# Interaction between drugs and water-soluble polymers: 5. Binding position of indomethacin and its related compounds to bovine serum albumin

**Masami Tanaka* and Yutaka Asahi**
*Faculty of Pharmaceutical Science, Tokushima Bunri University, Tokushima 770, Japan*

**and Seizo Masuda**
*Department of Applied Chemistry, Technical College, The University of Tokushima, Tokushima 770, Japan*
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The interaction between drugs (indomethacin (ID), *p*-chlorobenzamide (CBA) and 5-methoxy-2-methyl-3-indoleacetic acid (MMI)) and bovine serum albumin (BSA) was investigated by equilibrium dialysis and nuclear magnetic resonance (n.m.r.) spectroscopy. The binding of ID and MMI to BSA was concluded to be hydrophobic and hydrophilic, respectively, on the basis of the dependence of the binding constants on temperature and ionic strength. In $^1H$ n.m.r. spectra of ID, there were no significant shifts with change in the concentration and on addition of BSA. The spin–lattice relaxation time ($T_1$) and spin–spin relaxation time ($T_2$) of the respective protons of ID were independent of concentration, but depended on the concentration of BSA added. The binding position was determined from the ratio of the spin–spin relaxation rates of ID bound to BSA and free ID. ID and MMI were found to bind to BSA through the aromatic moiety and the carboxyl group as the substituent, respectively. The binding property of ID was known to be governed by the competition between the hydrophobic effect of the chlorophenyl group and the hydrophilic effect of the carboxyl group in the molecule.

**(Keywords: polymer–drug interaction; bovine serum albumin; binding position)**

## INTRODUCTION

In n.m.r. spectroscopic investigations of the interaction between proteins and drugs, the conventional method is based on measurement of peak width at half height[1–4]. However, this method is considered questionable from the viewpoint of precision. Ueda *et al.*[5] have discussed the interaction between bovine serum albumin (BSA) and sulfonylureas on the basis of n.m.r. relaxation time $T_{1\rho}$, instead of $T_2$.

It is a matter of course that direct measurement of relaxation time $T_2$ is better. The Carr–Purcell–Meiboom–Gill (CPMG)[6] method is the most useful one. However, there are two controversial points in the direct measurement of $T_2$. One is the deformation of phase by $J$ modulation, and the other is the induction of a sort of modulation action by repeated irradiation of the $\pi$ pulse[7].

This paper describes the binding of the anti-inflammatory drug indomethacin (ID) and its related compounds, *p*-chlorobenzamide (CBA) and 5-methoxy-2-methyl-3-indoleacetic acid (MMI), to BSA. The binding constant and binding position were determined by equilibrium dialysis and n.m.r. relaxation (CPMG) techniques.

In our experiments, no modulation phenomenon was observed because of very fast chemical exchange between free and bound drugs when the repeated $\pi$ pulse was irradiated at short time interval.

*To whom correspondence should be addressed

## EXPERIMENTAL

### *Materials*

ID, CBA and MMI were of special reagent grade from Sigma, and were used without further purification. BSA with molecular weight of $6.9 \times 10^4$ was from Wako Pure Chemical Industrials Ltd. Other reagents were commercially available and used without further purification.

### *Equilibrium dialysis*

The procedure of equilibrium dialysis was described previously[8]. Temperature was regulated within 0.2°C during all experiments. The drug concentration was determined by the u.v. method. U.v. spectra were recorded on a Shimadzu UV-190 spectrometer; $\lambda_{max}$ nanometres at pH 7 ($\varepsilon$): ID, 318.5 (6800); CBA, 236.5 (13 400); MMI, 286.5 (8630).

### *N.m.r. measurements*

The n.m.r. spectra were measured in deuterium oxide ($D_2O$, phosphate buffer, 0.1 M, pH 7) on a JEOL GX-400 spectrometer (radiofrequency, 400 MHz; $\pi/2$ pulse, 11.1 $\mu$s) at $40.0 \pm 0.5$°C. The spin–lattice

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relaxation time ($T_1$) was obtained by the inversion-recovery method[9]:

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

where $t$ is the interval between $\pi$ 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[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 intensity of a spin echo at $t$. The pulse delay time (20 s), when the next pulse was applied after observation of FID, was necessarily longer than the relaxation time $T_1$ by a factor of 5 or more. The homo-gated irradiation technique was used to depress the HDO peak in $D_2O$.

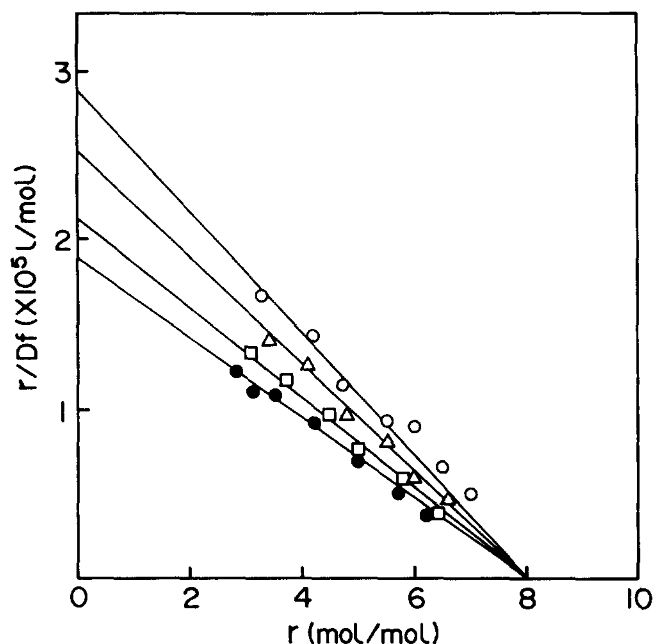



**Figure 1** Scatchard plots for the binding of ID to BSA ($1.45 \times 10^{-2}$ mM) in 0.1 M phosphate buffer (pH 7) at 30°C (○), 35°C (△), 40°C (□) and 45°C (●)

## RESULTS AND DISCUSSION

### *The binding constant of ID to BSA*

The binding of the drug (ID) to protein (BSA) was examined by equilibrium dialysis in the temperature range of 30 to 45°C. The free drug concentration ($D_f$) and the number of molecules of the drug bound to a molecule of BSA ($r$) were estimated from the concentration of the dialysed drug. A plot of $r$ vs. $r/D_f$ gave a linear relationship, i.e. the plot obeyed the Scatchard equation[10]:

$$r/D_f = Kn - Kr \quad (3)$$

where $n$ is the number of binding sites per molecule of BSA, and $K$ represents the binding constant of the drug to BSA. The values of $n$ and $K$ were calculated from the intercept and slope of the straight line. *Figure 1* reveals that the binding site is of one class and the number of sites is $n=8$. Kaneo *et al.*[11] have also reported that the interaction between ID and BSA involves one-class binding ($n=6.09$, $K=3.43\times10^4$ M$^{-1}$). The results obtained in this work agreed well with those of Kaneo *et al.* except for the number of binding sites.

The number of binding sites of ID on BSA is larger than that of a site I drug (phenylbutazone[12] (PB), $n=3$) and a site II drug (ibuprofen[13] (IB), $n=3$). Sjoholm *et al.*[14] have shown that ID can bind to both sites I and II on human serum albumin (HSA). BSA is homologous with HSA. Therefore, it seems reasonable that the number of binding sites of ID on BSA is larger than that of PB or IB. The number of binding sites of ID on BSA was independent of temperature, similarly to the cases of PB[12], IB[13] and azathioprine (AZ)[8].

*Table 1* summarizes the thermodynamic parameters calculated from the linear relationship between ln $K$ and

**Table 1** Thermodynamic parameters for the binding of compounds to BSA[a]

| Compound | Temp. (°C) | $K\times10^{-3}$ (M$^{-1}$) | $\Delta G^\circ$ (kJ mol$^{-1}$) | $\Delta H^\circ$ (kJ mol$^{-1}$) | $\Delta S^\circ$ (J mol$^{-1}$ K$^{-1}$) | $n$ |
|---|---|---|---|---|---|---|
| ID[b] | 30 | 36.2 | −26.44 | −23.16 | 10.79 | 8 |
| | 35 | 31.8 | −26.54 | | 11.00 | |
| | 40 | 26.6 | −26.51 | | 10.71 | |
| | 45 | 23.9 | −26.65 | | 10.96 | |
| | | | | | 10.88 (av.) | |
| CBA[c] | 30 | 1.99 | −19.14 | −17.82 | 4.36 | 1 |
| | 35 | 1.89 | −19.31 | | 4.84 | |
| | 40 | 1.65 | −19.28 | | 4.66 | |
| | 45 | 1.44 | −19.23 | | 4.43 | |
| | | | | | 4.48 (av.) | |
| MMI[d] | 30 | 9.16 | −22.98 | −43.17 | −66.63 | 1 |
| | 35 | 7.37 | −22.80 | | −66.14 | |
| | 40 | 5.76 | −22.54 | | −66.91 | |
| | 45 | 4.06 | −21.97 | | −66.67 | |
| | | | | | −66.34 (av.) | |

[a] [Phosphate buffer] = 0.1 M, pH 7
[b] [BSA] = $1.45\times10^{-2}$ mM
[c] [BSA] = $7.25\times10^{-1}$ mM
[d] [BSA] = $7.25\times10^{-2}$ mM

the reciprocal of absolute temperature. For the ID–BSA system, the free-energy changes ($\Delta G^\circ$) and standard enthalpy changes ($\Delta H^\circ$) were large and negative, and the standard entropy changes ($\Delta S^\circ$) were positive. It could be suggested that the binding of ID to BSA was enthalpy-controlled. Furthermore, it was also presumed from the positive $\Delta S^\circ$ values for ID and CBA and the negative $\Delta S^\circ$ value for MMI that the binding of the first two was hydrophobic and that of the last one hydrophilic.

The binding constants of ID, CBA and MMI to BSA are listed in *Table 2*. Decrease in the ionic strength resulted in reduced binding constants for ID and CBA, whereas it produced the opposite effect for MMI. These findings also supported that the binding of the first two and the last one were hydrophobic and hydrophilic, respectively.

*N.m.r. chemical shift of ID*

As is shown in *Table 3*, both 10 times dilution and addition of BSA ($1.45 \times 10^{-5}$ M) resulted in a slight shift of the resonance peaks of ID. Therefore, it was difficult to determine the binding position from the change in the chemical shift.

**Table 2** Dependence of the binding constant $K$ on the concentration of phosphate buffer[a]

| [Phosphate buffer] (M) | $K \times 10^{-3}$ ($M^{-1}$) | | |
|---|---|---|---|
| | ID[b] | CBA[c] | MMI[d] |
| 0.1 | 26.6 | 1.65 | 5.76 |
| 0.05 | 23.5 | 1.38 | 9.14 |
| 0.025 | 19.8 | 1.16 | 17.81 |
| 0.0125 | 19.2 | 0.60 | 30.56 |

[a] pH 7, 40°C
[b] [BSA] = $1.45 \times 10^{-2}$ mM
[c] [BSA] = $7.25 \times 10^{-1}$ mM
[d] [BSA] = $7.25 \times 10^{-2}$ mM

*Spin–lattice relaxation time*

*Table 4* shows the spin–lattice relaxation time ($T_1$) of ID. The measurements were made by the inversion-recovery method. The addition of BSA caused a significant change in the relaxation time, i.e. the ratios of $T_1$ in the presence and absence of BSA are 0.887 (1-$CH_3$), 1.017 (2-$CH_2$), 0.779 (3-$CH_3$), 0.603 (4-CH), 0.597 (5-CH), 0.555 (6-CH), 0.502 (7-CH) and 0.571 (8-CH). Therefore, it can be predicted that the aromatic part of ID interacts with BSA rather than the aliphatic part.

*Spin–spin relaxation time*

The spin–spin relaxation time ($T_2$) of ID was measured by the CPMG method[6]. A series of spectra and $T_2$ values are shown in *Figure 2* and *Table 5*, respectively. Dilution of ID solution gave a slight increase in $T_2$ values. Addition of BSA led to a remarkable decrease in $T_2$, i.e. the $T_2$ values of aromatic and aliphatic protons decreased to 5–9% and 11–14% of their original values, respectively. It was therefore proved that ID bound to BSA through the hydrophobic phenyl moiety. In addition, $T_2$ values decreased greatly compared with $T_1$ values. Thus, $T_2$ can be concluded to be a useful parameter to detect a small change of the molecular environment.

*Spin–spin relaxation rate*

Owing to the lack of effect of BSA on chemical shift, slow exchange between free and bound states was reported to cause the superposed narrow and broad peaks in the n.m.r. spectrum[1]. In this work, however, rapid

**Table 3** Chemical shifts $\delta$ of ID[a]

| [ID] (mM) | [BSA] (mM) | 1-$CH_3$ | 2-$CH_2$ | 3-$CH_3$ | 4-CH | 5-CH | 6-CH | 7-CH | 8-CH |
|---|---|---|---|---|---|---|---|---|---|
| 5 | 0 | 2.137 | 3.488 | 3.798 | 6.659 | 6.956 | 6.993 | 7.521 | 7.607 |
| 0.5 | 0 | 2.176 | 3.528 | 3.826 | 6.718 | 7.037 | 7.052 | 7.558 | 7.665 |
| 5 | $1.45 \times 10^{-2}$ | 2.143 | 3.493 | 3.810 | 6.654 | 6.954 | 6.999 | 7.519 | 7.606 |

[a] From TMS (external reference), pH 7, [phosphate buffer] = 0.1 M

**Table 4** Spin–lattice relaxation time $T_1$ (s) of ID[a]

| [ID] (mM) | [BSA] (mM) | 1-$CH_3$ | 2-$CH_2$ | 3-$CH_3$ | 4-CH | 5-CH | 6-CH | 7-CH | 8-CH |
|---|---|---|---|---|---|---|---|---|---|
| 5 | 0 | 1.15 | 0.60 | 1.72 | 2.34 | 2.43 | 2.38 | 3.21 | 2.80 |
| 0.5 | 0 | 1.15 | 0.64 | 1.75 | 1.35 | 2.61 | 2.56 | 3.45 | 2.96 |
| 5 | $1.45 \times 10^{-2}$ | 1.02 | 0.61 | 1.34 | 1.41 | 1.45 | 1.32 | 1.61 | 1.60 |

[a] pH 7, [phosphate buffer] = 0.1 M

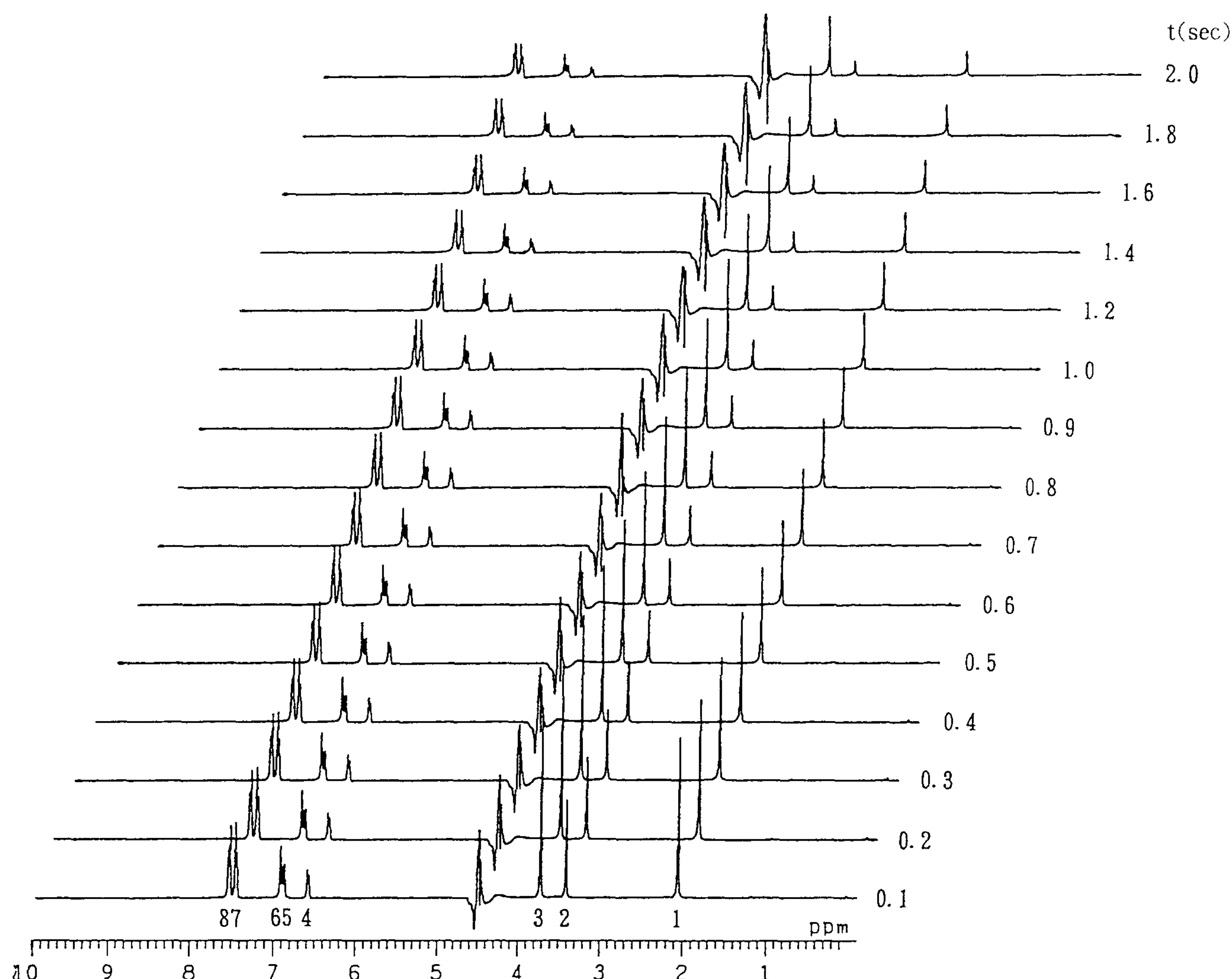



**Figure 2** Spin–spin relaxation traces obtained by the Carr–Purcell–Meiboom–Gill method for protons of ID at 40°C

**Table 5** Spin-relaxation time $T_2$ (s) of ID[a]

| [ID] (mM) | [BSA] (mM) | 1-$CH_3$ | 2-$CH_2$ | 3-$CH_3$ | 4-CH | 5-CH | 6-CH | 7-CH | 8-CH |
|---|---|---|---|---|---|---|---|---|---|
| 5 | 0 | 0.727 | 0.648 | 1.030 | 1.445 | 1.196 | 1.628 | 1.608 | 2.235 |
| 0.5 | 0 | 0.864 | 0.882 | 1.431 | 1.705 | 1.812 | 2.319 | 1.945 | 2.645 |
| 5 | $1.45 \times 10^{-2}$ | 0.088 | 0.071 | 0.144 | 0.079 | 0.106 | 0.141 | 0.110 | 0.111 |

[a] pH 7, [phosphate buffer] = 0.1 M

exchange between both states was understood, since one peak was observed as the weighted average of both states. In a slow exchange system, $T_2$ measurement by the CPMG method was found to be difficult because of modulation action by repeated irradiation of the $\pi$ pulse[7]. In this work, a series of echoes measured by the CPMG method decayed according to equation (2) without modulation. Therefore, the exchange between free and bound states in our system was suggested to be rapid, similarly to the case reported by Jardetzky[15]. The spin–spin relaxation rate of drugs bound to BSA, $(1/T_2)_b$, was calculated according to the equation proposed by Jardetzky[15]:

$$1/T_2 = (1-B)(1/T_2)_f + B(1/T_2)_b \quad (4)$$

where $(1/T_2)_f$ 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 ($n$) obtained by equilibrium dialysis.

*Table 6* lists the spin–spin relaxation rates of the respective protons of ID. The values of $(1/T_2)_b$ were calculated according to equation (4). However, the discussion of the binding position on the basis of $(1/T_2)_b$ values was not reasonable, since the relaxation rates of the respective protons of free ID, $(1/T_2)_f$, were different from each other. The ratio $(1/T_2)_b/(1/T_2)_f$ has a significant meaning for determination of the binding position. As can be seen from *Table 6*, the 8-CH proton had the largest value of $(1/T_2)_b/(1/T_2)_f$, followed by the 4-CH proton. It was therefore concluded that the binding position of ID to BSA was at the phenyl group and the aromatic moiety in the indole ring.

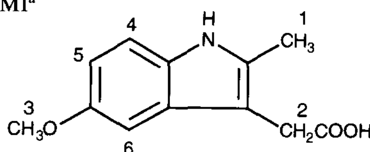
Since the drug/protein ratio obtained by n.m.r. was

**Table 6** Spin–spin relaxation rates $1/T_2$ ($s^{-1}$) of ID[a]

| | | | $n=8$ (dialysis) | | $n=100$ (assumed) | |
|---|---|---|---|---|---|---|
| Proton | $1/T_2$ ($s^{-1}$) | $(1/T_2)_f$ ($s^{-1}$) | $(1/T_2)_b$ ($s^{-1}$) | $(1/T_2)_b/(1/T_2)_f$ | $(1/T_2)_b$ ($s^{-1}$) | $(1/T_2)_b/(1/T_2)_f$ |
| 1-$CH_3$ | 11.33 | 1.375 | 434 | 316 | 40.1 | 29.2 |
| 2-$CH_2$ | 14.02 | 1.544 | 544 | 352 | 50.1 | 32.4 |
| 3-$CH_3$ | 6.95 | 0.971 | 261 | 269 | 24.2 | 25.0 |
| 4-CH | 12.69 | 0.692 | 522 | 755 | 47.4 | 68.5 |
| 5-CH | 9.46 | 0.836 | 376 | 450 | 34.4 | 41.1 |
| 6-CH | 7.09 | 0.614 | 282 | 460 | 25.8 | 42.0 |
| 7-CH | 9.09 | 0.622 | 369 | 593 | 33.6 | 54.0 |
| 8-CH | 8.98 | 0.447 | 371 | 831 | 33.6 | 75.3 |

[a] The rates were measured at ID concentration of 5 mM in the presence or absence of BSA. $1/T_2$, values found in the presence of BSA ($1.45 \times 10^{-2}$ mM); $(1/T_2)_f$, values found in the absence of BSA; $(1/T_2)_b$, values calculated according to equation (4); $n$, number of binding sites on BSA

**Table 7** Spin–spin relaxation rates $1/T_2$ ($s^{-1}$) of MMI[a]




| | | | $n=1$ (dialysis) | | $n=100$ (assumed) | |
|---|---|---|---|---|---|---|
| Proton | $1/T_2$ ($s^{-1}$) | $(1/T_2)_f$ ($s^{-1}$) | $(1/T_2)_b$ ($s^{-1}$) | $(1/T_2)_b/(1/T_2)_f$ | $(1/T_2)_b$ ($s^{-1}$) | $(1/T_2)_b/(1/T_2)_f$ |
| 1-$CH_3$ | 3.25 | 1.366 | 673 | 493 | 31.8 | 23.3 |
| 2-$CH_2$ | 4.41 | 1.313 | 1105 | 842 | 51.4 | 39.2 |
| 3-$CH_3$ | 2.68 | 1.230 | 517 | 420 | 24.6 | 20.0 |
| 4-CH | 2.36 | 0.964 | 499 | 518 | 23.6 | 24.5 |
| 5-CH | 2.52 | 1.120 | 499 | 446 | 23.7 | 21.2 |
| 6-CH | 2.22 | 1.182 | 371 | 314 | 18.0 | 15.2 |

[a] The rates were measured at MMI concentration of 5 mM in the presence or absence of BSA. $1/T_2$, values found in the presence of BSA ($1.45 \times 10^{-2}$ mM); $(1/T_2)_f$, values found in the absence of BSA; $(1/T_2)_b$, values calculated according to equation (4); $n$, number of binding sites on BSA

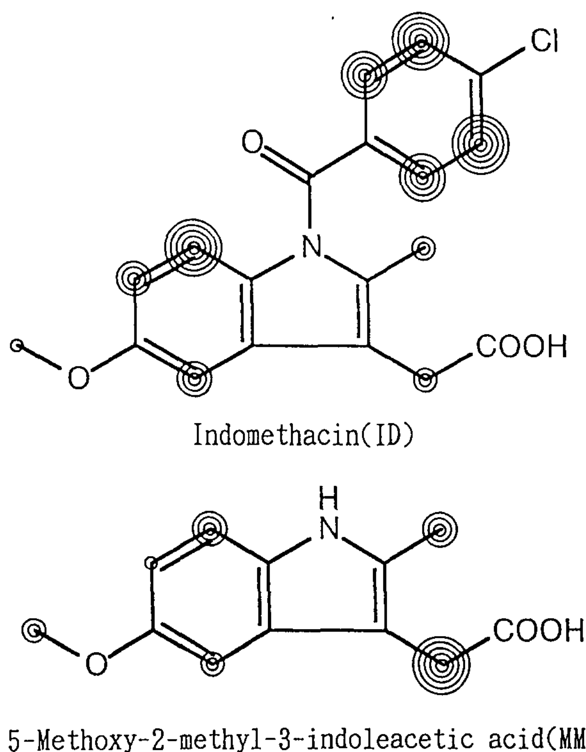



**Figure 3** Contour plot of the ratio of spin–spin relaxation rate of bound drugs to free drugs

larger than that by equilibrium dialysis or other methods, the binding site obtained by n.m.r. was secondary, in other words, non-specific. Even if the number of non-specific sites ($n$) is assumed to be sufficiently large (for example, $n=100$, an arbitrary figure), the relative relaxation rates of the respective protons remain constant, though the values themselves decrease.

*Table 7* shows the spin–spin relaxation rate of MMI. The 2-$CH_2$ proton had the largest value of $(1/T_2)_b/(1/T_2)_f$. This observation suggested that MMI bound to BSA through the carboxyl group of MMI, which was the most hydrophilic.

*Figure 3* illustrates the counter plots of the $(1/T_2)_b/(1/T_2)_f$ values. These illustrations were a useful method for visible representation of the binding position.

As described above, the binding of ID and MMI to BSA was found to be due to hydrophobic and hydrophilic interactions, respectively. ID exhibited different binding properties from MMI, though they have the same basic structure. The competition between the hydrophilic effect of the carboxyl group and the hydrophobic effect of the aromatic group was concluded to be the main factor governing the binding property of ID.

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