Dissociation Constant of Chlorpromazine Incorporated in Lecithin Vesicles Determined by ^{1}H Nuclear Magnetic Resonance Titration

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The pK_a value of chlorpromazine incorporated in lecithin vesicles, denoted by pK_{a_v}, was determined by proton nuclear magnetic resonance (^{1}H-NMR) spectrometric titration in D_2O containing 50 mM NaCl and 2.5 mM $Na_2B_4O_7 \cdot 10H_2O$ at 25 °C for the drug and lipid concentrations of 1.76—3.5 mM and 20—67 mM, respectively. The pK_{a_v} value was calculated from the relation between the chemical shift of the *N*-dimethyl signal of the incorporated chlorpromazine and the bulk pH value using a nonlinear least-squares method. Six experiments gave the mean value of pK_{a_v} 8.48 ± 0.07 (S.D.). The value was significantly lower than pK_a 9.8 in D_2O, which was determined by extrapolating the pK_a's obtained in mixtures of methanol-d_4 and D_2O by the same ^{1}H-NMR titration method. The results indicated that about 10% of chlorpromazine incorporated in lecithin vesicles was in a deprotonated free base state at physiological pH 7.5, although it was considered to be mostly in a protonated cationic state in aqueous solution of the same pH.

Keywords chlorpromazine; dissociation constant; ^{1}H-NMR; lecithin vesicle; liposome; drug–membrane interaction

The pK_a value of chlorpromazine, a widely used tranquillizer, is reported to be 9.2—9.36 (25 °C).[1,2] This value indicates that most chlorpromazine in an aqueous solution is in a protonated cationic state (Chart 1) at the physiological pH of 7.5.

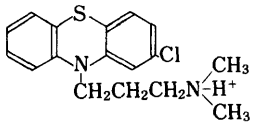

Chart 1. Chlorpromazine in Protonated Cationic State

We have so far studied the interaction of chlorpromazine with lecithin vesicles by using ^{1}H and ^{13}C nuclear magnetic resonance (^{1}H- and ^{13}C-NMR) spectroscopy and reported that the drug interacts with lecithin vesicles on the surface to displace metal cations, such as Mn^{+2}, Eu^{+3} and Pr^{+3}.[3,4] The ring current effect[5] and intermolecular nuclear Overhauser effect (NOE)[6] proved that the phenothiazine ring of chlorpromazine is located near the α-methylene moiety, *i.e.*, the first methylene from the ester group of the lecithin molecule. Thus, chlorpromazine incorporated in lecithin vesicles inserts its phenothiazine ring into the hydrophobic region constructed by acyl chains of lecithin and locates its *N*-dimethyl group in the polar region formed by the polar head group of lecithin.

These conditions of the incorporated chlorpromazine might affect the dissociation of a proton from its *N*-dimethyl group, and consequently the equilibrium between the protonated cationic state and the deprotonated free base would change from that predicted by the pK_a obtained in aqueous solutions.

In this work, we investigated the apparent pK_a value of chlorpromazine incorporated in lecithin vesicles by using ^{1}H-NMR spectrometry.

Experimental

Materials Chromatographically purified egg yolk lecithin was sonicated[3-5] at 20—67 mM concentration in D_2O containing 50 mM NaCl and 2.5 mM $Na_2B_4O_7 \cdot 10H_2O$ (see pH measurements). Sonication was performed in an ice-water bath under a nitrogen stream. Chlorpromazine hydrochloride purchased from Sigma Co. was recrystallized from chlorobenzene and dissolved in D_2O to make a stock solution. Several microliters of the stock solution was added to 1 ml of the lecithin vesicle D_2O solution with a microsyringe so that the chlorpromazine concentration in the solution was adjusted to be 1.76—3.5 mM.

^{1}H-NMR ^{1}H-NMR spectra were measured at 300 MHz locked on the D_2O signal with a Varian XL-300 NMR spectrometer. Probe temperature was 25 ± 1 °C. The signal of methylenes in the acyl chains of lecithin vesicles was used as an internal reference. For the measurements of chlorpromazine in mixtures of methanol-d_4 and D_2O, tetramethylsilane was used as the reference.

pH Measurements The pH of sample solutions was measured directly in NMR sample tubes by using a pH-meter (Orion SA520) with a combination electrode (CE-103, Toko Kagaku Kenkyusho) at 25 ± 1 °C. If the vesicle solution was prepared in D_2O containing NaCl alone, the pH value under alkaline conditions was decreased by *ca.* 0.05 unit from the previous value, whenever the measurement was repeated. The phenomenon would be caused by a small amount of water adsorbed on the outer surface of the electrode; since the internal diameter of the NMR sample tube was 4.2 mm and the outer diameter of the electrode was 3 mm, the solution had a large contact area with the outer surface of the electrode in comparison with the solution volume. By using the solution containing $Na_2B_4O_7 \cdot 10H_2O$ as described in Materials, the pH decrease was almost entirely suppressed owing to the buffer ability. The pH calibration was done by using standard buffer solutions. The pH of a sample solution was altered by adding a small amount of DCl or NaOD D_2O solutions to the sample solution with a glass capillary. The NMR spectrum was measured 1 h after the pH of the sample had been changed. The pH value employed was the average of those measured just before and after the NMR spectrum was taken. The difference of pH values between before and after the measurement of the NMR spectrum was within 0.02. The pH-meter readings were used without correction for isotope effects.

Ultraviolet (UV) Spectra The UV spectrum of 3.5 mM chlorpromazine in 40 mM lecithin vesicle D_2O solution containing 50 mM NaCl was measured with a 0.1 mm light-path length provided by a lead spacer sandwiched between a pair of quartzs plates (1 mm thickness). The reference solution was 40 mM lecithin vesicle D_2O solution containing 50 mM NaCl.

Calculation of pK_a Value Calculation of the pK_a value by a nonlinear least-squares method (BASIC program) was executed on a personal computer (NEC PC-9801F2).

Results and Discussion

The solubility of chlorpromazine free base (deprotonated at the *N*-dimethyl group in Chart 1) in water is very low; it was reported to be 8×10^{-6} M (25 °C).[1] Hence, when the pH of an aqueous solution containing a few milimolar chlorpromazine hydrochloride was raised to 7, the solution formed a white turbid suspension due to the formation of

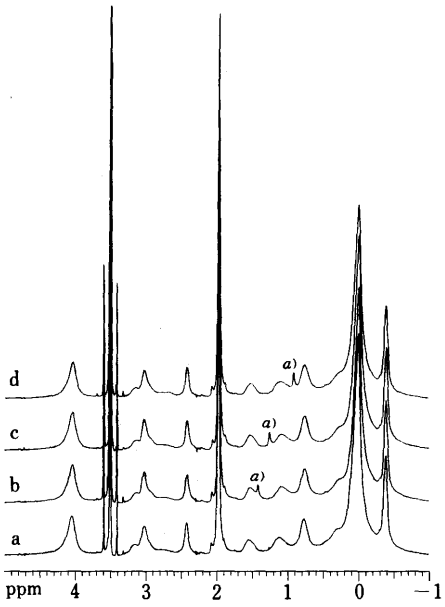

Fig. 1. ^{1}H-NMR Spectra of Chlorpromazine-Containing Lecithin Vesicle Solution at Several Bulk pH Values

Lecithin vesicle D_2O solution (40 mM) containing 50 mM NaCl and 2.5 mM $Na_2B_4O_7 \cdot 10H_2O$ (a). Chlorpromazine (3.5 mM)-containing vesicle solution, pH 7.77 (b); pH 8.29 (c); pH 9.98 (d). *a*) *N*-Dimethyl proton signal of chlorpromazine.

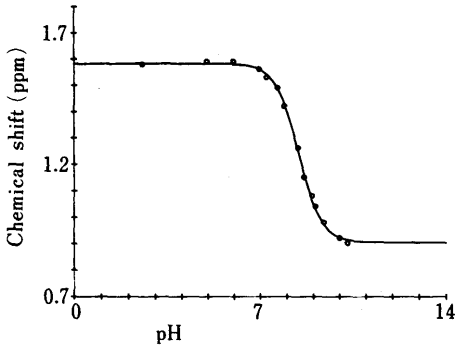

Fig. 2. NMR Titration of Chlorpromazine Incorporated in Lecithin Vesicles

The chemical shift of *N*-dimethyl protons of chlorpromazine (3.5 mM) in 40 mM lecithin vesicle D_2O solution containing 50 mM NaCl and 2.5 mM $Na_2B_4O_7 \cdot 10H_2O$ was plotted *versus* the bulk pH. The solid line is the best fit curve obtained by a nonlinear least-squares method.

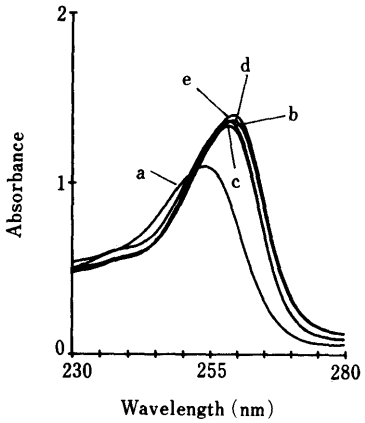

Fig. 3. UV Spectral Change of Chlorpromazine in Lecithin Vesicle Solution Induced by the Variation of Bulk pH Value

Chlorpromazine (3.5 mM) in D_2O containing 50 mM NaCl (a), in 40 mM lecithin vesicle D_2O solution containing 50 mM NaCl at pH 2.87 (b); pH 5.94 (b); pH 8.92 (c); pH 10.1 (e).

insoluble chlorpromazine free base, though the amount of free base formed at pH 7 was estimated to be only 1% or less of chlorpromazine in the solution. But, when a similar amount of chlorpromazine was dissolved in an aqueous solution containing a few percent of lecithin vesicles, no turbidity was observed at pH higher than 7. It could be considered that most of the chlorpromazine in the vesicle solution was incorporated into lecithin vesicles under these conditions. Therefore, we could measure the ^{1}H-NMR spectra of chlorpromazine-added lecithin vesicle D_2O solution at pH higher than 7 without the problem of the formation of insoluble free base in the aqueous phase.

Some of the ^{1}H-NMR spectra measured at various pH's are shown in Fig. 1. As previously reported,[3,5,6] the signals of chlorpromazine in a lecithin vesicle solution were greatly broadened due to the interaction with the vesicles, but the signal arising from the *N*-dimethyl group was still clearly detectable, as seen in Fig. 1 (denoted by *a*)). The *N*-dimethyl signal showed an upfield shift depending on the elevation of pH in the sample solution.

The elevation of pH in the solution caused an increase in the free base of chlorpromazine both incorporated in the lecithin bilayer and in the aqueous phase. It is well known that the chemical shift of the *N*-dimethyl signal in the deprotonated state is at higher field than that in the protonated state. Therefore, since the *N*-dimethyl signal appears at the averaged position of its four possible chemical shifts corresponding to the protonated and free base states of chlorpromazine incorporated and not incorporated in lecithin vesicles, the increase in the free base states owing to the elevation of pH in the solution caused the upfield shift of the *N*-dimethyl signal.

A typical plot of the chemical shift of the *N*-dimethyl signal of chlorpromazine in a vesicle D_2O solution *versus* its bulk pH is shown in Fig. 2.

In order to calculate the pK_a value of chlorpromazine incorporated in lecithin vesicles by using the titration data as shown in Fig. 2, it is necessary to estimate the fraction of chlorpromazine incorporated into lecithin vesicles or, alternatively not incorporated, *i.e.*, that exists in the aqueous phase.

The partition coefficient of chlorpromazine to lecithin vesicle was investigated by Welti *et al.*[7] by difference spectrophotometry at drug concentrations of 5—100 μM. With their partition coefficient obtained at the chlorpromazine concentration of 100 μM and pH 7.4, a calculation for 3.5 mM chlorpromazine in 40 mM lecithin vesicle solution suggested that 99% of chlorpromazine was incorporated into the vesicles at this pH. But, according to their results, an increase in chlorpromazine concentration from 10 to 100 μM decreases the partition coefficient from 4.4×10^5 to 1.9×10^5. If the relation could be extended to cover the increase in chlorpromazine concentration from 100 μM to 1 mM, and from 1 to 10 mM, the partition coefficient would be 0.82×10^5 for 1 mM chlorpromazine and would be 0.35×10^5 for 10 mM. Using the partition coefficient of 0.82×10^5, the incorporation of 3.5 mM chlorpromazine into the lecithin vesicles in the 40 mM solution was estimated to be 98%, and the corresponding results using 0.35×10^5 was 96%.

These calculations indicated that most of the chlorpromazine was incorporated into the vesicles in our experiments. The results of calculation were experimentally endorsed by the following observations.

The UV spectra of 3.5 mM chlorpromazine in 40 mM lecithin vesicle solution at several pH's were measured at 0.1 mm liquid thickness and are shown in Fig. 3.

It is known that the incorporation of chlorpromazine into lecithin vesicles induces a red shift in the absorption of chlorpromazine as compared to that in aqueous solution.[7,8] In Fig. 3, the absorption of chlorpromazine in lecithin vesicle solution showed a similar red shift at every pH.

The dissociation of chlorpromazine in water can be written as

$$BH^+ \rightleftarrows B + H^+$$

where BH^+ represents protonated cationic chlorpromazine and B represents the free base of chlorpromazine. Thus pK_a of chlorpromazine in water can be described as

$$pK_a = -\log([B][H^+]/[BH^+]) \quad (1)$$

where brackets represent the concentration of each species.

From Eq. 1, the concentration of protonated chlorpromazine $[BH^+]$ in the aqueous phase is given as

$$[BH^+] = 10^{pK_a - pH + \log[B]} \quad (2)$$

Under alkaline conditions, the free base in the aqueous phase will be saturated, so that [B] may be considered to be 8×10^{-6} M. Introducing 8×10^{-6} as [B] and the reported value of 9.3 for pK_a in Eq. 2, possible $[BH^+]$ in the aqueous phase is obtained as

$$[BH^+] = 10^{4.2 - pH}$$

The total concentration of chlorpromazine in the aqueous phase is,

$$[BH^+] + [B] = 10^{4.2 - pH} + 8 \times 10^{-6} \quad (3)$$

From Eq. 3, the amount of chlorpromazine that could exist in the aqueous phase at the highest pH of 10.1 in Fig. 3 was calculated to be 0.01 mM, which corresponds to about 0.3% of the added chlorpromazine (3.5 mM). As the sample solution did not show cloudiness due to the formation of water insoluble free base at pH 10.1, the rest of the chlorpromazine added was considered to be incorporated into lecithin vesicles at this pH. This was confirmed by the fact that the maximum absorbance at pH 10.1 did not decrease as compared with those at lower pH's in Fig. 3; if insoluble free base had been formed, the absorbance of chlorpromazine at pH 10.1 would have decreased.

Figure 3 also shows that the red shifts at pH's lower than 10.1 had similar values to that at pH 10.1. In the case of pH 2.78, where the red shift was smallest, it was roughly estimated to be still 80% of the maximum value at pH 10.1. Consequently, it could be assumed that the ^{1}H-NMR signal of the *N*-dimethyl protons of chlorpromazine in lecithin vesicle D_2O solution was mostly derived from that incorporated into lecithin vesicles at any pH value in our experiment. On the basis of this result, the calculation of pK_a of chlorpromazine incorporated into the lecithin bilayer was performed as follows.

When the chemical shift of the *N*-dimethyl signal from the incorporated BH^+ was represented as S_c and that of the incorporated B as S_n, and their populations were taken as P_c and P_n respectively, the chemical shift of the *N*-dimethyl signal to be observed (S_o) could be expressed as

$$S_o = P_c \cdot S_c + P_n \cdot S_n$$

Then, P_c and P_n were represented by the concentrations of incorporated BH^+ and B,

$$P_c = [BH^+]_v/([BH^+]_v + [B]_v),$$

$$P_n = [B]_v/([BH^+]_v + [B]_v)$$

where the subscript v denotes incorporation into vesicles. Thus, S_o is given by:

$$S_o = [BH^+]_v \cdot S_c/([BH^+]_v + [B]_v) + [B]_v \cdot S_n/([BH^+]_v + [B]_v) \quad (4)$$

On the other hand, the dissociation constant of incorporated chlorpromazine, K_{a_v}, and pK_{a_v} can be defined as

$$K_{a_v} = [H^+][B]_v/[BH^+]_v \quad (5)$$

$$pK_{a_v} = -\log([H^+][B]_v/[BH^+]_v)$$

where $[H^+]$ represents the proton concentration in the vicinity of the vesicle surface.

If there is a some electrical charge on the vesicle surface, the proton concentration near the vesicle surface would be different from that in the bulk. However, the positive charge on the surface that would be formed by the incorporation of protonated chlorpromazine is rather small since the amount of added chlorpromazine was about 3—9% of the amount of lecithin in the sample solution. As to Cl^- ion derived from NaCl, the binding of Cl^- to the vesicle surface was reported to be small.[9] Under these conditions, we used the bulk proton concentration for $[H^+]$.

From Eqs. 4 and 5, S_o is expressed as

$$S_o = ([H^+]S_c + K_{a_v}S_n)/([H^+] + K_{a_v})$$

using pH and pK_{a_v},

$$S_o = (10^{-pH} \cdot S_c + 10^{-pK_{a_v}} \cdot S_n)/(10^{-pH} + 10^{-pK_{a_v}}) \quad (6)$$

From Eq. 6, S_o could be regarded as a function of pH with the parameters of S_c, S_n and pK_{a_v}. Therefore, the values of S_c, S_n and pK_{a_v} could be determined by fitting the

Table I. Calculation Results for pK_{a_v}, S_c and S_n

No.	PC[a] (mM)	Chlorpromazine (mM)	n[b]	pK_{a_v}	S_c (ppm)	S_n (ppm)	S.D. for S_o[c] (ppm)
1	40	1.76	9	8.46	1.60	0.92	0.01
2	20	1.76	10	8.46	1.57	0.89	0.01
3	40	3.5	14	8.43	1.58	0.90	0.01
4	40	1.76	14	8.58	1.58	0.89	0.01
5	67	1.76	10	8.54	1.58	0.90	0.01
6	40	3.5	10	8.38	1.58	0.90	0.02
			Mean	8.48	1.58	0.90	
			S.D.	0.07	0.01	0.01	

a) Concentration of lecithin vesicles expressed as mM phosphatidylcholine in the sample solution. *b*) Number of data. *c*) Standard deviation of S_o between experimentally obtained and calculated values from Eq. 5 by using pK_{a_v}, S_n and S_c in each row.

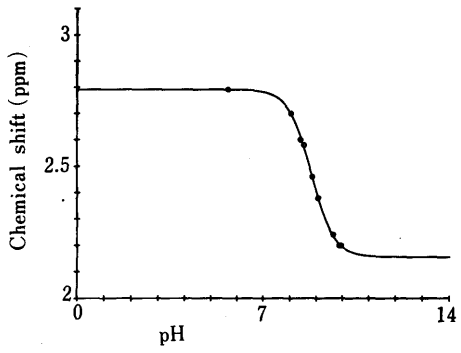

Fig. 4. NMR Titration of Chlorpromazine in a Mixture of Methanol-d_4 and D_2O

The chemical shift of *N*-dimethyl protons of chlorpromazine (1 mM) in a mixture of methanol-d_4 and D_2O (44.5 : 55.5 by weight) was plotted *versus* the bulk pH. The solid line is the best fit curve obtained by a nonlinear least-squares method.

TABLE II. pK_a Values of Chlorpromazine Determined in Mixtures of Methanol-d_4 and D_2O

Concentration of methanol-d_4 (%)	25.6	34.8	44.5	54.6
pK_a	9.22	9.09	8.80	8.59

experimental values of S_o and pH to Eq. 6 using a nonlinear least-squares method.[10] The condition for convergence in the iterative calculation of the nonlinear least-squares method was that the corrected values for each of the parameters (S_c, S_n, pK_{a_v}) at each iteration differed by less than 0.1% of their latest values.

The calculated results for the six experiments are summarized in Table I. The obtained values for each of pK_{a_v}, S_c and S_n showed good concurrence in all experiments. Most of the standard deviations for S_o between experimentally obtained and calculated values were within 0.01 ppm. The solid line in Fig. 2 was drawn by calculation using Eq. 6 and showed a good fit to the plotted data.

The obtained pK_{a_v} value of 8.5 is significantly lower than the pK_a values of 9.2—9.36 that have been measured in aqueous solutions.[1,2] To compare these values exactly, the isotope effect should be taken into account, *i.e.*, pK_a of chlorpromazine in D_2O should be measured.

As the solubility of chlorpromazine free base in D_2O is very low, the ordinary potentiometric titration in D_2O was not available for the determination of pK_a in D_2O. Further, the UV absorption spectrum of chlorpromazine did not vary between the protonated state and the free base. Therefore, we measured the pK_a of chlorpromazine in mixtures of D_2O and methanol-d_4 by the same ^{1}H-NMR titration method as in the case of vesicle solution. Typical titration results are plotted in Fig. 4. The values of pK_a in mixtures with various amounts of methanol-d_4 were determined and are listed in Table II. Extrapolation to zero methanol-d_4 concentration of the linear plot of pK_a *versus* methanol-d_4 concentration (data in Table II) gave the pK_a value of 9.8 for chlorpromazine in D_2O.

As it has been reported that a pH value in D_2O solution is higher than the corresponding pH value in H_2O solution by about 0.4,[11] the obtained pK_a value of 9.8 in D_2O is consistent with a value of 9.3 in H_2O.

Thus, the apparent pK_a value of chlorpromazine incorporated in lecithin vesicles is explicitly lower than the value in D_2O solution by more than one unit at 25 °C. Rooney and Lee,[12] to interpret the zeta potential of drug-binding liposomes theoretically, assumed a decrease in pK_a by 1.5 for chlorpromazine bound to lecithin liposomes in their microelectrophoretic study. We can offer a direct observation of the decrease in pK_a of chlorpromazine incorporated in lecithin vesicles.

The results indicated that about 10% of chlorpromazine incorporated in lecithin vesicles is in the deprotonated nonionic state at the physiological pH of 7.5 and 25 °C, though chlorpromazine in an aqueous solution under the same conditions is considered to be mostly in the protonated cationic state.

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