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Dissociation constants of phenothiazine drugs incorporated in phosphatidylcholine bilayer of small unilamellar vesicles as determined by carbon-13 nuclear magnetic resonance spectrometric titration

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Abstract

The dissociation constants (pK_ms) of the phenothiazine drugs promazine, chlorpromazine, and triflupromazine, incorporated in the phosphatidylcholine (PC) bilayer of small unilamellar vesicles (SUV), were investigated by a ^{13}C nuclear magnetic resonance (NMR) titration method employing their *N*-$^{13}CH_3$ (ionizable group) labelled derivatives. Use of the labelled drugs enabled direct observations of the ionization equilibrium of the *N*-dimethyl group. A second derivative spectrophotometric study proved that 95–98% of the phenothiazine species in the sample solutions (200 μM phenothiazine in the presence of 27 mM PC SUV) were incorporated into the PC bilayer, which simplified the calculation of pK_m values by allowing that the phenothiazines in the aqueous phase could be neglected. The pK_m values were calculated from the chemical shift dependence of the *N*-dimethyl ^{13}C NMR signal on the pH value of sample solutions. The pK_m values obtained were smaller than those measured in aqueous solutions by about one unit. The existence of cholesterol (30 mol%) in the PC bilayer showed little effect on the pK_m values, suggesting that cholesterol in the bilayer does not largely affect the interfacial region where the *N*-dimethyl group of the incorporated phenothiazines is located. The results offered clear evidence for the pK_m decrease and provided their precise values.

Keywords: Dissociation constant; Phenothiazine; Phosphatidylcholine bilayer; ^{13}C NMR; Liposome; Cholesterol

1. Introduction

The affinity of a drug for biomembranes is related to the absorption, membrane transport, distribution, and accumulation of the drug in the body. The phospholipid bilayer is a fundamental structure of biomembranes, and the affinity of a drug for the phospholipid bilayer is an important index of its affinity for biomembranes. When a drug has ionizable group(s) in its structure, the affinity of the drug for biomembranes depends on its dissociation constant (p*K*), since the lipophilicity of the drug in the neutral state is usually far higher than that in the ionic state. Thus, the p*K* is a very important physicochemical property of drugs.

The p*K* values of local anesthetics decrease upon their incorporation into the phosphatidylcholine (PC) bilayer [1,2]. The interfacial p*K* shift (Δp*K*) was first obtained for procaine by a theoretical calculation using the partition coefficients of its ionic and neutral forms [3]. Subsequently, the Δp*K* values of several hydrophobic amine drugs were calculated similarly and reported [4,5]. The decrease in p*K* has been considered to be derived from a decrease in the dielectric constant of the interfacial regions between the surface of the PC bilayer membrane and bulk water, where the ionizable amino group of incorporated drugs is located [1,2,9]. The experimental determination of the Δp*K* value of tetracaine has been performed by electrometric titration [6], electron paramagnetic resonance (EPR) spectrometry of spin-labelled tetracaine [6,7], and ^{2}H nuclear magnetic resonance (NMR) [8]. In recent papers, the Δp*K* values (Δp*K* = − 1) of benzodiazepines [9,10] were determined spectrophotometrically.

The interfacial p*K* shift means that the ratio of ionic and neutral forms of a drug in the membrane is different from that calculated with the p*K* value measured in an aqueous

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solution. The interactions of ionic and neutral forms of a drug with biomembranes are different, and thus the determination of the interfacial p*K* shifts is very important [1,2].

In this study, we determined the p*K* values of the clinically widely used phenothiazine psychotropic drugs promazine, chlorpromazine, and trifluprazine, incorporated into the PC bilayer membranes of small unilamellar vesicles (SUV), by using a ^{13}C NMR titration method. The dissociation extent of the ionizable *N*-dimethyl group of the incorporated phenothiazines was directly observed by their ^{13}C NMR chemical shift change according to the pH value of the sample solution. The phenothiazine drugs used in this study were those in which the one methyl group in the ionizable *N*-dimethyl group was labelled by ^{13}C. Therefore, their *N*-dimethyl signals could be detected easily without the interference of the background signals of PC SUV even at a low drug concentration (200 μM), which we employed to avoid micelle formation of the drugs.

While ^{1}H NMR is far superior in sensitivity to ^{13}C NMR, large background signals arising from biological substrates interfere with observation of the ^{1}H NMR signals in the drugs of interest, particularly when the drug concentration is much lower relative to those of the substrates.

We also investigated the effect on the pK_m values of the existence of cholesterol in the PC bilayer.

The proposed ^{13}C NMR method is a more direct determination method than other methods mentioned above, so it can provide clear experimental evidence of the interfacial p*K* shift.

2. Materials and methods

2.1. Reagents

Promazine hydrochloride (PZ), chlorpromazine hydrochloride (CPZ), and trifluprazine hydrochloride (TFZ) were purchased from Sigma. [*N*-$^{13}CH_3$] promazine [*N*-([^{13}C] methyl)-*N*-methyl-10*H*-phenothiazine-10-propanamine] (^{13}C-PZ), [*N*-$^{13}CH_3$] chlorpromazine [2-chloro-*N*-([^{13}C] methyl)-*N*-methyl-10*H*-phenothiazine-10-propanamine] (^{13}C-CPZ), and [*N*-$^{13}CH_3$] trifluprazine [*N*-([^{13}C]methyl)-*N*-methyl-2-(trifluoromethyl)-10*H*-phenothiazine-10-propanamine] (^{13}C-TFZ) were synthesized and purified according to the method described in our previous report [11]. Egg yolk L-α-phosphatidylcholine was supplied as part of a 5% (w/v) chloroform solution from Avanti-Polar Lipid Inc. (USA) and stored at −30 °C. Cholesterol (Tokyo Kasei) was recrystallized from ethyl acetate and stored as a 5% (w/v) chloroform solution at −30 °C. The purity of PC and cholesterol was confirmed by thin-layer chromatography.

2.2. SUV preparation

An appropriate amount of the PC stock solution or a mixture of appropriate amounts of PC and cholesterol stock solutions was dried by using a rotary evaporator and then a vacuum pump. To the residue, 5 ml of a buffer of suitable pH was added to yield ca. 40 mM PC concentration. The mixture was vortexed to produce multilamellar vesicles. PC SUV suspensions were prepared by a sonication method described previously [12], and the size distribution of PC SUV measured by a dynamic light scattering method [13] showed that about 90% of the PC SUV had a diameter of 20–30 nm. The PC SUV suspensions were freshly prepared for each pH value to be examined. The PC concentration in PC SUV suspensions was calculated by phosphorus determination [14]. The buffers used for ^{13}C NMR titration experiments were 0.1 M KH_2PO_4–0.1 M NaOH for pH 5.5–7.9 and 0.1 M H_3BO_3–0.1 M NaOH for pH higher than 8.0. For the ultraviolet (UV) absorption measurements, a Hepes buffer containing 10 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid and 50 mM NaCl (pH 7.4) was used.

2.3. ^{13}C NMR experiments

To a 2-ml volumetric flask containing an amount (50 or 100 or 150 μl) of 8 mM ^{13}C-PZ or ^{13}C-CPZ or ^{13}C-TFZ aqueous stock solution, a suitable aliquot of a PC SUV suspension having a certain pH value to be examined was added to obtain a final drug concentration of 200 μM (or 400 or 600 μM) and a PC concentration of 27 mM. Then the same buffer as that used to prepare the PC SUV suspension was further added to volume. After shaking the flask for a short time, ca. 1 ml of the mixture in the flask was transferred to a 5 mm NMR tube, and a coaxial internal tube containing D_2O was carefully inserted into the NMR tube. ^{13}C NMR spectra were measured by a DEPT mode using a Varian XL-300 spectrometer at 75.429 MHz locked on D_2O contained in the internal tube. The number of FID accumulations to enhance the signal-to-noise ratio was 3000. The internal reference was the PC acyl chains terminal methyl signal. The probe temperature was 21–23 °C.

2.4. pH measurement

The pH value of sample solutions was measured directly in NMR tubes at 21–23 °C by using a pH meter (TP-95 Toko Kagaku Kenkyusho, Japan) with a combination electrode (CE 103C-SR, Toko Kagaku Kenkyusho). The pH value employed was the average of those values measured just before and after the NMR spectrum was obtained. The pH difference between before and after the measurement of NMR spectrum was within 0.02 pH units.

2.5. UV absorption and second derivative UV spectra measurements

The UV sample solutions containing 200 μM PZ, or CPZ, or TFZ and various amounts of PC SUV (0–27 mM) were prepared in a similar manner to that used in the NMR

sample preparation. The reference solutions were those prepared without the drug. The absorption spectra were measured using a spectrophotometer (Hitachi U-3210) connected to a personal computer (NEC PC-9801VX) via an RS-232C interface. The absorption spectra were measured against the reference solution using 1 mm light-path length cuvettes with a slit width of 1 nm and a wavelength interval of 0.1 nm at 21–23 °C. The second derivative spectra were calculated as previously reported [12].

2.6. Calculation of fraction of phenothiazines incorporated in PC SUV

The molar partition coefficient (K_p) of phenothiazine between the PC SUV and water was defined as

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

where $[P_m]$ and $[P_W]$ represent the concentrations of phenothiazine in the PC bilayer of SUV and water, respectively, and $[P_t]=[P_m]+[P_W]$, and $[L]$ and $[W]$ are molar concentrations of PC and water (55.6 M at 22 °C), respectively [12,13,15]. The fraction (α) of phenothiazine in the PC bilayer of SUV at a $[L]$ is derived from Eq. (1) and given as [12,13,15]:

$$\alpha = [P_m]/[P_t] = \frac{K_p[L]}{[W] + K_p[L]} \quad (2)$$

When the background signal effect due to the PC SUV is eliminated in the second derivative spectra, the derivative intensity difference (ΔD) of phenothiazine before and after the addition of PC SUV at a specific wavelength is proportional to the concentration of phenothiazine partitioned in the PC bilayer of SUV [12,13]. Then, as described in previous papers [12,13], Eq. (3) can be derived from Eq. (1) as follows:

$$\Delta D = \frac{K_p \Delta D_{max}[L]}{[W] + K_p[L]} \quad (3)$$

where ΔD_{max} is the ΔD value when all of the phenothiazine species in the sample solution are assumed to partition into the PC bilayer of SUV. The values of K_p and ΔD_{max} were calculated from the experimental $[L]$ and ΔD values by applying a nonlinear least-squares calculation to Eq. (3) [12,13].

2.7. Calculation of dissociation constants

In a sample solution containing a phenothiazine drug and PC SUV, a condition under which most of the phenothiazine species are incorporated into the PC bilayer of SUV may be attained by increasing the PC SUV concentration. If this condition is satisfied, the dissociation equilibrium of phenothiazine in the sample solution may be considered to be that of the phenothiazine incorporated into the PC bilayer membrane, and can be written as

$$BH_m^+ \leftrightarrows B_m + H^+ \quad (4)$$

where BH^+ and B represent protonated cationic and neutral forms of phenothiazine, respectively, and the subscript m denotes the incorporated state. Thus, the dissociation constant of the incorporated phenothiazine, K_m (pK_m), can be defined as

$$K_m = [B]_m[H^+]/[BH^+]_m \quad (5)$$

$$pK_m = -\log([B]_m[H^+]/[BH^+]_m) \quad (6)$$

where brackets represent the concentration of each species and $[H^+]$ is the hydrogen ion concentration in the vicinity of the PC SUV surface.

In these sample solutions, the *N*-dimethyl ^{13}C NMR signal of phenothiazine can be considered to be derived from the portion of the drug incorporated into the PC bilayer. Thus, the signal will not be influenced by the exchange between the free and incorporated states of the drug. Therefore, when the chemical shift of the *N*-dimethyl ^{13}C NMR signal from the incorporated BH^+ is represented as S_c and that of the incorporated B as S_n, and their populations are shown as P_c and P_n, respectively, and with the fact that the rate of the dissociation process is extremely high in the NMR time scale, the chemical shift of the *N*-dimethyl signal to be observed (S_o) can be expressed as

$$S_o = P_c S_c + P_n S_n \quad (7)$$

In that case, P_c and P_n are represented by $[BH^+]_m$ and $[B]_m$, respectively, as follows:

$$P_c = [BH^+]_m/([BH^+]_m + [B]_m) \quad (8)$$

and

$$P_n = [B]_m/([BH^+]_m + [B]_m) \quad (9)$$

Thus, S_o is given as

$$S_o = S_c[BH^+]_m/([BH^+]_m + [B]_m) + S_n[B]_m/([BH^+]_m + [B]_m). \quad (10)$$

From Eqs. (5) and (10), S_o is expressed as

$$S_o = ([H^+]S_c + K_m S_n)/([H^+] + K_m), \quad (11)$$

and then, using pH and pK_m,

$$S_o = (10^{-pH}S_c + 10^{-pK_m}S_n)/(10^{-pH} + 10^{-pK_m}). \quad (12)$$

Therefore, the values of pK_m, S_c, and S_n can be calculated from the experimental values of S_o and pH by applying a

nonlinear least-squares method (accompanying a Taylor expansion) to Eq. (12).

3. Results and discussion

3.1. Second derivative spectrophotometric results

As a typical example of the results obtained with the three phenothiazine drugs studied, Fig. 1(a) and (b) show the absorption and second derivative spectra, respectively, of 200 μM PZ in a buffer solution containing various amounts of PC SUV. The increase of PC SUV concentration in the sample solution caused a bathochromic shift and an intensity increase of the absorption maximum of PZ. However, no isosbestic point could be observed in the absorption spectra because of the incomplete baseline compensation due to the intense light scattering of PC SUV [13]. Meanwhile, the derivative spectra in Fig. 1(b) obtained from the absorption spectra in Fig. 1(a) clearly show three derivative isosbestic points at 242.3, 255.7, and 264.4 nm, confirming that the baseline correction is completely attained in these derivative spectra. Similar results were obtained for CPZ and for TFZ. Thus, calculation of the K_p values of the three phenothiazines was performed by using these derivative spectra.

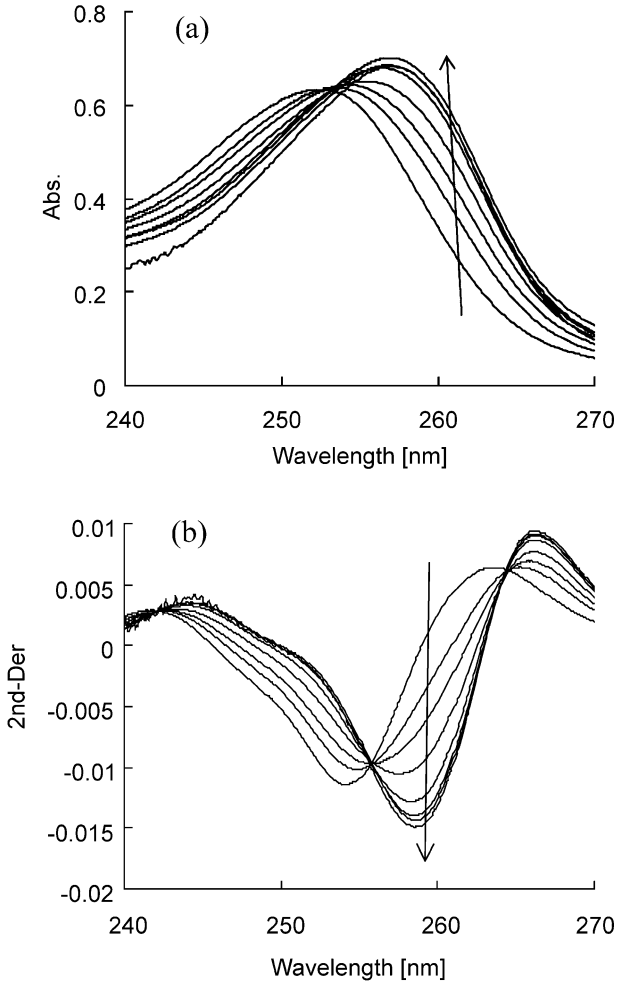

Fig. 1. Absorption (a) and second derivative spectra (b) of PZ (200 μM) in phosphate buffer (pH 7.4) containing various amounts of PC SUV at 23 °C. PC concentration: 0, 0.5, 1.0, 2.0, 4.0, 8.0, 16.0, 27.0 mM (in the direction of the arrow).

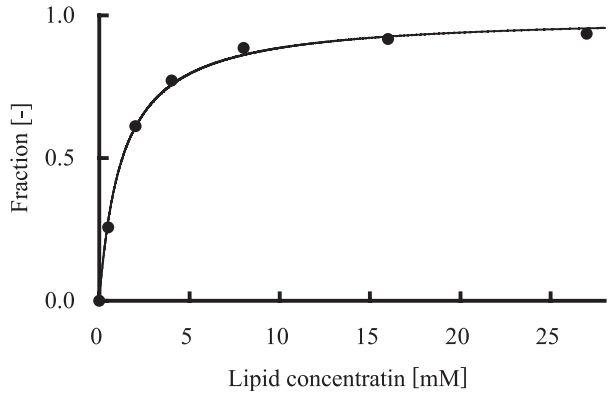

Fig. 2. Fraction (α) of PZ (200 μM) in PC SUV bilayer membranes as a function of PC concentration in phosphate buffer (pH 7.4) at 23 °C. The solid line shows the theoretical curve calculated from Eq. (2) using the obtained K_p value. The closed circles are experimental α ($\Delta D/\Delta D_{max}$) values.

The fraction of incorporated phenothiazine at a given PC SUV concentration [L] was calculated from Eq. (2) with the obtained K_p values, and a typical result for PZ ($K_p = 4.3 \times 10^4$) is shown as a curve in Fig. 2. The experimental

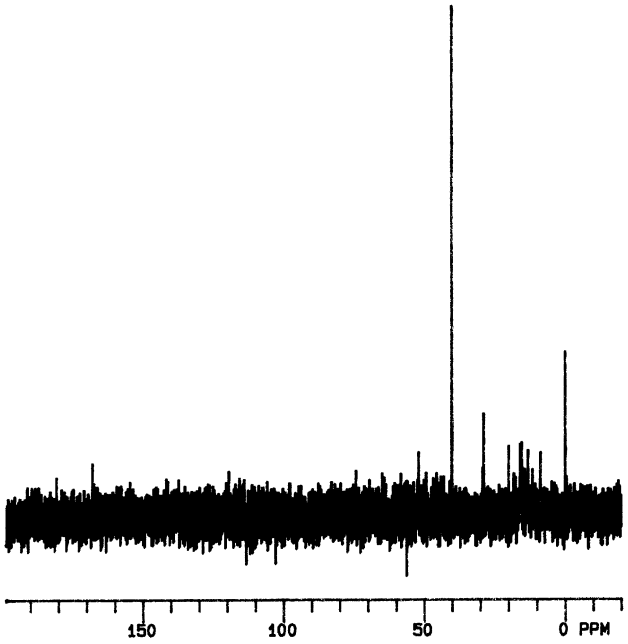

Fig. 3. ^{13}C NMR spectrum of 200 μM ^{13}C-PZ in phosphate buffer (pH 7.4) containing 27 mM PC SUV at 21 °C.

Table 1
Dissociation constants (pK_ms) of the phenothiazine drugs incorporated in PC SUV

Drug	Concentration (μM)	pK_m		pK
		PC	PC + cholesterol (30 mol%)	
PZ	200	8.59	8.53	
	400	8.59	8.53	
	600	8.56	8.66	
		8.58 ± 0.02[a]	8.57 ± 0.07[a]	9.40
CPZ	200	8.26	8.39	
	400	8.27	8.33	
	600	8.33	8.29	
		8.29 ± 0.04[a]	8.33 ± 0.05[a]	9.35
TFZ	200	8.37	8.25	
	400	8.26	8.20	
	600	8.32	8.22	
		8.32 ± 0.06[a]	8.22 ± 0.03[a]	9.21

pK represents the value obtained in aqueous solutions [17].
[a] Mean ± standard deviation.

values, $\alpha = \Delta D/\Delta D_{max}$, show good agreement with the theoretically calculated curve.

The above calculation showed that the fraction of PZ incorporated into PC SUV at 27 mM was 95%; that is, the fraction of free PZ in the bulk aqueous phase was 5%. The fractions of CPZ ($K_p = 12.5 \times 10^4$) and TFZ ($K_p = 13.2 \times 10^4$) in the bulk aqueous phases of the 27 mM PC SUV suspensions were calculated to be 2% for both.

These results confirmed that under the experimental condition of using 27 mM PC SUV suspensions, the contribution of each phenothiazine in the bulk aqueous phase could be neglected in the calculation of the pK_m values.

3.2. ^{13}C NMR titration results

Fig. 3 shows a typical ^{13}C NMR spectrum of a sample solution containing 200 μM ^{13}C-PZ and 27 mM PC SUV at pH 7.4. The signal at 0 ppm (internal reference) is the terminal methyl of acyl chains of PC constructing the SUV membranes. The signal at about 30 ppm is the *N*-dimethyl of the ^{13}C-PZ incorporated into the PC SUV membranes, and it showed an upper field shift according to the increase in pH value of the sample solutions. However, the signal did not reveal significant line broadening at any pH value employed. This proved that the exchange rate between the cationic and neutral forms of the incorporated ^{13}C-PZ was fast enough in the NMR time scale. The signal at 40.1 ppm is choline methyl of PC in the SUV membranes, and it did not show a shift change for any pH value employed.

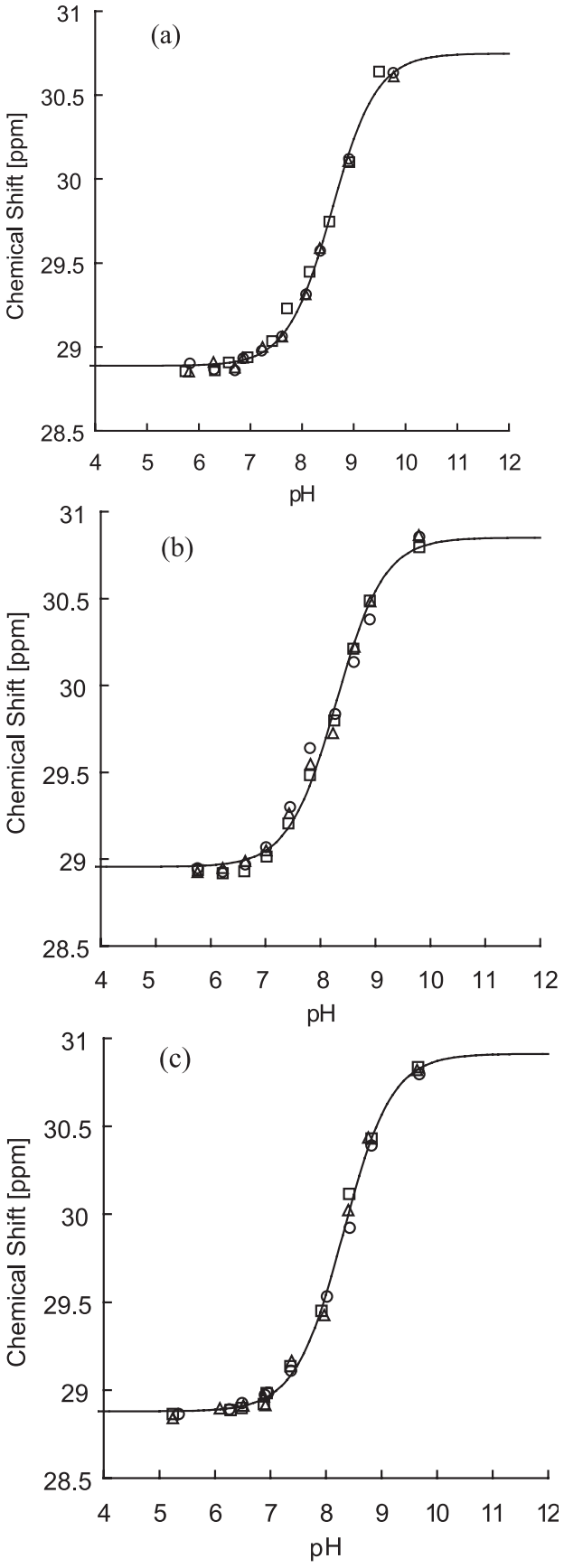

Fig. 4. Titration curves of phenothiazine drugs. (a) ^{13}C-PZ, (b) ^{13}C-CPZ, (c) ^{13}C-TFZ at 21–23 °C. The solid lines show the theoretical curves calculated from Eq. (12) using the obtained pK_m, S_c, and S_n values. The symbols indicate the experimental values obtained with drug concentrations of 200 μM (○), 400 μM (□), and 600 μM (△).

The chemical shift (S_o) of the *N*-dimethyl ^{13}C NMR signal of the ^{13}C-labelled phenothiazine drugs incorporated in the PC SUV membranes was measured at several pH values ranging from pH 5 to pH 10. Using the measured S_o and pH values, the pK_m values were calculated from Eq. (12) by a nonlinear least-squares method. In the calculation, proton concentration in the bulk aqueous phase was used for the proton concentration near the PC SUV surface, $[H^+]$. If there is significant electrical charge on the vesicle surface, $[H^+]$ will be different from the proton concentration in the bulk aqueous phase. However, the positive charge that could be formed on the surface by the incorporation of protonated phenothiazine drugs in this study was rather small, given that the amount of added phenothiazine was about 0.7–2% of that of the PC in the sample solution. With regard to Cl^- ions derived from NaCl, the binding of Cl^- to the PC SUV surfaces has been reported to be small [16]. Thus, we used the bulk proton concentration for $[H^+]$.

The pK_m values of the three phenothiazines were determined for three different concentrations (200, 400, and 600 μM) to confirm that neglecting the phenothiazine drugs in the aqueous phase was acceptable at drug concentrations higher than 200 μM. The results are summarized in Table 1. PZ, CPZ, and TFZ each showed similar pK_m values for the three different concentrations, confirming the validity of the assumption that under our experimental condition, the phenothiazine in the aqueous phase can be neglected in the calculation of pK_m.

Fig. 4 illustrates the ^{13}C NMR titration curves of these phenothiazine drugs. The solid lines represent theoretical calculations using Eq. (12) with the obtained pK_m, S_c, and S_n values. The plotted experimental values of S_o fall closely along the calculated curves, indicating that the obtained pK_m values were highly accurate.

The reported p*K* values of these three phenothiazine drugs measured in aqueous solutions [17] are also listed in Table 1. The results show that the incorporated phenothiazine drugs have p*K* decreases of about one p*K* unit from their values in aqueous solutions. These p*K* decreases are within the reported values (-0.4 to -1.5) for the drugs having an ionizable amino group [3–8].

3.3. Effect of cholesterol

The pK_m values for the PC SUV containing 30 mol% cholesterol were examined to see the effect of cholesterol on the p*K* shift of the phenothiazine drugs. The results listed in Table 1 show that the existence of 30 mol% cholesterol in the PC bilayer does not induce much difference in the pK_m values. The decrease of p*K* values of incorporated phenothiazines can be considered to be derived from a decrease in the dielectric constant of the region near the PC SUV surface where the *N*-dimethyl group of the phenothiazine drugs is located. Therefore, it can be deduced that the incorporation of cholesterol into the PC bilayer does not have much effect on the location of the *N*-dimethyl group of the incorporated phenothiazine and on the dielectric constant of this region.

In this study, we directly measured the change in dissociation constants of phenothiazine drugs induced by their incorporation into PC SUV bilayer membranes by observing the *N*-dimethyl ^{13}C NMR signal of the ^{13}C-labelled phenothiazine drugs. Thus, our results offer clear evidence of the p*K* shifts and provide accurate pK_m values of the three phenothiazine drugs.

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