IMPACT: International Journal of Research in Applied, Natural and Social Sciences (IMPACT: IJRANSS)
ISSN(E): 2321-8851; ISSN(P): 2347-4580
Vol. 3, Issue 10, Oct 2015, 81-88
© Impact Journals

ANALYSIS OF INTERACTION BETWEEN DIPHENHYDRAMINE AND HUMAN SERUM ALBUMIN

MASAMI TANAKA[1], KEIJI MINAGAWA[2], YUKIHIRO ARAKAWA[3] & YASUSHI IMADA[4]

[1]Faculty of Pharmaceutical Sciences, Tokushima Bunri University, Tokushima, Japan

[2,3,4] Institute of Technology and Science, Tokushima University, Tokushima, Japan

ABSTRACT

The interaction between an antihistamine drug diphenhydramine (DPH; 2-(diphenylmethoxy)-*N,N*-dimethylethylamine) and human serum albumin (HSA) was investigated by means of equilibrium dialysis and NMR relaxation analysis. The binding constant of DPH to HSA determined from equilibrium dialysis was independent of ionic strength and decreased with the addition of fatty acid, suggesting that hydrophobic interaction predominates for the binding of DPH to HSA. It was difficult to determine the binding position from the chemical shifts in ^{1}H-NMR spectra of DPH, because they were almost independent of the concentration of DPH and HSA added. On the other hand, the relaxation analyses gave information on the interaction. The spin-lattice relaxation time (T_1) and spin-spin relaxation time (T_2) of respective protons of DPH were independent of the concentration of itself, but depended on the concentration of HSA added. The ratio of spin-spin relaxation rates ($1/T_2$) of DPH bound to HSA and free DPH indicated that the binding position of DPH to HSA involved hydrophobic aromatic moiety of DPH.

KEYWORDS: Hydrophobic Interaction, Diphenhydramine, Human Serum Albumin, Spin-spin Relaxation Rate

INTRODUCTION

In the previous study concerning interaction between drugs and biomedical materials, equilibrium dialysis was commonly used to study the binding constant (K), the number (n) of binding sites, and nature of binding (hydrophobic or hydrophilic). On the other hand, NMR especially the ratio of the spin-spin relaxation rate ($1/T_2$) of the free drug to that of the bound drug was the most useful parameter to determine the binding position of the drug with serum albumin (Tanaka, 2012), which is based on the modification of spin-echo method (Hahn, 1950) and the Carr-Purcell-Meiboom-Gill (CPMG) method (Meiboom and Gill, 1958). In the present paper, the binding position of antihistamine drug diphenhydramine (DPH; 2-(diphenylmethoxy)-*N,N*-dimethylethylamine) to human serum albumin (HSA) was studied by examining NMR relaxation time to clarify the essence of binding microscopically.

EXPERIMENTAL

Equilibrium Dialysis

DPH was of special reagent grade from Sigma, and used without further purification. HSA (mol. wt., 6.9×10^4) was from Sigma. Other reagents were commercially available and used without further purification. The procedure of equilibrium dialysis was as follows. Dialysis membrane (0.09 mm in thickness) supplied by Visking Company was boiled four times for 5 min each, and interposed between two parts of a dialysis cell made of poly(methyl methacrylate). The HSA

solution or phosphate buffer solution (0.1M, pH 7, for the control experiment) was injected into one side of the cell, and the drug solution into the other side, the volume of each side being 0.8 ml. After the cell had been shaken in a thermostat at a given temperature regulated within 0.2°C for 24 h, the absorbance of the drug without HSA was measured on a UV spectrometer (Shimadzu UV-190 spectrometer). Parameters of DPH at pH 7 are as follows: $_{max}$ in nm ( ): 223 (9160).

NMR Measurements and Analyses

The NMR spectra were measured in deuterium oxide (D_2O, phosphate buffer, 0.1M, pH 7) on a Varian Inova-600 spectrometer (radio frequency, 600MHz, /2 pulse, 10.9 sec) at 40°C. Isotope effects on binding constant were uncorrected. The spin-lattice relaxation time (T_1) was obtained by inversion recovery method (Eq.1):

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

Where t is the interval between and /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 CPMG method (Eq.2):

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

Where t is the time when a free induction decay (FID) observed after application of /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 the FID, was longer than the relaxation time T_1 by a factor of five or above, as required to avoid saturation. A homo-gated irradiation technique was used to depress the HDO peak in D_2O.

RESULTS AND DISCUSSIONS

Binding Constants of DPH to HAS

The binding of the drug (DPH) to protein (HSA) was examined by equilibrium dialysis in the temperature range of 20°C to 40°C. Free drug concentration (D_f) and the number of the drug bound to a molecule of HSA (r) were estimated from the concentration of the dialyzed drug. A plot of r versus r/D_f produced a linear relationship, as shown in Figure 1, and satisfied Eq.3 proposed by Scatchard (1949)

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

where n is the number of binding sites per molecule of HSA, and K represents the binding constant of the drug to HSA. The values of n and K were calculated from intercept and slope of the line.

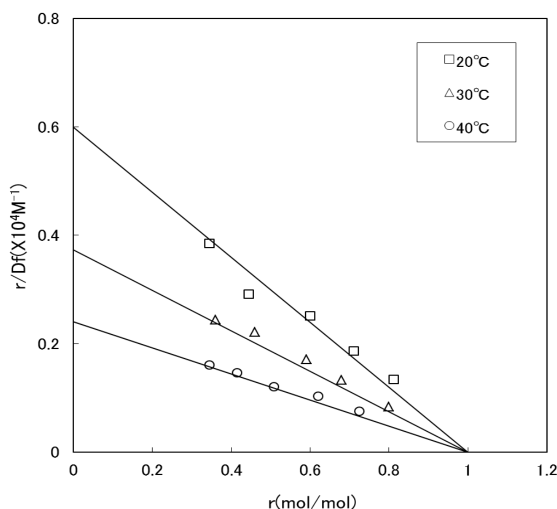

Figure 1: Scatchard Plots for the Binding of DPH to HSA (7.25×10^{-5}M) in 0.1M phosphate buffer (pH 7) at 20°C(○), 30°C(△), 40°C(□)

Table 1 summarizes thermodynamic parameters calculated from the linear relationship between ln K and the reciprocal of absolute temperature. The free energy changes (G°) and standard enthalpy changes (H°) were negative, and standard entropy changes (S°) were also negative. The binding of DPH to HSA would be governed by advantageous enthalpy. The binding constant was independent of ionic strength (Figure 2), and decreased with the addition of short-chain fatty acid as shown in Table 2. These results suggest that the binding of DPH to HSA was due to a hydrophobic mechanism.

Table 1: Thermodynamic Data for the Binding of DPH with HSA

Temp. (°C)	K (×10^{3}M^{-1})	G° (kJ/mol)	H° (kJ/mol)	S° (J mol^{-1}K^{-1})
20	5.99	-21.19	-34.85	-46.61
30	3.73	-20.72		-46.62
40	2.40	-20.26		-46.61

[HSA]=7.25×10^{-5}M; phosphate buffer, 0.1M, pH=7.

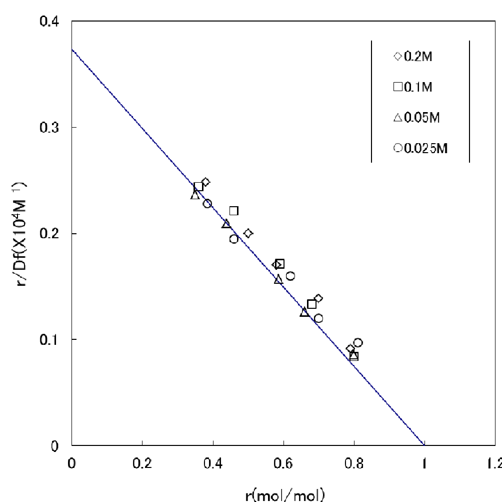

Figure 2: Scatchard Plots for the Binding of DPH to HSA (7.25×10^{-5}M) in Phosphate Buffer (0.025M;○, 0.05M;△, 0.1M;□, 0.2M;◇, pH 7) at 30°C

Table 2: Effect of Fatty Acid on the Binding Constant *K* of DPH to HSA

Fatty Acid	C_n	K_a *($\times10^{-5}$)	K ($\times10^3 M^{-1}$)
			3.73
Butylic acid	C_4	1.50	2.96
Valeric acid	C_5	1.56	2.53
Caproic acid	C_6	1.46	2.42
Enanthic acid	C_7	1.46	2.16
Caprylic acid	C_8	1.44	2.09

[Fatty acid]=1mM, [HSA]=7.25×10^{-5}M, [Phos.buf.]=0.1M, pH = 7, 30℃.

*Acid dissociation constant (Encyclopaedia Chimica, Vol. 4, 30th Ed., Kyoritsu, Tokyo, 1987.)

Chemical Shift of DPH

The interaction between DPH and HSA may affect the elctronic environment around each proton. If it occurs, the DPH concentration and/or addition of HAS may induce some changes in the chemical shifts of DPH proton signals. Table 3 shows the comparison of chemical shifts of DPH proton signals measured under different DPH concentrations or in the presence of HSA. It was found that both 10-times dilution of DPH and addition of HSA (7.25×10^{-5} M) slightly affected the chemical shifts, but the absolute values of the changes were very small; The largest shift was only 0.021 ppm at 4-CH. This result indicateds that the changes in the chemical shifts hardly give us the information on the binding position. Therefore another method is necessary to analyze the interaction in detail.

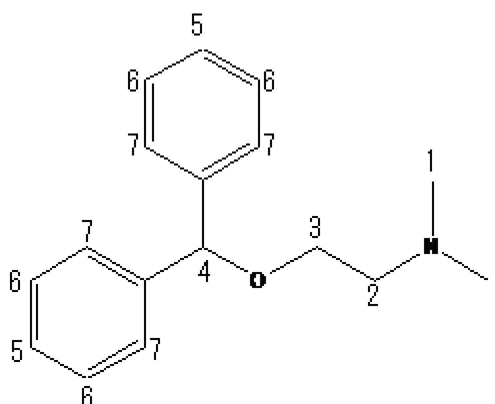

Figure 3

Table 3: Chemical Shifts (δ, ppm) of DPH*

	$1\text{-}CH_3$	$2\text{-}CH_2$	$3\text{-}CH_2$	4-CH	5-CH	6-CH	7-CH
	(s)	(t)	(t)	(s)	(t)	(t)	(d)
1mM DPH	2.923	3.450	3.872	5.662	7.418	7.484	7.519
10mM DPH	2.923	3.437	3.867	5.651	7.413	7.478	7.512
10mM DPH/7.25×10^{-5}M HSA	2.923	3.436	3.860	5.641	7.401	7.469	7.503

* External reference, tetramethylsilane; pH=7, 40°C; (s), singlet; (d), doublet; (t), triplet.

Spin-Lattice Relaxation Time of DPH

Spin-lattice relaxation time (T_1) of DPH was independent of the concentrations (1 - 10mM)(Table 4). The addition of HSA (7.25×10^{-5}M) to DPH (10mM) caused a significant decrease in the relaxation time. The T_1 values of aromatic and aliphatic protons decreased to 32.8-43.9% and 56.7-78.4% of their original values, respectively. Singnificant decrease in the T_1 values of aromatic protons proved that DPH bound to HSA through the hydrophobic phenyl moiety.

Table 4: Spin-Lattice Relaxation Time (T_1,s) of DPH*

	1-CH_3	2-CH_2	3-CH_2	4-CH	5-CH	6-CH	7-CH
1mM DPH	1.669	1.261	1.266	2.354	5.337	4.130	3.930
10mM DPH	1.590	1.190	1.214	2.267	5.099	3.873	3.720
10mM DPH/7.25×10^{-5}M HSA	1.161	0.933	0.934	1.285	1.672	1.677	1.634

*pH=7, 40°C

Spin-Spin Relaxation Rate

Spin-spin relaxation time (T_2) of DPH was measured by CPMG method (Figure 3). Spin-spin relaxation rates ($1/T_2$) of DPH were almost independent of the concentrations (1 - 10 mM) (Table 5). The addition of HSA (7.25×10^{-5}M) to DPH (10 mM) caused a significant increase in the relaxation rate. Owing to the lack of HSA on chemical shift, slow exchange between free and bound states was reported to cause the superposed narrow and broad peaks in the NMR spectrum. In this work, however, rapid exchange between both sites was understood, since one peak was observed as the weighted average of two states. In a slow exchange system, T_2 measurement by CPMG method was found to be difficult because of modulation action by repeated irradiation of the pulse. In this work, a series of echoes measured by CPMG method decayed according to Eq.2 without modulation. Therefore, the exchange between free and bound states in our system was suggested to be rapid, similar to the case reported by Jardetzky (1964). The spin-spin relaxation rate of drug bound to albumin, $(1/T_2)_b$, was calculated according to the equation proposed by Jardetzky:

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

Here $(1/T_2)_f$ is the spin-spin relaxation rate of the free drug, and B is the proportion of the drug bound to albumin. The B value can be calculated from the binding constant (K) and the number of binding sites (n) obtained by equilibrium dialysis. Fehske et al. (1979) reported that the binding sites of two hydrophobic drugs, warfarin and diazepam, were tryptophan and tyrosine residues on HSA, respectively, and that a maximum 10.7 units of 18 units of tyrosine in HSA were modified. Therefore, the largest number of hydrophobic binding sites on HSA was assumed to be 12, which consisted of 1 unit tryptophan and 11 units tyrosine. The values of $(1/T_2)_b$ were calculated according to Eq.4. However, the discussion of the binding position on the bases of $(1/T_2)_b$ values was not reasonable, since the relaxation rates of the respective protons of DPH, $(1/T_2)_{f2}$, were different from each other. The ratio $(1/T_2)_b/(1/T_2)_{f2}$ has a significant meaning for determination of the binding position, since the sequence of the ratio was independent of the arbitrary n-values (1 or 12), although the absolute values of the ratio depended on these n-values. As can be seen from Table 4, the 6-CH proton had the largest value of $(1/T_2)_b/(1/T_2)_{f2}$, followed by the 5-CH proton. It was therefore concluded that the binding position of DPH to HSA was at the hydrophobic aromatic moiety. In Figure 4, the contour plot of the $(1/T_2)_b/(1/T_2)_{f2}$ values was illustrated for visual representation of the binding position.

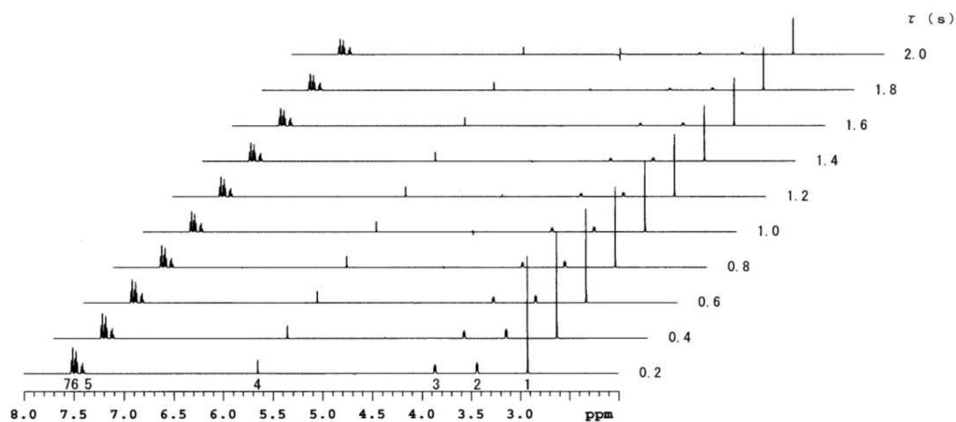

Figure 4: Spin-Spin Relaxation Traces Obtained by the CPMG Method for the Protons of DPH

Table 5: Spin-Spin Relaxation Rates ($1/T_2$, s^{-1}) of DPH*

				***n* = 1**		***n* = 12**	
Peak	**$(1/T_2)_{f1}$ (s^{-1})**	**$(1/T_2)_{f2}$ (s^{-1})**	**$1/T_2$ (s^{-1})**	**$(1/T_2)_b$ ($\times 10^3 s^{-1}$)**	**$(1/T_2)_b/(1/T_2)_{f2}$ ($\times 10^3$)**	**$(1/T_2)_b$ ($\times 10^3 s^{-1}$)**	**$(1/T_2)_b/(1/T_2)_{f2}$ ($\times 10^3$)**
1-CH_3	0.41	0.37	4.59	6.07	1.65	7.46	2.03
2-CH_2	0.85	0.84	10.29	13.59	1.62	16.70	1.99
3-CH_2	1.05	0.89	9.62	12.56	1.41	15.45	1.74
4-CH	0.21	0.23	10.82	15.22	6.64	18.63	8.14
5-CH	0.14	0.09	4.37	6.15	6.71	7.53	8.22
6-CH	0.26	0.16	8.18	11.53	7.30	14.11	8.93
7-CH	0.34	0.21	9.02	12.66	6.00	15.50	7.35

* $(1/T_2)_{f1}$, free (1mM) observed; $(1/T_2)_{f2}$, free (10mM) observed; $1/T_2$, overall observed (10mM DPH/7.25×10^{-5}M HSA); $(1/T_2)_b$ DPH bound to HSA calculated; *n*, number of binding sites on HSA.

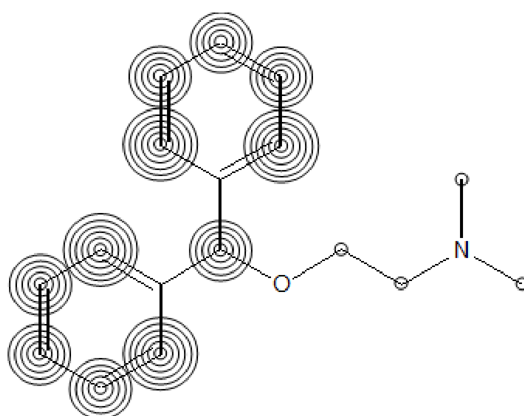

Figure 5: Contour Plot of the Ratio of the Spin-Spin Relaxation Rate of Bound DPH to Free DPH

REFERENCES

1. M. Tanaka, K. Minagawa, M. R. Berber, I. H. Hafez, and T. Mori, Interaction between drugs and biomedical materials I: Binding position of bezafibrate to human serum alubmin, *Int. J. Mod. Phys. Conf. Ser.* Vol. 6, 2012, pp.751-756.
2. E. L. Hahn, Spin echoes, Phys. Rev. Vol. 80, 1950, pp.580-594.
3. S. Meiboom and D. Gill, Modified spin-echo method for measuring nuclear relaxation times, *Rev. Sci. Instrum.* Vol. 29, 1958, pp.688-691.
4. G. F. Scatchard, The attractions of proteins for small molecules and ions, *Ann. N. Y. Acad. Sci.* Vol. 51, 1949, pp.660-672.
5. O. Jardetzky, in: J. Duchesne (Ed.), *Advances in Chemistry and Physics,* Vol. VII, Interscience, New York, 1964, p.499.
6. K. J. Fehske, W. E. Müller, and U. Wollert, A highly reactive tyrosine residue as part of the indole and benzodiazepine binding site of human serum albumin, *Biochim. Biophys. Acta* Vol. 577, 1979, pp.346-359.
7. K. J. Fehske, W. E. Müller, U. Wollert, and L. M. Velden, *Mol. Pharmacol.* Vol. 16, 1979, pp.778-789.