CHAPTER IV

RESULTS AND DISCUSSION

Generally, lipophilic amines are able to permeate through membranes in unprotonated form [Mayer, 1988]; thus, uptake in response to ΔpH relies on permeation of the unprotonated species and subsequently by reprotonation in the acidic internal medium which will deplete the internal proton pool.

Propranolol was determined by fluorescence spectroscopy. Figure 9 showed fluorescence spectra of propranolol in methanol between 250 and 400. The excitation wavelength was 289.5 nm and emission wavelength was 340 nm.

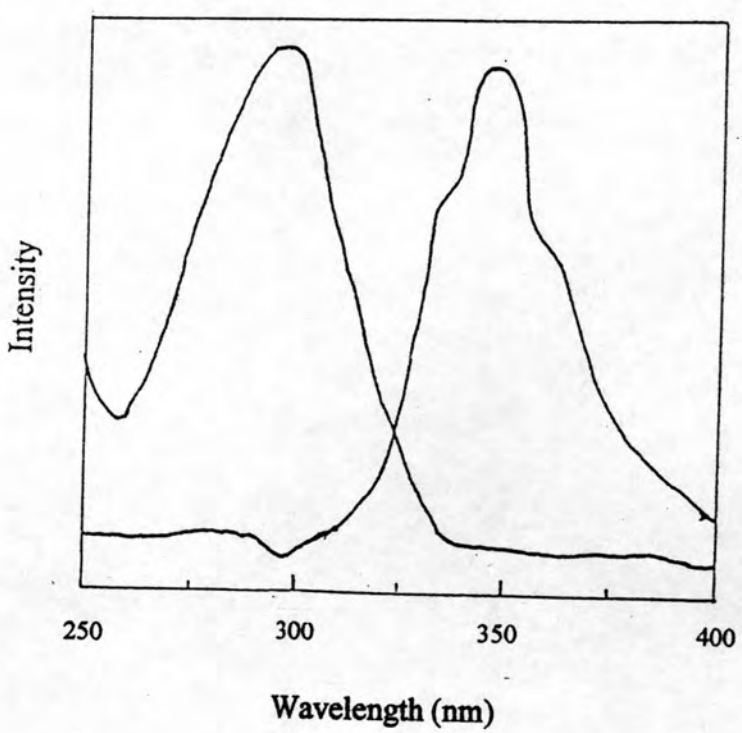


Figure 9. Fluorescence spectra between 250 and 400 nm for propranolol in methanol.

Influence of ΔpH on propranolol uptake.

It had previously been reported that propranolol could be accumulated inside lipid vesicles in response to a transmembrane pH gradient [Madden, 1990]. Therefore, experiments involving ΔpH were performed to find suitable pH (inside and outside vesicles) that could be used efficiently for significant accumulation of drug in vesicles

As demonstrated in Table 1 and 2, the uptake of propranolol into vesicles was maximum when pH outside liposome (pH_{ex}) was 7.5 and pH inside (pH_{in}) was 4.0; however, pH gradient ($\Delta pH = pH_{ex} - pH_{in}$) for maximum uptake of drug remained constant around 3.5 pH units, whatever the pH_{ex} or pH_{in} was. In the absence of a pH gradient ($pH_{ex} = pH_{in}$, e.g. at pH 4.0 and pH 7.5), very low uptake of drugs were observed.

As described previously in Chapter II, when propranolol was incubated in a medium containing liposome exhibiting a transmembrane pH gradient, rapid permeation of the neutral (deprotonated) form of the amine across membrane occurred, and subseqent transmembrane redistribution dictated by the Henderson-Hasselbach relation although its pK_a value (9.5) was higher than the exterior pH of the vesicles (pH_{ex} 7.5, pH_{in} 4.0 for maximum entrapment). Since pH gradient (ΔpH) for maximum uptake of propranolol also obtained approximately 3.5 pH units, this indicated almost equilibrium concentration of propranolol was trapped inside the vesicle in protonated form, comparing with the outside one. The results presented here were similar with other basic drugs [Bally, 1988; Nichols, 1976; Mayer, 1990; Boman, 1993]

Table 1. Influence of pH_{in} on propranolol uptake when pH_{ex} was 7.5.

pH_{in}	Entrapped propranolol/lipid (nmol/μmol)
7.5	1.0511 ± 0.0461
7.0	8.9282 ± 0.1111
6.5	15.7216 ± 0.2406
6.0	49.9606 ± 0.4301
5.5	90.3560 ± 0.7439
5.0	149.6849 ± 1.1420
4.5	179.6085 ± 1.1713
4.0	197.7380 ± 0.8906
3.5	125.7737 ± 1.0634
3.0	18.1233 ± 0.2176

Table 2. Influence of pH_{ex} on propranolol uptake when pH_{in} was 4.0.

pH_{ex}	Entrapped propranolol/lipid (nmol/μmol)
4.0	0.6513 ± 0.0101
4.5	9.0265 ± 0.0854
5.0	17.9517 ± 0.1881
5.5	60.0217 ± 0.2765
6.0	104.0083 ± 0.2371
6.5	145.6903 ± 0.3759
7.0	189.9691 ± 0.4223
7.5	197.7380 ± 0.8906
8.0	145.1902 ± 0.2295
8.5	18.7913 ± 0.3725

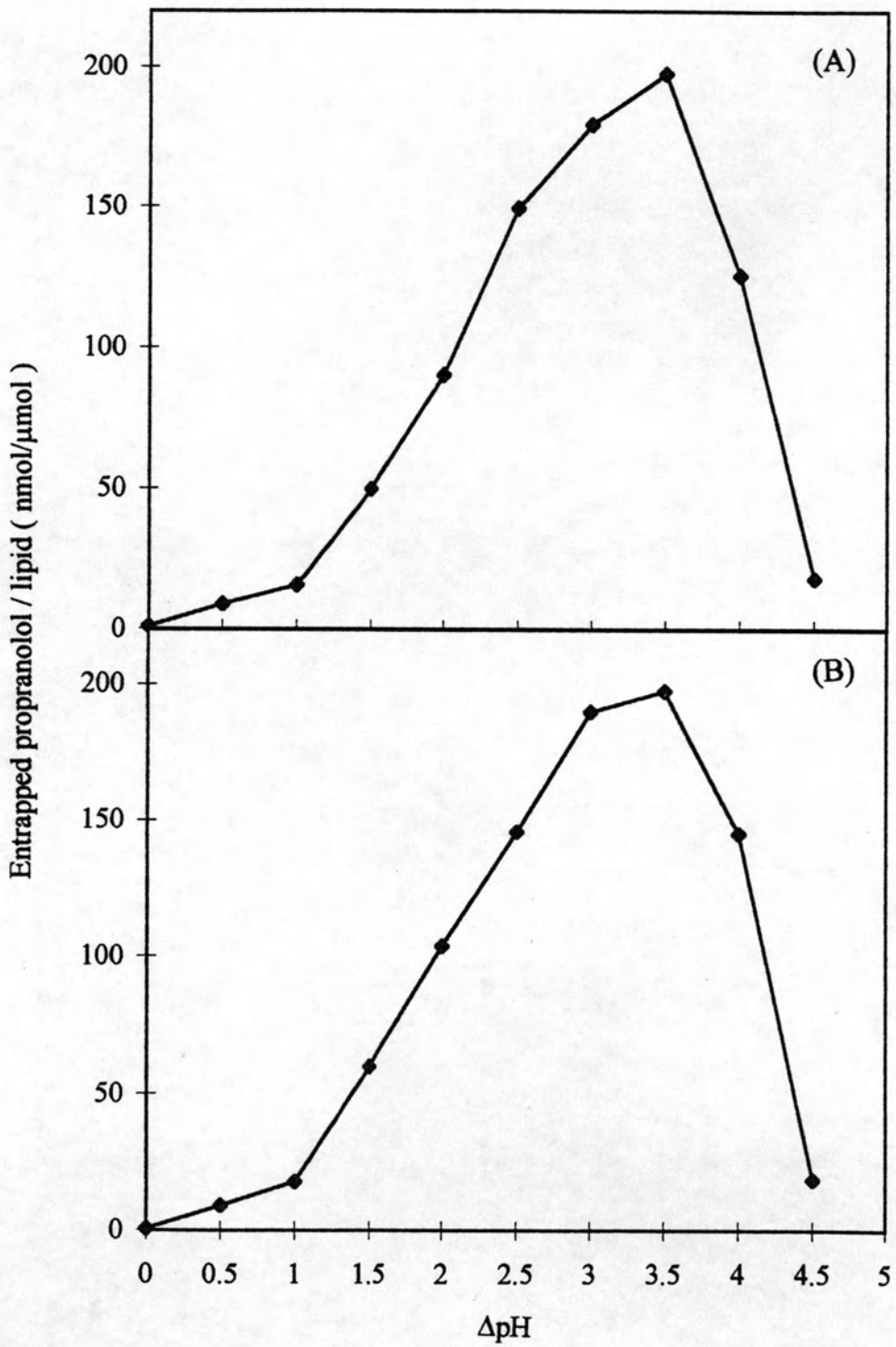


Figure 10. Influence of ΔpH on propranolol uptake into EPC liposomes when pH_{ex} was 7.5 (A) and when pH_{in} was 4.0 (B).

As illustrated in Table 1 and Figure 10, the minimum pH in this investigation was performed at 3.0 since further decrease in the pH would cause a problem to lipid stability, associated with acid catalyzed hydrolysis of acyl chains in the phospholipid component. Higher pH than 8.5 resulted in decreasing solubility of propranolol HCl as well as instability of phospholipid owing to base catalyzed hydrolysis. Lysophospholipid, the hydrolysis product, with an effectively wide head group and a narrow hydrocarbon chain would increased curvature in membrane. As a consequence, pore might be formed; thus, enhancement of permeability of entrapped was obtained as lipid assembly changed from a lamellar into a micellar system.

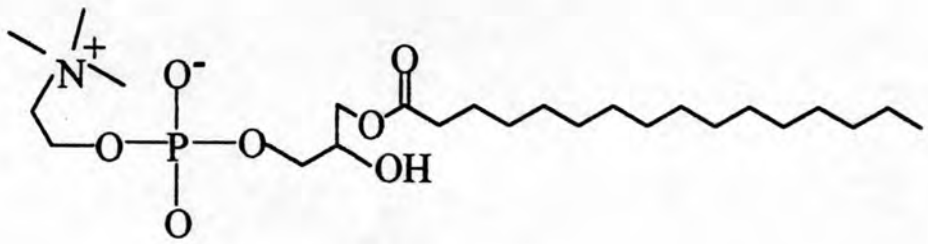


Figure 11. Chemical structure of lysolecithin.

Influence of interior buffering capacity on propranolol uptake.

The vesicle uptake of lipophilic amines in response to transmembrane pH gradient resulted in a depletion of the internal proton pool as the neutral species were protonated upon exposure to the acidic intravesicular medium. Consequently, pH gradient-mediated drug encapsulation was depended on the entrapped buffering capacity and a major determinant of this parameter was buffer composition. The three important factors in this area were buffer chemical composition, buffer concentration, and the preuptake pH relative to the pK_a of the selected buffer.

For liposomal systems exhibiting transmembrane pH gradients (inside acidic) in which the interior pH was less than 5.0, it was found that citric acid was the buffer of choice [Mayer, 1992]. It had several advantages over other buffers which were particularly applicable for drug delivery applications. Firstly, it was widely application as pharmaceutically excipient in injection. Secondly, citric acid was a triprotic buffer that exhibited a wide buffering range (pH 3.0 to 6.5). Thirdly, because citric acid had three titratable groups, osmotic contributions on a per proton equivalent basis was reduced as compared to diprotic and monoprotic buffers such as oxalic acid and acetic acid. This was an important consideration since liposomal systems exhibiting hyperosmolar intravesicular media were susceptible to serum- and plasma-induced leakage of entrapped contents.

As had been well described elsewhere for doxorubicin [Harrigan, 1993], the ability to accumulated high levels of weak base drugs in response to transmembrane pH gradient was a sensitive function of the interior buffering capacity. This was because the molecules permeated across the bilayer in the neutral form and were protonated on reaching the interior, thus consuming a proton and raising the interior pH. This would, in turn, limited the equilibrium uptake of drug. Thus, uptake in response to transmembrane pH gradient relied on permeation of the neutral species and subsequent reprotonation in the acidic internal medium would depleted the internal proton pool. The resulting dependence for propranolol on the interior buffering capacity was studied by monitoring drug accumulation while varying the concentration of citric acid inside the liposome. Table 3 and Figure 12 showed that for the lipophilic amine drug, propranolol, pH gradient-mediated drug entrapment was depended on the buffer concentration in the entrapped aqueous compartment. For an initial propranolol-to-lipid ratio 0.25:1 (mol/mol), increasing the citric acid

concentration from 10 mM to 100 mM produced an increase in the propranolol-to-lipid ratio from 132 nmol/μmol to 247 nmol/μmol. Further increased in the entrapped citric acid concentration above 100 mM resulted in propranolol-to-lipid ratio of approximate 248 nmol/μmol. Again, this was consistent with the consumption of entrapped protons during the uptake process, where citric acid concentration belowed 100 mM resulted in a collapse of the transmembrane pH gradient and inhibition of further propranolol uptake. The relationship between drug uptake and buffer concentration would also depend on the vesicle aqueous captured volume and the number of titratable group per drug molecule.

The buffering capacity inside the liposomes also could be increased by lowering the pH of the intravesicular media; thus the pH gradient remaining subsequent to the uptake process was increased. However, this approach was prohibited by acid catalyzed hydrolysis of phospholipid occurred at low pH value.

Large unilamellar liposomes, preparing by reversed-phase evaporation method were used in the study to assure sufficient aqueous captured volume for maintaining entrapped buffering capacity.

For pervention of osmotically induced lysis of liposomes in blood circulation while maintaining adequate transmembrane pH gradient, 300 mM citric acid was selected as a appropriate interior buffer concentration.

Table 3. Influence of citric acid concentration on propranolol uptake.

Citric acid concentration (mM)	Entrapped propranolol/lipid (nmol/μmol)
10	132.8202 ± 0.8775
20	195.6534 ± 1.7017
50	235.0803 ± 1.6776
100	247.2058 ± 1.4173
150	248.1027 ± 0.8468
200	247.6484 ± 0.5006
250	247.8528 ± 1.3230
300	247.9350 ± 0.7331
350	247.8957 ± 1.2796
400	248.3902 ± 0.8372

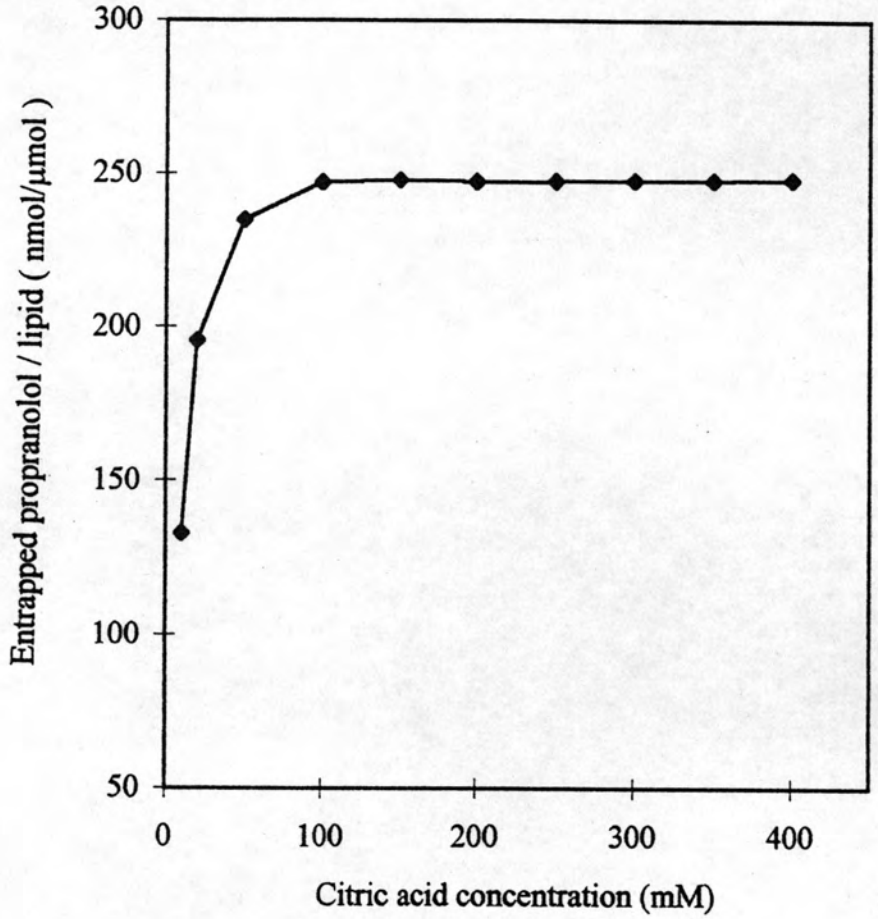


Figure 12. Influence of interior buffering capacity on propranolol uptake into EPC liposomes.

Influence of incubation temperature and time on propranolol uptake.

Incubation temperature, sometimes, has a marked influence on drug uptake into liposomes. As shown in Table 4 and Figure 13, maximumum uptake level was obtained when EPC liposomes (2 μmole) were incubated with 580.8495 nmole propranolol for about 15 minutes at temperature 25 °,37 ° and 40 ° C and remained constant for another 30 minutes except temperature 25 °C for another 45 minutes after that gradually declined. Since relatively stable maximum uptake level of propranolol was observed between 15 and 60 minutes for incubation temperature 25 °C, then it was selected as the standard experimental condition with incubation time 30 minutes.

The result demonstrated a significant correlation between incubation temperature and time on propranolol uptake within liposomal system exhibiting transmembrane pH gradients (inside acidic). A slightly low uptake level of drug obtaining at incubation temperature 10 °C comparing with higher ones might be related to low uptake rate occurring at low temperature. Rapid uptake rate was found at high temperature (37 ° and 40 °C); however, a rapid release of entrapped drug was also observed. This might be due to greater fluidity of bilayer membrane at high temperature, consequently by enhancement of permeability of drug across membrane. Furthermore, longer incubation time at high temperature might promote acid-catalyzed hydrolysis of internal bilayer's.

Table 4. Influence of incubation temperature and time on propranolol uptake.

Time (min)	Entrapped propranolol/lipid (nmol/μmol)			
	10 °C	25 °C	37 °C	40 °C
5	153.5327 ± 0.8435	189.5420 ± 1.0388	201.3635 ± 0.6445	213.2391 ± 0.6405
10	188.8456 ± 1.0669	208.3502 ± 0.8144	218.4642 ± 0.8665	234.1483 ± 0.7534
15	235.6617 ± 1.2544	247.0785 ± 1.1023	245.5431 ± 0.7380	246.9509 ± 0.1643
30	241.9782 ± 1.0238	248.5952 ± 1.0186	248.5268 ± 0.4630	248.1497 ± 0.3245
45	242.2390 ± 1.4185	245.2112 ± 1.4506	237.7560 ± 0.6390	237.0082 ± 0.9400
60	241.1618 ± 0.9349	243.9585 ± 1.7991	224.0902 ± 0.8489	223.6343 ± 0.6036
75	234.8713 ± 1.8075	239.8075 ± 1.5727	211.5200 ± 0.7122	207.0797 ± 0.5865
90	230.6524 ± 2.0940	235.5210 ± 0.7373	198.0290 ± 0.8483	193.6009 ± 0.4846
120	223.8340 ± 1.0224	226.6786 ± 0.9052	171.6330 ± 0.5730	167.3971 ± 0.4899

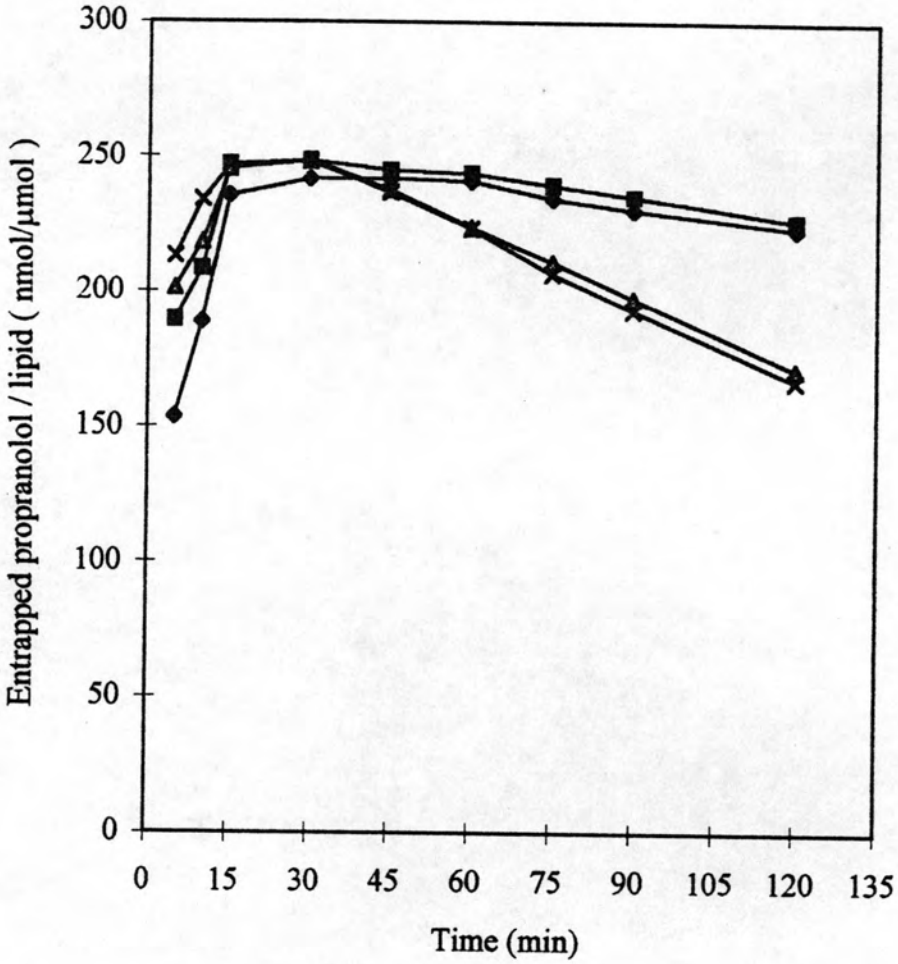


Figure 13. Influence of incubation temperature and time on propranolol uptake into EPC liposomes.

◆ 10°C; ■ 25°C; ▲ 37°C; ✕ 40°C

Influence of propranolol concentration on propranolol uptake.

As explained earlier, drug uptake by the liposome will be accompanied by a reduction in the transmembrane pH gradient as intravesicular protons are bound by the accumulated base; hence, maximum drug uptake obtainable while maintaining a significant postencapsulation pH gradient is necessary examine by monitoring trapping efficiency, liposomal drug uptake as a function of drug:lipid ratio in the incubation mixture. As shown in Table 5 and figure 14, 15, addition of propranolol from 25.3 to 251.1 nmole (per μmole lipid) resulted in increasing entrapped propranolol from 24.2 to 247.9 nmole (per μmole lipid), further addition of propranolol exhibited no improvement on drug entrapment, incontrast with reduction of trapping efficiency. Since denominator (propranolol added) increased while maximum uptake of propranolol (numerator) remained constant, as a consequent, decreasing of trapping efficiency was achieved. Thus, trapping efficiency leaded to misinterpreting and not suitable for use in this study.

It was proposed that the non-protonated propranolol species was membrane permeable and, therefore, present at the same concentration on both side of the membrane. Since the protonated form impermeable, and the pKa was the same on both sides of the membrane, thus the influence of a transmembrane pH gradient on the intravesicular and external propranolol concentration could be derived from the Henderson-Hasselbach equation as :

$$[HA^+]_{in}/[HA^+]_{out} = [H^+]_{in}/[H^+]_{out} \qquad (2)$$

where $[HA^+]$ was the concentration of the protonated propranolol inside or outside the liposome and $[H^+]$ was the proton concentration. From the experiment, propranolol were accumulated to a much greater extent than those

mentioned above, exhibited concentration gradients which far exceed what would be expected based simply on the Henderson-Hasselbach equation. If 95% of the available propranolol was taken up, for example, this represented a concentration gradient which was an order of magnitude greater than the proton gradient, two parameters which might affected for this anomalously high accumulation were considered [Madden, 1990].

First, according to relatively high membrane surface area: aqueous volume ratio of the vesicle interior, a significant portion of entrapped propranolol might associate or partition into liposome membrane due to its lipophilic character since the level of drug uptake depends largely on the lipophilicity of drug, the pH of the vesicle interior, and the maximum capacity of the membrane for drug insertion. Consequently, total uptake levels of propranolol would be expected to surpass those predicted by the Handerson-Hasselbach relationship as the membrane-bound drug fraction was significant relative to the intravesicular soluble drug fraction.

Second, many lipophilic, cationic drugs have maximum solubilities far below entrapped concentration, consequently, it is unlikely that these accumulation levels represent actual drug concentration that can be related directly to the transmembrane proton concentration gradient. Hence, the ability of these drugs to form microprecipitates or alternate phases inside liposomes will also increase equilibrium uptake levels since the exterior drug concentration will need to be depleted until $[\text{soluble } AH^+]_{in}/[\text{soluble } AH^+]_{out} = [H^+]_{in}/[H^+]_{out}$. Nevertheless, this explanation may be unlikely suited with propranolol because its apparent maximum solubility in 300 mM citrate buffer pH 5.0 was 326 mM.

Table 5. Influence of propranolol concentration on propranolol uptake.

propranolol added/lipid (nmol/μmol)	Entrapped propranolol/lipid (nmol/μmol)	%Trapping efficiency
25.2583	24.1741 ± 0.2494	95.7067 ± 0.8205
48.9562	46.7256 ± 0.4703	95.4436 ± 0.9489
99.2327	97.8383 ± 0.7044	98.5948 ± 0.6181
150.1567	147.3875 ± 0.5049	98.1558 ± 0.3212
203.3731	198.1081 ± 0.7399	97.4110 ± 0.2074
225.7122	223.0717 ± 0.4093	98.8301 ± 0.1101
251.1091	247.8603 ± 0.3332	98.7060 ± 0.0708
273.7931	247.7856 ± 0.7801	90.5009 ± 0.2225
296.6913	247.3824 ± 0.4878	83.5140 ± 0.2734

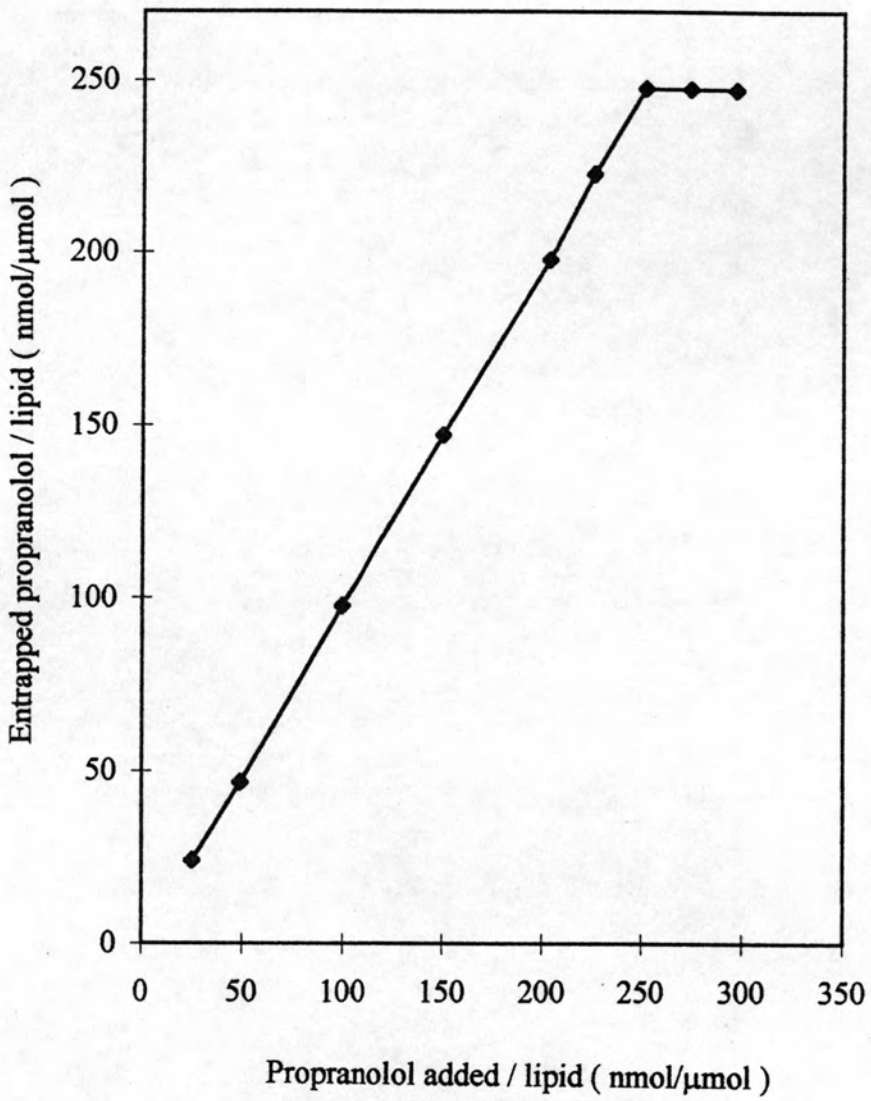


Figure 14. Influence of propranolol concentration added on propranolol uptake into EPC liposomes.

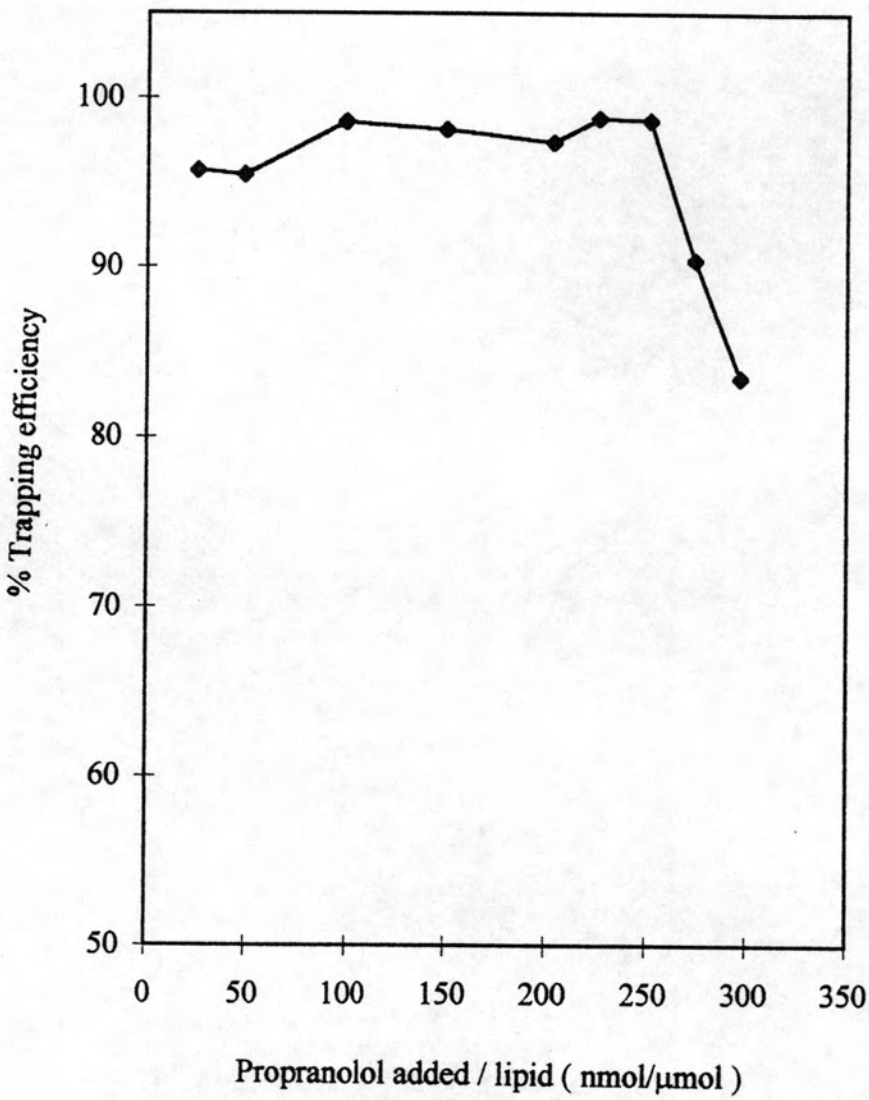


Figure 15. Influence of propranolol concentration added on % trapping efficiency of propranolol uptake into liposomes.

Influence of acyl chain length on propranolol uptake.

Alteration in lipid composition of liposome may affect chemical and physical properties of liposomal membrane that influence relative rates of drug uptake and drug retention. As demonstrated in Table 6 and Figure 16, increasing of acyl chain length resulted in an increase of propranolol uptake. Liposomes preprared from the shortest acyl chain length phospholipid (DLPC, C12:0) showed minimum uptake of about 4.7 nmole (1.9% trapping efficiency) while the longest one (DSPC, C18:0) obtained about 247.4 nmole (98.7%) at ratio of drug to lipid 0.25:1 and 443.1 nmole (97.6%) at ratio 0.45:1, comparing with EPC which was the mixture of saturated and unsaturated lipid with various acyl chain length 247.8 nmole (98.7%). Incorporation of cholesterol to DSPC liposomes in mole ratio of 2:1 and 1:1 (lipid:sterol) resulted in lowering uptake level of DSPC to 383.1 nmole (87.3%) and 390.4 nmole (89.2%), respectively.

In regard to the length of acyl chain of phospholipid, an increase of membrane thickness is achieved in case of the longer acyl chain, consequently by greater in vesicle size; thus, enhancing interior aqueous phase of vesicles. As a result, enlargement of entrapped propranolol was observed with DSPC liposomes as could be seen from increasing %trapping efficiency from 1.8 % of DLPC liposome to 98.7% of DSPC liposomes at molar ratio of drug to lipid 0.25:1. In case of DSPC liposomes, the amount of incubated propranolol was increased from 0.25:1 to 0.45:1 (drug:lipid, by mol) to assure for appropriate propranolol concentration used, since added drug was nearly complete entrapped in liposome (added propranolol 250.4 nmole, entrapped 247.3 nmole) in case of molar ratio 0.25:1. It was found that much more propranolol was entrapped in DSPC liposome when added propranolol was increased to 450.9

nmole (Table 6, entrapped propranolol 443.1 nmole); thus, emphasizing the greater interior aqueous phase in DSPC liposomes.

Increasing in trapping efficiency was observed in oder of DSPC ≈ EPC > DSPC:Chol (1:1) > DSPC:Chol (2:1) > DPPC >> DMPC > DLPC when propranolol was incubated with lipid in molar ratio 0.25:1. Since this parameter can be used efficienctly for various liposome preparations under the same experimental condition; thus, trapping efficiency obtaining from incubation of propranolol with DSPC liposomes in molar ratio 0.45:1 could not be used for comparison with the others described aboved owing to difference in amount of incubated propranolol.

In addition to influencing the pH gradient-dependent uptake process, vesicle lipid composition also exerts effects on drug retention properties of the liposome subsequent to encapsulation. At experimental temperature 25 °C, acyl chains of DPPC (T_c 41 °C) and DSPC (T_c 58 °C) molecules were arranged in trans conformation, therefore, DPPC and DSPC molecules were lengthen and narrow. As a consequent, the liposome membrane thickened and in gel state. Therefore, an increased lipid-water partition coefficient for propranolol would be obtained for the outer monolayer, conversely with tighter packing in the head group region of lipid in inner monolayer that reduced leakage of entrapped drug. In contrast with DLPC (T_c 0 °C) and DMPC (T_c 23 °C) which their acyl chains were arranged in mixed forms of trans and gauche conformations, therefore the thickness of the membrane was reduced and membrane was said to be in fluid state [De Gier, 1986; Inoue, 1974]. In summary, membrane permeability was increased in the order of DLPC>DMPC>DPPC>DSPC [Blok, 1975]; thus, great enhancement latency of entrapped propranolol in DSPC was observed. Addition of cholesterol into DSPC liposomes reduced Van der Waals attraction between acyl chains ; thus, phospholipid hydrocarbon chains

tended to kink and bend, producing fluidizing effect on DSPC bilayer [De Gier, 1986; Inoue, 1974]. As a result, reduction of entrapped propranolol was achieved in DSPC:cholesterol (2:1, by mole). However, increasing amount of cholesterol to DSPC liposome (1:1, by mole) resulted in a little increase of entrapped drug (383.1 nmole for 2:1 to 390.4 nmole for 1:1). This might be due to condensation of membrane caused by increasing amount of cholesterol, therefore, producing a balance between condensation and fluidizind effect in membrane.

EPC liposomes exhibited high entrapped propranolol uptake (247 nmole) although it prossessed low phase transition temperature (-15 °C), then, it should be in fluidizing state at experimental temperature. Nevertheless, EPC is a mixture of heterogeneous lipids which its major components are phospholipids with long acyl chain length (C 18:1 39%, C 16: 0 27%, C 18:2 14%, C 18:0 11%, other 9%, by wt [Lentz, 1980]), therefore, interior aqueous volume might be increased as a result of large size.

Table 6. Influence of acyl chain length on propranolol uptake.

Lipid	Entrapped propranolol/lipid (nmol/μmol)	%Trapping efficiency
EPC	247.8603 ± 0.3332	98.7060 ± 0.0708
DLPC (C 12:0)	4.7048 ± 0.7076	1.8724 ± 0.2900
DMPC (C 14:0)	8.2154 ± 1.0211	3.2804 ± 0.4052
DPPC (C 16:0)	228.6832 ± 1.7748	91.3735 ± 0.8376
DSPC (C 18:0)[a]	247.3573 ± 1.1691	98.7372 ± 0.3389
DSPC (C 18:0)[b]	443.1112 ± 1.4751	97.5892 ± 1.7246
DSPC:Chol(2:1)	383.1488 ± 0.6726	87.2696 ± 0.2636
DSPC:Chol(1:1)	390.3675 ± 2.0470	89.1847 ± 0.4941

[a] Incubation with drug:lipid ratio 0.25:1 (in mole).

Each data represented the mean of three samples ± S.D..

[b] Incubation with drug:lipid ratio 0.45:1 (in mole).

Each data represented the mean of five samples ± S.D..

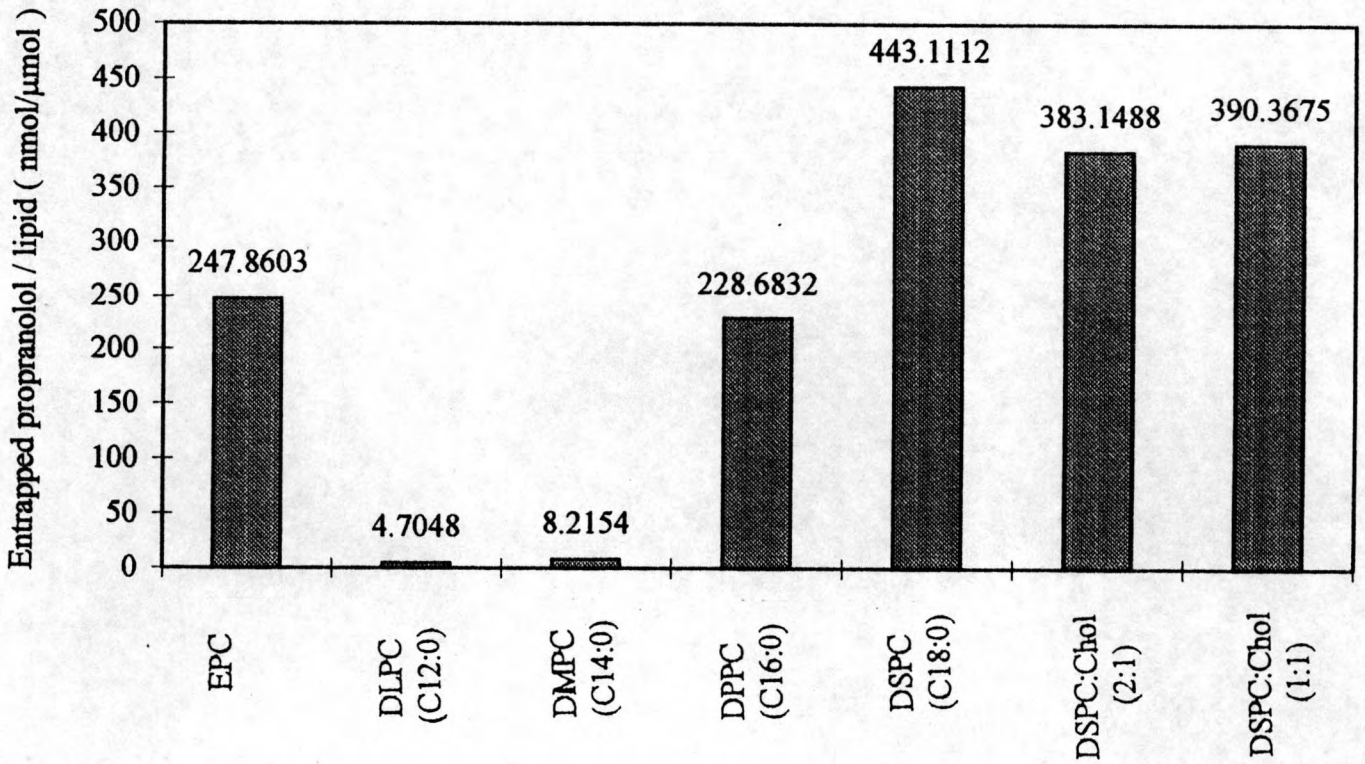
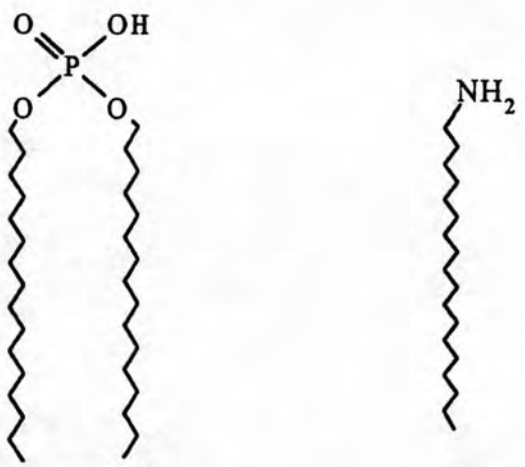


Figure 16. Influence of acyl chain length on propranolol uptake.

Major components of EPC were C 18:1 39%, C 16:0 27%, C 18:2 14%, C 18:0 11%, other 9%, by wt [Lentz, 1980].

Influence of charged amphiphiles on propranolol uptake.

Charged amphiphile is the one important parameter determining the amount of entrapped drug in liposome. The experiment was performed by addition of either dicetylphosphate as a negatively charged amphiphile or stearylamine as a positively charged amphiphile into bilayer of EPC:cholesterol (2:1, by mole).



(A) (B)

Figure 17. Structures of dicetylphosphate (A) and stearylamine (B).

As demonstrated in Table 7 and Figure 18, the entrapment was observed in the following sequence, DCP > SL > neutral with entrapped propranolol 247.5, 217.9 and 99.0 nmole, respectively. Since, nearly complete entrapment of propranolol added was observed in DCP liposome with incubation concentration of drug to lipid 0.25:1 (by mole); then, to as certain the appropriate concentration of drug added, another incubation concentration of drug was tested at molar ratio 0.43:1. As predicted, a large increment of propranolol were found to be entrapped inside the vesicle (394.7 nmole), demonstrating the larger internal aqueous volume of DCP liposome, comparing with the neutral one (EPC:Chol).

Table 7. Influence of charged amphiphile on propranolol uptake. DCP was negatively charged amphiphile and SL was positively charged amphiphile.

Lipid	Entrapped propranolol/lipid (nmol/μmol)	%Trapping efficiency
EPC	247.8603 ± 0.3332	98.7060 ± 0.0708
EPC:Chol(2:1)	98.9568 ± 2.2554	38.9751 ± 1.2255
EPC:Chol(1:1)	201.0247 ± 0.8328	80.2633 ± 0.5756
EPC:Chol:DCP(2:1:0.1)[a]	247.4763 ± 1.2923	98.2011 ± 0.4816
EPC:Chol:DCP(2:1:0.1)[b]	394.6610 ± 2.5060	91.1844 ± 0.6419
EPC:Chol:SL(2:1:0.1)	217.9150 ± 1.1786	87.8758 ± 0.5518

[a] Incubation with drug:lipid ratio 0.25:1 (in mole).
Each data represented the mean of two samples ± S.D..

[b] Incubation with drug:lipid ratio 0.43:1 (in mole).
Each data represented the mean of five samples ± S.D..

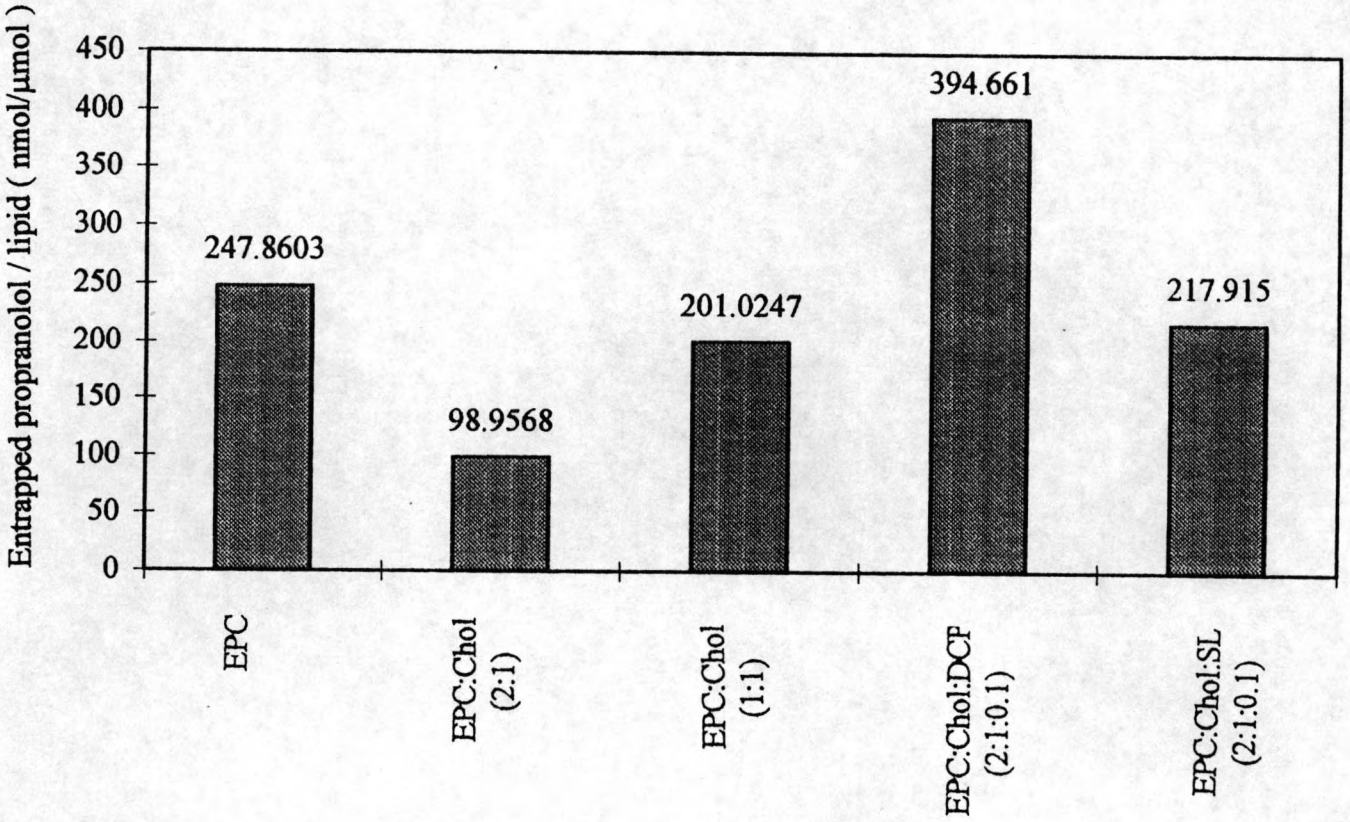


Figure 18. Influence of charged amphiphiles on propranolol uptake. DCP was negatively charged amphiphile and SL was positively charged amphiphile.

Charged amphiphile is one factor that governs the properties of liposome bilayer. When charged amphiphiles are incorporated into liposomes prepared by passive or conventional method, they will repel each other electrostatically, as a consequent, they are distributed relatively homogeneously throughout liposomal membrane. However, this event may be different in liposomes prepared by active or pH gradient method as in the present experiments. Sinces the exterior pH of vesicles was 7.5 and that of the interior pH was 4.0, DCP molecules in the outer monolayer would be fully ionized which DCP molecules in the inner monolayer were partially ionized ($pK_a \sim 4.5$-4.7 in EPC bilayer) [Zuidam, 1995]. Then, there was greater electrostatic repulsion between phosphate head group of phospholipid and phosphate head group of DCP in the outer monolayer than between partially ionized DCP molecules in the inner monolayer. Based on these electrostatic considerations a packing gradient was visualized to exist along the bilayer normal with the inner DCP monolayer being more tightly packed than the outer one. Due to electrostatic repulsion in the outer monolayer, lateral expansion of membrane might be occurred, leading to an increase of vesicle size; as a result, the large entrapment of propranolol was achieved in DCP liposome. Loosely packing in the outer monolayer of DCP liposomes was also one factor that permitted more entrapment of drug.

Conversely behavior should be found with SL, since SL was a weak base (with a $pK_a \sim 9$ for amino group). However, in the other way, SL might exhibit transbilayer localization. An acidic pH would lead to decrease availability of SL on the exterior membrane since they might be redistributed across the bilayer according to Henderson-Hasselbach relation, then vesicles with an interior pH of 4.0 and exterior pH of 7.5 should experience a $\sim 10^{3.5}$ fold reduction in the surface charge at the external surface. Hence, an obvious

consequence of the localization to the inner monolayer of SL in LUV systems should result in a dramatic reduction in the exterior surface charge. For DCP liposomes, the transbilayer localization that caused DCP molecules to migrate to the outer monolayer might be unlikely occured since they acquired a high energy for the transport of phosphate group. Nevertheless, incorporation of SL might also produced lateral expansion due to charged repulsion since a large amount of SL were existed in the inner monolayer. As a consequence, a greater entrapped propranolol was obtained comparing with the neutral one (Table 7).

Influence of cholesterol and cholesterol analogues on propranolol uptake.

Several investigations have been taken to deliver selectively liposome-encapsulated materials to specific organs or types of tissue thus integrity of liposome in blood circulation before reaching target organ is necessary, as well as their high trapping efficiency. Cholesterol is one of the lipid components that has the most profound influence on structural and dynamic bilayer properties [Agawal, 1986; Allen, 1980; McIntosh, 1978]. As presented in Table 8 and Figure 19, incorporation of cholesterol into EPC liposomes resulted in decreasing of entrapped propranolol from 247.9 nmole to 99.0 and 201.0 nmole for EPC:Chol (2:1) and EPC:Chol (1:1), respectively. However, replacement of cholesterol by cholesterol analogues exhibited different effect depending on their head group and chain length. Hydroxyl cholesterol analogues liposomes entrapped propranolol 192.5, 222.7 and 210.6 nmole for analogues 0, I, II, respectively. With amino cholesterol analogues, however, aggregations were observed during preparation of analogues V and VI; thus, the liposomes could be prepared only from analogues III and IV with entrapped propranolol 94.3 and 53.1 nmole, respectively.

Table 8. Influence of cholesterol and cholesterol analogues on propranolol uptake.

Lipid	Entrapped propranolol/lipid (nmol/μmol)	%Trapping efficiency
EPC	247.8603 ± 0.3332	98.7060 ± 0.0708
EPC:Chol(2:1)	98.9568 ± 2.2554	38.9751 ± 1.2255
EPC:Chol(1:1)	201.0247 ± 0.8328	80.2633 ± 0.5756
EPC:Cpd O(2:1)	192.4877 ± 0.7891	75.6432 ± 0.3933
EPC:Cpd I(2:1)	222.7157 ± 0.9352	88.3187 ± 0.4017
EPC:Cpd II(2:1)	210.5946 ± 1.8941	85.5227 ± 0.8000
EPC:Cpd III(2:1)	94.3323 ± 1.3671	37.1319 ± 0.5528
EPC:Cpd IV(2:1)	53.0609 ± 0.9505	21.1455 ± 0.4011

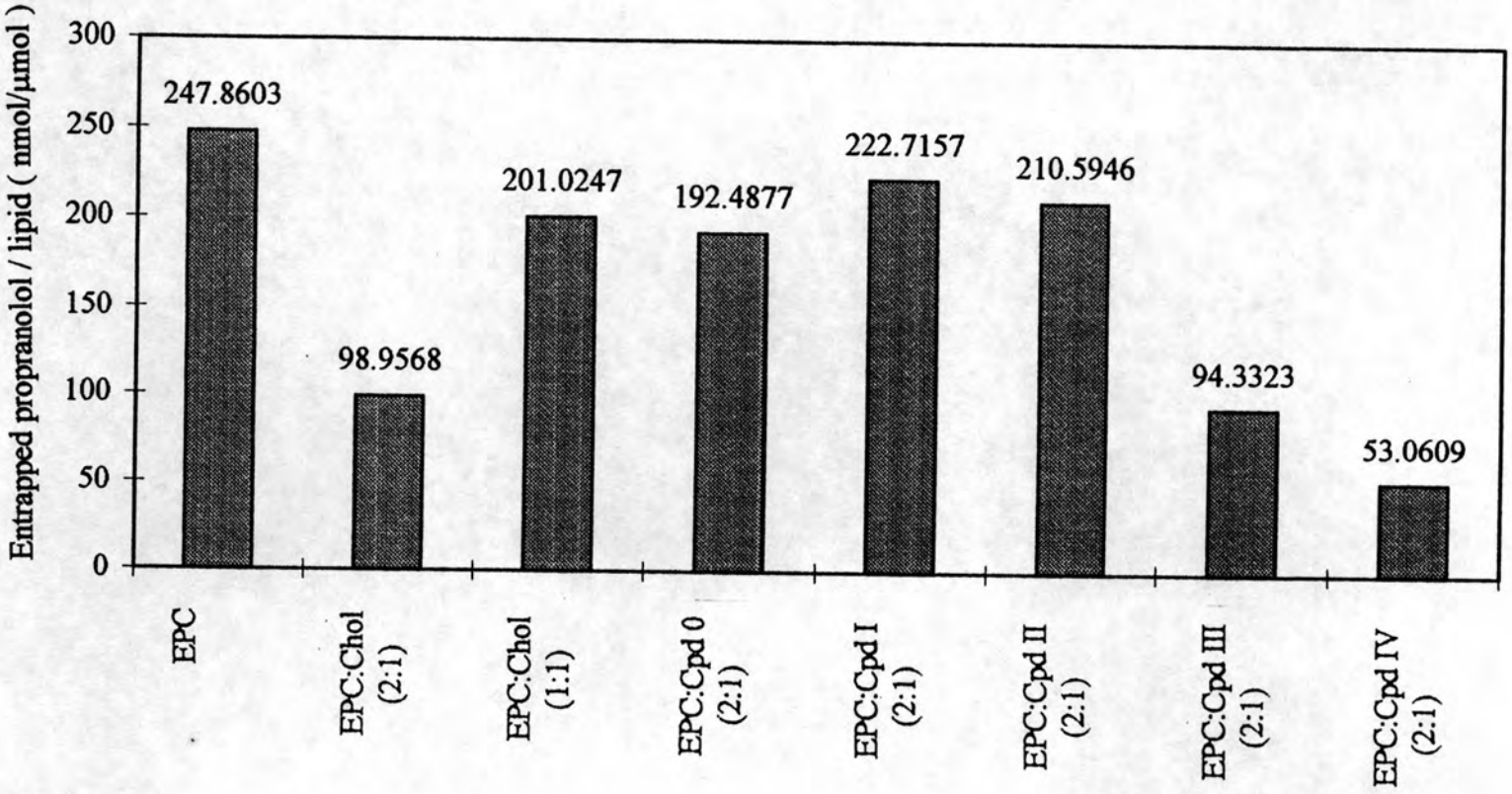


Figure 19. Influence of cholesterol and cholesterol analogues on propranolol uptake.

When lipid molecules of different shapes fit together in a bilayer membrane, their arrangement depends on interaction forces of the hydrophilic head groups and of the hydrocarbon chains. Both have attractive and repulsive forces, and the result is that, despite their movement, they tend to pack in quite specific ways. In general, chain length plays a much smaller role than the properties of lipid heads, but chain packing parameters do matter. Head groups have a profound influence on the different ways in which lipid molecules pack into bilayers; the more important reasons are:

1) the presence of charges and the possible interaction (attractive or repulsive) of the charged groups

2) the hydration shell which will accompany both the charges and any hydroxyl group so that the water molecule, being locally oriented and not in random motion, will fill the space between the covalently bounded atoms of the hydrophilic heads and thus enlarge the effective head group area

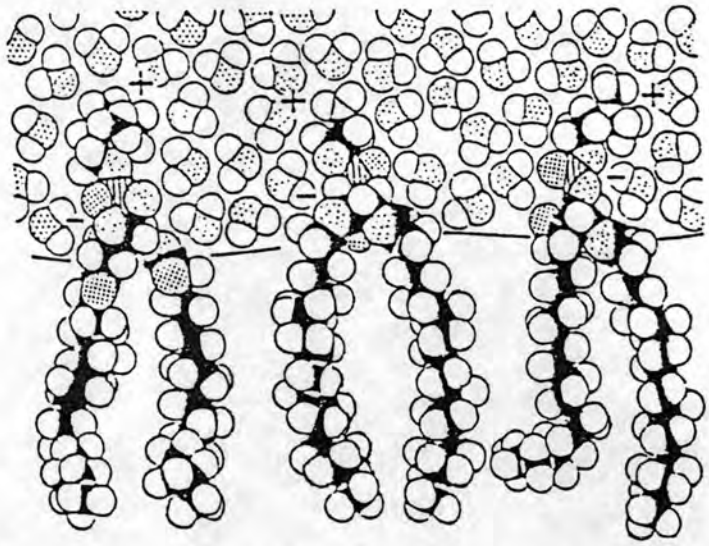


Figure 20. Water molecules are attracted to the polar heads of lipids, particularly by the charges. Water dipoles align with their oxygens towards the positive charges and their hydrogens towards the negative.

3) hydrogen bonds between adjacent hydrophilic heads may contribute to reduce the effective area of the hydrophilic group.

Cholesterol is an ubiquitous structural and functional component of most eukaryotic plasma membranes. The structure of cholesterol has most likely evolved in order to allow for an optimal interaction of the sterol with various phospholipids and proteins in the membranes. The cholesterol backbone is the rigid planar transfused tetracyclic ring structure of a steriod which reaches up to 9 or 10 carbons of extended alkyl chains and to somewhat deeper level in the hydrophobic region of the membrane in the liquid crytalline phase. The 3β-hydroxyl head group locates at carbonyl group of glycerol backbone of phospholipid. Cholesterol has a dual effect (condensing and fluidizing effects) on phospholipid depending on its phase transition temperature, whereby a 3β-hydroxyl and planar steriod nucleus play an important structure role in this interaction [Marsh, 1973].

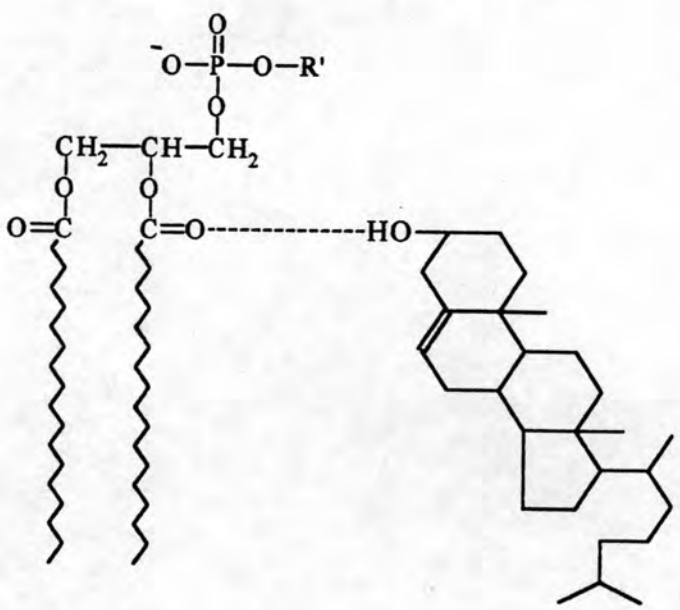


Figure 21. Possible hydrogen bonding between cholesterol and phospholipid.

Since EPC is itself a complex mixture having no well-defined structural transitions. As described by Ladbrooke (1968), the presence of cholesterol in egg yolk lecithin at 33.5 °C (well above the lipid transition temperature, T_c = -15 °C) caused some inhibition of the lipid chain motion. Thus, at a particular temperature (T), the presence of cholesterol kept the hydrocarbon chains of differing phospholipid molecules in an intermediate fluid condition. Some lipids which would normally be above their limiting transition temperature ($T>T_c$) might have a certain amount of inhibition of chain motion since rigid cholesterol molecule immobilized the adjacent phospholipids' hydrocarbon chains by Van der Waals attraction force thus lipid molecule lenthened (gel state). This inhibition might be depend upon how unsaturated were the lipid chains. Those lipids which would normally be in a gel condition ($T_c>T$) were, however, given much greater fluidity by disturbing the cohesive forces between the adjacent hydrocarbon chains. However, the state of EPC membrane also depended on amount of cholesterol presented; since investigation of the phase behavior of EPC-cholesterol bilayers, using 1,6-diphenyl-1,3,5-hexatriene (DPH) and freeze-fracture electron microscopy demonstrated coexisting liquid and amorphous, gel-like phases upon addition of cholesterol up to 36 mol% which exhibited inflection point, revealing a boundary between a liquid phase and coexisting liquid and amorphous, gel-like phases. Cholesterol rich phase was obtained upon addition of cholesterol to 50 mol% [Lentz,1980]. Previously explanation might be used to clarify the lower entrapment found in EPC liposome incorporated cholesterol (Table 8). Since upon addition of cholesterol into EPC liposomes in molar ratio 1:2 (~ 33 mol% Chol) resulted in decreasing of propranolol entrapment from 247.9 nmole (EPC liposomes) to 99.0 nmole (Chol:EPC, 1:2 by mol). This might be due to inhomogeneity, caused by coexisting of liquid and gel-like phases in liposomal membrane that disturbed permeation of propranolol across membrane. Since

cholesterol rich phase was achieved upon addition of cholesterol to EPC in molar ratio 1:1; therefore, increasing amount of entrapped drug was obtained (201.0 nmole) owing to ease of permeation.

Substitution of amino cholesterol analogues in place of cholesterol in EPC liposome reduced amount of entrapped propranolol for analogues III, IV and no liposome formed for analogues V,VI. Strong interaction might be occurred between amino group of analogues V,VI with lipid phosphate group or citrate group of buffer at pH 4.0, in process of liposome preparation, leading in aggregation as shown in Figure 22; therefore, liposome could not be form. Since, low basicity of amino group might be found in analogues III, IV; then, liposomes could be prepared, however, with low entrapment (94.3 and 53.1 for anologues III and IV, respectively). In outer monolayer (pH_{ex} 7.5), amino group of analogue III might present in neutral form or partially ionized, then hydrogen bond might be formed between amino group of analogue III and carbonyl moiety of glycerol backbone or polar-polar interaction between protonated form and phosphate group of phospholipid. For inner monolayer (pH_{in} 4.0) amino groups of analogue III were protonated, inducing to charge repulsion due to tighter packing in the head group region of lipid in inner monolayer resulted in closely pack of protonated forms. However, the attractive force in outer monolayer and repulsive force in inner monolayer might be not strong enough to produce any change to liposomal membrane, comparing with cholesterol since amount of entrapped propranolol was nearly equal in both types. In contrast with analogue IV, the longer one, exhibited lower entrapment of drug. This might be explained as electrostatic repulsion between long chain primary amino group of analogue IV with positive charged choline moiety of lipid in outer monolayer, corresponding with electrostatic repulsion in inner monolayer; hence, loosely packing was obtained in both side of bilayer, enhancing of

leakliness. On the other hand, purturbation around surface head groups of bilayer membrane resulted from steric factor of long chain head group of analogue IV might also occur, subsequently by increasing of membrane instability. Transbilayer localization to inner monolayer as stearylamine seemed not to occur for amino cholesterol analogues owing to their bulky, rigid steroid nucleus.

Figure 22. Aggregation of EPC and cholesterol analogues compound V or VI during REVs preparation (2).

Hydroxyl cholesterol analogues exhibited greater entrapment of propranolol comparing with cholesterol (99.0, 192.5, 222.7, 210.6 nmole for cholesterol, analogue 0, I and II, respectively). Since hydroxyl group is very weak acidic group, enhancement of entrapped propranolol might be attributed from polar-polar interaction with choline moiety of phosphate head groups and

hydroxyl head group or ether group of hydroxyl cholesterol analogues. Moreover, intermolecular hydrogen bonding migh be formed between carbonyl group of phospholipids and hydroxyl head group of hydroxyl cholesterol analogues. These interactions might possibly produce two effects. First, the interaction, if occurred between adjacent lipid molecules, would hold them together, leading to reduction in fluidity of hydrocarbon chains. As a result, acyl chain increased in length and the membrane thickened, subsequently by greater of entrapped aqueous space in vesicles. Second, they might stabilized the integrity of liposomal membrane since membrane became densely. However, the least entrapment of drug found in analogue II might be caused by steric effect, produced by long, bulky head group, that disturbed arrangement of membrane bilayer, subsequently by a slightly increase in membrane permeability.

Integrity of liposomes entrapped propranolol.

Previous studies on doxorubicin [Mayer, 1990] and vincristine [Boman, 1993] had shown that drug retention after loading in response to a pH gradient (inside acidic) was over 24 hr. As indicated previously in Chapter II and III, a model based on the assumption that only the neutral form of the propranolol was membrane permeable. To test for integrity of liposome, drug retention in vesicles was monitored at temperature 10 °, 25 ° and 37 °C, as shown in Table 9-26 and Figure 23-28. For all of REVs system tested, drug release were increased as temperatures increased. Figure 28-30 compared leakage of entrapped propranolol from liposomes at 10 °C, 25 °C and 37 °C, respectively in term of drug retention. No significant release of propranolol was observed over 40 hr upon incubation of EPC:Chol (2:1) liposomes at 10 ° and 25 °C. For liposome studied here, EPC:Chol (2:1) exhibited the best for

retaining entrapped propranolol inside the vesicles. The high entrapped propranolol/lipid ratios and stable drug retention were closely related to the ability of the liposomes to maintain a significant transmembrane pH gradient. The proton-hydroxide flux should have a high activation energy; thus, the liposomes had a high intrinsic proton permeability. The relatively large activation energy supported the concept that the flux mechanism may include proton-hydroxide transfer along water structure associated by H-bonding [Deamer, 1983].

An increase in the surface charged density (by incorporation charged amphiphiles) would increase the intermolecular electrostatic energy of repulsion, giving rise to a time-average increase in pore size existing normal to the plane of the lipid lamellae. Any increase in the size of these pores, the effective diameter of which must be of the order of ionic radii, could significantly increase the diffusion rate.

Table 9. Retention of propranolol in EPC liposomes at 10 °C.

Time (hr)	Entrapped propranolol/lipid (nmol/μmol)
0	248.4287 ± 0.3760
1	233.1812 ± 0.5914
2	224.2047 ± 0.3673
4	207.5548 ± 0.4353
6	174.5663 ± 0.5141
8	156.1271 ± 1.0723
10	114.9092 ± 0.8486
20	90.9150 ± 0.8906
24	80.6745 ± 1.5907

Table 10. Retention of propranolol in EPC liposomes at 25 °C.

Time (hr)	Entrapped propranolol/lipid (nmol/μmol)
0	248.0751 ± 0.4210
0.5	243.2101 ± 0.2048
1	234.4451 ± 0.3549
2	219.6438 ± 0.4948
3	188.6333 ± 0.7664
4	154.6139 ± 0.3228
5	120.3111 ± 0.3448
6	95.2992 ± 1.1130
8	44.0091 ± 2.3575
10	17.4417 ± 7.4346

Table 11. Retention of propranolol in EPC liposomes at 37 °C.

Time (min)	Entrapped propranolol/lipid (nmol/μmol)
0	248.2025 ± 0.2598
15	240.2946 ± 0.3446
30	228.9212 ± 0.3185
45	215.9012 ± 0.6151
60	199.2292 ± 0.3109
90	168.4399 ± 0.7109
120	119.7910 ± 1.9955
180	86.6575 ± 1.4243
240	64.3415 ± 0.7344

Table 12. Retention of propranolol in EPC:Chol:DCP liposomes (2:1:0.1, by mol) at 10 °C.

Time (hr)	Entrapped propranolol/lipid (nmol/μmol)
0	397.5066 ± 0.1243
0.25	395.9219 ± 0.3019
0.50	394.6542 ± 0.3130
0.75	393.4153 ± 0.3499
1	391.9610 ± 0.1917
1.5	390.2652 ± 0.3257
2	367.1036 ± 0.4260
3	331.9951 ± 0.3458
4	298.8666 ± 0.4735
8	198.7246 ± 1.7246
12	112.5436 ± 1.9191

Table 13. Retention of propranolol in EPC:Chol:DCP liposomes (2:1:0.1, by mol) at 25 °C.

Time (min)	Entrapped propranolol/lipid (nmol/μmol)
0	397.4652 ± 0.1380
5	395.6055 ± 0.2831
15	325.3166 ± 0.3832
30	234.9849 ± 0.4541
45	192.4369 ± 0.6607
60	134.3694 ± 0.7263
90	100.1439 ± 1.0834
120	94.6252 ± 0.9702
180	79.1029 ± 1.4916
240	61.5438 ± 1.6422
300	22.5107 ± 6.4922

Table 14. Retention of propranolol in EPC:Chol:DCP liposomes (2:1:0.1, by mol) at 37 °C.

Time (min)	Entrapped propranolol/lipid (nmol/μmol)
0	395.6231 ± 0.3904
5	279.9439 ± 0.4638
15	145.2946 ± 0.8806
30	51.3402 ± 2.2490
45	18.8702 ± 4.8459
60	15.9344 ± 2.9656
75	15.4258 ± 3.6952
90	13.6954 ± 5.5767
120	12.8362 ± 4.8011

Table 15. Retention of propranolol in EPC:cholesterol liposomes (2:1, by mol) at 10 °C.

Time (hr)	Entrapped propranolol/lipid (nmol/μmol)
0	98.4113 ± 1.1415
5	94.9997 ± 0.9894
10	93.8722 ± 1.2310
15	94.2696 ± 0.9587
20	94.2355 ± 1.2353
24	94.3170 ± 1.5247
30	93.5130 ± 1.4401
36	92.7863 ± 1.4953
40	91.7153 ± 1.4499
45	89.9504 ± 1.6780
48	88.8110 ± 1.4585
50	86.9749 ± 1.4680

Table 16. Retention of propranolol in EPC:cholesterol liposomes (2:1, by mol) at 25 °C.

Time (hr)	Entrapped propranolol/lipid (nmol/μmol)
0	98.6382 ± 1.6674
2	95.4993 ± 0.6913
5	93.0024 ± 0.9318
10	91.5302 ± 1.5401
15	86.5718 ± 1.0842
20	83.9803 ± 1.9567
24	73.6153 ± 2.1353
30	66.2082 ± 2.8176
36	52.4297 ± 2.1938

Table 17. Retention of propranolol in EPC:cholesterol liposomes (2:1, by mol) at 37 °C.

Time (min)	Entrapped propranolol/lipid (nmol/μmol)
0	138.7209 ± 0.2523
15	133.7573 ± 0.4103
30	128.6200 ± 0.7397
90	122.6009 ± 0.8706
150	109.6474 ± 0.4359
210	93.4116 ± 0.8191
270	78.8736 ± 1.0816
330	54.3770 ± 0.8755
560	20.2908 ± 5.3352

Table 18. Retention of propranolol in EPC:Cpd I liposomes (2:1, by mol) at 10 °C.

Time (hr)	Entrapped propranolol/lipid (nmol/μmol)
0	222.1183 ± 0.5607
2	212.6996 ± 0.5524
4	205.1889 ± 0.8666
6	190.4339 ± 1.0166
8	163.1371 ± 0.7307
10	142.7838 ± 1.4890
12	94.4637 ± 1.2778
18	41.1631 ± 1.631
24	22.3056 ± 4.9868

Table 19. Retention of propranolol in EPC:Cpd I liposomes (2:1, by mol) at 25 °C.

Time (hr)	Entrapped propranolol/lipid (nmol/μmol)
0	222.6587 ± 0.7085
1	204.1205 ± 0.6464
2	154.0601 ± 0.6313
4	63.3140 ± 1.4119
6	48.0919 ± 2.4489
8	42.9390 ± 1.7000
10	32.6825 ± 1.9385

Table 20. Retention of propranolol in EPC:Cpd I liposomes (2:1, by mol) at 37 °C.

Time (min)	Entrapped propranolol/lipid (nmol/μmol)
0	222.4438 ± 0.3623
15	169.7394 ± 0.6934
30	93.3416 ± 0.8854
45	61.2516 ± 1.9229
60	46.5367 ± 2.4113
90	33.7864 ± 2.6162
120	29.8822 ± 3.0840
180	22.6829 ± 2.7714
240	17.4023 ± 5.5255

Table 21. Retention of propranolol in EPC:Cpd II liposomes (2:1, by mol) at 10 °C.

Time (hr)	Entrapped propranolol/lipid (nmol/μmol)
0	209.7029 ± 0.6303
2	207.7158 ± 0.7691
4	203.5860 ± 0.5679
6	186.0129 ± 0.4798
8	161.6360 ± 0.9479
10	141.2774 ± 1.3506
12	97.3701 ± 0.7526
18	70.4589 ± 2.9290
24	41.1047 ± 3.8678

Table 22. Retention of propranolol in EPC:Cpd II liposomes (2:1, by mol) at 25 °C.

Time (hr)	Entrapped propranolol/lipid (nmol/μmol)
0	209.6311 ± 0.6129
1	181.6705 ± 0.6244
2	149.3933 ± 1.0386
3	73.9637 ± 1.1584
4	56.1134 ± 2.2193
6	39.0176 ± 2.8564
8	35.1540 ± 2.9985
10	33.4314 ± 3.4721

Table 23. Retention of propranolol in EPC:Cpd II liposomes (2:1, by mol) at 37 °C.

Time (min)	Entrapped propranolol/lipid (nmol/μmol)
0	210.0339 ± 0.3413
15	96.4193 ± 0.6871
30	68.3165 ± 1.2861
45	60.4701 ± 1.6807
60	46.0877 ± 1.4976
90	37.3187 ± 1.5945
120	35.2156 ± 1.0997
180	33.6876 ± 1.6116
240	20.5030 ± 2.5390

Table 24. Retention of propranolol in EPC:Cpd III liposomes (2:1, by mol) at 10 °C.

Time (hr)	Entrapped propranolol/lipid (nmol/μmol)
0	96.8161 ± 1.9898
2	90.8303 ± 1.9683
4	74.9448 ± 1.8927
6	65.7384 ± 2.2416
8	58.8398 ± 2.1430
10	42.9353 ± 3.8371
12	31.3912 ± 3.7646
24	22.9028 ± 2.5731

Table 25. Retention of propranolol in EPC:Cpd III liposomes (2:1, by mol) at 25 °C.

Time (hr)	Entrapped propranolol/lipid (nmol/μmol)
0	96.1058 ± 1.3348
1	88.9121 ± 1.4374
2	80.2652 ± 1.6071
3	64.2699 ± 1.1576
4	54.1286 ± 3.0051
5	30.4015 ± 3.3256
6	29.8206 ± 4.6441

Table 26. Retention of propranolol in EPC:Cpd III liposomes (2:1, by mol) at 37 °C.

Time (min)	Entrapped propranolol/lipid (nmol/μmol)
0	93.9665 ± 0.7720
15	78.1523 ± 1.4503
30	53.7720 ± 0.7699
45	48.3470 ± 1.0972
60	42.5909 ± 1.6106
90	36.3274 ± 1.8758
120	29.8278 ± 2.3186
180	21.9821 ± 2.5781
240	17.9587 ± 1.6404

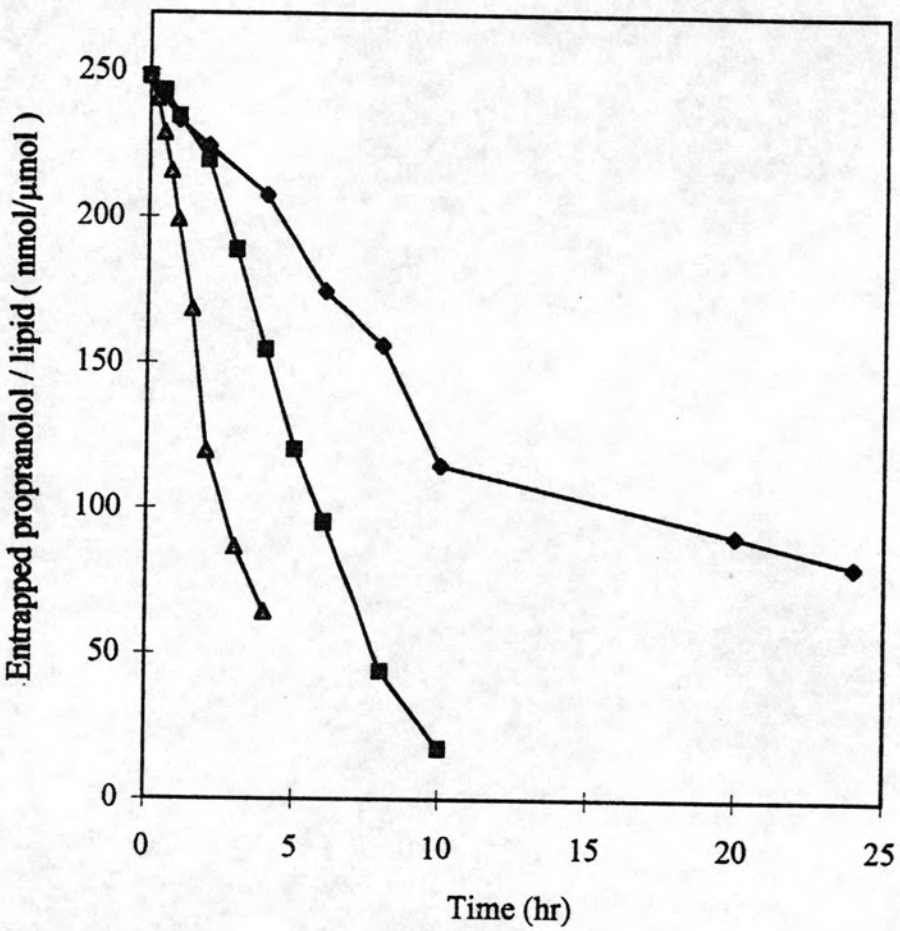


Figure 23. Retention of propranolol in EPC liposomes.

◆,10°C; ■,25°C; ▲,37°C

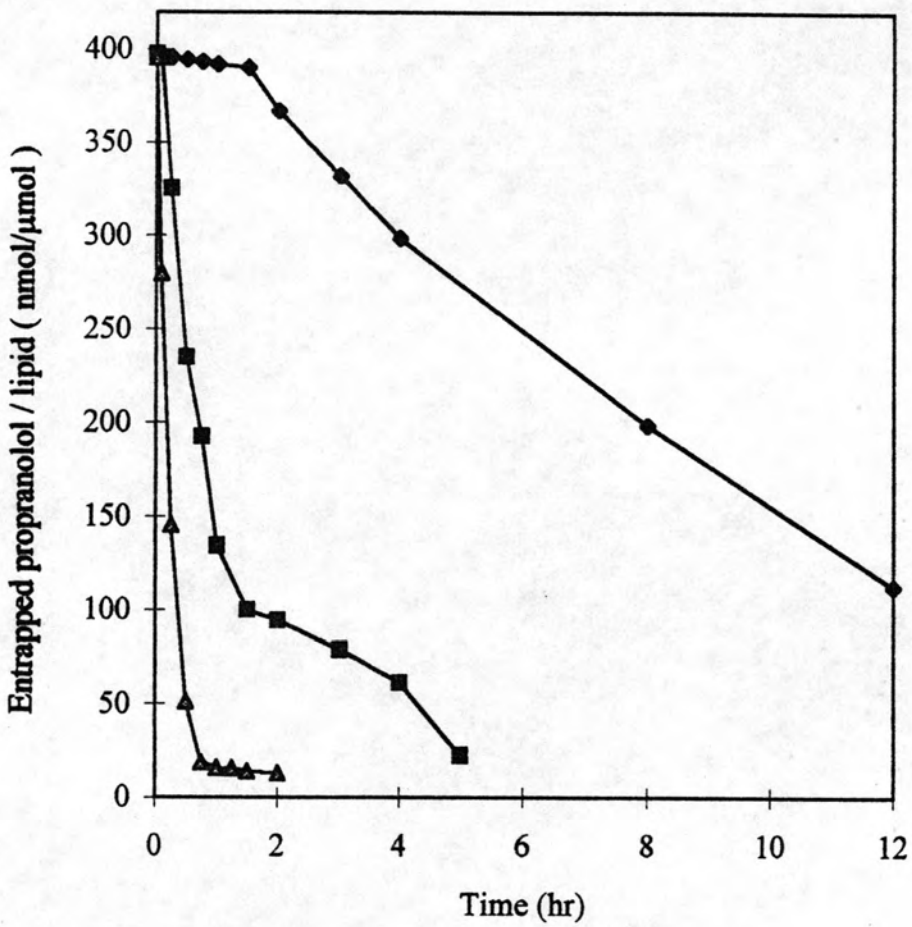


Figure 24. Retention of propranolol in EPC:Chol:DCP liposomes (2:1:0.1,by mol).

—◆—,10°C; —■—,25°C; —▲—,37°C

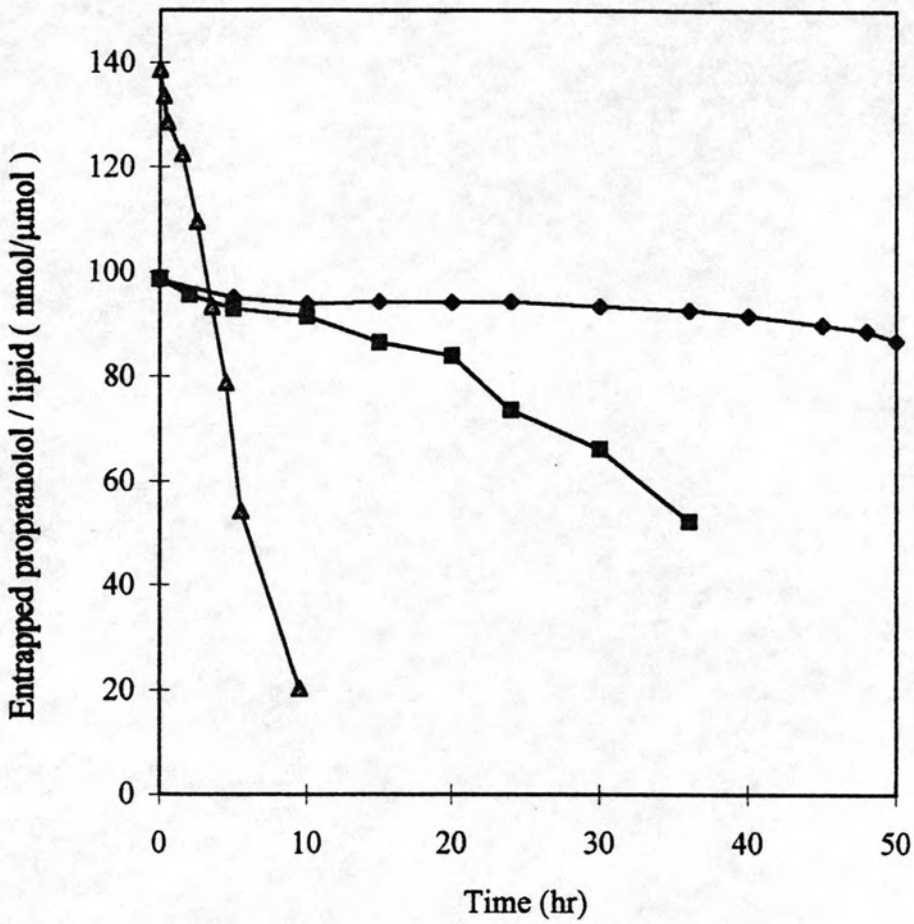


Figure 25. Retention of propranolol in EPC:Chol liposomes (2:1, by mol).

◆,10°C; ■,25°C; ▲,37°C

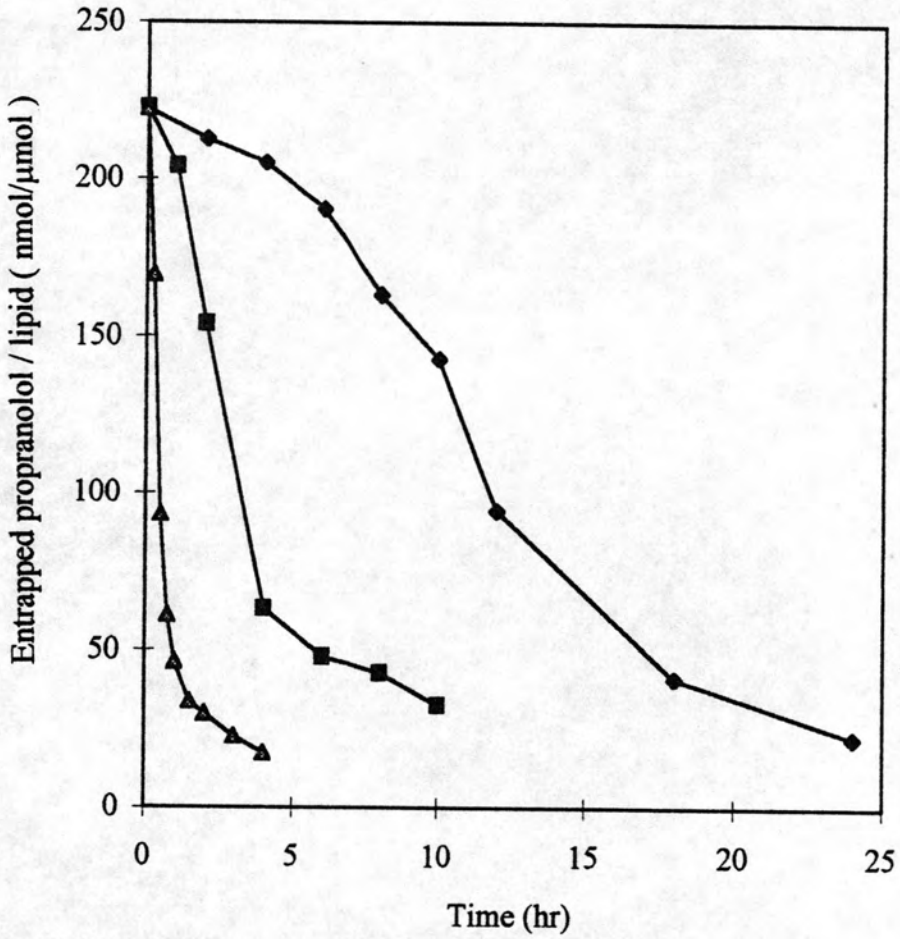


Figure 26. Retention of propranolol in EPC:Cpd I liposomes (2:1, by mol).

◆,10°C; ■,25°C; ▲,37°C

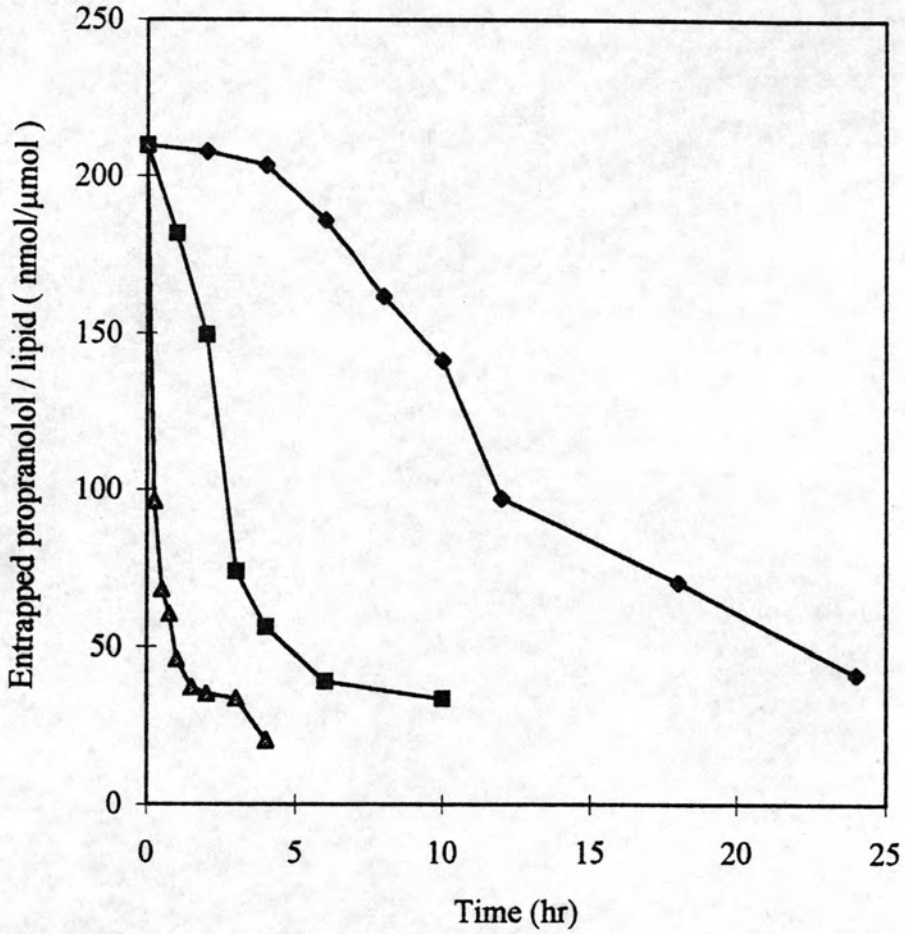


Figure 27. Retention of propranolol in EPC:Cpd II liposomes (2:1, by mol).

◆,10°C; ■,25°C; ▲,37°C

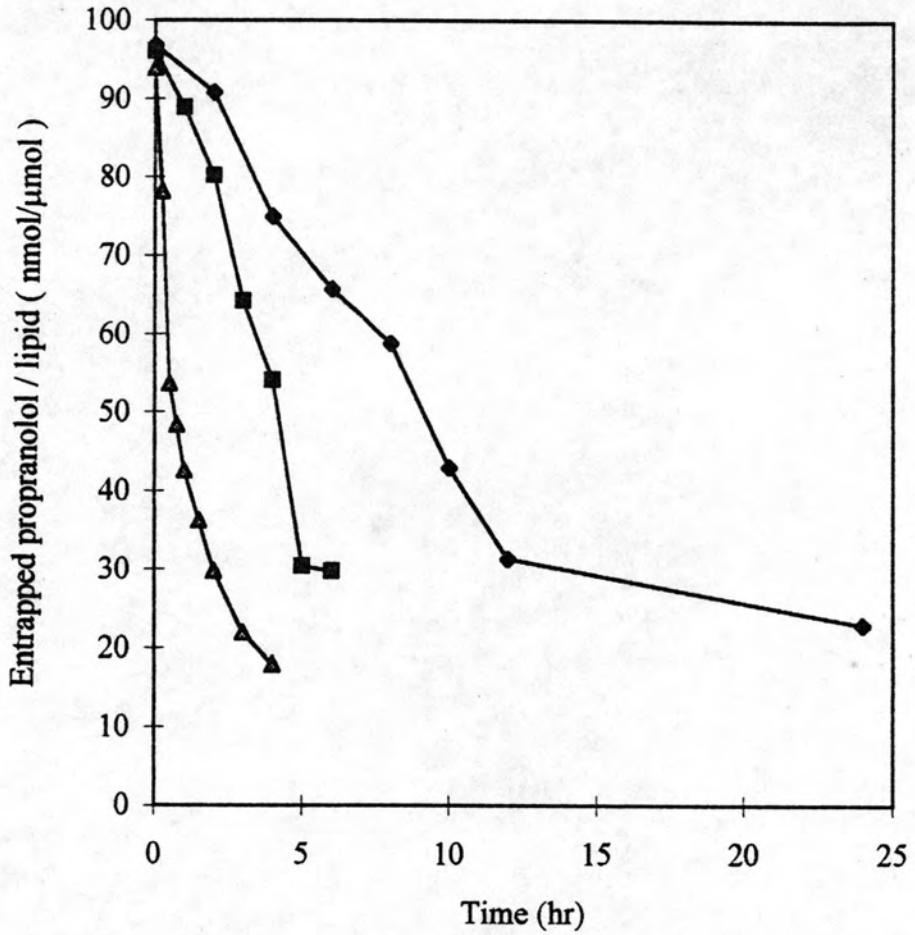


Figure 28. Retention of propranolol in EPC:Cpd III liposomes (2:1, by mol).

—◆—,10°C; —■—;25°C; —▲—,37°C

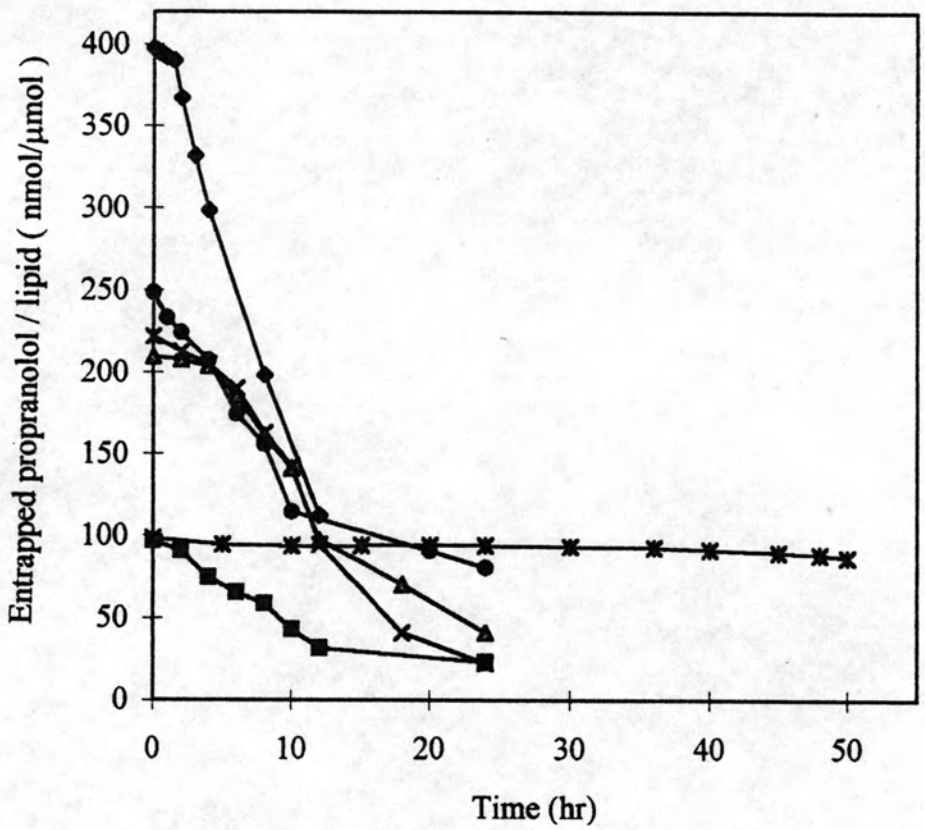


Figure 29. Retention of propranolol in liposomes at 10 °C.

All of liposome preparations were preprared in molar ratio.

—●— , EPC; —◆— , EPC:Chol:DCP (2:1:0.1);

—✱— , EPC:Chol (2:1); —✕— , EPC:Cpd I (2:1);

—▲— , EPC:Cpd II (2:1); —■— , EPC:Cpd III (2:1).

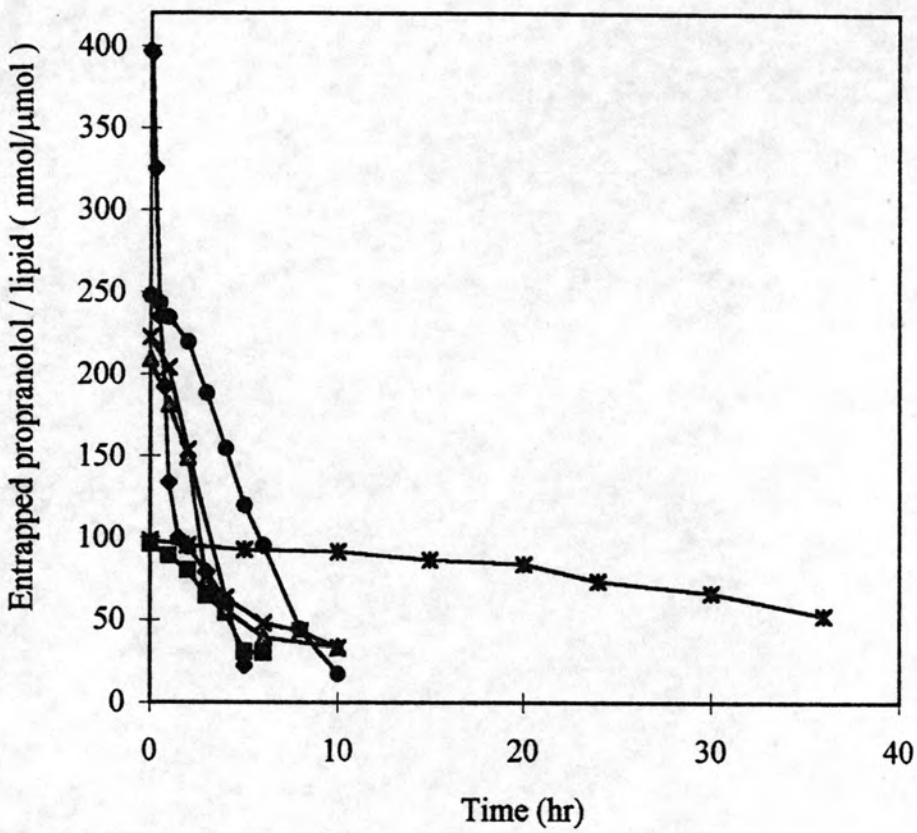


Figure 30. Retention of propranolol in liposomes at 25 °C.

All of liposome preparations were preprared in molar ratio.

—●— , EPC; —◆— , EPC:Chol:DCP (2:1:0.1);

—✱— , EPC:Chol (2:1); —✱— , EPC:Cpd I (2:1);

—▲— , EPC:Cpd II (2:1); —■— , EPC:Cpd III (2:1).

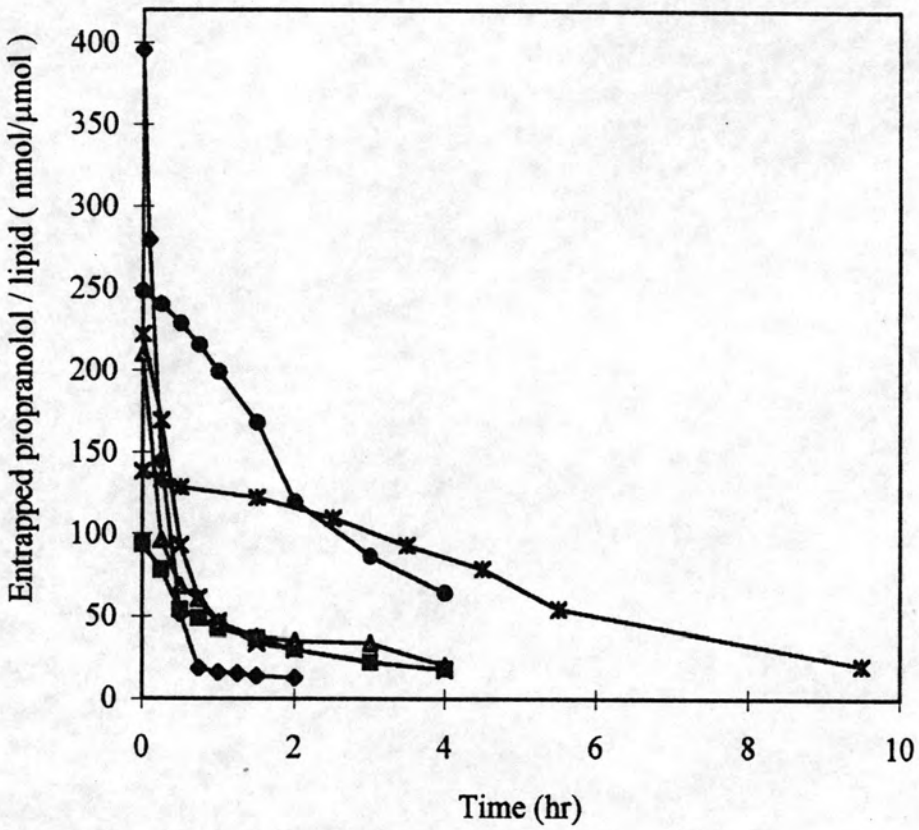


Figure 31. Retention of propranolol in liposomes at 37 °C.

All of liposome preparations were preprared in molar ratio.

—●— , EPC; —◆— , EPC:Chol:DCP (2:1:0.1);

—✱— , EPC:Chol (2:1); —✕— , EPC:Cpd I (2:1);

—▲— , EPC:Cpd II (2:1); —■— , EPC:Cpd III (2:1).