Effects of Phosphatidylserine and Phosphatidylethanolamine Content on Partitioning of Triflupromazine and Chlorpromazine between Phosphatidylcholine–Aminophospholipid Bilayer Vesicles and Water Studied by Second-Derivative Spectrophotometry

Shigehiko TAKEGAMI, Keisuke KITAMURA,* Tatsuya KITADE, Miwa TAKASHIMA, Mika ITO, Eiko NAKAGAWA, Midori SONE, Rie SUMITANI, and Yumiko YASUDA

Kyoto Pharmaceutical University; 5 Nakauchicho, Misasagi, Yamashina-ku, Kyoto 607–8414, Japan.
Received October 8, 2004; accepted October 21, 2004

To assess the affinity of psychotropic phenothiazine drugs, triflupromazine (TFZ) and chlorpromazine (CPZ), for the membranes of central nervous system and the other organs in the body, the partition coefficients (K_ps) of these drugs to phosphatidylcholine (PC)–phosphatidylserine (PS) and PC–phosphatidylethanolamine (PE) small and large unilamellar vesicles (SUV, LUV) were examined by a second-derivative spectrophotometric method, since PS is abundantly contained in the membranes of the central nervous system and PE is distributed widely in the membranes of the organs in the body. Size and preparation methods of the vesicles did not affect the K_p values at each aminophospholipid content suggesting that the partition of the phenothiazine drugs was not affected by the structural differences in the vesicles such as their curvature or asymmetric distribution of the phospholipids between the outer and inner layers of the bilayer membranes. However, the K_p values of both drugs increased remarkably according to the PS content in the bilayer membranes, *i.e.*, the K_p values for the vesicles of 30 mol% PS content were about 3 times of that for the vesicles of PC alone, while both K_p values slightly reduced with the increase in the content of PE in the bilayer membranes of PC–PE vesicles. The results indicate that both drugs have higher affinity for the PC–PS bilayer membranes than for the PC and PC–PE membranes, which can offer an evidence for the fact that TFZ and CPZ are predominantly distributed and accumulated in the brain and nerve cell membranes that contain PS abundantly.

Key words phenothiazine; partition coefficient; aminophospholipid; second-derivative spectrophotometry; liposome

Partition coefficients of drugs between lipid bilayer vesicles (liposomes) and water provide fundamental information relating to the drug interactions with biomembranes. Especially, information on the drug partitioning helps understanding the pharmacodynamics and pharmacokinetics of drugs, because most of drugs usually partition into cell membrane by passive diffusion. In the quantitative structure–activity relationship (QSAR) studies of drugs, it has been suggested that the partition coefficients obtained for the liposome system are more effective than those obtained for the *n*-octanol/water system.[1—3)]

Triflupromazine (TFZ) and chlorpromazine (CPZ) are known as major antipsychotropic drugs of the phenothiazine derivatives and exert their action by antagonizing neuronal D_2 receptor in the brain. Recently, CPZ has been shown to hold promise as a pharmacotherapeutic agent for prion-based afflictions.[4)] We previously reported the partition coefficients (K_ps) of some phenothiazine drugs including TFZ and CPZ for phosphatidylcholine (PC) small unilamellar vesicles (SUV) determined by second-derivative spectrophotometry.[5)] The derivative method has been recognized to eliminate the effect of background signals[6,7)] and usefully applied to the determination of the partition coefficients of drugs between lipid vesicles and water without the troublesome separation procedures[8—10)] that may disturb the equilibrium states. Using SUV and large unilamellar vesicles (LUV) prepared from PC and cholesterol, the effects of vesicle size and cholesterol content in the bilayer membranes on the K_p values of CPZ and TFZ were also studied by the second-derivative spectrophotometry.[11)]

A recent fluorescence study by Chen *et al.* provides that the hydrophobic nature of CPZ drives its general association with membranes, while the cationic nature of CPZ promotes its preferential association with phosphatidylserine (PS) in the bilayer membranes.[12)] Also, Elferink[13)] and Dachary-Prigent[14)] reported that CPZ bound preferentially to PC liposomes containing PS, compared to PC liposomes.

PS is contained abundantly in the brain and nerve cell membranes as compared to the other organs. While phosphatidylethanolamine (PE), an aminophospholipid as PS and the second major phospholipid component after PC, is distributed widely all over the organs in the body and concerned with the membrane fusion and permeability.

Therefore, the effects of these aminophospholipids, PS and PE, on the interactions of phenothiazine drugs with phospholipid bilayer vesicles should be investigated quantitatively, since the quantitative evaluation of the difference in the affinity of the drugs for PC, PS and PE will offer important information to understand their distribution and accumulation in the body.

In this study we examined the effects of aminophospholipid contents and vesicle size on the partitioning of TFZ and CPZ into the vesicles (SUV and LUV) by using second-derivative spectrophotometry.

Experimental

Calculation of Molar Partition Coefficients The molar partition coefficient (K_p) of phenothiazine between the vesicles and water is defined as,[5,15)]

$$K_p = \frac{([P_m]/[P_t])/[L]}{([P_W]/[P_t])/[W]} \quad (1)$$

where $[P_m]$ and $[P_W]$ represent the concentrations of phenothiazine in the vesicles and water, respectively, and $[P_t]=[P_m]+[P_W]$, and [L] and [W] are

* To whom correspondence should be addressed. e-mail: kitamura@mb.kyoto-phu.ac.jp

Table 1. Mean Diameter (nm) of the Several Kinds of Vesicles Determined by the DLS Method

Vesicle preparation method	Membrane pore size (nm)	Content of aminophospholipid						
		PS (mol%)			PE (mol%)			
		10	20	30	10	20	30	0[a]
Extrusion	50	72.8	74.8	63.1	76.5	82.0	77.5	64.7
	100	115.9	108.5	115.4	119.5	129.2	141.7	107.7
	200	186.8	186.4	174.4	210.1	225.2	231.0	184.8
Sonication		25.3	24.8	23.9	29.7	23.7	25.2	24.4

a) Refer to ref. 11.

molar concentrations of phospholipid in the vesicles and water (55.3 M at 37 °C), respectively.

As the background signal effect based on the vesicles can be eliminated in the second derivative spectra, the derivative intensity difference (ΔD) of phenothiazine before and after the addition of the vesicles measured at a specific wavelength is proportional to the concentration of phenothiazine in the vesicles, and thus we can get Eq. 2 from Eq. 1 as described in a previous paper,[5)]

$$\Delta D = \frac{K_P \Delta D_{max}[L]}{[W] + K_P[L]} \quad (2)$$

where ΔD_{max} is the maximum ΔD value assuming all phenothiazines are partitioned in the vesicles. The values of K_p and ΔD_{max} were calculated from the experimental values of [L] and ΔD (measured at 256.0 and 254.5 nm for TFZ and CPZ, respectively[11)]) by applying a non-linear least-squares calculation to Eq. 2.[5)]

Reagents TFZ hydrochloride and CPZ hydrochloride were purchased from Sigma and used without further purification. The buffer used was 50 mM NaCl–10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes buffer, pH 7.4). L-α-PC (egg yolk) of 99% purity was supplied as a 2% (w/v) chloroform solution from Avanti Polar-Lipids Inc. (U.S.A.) and L-α-PS (bovine brain) was obtained as a 2% (w/v) chloroform solution from Doosan Serdary Research Laboratories (Canada). L-α-PE was purchased from Nof Corporation (Japan) and prepared as a 2% (w/v) chloroform solution. All solutions were stored at −30 °C. The purity of PC, PS and PE was confirmed by thin-layer chromatography and it showed a single spot.

Vesicle Preparation Appropriate amounts of the PC and PS or PE stock solutions were mixed and evaporated by a rotary evaporator and a vacuum pump. Thereafter, 5 ml of the buffer were added so as to yield *ca.* 24 mM phospholipid concentration and the mixture was vortexed to produce multilamellar vesicles. Then the vesicles were prepared by the sonication or extrusion methods as previously reported.[5,11)] The content of PS and PE (mol%) in the phospholipid mixture was calculated as aminophospholipid/(PC+aminophospholipid)×100 using the volume of each stock solution withdrawn and each concentration.

Measurement of Mean Diameter of the Vesicles The size distribution of the vesicles was determined by a dynamic light scattering (DLS) method using a submicron particle analyzer (Nicomp Model 380, Particle Sizing Systems, Santa Barbara, CA, U.S.A.).[11)] In the sonicated SUV, it was confirmed to be that the diameters of more than 90% of the vesicles were in the range of 20—30 nm.

Phosphorus Determination The exact phospholipid concentration in the vesicle suspensions was calculated from phosphate analysis according to the phosphovanadomolybdate method.[16)]

Measurements of Absorption and Second Derivative Spectra The sample solutions containing 15 μM of TFZ or CPZ and various amounts of the vesicle suspension were prepared in a similar manner to that of the previous papers.[11,17,18)] The reference solutions were those prepared without the drug. Absorption and second derivative spectra were obtained by similar ways as in the previous papers.[11,17—19)]

Results and Discussion

Particle Size of PC–PS and PC–PE Vesicles The observed mean diameters of PC–PS or PC–PE vesicles prepared by sonication and extrusion methods are summarized in Table 1. The mean diameters of the vesicles prepared by the extrusion method showed the different tendency between PS and PE. The PC–PS vesicles had similar diameters to the corresponding PC vesicles, but the PC–PE vesicles showed larger mean diameter than the pore size of the membrane filters used in the extrusion method, and which increased markedly according to the increase of the PE content. This can be accounted for that since PE has a small and flexible polar head group, its intermolecular interaction with neighboring phospholipids in the bilayer membranes is weaker than that of PS,[20)] so that the PC–PE vesicles of which mean diameter is larger than the pore size of the membrane filter can pass through it.

Absorption and Second Derivative Spectra The absorption and second derivative spectra of TFZ in the sample solutions containing various amounts of PC–PS (30 mol% PS) LUV of diameter 200 nm at 37 °C are shown in Figs. 1a and b, respectively, as an example. The absorption maximum of TFZ in Fig. 1a exhibits a bathochromic shift according to the increase in phospholipid concentration indicating the partition of TFZ to the PC–PS bilayer of LUV.

The second derivative spectra in Fig. 1b calculated from the absorption spectra in Fig. 1a clearly show three derivative isosbestic points, confirming that the influences of the residual background signal of LUV observed in Fig. 1a are entirely eliminated in the second derivative spectra, and that TFZ exists in two states,[21)] *i.e.*, in the bulk water and the PC–PS bilayer of LUV. Similar results were obtained for CPZ and also for PC–PE vesicles.

By using the ΔD values obtained from these second derivative spectra, the K_p values of both drugs were calculated from Eq. 2 and summarized in Table 2. All of the K_p values were obtained with the R.S.D of below 10%, confirming a good precision of the second derivative method.

Effect of Particle Size on K_p Values In Table 2, either of the K_p values of TFZ and CPZ does not show obvious difference for the vesicle size ranging from 25 (sonicated SUV) to 200 nm in diameter at each lipid composition. Also the method of vesicle preparation, sonication or extrusion, does not affect the K_p values. These results are similar to the results of PC or PC–cholesterol vesicles previously reported.[11)]

It has been recognized that phospholipids are distributed asymmetrically across the bilayer of biological membranes.[22,23)] Using SUV and/or LUV, many previous studies investigated the asymmetric distribution of phospholipids in the bilayer membranes,[24—29)] and have reported that both PS and PE are predominantly distributed in the outer layer of the vesicles. Also, it has been theoretically predicted[30)] and confirmed that the asymmetric distribution of the aminophos-

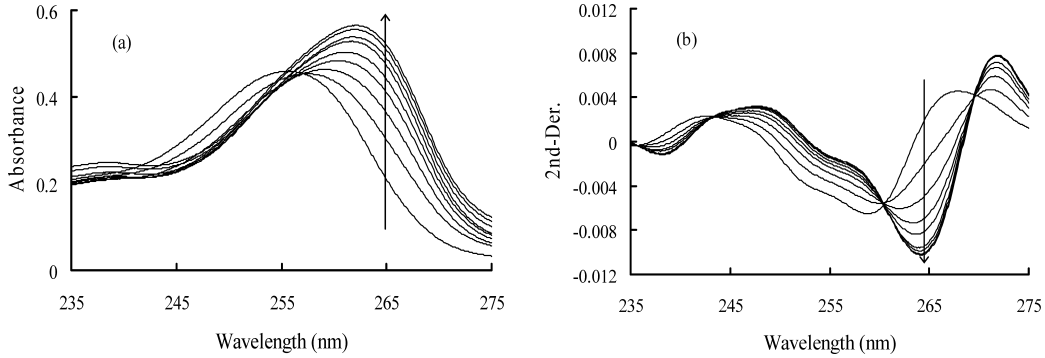

Fig. 1. Absorption (a) and Second Derivative Spectra (b) of 15 μM TFZ in Hepes Buffer (pH 7.4, 37 °C) Containing Varying Concentrations of PC–PS (30 mol% PS) LUV of Diameter 200 nm

PC–PS concentration: 0, 0.023, 0.046, 0.068, 0.091, 0.137, 0.182, 0.228, 0.273 mM (in the direction of the arrow).

Table 2. K_p Values of TFZ and CPZ for PC–PS or PC–PE Vesicles at Several Aminophospholipid Contents

Membrane pore size (nm)	K_p ($\times 10^{-5}$)[a]							
	TFZ				CPZ			
	PS content in PC–PS vesicles (mol%)							
	0[b]	10	20	30	0	10	20	30
50	4.32±0.12	5.21±0.23	8.47±0.36	12.64±1.02	2.90±0.20[b]	3.47±0.31	4.26±0.15	6.54±0.52
100	4.19±0.19	5.46±0.35	8.89±0.56	12.96±0.35	2.80±0.12[b]	3.60±0.24	5.33±0.43	6.03±0.17
200	3.66±0.19	5.75±0.53	6.90±0.56	11.67±0.72	2.78±0.05[b]	3.50±0.03	5.41±0.20	6.05±0.26
Sonication	4.36±0.32	5.62±0.29	7.18±0.68	10.07±0.95	2.93±0.11[c]	4.29±0.23	6.33±0.09	7.39±0.10
	PE content in PC–PE vesicles (mol%)							
		10	20	30		10	20	30
50		3.39±0.21	3.27±0.13	3.13±0.12		2.35±0.10	2.51±0.08	2.39±0.11
100		3.79±0.12	3.05±0.28	3.10±0.16		2.30±0.16	2.30±0.07	2.29±0.22
200		3.47±0.20	3.38±0.15	3.04±0.28		2.23±0.09	2.36±0.14	2.51±0.10
Sonication		3.72±0.21	3.33±0.13	3.19±0.23		3.05±0.30	2.63±0.09	2.58±0.07

a) Each value is expressed as the mean±S.D. (n=3). *b*) Refer to ref. 11. *c*) Refer to ref. 5.

pholipids increases with decreasing radius of curvature of vesicles,[29] *i.e.*, the distribution comes to be symmetric according to the increase of the vesicle size. The results in Table 2 reveal that the structural difference between SUV and LUV, *i.e.*, the difference in the distribution of aminophospholipids between the outer and inner layers of the vesicles, does not affect the partitioning of TFZ and CPZ.

Effect of Aminophospholipid Content on K_p Values In Fig. 2, the value of K_{pm}/K_{p0}, where K_{pm} shows a mean value of K_p values for the vesicles containing the same amount of PS (or PE), and K_{p0} is that of the K_p values for the PC vesicles, is plotted as a function of the aminophospholipid content. The results in Fig. 2 show that the K_{pm}/K_{p0} values of both TFZ and CPZ apparently increase according to the increase in the content of PS in the bilayer membranes of the vesicles. This proves that both drugs have higher affinity to PS than to PC, *e.g.*, at 30 mol% PS content both drugs have more than 2—3 times as high affinity as that for PC alone.

On the contrary, the K_{pm} values of both drugs slightly reduced with the increase in the content of PE in the bilayer membranes of PC–PE vesicles. At 30 mol% aminophospholipid contents, the K_{pm} values of TFZ and CPZ for PC–PS vesicles are 3.8 and 2.7 times as high as those for PC–PE

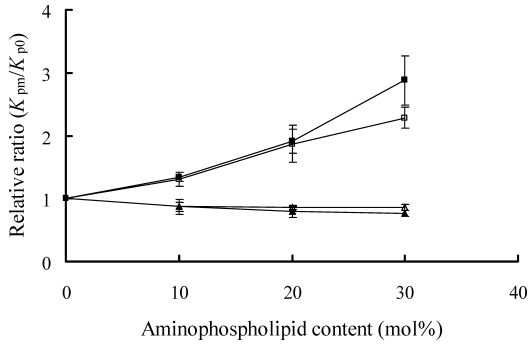

Fig. 2. Relative Ratio (K_{pm}/K_{p0}) of TFZ (closed) and CPZ (open) at Various PS (square) and PE (triangle) Contents

vesicles, respectively. Consequently, the affinity of TFZ and CPZ to phospholipid can be considered to be in the order of PS⩾PC≥PE. The results support the fact that TFZ and CPZ are predominantly distributed and accumulated in the brain and nerve cell membranes which contain PS abundantly.

These results can be accounted for as follows. At physiological pH of 7.4, both TFZ (pK_a=9.21) and CPZ (pK_a=9.35) are in their cationic forms.[6] On the other hand,

the net charge of PC is neutral, and that of PE is also neutral, as the pK_a value of amino group of PE is 9.6. While PS is an anionic (−1), since it has pK_a values of 3.6 and 9.8 for carboxyl and amino groups, respectively.[31] Accordingly, the surfaces of bilayer membranes of PC and PC–PE vesicles are neutral, however, that of PC–PS vesicles are negatively charged. Thus, the electrostatic interaction between the cationic TFZ and CPZ with the negatively charged surfaces of the PC–PS vesicles largely contributes for the partition of TFZ and CPZ than the neutral surface of the PC or PC–PE vesicles. This also accounts for the above result that the K_p values for the PC–PS vesicles do not depend on the PS distribution between the outer and inner layers of the vesicles. Consequently, it can be considered that for the partition of TFZ and CPZ to biomembranes their electrostatic interaction with biomembranes is also important as well as their lipophilicity.

As the psychotropic benzodiazepine drugs, diazepam and flurazepam, also have higher affinity for PS than PC,[32] it may be deduced that for the psychotropic drugs, a high affinity for PS is an important physicochemical nature to be distributed in the brain and central nervous system with a high concentration as compared to the other organs. Therefore, the partition coefficients of psychotropic drugs to PC–PS liposomes will be a good index of their affinity for biomembranes of the central nervous system.

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