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Journal of Pharmaceutical and Biomedical Analysis
34 (2004) 247–254
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
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Analysis of competitive binding of ligands to human serum albumin using NMR relaxation measurements

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Received 27 July 2003; received in revised form 14 August 2003; accepted 2 September 2003

Abstract

The competitive binding of two ligands, ibuprofen (IBP) and salicylic acid (SAL), to human serum albumin (HSA) was studied by using nuclear magnetic resonance (NMR) relaxation measurements. When the concentration of one ligand was increased in the solution containing IBP, SAL and HSA, the fractions of free IBP and SAL were increased because of the competitive binding. The ^{1}H relaxation rates (R_1) of both ligands were subsequently decreased. If a ligand is in fast exchanging between the free and bound forms, the observed ^{1}H relaxation rate is a weighted average of that for the free ligand and the protein–ligand complex. The concentrations of the free and bound ligands can be quantitatively derived from the relaxation rates. The results presented in this work revealed that IBP and SAL shared certain low-affinity binding sites on the HSA molecule, in addition to the same high-affinity binding site of AIII.

Keywords: NMR spectroscopy; ^{1}H relaxation rate; Competitive binding; Ibuprofen; Salicylic acid; Human serum albumin

1. Introduction

Human serum albumin (HSA) is a principal binding protein in blood plasma for a large number of drugs [1–8]. The binding process controls the concentration of the free or bioactive, drug, and hence affects the drug's pharmacokinetics, storage, toxicity, transportability to the tissue and through cell membranes [6,7]. It is, therefore, of great interest to study HSA–drug interactions. HSA has three high-affinity binding sites, named the phenylbutazone binding site (Site I), the diazepam binding site (Site II) and the digitoxin binding site (Site III), respectively [8,9]. Sites I and III are also known as the warfarin binding site [8]. The name of the binding site is taken originally from the model compound used to probe the site. In addition, there are many low-affinity binding sites on the HSA molecule. The low-affinity binding generally has higher binding capacity and results in many molecules bound simultaneously to the HSA molecule. If two kinds of drug, with similar molecular structure, are co-administrated, competitive binding is expected, and the concentration

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doi:10.1016/S0731-7085(03)00579-X

ratio of the free drugs in the blood may be different from that administered. Some of the metabolites in serum may also compete for the same binding sites of a drug and, therefore, affect the binding process of the drug.

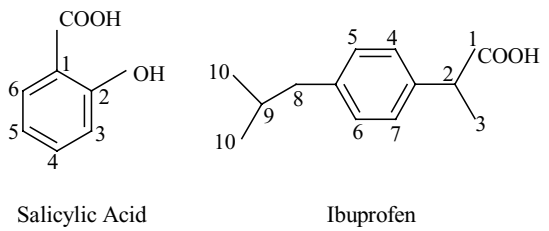
The competitive binding of drugs to serum albumin has been studied using various techniques. Recently, the extent and nature of cosalane, a potent inhibitor of HIV replication, binding to mucin, α-(1)-acid glycoprotein (AAG), plasma, and human and bovine serum albumin, has been examined via competitive inhibition studies in the presence of salicylic acid (SAL) by a gel filtration technique [10]. The binding constant of several drugs toward IIIA subdomain of HSA was determined using near-infrared dye-displacement capillary electrophoresis [11]. Another study using equilibrium dialysis showed [12] that the HSA Site II-ligand indoxyl sulfate influenced the binding of dansyl-L-asparagine at the azapropazone binding area in Site I, but did not affect the warfarin binding area of Site I. The pH-profile showed that interaction between indoxyl sulfate and dansyl-L-asparagine was very sensitive to the N-conformer to B-conformer transition of HSA and competitive interaction was observed for binding of the two ligands to the N-conformer (pH 6.5), whereas in the B conformation (pH 8.5), binding of these molecules was nearly "independent" [12]. Sulbenicillin isomers can displace each other competitively at high-affinity binding site (Site I, stereoselective) and low-affinity binding sites (non-stereoselective) on HSA by using site marker ligands [13]. Nuclear magnetic resonance (NMR) spectroscopy is used here as an alternative approach to analyze the competitive binding of two drugs to HSA.

Ibuprofen (IBP) is a non-steroidal anti-inflammatory drug and can bind to HSA at Site III [2] with a dissociation constant of about 10^{-6} M [9]. Because of its high binding affinity and selectivity, IBP has been used extensively as a model compound in the study of drug–HSA interaction [14–19]. Salicylic acid binds to HSA at Sites II and III with nearly equal distribution [2]. A recent study using NMR spectroscopy has shown that both IBP and SAL have tens of low-affinity binding sites on HSA [20]. Sharing the same high-affinity binding Site III and having large number of low-affinity binding sites on HSA, IBP and SAL are expected to interfere with each other in the binding process and may be used as an ideal system for studying the competitive low-affinity binding of ligands to HSA.

In the present work, we demonstrate that NMR spectroscopy can be used as an alternative approach to study the competitive binding of two ligands (IBP and SAL) to protein (HSA) at the low-affinity binding sites. We utilized an excess of ligands, IBP and SAL, over the albumin in order to saturate the high-affinity binding sites and to ensure that the competitive binding of IBP and SAL to HSA mainly occurs at the low-affinity binding sites. The competitive binding was analyzed quantitatively using NMR based ^{1}H spin-lattice relaxation (R_1) measurements. The values of R_1 were used to derive the fractions and concentrations of the bound and free ligands.

2. Experimental

Human serum albumin (fraction V), ibuprofen sodium salt and salicylic acid were bought from Sigma (Poole, Dorset, UK) and used without purification. For convenience, the numbering systems and molecular structures of SAL and IBP are shown in Scheme 1. Three groups of samples were prepared in phosphate buffer (pH 7.4, 0.2 M), in which 10% D_2O was added for the NMR spectrometer frequency lock. The first group of the samples contained only IBP and SAL and was used to measure ^{1}H relaxation rates of the free ligands (R_{1f}). The second group consisted of HSA (0.2 mM) and one of the ligands of variable concentrations (IBP: 4.0–60 mM or SAL: 4.0–65 mM). The samples in this group were used to extrapolate ^{1}H relaxation rates of the bound form (R_{1b}). The third group consisted of two sets of samples and was used to study the competitive binding of IBP and SAL to HSA. In the first set, the concentrations of HSA and SAL were 0.2 and 8.0 mM, and the contents of IBP



Scheme 1. The molecular structures and numbering systems of SAL and IBP.

were varied: 4.0, 8.0, 12.0, 16.0 and 20.0 mM. In the other set, the contents of HSA (0.2 mM) and IBP (8.0 mM) were fixed, and the concentrations of SAL were varied: 4.0, 8.0, 12.0, 16.0, 20.0, 24.0 mM.

All NMR experiments were carried out at 298 K on a Varian Inova-500 instrument, operating at proton frequency of 500.12 MHz. The longitudinal relaxation rates (R_1) of the protons were measured using standard inversion-recovery methods with solvent saturation pulses being implemented into the pre-scan delay and recovery delay periods. The 16 recovery delays randomly ranged from 0.1 to 4 s and 0.1 to 20 s were used for the solutions with and without HSA, respectively. Typically, 32 transients were acquired into 16 k complex data points over a spectral window of 6000 Hz. These data were multiplied by a cosine-shaped window function ($0 \sim \pi/2$) to improve the signal-to-noise ratio and were zero-filled by a factor of two prior to Fourier transformation. The areas of the NMR peaks were used to derive the relaxation rate using a three parameter equation of $A(t) = A_0 - [A_0 - A(0)]\exp(-R_1 t)$, where $A(t)$, $A(0)$ and A_0 are the peak areas at the recovery time of t, 0 and at the thermal equilibrium, respectively.

3. Results and discussion

Fig. 1 shows the aromatic region of one-dimensional ^{1}H NMR spectra of IBP and SAL with and without HSA. In solutions of IBP and SAL (Fig. 1a), there were no observable changes in ^{1}H NMR chemical shifts or lineshapes when the concentration of either IBP or SAL varied. There were no intermolecular NOE between IBP and SAL in the NOESY spectrum with mixing times as long as 1 s (data not shown). These results revealed that the molecular dynamics of ibuprofen and salicylic acid were independent in the mixture without HSA under the experimental conditions. The relaxation rates measured in the solutions without HSA were assigned to the free forms (R_{1f}): $0.486 \pm 0.018\,s^{-1}$ (H4, 7), $0.53 \pm 0.02\,s^{-1}$ (H5, 6), $1.35 \pm 0.02\,s^{-1}$ (H3), $0.99 \pm 0.02\,s^{-1}$ (H10) for IBP, and $0.19 \pm 0.01\,s^{-1}$ (H6), $0.19 \pm 0.01\,s^{-1}$ (H4), $0.19 \pm 0.01\,s^{-1}$ (H3, 5) for SAL.

Significant NMR line broadening and chemical shift up-field drift were observed when 0.2 mM HSA was present in solutions containing of IBP and SAL (Fig. 1b and 1c). It can be seen from the figure that the IBP ^{1}H NMR line-widths were broader than those of SAL at the same molar ratio of IBP to HSA and SAL to HSA (Fig. 1b). When the IBP concentration was increased from 8.0 mM (Fig. 1b) to 20.0 mM (Fig. 1c), the peaks of both IBP and SAL became sharper and the chemical shifts drifted toward the values of the free forms. These results are listed in Table 1. The sharpness of IBP resonances can be understood as an increased fraction of the free form, or decreased proportion of the bound form, at the higher

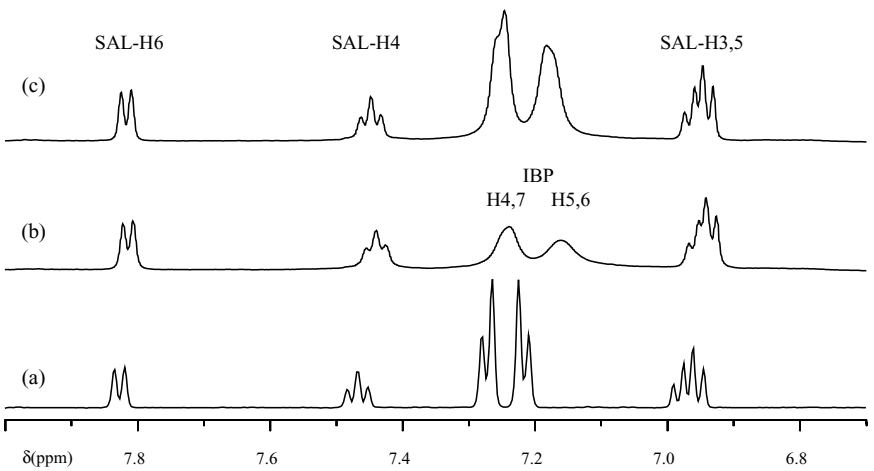


Fig. 1. Aromatic regions of 1D ^{1}H NMR spectra of IBP and SAL in solutions containing (a) 4.0 mM IBP and 4.0 mM SAL, (b) 8.0 mM IBP, 8.0 mM SAL and 0.2 mM HSA, (c) 20.0 mM IBP, 8.0 mM SAL and 0.2 mM HSA. The systematic line broadening and chemical shifts up-field drifts revealed the competitive binding of IBP and SAL to HSA.

Table 1
Relative chemical shift changes ($\Delta\delta$) of the major resonances of IBP and SAL in the absence and presence of 0.2 mM HSA, at different concentrations of IBP and SAL

Concentration (mM)		SAL ($\Delta\delta$, Hz)			IBP ($\Delta\delta$, Hz)			
IBP	SAL	H6	H4	H3, 5	H4, 7	H5, 6	H3	H10
24.0	8.0	0.05	4.96	3.20	8.55	9.70	2.36	9.09
20.0	8.0	0.69	6.06	3.93	8.91	11.90	3.82	10.56
16.0	8.0	1.05	6.79	4.66	10.01	13.73	4.92	12.39
12.0	8.0	1.42	7.52	5.03	10.74	15.93	6.02	13.85
8.0	8.0	2.15	9.72	6.50	12.94	22.52	8.58	18.98
4.0	8.0	3.98	14.85	9.43	18.43	34.60	12.25	27.77
8.0	24.0	3.91	9.70	6.93	9.98	26.16	13.93	19.57
8.0	20.0	3.82	10.21	7.15	10.12	26.99	14.34	19.63
8.0	16.0	4.74	10.53	7.15	10.92	27.60	13.72	19.63
8.0	12.0	4.03	10.59	7.52	10.10	26.66	14.18	19.85
8.0	8.0	4.74	12.22	8.07	9.73	26.37	12.64	20.21

molar ratio of IBP to HSA. The increased resolution of SAL peaks at higher concentrations of IBP revealed that a noticeable fraction of the bound SAL had been replaced by IBP. Similar results were observed when excess SAL was added to solutions containing IBP and HSA. It was noticed that in the system containing IBP, SAL and HSA the chemical shifts of IBP and SAL resonances showed a larger-dependence on the concentration of IBP than that of SAL (Table 1). The results indicated that IBP bound to HSA at higher affinity than SAL and provided the evidence of competitive binding of IBP and SAL to HSA.

Since there were no additional peaks observed in the solutions containing the two ligands and HSA, the binding reaction could be considered as a fast process on the NMR time scale. The competitive binding process can, therefore, be expressed using a fast reversible equilibrum

$$\mathrm{IBP} + \mathrm{HSA}\cdot\mathrm{SAL} \rightleftharpoons \mathrm{HSA}\cdot\mathrm{IBP} + \mathrm{SAL} \quad (1)$$

where HSA·IBP and HSA·SAL represent binary molecular complexes. When the concentration of IBP is increased, the above equilibrium will move to the right side, which leads to increment of the free SAL fraction.

For low-affinity binding, it is also possible to form ternary complexes, IBP·HSA·IBP, IBP·HSA·SAL and SAL·HSA·SAL, if the binding site is large enough. In this case, the first and the secondary disassociation constants are expected to be different for each of the complexes. In this work, we did not attempt to distinguish the binary and ternary complexes for the reason of simplicity.

For a fast reversible binding process, the observed ^{1}H longitudinal relaxation rate (R_{1ob}) of ligand can be regarded as a weighted average of that of the free (R_{1f}) and bound (R_{1b}) forms [21–23]:

$$R_{1ob} = f_f R_{1f} + f_b R_{1b} \quad (2)$$

where f_f (=$[L_1]/C_L$) and f_b (=$1 - f_f$) represent the molar fractions of a ligand in the free and bound forms, respectively. C_{L1} (=$[L_1] + [P\cdot L_1]$) is the total concentration of the ligand L_1. It is assumed here that the value of R_{1b} is independent of the binding site [21–23]. This assumption is based on the fast binding process and the hydrophobic binding mechanism of IBP and SAL to HSA. The assumption also makes the analysis easier for such a complex system with multiple binding sites and two ligands. The ternary site was treated simply as two independent binary sites. Under such a binding model, the fraction of the bound ligand can be derived directly from the relaxation rate [21–23]:

$$f_b = \frac{R_{1ob} - R_{1f}}{R_{1b} - R_{1f}} \quad (3)$$

Fig. 2a and 2b showed the binding induced ^{1}H relaxation rate changes ($\Delta(R_{1ob} - R_{1f})$) of IBP and SAL as a function of their concentration, respectively. From these data, it was possible to extrapolate the relaxation rate of the bound ligand, R_{1b}. The R_{1b} values were $1.90 \pm 0.14\,s^{-1}$ (SAL H6), $1.94 \pm 0.18\,s^{-1}$ (SAL H4),

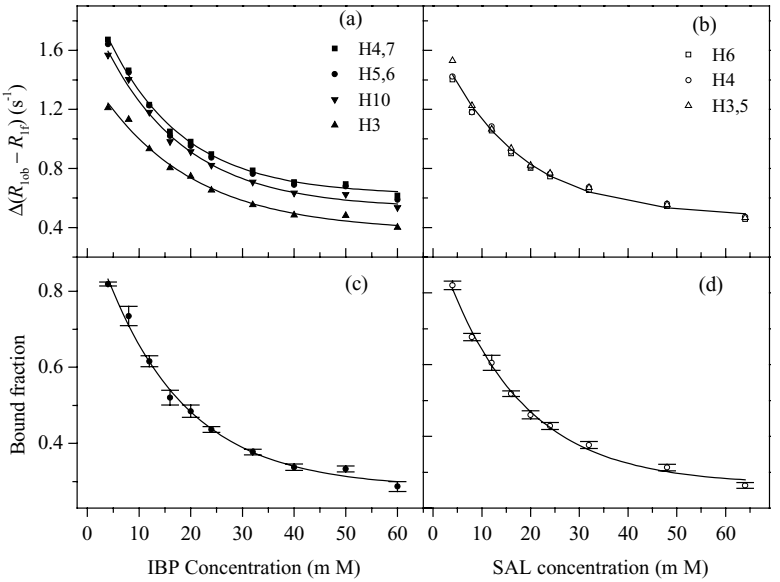


Fig. 2. Plots of proton longitudinal relaxation rate changes $\Delta(R_{1ob} - R_{1f})$ of IBP (a) and SAL (b) in solutions without and with 0.2 mM HSA as function of their concentration, respectively. The corresponding fraction of the bound IBP (c) and SAL (d) were derived from the relaxation data.

$2.02 \pm 0.14\,s^{-1}$ (SAL H3, 5), $3.63 \pm 0.18\,s^{-1}$ (IBP H5, 6), $3.56 \pm 0.23\,s^{-1}$ (IBP H4, 7), $4.15 \pm 0.28\,s^{-1}$ (IBP H3) and $4.21 \pm 0.25\,s^{-1}$ (IBP H10). Based on the values of R_{1f}, R_{1b} and R_{1ob}, it was possible to derive the fraction of the bound ligand using Eq. (3). The results are shown in Fig. 2c for IBP and Fig. 2d for SAL. After binding to HSA, the relaxation rate changes of the four SAL protons were identical (Fig. 2b) since SAL is a relatively small ligand. In contrast to SAL, the relaxation rate changes of IBP protons showed significantly asymmetry (Fig. 2a). The smaller ΔR_1 of $CH_3(3)$ protons indicated that the *iso*-propionic acid chain (IBP) was away from the binding center. This agreed with the observation of the binding of IBP to HSA at different pH [14]. Although ΔR_1 of the $CH_3(3)$ protons was smaller than that of the other protons of IBP, the bound fraction derived was quite similar to that derived from the other protons (Fig. 2c). This was also true for the binding of SAL to HSA (Fig. 2d). Using the well established binding model [21–23] of

$$\mathrm{HSA \cdot L} \rightleftharpoons \mathrm{HSA + L} \tag{4}$$

it is possible to calculate the apparent dissociation constant (K_d) and number of binding sites (n):

$$K_d = \frac{[\mathrm{HSA}][\mathrm{L}]}{[\mathrm{HSA \cdot L}]} = \frac{(nC_P - C_L f_b)(1 - f_b)}{f_b}, \tag{5}$$

or

$$C_L = \frac{nC_P}{f_b} - \frac{K_d}{1 - f_b} \tag{6}$$

where C_P and C_L represent the total concentration of HSA and ligand (IBP or SAL). From the data in Fig. 2c and 2d, K_d and n can be derived according to Eq. (6) using ORIGIN (Microcal Software, Inc. Version 5.0). The derived apparent dissociation constant (K_d) and number of the binding sites (n) were 1.39 ± 0.16 mM and 33.2 ± 1.5 for IBP, and 4.27 ± 0.48 mM and 35.0 ± 2.3 for SAL, which agree well with previously reported values [18,19]. These results revealed that binding properties, such as the orientation of the ligand molecule, were consistent in the concentration range studied and that the above equations were applicable.

When the concentration of IBP increased from 4.0 to 20.0 mM in the solutions which contained 4.0 mM SAL and 0.2 mM HSA (Fig. 3a) the fractions of free IBP (■) and SAL (●) increased from 0.35 to 0.66 and 0.59 to 0.83, respectively. A similar phenomenon was observed when the content of SAL was increased from 4.0 to 24 mM in the solutions that contained 8.0 mM

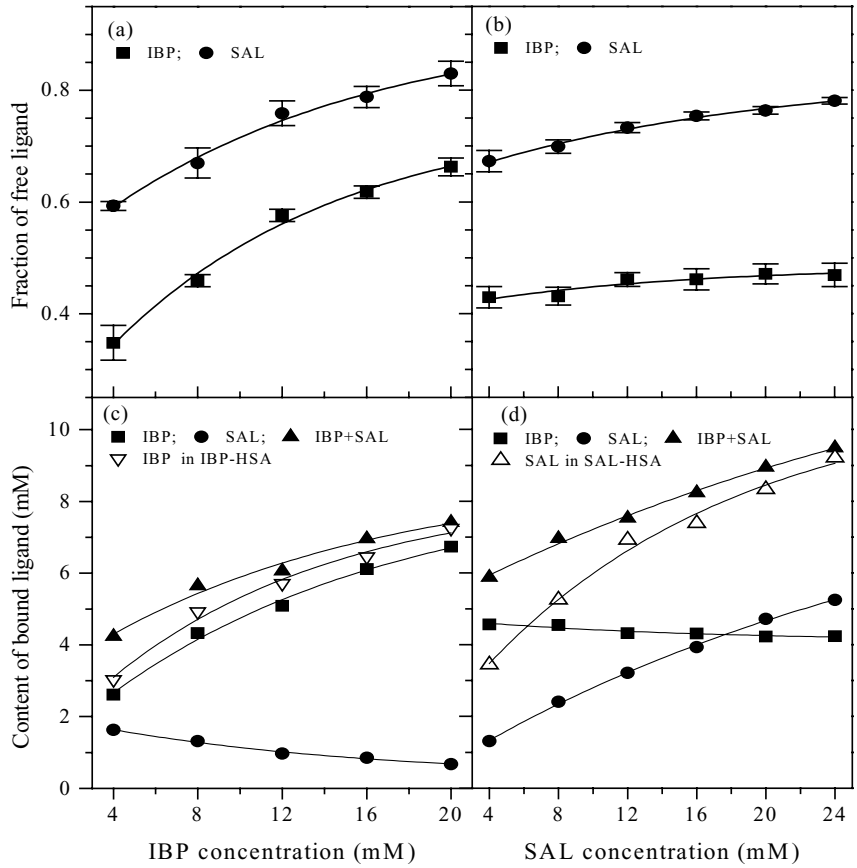


Fig. 3. The fraction of the free ligands (a, b) and contents of the bound ligands (c, d) plotted as a function of the concentration of IBP (a, c) and SAL (b, d) in solutions of IBP, SAL and HSA. The concentrations of the bound IBP and SAL in the system IBP–SAL–HSA (▲), bound IBP in the system IBP–HSA (∇), and bound SAL in system SAL–HSA (Δ) were also shown for comparison.

IBP and 0.2 mM HSA (Fig. 3b). These results revealed that IBP and SAL shared certain binding sites on HSA.

The fraction of bound or free ligand can be converted into the concentration ($C_b = f_b C_{total}$). The results are shown in Fig. 3c for the solutions containing variable concentrations of IBP, 8.0 mM SAL and 0.2 mM HSA, and in Fig. 3d for the solutions consisting of variable SAL, 8.0 mM IBP and 0.2 mM HSA. The concentrations of the total bound ligands ($= C_{b,IBP} + C_{b,SAL}$) in the three-component system of IBP–SAL–HSA (▲), bound IBP ($C_{b,IBP}$) and SAL ($C_{b,SAL}$) in the two-component systems of IBP–HSA (Δ) and SAL–HSA (∇) were given in the figure for the comparison. In the solutions containing variable IBP and 0.2 mM HSA (Fig. 3c), the concentration of the bound IBP (■) was increased when its content was increased because of the large low-affinity binding capacity of HSA. When 8.0 mM SAL was present in the system, the concentration of the bound IBP (■) was reduced by about 0.5 mM in the titration range. This can be understood as some of the IBP binding sites were occupied by SAL. When the content of IBP was increased from 4.0 to 20 mM, the concentration of bound SAL (●) was, correspondingly, reduced from 1.6 to 0.7 mM. Since the concentration of HSA was fixed in the solutions, the increased bound IBP meant decrement of the free binding sites. This, in turn, caused disassociation of the HSA·SAL complex. Although similar phenomena were observed during the titration using SAL (Fig. 3d), the bound SAL (●) was significantly reduced (1.1–4.0 mM) when 8.0 mM IBP was present in the solutions. The concentration

of bound IBP (■) was reduced slightly from 4.6 to 4.2 mM when the SAL concentration was increased from 4.0 to 24.0 mM. This can be explained by the higher binding affinity of IBP to HSA than that of SAL to HSA.

It was noticed that the total concentration of bound ligands (▲) was higher in the solutions of HSA–IBP–SAL than that in the solutions of either HSA–IBP (△) or SAL–HSA (▽). This indicated that each of IBP and SAL may have their specific binding sites in addition to the mutual ones. In the other words, the binding sites of IBP and SAL on HSA are not fully overlapped. The competitive binding happens only at those mutual binding sites.

Theoretically, chemical shift change can also be used to derive the bound fraction of IBP or SAL in a similar manner. However, multiple line-shapes and large line broadening caused by the binding make it difficult to measure the accurate chemical shift of the IBP and SAL resonances, especially at low ligand to HSA ratio.

There are several sources of errors in the measurements. The uncertainty in the relaxation rate determination using inverse-recovery approach is about 5%. For the current system, the cross-relaxation between the protons of the ligand and HSA, also known as the origin of the inter-molecular NOE, may affect the values of R_{1ob}. This problem has been addressed in the study of ligand–protein interaction using diffusion based NMR experiment [24,25]. There is no attempt to reduce such effects in this work since the majority of the ligand is in the bound form, especially at the lower ligand to HSA ratios, and this reduces the relaxation rate differences between the ligand and HSA protons and the effect of cross-relaxation as well. The ^{1}H relaxation rates of the free ligand (R_{1f}) were measured in solution without HSA. The values were significantly smaller than that extrapolated from the data of Fig. 2. The extrapolated R_{1f} may exclude the effect of conditional changes of the solution, such as viscosity, but enhances the cross-relaxation because the difference between R_1 of the ligand and HSA protons are enlarged. The accuracy of extrapolated R_{1b} could be improved if more data points with low ligand to HSA ratio were added. However, the overlapped peaks of the ligands (IBP and SAL) and HSA may cause another problem at the lower ligand to HSA ratio. To balance the problem, the minimum ratio of 20:1 (4 mM IBP or SAL, 0.2 mM HSA) may be the best choice. It is more likely that the two ligands compete for the high-affinity binding Site III on HSA, which may affects the results presented in this work. However, considering the high concentration ratio of the ligand to HSA and large number of low-affinity bind sites, the effect of the high-affinity binding is well within the experimental errors.

4. Conclusions

The competitive low-affinity binding of ibuprofen and salicylic acid to human serum albumin was observed and studied using NMR relaxation measurements. It was demonstrated that the fractions and concentrations of bound ligands can be calculated from the R_1 data based on the model of fast reversible binding reaction. The relaxation rate changes during the titration using either IBP or SAL revealed that a majority of the low-affinity binding sites of IBP and SAL on HSA were mutual for the two ligands, in addition to the specific binding Site III. The approach presented here may be used as an alternative method to analyze the competitive binding of two ligands to a protein. The possible errors in the analysis are addressed. The number of the overlapped binding sites and the dynamics need to be further studied.

Acknowledgements

This work is supported by the grants from the Natural Science Foundation (NSFC) and 973 Project of China and Multidisciplinary Research Program of Chinese Academy of Sciences.

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