CHAPTER III

EXPERIMENTAL

Instruments

1. Refrigerated centrifuge (Hitachi Model CR20B3)
2. Spectrofluorometer (Jasco FP-777) connected with temperature controller (Eyela cool ACE CA 1100)
3. Ultraviolet-visible spectrophotometer (Jasco Model 7800)
4. pH meter (Orion Model 420 A)
5. Water bath shaker (Heto Model TB SH 02)

Materials

1. Phospholipids

1.1 Egg phosphatidylcholine (EPC)

EPC was isolated from hen egg yolks employing standard procedure [New, 1990] with purity greater than 99% as detected by thin layer chromatography.

1.2 Synthetic phospholipids

Dilauroylphosphatidylcholine (DLPC, C12:0), dimyristoyl-phosphatidylcholine (DMPC, C14:0), and distearoylphosphatidylcholine (DSPC C18:0) were purchased from Sigma Chemical Co., St.Louis, dipalmitoylphos-phatidylcholine (DPPC, C16:0) was from Nippon Oil & Fat Co., Ltd., Tokyo. Thin-layer chromatography of all lipids on silica gel in a chloroform : methanol

: water (65:25:4 v/v) with iodine vapor as a detecting agent showed a single spot, thus they were used without further purification.

2. Cholesterol and cholesterol analogues.

2.1 Cholesterol was obtained from Koso Chemical Co., Ltd., Tokyo., and purified by recrystallization from hot methanol for several times.

2.2 Cholesterol analogues

All of the cholesterol analogues used in this experiment were the gift from Dr. Usa Glagasigij (Department of Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, Chulalongkorn University). They were synthesized by the method of Patel et. al., (1985) and Glagasigij et al., (1988).

Hydroxyl cholesterol analogues having side chain of 1-hydroxy propyl, 1-hydroxy ethyl, 1-hydroxy-3,6-dioxa-octyl at the 3-position of the cholestene nucleus were named Cpd 0, Cpd I, Cpd II while amino cholesterol having side chain of 1,2-diamino-ethyl, 1,6-diamino-hexyl , N-(2 aminoethyl)-1,3-propanediamino and triethylenetretra-amino at the 3-position of the cholestene nucleus were named Cpd III, Cpd IV, Cpd V, and Cpd VI, respectively.

3. Charged amphiphiles

Dicetylphosphate (DCP) and stearylamine (SL) were purchased from Sigma chemical Co., St. Louis and used as negatively and positively charged amphiphiles, respectively.

4. Propranolol : 20 μmol/ml propranolol pH 7.5

Propranolol HCl was the gift from Department of Pharmaceutical Manufacturing, Faculty of Pharmaceutical Sciences, Chulalongkorn University and prepared by dissolving 59.16 mg propranolol HCl in buffer pH 7.5 to 10 ml.

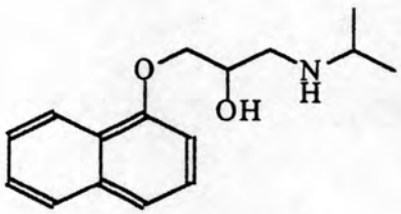

Figure 7. Chemical structure of propranolol

5. Citrate buffer : 300 mM citric acid pH 4.0

57.636 g of citric acid was dissolved in 1000 ml distilled water and adjusted to pH 4.0.

6. Hepes buffer : 20 mM Hepes , 150 mM NaCl, pH 7.5

4.766 g of Hepes (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) and 8.775 g of NaCl was dissolved in 1000 ml distilled water and adjust to pH 7.5

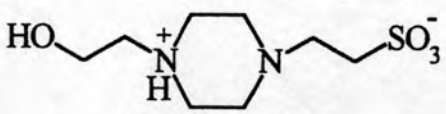

Figure 8. Chemical structures of N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid

7. Other reagents

Other reagents used in the experiments were of reagent grade or better.

Methods

1. Preparation of large unilamellar liposomes entrapped propranolol

Large unilamellar liposomes (LUVs) were prepared by reversed-phase evaporation (REVs) method [Duzgunes, 1983]. 10 μmol thin dried lipid film was prepared from egg yolk lecithin or synthetic phosphatidylcholine or its mixture with other components. Then, mixture of 2 ml chloroform and 1 ml of diethylether was added, followed by 1 ml of 300 mM citric acid pH 4.0 and the mixture was vigorously vortexed and followed by sonication in a bath-type sonicator. This procedure was repeated until good emulsion was obtained. The organic solvent was evaporated off under slighly reduced pressure until clear suspension was obtained. Traces of organic solvent was eliminated by continuing evaporation under highly reduced pressure for another 4 hr. All of the experiments were performed at temperature above the transition temperature of each phospholipid e.g., -15 ^{o}C for egg york lecitin, 0 ^{o}C for DLPC, 23 ^{o}C for DMPC, 41 ^{o}C for DPPC, and 58 ^{o}C for DSPC. Transmembrane pH gradient was established by titrating the exterior of vesicles to pH 7.5 or alternatively passed the vesicles over a Sephadex G-50 column equilibrated in 20 mM Hepes, 150 mM NaCl (pH 7.5). Samples were then diluted to concentration as indicated in the following experiment with buffer pH 7.5. Uptake of drug was performed by incubation of propranolol with liposome (0.25:1, by mol) at 25 °C for 30 min.

Then, liposomes were separated from untrapped drug by centrifugation ,1 ml aliquots of the incubation mixture , through 5 ml minicolumn of Sephadex G-50 (medium). Liposomes entrapped drugs were come out in the first fraction.

Phosphorus content of lipid was determined by method of Barlett. The amount of propranolol uptake was determined by fluorescence spectroscopy after lysis of vesicles with methanol, using excitation and emission wavelengths at 289.5 and 340 nm, respectively.

All of the following experiments were performed with five preparation of liposomes which each of these was repeated for five determinations. Data in table and figure showed mean $\pm$ S.D..

2. Influence of ΔpH on propranolol uptake

Effect of ΔpH was studied by varying pH inside and outside of EPC liposome mixture. For variation of inside pH, liposomes were preprared in 300 mM citric acid (with pH varying from 3.0 to 7.0) and then outside pH of liposomes was titrated to pH 7.5 by 0.2 M sodium carbonate solution. For variation of outside pH, liposomes were prepared in 300 mM citric acid pH 4.0 and titrated to desired pH (varying from pH 4.5 to 8.5). After that, liposomes were diluted to final concentration of 3 μmol/ml phospholipid and incubated with propranolol at drug-to-lipid ratio of 0.25:1, by mol, at 37 °C for 90 min. Then 1 ml of aliquot was passed over Sephadex G-50 minicolumn to separate untrapped drug.

3. Influence of interior buffering capacity on propranolol uptake

EPC liposomes were prepared in various concentrations of citric acid (10-400 mM) at pH 4.0. Then, liposomes (2 μmol/ml phospholipid) were incubated with propranolol at drug-to-lipid mole ratio 0.25:1 at 25 °C for 30 min after the liposomes external medium was brought to pH 7.5. Aliquot was passed through Sephadex G-50 minicolumn for separating of untrapped drug.

4. Influence of incubation temperature and time on propranolol uptake

EPC liposomes were prepared with 300 mM citric acid pH 4.0 inside and pH 7.5 outside vesicles. Liposomes (2 μmol/ml phospholipid) were incubated with a final propranolol concentration of 500 nmol/ml (propranolol : lipid 0.25:1, by mol) at temperature 10 °, 25 °, 37 ° and 40 °C. At various time up to 120 min , aliquot of the mixture was taken and untrapped drug was separated.

5. Influence of propranolol concentration on propranolol uptake

EPC liposomes were prepared with 300 mM citric acid pH 4.0 inside and pH 7.5 outside vesicles. Liposomes (2 μmol/ml phospholipid) were incubated at 25 °C for 30 min in presence of propranolol at the desired drug-to-lipid ratios (0.025:1 to 0.3:1, by mol). Aliquots of the mixture was taken and untrapped drug was separated. Trapping efficiency was calculated as follow:

$$\% \text{ Trapping efficiency} = \frac{\text{entrapped propranolol/lipid}}{\text{propranolol added/lipid}} \times 100$$

6. Influence of acyl chain length on propranolol uptake

Liposomes were prepared from synthetic phosphatidylcholine with different acyl chain length, e.g. C12:0 for DLPC, C14:0 for DMPC, C16:0 for DPPC and C18:0 for DSPC with 300 mM citric acid pH 4.0 inside and pH 7.5 outside vesicles. Liposomes (2 μmol/ml phospholipid) were incubated with propranolol at desired drug-to-lipid ratio e.g. 0.25:1 for DLPC, DMPC, DPPC and DSPC (with additional molar ratio of 0.45:1 for DSPC) at 25 °C for 30 min.

7. Influence of charged amphiphile on propranolol uptake

EPC liposomes were prepared from EPC, cholesterol and charged amphiphile in molar ratio of 2:1:0.1 with 300 mM citric acid pH 4.0 inside and pH 7.5 outside vesicles. Dicetylphosphate was used for provideing negative charge while stearylamine was a positive one. Liposomes (2 μmol/ml all lipid) were incubated in present of propranolol at drug to lipid molar ratio 0.25:1 (with additional molar ratio of 0.43 : 1 for DCP) at 25 ^{o}C for 30 min.

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

Liposomes were prepared from EPC and cholesterol or cholesterol analogues on molar ratio of 2:1 with 300 mM citric acid pH 4.0 inside and pH 7.5 outside vesicles. Liposomes (2 μmol/ml all lipid) were incubated in present of propranolol at drug to lipid molar ratio 0.25:1 at 25 ^{o}C for 30 min.

9. Integrity of liposomes entrapped propranolol

Stability of liposomes entrapped propranolol were investigated by storaging them at temperature (10 °, 25 ° and 37 °C). Leakage of propranolol from vesicles was measured by taking aliquot from liposome preparation at various time interval and passed through Sephadex G-50 minicolumn.