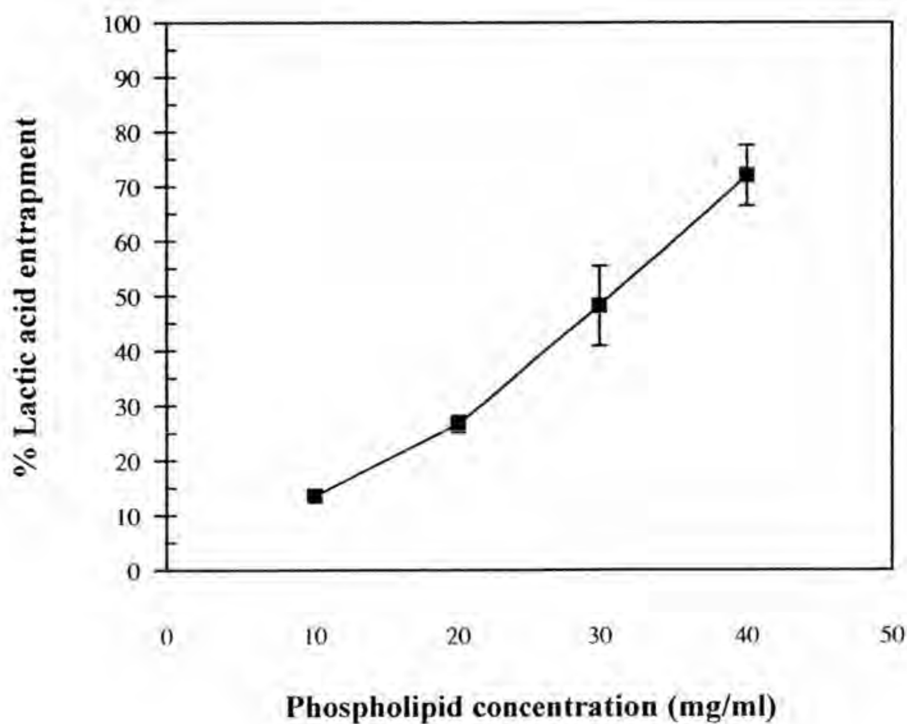


Figure 16. Effect of phospholipid concentration on lactic acid entrapment.

Table 12. Effect of phospholipid concentration on encapsulation efficiency of lactic acid in neutral liposomes.

Phospholipid concentration (mg/ml)	Lactic acid entrapped/Lipid (mol/mol)*
10.00	1.28 ± 0.12
20.00	1.25 ± 0.13
30.00	1.31 ± 0.17
40.00	1.28 ± 0.03

* Mean ± SD, n=3

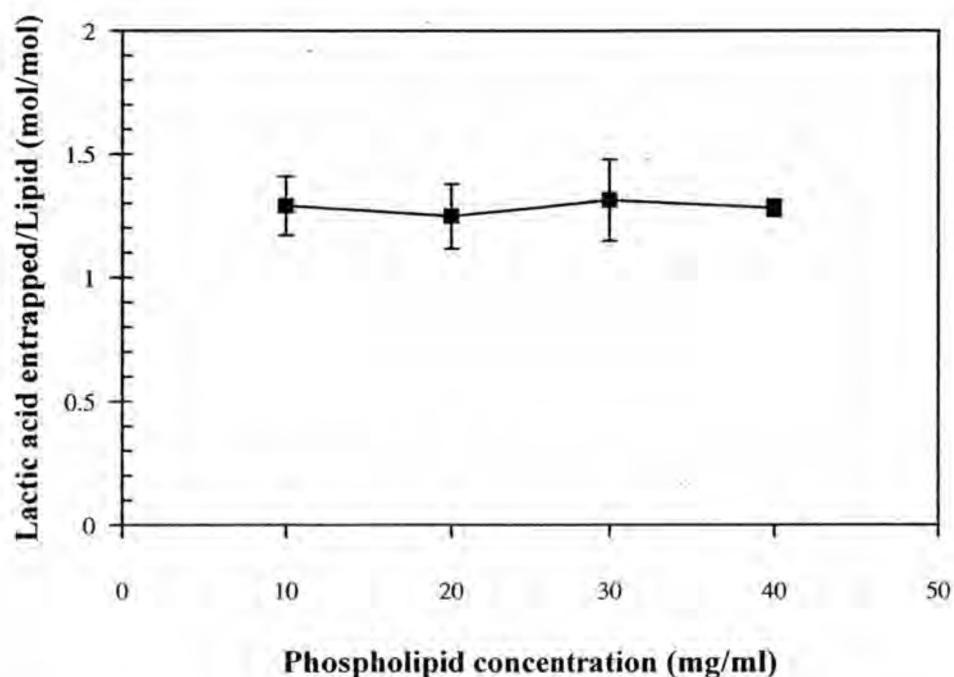


Figure 17. Effect of phospholipid concentration on encapsulation efficiency of lactic acid in neutral liposomes.

encapsulation efficiency by calculating from amount of lactic acid entrapped per mole of lipid.Therefore, in following experiments, entrapment of lactic acid was evaluated from mole of lactic acid per mole of lipid.

4.2 Effect of lactic acid concentration on lactic acid entrapment

Table13 shows the encapsulation efficiency of lactic acid in liposomes with various concentrations of lactic acid when total lipid concentration was fixed at 10 mg/ml. The encapsulation efficiency of lactic acid increased almost linearly when the lactic acid concentration was increased from 10 to 80 mg/ml due to the increasing density of lactic acid molecules in the aqueous compartment. However, when lactic acid concentration was further increases to 100 mg/ml, the encapsulation efficiency of lactic acid could not be determined due to dramatic changes in the appearance of resultant suspensions. The suspensions were more translucent, and examination under light microscope revealed reduced number of vesicles formed. These changes may have been caused by excessive acidity of lactic acid which can interfere with liposome formation . Efficient capture of the liposome depends on the use of drug at concentrations which do not exceed the saturatation limit of the drug in the aqueous compartment (for polar drugs) or the lipid bilayers (for nonpolar drugs) (Weiner et al., 1989). Hence, the results suggested that the maximum concentration of lactic acid allowed for EPC liposomes prepared by reverse phase evaporation method was somewhere between 80-100 mg/ml.

4.3 Effect of liposomal charge, pH and ionic strength on lactic acid entrapment

Because lactic acid is an ionizable species, it was used to study the effects of liposomal charge, pH and ionic strength on encapsulation efficiency of phospholipid liposomes. These factors can be described by a factorial experimental design (3x3x3) to examine whether they would have interaction effects on

Table 13. Effect of lactic acid concentration on lactic acid entrapment.

Lactic acid concentration (mg/ml)	Lactic acid entrapped/Lipid (mol/mol)*
10.02	1.28 ± 0.12
20.28	2.63 ± 0.10
40.44	7.43 ± 0.26
60.40	9.54 ± 0.57
80.53	16.08 ± 1.82

* Mean ± SD, n=3

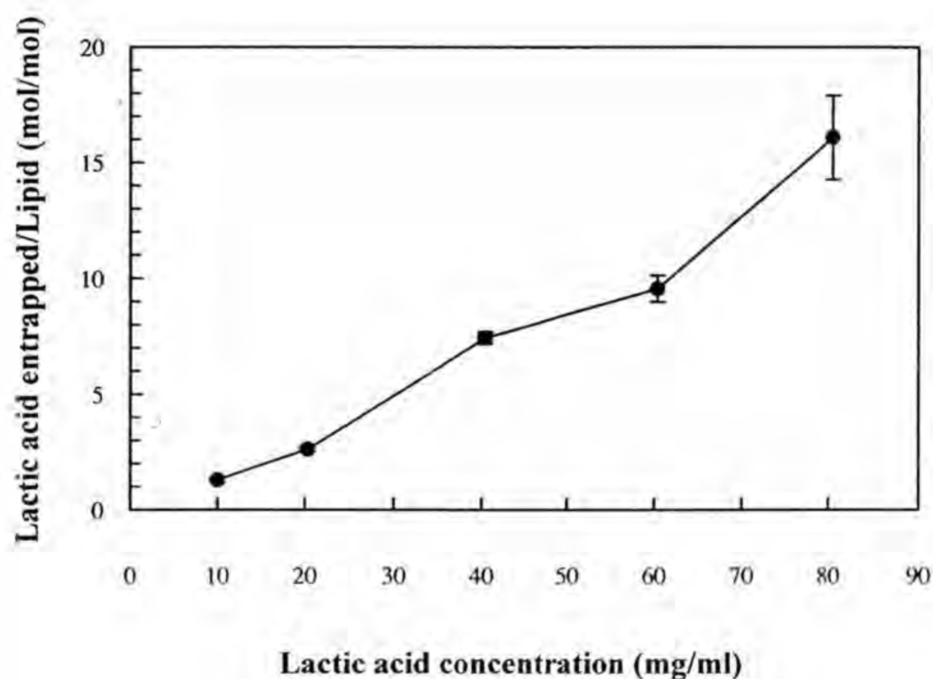


Figure 18. Effect of lactic acid concentration on lactic acid entrapment.

encapsulation efficiency of lactic acid. Statistical analysis used was ANOVA and Duncan' s multiple range tests preformed on MSTAT program (Nissen, O., 1986). The data in Table 14 shows that the difference in encapsulation efficiency of lactic acid in liposomes was the result of interaction effects of liposomal charge, pH, and ionic strength ($p<0.05$). Encapsulation efficiency of lactic acid changed when liposomal charge, pH, or ionic strength was varied. Both pH and ionic strength affected ionization of lactic acid resulting in changes in the binding between the lipid and the lactic acid molecules, causing different encapsulation efficiencies.

From Table 15 and Figure 19, for positive and neutral liposomes at ionic strength of 0.1, highest encapsulation efficiencies were achieved at pH 4. On the contrary, pH had no effect on encapsulation efficiency of negatively charged liposomes. An increase in the encapsulation efficiency at pH 4 may be related to the existence of appropriate forms of lactic acid for encapsulation. The results indicate that both unionized and ionized forms were equally important for the entrapment since the entrapment was maximized at the pH equal to pK_a of the drug, where both forms are present at the same concentration. At pH 3, lactic acid was mostly unionized, and at pH 5 it was mostly ionized resulting in lower encapsulation efficiencies than at the pH 4 that provides both the unionized form and the ionized form of lactic acid. Mechanisms for encapsulating of drugs into liposomes include entrapping in the aqueous compartment , binding to the hydrophilic head groups of the liposomal membrane, or interacting with the hydrophobic region of the bilayer (Gregoriadis, 1976). It is likely that all three of the above mechanisms can be applied to the encapsulation of lactic acid in phospholipid liposomes. The unionized form of lactic acid could be associated with liposomal bilayer, whereas the ionized form stayed in the aqueous core of liposomes with or without binding to the hydrophilic head groups of phospholipids via electrostatic interaction. The ionized lactic acid molecules could also interact with stearylamine in positively charged liposomes. This electrostatic interaction has been observed with the positively charged drug, such as tetracaine (Foldvari et al., 1993). The entrapment of tetracaine increases by including negative charges in lipid composition when the drug is in ionized form.

Table 14. Effect of pH and ionic strength on lactic acid entrapment in different liposomes.

Liposomal Type	pH	ionic strength (μ)	Lactic acid entrapped/Lipid(mol/mol) (Mean±SD, n=3)*
Neutral (EPC)	3	0.1	1.21 ± 0.05^{b}
		0.3	1.15 ± 0.17^{d}
		0.5	1.21 ± 0.07^{c}
	4	0.1	1.36 ± 0.07^{a}
		0.3	1.32 ± 0.07^{a}
		0.5	1.34 ± 0.21^{a}
	5	0.1	1.03 ± 0.06^{g}
		0.3	0.88 ± 0.13^{i}
		0.5	1.40 ± 0.03^{a}
Positive (EPC:SA)	3	0.1	1.21 ± 0.07^{b}
		0.3	1.22 ± 0.07^{b}
		0.5	1.19 ± 0.17^{c}
	4	0.1	1.40 ± 0.09^{a}
		0.3	1.16 ± 0.08^{d}
		0.5	1.32 ± 0.07^{a}
	5	0.1	1.19 ± 0.15^{c}
		0.3	1.08 ± 0.06^{g}
		0.5	1.03 ± 0.13^{g}
Negative (EPC:PG)	3	0.1	1.11 ± 0.06^{g}
		0.3	1.13 ± 0.07^{g}
		0.5	1.13 ± 0.09^{f}
	4	0.1	1.06 ± 0.07^{g}
		0.3	0.88 ± 0.04^{h}
		0.5	1.05 ± 0.02^{g}
	5	0.1	1.12 ± 0.08^{g}
		0.3	1.10 ± 0.13^{g}
		0.5	1.13 ± 0.07^{e}

* The mean values are statistically different at α=0.05 if the superscriptions are different

Table 15. Effect of pH on lactic acid entrapment in the different liposomes (μ = 0.1).

Lipid composition	Lactic acid entrapped/Lipid(mol/mol)*		
	pH3	pH4	pH5
EPC	1.21±0.05	1.36±0.07	1.03±0.06
EPC:SA(9:1)	1.21±0.07	1.40±0.09	1.19±0.15
EPC:PG(9:1)	1.11±0.06	1.06±0.07	1.12±0.08

* Mean±SD, n=3

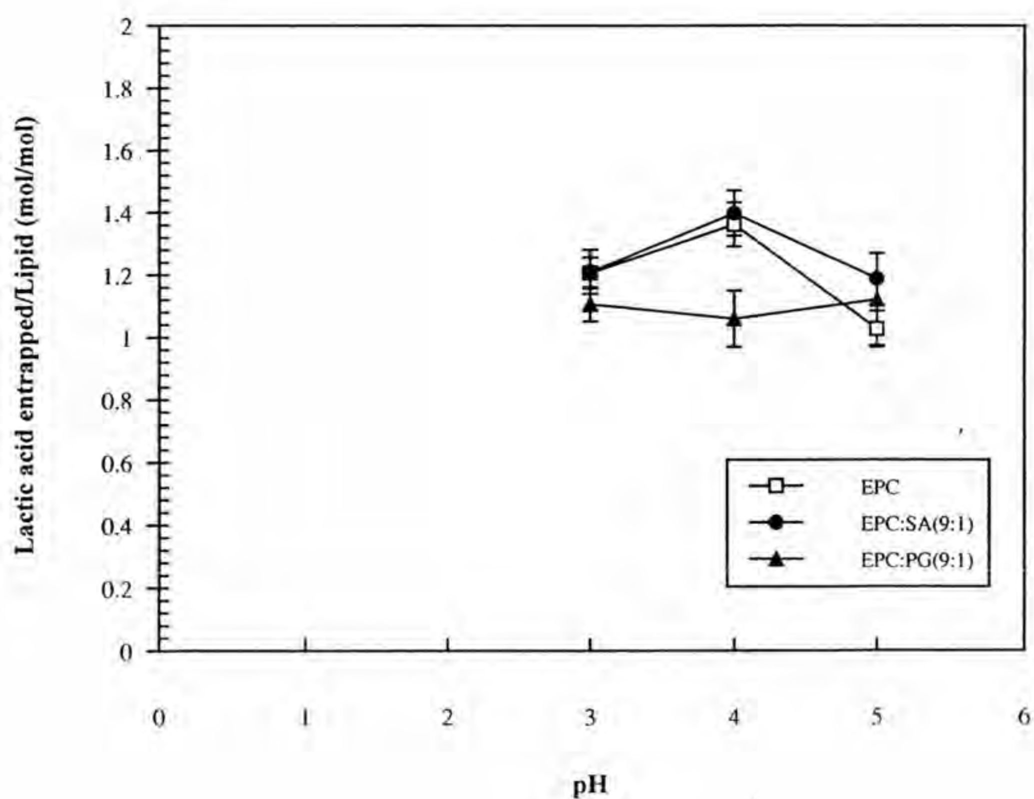


Figure 19. Effect of pH on lactic acid entrapment in the different liposomes (μ = 0.1).

However, inclusion of positive charges (10 mol% stearylamine) in liposomes at pH 4 (μ=0.1) had minor effects on encapsulation compared with the inclusion of neutral species since their encapsulation efficiencies were not statistically different (Table 14). At pH 5 (μ=0.1) the effect of charge interaction between the positively charged lipids and the ionized lactic acid was more obvious than at pH 4 (μ=0.1). The encapsulation efficiencies of lactic acid in the neutral and positive liposomes were statistically different at α=0.05.

For negatively charged liposomes, the difference in encapsulation efficiency of lactic acid at different pH values was minimal. The ionized lactic acid molecule, being negatively charged, can not bind to the similarly charged head group of liposomes. Electrostatic repulsive force between liposomal bilayers and ionized lactic acid molecules may also nullify the high encapsulation efficiency expected at pH equal to the pK_a. However, one cannot rule out the effect of particle size on encapsulation because there was no guarantee that the particle size of liposomes formed under different conditions would stay the same. For unilamellar vesicles, bigger vesicles correspond to higher capture volumes (Hope et al., 1985).

When the ionic strength was increased to 0.5, the overall trend of encapsulation efficiency stayed the same for both positively and negatively charged liposomes (Figure 20). However, in neutral liposomes, increasing the ionic strength to 0.5 resulted in an increase in the encapsulation efficiency at pH 5. In neutral liposomes, lactic acid entrapment depended only on the partition of lactic acid molecules between the aqueous compartment and the nonpolar region of the bilayer and not on ionic interaction between the drug molecule and the bilayer. At low ionic strength, lactic acid was almost completely ionized at pH=5, which is one pH unit above its pK_a, and the ionized form could not intercalate into the nonpolar region of the bilayer. The space available for encapsulation of lactic acid was then limited only to the aqueous compartment of liposomes, without any contribution from the bilayer. At high ionic strength, a strong electrolyte may be completely ionized, yet

Table 16. Effect of pH on lactic acid entrapment in the different liposomes (μ=0.5).

Lipid composition	Lactic acid entrapped/Lipid(mol/mol)*		
	pH3	pH4	pH5
EPC	1.20±0.07	1.34±0.21	1.40±0.03
EPC:SA(9:1)	1.19±0.17	1.32±0.07	1.03±0.13
EPC:PG(9:1)	1.13±0.09	1.05±0.02	1.13±0.07

* Mean±SD, n=3

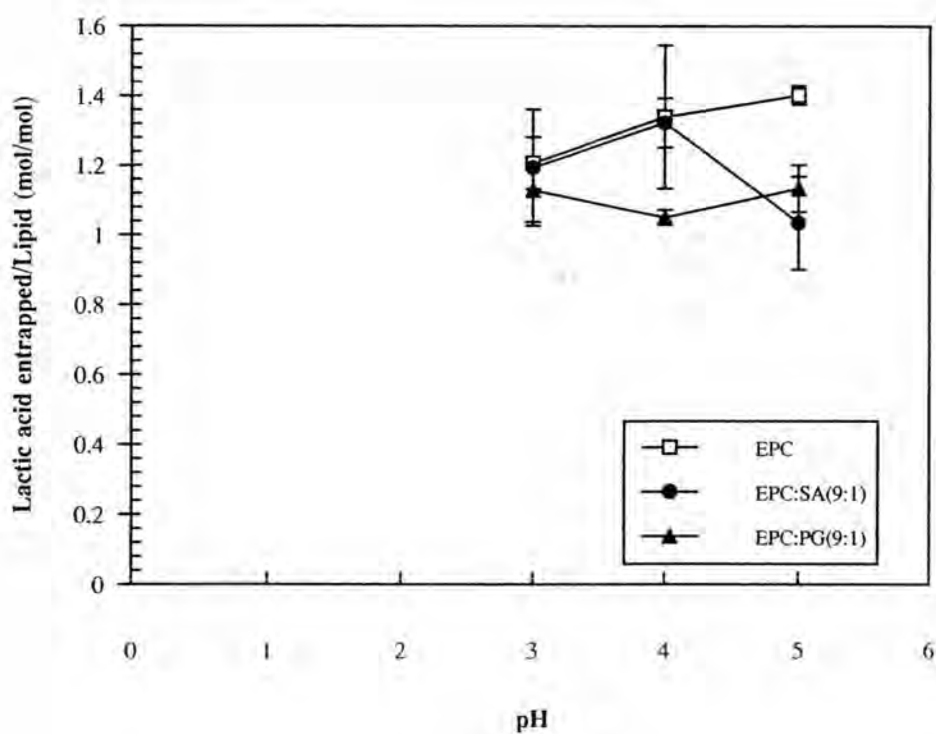


Figure 20. Effect of pH on lactic acid entrapment in the different liposomes (μ = 0.5).

incompletely dissociated into free ions because of the interionic attraction and ion association caused by the large number of oppositely changed ions present in the solution (Martin et al., 1983). Hence at μ=0.5 at pH 5, lactic acid might not be completely dissociated into free ions as it does at low ionic strength. The undissociated species of lactic acid may be able to interact with the non polar region of the bilayer, whereas the dissociated form stayed in the aqueous compartment of the neutral liposomes. Consequently, the encapsulation efficiency at pH 5 was high due to the presence of both species of lactic acid, making both the aqueous compartment and the bilayer available for drug encapsulation.

On the contrary, at pH 5, the ability of the positively charged liposomes to entrap lactic acid was not increased (Figure 21). As the ionic strength increased, there was a decrease in encapsulation efficiency because stearylamine could not impose positive charges to the bilayer as strongly as it did at lower ionic strength. Hence, there was less binding of ionized lactic acid molecules to the bilayer. These results are comparable to those reported by Szoka and Papahajopoulos (1978). These investigators reported that the encapsulation of cytosine arabinoside in PG/EPC/Chol (1:4:5) depends on the ionic strength of the buffer. At higher ionic strength, there is a reduction in the percent encapsulation of cytosine arabinoside.

4.4 Effect of cholesterol on lactic acid entrapment

To study the effect of cholesterol on lactic acid entrapment, two of the conditions which gave highest encapsulation were selected. The selection was made such that representations of all different parameters (charge, ionic strength, and pH) were included. The results are shown in Table 17. Inclusion of cholesterol in the bilayer significantly reduced lactic acid encapsulation. Addition of cholesterol to the neutral or positively charged liposomes above their T_m (phase- transition temperature) may increase rigidity and thickness of the bilayer resulting in smaller internal volume of liposomes to embrace ionized lactic acid molecules. In addition, the hydrophobic

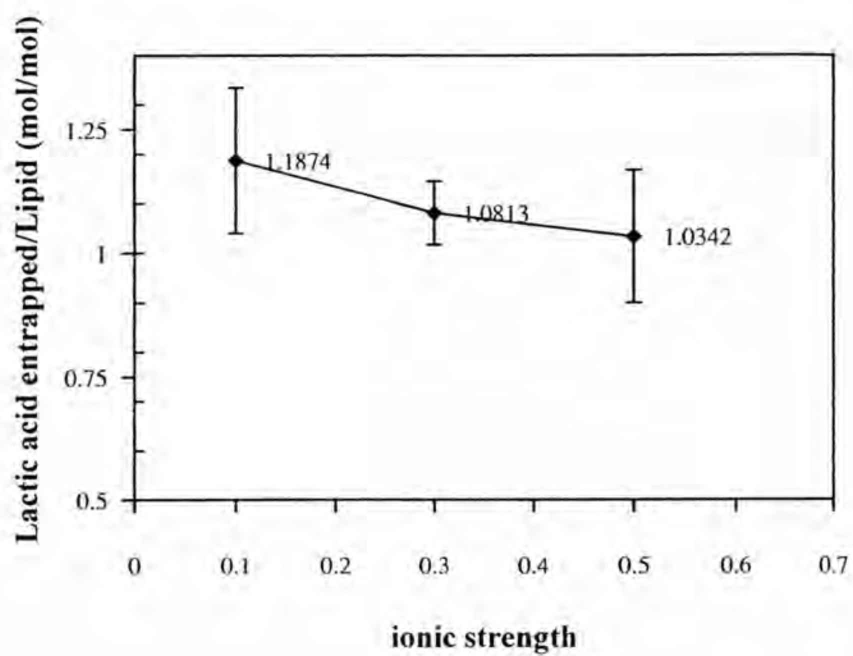


Figure 21. Effect of ionic strength on lactic acid entrapment in positive liposomes at pH 5.

Table 17. Effect of cholesterol on lactic acid entrapment.

Lipid composition	pH	u	Lactic acid entrapped/Lipid (mol/mol)*
EPC	5	0.5	1.40 ± 0.03
EPC:CHOL (1:1)	5	0.5	0.84 ± 0.05
EPC:SA (9:1)	4	0.1	1.40 ± 0.09
EPC:CHOL:SA (4.5:4.5:1)	4	0.1	0.88 ± 0.17

* Mean ± SD, n=3

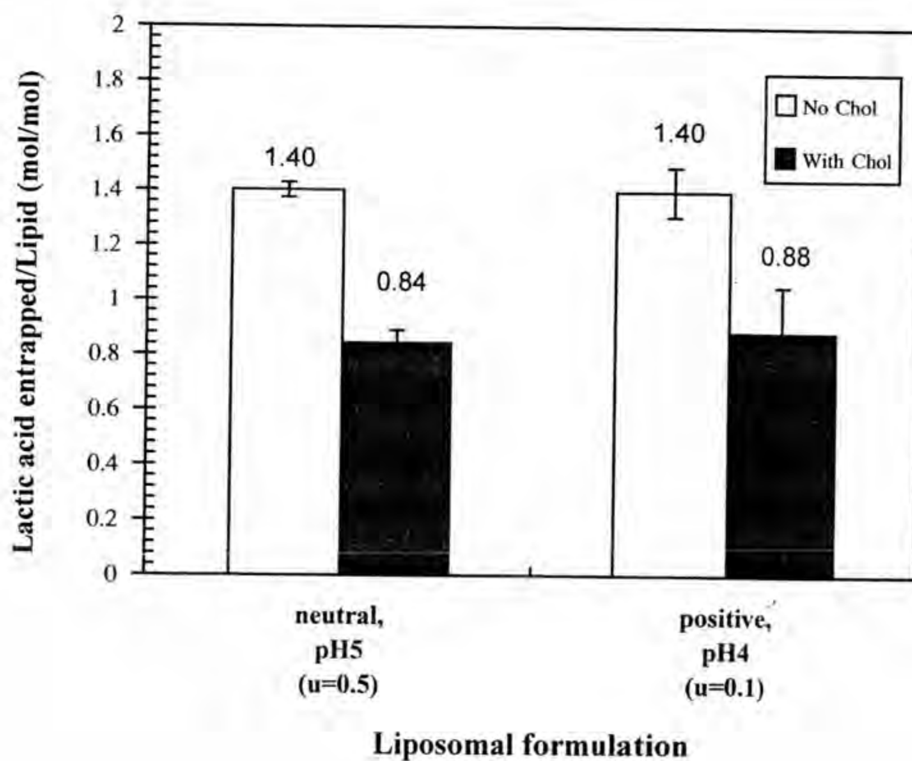


Figure 22. Influence of cholesterol on lactic acid entrapment

regions in the bilayer that had already intercalated with cholesterol molecule may have less capacity to accommodate unionized lactic acid molecules. These results agreed with the results reported by Ganapathi and Krishan (1984) which suggested that incorporation of a high amount of cholesterol decreased encapsulation of adriamycin possibly due to reduced intercalation and binding of adriamycin to less fluid bilayers.

5. *In vitro* release of lactic acid from liposomes

An important point in the evaluation of a drug delivery system, is the rate at which the drug is released from the carrier. Dissolution and release tests are generally used not only in the quality control of the drug formulation, but also to predict *in vivo* behavior and to study the structure of the dissolving matrix (Henriksen et al., 1995).

However, it is rather difficult to characterize drug release from liposomal carriers because of the physical obstacles associated with the extremely small size of the dispersed particles. Since liposomes have non-solid and flexible structures, they will suffer from stability problems during the time-course of the release study which are different from other colloidal carriers. Thus, much consideration is needed in selecting an appropriate experiment setup for release studies for liposomal systems.

A large number of methods have been developed in order to characterize the release profiles of drugs from colloidal disperse systems. The membrane diffusion techniques using either diffusion cells (Hashida et al.,1980; Benita et al.,1986; Miyazaki et al.,1986; Lostritto and Silvestri, 1987) or dialysis sacs (Ammoury et al.,1989, 1990) were criticized by Washington (1989) that these kinetic experiments were not performed under perfect sink conditions, since the colloidal carrier was not directly diluted in the release medium but rather seperated from the

release solution by a membrane. As a result, the rate of drug appearance in the release solution does not reflect its real release profile, but rather the concentration gradient between the continuous phase of the colloidal dispersion and the release solution.

Another method for release studies is the socalled " sample-and-seperate" method. In this method, the carrier is diluted with buffer, and samples are taken at intervals. The carrier is then separated from the continuous phase by filtration or centrifugation (Tsukada et al., 1984; Taylor et al., 1990), and the drug released is assayed. This method is efficient only if a sucessful and quick separation of the diluted dispersed phase from the continuous phase is achieved. This is likely to be difficult, especially when particle size of the dispersed phase is smaller than one micron as is the case of liposomes. The time required for separation by ultracentrifugation may be too long while release rate of the drug from the colloidal carrier can be very rapid (Washington, 1990).

Some other methods for *in vitro* release profile estimation of colloidal carriers include continuous flow filtration which was described by a number of researchers (Burgess et al., 1987; Koosha et al., 1988). However, this method may lack the ability to determine the real release profile since the method needs infinitely rapid procedures to remove and replace sink phase, which may not be possible with the commonly used sampling technique.

In this present study, the prepared liposome suspensions consisted of very small particles (below 1 μm). The extremely small particle size did not justify the "sample-and-seperate" method, which may give most accurate release profiles, since filtration of such small particles was not practical, and the time to sediment them by centrifugating was too long (2 hr) while the release profile could be much faster. Thus, the membrane diffusion techniques using diffusion cells was selected to evaluate the *in vitro* release of lactic acid from liposomes in this present study. Though the experiment was not performed under perfect sink conditions, and it had

limited value in predicting the *in vivo* behavior of colloidal carriers, it has been used sucessfully to study the shelf-life of liposomes/drug systems with respect to drug retention (Margalit et al., 1991).

All formulations investigated in 4.4 were studied for their release profiles. The HPLC chromatograms of the sample solutions of lactic acid in the receptor cell containing the iso-osmotic phosphate buffer at the pH of 4 and 5 are shown in Figure 23. The results show that phosphate buffer did not interfere with the assay. The release profiles of encapsulated lactic acid from different types of liposomes at 37°C are shown in Figure 24. Depending on the lipid composition, the liposomes released the entrapped lactic acid in different amounts during the time-course of the experiments. About 80 % of the drug was released within 8 hours and the remaining being gradually released during the following 8-24 hours from positively charged liposomes either with or without cholesterol. Slower release profiles were obtained from neutral liposomes, regardless of cholesterol content. These findings may be explained by the difference in phospholipid packing in the bilayer of liposomes.

An increase in the surface charge density on the bilayer will increase the intermolecular electrostatic energy of repulsion which may result in the packing constraint as well as the reduction of vesicle size of liposomes. Suspensions of positive liposomes of lactic acid were more translucent than those of neutral liposomes, implying a smaller particle size. A decrease in the vesicle size increases the overall surface area for drug release resulting in the higher release rate. The packing constraint may also cause some defects in the bilayer which allow faster escape of entrapped molecules. These two factors may offset the electrostatic interaction between ionized lactic acid molecules and the positively charged bilayer. This was possible because most of ionized lactic acid molecules were expected to be in the aqueous compartment of the liposomal structure with only a small portion had ionic interaction with the charged bilayer. The ionic interaction with the bilayer was

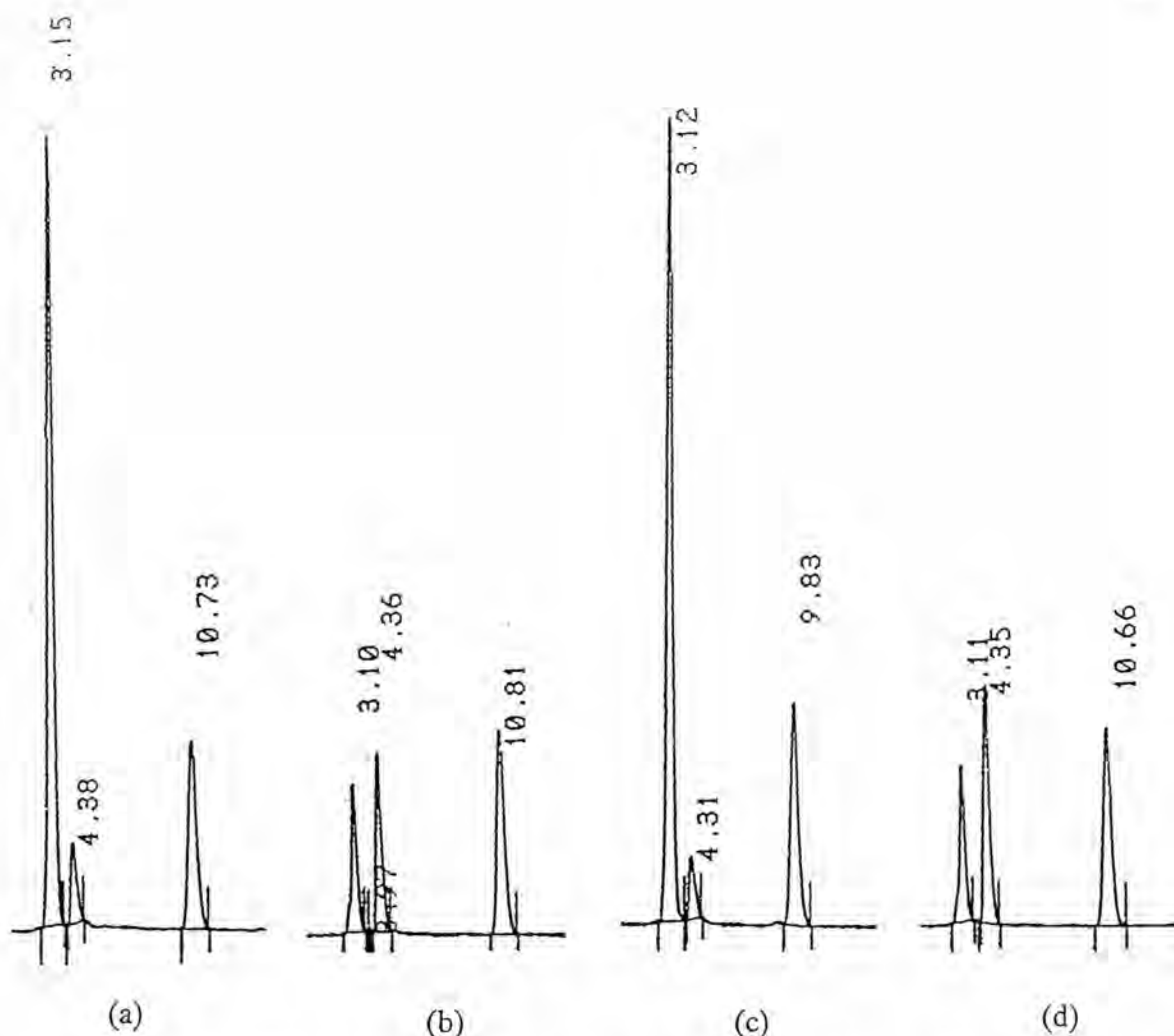


Figure 23. The HPLC chromatograms of lactic acid released from different types of liposomes in the receptor cell containing the iso-osmotic phosphate buffer at the pH 4 and 5.

(a) Neutral liposomes redispersed in iso-osmotic phosphate buffer pH 5; lactic acid (4.24-4.28 min); internal standard (9.45-10.10 min).

(b) Positive liposomes redispersed in iso-osmotic phosphate buffer pH 4; lactic acid (4.24-4.28 min); internal standard (9.45-10.10 min).

(c) Neutral liposomes with Chol redispersed in iso-osmotic phosphate buffer pH 5; lactic acid (4.24-4.28 min); internal standard (9.45-10.10 min).

(d) Positive liposomes with Chol redispersed in iso-osmotic phosphate buffer pH 4; lactic acid (4.24-4.28 min); internal standard (9.45-10.10 min).

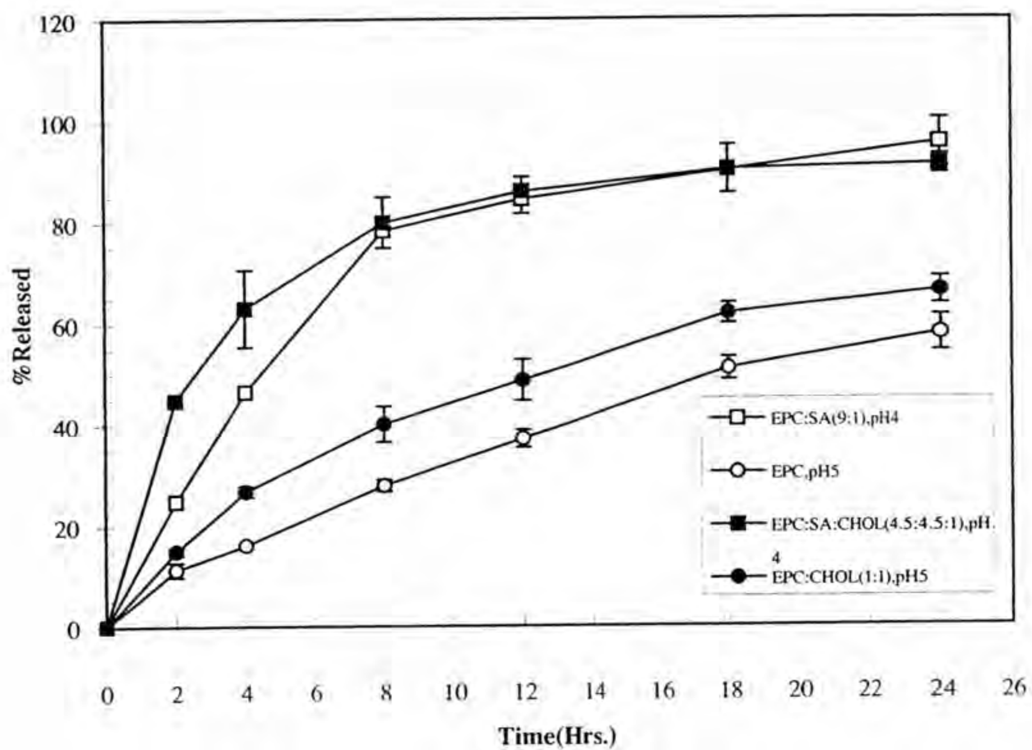


Figure 24. Release profiles of entrapped lactic acid from different types of liposomes at 37° C.

less dominant because these liposomes were unilamellar and had limited surface charges. Hence, lactic acid in positively charged liposomes had a faster release rate when compared with neutral liposomes .

Surprisingly, incorporation of cholesterol (50mol%) into liposomal membrane of both positive and neutral liposomes caused faster release than the formulations without cholesterol as shown in Figure 24. This was seen at both 4°C and 37°C. Cholesterol concentration higher than 50% are known to disrupt regular structure of liposomes (New, 1990). Since the amount of cholesterol used in these preparations was at its borderline, sudden changes in the environment of these liposomes in the experimental set-up, such as ultracentrifugation and redispersion of the pellets with lactic acid-free buffer might induce phase inversion and/or phase separation of the bilayer. This speculation could not be clarified since techniques used to study these changes in the structure of the bilayer, such as freeze-fracture EM, were not readily available.

Figure 25 presents the release profiles of lactic acid from liposomes at 4°C. As expected, the release rate increased with increased temperature (see also Figure 24) because an increase in temperature enhanced the fluidity and permeability of the bilayer and also increased the diffusivity of lactic acid in both the membrane and the aqueous medium . It is then likely that the temperature of 4°C is more suitable for the storage of lactic acid-containing liposomes. However, almost 20% of the total entrapped lactic acid in neutral liposomes was also released after 24 hours at 4°C . Because lactic acid was a water-soluble drug , its leakage from liposomes occurred much more easily than that of hydrophobic drugs (Weiner et al., 1989). Besides, the experiment set-up where lactic acid liposomes were resuspended in a drug -free buffer might impose a steep lactic acid concentration gradient across the bilayer leading to the apparently fast release.

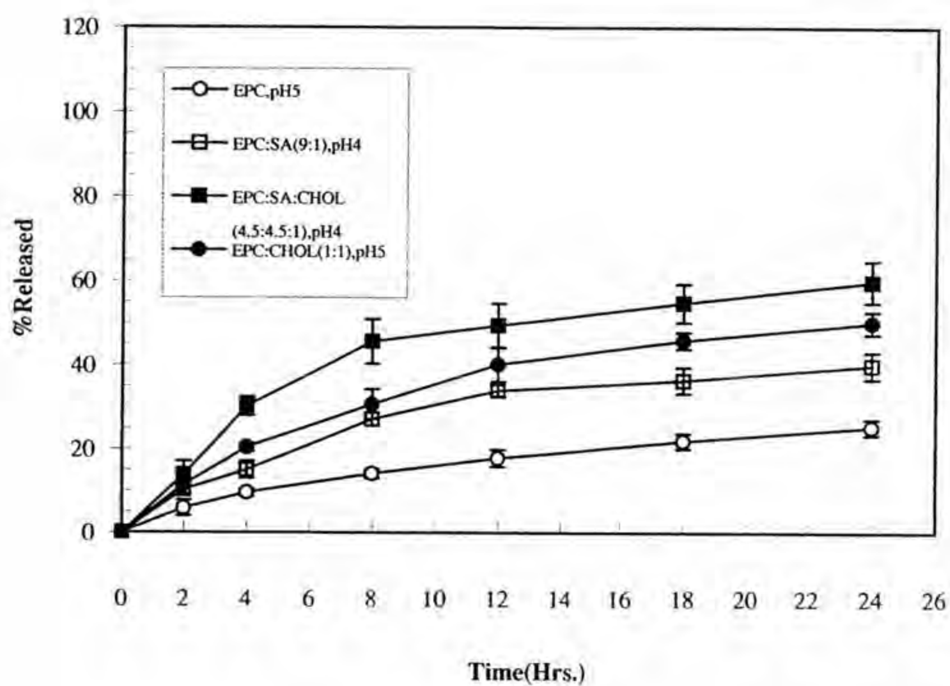


Figure 25. Release profiles of entrapped lactic acid from different types of liposomes at 4° C.

To examine the mechanism of release of lactic acid from liposomes, the percentage of release was plotted against the square root of time as shown in Figure 26-29. The plots indicates that the release of entrapped lactic acid from neutral liposomes is a diffusion-controlled process (Higuchi, 1961), and that the release from positive liposomes may not follow the same pattern.

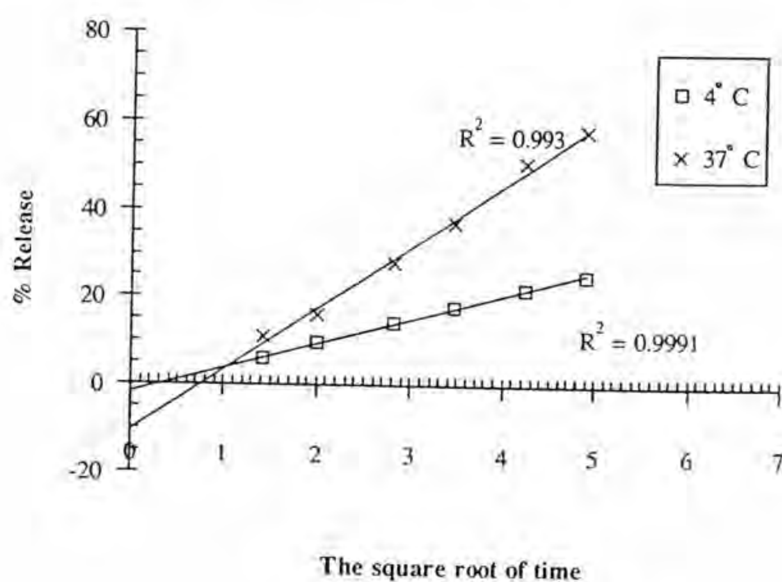


Figure 26. Release profiles plotted as the percentage of lactic acid released from neutral liposomes without cholesterol (pH5, μ=0.5) against the square root of time

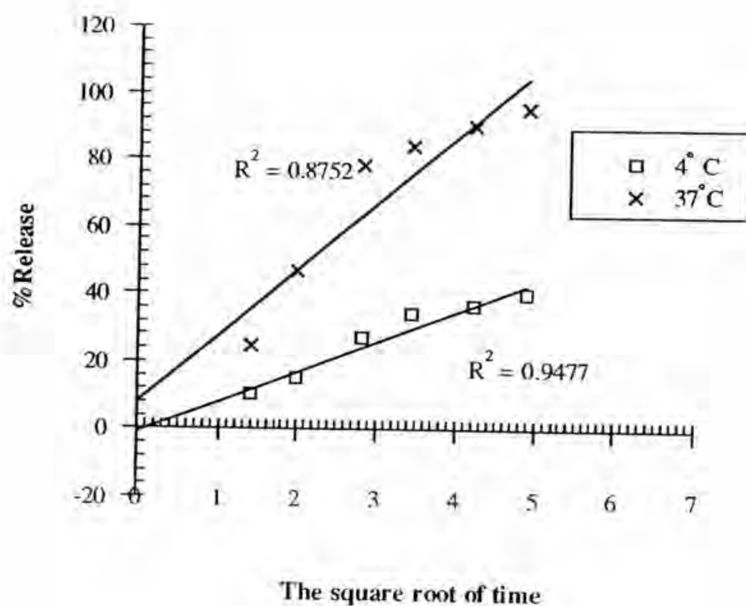


Figure 27. Release profiles plotted as the percentage of lactic acid released from positivel liposomes without cholesterol (pH4, μ=0.1) against the square root of time

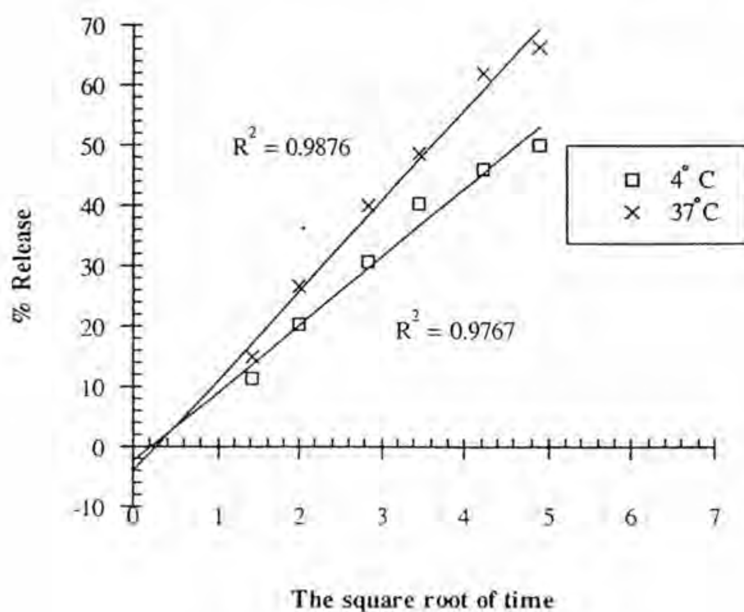


Figure 28. Release profiles plotted as the percentage of lactic acid released from neutral liposomes with cholesterol (pH5, μ=0.5) against the square root of time

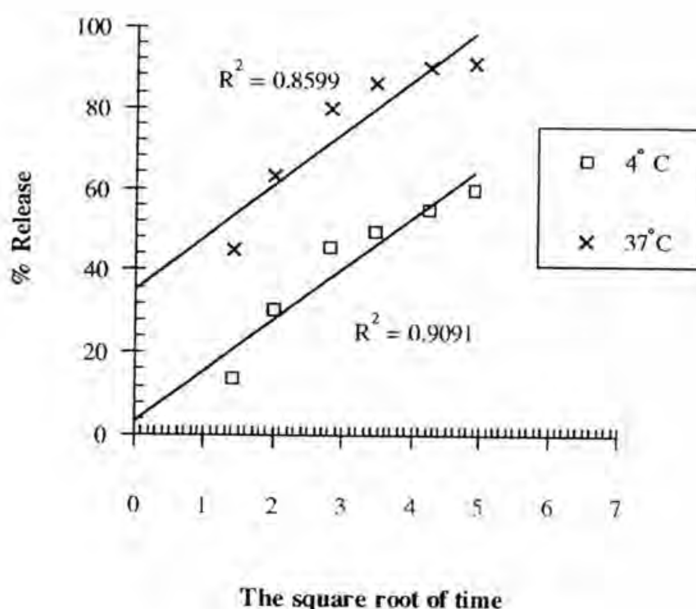


Figure 29. Release profiles plotted as the percentage of lactic acid released from positive liposomes with cholesterol (pH4, μ=0.1) against the square root of time

6. <u>Effect of composition on stability of lactic acid liposomes</u>

In this study, the ability of the liposomal preparation to retain the entrapped lactic acid after one week of storage in a refrigerator was used to evaluate the effects of liposomal composition on stability of the liposomes.

Figure 30 shows the percentage of remaining of lactic acid in different types of liposomes when they were stored in a refrigerator for one week. Statistical analysis used was Kruskal-wallis Test (one-tailed). There is a statistical difference of the percentage of remaining of lactic acid of at least one pair among the eight formulae (at α=0.05). The remaining lactic acid in the neutral liposomes with both cholesterol and α-tocopherol was the highest comparing with other liposomal formulations. The leakage of lactic acid in the positively charged liposomes was more than that of the neutral liposomes. The presence of charge intercalated to the lipid

bilayer resulted in the higher leakage of lactic acid since inclusion of stearylamine in lipid membrane may induce loose packing of the membrane resulting in the increase of permeability of the bilayer as also seen in the release studies. In addition, since the prepared vesicles were LUVs, the interaction between positive charged on the bilayer and the ionized form of lactic acid had a minor effect in decreasing of leakage of lactic acid since most leakage occurred from the ionized lactic acid in aqueous compartment . Retardation of leakage of charged molecules from oppositely charged liposomes has been reported in the literature mostly with multilamellar liposomes where lipid bilayers were sufficiently abundant for the electrostatic interaction to dominate and control overall leakage (Law et al., 1994).

Incorporation of cholesterol in both neutral and positive liposomes decreased leakage of lactic acid during storage because cholesterol increased bilayer rigidity (Gregoriadis and Davis, 1979). These results are in contrast with the results in the release studies, probably because of the difference in the experimental set-up. In the release studies, the liposomes were subjected to several environmental changes as previously described, whereas in these stability studies the liposomes were kept in the medium where free lactic acid was present at the same concentration as in the aqueous compartment of the liposomes.

The effect of α-tocopherol on stability of liposomes was observed in neutral liposomes. The leakage of lactic acid decreased when α-tocopherol was added to the bilayer. α-tocopherol is known to stabilize liposomal bilayers by retardation of lipid oxidation (Villalain et al., 1986). This effect was not clearly seen in positively charged liposomes where bilayers were already leaky, and oxidation of lipids did not play such a major role.

Eventhough the time span of the experiment was short , these results can still show some effects of the difference between liposomal compositions. The neutral liposomes with cholesterol and α-tocopherol (Formula 8, EPC:Chol 1:1 with

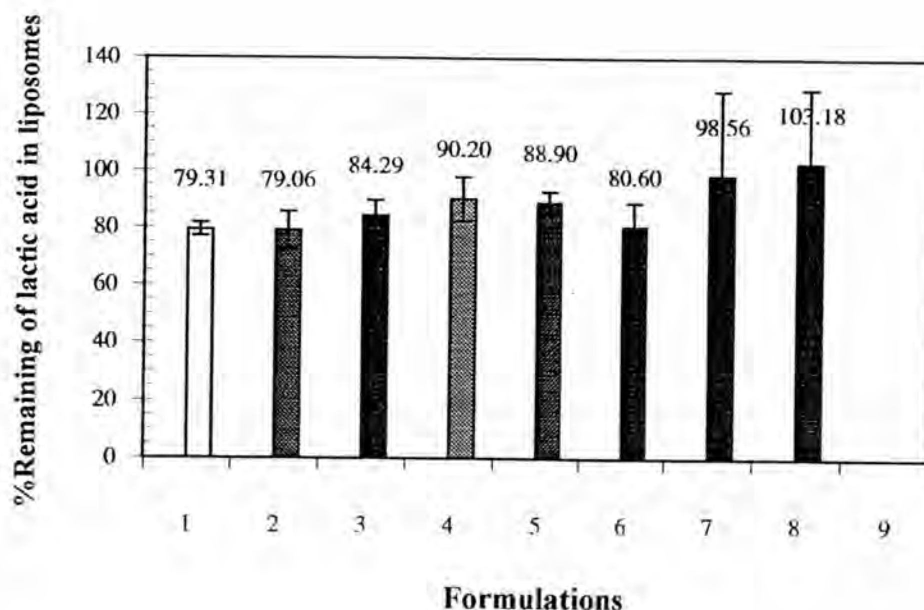


Figure 30. Retention of lactic acid in different liposomes at 4°C for one week;

1 = EPC:SA (9:1) 2 = EPC:SA:vit E (9:1:0.1)
3 = EPC 4 = EPC:vit E (1:0.1)
5 = EPC:Chol:SA (4.5:4.5:1) 6 = EPC:Chol:SA:vit E (4.5:4.5:1:0.1)
7 = EPC:Chol (1:1) 8 = EPC:Chol: vit E (1:1:0.1)

α-tocopherol) gave the highest stability. These results, however, show only the trend of stability of lactic acid liposomes. From practical point of view, long term stability of lactic acid liposomes should be investigated by selecting apparently most stable formula(e) from these preparations and determining the percentage of remaining of lactic acid at appropriate intervals for at least 12 months.