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INTERACTION BETWEEN DRUGS AND WATER-SOLUBLE POLYMERS. VII. BINDING OF BERBERINE WITH BOVINE SERUM ALBUMIN

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ABSTRACT

The interaction between berberine chloride (BC) and bovine serum albumin (BSA) was studied by equilibrium dialysis. Since the number (n) of binding sites per mole of BSA was large, the binding was nonspecific. Then the binding capacity (nK) was evaluated instead of the binding constant (K). According to the dependence of the NK value on ionic strength, the interaction was due to hydrophilic binding. Intermolecular interaction of BC was observed by the concentration (1–10 mmol/L) dependence of ^{1}H-NMR parameters; that is, the respective signals shifted to a higher magnetic field, the spin-lattice relaxation time (T_1) decreased, and the spin–spin relaxation rate (T_2^{-1}) increased. The interaction of BC with BSA (1.45×10^{-4} mol/L) resulted in a decrease in T_1, an increase in T_2^{-1}, and little variation of the chemical shift. On the basis of the ratio of the spin–spin relaxation rate of bound BC to free BC (T_{2b}^{-1}/T_{2f}^{-1}), the binding position of BC to BSA was considered to be spread over the entire BC molecule because of the rigid ring structure of BC even if the binding was nonspecific and hydrophilic.

INTRODUCTION

In a series of studies concerning the interaction between drugs and water-soluble polymers, the bindings of phenylbutazone (PB) [1], ibuprofen (IB) [2], azathioprine (AZ) [3], and indomethacin (ID) [4] to bovine serum albumin (BSA) were ascribed to hydrophobic binding. Furthermore, the binding positions of drugs to BSA were elucidated by means of the NMR spin–spin relaxation rate (T_2^{-1}): (1) PB, IB, and ID bound to BSA through the phenyl group; (2) AZ bound to BSA through the methylimidazole group.

In the present paper the protein binding of a hydrophilic antidiarrheal drug, berberine chloride (BC), was investigated by means of equilibrium dialysis and NMR relaxation time. The binding of BC to BSA was nonspecific and hydrophilic. However, the binding position of BC to BSA was not only to the hydrophilic part but also to the hydrophobic part because of a rigid ring structure. Furthermore, interaction among BC molecules was observed by NMR.

EXPERIMENTAL

Materials

BC was of special reagent grade from Tokyo Kasei and used without further purification. BSA with a molecular weight of 6.9×10^4 was from Wako Pure Chemical Industries. Other reagents were commercially available and used without further purification.

Equilibrium Dialysis

The procedure of equilibrium dialysis was described previously [4]. Temperatures were regulated within 0.2°C during all experiments. The drug concentration was determined by absorbance on a Shimadzu UV-190 spectrometer connected to an Iwatsu VOAC-7513 digital multimeter and an NEC PC-9801E microcomputer. λ_{max} in nm at pH 7 (ϵ) for BC was 344 (26,700).

NMR Measurements

The NMR spectra were measured in deuterium oxide (D_2O, phosphate buffer, 0.1 mol/L, pH 7) on a JEOL GX-400 spectrometer (radio frequency, 400 MHz; $\pi/2$ pulse, 11.1 μs) at 40.0 ± 0.5°C. The spin–lattice relaxation time (T_1) was obtained by the inversion recovery method (Eq. 1) [5]:

$$\ln(M_0 - M_t) = -t/T_1 + \ln(2M_0) \quad (1)$$

where t is the interval between π and $\pi/2$ pulses, and M_0 and M_t represent equilibrium magnetization at $t = 0$ and macroscopic magnetization at t, respectively. The spin–spin relaxation time (T_2) was determined according to the CPMG method (Eq. 2) [6]:

$$\ln(M_t) = \ln(M_0) - t/T_2 \quad (2)$$

where t is the time when a free induction decay (FID) is observed after irradiation of $\pi/2$ pulse, and M_t is the intensity of a spin echo at t. The pulse delay time (20 seconds), when the next pulse was applied after observation of FID, had to be longer than the relaxation time T_1 by a factor of 5 or above. Homo-gated irradiation technique was used to depress the HDO peak in D_2O.

RESULTS AND DISCUSSION

The Binding Constant of BC to BSA

The binding of BC to BSA was examined by equilibrium dialysis at 20–40°C. The free drug concentration (Df) was determined from the residual drug concentration, and the number of moles of the drug binding to 1 mol BSA (r) was estimated from the decrease in drug concentration. The plot of $1/Df$ vs $1/r$ produced a linear relationship, as shown in Fig. 1, and satisfied Eq. (3) proposed by Klotz [7]:

$$\frac{1}{r} = \frac{1}{nKDf} + \frac{1}{n} \tag{3}$$

where n is the number of binding sites per mole of BSA and K is the binding constant between BC and BSA. The values of n and K can be calculated from the intercept and slope of the straight line. However, the n-value was numerically large and could not be estimated accurately because the intercept was nearly zero, although Nahar et al. [8] reported an apparent n value ($n = 32$) for the BC–BSA system. This system was considered to have nonspecific binding. Takagishi et al. [9] evaluated the magnitude of binding in terms of the nK-value (binding capacity) rather than the K-value estimated from the uncertain n-value. Then the nK values were estimated in our system as shown in Table 1.

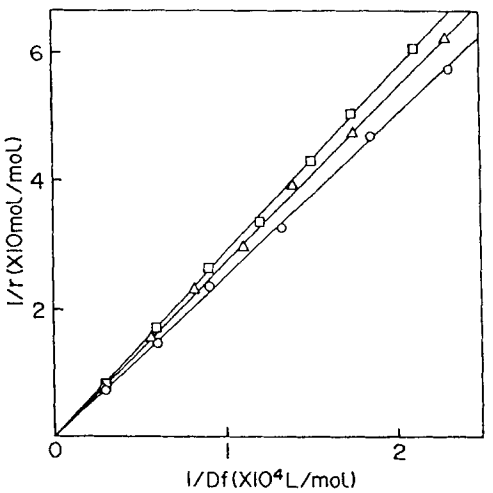


FIG. 1. Klotz plots for the binding of BC to BSA (1.45×10^{-4} mol/L) in 0.1 mol/L phosphate buffer (pH 7) at 20°C (△), 30°C (○), and 40°C (□).

TABLE 1. Binding Capacity (nK) for the Interaction of BC with BSA[a]

Temperature, °C	$nK \times 10^2$ L/mol
20	3.98
30	3.69
40	3.50

[a][BSA] = 1.45×10^{-4} mol/L, [phosphate buffer] = 0.1 mol/L, pH 7.

Assuming that the number of binding sites (n) was independent of temperature, the standard increase of enthalpy ($\Delta H° = -4.58$ kJ/mol) was estimated from the slope of the linear relationship ($r^2 = 0.99$) between ln nK and the reciprocal absolute temperature ($1/T$). It is believed that the binding of BC to BSA was advantageous for enthalpy because of the negative value of $\Delta H°$. The nK-value decreased with the increase of ionic strength (Table 2). From this fact, the binding of BC to BSA was ascribed to hydrophilic binding.

Intermolecular Interaction of BC

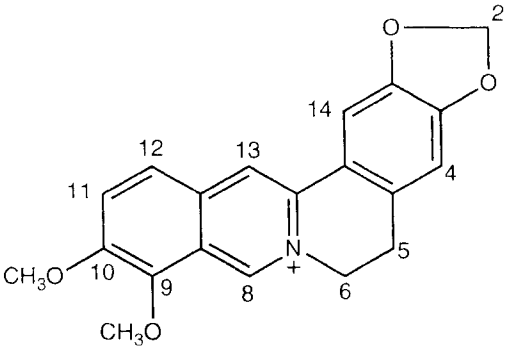
The chemical shifts of BC were shifted to high field by an increase in concentration (1–10 mmol/L) of BC (Table 3). This shift was probably due to a ring current effect with stacking of BC molecules. The spin-lattice relaxation time (T_1) of BC was measured by the inversion recovery method (Table 4). When the concentration of BC increased from 1 to 10 mmol/L, the T_1-value of each proton decreased by 0.8–0.6 times. The spin-spin relaxation rate (T_2^{-1}) of BC was measured by the CPMG method (Fig. 2, Table 5). When the concentration of BC increased from 1 to 10 mmol/L, the spin-spin relaxation rate of each proton in the free drug (T_{2f}^{-1}) increased by 1–2.5 times. It was again believed that the variation of T_1 and T_{2f}^{-1} values was due to the intermolecular interaction of BC.

TABLE 2. Dependence of the Binding Capacity (nK) on the Concentration of Phosphate Buffer[a]

[Phosphate buffer], mol/L	$nK \times 10^2$ L/mol
0.025	4.92
0.05	4.22
0.1	3.69
0.2	3.28

[a][BSA] = 1.45×10^{-4} mol/L, [phosphate buffer] = 0.1 mol/L, pH 7.

TABLE 3. Chemical Shifts (δ) of BC:[a]



Proton	1 mmol/L BC	10 mmol/L BC	10 mmol/L BC/1.45 × 10^{-4} mol/L BSA
8-CH	9.549	9.442	9.441
13-CH	8.407	8.043	8.046
11-CH	7.971	7.813	7.814
12-CH	7.878	7.639	7.640
14-CH	7.254	7.087	7.089
4-CH	6.896	6.768	6.768
2-CH_2	6.033	5.953	5.953
6-CH_2	4.798	4.701	4.701
9-OCH_3	4.052	4.007	4.006
10-OCH_3	4.030	3.938	3.938
5-CH_2	3.161	3.061	3.061

[a]From TMS (external reference), pH 7, [phosphate buffer] = 0.1 mol/L.

TABLE 4. Spin-Lattice Relaxation Time (T_1, s) of BC[a]

Proton	1 mmol/L BC	10 mmol/L BC	10 mmol/L BC/1.45 × 10^{-4} mol/L BSA
8-CH	1.367	1.015	0.903
13-CH	0.991	0.732	0.732
11-CH	1.294	0.913	0.800
12-CH	1.605	1.082	0.955
14-CH	1.407	0.965	1.001
4-CH	2.891	1.923	1.827
2-CH_2	1.220	0.755	0.752
6-CH_2	0.428	0.338	0.316
9-OCH_3	2.591	1.778	1.757
10-OCH_3	1.385	0.965	0.931
5-CH_2	0.501	0.396	0.353

[a]pH 7, [phosphate buffer] = 0.1 mol/L.

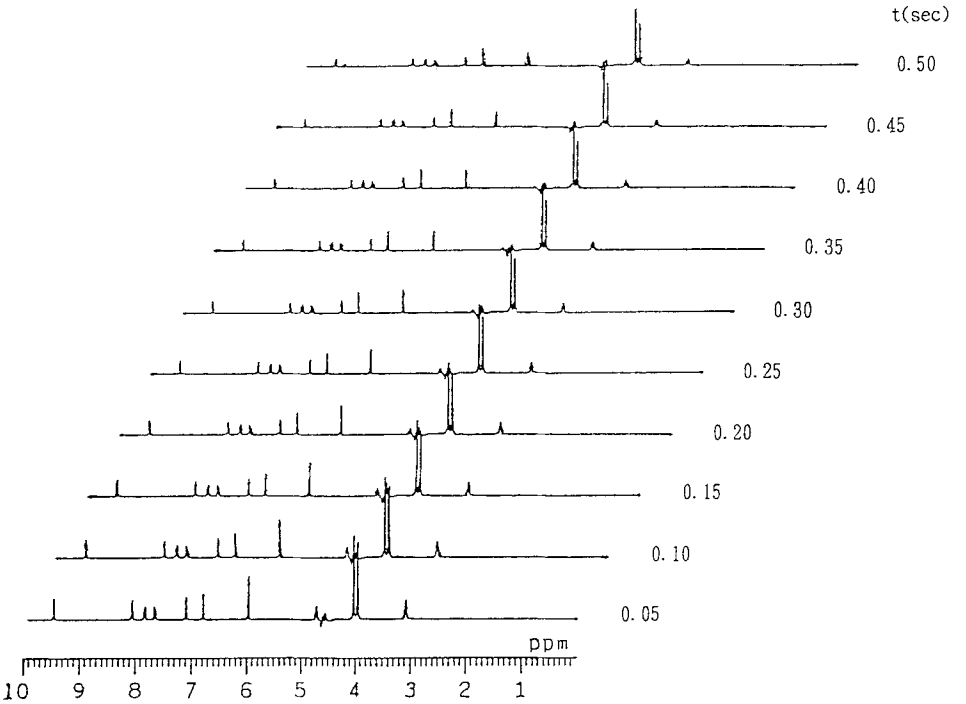


FIG. 2. Spin-spin relaxation traces obtained by the Carr-Purcell-Meiboom-Gill method for protons of BC at 40°C.

Interaction of BC with BSA

The chemical shifts of BC (10 mmol/L) were not affected more than 0.003 ppm by the addition of BSA (1.45×10^{-4} mol/L) (Table 3). The T_1 values of the protons of BC varied less than 13% in the presence of BSA (1.45×10^{-4} mol/L) (Table 4). However, the variation of ^{1}H-NMR parameters was smaller than those of drugs (PB, IB, AZ, ID) reported previously [1-4]. The T_2^{-1} values of protons in BC (10 mmol/L) in the presence of BSA (1.45×10^{-4} mol/L) were larger than the T_{2f}^{-1} values of BC (10 mmol/L) by factors from 1.12 to 1.85 (Table 5). Thus, interaction of BC with BSA was observed by the variation of T_1 and T_2^{-1} values.

Binding Position of BC to BSA

Because of the lack of an effect of protein on chemical shift, slow exchange between free and bound states was reported to cause the superposed narrow and broad peaks in NMR spectrum [10]. In a slow exchange system, T_2-measurement by CPMG method was found to be difficult because of the modulation action following repeated irradiation of the π pulse [11]. In this work, however, one peak was observed as the weighted average of both states. Furthermore, a series of echos measured by the CPMG method decayed according to Eq. (2) without the modulation. Therefore, the exchange between free and bound states in our system is sug-

TABLE 5. Spin–Spin Relaxation Rates (T_2^{-1}, s^{-1}) of BC[a]

	Observed			Calculated			
				$n = 32$		$n = 100$	
Proton	$T_{2f'}^{-1}$, (s^{-1})	T_{2f}^{-1} (s^{-1})	T_2^{-1} (s^{-1})	T_{2b}^{-1} (s^{-1})	T_{2b}^{-1}/T_{2f}^{-1}	T_{2b}^{-1} (s^{-1})	T_{2b}^{-1}/T_{2f}^{-1}
8-CH	1.42	2.27	2.55	8.71	3.84	8.31	3.66
13-CH	0.92	1.27	1.71	11.3	8.96	10.7	8.47
11-CH	0.66	1.00	1.51	12.6	12.6	11.9	11.9
12-CH	0.71	1.18	1.70	13.0	11.0	12.3	10.4
14-CH	0.76	1.57	1.93	9.84	6.25	9.33	5.93
4-CH	0.49	0.84	1.47	15.2	18.0	14.3	17.0
2-CH_2	0.69	4.79	6.90	53.1	11.1	50.1	10.5
6-CH_2	0.61	7.47	11.9	109	14.6	103	13.8
9-OCH_3	0.55	0.81	1.23	10.5	13.1	9.94	12.3
10-OCH_3	0.81	1.11	1.76	16.0	14.4	15.0	13.6
5-CH_2	0.98	1.04	1.94	21.6	20.8	20.8	19.6

[a] $T_{2f'}^{-1}$, 1 mmol/L BC; T_{2f}^{-1}, 10 mmol/L BC; T_2^{-1}, 10 mmol/L BC/1.45 × 10^{-4} mol/L BSA; T_{2b}^{-1}, BC (10 mmol/L) bound to BSA; n, assumed number of binding site on BSA.

gested to be rapid, similar to the case reported by Jardetzky [12]. The spin-spin relaxation rate of drugs bound to BSA (T_{2b}^{-1}) was calculated according to Eq. (4) proposed by Jardetzky [12]:

$$T_2^{-1} = (1 - B)T_{2f}^{-1} + BT_{2b}^{-1} \quad (4)$$

where T_{2f}^{-1} is the spin-spin relaxation rate of the free drug, and B is the proportion of the drug bound to BSA. The B-value can be calculated from the binding constant (K) and the number of binding sites (n).

The n-value for the BC and BSA system was not determined accurately, although Nahar et al. [8] assumed $n = 32$ for the BC and BSA system. The hydrophilic binding of BC to BSA was determined from the results in Table 2. Assuming hydrophilic binding between the cationic center (N^+) of BC and the acidic residues of BSA, which includes aspartic acid (64) and glutamic acid (43) [13], the n-values may be estimated to be 107. In this work the relaxation rates were calculated using $n = 32$ and $n = 100$ according to Eq. (4) (Table 5). However, it was hard to determine the binding position based on the T_{2b}^{-1} values obtained, because the T_{2f}^{-1} values of the respective protons differed from each other. On the other hand, in our previous papers [1-4] the ratio T_{2b}^{-1}/T_{2f}^{-1} was the most useful parameter for determination of the binding position. In this case the number of binding sites on BSA (n) was large and uncertain. However, similar ratios for the respective protons were obtained even if different n-values (32 and 100) were used.

The contour plot of the ratio (T_{2b}^{-1}/T_{2f}^{-1}) is illustrated in Fig. 3. The binding position of BC to BSA was not only in the vicinity of the ionic center (immonium cation), but also the other hydrophobic moieties. When the ionic center was BC

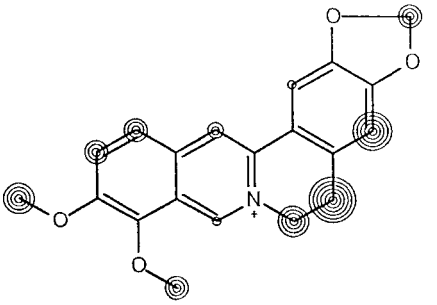


FIG. 3. Contour plot of the ratio of spin–spin relaxation rate of bound BC to free BC.

bound to BSA, other atoms or groups were also close to the BSA molecule. Therefore, it was believed that the T_2^{-1}-value of almost all protons of BC bound to the nonspecific sites of BSA increase according to a slow motion of BSA.

CONCLUSION

The dependency of binding capacity (nK) on the ionic strength of BC binding to BSA is ascribed to nonspecific and hydrophilic binding. The binding position of BC to BSA is spread over the whole BC molecule as illustrated by the contour plot of the ratio of relaxation rates (T_{2b}^{-1}/T_{2f}^{-1}) because the hydrophilic binding of BC to BSA forces a decrease in the mobility of the whole molecule. Stacking of BC molecules was postulated from the concentration dependency of NMR parameters.

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