NMR Study on the Low-Affinity Interaction of Human Serum Albumin with Diclofenac Sodium

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The low-affinity interaction between human serum albumin (HSA) and Diclofenac sodium (DCF) was studied using NMR techniques. Both ^{13}C-NMR chemical shift and linewidth show that the dichlorophenyl ring in DCF molecule plays a primary role in its interaction with HSA. Langmuir adsorption isotherm was applied to evaluate the association constant *K* and the number of binding sites *n* of the drug/HSA complex through ^{1}H-NMR spin-lattice relaxation measurement. The results indicate that Langmuir isotherm can perfectly explain the capacity of low-affinity binding of proteins for the ligands.

Key words Diclofenac sodium; human serum albumin; NMR; spin-lattice relaxation rate; Langmuir adsorption isotherm

Human serum albumin (HSA) is the major plasma protein responsible for the reversible binding of a wide range of drugs.[1)] Drug–HSA interaction has been intensively studied because this interaction can serve as a means of drug storage, control of the drug delivery to tissue receptors, and prevention of the drug from being metabolized rapidly. This is important for the biological distribution of the drug compounds as well as their excretion, therapeutic activity and toxicity.

HSA interacts with drugs through its binding sites. There are mainly two classes of binding sites on the HSA molecule.[2)] One is the high-affinity binding sites and the other is the low-affinity binding sites. The high-affinity binding sites, which have the properties of low capacity, have been the main topic of study for a long time by various analytical techniques, including equilibrium dialysis and ultrafiltration.[3)] Recently, high-performance liquid chromatography (HPLC), capillary electrophoresis, microdialysis and some spectroscopy methods, such as circular dichroism and fluorescence, were added to this list of the applied technique.[4)] The high-affinity binding sites have been further divided into several groups according to their selectivity to the drugs. Sudlow *et al.*[5,6)] identified two distinct binding sites on albumin for acidic drugs based on their abilities to displace the fluorescent dansylamino acid probes 5-(dimethylamino)-naphthalene-1-sulfonamide (DNSA, site I) and dansylsarcosine (site II). Sudlow's site I and II are commonly known as the warfarin site and the diazepam site, respectively. In a recent report,[7)] the site I is further divided into site I (the phenylbutazone binding site) and site III (the digitoxin binding site). The low-affinity binding sites have the properties of high capacity.[8,9)] It means that in this type of binding one HSA molecule can bind many drug molecules but the interaction is very weak. It has been found that many drugs are involved in this type of low-affinity binding interaction, especially when the drug concentration is much higher than that of HSA in blood plasma. In this case the drug is bound first to the high-affinity binding sites and then the drug in excess is bound to the low-affinity binding sites.[10)] Both mechanisms of binding contribute to the biological effect of drugs.

Diclofenac sodium (DCF) (Fig. 1) is a nonsteroidic antiinflammatory drug (NSAID) from the group of the arylalcanoic acid derivatives and with large therapeutic applicability in the symptomatic standard treatment of the rheumatic affections.[11)] It has been pointed out that the HSA binding is a major determinant of the pharmacokinetics and pharmacodynamics of this class of compounds.[12)] The high-affinity binding of DCF to HSA has been a subject of several publications.[13—16)] It is supposed that two high-affinity binding sites exist on HSA for DCF with one site each.[15)] One ($K_1=5\times10^5\ \mathrm{M}^{-1}$) is likely to be the benzodiazepine binding site, and the other ($K_2=0.6\times10^5\ \mathrm{M}^{-1}$) is the warfarin site. But so far there is no information about the drug's low-affinity binding to HSA.

NMR has been extensively used as a useful method for obtaining information on the interactions between macromolecules and small ligand molecules.[10,17—24)] However, in studies on the high-affinity binding between proteins and drug molecules, the application of this spectroscopic method has been limited to small proteins.[17)] When the drug molecules are tightly bound to the high-affinity sites of a large protein as HSA, the line-broadening effect makes the drug NMR signal non-observable.[10)] However, NMR is suitable for studies of the weak low-affinity interaction, where the drug molecules in free and bound states are in fast exchange on the NMR time scale. In such a case, NMR parameters of drug molecules, such as chemical shifts, relaxation rates and self-diffusion coefficients are the weighted-average of the free and bound states. By the measurement of these ^{1}H-NMR parameters both the binding position of a drug to albumin[18,19)] and the dynamic parameters of this interaction can be determined.[20—22)] ^{13}C-NMR can also be applied in the field. But till now only ^{13}C-enriched drug molecules have been used.[23,24)]

In this paper, we studied the low-affinity interaction between HSA and DCF with NMR technique. ^{13}C-NMR chemical shift and linewidth were used to determine the binding

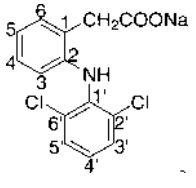

Fig. 1. The Chemical Structure of DCF

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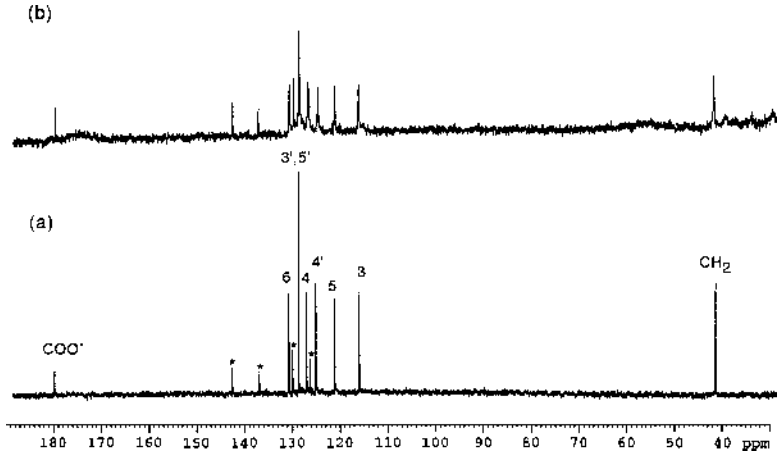

Fig. 2. ^{13}C-NMR Spectra of (a) a Solution of DCF (40 mM) with Resonance Assignments (*: Signals from C-1, -2, -1′, -2′, -6′) and (b) the Same as (a) But in the Presence of HSA (0.5 mM)

Chemical shifts are referenced to TMS (external reference).

position on DCF because this method, while necessitating long accumulation times, has the advantage over ^{1}H-NMR by affording less complicated spectra. We also use ^{1}H-NMR relaxation measurement to infer dynamic parameters of the interaction from the Langmuir adsorption isotherm.

Experimental

HSA (fraction V) was purchased from Sigma Chemical Co. and used without further purification. DCF was obtained from Wuhan Pharmaceutical Co., Ltd. (Wuhan) and purified by recrystallization. Eleven NMR samples were prepared by quantitatively dissolving the substances in phosphate buffer at pH 7.4. The concentration of HSA (C_P) was kept 0.5 mM for all samples, while the concentration of DCF varied. For lock purposes, 10% D_2O (v/v) was added to the solution.

All NMR experiments were performed on a Bruker ARX-500 spectrometer at 37 °C (310 K), using a probe tuned at 500.13 MHz and 125.77 MHz for ^{1}H and ^{13}C, respectively. Spin lattice relaxation times were measured using the standard inversion-recovery method. The water signal was effectively suppressed using phase-shift presaturation during both the pre-pulse delay and the recovery period.[25]

Results and Discussion

Measurements of ^{13}C-NMR Chemical Shifts and Linewidths The ^{13}C-NMR spectra of DCF with and without HSA are shown in Fig. 2. The assignment was confirmed by ^{1}H-detected heteronuclear multiple quantum coherence (HMQC) measurement (data not shown), as indicated in the figure (Fig. 2a). The chemical shifts at 142.6, 136.9, 129.9, 126.3 ppm are signals from some quaternary carbons (C-1, -2, -1′, -2′, -6′) and are not assigned. It is obvious that the lines are broadened in the presence of HSA compared with the absence of HSA. The linewidths at half-height ($\Delta\nu_{1/2}$) and the chemical shifts (δ) of DCF signals are shown in Table 1, where $\Delta\delta$ and X denote the variation of chemical shifts (δ) and the ratio of linewidth at half-height ($\Delta\nu_{1/2}$) of DCF in the presence and absence of HSA, respectively. The $\Delta\nu$ and X values of C-1, -2, -1′, -2′, -6′ are respectively in the range of ±12.6—25.2 Hz and 2.8—3.5 and are not shown in the Table 1. The single peak of each DCF carbon in the presence of HSA could be best explained by assuming a rapid exchange between bound and free DCF.[26] The change in ^{13}C chemical shift of DCF induced by HSA–DCF interaction is very small, which could be attributed to the high DCF/HSA molar ratio (80 : 1). Although small, the variation of chemical shift still gives some information. From the chemical structure of DCF (Fig. 1) , the DCF molecule is composed of a phenylacetate group and a dichlorophenyl ring where C-4′ is located. The $\Delta\delta$ value of C-4′ is −62.9 Hz, whereas that of other carbons are in the range of ±12.6—25.2 Hz. From these results, it could be assumed that the dichlorophenyl ring attaches to HSA.

Table 1. Chemical Shift and Linewidth at Half-Height of the ^{13}C-NMR of DCF and the Effects of Binding to HSA

Assignment	δ (ppm)	DCF-HSA[a] / δ (ppm)	$\Delta\delta$ (Hz)[b]	$\Delta\nu_{1/2}$ (Hz)	DCF-HSA[a] / $\Delta\nu_{1/2}$ (Hz)	X[c]
COO^-	179.7	179.5	−25.2	5.4	12.9	2.4
C-6	130.7	130.6	−12.6	5.1	26.8	5.3
C-3′, 5′	128.7	128.6	−12.6	5.1	26.2	5.1
C-4	127.0	126.8	−25.2	5.1	26.5	5.2
C-4′	125.1	124.6	−62.9	4.8	36.1	7.5
C-5	121.1	121.0	−12.6	5.7	29.9	5.2
C-3	115.9	116.0	12.6	5.4	31.7	5.9
CH_2	41.3	41.5	25.2	5.4	30.8	5.7

a) The concentration ratio of DCF: HSA is 80 : 1. *b*) Difference in chemical shift between DCF in the presence and absence of HSA. *c*) The ratio of linewidth at half-height ($\Delta\nu_{1/2}$) of DCF in the presence of HSA to that in the absence of HSA.

The ^{13}C-NMR line broadening of the DCF resonances also supports the assumption. The X value for C-4′ (7.5) is larger than that of other carbons (2.4—5.9). This indicates that the dichlorophenyl ring plays an important role in the binding of DCF to HSA.

The phenylacetate group, with an intramolecular hydrogen bond between the carboxyl oxygen and the amino hydrogen,[27] is rather hydrophilic. Because the low-affinity binding is mainly a kind of hydrophobic interaction,[18] it is not difficult to understand that the dichlorophenyl ring has a stronger affinity than the phenylacetate group to HSA. It is noted that C-3′ and C-5′, also located at the dichlorophenyl ring, have both $\Delta\delta$ (−12.6 Hz) and X (5.1) values smaller than that of C-4′. This can be explained by the fact that both of C-3′, -5′ carbon atoms are adjacent to the chlorine atoms (see Fig. 1), which may hinder and weaken the interaction of them with HSA.

It is noted that few applications of ^{13}C-NMR in study on the drug–protein interaction because of the low natural abundance and poor relative sensitivity of ^{13}C nucleus. But this method has some advantages over ^{1}H-NMR. It gives larger disperse chemical shifts that makes the signals less overlapped and the interpretation of observed spectra relatively easy. In addition, there is no background ^{13}C signal in water and the solvent suppression is unnecessary. It is expected that when either ultra-high field NMR spectrometer or ^{13}C-enriched drugs are available, ^{13}C-NMR can also be applied to the process described below to evaluate dynamic parameters of the drug–protein interaction.

Langmuir Adsorption Isotherm for Drug–Protein Interaction Langmuir isotherm is an equation used to describe the dynamical equilibrium between adsorption and desorption of gaseous molecules at solid surfaces. It is known that this equation can be applied not only to gas–solid interaction but also to liquid–solid interaction. Two assumptions have been adopted for the Langmuir isotherm: (i) uniform monolayer adsorption takes place; (ii) there is no interaction between adsorbate molecules, which means that all the binding sites at the surface are equivalent and the ability of a gas molecule to bind to one site is independent of whether or not the neighboring sites are occupied.[28)]

Compared to the small drug molecule, the HSA molecule will provide an enormous surface to adsorb (or bind) the drug molecules. Because the low-affinity binding is weak and the free and bound drugs are in the fast exchange, it is reasonable to assume that the two assumptions for Langmuir isotherm are also applicable for the low-affinity binding between a drug and HSA. The binding process can be described by the equation

$$\text{drug}+\text{HSA} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{complex} \tag{1}$$

Suppose that each HSA molecule has a maximum of n sites available for accepting ligand molecules, and the total concentration of HSA and ligand in solution are C_P and C_L, respectively. We have the following relations:

$$nC_P=[L_B]+[P] \tag{2}$$

$$C_L=[L_F]+[L_B] \tag{3}$$

where [P] is the concentration of free binding sites of HSA, $[L_F]$, $[L_B]$ are the concentration of free and bound ligand, respectively. From the two assumption of the Langmuir isotherm, the following equations can be derived:

$$\text{rate of adsorption}=k_1[L_F](nC_P-[L_B]) \tag{4}$$

$$\text{rate of desorption}=k_{-1}[L_B] \tag{5}$$

When the equilibrium between adsorption and desorption occurs, the right side of the above two equations must equal. Then we can get a relation analogous to the Langmuir isotherm:

$$[L_B]/nC_P=K[L_F]/(1+K[L_F]) \tag{6}$$

or

$$[L_B]=nC_PK[L_F]/(1+K[L_F]) \tag{7}$$

where $K=k_1/k_{-1}=[L_B]/[L_F](nC_P-[L_B])$, is the association constant for the formation equilibrium of the drug/HSA complex. $[L_B]$ and $[L_F]$ are the equilibrium concentration of bound and free ligand, respectively.

When data for $[L_B]$ and $[L_F]$ are available, plotting $[L_B]$ *versus* $[L_F]$ and fitting the curve with Eq. 7 will simultaneously yield the two parameters, n and K.

Data for $[L_B]$ and $[L_F]$ can be obtained through NMR measurement. The observed NMR parameters, the relaxation rates or self-diffusion coefficients, should be a weighted average of the contributions from bound and free molecules and should be expressed in the form:[29)]

$$Y_{obs}=X_BY_B+X_FY_F=X_B(Y_B-Y_F)+Y_F \tag{8}$$

where Y is relaxation rates or self-diffusion coefficients, and X_B and X_F are the fractions of the bound and free drug molecules defined by $[L_B]/C_L$ and $[L_F]/C_L$, respectively. By measuring a series of Y_{obs} data as a function of the concentration ratio C_P/C_L and by extrapolating the observed Y_{obs} data respectively to zero and infinite C_P/C_L, Y_B and Y_F, $[L_B]$ and $[L_F]$ can be determined.

The ^{1}H-NMR spectra of DCF in the absence and presence of HSA are shown in Fig. 3. For all concentrations, signals from HSA are negligibly weak because of its low concentration (0.5 mM) and the naturally broadened line shapes. Meanwhile, all signals from DCF are serious broadened due to the high viscosity of the solution and the exchange between the free and bound molecules (Fig. 3b). Because the signal from 4′-H is overlapped with that from 4-H, signals from other aromatic protons (3′,5′-, 6-, 5-, 3-H) of DCF are monitored. The measured longitudinal relaxation rates $R_{1\,obs}$ for 3′,5′-, 6-, 5- and 3-H at various concentrations are showed in Fig. 4. The results show that as the ratio of C_P/C_L is increased, the observed relaxation rates $R_{1\,obs}$ are correspondingly increased. Take 5-H for example. Extrapolating $R_{1\,obs}$ to infinite C_P/C_L gives $R_{1B}=2.71\ s^{-1}$ and to zero C_P/C_L gives $R_{1F}=1.16\ s^{-1}$. Based on the fast exchange model in Eq. 8 and the definition of X_B and X_F, a set of values of $[L_B]$ and $[L_F]$ can be determined. The plot of the values of $[L_B]$ *versus* $[L_F]$ for 5-H of DCF is shown in Fig. 5, which can be fitted by Langmuir adsorption isotherm, Eq. 7. Doing so, the association constant K and the number of binding sites n are simultaneously obtained. Table 2 shows the apparent association constant of DCF binding to HSA and number of binding sites on HSA by NMR relaxation measurements of 3′,5′-, 6-, 5- and 3-H of DCF molecule. The average association constants and number of binding sites are $K=(1.70\pm0.23)\times10^{2}\ M^{-1}$, $n=81\pm1$.

It should be pointed out that the model used in this study could only be used for fast ligand exchange between the free and bound states. In the case of slow exchange, Eq. 8 is not valid any more, because $R_{1\,obs}$ can not be defined and R_{1B} could not be measured due to extremely broadened NMR signals. X-ray studies of complexes of HSA with various kinds of ligands has proven that there are hydrophobic cavities in the subdomains IIA and IIIA of HSA, which can host drug molecule.[30,31)] It has been concluded that Sudow's site I corresponds to the subdomain IIA binding cavity and site II to the subdomain IIIA binding cavity.[32)] This conclusion is in good agreement with many spectroscopic studies that the number of the high-affinity binding sites for most drug molecules to HSA are between 1 and 2. Since the cavities well fit the small ligand molecules, it is easy to understand that the

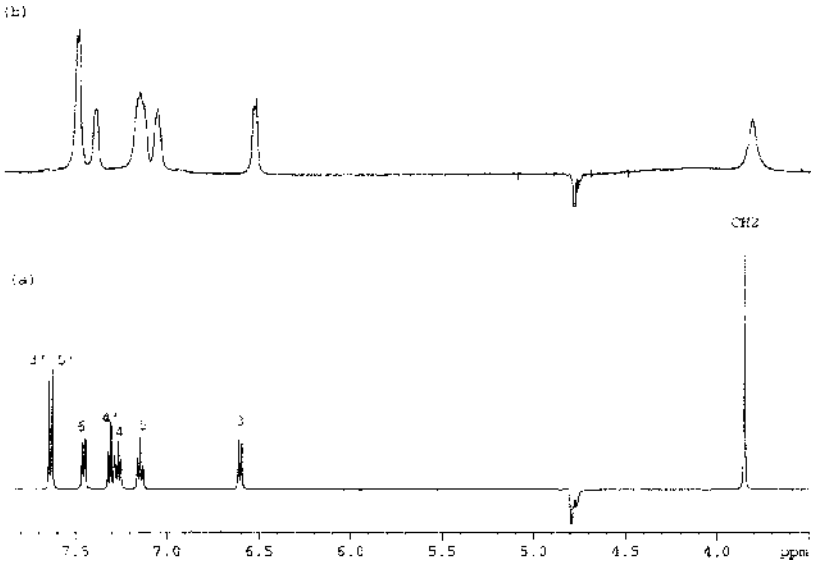

Fig. 3. ^{1}H-NMR Spectra of (a) a Solution of DCF (75 mM) with Resonance Assignments and (b) the Same as (a) But in the Presence of HSA (0.5 mM)
Chemical shifts are referenced to H_2O (4.800 ppm).

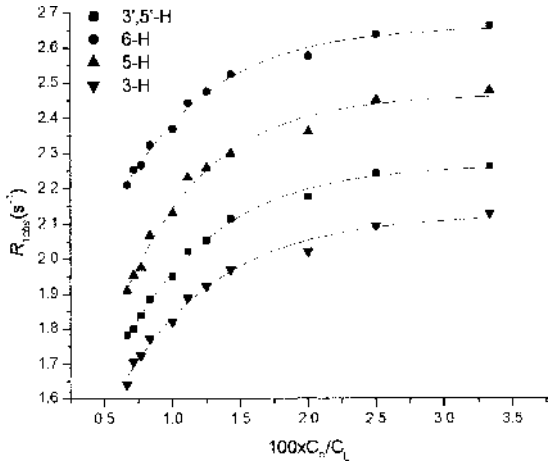

Fig. 4. Relaxation Rates of Aromatic Protons in DCF, 3′,5′-, 6-, 5- and 3-H, as a Function of the Concentration Ratio $100\times C_P/C_L$, Where C_P Is the Total Concentration of HSA and C_L Is the Total Concentration of DCF

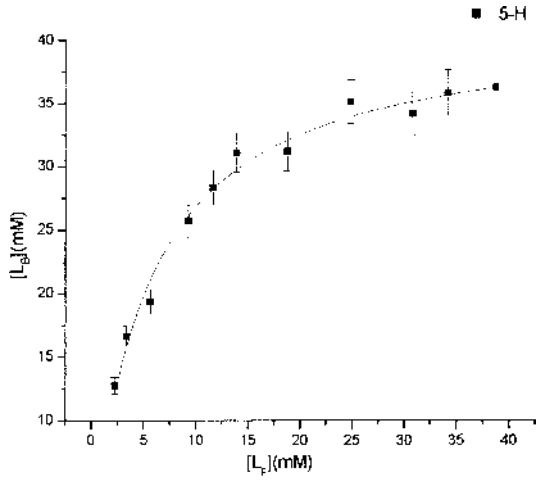

Fig. 5. Plots of $[L_B]$ *versus* $[L_F]$ for the Binding Interaction between HSA and DCF
The data of $[L_B]$ and $[L_F]$ are from the relaxation rate measurements. Solid curve is the simulated result using Langmuir isotherm equation, Eq. 7.

associate constants, *K*, of this kind of HSA/drug complexes are very large, usually in the order of 10^5—10^6 M^{-1} and the binding process must be in slow exchange. However, as mentioned above this kind of binding can not be observed in our study. We used high concentration ratios of drug to HSA to ensure that the high-affinity binding sites were fully saturated. So, although both the high-affinity binding and the low-affinity binding occurred at the same time in this case, only the information of low-affinity binding can be obtained in our study.

As macro biomolecules, not only proteins but also DNA, RNA or enzymes, can interact with small molecules, it is expected that the Langmuir isotherm will be widely used in the studies of the binding dynamics of small molecules to biomolecules.

Since only free or unbound drug is pharmacologically active and the concentration of unbound drug in serum is determined by not only high-affinity but also low-affinity interaction between drug and serum protein,[8,9] the pharmacological activity of a drug is closely related to its low-affinity binding to HSA. On the other hand, it is known that HSA is in charge of storing and transporting drug molecules in human body, and the stored drugs in the HSA–drug complex can be released whenever and wherever they are needed. As we know the high-affinity interaction between drug and HSA is extremely strong with an enormously large association con-

Table 2. Determination of Apparent Association Constant (*K*) of DCF Binding to HSA and Number of Binding Sites (*n*) on HSA by NMR Relaxation (*R*) Measurements

	3′,5′-H	6-H	5-H	3-H	Average
K (10^2 M^{-1})	1.50	1.93	1.86	1.49	1.70±0.23
n	80	80	83	81	81±1

stant. In this case the drug molecule attaches to HSA so tightly as to it could hardly be released from the high-affinity drug–HSA complex and HSA would not play the role of transporting drug molecules. In contrast, the low-affinity interaction can account for the ability of HSA to transport drugs after the drugs are orally administered because only when the interactions between drug and HSA are very weak can a drug/HSA complex easily release drug molecules. From these points of view, the study of low-affinity interaction between drugs and macromolecules is of great significance in the pharmacology and pharmacokinetics studies. The approach described here will be helpful to the researchers in this field.

Conclusions

The low-affinity interaction between DCF and HSA has been studied. Useful information has been obtained from ^{13}C-NMR chemical shift, linewidth and ^{1}H-NMR relaxation measurement. Undoubtedly, both the binding position of a drug molecule to HSA and accurate determination of the binding isotherm of drug–HSA complexes over a wide range of drug concentration are crucial for understanding the formation process of this kind of interaction. ^{13}C-NMR can be used for determining the binding position of a drug molecule. As a means of describing the dynamical equilibrium between the adsorption and the desorption of large surface on small molecules, Langmuir isotherm can perfectly explain the capacity of low-affinity binding between proteins and ligands.

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