http://www.pjbs.org

PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Pakistan Journal of Biological Sciences 7 (12): 2099-2104, 2004
ISSN 1028-8880

Tetracycline Hydrochloride Binds with High Affinity to Warfarin Site (Site-I) on Bovine Serum Albumin: Temperature and pH Influence the Binding Process

[1]S.M. Mahbubul Alam, [1]M.M. Rahman, [2]M.H. Rahman and [2]N.N. Rahman
[1]Pharmacy Discipline, Life Science School, Khulna University, Khulna 9208, Bangladesh
[2]Faculty of Pharmacy, University of Dhaka, Dhaka-1000, Bangladesh

Abstract: The interaction of tetracycline hydrochloride to Bovine Serum Albumin (BSA) at various temperatures and pH values using Equilibrium Dialysis (ED) method was studied. Scatchard analysis of the binding data revealed the presence of one high affinity binding site with k_1 value of 1.67×10^6 M^{-1} and six low affinity binding sites with k_2 value of 1.44×10^5 M^{-1} at pH 7.4 and 25°C. Site-specific probe displacement data suggested that warfarin site (site-I) is the high affinity binding site and benzodiazepine site (site-II) is the low affinity binding site on BSA for this drug. The high affinity binding site was found to be affected by temperature and pH of the medium. The thermodynamic data indicated that the binding process of tetracycline hydrochloride to BSA is spontaneous, exothermic and entropically driven. Electrostatic forces, hydrogen bonding, hydrophobic interactions and van der Waals forces are probably involved in the overall binding process of tetracycline hydrochloride to BSA. The affinity of this drug to BSA is dependent on the conformational changes of BSA caused by N-B transition.

Key words: Tetracycline hydrochloride, albumin, equilibrium dialysis, binding site

INTRODUCTION

Plasma protein binding properties are primary determinants of the pharmacokinetic properties of most of the drugs such as plasma clearance, half life, apparent volume of distribution and the duration and intensity of pharmacologic effect of most of the drugs[1]. The serum albumin as one of the abundant carrier proteins plays an important role in the treatment and disposition of variety of endogenous and exogenous ligands in blood[2-5].

The early work of Klotz *et al.*[6] and Scatchard[7] formed the basis for investigation of drug protein binding that has been carried out during subsequent decades. To understand the nature of drug protein interaction the affinity of the drug for protein and number of binding sites are important. The binding affinity is quantified in terms of association constant. Since the overall distribution[8], metabolism and efficacy[9] of many drugs in the body are correlated with their affinities towards serum albumin, the investigation on drugs with respect to albumin-drug binding interaction is imperative and of functional importance.

On the basis of probe displacement method, it has been detected that there exist at least three relatively high affinity binding sites on serum albumin. These sites are commonly called the warfarin, the benzodiazepine and the digoxin sites which are also denoted as site I, site II and site III, respectively[2,10,11]. Albeit, the high resolution crystal structures revealed the major ligand binding site on serum albumin[4,12] the exact location of many ligands is still obscure.

The binding characteristics of several drugs and therefore also the degree of binding to albumin, are dependent on the composition of the solvent[13,14]. This is partly due to the occurrence of a conformational change in the albumin molecule around physiological pH. This conformational change is known as the neutral-to-base or N-B transition[15,16]. To study the effect of N-B transition on the binding of tetracycline hydrochloride to BSA, the association constants for binding of tetracycline hydrochloride to BSA are calculated at different pH values by Scatchard[7] analysis.

The affinity of drug binding with serum albumin is inversely related to the temperature within the range of 10 to 40°C[17]. So the binding parameters for the binding of tetracycline hydrochloride to BSA have been studied at pH 7.4 as a function of temperature 10, 20, 30 and 40°C.

The purpose of the present study is to estimate the values of association constants and to characterize the binding sites on BSA. The work also aimed at the study of the binding mode involved in the drug protein interaction and effect of pH on the binding affinity.

Corresponding Author: Md. Habibur Rahman, Professor, Faculty of Pharmacy, University of Dhaka, Dhaka-1000, Bangladesh
Tel: +880 (0)2-9661900-59/4799 Fax: +880-(0)2-861 5583 E-mail: hrahman82@yahoo.com

MATERIALS AND METHODS

Drug International Ltd., Dhaka, Bangladesh supplied tetracycline hydrochloride and Gaco Pharmaceuticals Ltd., Bangladesh supplied probes (warfarin sodium and diazepam). Dialysis membrane was purchased from Medical International Ltd., 239 Liverpool Road, London and BSA from the Sigma Chemical Co. Ltd., USA.

Estimation of binding parameters: The association constants and the number of corresponding binding sites of tetracycline hydrochloride for BSA were studied by Scatchard[7] method of analysis using equilibrium dialysis technique[18].

Three milliliter of previously prepared $2x10^{-5}$ M BSA solution buffered at pH 7.4 was taken into each of the ten test tubes. Tetracycline hydrochloride solution (either 10^{-2}M or 10^{-3}M) was added with increasing volume to 9 out of the 10 test tubes containing BSA solution in each so that the final concentrations of tetracycline hydrochloride were $0.5x10^{-5}$, $1x10^{-5}$, $2x10^{-5}$, $4x10^{-5}$, $6x10^{-5}$, $7x10^{-5}$, $8x10^{-5}$, $9x10^{-5}$, $10x10^{-5}$ and $12x10^{-5}$ M. The tenth test tube containing only BSA solution was marked as 'control'. After proper mixing 2.0 mL of solution was pipetted out from each test tube and poured into previously prepared ten different semipermeable membrane tubes. The tubes containing drug-protein mixture were then immersed in ten separate 50 mL conical flasks containing 30 mL phosphate buffer solution of pH 7.4. These flasks were then placed in a metabolic shaker and shaking was continued for 10 h at 20 rpm and 25°C to complete dialysis. The concentrations of free tetracycline outside the membrane were measured by a UV spectrophotometer (Pye Unicum, England) at a wavelength of 380 nm[19]. The shaking time was set at 10 h, which was determined experimentally by trial and error method. This time was found to be adequate to complete the dialysis of tetracycline hydrochloride under the same experimental condition.

In order to assess the effect of temperature on tetracycline binding to BSA, the similar experiment was carried out at various temperatures (10, 20, 30 and 40°C) at pH 7.4. To study the N-B transition the binding was studied at pH 6.4, 7.4 and 8.4 and at 25°C.

Characterization of binding site of tetracycline hydrochloride using site specific probe: The binding sites of tetracycline on BSA molecule were identified using the site-specific probes, warfarin for site-I[20,21] and diazepam for site-II[22]. Warfarin sodium solution (0.001M) was added to seven out of the eight test tubes containing 3 mL of $2x10^{-5}$ M BSA solution in each so that the final protein-warfarin ratio was 1:1($2x10^{-5}$ M: $2x10^{-5}$ M). The eighth test tube was marked as 'control', which contained only BSA solution. Tetracycline hydrochloride solution (either 10^{-2}M or 10^{-3}M) was then added with increasing concentrations into seven out of eight test tubes containing protein-warfarin (1:1) mixture to have the final ratios of protein-warfarin-tetracycline hydrochloride: 1:1:0, 1:1:1, 1:1:2, 1:1:3, 1:1:4, 1:1:5 and 1:1:6. That is, tetracycline was not added into the first test tube. After proper mixing 2.0 mL of solution was taken from each test tube and poured into eight different semi permeable membrane tubes. The tubes were then immersed in seven separate 50 mL conical flasks containing 30 mL phosphate buffer solution (pH 7.4). After proper shaking in a metabolic shaker for 10 h at 20 rpm and 25°C, concentrations of free warfarin were measured by a UV spectrophotometer (Pye Unicum, England) at a wavelength of 308 nm[23]. The reverse experiment was also carried out to observe the effect of warfarin on tetracycline binding to BSA.

Similar method was followed for diazepam. The concentrations of free diazepam were measured by UV spectrophotometric method at a wavelength of 242 nm[24].

Estimation of thermodynamic parameters: Thermodynamic parameters of tetracycline hydrochloride-BSA interaction were determined by the method of Pedersen[25] using the van't Hoff plots constructed at four temperatures 10, 20, 30 and 40°C. The dependence of high affinity association constants on temperature made it possible to calculate the values for thermodynamic parameters involved in the binding process.

RESULTS AND DISCUSSION

Estimation of binding parameters: Scatchard analysis of tetracycline hydrochloride at pH 7.4 and at 25°C is shown in Fig. 1. Scatchard plot of the ED data resulted in a non-linear curve illustrating the presence of both high affinity and low affinity binding sites on the protein molecule. It is evident from the Fig. 1 that the number of high affinity binding site (n_1) for tetracycline hydrochloride is approximately one (low capacity) and the number of low affinity binding site (n_2) is approximately six (high capacity). The high affinity association constant (k_1) and the low affinity association constant (k_2) were found to be $1.67x10^6$ M^{-1} and $1.44x10^5$ M^{-1}, respectively. The association constant for high affinity binding site was about 12 times higher than that for low affinity binding site at the experimental temperature and pH. Binding parameters of tetracycline hydrochloride to BSA at various pH values and temperatures are shown in Table 1 and 2.

Table 1: Binding parameters of tetracycline hydrochloride bound to BSA at different pH values at 25°C

	Association constants		Number of binding sites	
pH	k_1 (high affinity) x10^6 M^{-1}	k_2 (low affinity)x10^5 M^{-1}	n_1 (high affinity)	n_2 (low affinity)
6.4	2.45±0.30	1.70±0.30	1.10±0.10	5.30±0.50
7.4	1.67±0.10	1.44±0.40	1.40±0.10	5.70±0.60
8.4	2.20±0.30	2.20±0.30	1.00±0.09	4.40±0.50

Table 2: Binding parameters of tetracycline hydrochloride bound to BSA at pH 7.4 and different temperatures

	Association constants		Number of binding sites	
Temperature	k_1 (high affinity) x10^6 M^{-1}	k_2 (low affinity)x10^5 M^{-1}	n_1 (high affinity)	n_2 (low affinity)
10°C	2.55±0.30	2.30±0.30	1.00±0.10	5.20±0.0.40
20°C	2.00±0.10	4.30±0.10	1.60±0.10	3.90±0.20
30°C	1.67±0.20	1.40±0.30	1.40±0.04	5.90±0.30
40°C	1.62±0.30	2.30±0.10	2.10±0.05	5.80±0.30

Table 3: Thermodynamic parameters for tetracycline hydrochloride–BSA interaction at pH 7.4 and 25°C

Drug	Free energy change, ΔG Cal $mole^{-1}$	Enthalpy, ΔH Cal $mole^{-1}$	Entropy, ΔS Cal $mole^{-1}°K^{-1}$
Tetracycline hydrochloride	(-) 8468±6.25	(-)3497±0.6	(+) 16.68±0.35

Each value represents the average value±SD from three experiments

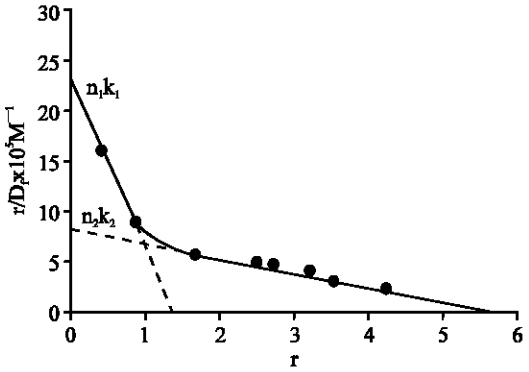

Fig. 1: Scatchard plot for the binding of tetracycline to BSA at pH 7.4 and 25°C
Concentrations used: [BSA]=2x10^{-5}M; [Tetracycline]=2-8x10^{-5}M

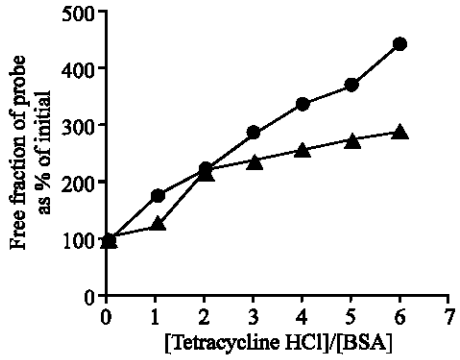

Fig. 2: Free fraction of warfarin (•) and diazepam (▲) as % of initial upon addition of tetracycline at pH 7.4 and 25°C
Concentrations used: [Warfarin sodium] = [diazepam] = [BSA] = 2×10^{-5} M; [tetracycline] = 0-12×10^{-5} M

Identification of binding site: In the present study, binding sites of tetracycline were determined by using probe displacement method where warfarin and diazepam were used as site-I and site-II specific probes, respectively. To characterize the binding sites the free concentration of warfarin and diazepam was measured upon addition of tetracycline hydrochloride. The experimental results are summarized in Fig. 2, which shows the change in free concentrations of warfarin and diazepam by tetracycline. Free concentration of warfarin bound to BSA (1:1) was increased from 100% (as % of initial) to 436.7% by tetracycline hydrochloride when the tetracycline hydrochloride-protein ratio was increased to 6:1. In contrast, under the same experimental condition the free concentration of diazepam bound to BSA (1:1) was increased from 100% (as % of initial) to 286% by tetracycline hydrochloride. This was evident from the Fig. 2 that increment in the free concentration of warfarin sodium was obviously greater than that of diazepam by tetracycline hydrochloride. This suggests that tetracycline hydrochloride preferably binds strongly to site I (warfarin site) compared to site II on the BSA molecule. As the displacement of diazepam is also quite enough this further suggests that tetracycline hydrochloride also binds to site II on BSA molecule to a lesser extent.

To characterize the binding sites, the reverse experiment was also carried out where free concentration of tetracycline was measured upon addition of warfarin and diazepam respectively as shown in Fig. 3. Free concentration of tetracycline by warfarin was increased from 100% (as % of initial) to 470% when the warfarin-protein ratio was increased to 6:1. In contrast, under the same experimental condition the free tetracycline by

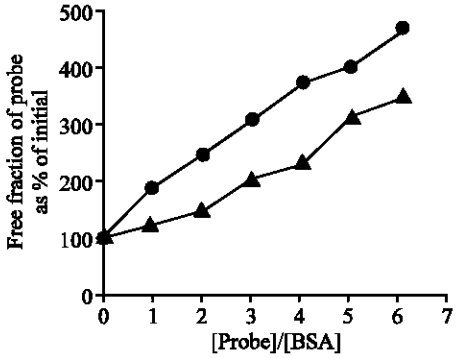

Fig. 3: Free fraction of tetracycline as % of initial upon addition of warfarin (•) and diazepam (▲) at pH 7.4 and 25°C (Reverse Experiment)
Concentrations used: [Tetracycline]= [BSA]=2×10^{-5} M; [warfarin sodium] = [diazepam]= 0-12×10^{-5} M

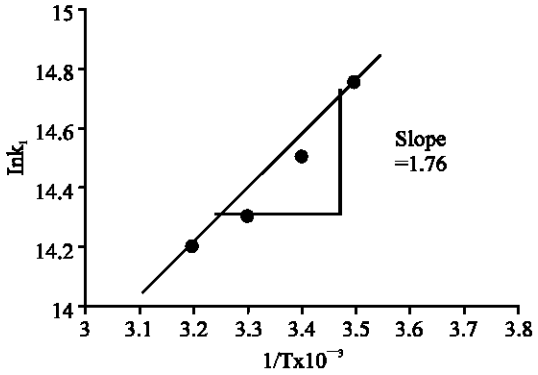

Fig. 4: Effect of temperature on high affinity association constant of tetracycline bound to BSA at pH 7.4

diazepam was increased from 100% (as % of initial) to 350%. Here it is obvious that the increment in the free tetracycline concentration due to displacement by warfarin is higher than that due to displacement by diazepam. This reverse experiment also supports the result of the previous experiment.

Effect of pH on association constants: Binding parameters for the binding of tetracycline hydrochloride to BSA at various pH values (6.4, 7.4 and 8.4) at 25°C were studied and the results are summarized in Table 1. For tetracycline hydrochloride the value of high affinity association constant, k_1, decreased with the increase of pH from 6.4 to 7.4 and then increased when the pH was further increased to 8.4. BSA is said to undergo conformational changes at the physiological pH range (pH 6.4 to 9), commonly referred to as N-B transition[15]. At pH 6 BSA is almost entirely in neutral form, whereas it is almost entirely in the basic form at pH 9. When the protein is in the B–conformation, fewer protons are bound to BSA than that in the N-conformation. The high affinity binding of tetracycline hydrochloride seems to be affected by N-B transition. This is why the binding of tetracycline hydrochloride to BSA has been affected by pH changes of the medium from pH 6.4 to 8.4. Besides, the differences in effect of pH may be due to structural modification of protein molecule. For this reason, at a certain pH the binding site for drug are more suitable or properly accommodating and at other pH values these binding sites become less convenient and less accommodating to the drug in concern.

Effect of temperature on binding parameters: Binding parameters of tetracycline hydrochloride bound to BSA were also determined as a function of temperature (10, 20, 30 and 40°C) at physiologic pH 7.4 (Table 2). It has been observed that the high affinity association constant of tetracycline hydrochloride at pH 7.4 decreases as the temperature increases from 10 to 40°C (Fig. 4). A linear relationship was found between temperature and the value of high affinity association constant. This result complies with the previous results obtained for other drugs bound to BSA that the affinity of drug binding is inversely related to the temperature within the range of 10 to 40°C[17]. And it can be inferred that high affinity binding of tetracycline on the protein molecule takes place more firmly at low temperature than at high temperature.

Mode of binding: The binding mode of tetracycline hydrochloride has been evaluated on the basis of thermodynamic data as shown in Table 3. There was essentially four types of non-covalent interactions that are involved in ligand binding to proteins. These were hydrogen bonds, van der Waals forces, hydrophobic forces and electrostatic interactions[26,27]. Table 3 shows that for high affinity binding of tetracycline hydrochloride to BSA, ΔH (enthalpy change) is negative which implies that the binding process of this drug to BSA is exothermic. The negative sign of ΔG (Gibbs free energy change) values indicate that the binding of tetracycline was spontaneous. The high positive value of ΔS indicates that the unfolding of albumin molecule during ligand-albumin binding process requires energy for breaking or bending some bonds of the albumin molecules. Thus the reaction would be endothermic. But negative value of ΔH stands against this possibility of unfolding the protein molecule at the time of drug's binding. For typical hydrophobic interactions, both ΔH and ΔS, should be positive, whereas for van der Waals forces and hydrophobic bonding formation in low

dielectric medium, these enthalpy and entropy changes should be negative[28]. For actual and true electrostatic interactions, ΔH is expected to be negative or very small or zero[29]. Therefore the very low and negative value of ΔH suggests that high affinity binding of tetracycline hydrochloride to BSA might involve electrostatic interactions. The negative value of ΔH also indicates the clue that van der Waals force and hydrogen bonding may be present. The positive value of ΔS indicates the presence of hydrophobic interaction.

The value of ΔS was found to be positive and the major contribution to ΔG arose from ΔS term rather than from ΔH. This suggests that the high affinity binding of tetracycline hydrochloride to BSA was entropically driven.

REFERENCES

1. Jiunn, H., L. David, M. Cochetto and D.E. Duggan, 1987. Protein binding as a primary determinant of the clinical pharmacokinetic properties of non-steroidal anti-inflammatory drugs. Clinical Pharmacokinetics, 12: 402-432.
2. Fehske, K.J., W.E. Muller and U. Wollert, 1981. The location of drug binding sites in human serum albumin. Biochem. Pharmacol., 30:687-692.
3. Kragh-Hansen, U., 1981. Molecular aspects of ligand binding to serum albumin. Pharmacol. Rev., 33: 17.
4. He, X.M. and D.C. Carter, 1992. Atomic structure and chemistry of human serum albumin. Nature, 353: 209-215.
5. Carter, D.C. and J.X. Ho, 1994. Structure of serum albumin. Adv. Protein Chem., 45:153-203.
6. Klotz, I.M. and J.M. Urquhart, 1949. Binding of organic ions by proteins: effects of temperature. J. Am. Chem. Soc., 71: 847-851
7. Scatchard, G., 1949. The attraction of proteins for small molecules and ions. Annal. New York. Acad. Sci., 51: 660-673.
8. Merrikin, D.J., 1983. Effect of protein binding on antibiotic activity *in vivo* J. Antimicrob. Chemothera. 11: 233-238.
9. Shyu, W.C., R. Quintiliani., C.H. Nightingale and M.N. Dudley, 1988. Effect of protein binding on drug penetration into blister fluid Antimicrob. Agents Chemother., 23: 128-130.
10. Sudlow, G., D.J. Birkett and D.N. Wade, 1975. The characterization of two specific binding sites on human serum albumin. Mol. Pharmacol., 11: 824-832
11. Sudlow, G., D.J. Birkett and D.N. Wade, 1976. Further characterization of two specific binding sites on human serum albumin. Mol. Pharmacol., 12: 1052-1061.
12. Curry, S., P. Brick and N. Franks, 1999. Fatty acid binding to human serum albumin: New insights from crystallographic studies. Biochim. Biophys. Acta., 1441: 131-140.
13. Harmsen, B.J.M., S.H. de Bruin, L.H.M. Janssen, J.F. Rodrigues de Miranda and G.A.J. van Os, 1971. pK change of imidazole groups of bovine serum albumin due to the conformational change at neutral pH. Biochemistry, 10: 3217-3221.
14. Wilding, G., R.C. Feldhoff and E.S. Vesell, 1977. Concentration-dependent effects of fatty acids on warfarin binding to albumin. Biochem. Pharmacol., 26: 1143-1146.
15. Peters, T.Jr., 1985. Serum albumin. Advances in Protein Chemistry, 37: 161-245. Scatchard, G., 1949. The attraction of proteins for small molecules and ions. Annal. New York. Acad. Sci., 51: 660-673.
16. Foster, J.F., 1977. Some Aspects of the Structure and Conformational Properties of Serum Albumin. The Albumin Structure, Function and Uses (Eds.) Rosenoer V.M., M. Oratz, M.A. Rothshild. Pergamon Press, Oxford, pp: 53-84.
17. Rahman, M.H., K. Yamasaki, Y.H. Shin, C.C. Lin and M. Otagiri, 1993. Characterization of high affinity binding sites of non-steroidal anti-inflammatory drugs with respect to site-specific probes on human serum albumin. Biol. Pharm. Bull., 16: 1169-1174.
18. Singlas, E., 1987. Protein Binding of Drugs: Definition, Modalities, Effects, Changes. 2nd Edn. F. Hoffmann-La Roche and Co. Limited, Basle, Switzerland, pp: 25-33.
19. The United States Pharmacopoeia, 2000. Twinbrook, Parkway, Rockville, MD., pp: 1620.
20. Sjoholm, I., B. Ekman, A. Kober, I. Ljungsted-Pahlman, B. Seiving and T. Sjodin, 1979. Binding of drugs to human serum albumin: XI. The specificity of three binding sites as studied with albumin immobilized in microparticles. Mol. Pharmacol., 16: 767-777.
21. Fehske, K.J., U. Schlafer, U. Wollert and W.E. Muller, 1982. Characterization of an important drug binding area on human serum albumin including the high-affinity binding sites of warfarin and azapropazone. Mol. Pharmacol., 21: 387-393.
22. Yamasaki, K., T. Maruyama, U. Kragh-Hansen and M. Otagiri, 1996. Characterization of site I on human serum albumin: Concept about the structure of a drug binding site. Biochem. Biophys. Acta, 1295: 147-147.
23. The British Pharmacopoeia, 2000. HMSO Publication Centre, London, pp: 1593.
24. The British Pharmacopoeia, 2000. HMSO Publication Centre, London, pp: 523.

25. Pedersen, A.O., B. Honore and R. Brodersen, 1990. Thermodynamic parameters for binding of fatty acids to human serum albumin. Eur. J. Biochem., 190: 497-502.
26. Klotz, I.M., 1973. Physicochemical aspects of drug-protein interactions: A general perspective. Ann. NY Acad. Sci., 226: 18.
27. Timaseff, S.N., 1972. Thermodynamics of Protein Interactions. In: Proteins of Biological Fluids` Ed. By Peters, H., Pergaman Press, Oxford, pp: 511-519.
28. Aki, H. and M. Yamamoto, 1989. Thermodynamics of the binding of phenothiazines to human plasma, human serum albumin and alpha-acid glycoprotein. A calorimetric study. J. Pharm. Pharmacol., 41: 674-679.
29. Ross, P.D. and S. Subramanian, 1981. Thermodynamics of protein association reactions. Forces contributing to stability. Biochemistry, 20: 3096-3102.