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A competitive low-affinity binding model for determining the mutual and specific sites of two ligands on protein

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Abstract

A competitive low-affinity binding model was proposed for determining the number of mutual (overlapped) and specific binding sites of two ligands (A, B) on a protein (P). To use the model, one needs to carry out a titration experiment by adding either ligand A or B into a three-component system (A–B–P), and to monitor the spectroscopic parameter changes. Fitting the titration curve to the proposed model, one can get the mutual and specific binding sites of the two ligands on the protein. The model was examined by using human serum albumin (HSA) as a receptor and tolmetin (TOL) and salicylic acid (SAL) as ligands. Proton longitudinal relaxation rates (R_1) were measured on a 500-MHz NMR spectrometer during the titration and used to derive the mutual binding sites. It was found that among the binding sites of 32 ± 4 for SAL and 28 ± 2 for TOL on HSA, there were 17 ± 5 mutual sites for the two ligands. This result indicates that, although HSA has large binding capacities for most ligands, there are still a reasonable amount of the low-affinity binding sites that are structure selective.

Keywords: Competitive binding; Mutual binding sites; NMR; Relaxation rate; Human serum albumin; Tolmetin; Salicylic acid

1. Introduction

Studies on the binding of drugs to protein are of great importance in biological, biomedical and pharmaceutical sciences. The binding affinity and capacity can be described by the dissociation constant of the molecular complex and the number of the binding sites on the protein. Any interaction that interferes with the binding of a drug to its receptor, such as competitive binding, may affect the pharmacological activity of the drug. The competitive binding represents the condition that two ligands compete for the same binding site or sites on a protein molecule. This may happen on the target protein and on the transport protein as well, such as serum albumin. The interactions between drug and the serum proteins have a strong impact on the drug's transportation, metabolism, excretion, and the concentration of free drug in the blood, which shows a better correlation to the pharmacological activity of the drug [1]. Over the last decades, several techniques have been developed and applied to study the binding of ligands to protein [2–19], such as equilibrium dialysis, ultrafiltration, HPLC, and so on. Nuclear magnetic resonance (NMR) spectroscopy has been recognized as a powerful tool in the study of interaction between protein and drug [10–17], such as identifying high-affinity ligands by linking low-affinity binding fragments, screening drugs that bind more tightly to a receptor in the presence of weak- to medium-affinity ligands, and determining the number of low-affinity binding sites and the corresponding dissociation constant. NMR is a non-invasive approach, which needs minimum sample preparation and requires neither chromophore nor radioactive-labeled ligand. Because the sample composition and equilibrium are not affected during the NMR experiment, the sample is ready for subsequent analysis by using the other methods. In addition, there are a number of NMR detectable parameters that are very sensitive

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to, and can be used to characterize, the molecular interaction. The main disadvantage of the NMR method is its inherent limitation of low sensitivity compared to other spectroscopic methods.

Most of the above mentioned methods are focused on qualitatively characterizing the competitive binding phenomena, particularly, on the high-affinity binding. Wang has presented a chemical model for analyzing the competitive binding of two different ligands on a single high-affinity site of a protein molecule [18]. And a similar mathematical expression has been tested using the displacement isothermal titration calorimetry method [19]. As for low-affinity binding, a fast and reversible chemical-reaction (FRC) model has been widely accepted and used to derive the number of binding sites and apparent dissociation constant (K_d) [20–25]. The competitive binding of two drugs on the low-affinity sites of human serum albumin (HSA) has also been investigated using NMR method [17]. For each ligand, the low-affinity binding sites can be divided into specific and non-specific or mutual sites. Competitive binding happens only on the mutual binding sites, which is expected to have significant effect on the interaction of ligand and protein. In this article, we propose a chemical model for determining the mutual and specific low-affinity binding sites. We use HSA as a model protein because it is a major drug carrier protein in blood and has large binding capacity for most drugs and endogenous metabolites [26–31]. Two non-steroidal anti-inflammatory drugs, tolmetin (TOL) and salicylic acid (SAL), were used as ligands. It has been shown that TOL has a primary binding site at site I [32] and has three classes of binding sites [33]. SAL can also bind to HSA at site I (in subdomain IIA) [34] and has tens of low-affinity binding sites [17,25,35]. It is, therefore, expected that TOL and SAL will interfere with each other in the binding process.

2. Experimental

Human serum albumin (fraction V, fatty acid free), tolmetin (sodium 1-methyl-5-*p*-toluoylpyrrole-2-acetate dihydrate) and salicylic acid were bought from Sigma (Poole, Dorset, UK) and used without purification. The numbering systems and molecular structures of SAL and TOL are shown in Scheme 1.

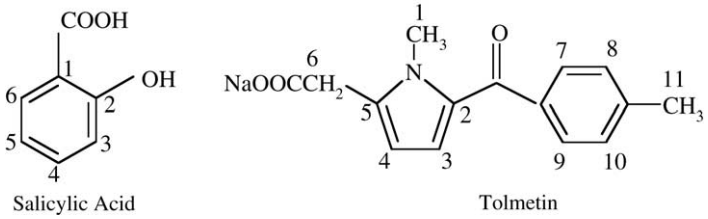
Two groups (G1 and G2), each containing two sets, of samples were prepared in phosphate buffer (pH 7.4) with 10% D_2O added for the NMR spectrometer frequency lock. In group G1, the concentration of TOL was varied: 4.0, 8.0, 12.0, 16.0, 20.0 and 24.0 mM in each of the two sets of solutions. Set 1 containing 0.2 mM HSA served as controls. Set 2 containing 4.0 mM SAL and 0.2 mM HSA, for the study of the competitive binding of SAL and TOL. The concentrations of SAL (4.0, 8.0, 12.0, 16.0, 20.0, 24.0 mM) were varied in group G2. The compositions of the samples in this group were Set 3, only 0.2 mM HSA; Set 4, 8.0 mM TOL and 0.2 mM HSA. Another sample containing TOL and SAL but without HSA was also prepared to obtain the relaxation rates of the free ligands (R_{1f}).

The ^{1}H longitudinal relaxation rates (R_1) were measured using a conventional inverse-recovery sequence on a Varian INOVA 500 NMR spectrometer, operating at proton frequency of 500.12 MHz at 298 K. Sixteen recovery delays ranging randomly from 0.01 to 5 s, and from 0.03 to 10 s were used for the measurement of R_1 of TOL and SAL for the samples with HSA, respectively, and 30 recovery delays ranging from 0.2 to 20 s were used for the samples without HSA. The number of scans was adjusted to gain enough signal-to-noise (S/N) ratio for the different samples. The free induction decay (FID) was acquired into 16k complex data points covering a spectral width of 6000 Hz. 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. In general, the experimental error for the R_1 is less than 5% [20,36].

3. Results and discussion

3.1. Competitive low-affinity binding model (CLAB)

To establish the competitive low-affinity binding model (CLAB) of two ligands (A and B) to protein (P), we used the assumptions that (a) all binding sites on the protein are independent; (b) the binding reaction, $PA + B \rightleftharpoons PB + A$, is reversible and fast on the time scale of the monitoring method (NMR in this case); (c) the observed parameter is the fraction-weighted average of the parameters of the free and bound states; (d) the dissociation constant is the same for the mutual and specific binding sites of each ligand, but different for ligand A and B ($K_{dB} \neq K_{dA}$). Apart from the binding reaction, the concept of the assumptions is similar



Scheme 1. The molecular structure and numbering system of SAL and TOL.

to that used for the low-affinity binding of a single ligand to protein [20–25,37,38].

For a competitive binding reaction of PA + B ⇌ PB + A, we have an equilibrium constant of

$$K = \frac{[PB][A]}{[PA][B]} \quad (1)$$

where [PA] and [A], [PB] and [B] represent the concentrations of the bound and free ligands A and B, respectively. The competitive binding reaction is, in fact, a combination of two reactions of PA ⇌ A+P and PB ⇌ P + B, with dissociation constants of K_{dA} (=[A][P]/[PA]) and K_{dB} (=[B][P]/[PB]), respectively. It must be indicated that K, K_{dA} and K_{dB} are the apparent constants and their relationship is

$$K = \frac{K_{dA}}{K_{dB}} \quad (2)$$

If there are N_A and N_B binding sites for A and B, respectively, and N_M mutual sites, the number of total binding sites (N) on the protein is

$$N = N_A + N_B - N_M \quad (3)$$

According to the mass conservation, we have

$$NC_P = [PA] + [PB] + [P] \quad (4)$$

$$C_A = [A] + [PA] \quad (5)$$

$$C_B = [B] + [PB] \quad (6)$$

where C_p, C_A and C_B are the total concentrations of protein P, ligands A and B, respectively, NC_P and [P] are the concentrations of the total and free binding sites on protein P, respectively.

For the fast binding equilibrium, the observed parameter, A_{obs}, is the fraction-weighted average of the parameters of the free state, A_f, and bound state, A_b:

$$A_{obs} = X_b A_b + X_f A_f \quad (7)$$

where X_b (=C_{bound}/C_{total}) and X_f (=$C_{free}/C_{total} = 1 - X_b$) represent the molar fractions of the bound and free ligand, respectively. Eq. (7) can be rewritten as

$$X_b = \frac{A_{obs} - A_f}{A_b - A_f} \quad (8)$$

From the above equations (1–6), one gets a cubic algebraic equation as following (with $K_{dA} \neq K_{dB}$):

$$X_{bA}^3 + aX_{bA}^2 + bX_{bA} + c = 0 \quad (9)$$

with

$$a = \frac{1}{K-1} - 1 + \frac{K}{K-1}\frac{C_B}{C_A} - \frac{K_{dB}}{C_A} - NC_A,$$

$$b = \frac{NC_P}{C_A} + \frac{1}{1-K}\left(\frac{NC_P}{C_A} + \frac{KC_B}{C_A} + \frac{K_{dA}}{C_A} + 1\right),$$

$$c = \frac{NC_P}{C_A(K-1)}$$

Solving Eq. (9) in a conventional manner [39], we get two meaningful solutions,

$$X_{bA1} = -\frac{a}{3} + \frac{2}{3}\sqrt{(a^2 - 3b)}\cos\left(\frac{2\pi + \theta}{3}\right), \quad \text{for } K_{dA} < K_{dB} \quad (10a)$$

$$X_{bA2} = -\frac{a}{3} + \frac{2}{3}\sqrt{(a^2 - 3b)}\cos\left(\frac{2\pi - \theta}{3}\right), \quad \text{for } K_{dA} > K_{dB} \quad (10b)$$

$$\text{with } \theta = \arccos\frac{-2a^3 + 9ab - 27c}{2\sqrt{(a^2 - 3b)^3}}$$

To utilize this model, one of the ways is to carry out a titration experiment using either ligand as titrating agent, and to monitor the binding fraction changes. With pre-knowledge of the dissociation constants of K_{dA} and K_{dB}, and the binding sites (N_A and N_B), one can obtain the total and mutual binding sites (N and N_M) for the two ligands, as well as the specific binding sites of ($N_A - N_M$) for ligand A and ($N_B - N_M$) for ligand B, by fitting the titration curve (X_{obs} versus C_A) to Eq. (10).

3.2. Results

Fig. 1 shows the low-field region (δ 5.8–8.0) of ^{1}H NMR spectra of SAL and TOL in the absence (Fig. 1a) and presence of 0.2 mM HSA (Fig. 1b–d). The figure shows the binding induced up-field chemical shift-drift and line broadening. When increasing the concentration of either SAL or TOL, the line-width and chemical shift changes of both ligands were reduced (Fig. 1c and d) because of the competitive binding [17]. In the spectrum of 8.0 mM SAL and 8.0 mM TOL (Fig. 1a), there were no observable changes in ^{1}H NMR chemical shifts or line shapes compared to the spectrum of pure TOL or SAL. There were no significant differences between ^{1}H relaxation rates of TOL or SAL in the mixture and pure solutions. These results revealed that the molecular dynamics of tolmetin and salicylic acid were independent in the mixture without HSA under the experimental conditions. Therefore, the relaxation rates measured in the solutions without HSA were assigned to that of the free forms (R_{1f}): $0.48 \pm 0.01\,s^{-1}$ (H7, 9), $0.51 \pm 0.03\,s^{-1}$ (H8, 10), $0.46 \pm 0.02\,s^{-1}$ (H3), $0.42 \pm 0.02\,s^{-1}$ (H4) for TOL, and $0.189 \pm 0.004\,s^{-1}$ (H6), $0.193 \pm 0.004\,s^{-1}$ (H4), $0.187 \pm 0.004\,s^{-1}$ (H3, 5) for SAL. Due to the peak overlapping, alkyl protons of TOL were excluded in the measurements.

We used the FRC model [20–25] to measure the number of binding sites and the apparent dissociation constants of SAL and TOL to HSA. The ^{1}H relaxation rates (R_{1obs}) of SAL and TOL were measured using samples Set 1 and

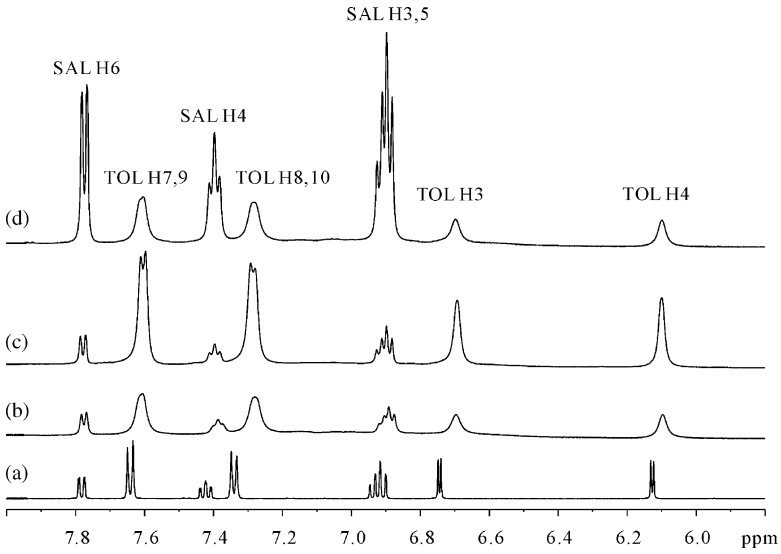


Fig. 1. Down-field regions of 1D ^{1}H NMR spectra of TOL and SAL in the solutions containing (a) 8.0 mM TOL and 8.0 mM SAL, (b) 8.0 mM TOL, 4.0 mM SAL and 0.2 mM HSA, (c) 16.0 mM TOL, 4.0 mM SAL and 0.2 mM HSA, (d) 8.0 mM TOL, 20.0 mM SAL and 0.2 mM HSA. The systematic line broadening and chemical shifts up-field drifts reveal the competitive binding of TOL and SAL to HSA.

Set 3, respectively. The results are shown in Fig. 2a (SAL) and Fig. 2b (TOL). The concentration dependence of the ligand relaxation rate and chemical shift suggests that the chemical exchange of free and bound ligand is on the fast NMR time scale. From these data, it was possible to extrapolate the relaxation rate of bound ligand, R_{1b}. The R_{1b} values were $2.67 \pm 0.23\,s^{-1}$ (H7, 9), $2.77 \pm 0.34\,s^{-1}$ (H8, 10), $2.90 \pm 0.25\,s^{-1}$ (H3), $2.87 \pm 0.26\,s^{-1}$ (H4) for TOL, and $2.80 \pm 0.25\,s^{-1}$ (H6), $2.83 \pm 0.46\,s^{-1}$ (H4), $2.90 \pm 0.27\,s^{-1}$ (H3, 5) for SAL. The R_{1b} values of SAL were larger than that shown in Ref. [17], which could be due to the fatty acid free HSA used here. The number of binding sites (n) and the dissociation constant (K_d) of each ligand on HSA can be obtained by fitting the data to the well-established FRC model for HAS–ligand binary system [17,20,37,38]:

$$2X_b = 2(R_{1,obs} - R_{1f})(R_{1b} - R_{1f}) = 1 + \left(\frac{K_d}{C_P}\right)\left(\frac{C_P}{C_L}\right) + n\left(\frac{C_P}{C_L}\right) - \left\{\left[1 + \left(\frac{K_d}{C_P}\right)\left(\frac{C_P}{C_L}\right) + n\left(\frac{C_P}{C_L}\right)\right]^2 - 4n\left(\frac{C_P}{C_L}\right)\right\}^{1/2} \quad (11)$$

The optimized values of K_d and n were 2.68 ± 0.48 mM and 32 ± 4 for SAL, and 1.75 ± 0.24 mM and 28 ± 2 for TOL. The results obtained here agree well with those of previous study for SAL [17]. The dissociation constant of TOL is in the same order of K_{d3} (K_d of the third class of binding sites) measured at 23 °C and about seven times smaller than K_{d3} measured at 37 °C, using an equilibrium dialysis method, and the number of binding sites is within the range of n_3 (number of the third class of binding sites) [33]. This may be due to the method and temperature dependent and the limited number of the experimental data points. Since the NMR method cannot separate the different classes (the first, second and third) of binding sites, all of the binding sites are

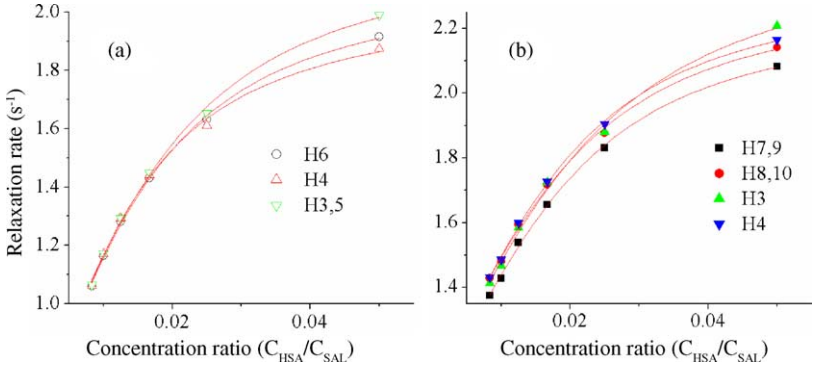


Fig. 2. Plots of proton longitudinal relaxation rates of SAL (a) and TOL (b) in HSA solutions as a function of the concentration ratio of C_{HSA}/C_{Ligand}. The curves represent the best fit to Eq. (11).

treated equally and assumed to be in the fast exchange regime on the NMR time scale. Therefore, the K_d and n determined here are the apparent values or the weighted average of all possible binding classes.

To determine the mutual binding sites, we measured the ^{1}H relaxation rates of SAL and TOL in the three-component system (HSA–SAL–TOL) at different concentrations (4.0–24.0 mM) of either TOL or SAL (samples Set 2 and Set 4). As expected, the ^{1}H relaxation rates of TOL and SAL in the tertiary system were both smaller than those in the binary system (samples Set 1 and Set 3). When TOL concentration was increased from 4.0 to 24.0 mM (Set 2), the relaxation rates of the protons of TOL changed towards those of free state protons, and $R_{1,obs}$ of SAL was decreased too, which suggested that more and more free SAL had been displaced by TOL. These were obvious evidence for the competitive binding of these two ligands on the low-affinity binding sites of HSA. The molar fraction of bound TOL ($X_{b,TOL}$) could be obtained from Eq. (8) using the observed relaxation rates of TOL. There was no significant difference of the values of $X_{b,TOL}$ calculated using the relaxation rates of different protons. The average $X_{b,TOL}$ versus the variation of TOL concentrations could be fitted to Eq. (10a) to determine the number of total independent binding sites since $K_{dA} < K_{dB}$. The best fitting to Eq. (10a) was shown in Fig. 3(a). The optimized value of N was 43 ± 2. The number of the overlapped binding sites (N_m) of SAL and TOL on HSA was 16 ± 5 ($=N_{SAL} + N_{TOL} - N$). The result was reasonable according to N_{TOL} and N_{SAL}. Using the same principle, we obtained the total and overlapped binding sites of 42 ± 4 (N) and 18 ± 6 (N_m), respectively, by fitting the titration curve of $X_{b,SAL}$ versus C_{SAL} to Eq. (10b) (Fig. 3b). Combining the two measurements, we got the average values of N and N_m as 42 ± 3 and 17 ± 5, respectively, for SAL and TOL on HSA. It is interesting to note that about half of the 32 ± 4 binding sites for SAL are shared with TOL and the other half are specific, while the mutual binding sites occupy about 60% of the 28 ± 2 sites for TOL. This result indicates that although HSA has large binding capacities for most ligands, there are still a reasonable amount of the low-affinity binding sites that are structure selective.

3.3. Discussions

The CLAB model presented here can be easily utilized to analyze the competitive low-affinity binding of ligands to macromolecules through measurement of the changes of spectroscopic parameters. To investigate the competitive binding conveniently, a simplified chemical model is proposed here, which may neglect some information. For instance, the co-binding of ligands on HSA may exist if the binding site is large enough. Therefore, it is possible to form ternary complexes like SAL, HSA and TOL on a single site. In the present work, only binary complex was concerned, because it will be very complicated to solve the equation if the first and secondary dissociation constants are taken into account.

It has been shown that SAL can decrease tolmetin binding [33] to HSA. This might be interpreted by the fact that they share a primary binding site at HSA [32,34]. Theoretically, the model presented here can also be applied to the competitive medium-to-high affinity binding, if the exchange between free and bound state of the ligands is fast on the time scale of the monitoring method. It must be indicated that the NMR approach presented here focuses on distinguishing between the mutual and specific binding sites. Since different classes of binding sites are treated equally by the NMR method, N_M obtained here is the apparent number of mutual binding sites.

In addition to the competitive binding, a number of other factors, such as pH, temperature, ionic strength, allosteric effect, etc., may cause NMR parameter changes. To minimize those effects, we prepared the sample in phosphate buffer and carried out the experiment at the same temperature. The

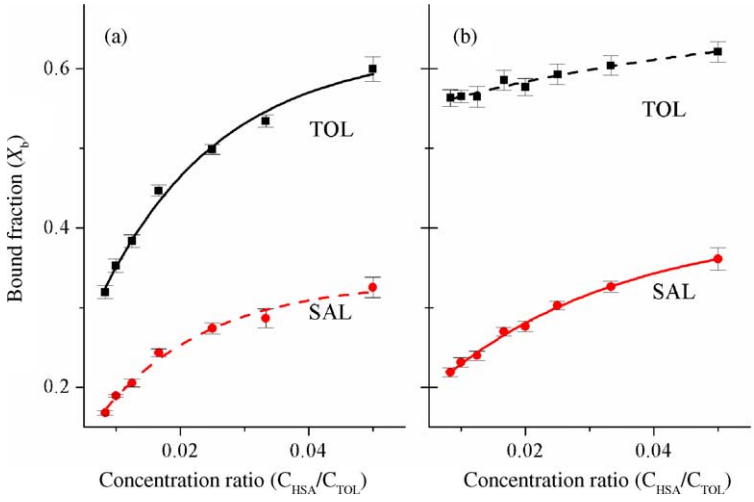


Fig. 3. The averaged bound fraction of TOL (■) and SAL (●) in the system of TOL–SAL–HSA as a function of C_{HSA}/C_{TOL} (a) and C_{HSA}/C_{SAL} (b) are diagramed. The solid curves represent the best fit to Eq. (10a) (■) and Eq. (10b) (●), respectively.

high concentration ratio of ligand to protein was expected to attenuate the allosteric effect and to enhance the low-affinity binding induced relaxation changes. Considering that the concentration of the compounds in blood may range from micromole to tens or even hundreds of millimole, the ligand concentration used in this experiment is acceptable.

There are several sources of errors in the analysis. The uncertainty in the relaxation rate determination using inverse-recovery approach is about 2–6%. The background resonance of protein [21] and inter-molecular NOE [40,41] may affect the values of R_1. The error in extrapolation of R_{1b} is about 10%, and in simulation of N_{TOL} or N_{SAL} is approximately 15% [20], which result in on uncertainty of about 30% in the determination of N_M. The results will be improved if more data points are concerned.

4. Conclusions

A chemical model CLAB was established for describing competitive low-affinity binding of ligands to the receptor in solution. It was examined with two drugs (SAL and TOL) and HSA as model ligands and receptor, respectively. The numbers of overlapped binding sites of these two drugs on HSA can be determined when the dissociation constants and numbers of binding sites of them are obtained from the binary system of HSA–SAL and HSA–TOL.

We measured the ^{1}H longitudinal relaxation rates of the ligands in the HSA–SAL–TOL system at different concentrations of either SAL or TOL. The similar results obtained from different varied ligand sets demonstrated that the method was able to analyze the competitive binding, though the chemical model used here was simplified by neglecting the co-binding of two ligands, which needs to be studied further.

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References

[1] J. Oravcova, B. Bohs, W. Lindner, J. Chromatogr. B: Biomed. Appl. 677 (1996) 1–28.
[2] J.J. Vallner, J. Pharm. Sci. 66 (1977) 447–465.
[3] G.M. Pacifici, A. Viani, Clin. Pharmacokinet. 23 (1992) 449–468.
[4] M.L. Ye, H.F. Zou, Z. Liu, Z.D. Lei, J.Y. Ni, J. Pharm. Biomed. Anal. 27 (2002) 651–660.
[5] S. Watanabe, T. Saito, Biochem. Pharmacol. 43 (1992) 931–935.
[6] L. Bagattoli, S. Kivatiniz, F. Aguilar, M. Soto, P. Sotomayor, G. Fidelio, J. Fluoresc. 6 (1996) 33–40.
[7] D. Essassi, R. Zini, J.-P. Tillement, J. Pharm. Sci. 79 (1990) 9–13.
[8] G. Sudlow, D. Birkett, D. Wade, Mol. Pharmacol. 11 (1975) 824–832.
[9] A. Angelakou, G. Valsami, P. Macheras, M. Koupparis, Eur. J. Pharm. Sci. 9 (1999) 123–130.
[10] S.B. Shuker, P.J. Hajduk, R.P. Meadows, S.W. Fesik, Science (Washington, DC) 274 (1996) 1531–1534.
[11] P.J. Hajduk, S. Boyd, D. Nettesheim, V. Nienaber, J. Severin, R. Smith, D. Davidson, T. Rockway, S.W. Fesik, J. Med. Chem. 43 (2000) 3862–3866.
[12] C. Dalvit, M. Fasolini, M. Flocco, S. Knapp, P. Pevarello, M. Veronesi, J. Med. Chem. 45 (2002) 2610–2614.
[13] C. Dalvit, M. Flocco, S. Knapp, M. Mostardini, R. Perego, B.J. Stockman, M. Veronesi, M. Varasi, J. Am. Chem. Soc. 124 (2002) 7702–7709.
[14] C. Dalvit, M. Flocco, B.J. Stockman, M. Veronesi, Comb. Chem. HTS 5 (2002) 645–650.
[15] W. Jahnke, P. Floersheim, C. Ostermeier, X. Zhang, R. Hemmig, K. Hurth, D.P. Uzunov, Angew. Chem. Int. Ed. 41 (2002) 3420–3423.
[16] A.H. Siriwardena, F. Tian, S. Noble, J.H. Prestegard, Angew. Chem. Int. Ed. 41 (2002) 3454–3457.
[17] Y.F. Cui, G.Y. Bai, C.G. Li, C.H. Ye, M.L. Liu, J. Pharm. Biomed. Anal. 34 (2004) 247–254.
[18] Z.X. Wang, FEBS Lett. 360 (1995) 111–114.
[19] B.W. Sigurskjold, Anal. Biochem. 277 (2000) 260–266.
[20] M.L. Liu, J.K. Nicholson, J.C. Lindon, Anal. Commun. 34 (1997) 225–228.
[21] T.S. Derrick, E.F. McCord, C.K. Larive, J. Magn. Reson. 155 (2002) 217–225.
[22] K.E. Price, L.H. Lucas, C.K. Larive, Anal. Bioanal. Chem. 378 (2004) 1405–1407.
[23] L.H. Lucas, C.K. Larive, Concepts Magn. Reson. A 20 (2004) 24–41.
[24] Z.S. Ji, H.Z. Yuan, M.L. Liu, J.M. Hu, J. Pharm. Biomed. Anal. 30 (2002) 151–159.
[25] R.S. Luo, M.L. Liu, X.A. Mao, Spectrochim. Acta A 55 (1999) 1897–1901.
[26] X.Y. Zhu, Pharmacology, 3rd ed., Public Health Press, Beijing, 1995.
[27] V.K. Cheruvallath, C.M. Riley, S.R. Narayanan, S. Lindenbaum, J.H. Perrin, J. Pharm. Biomed. Anal. 15 (1997) 1719–1724.
[28] L. Deschamps-Labat, F. Péhourcq, M. Jagou, B. Bannwarth, J. Pharm. Biomed. Anal. 16 (1997) 223–229.
[29] D. Zhou, F. Li, J. Pharm. Biomed. Anal. 35 (2004) 879–885.
[30] C.G. Zambonin, A. Aresta, J. Pharm. Biomed. Anal. 29 (2002) 895–900.
[31] K. Kitamura, M. Kume, M. Yamamoto, S. Takegami, T. Kitade, J. Pharm. Biomed. Anal. 36 (2004) 411–414.
[32] K. Matsuyama, A.C. Sen, J.H. Perrin, J. Pharm. Pharmacol. 39 (1987) 190–195.
[33] M.L. Selley, B.W. Madsen, J. Thomas, Clin. Pharmacol. Ther. 24 (1978) 694–705.
[34] X.M. He, D.C. Carter, Nature 358 (1992) 209–215.
[35] P. Balaram, A.A. Bothner-By, E. Breslow, J. Am. Chem. Soc. 94 (1972) 4017–4018.
[36] X.A. Mao, Appl. Magn. Reson. 4 (1993) 261–264.
[37] K.E. van Holde, W.C. Johnson, P.S. Ho, Principles of Physical Biochemistry, Prentice-Hall, New Jersey, 1998.
[38] R.L. Rich, Y.S.N. Day, T.A. Morton, D.G. Myszka, Anal. Biochem. 296 (2001) 197–207.
[39] J.W. Arehhold, Algebra, 4th ed., Pitman, London, 1970.
[40] S.V. Dvinskikh, I. Furo, J. Magn. Reson. 146 (2000) 283–289.
[41] W.H. Otto, C.K. Larive, J. Magn. Reson. 153 (2001) 273–276.