

Effects of Inorganic Ions on the Binding of Triflupromazine and Chlorpromazine to Bovine Serum Albumin Studied by Spectrometric Methods

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The effects of inorganic salts, NaCl, NaBr, NaI, Na₂SO₄, KCl, KBr, KI, on the binding constants (*K*s) of psychotropic phenothiazine drugs, triflupromazine (TFZ) and chlorpromazine, to bovine serum albumin (BSA) were examined by using second-derivative spectrophotometry. All of the salts examined, with the exception of Na₂SO₄, decreased the *K* values significantly, depending on the concentration of the salt, *e.g.*, the decrease in the *K* values of both drugs were about 40% for 0.1 M NaCl. The results obtained with Na₂SO₄ indicated that neither Na⁺ nor SO₄²⁻ had any effect on the binding of the phenothiazines to BSA. Based on the Na₂SO₄ results and the finding that the effect of each potassium salt on binding was quite similar to that of the corresponding sodium salt, the effects of these halogen salts can be considered to be derived from their anions, although the phenothiazines are positively charged at pH 7.4. The effectiveness of the anions was determined to occur in the following order: I⁻ ≫ Br⁻ > Cl⁻; these results coincided with the published order of the binding affinity of these anions to albumin. The ¹⁹F-NMR spectra of TFZ in the presence of each of these halogen salts revealed a concentration-dependent decrease in the intensity of the signal at 13.8 ppm that had previously been assigned to the TFZ bound to Site II. Consequently, the effects of these anions on the binding of positively charged phenothiazine drugs are thought to be local steric effects caused by the binding of these anions to Site II.

Key words drug-albumin binding; inorganic anions; second-derivative spectrophotometry; ¹⁹F-NMR; triflupromazine; chlorpromazine

Serum albumin is the most abundant protein (*ca.* 0.6 mM) in the blood, and it possesses strong ability to bind a large number of endogenous as well as exogenous substances. Thus, most of drugs administered are bound to serum albumin and are transported in the bound state in the circulating blood. It is also well known that the pharmacological activity of a drug is closely related to the free drug concentration in the blood. Therefore, the binding of drugs to serum albumin is pharmacokinetically and pharmacologically very important and has been extensively investigated.^{2,3)} However, less is known about the binding of cationic drugs to albumin, as it has been recognized that serum albumin binds both neutral and anionic drugs, whereas α₁-acid glycoprotein binds cationic drugs.^{2,3)}

However, we previously showed that chlorpromazine (CPZ) and triflupromazine (TFZ), both phenothiazine drugs that are widely prescribed psychotropic agents, bind to bovine serum albumin (BSA) with binding constants (*K* values) on the order of 10⁴ M⁻¹, despite the fact that these drugs are positively charged at the physiological pH of 7.4.⁴⁾ Furthermore, based on the results of an ¹⁹F-NMR study of TFZ which has a CF₃ group on the phenothiazine ring, we recently reported that TFZ exhibits two different types of binding to BSA and human serum albumin (HSA). One of these binding modes is specific to Sudlow's Site II, and the other is regarded as nonspecific binding.⁵⁾ Also, we demonstrated that the addition of 0.15 M NaCl displaced the bound TFZ from Site II.⁵⁾

Under physiological conditions, 0.15 M of Na⁺ and 0.1 M of Cl⁻ are present in the blood; therefore, it is very important to conduct quantitative investigations of the effects of inorganic ions on the binding constants of the phenothiazines to

albumin. In the present study, we used a second-derivative spectrophotometric method to investigate the effects of NaCl, NaBr, NaI, Na₂SO₄, KCl, KBr, and KI on the binding ability of BSA for TFZ and CPZ. The second-derivative spectrophotometric method used here has been applied for the determination of binding constants⁴⁾ and partition coefficients^{6–8)} of drugs to biological substances without requiring any separation procedures, since the derivative spectrophotometric methods^{9–12)} can eliminate the effects of background signals, enhance subtle spectral features, and allow the resolution of overlapped bands.

Furthermore, by using ¹⁹F-NMR spectroscopy, the effects of these salts on the binding of TFZ to BSA were investigated with respect to the binding site.

Experimental

Data Analysis If the effect of residual background signals is entirely eliminated in the derivative spectrum, the derivative intensity (*D*) of a phenothiazine drug in a sample solution at a specific wavelength is represented as follows:

$$D = E_b[C_b] + E_u[C_u],$$

where [C_b] and [C_u] represent the molar concentrations of the phenothiazine bound to BSA and unbound in the water phase, respectively, and E_b and E_u are their respective molar derivative intensities. With $E = E_b - E_u$ and $[C] = [C_b] + [C_u]$, *D* is written as

$$D = E_u[C] + E[C_b] \quad (1)$$

By rearranging Eq. 1, Δ*D* is introduced as

$$\begin{aligned} \Delta D &= D - E_u[C] \\ &= E[C_b] \end{aligned} \quad (2)$$

The Δ*D* represents the derivative intensity difference before and after the addition of BSA to a drug solution containing an inorganic salt, and its value is

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proportional to $[C_b]$

Thus, the fraction of a drug bound to BSA, α , can be given by

$$\alpha = \Delta D / \Delta D_{\max} \quad (3)$$

where $\Delta D_{\max} = E[C_u]$. The value of ΔD_{\max} will be obtained by extrapolating the total concentration of BSA added, $[P]$, to ∞ in the plot of $1/\Delta D$ versus $1/[P]$. Thus, the values of $[C_b]$ and $[C_u]$ are given by $\alpha[C_u]$ and $(1-\alpha)[C_u]$, respectively, and the number of moles of the drug bound per mole of BSA, r , is given by $\alpha[C_u]/[P]$. With these values, the interactions of the drug with BSA in the presence of each salt were analyzed using the Scatchard equation.

Calculation of the K Values For partition-like nonspecific binding, the binding constant, K , can be given as shown in Eq. 4.^{4,13)}

$$\frac{r}{[C_u]} = \frac{[C_b]}{[P][C_u]} = K \quad (4)$$

where $r = [C_b]/[P]$.

Using ΔD value, K can be written as follows:

$$K = \frac{(\Delta D / \Delta D_{\max})}{(1 - \Delta D / \Delta D_{\max})[P]} \quad (5)$$

From Eq. 5, we obtain

$$\Delta D = \frac{K[P]\Delta D_{\max}}{(1 + K[P])} \quad (6)$$

The K values were calculated by fitting the observed data regarding the ΔD value and the BSA concentration to Eq. 6 using a nonlinear least-squares method with a Taylor expansion (Visual Basic program).⁴⁾ In the calculation, the ΔD_{\max} value was also treated as a parameter, because the ΔD_{\max} values at hand had been obtained by the extrapolation.

Reagents Triflupromazine hydrochloride, chlorpromazine hydrochloride, and BSA (essentially fatty acid-free) were purchased from Sigma. Analytical reagent-grade sodium and potassium chloride, bromide, iodide, and sulphate (sodium only) were used. Filtered and then deionized tap water was distilled and used as a solvent to prepare the salt-containing sodium phosphate buffer (0.05 mol/l, pH 7.4). The pH of the salt-containing buffer was adjusted to 7.4 with H_3PO_4 or NaOH solutions at 37 °C. TFZ, CPZ, and BSA stock solutions were prepared by using the salt-containing buffer as a solvent. The concentration of BSA in a stock solution was determined by UV absorption using the absorptivity value $E_{1\text{cm}}^{1\%} = 6.67$ at 279 nm and the molecular weight of 66.4 kDa.

Second-Derivative Spectrophotometry To several 10-ml volumetric flasks, 4 ml of a salt-containing buffer and 1 ml of a 0.2 mM TFZ (CPZ) stock solution were added, such that the final drug concentration was 20 μM . Thereafter, a suitable aliquot of the BSA stock solution was added to each of the flasks, and the salt-containing buffer was added to the appropriate volume. The reference solutions were prepared as the same manner without the drug. Each flask was shaken for a short time and incubated at 37 °C for 30 min. The absorption spectrum (range: 230 to 310 nm) was then recorded in a 1-cm cuvette at 37 °C with a slit width of 2 nm and a wavelength interval of 0.1 nm using a double-beam spectrophotometer (Hitachi U-3210) equipped with a thermostatic cell holder. The spectrophotometer was connected to a personal computer (PC) through an RS-232C interface, and the spectral data were stored on the PC. Second-derivative spectra were calculated from the corresponding absorption spectra using a Visual Basic program based on the Savitzky-Golay method^{4,6-8)} with a cubic polynomial 17-point convolution employing a wavelength interval ($\Delta\lambda$) of 0.6 nm. The ΔD values of TFZ and CPZ were measured at 265 nm and 262 nm, respectively, as previously reported.⁴⁾

¹⁹F-NMR Measurements Sample solutions were prepared to contain 200 μM TFZ and 600 μM BSA by using 10% D_2O -phosphate buffer (pH 7.4) containing a suitable amount of one of the inorganic salts studied. To the sample solutions a small amount of 2,2,2-trifluoroethanol (TFE) was added for the ¹⁹F-NMR reference signal. The ¹⁹F-NMR spectra were measured by using a Varian ^{unity}Inova spectrometer locked on the D_2O signal of the sample solutions operating at 376.21 MHz without proton decoupling. The probe temperature was 30 °C. The number of FID accumulations used to improve the signal-to-noise ratio was 3000.

CD Measurements Twenty- μM BSA buffer solutions containing 0.15 M NaCl, NaBr, 0.15 M Na_2SO_4 and 0.01 M NaI, respectively, were prepared.

After the sample solutions were incubated at 37 °C in a constant temperature bath for 30 min, their CD spectra (range: 250 to 350 nm) were measured in a 1-cm cell at 37 °C with a scan speed of 20 nm/min and wavelength interval of 0.2 nm using a CD spectrophotometer (Nihon-Bunkou J-720WI) equipped with a thermostatic cell holder.

Results and Discussion

Absorption and Second-Derivative Spectra The absorption spectra of 20 μM TFZ with various amounts of BSA in the buffer solutions containing 0.15 M NaCl or 0.01 M NaI are depicted in Figs. 1a and b, respectively. Due to the strong light absorption of I^- , highly noisy spectra are observed in the short wavelength region in Fig. 1b. The absorption maxima in both Figures show bathochromic shifts according to the increase in the concentration of BSA, but the spectra in neither of the Figures exhibit distinct isosbestic points, due to the incomplete cancellation of the background signals caused by BSA.

The second-derivative spectra calculated based on the absorption spectra in Fig. 1 are depicted in Figs. 2a and b; these spectra clearly show derivative isosbestic points, which indicates that the residual background signal effects were entirely eliminated in the second-derivative spectra. This finding also confirmed that TFZ in the presence of the inorganic anions exists in two states that exhibit different derivative spectra, *i.e.*, the free state in the buffer phase, as well as the state of being bound to BSA.¹⁴⁾ Moreover, the derivative isosbestic points shown in Figs. 2a and b for NaCl and NaI, respec-

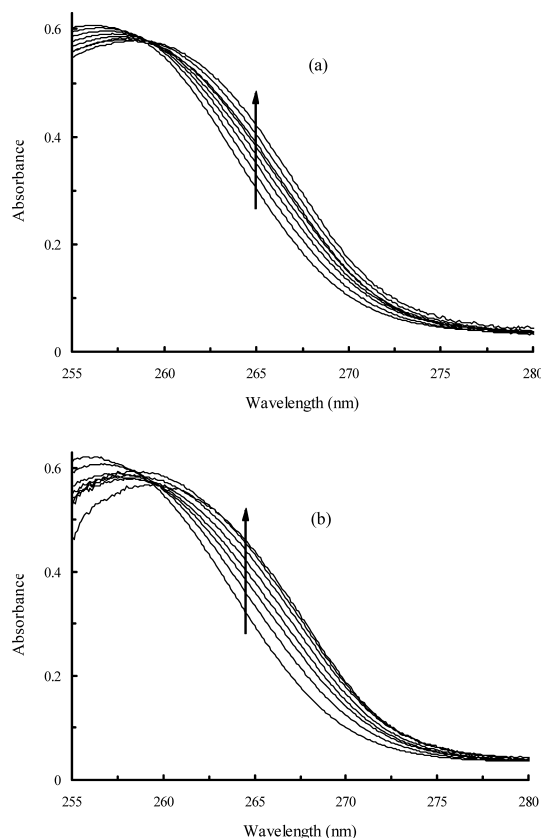


Fig. 1. Absorption Spectra of 20 μM TFZ in Phosphate Buffer Solutions (pH 7.4, 37 °C) Containing Various Amounts of BSA in the Presence of 0.15 M NaCl (a) and 0.01 M NaI (b)

BSA concentration: (a) 0, 6.6, 13.2, 19.8, 26.4, 33.0, 39.6, and 52.8 μM (in the direction of the arrow); (b) 0, 6.8, 13.6, 20.4, 27.2, 34.1, 40.1, and 54.5 μM (in the direction of the arrow).

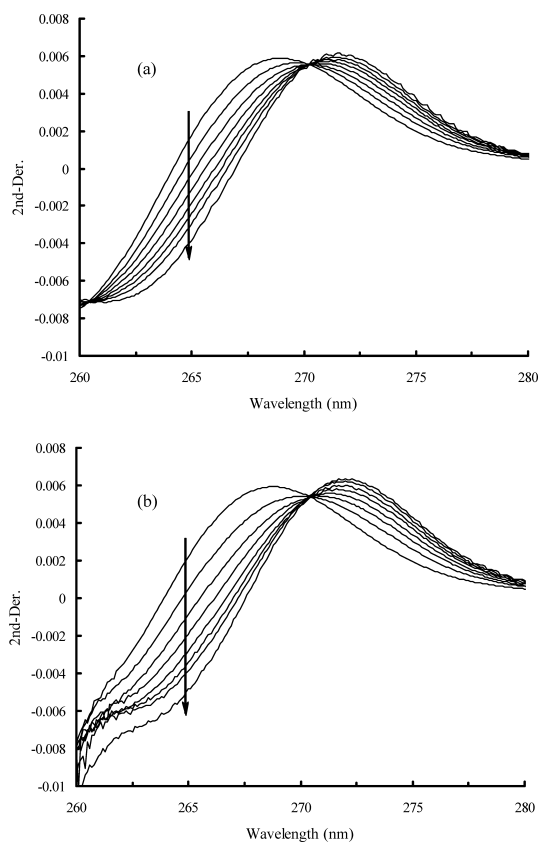


Fig. 2. Second-Derivative Spectra of TFZ Calculated Based on the Absorption Spectra in Figs. 1a and b

The curve labels are equivalent to those in Fig. 1.

tively, appeared at almost the same wavelength (270.1, 270.3 nm, respectively) and at the same intensity. All of the other salts examined here, including the potassium salts, gave the same results for TFZ. These results indicate that in the presence of any salt investigated, TFZ binds to BSA at the same region(s). Also, CPZ was found to exhibit the same tendency.

Scatchard Plot The ΔD values of TFZ and CPZ for each salt at each concentration were measured in each corresponding spectrum at 265 and 262 nm, respectively. Then, the ΔD_{\max} values were obtained by the extrapolation of the reciprocal plots of the ΔD values and the BSA concentrations, as described above. With the ΔD_{\max} values, the α values of TFZ in the presence of inorganic salts were calculated according to Eq. 3.

Using the obtained α values, the interactions of TFZ and CPZ with BSA in the presence of each inorganic salt were examined by Scatchard plot. The TFZ results for the sodium salts (the concentrations of which are indicated in the Figure legend) are depicted in Fig. 3, which shows a straight line parallel to the abscissa for each sodium salt studied. The remainder of the results for TFZ and all of the CPZ results showed features similar to those presented in Fig. 3.

It has been reported¹³⁾ that for partition-like nonspecific binding, the Scatchard plot gives a straight line parallel to the abscissa. The above results from the Scatchard analysis indicate that nonspecific binding can be adopted to account for the interactions of TFZ and CPZ with BSA in the presence of each inorganic salt examined. Our previous derivative spectrophotometric study⁴⁾ also showed that even without inor-

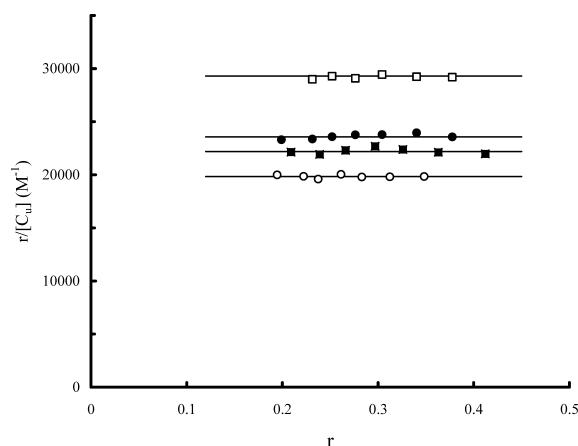


Fig. 3. Scatchard Plot for the Interactions of TFZ with BSA in the Presence of Different Anionic Contents

The symbols indicate the experimental values: (●) 0.15 M NaCl, (○) 0.15 M NaBr, (■) 0.01 M NaI, and (□) 0.15 M Na₂SO₄.

ganic salts, the phenothiazine binding to BSA could be treated as a partition-like nonspecific binding. On the contrary, our recent ¹⁹F-NMR results⁵⁾ revealed that the TFZ binding to BSA partially contains Site II binding, *i.e.*, the intensity of the TFZ signal relating to Site II binding was about one quarter of that of the signal relating to the nonspecifically binding TFZ. This can also be confirmed in Fig. 7a as the high- and low-field signals, respectively, in this study. As the contribution of Site II binding to the TFZ binding to BSA is small, the feature of specific binding was not reflected explicitly and, thus the TFZ binding to BSA appeared as a nonspecific binding in the derivative spectrophotometric study.

Consequently, the K values of TFZ and CPZ being obtained in this study should be considered to be apparent and overall binding constants.

Calculation of K Values The K values in the presence of each salt at several concentrations were calculated based on Eq. 6 with a nonlinear least-squares method. For each K value, three independent experiments were performed. All of the K values were obtained with precision (relative standard deviation, <8%).

To confirm the accuracy of the obtained K values, the fraction ($\alpha = \Delta D / \Delta D_{\max}$) of TFZ bound to BSA in the presence of NaCl (0.15 M), NaBr (0.15 M), NaI (0.01 M), or Na₂SO₄ (0.15 M) was calculated with the obtained K and ΔD_{\max} values at each experimental BSA concentration, as based on Eq. 6. The results are shown in Fig. 4 as four respective curves. All of the plotted experimental values fell close to calculated curves, thus demonstrating the reliability of the obtained K values. Similar results were obtained with the potassium salts, as well as in all of the CPZ experiments.

Effects of Inorganic Salts on K Values The effects of each inorganic salt on the K values of TFZ and CPZ are illustrated in Figs. 5 and 6 as a function of the respective sodium and potassium salt concentrations. Figures 5 and 6 reveal that both sodium and potassium salts of Cl⁻, Br⁻, and I⁻ led to decreases in the K values, depending on the salt concentration. However, the trial with Na₂SO₄ did not reveal any significant effect on the K values of either drug, as shown in Fig. 5. This finding indicates that neither the ionic strength of the sample solution, nor the Na⁺ cation or the SO₄²⁻ anion,

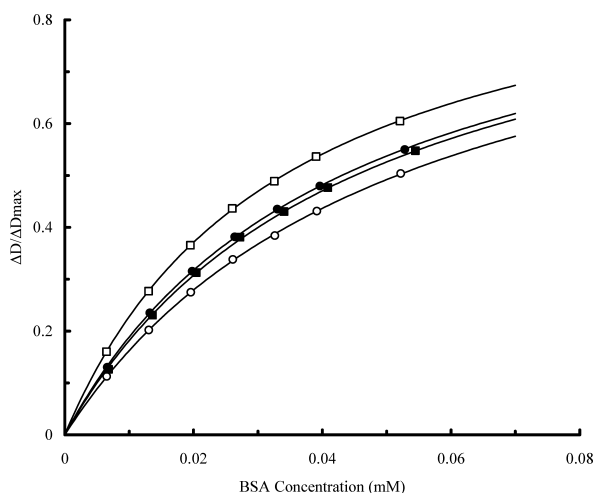


Fig. 4. Fraction of TFZ Binding to BSA in the Presence of Different Anionic Contents

The solid lines indicate the theoretical curves calculated using Eq. 6 with the obtained K and ΔD_{\max} values. The symbols indicate the experimental values: (●) 0.15 M NaCl, (○) 0.15 M NaBr, (■) 0.01 M NaI, and (□) 0.15 M Na_2SO_4 .

exerted any effect on the K values of the phenothiazines. That the ionic strength does not affect the K value suggests that the role of electrostatic interaction in the binding of phenothiazine to BSA can be considered to be small. Also previous reports^{14,15} that Na^+ and SO_4^{2-} do not bind to albumin reinforce our results for Na_2SO_4 . Note that the Na^+ cation was excluded as a candidate for binding inhibitor, and the results in Fig. 6 demonstrated that each potassium salt of these halogen anions exhibited a reductive effect on the K values that was quantitatively similar to that of the corresponding sodium salt in Fig. 5; hence, the inhibitory effects of the halogen salts on the binding of TFZ and CPZ to BSA should be considered to have been caused by the Cl^- , Br^- , and I^- anions, respectively, although the phenothiazine drugs were positively charged at pH 7.4.

Figures 5 and 6 also demonstrate the following order of effectiveness of anions in terms of inhibiting TFZ and CPZ binding to BSA: $\text{I}^- \gg \text{Br}^- > \text{Cl}^- \gg \text{SO}_4^{2-}$. It has previously been reported that Cl^- ,^{15–17} Br^- ,^{15,18} and I^- ^{15,19} anions bind to albumin at pH 7.4, and the order of binding ability of these anions to albumin has been shown to be $\text{I}^- \gg \text{Br}^- > \text{Cl}^- \gg \text{SO}_4^{2-}$.¹⁵ Furthermore, this order of effectiveness of the halogen anions was also shown to be correlated with ionic size. Therefore, it is reasonable to conclude that the effects of the Cl^- , Br^- and I^- ions on the reduction of the K values of the phenothiazines were the result of the binding of each of these anions to BSA.

On the other hand, the circular dichroism (CD) spectra (not shown) of 20- μM BSA buffer solutions containing 0.15 M NaCl, NaBr, 0.15 M Na_2SO_4 , or 0.01 M NaI did not show any detectable changes, as compared with the spectra obtained with a BSA buffer solution lacking these salts. Therefore, the binding of these anions is not expected to induce the conformational change in BSA required to reduce K values.

As mentioned above, our previous ¹⁹F-NMR study⁵ revealed that 0.15 M NaCl (Cl^-) completely inhibits the binding of TFZ to Site II. As TFZ and CPZ are positively charged at pH 7.4, the effects of Cl^- , Br^- , and I^- ions on the reduction

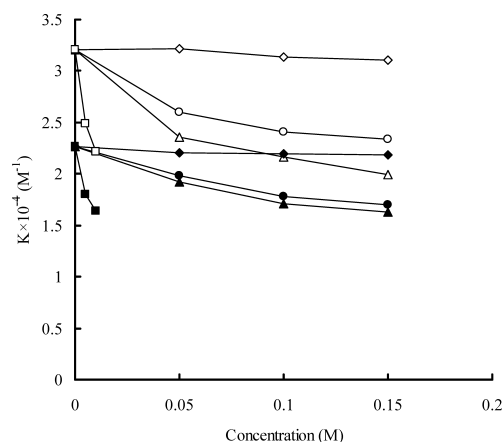


Fig. 5. Effect of Sodium Salts on the K Values of TFZ (Open Symbols) and CPZ (Closed Symbols) to BSA at 37 °C and pH 7.4

The sodium salts added were: (◇) Na_2SO_4 , (○) NaCl, (△) NaBr, and (□) NaI.

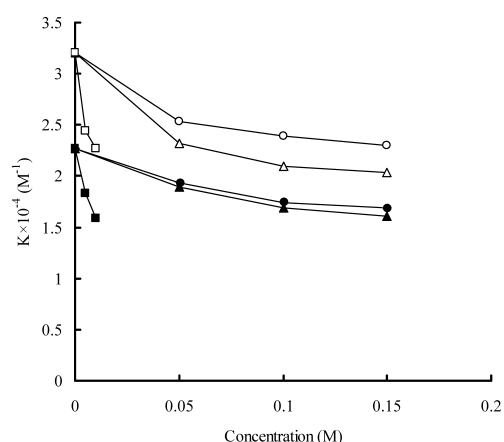


Fig. 6. Effect of Potassium Salts on the K Values of TFZ (Open Symbols) and CPZ (Closed Symbols) to BSA at 37 °C and pH 7.4

The potassium salts added were: (○) KCl, (△) KBr, and (□) KI.

in the K values of the phenothiazines could not be attributed to the binding competition between the phenothiazines and these anions at Site II (domain IIIA). The binding of these anions may lead to some local steric effects on TFZ binding in domain IIIA.

¹⁹F-NMR Spectra To obtain more detailed information regarding the effects of the halogen anions, an ¹⁹F-NMR study of TFZ binding to BSA was performed using various anion concentrations; the results are summarized in Fig. 7, which shows a CF_3 -associated signal change in TFZ in the BSA buffer solution caused by increasing the NaCl (a–d), NaBr (e–g), and NaI (h–i) concentrations in a step-wise manner. As mentioned above, the larger low-field signal in Fig. 7a measured without NaCl has been assigned to the signal arising from the TFZ nonspecifically bound to BSA, and the high-field small signal to a signal from TFZ bound to Site II.⁵ Of course, both signals would include a contribution from non-binding, free TFZ. Figure 7b and c show the results obtained at concentrations of 0.05 and 0.10 M NaCl, respectively, revealing a NaCl concentration-dependent decrease in the high-field signal intensity as well as the dissociation of TFZ from Site II caused by Cl^- binding. Also, a small up-field shift (*ca.* 0.15 ppm) of the low-field signal observed in Figs. 7a–d confirmed an increase of free fraction of TFZ ac-

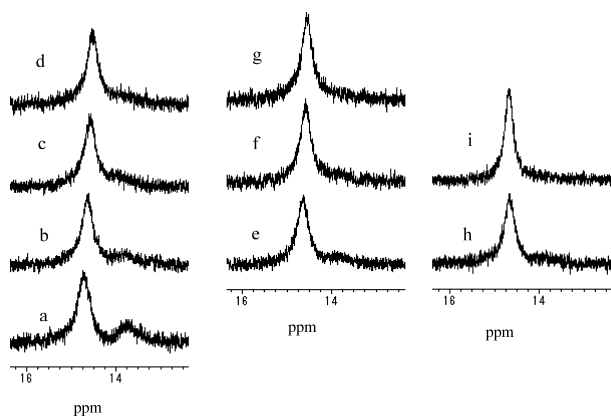


Fig. 7. The ^{19}F -NMR Spectra of TFZ in Phosphate Buffer Containing Various Amounts of NaCl (a—d), NaBr (e—g), and NaI (h—i)

Concentrations (M): (a) 0, (b) 0.05, (c) 0.10, (d) 0.15, (e) 0.05, (f) 0.10, (g) 0.15, (h) 0.01, and (i) 0.10.

ording to NaCl concentration increase. At a concentration of 0.15 M NaCl, the high-field signal entirely disappeared (Fig. 7d). However, Figs. 7c and d also demonstrate that an increase in the NaCl concentration from 0.10 to 0.15 M did not bring about a substantial difference between the two spectra. This finding coincides with the K value decrease determined by derivative spectrophotometry, which revealed that an increase in the NaCl concentration from 0.10 to 0.15 M induced only a slight decrease in the K value. These results are of interest, since they indicate that a slight Cl^- anion concentration change under conditions close to physiological conditions will not lead to a significant change in the free phenothiazine drug concentration.

The ^{19}F -NMR experiments for NaBr produced similar results to those obtained with NaCl, as seen in Figs. 7e—g. However, the results obtained with NaBr showed a slightly stronger effect than that of NaCl, and these results also corresponded to the K values obtained according to the derivative method, as shown in Fig. 5.

Figure 7h indicates the substantial effectiveness of I^- at inhibiting TFZ binding. Namely, in the presence of 0.01 M NaI, the high-field signal (*i.e.*, Site II-bound TFZ) disappeared, which was similar to the results shown in Fig. 7c and f, where 0.10 M NaCl and NaBr, respectively, were used at tenfold the NaI concentration. These findings also correspond to the decrease in K values observed with 0.01 M NaI, and those observed with 0.10 M NaCl and NaBr, as depicted in Fig. 5.

Although the signal of Site II-bound TFZ decreased and then disappeared in accord with increases in the concentration of halogen anions, the low-field signal of nonspecifically bound TFZ reflected small change in terms of shift and linewidth, as compared with the results shown in Fig. 7a, which were obtained in the absence of halogen anions. Therefore, it can be concluded that these halogen anions primarily displaced Site II-bound TFZ. The results thus corre-

spond to those in Figs. 5 and 6 showing no further significant decreases in the K values of TFZ and CPZ with concentration increases of NaCl (or NaBr) exceeding 0.1 M.

Figure 7i shows the effects of 0.10 M I^- , which was tenfold the concentration of 0.01 M used to obtain the results shown in Fig. 7h. However, there was little difference between the results shown in Figs. 7h and 7i, thus confirming that I^- did not exert a substantial effect on the low-field broadened signal that had been considered to be reflective of non-specific binding. Unfortunately due to the strong absorption of I^- in the ultraviolet region, the K value at 0.10 M I^- could not be obtained by the derivative spectrophotometric method.

In conclusion, the present study was the first to demonstrate that among the abundantly existing endogenous inorganic ions (Cl^- , Na^+ , and K^+) in the blood, negatively charged Cl^- inhibits the binding of positively charged phenothiazines to albumin by binding to subdomain IIIA, and in turn decreasing K values by approximately 40% at a physiological Cl^- concentration of 0.1 M.

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References and Notes

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