Journal of
Biological Sciences

Studies on Binding Parameters of Chloramphenicol on Bovine Serum Albumin (BSA)

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Abstract: In the current study on Pharmacokinetic parameter of Chloramphenicol especially emphasizing on binding parameters i.e. association constants, number of binding sites, forces involving in the drug-protein interactions was studied by equilibrium dialysis method. Two types of association constants characterize the binding of the drug on BSA: high affinity association constant (k_1) with lower number of binding sites (n_1) and low affinity association constant (k_2) with higher number of binding sites (n_2). The values for k_1 and k_2 at pH 7.4 and at temperature 25°C were found $(16.9\pm0.01)\times10^5$ and $(4.23\pm0.04)\times10^5$ M^{-1}, respectively and those for n_1 and n_2 were 1.8 ± 0.3 and 4.6 ± 0.4, respectively. Values of k_1 were found to decrease both with the increase of pH from 6.4 to 8.4 and temperature from 25 to 40°C. Thermodynamic data indicated that the protein-Chloramphenicol binding is exothermic, entropically driven and spontaneous. Electrostatic, hydrogen bonding, vander Waal's force and hydrophobic interactions are involved in the binding of Chloramphenicol to BSA.

Key words: Binding parameters, bovine serum albumin, chloramphenicol, equilibrium dialysis

INTRODUCTION

Among the pharmacokinetic parameters of a drug, plasma protein binding is important like absorption, distribution, bio-transformation and excretion. Serum albumin serves as a depot protein and transport protein for numerous compounds and decreases toxicities of many endogenous substances[1]. There is a striking homology in the sequences of BSA and HSA[2]. Due to the change of pH the isomeric from of albumin may also be changed accordingly. The warfarin binding to HSA increases as pH rises from 6.1 to 9.3 and that of diazepam increases as pH Changes from 5.5 to 9.5[3]. This dependence is partially due to the N-B conformational change[4-6]. By the thermodynamic parameters like entropy (ΔS), enthalpy (ΔH) and free energy (ΔG), the binding mode of drugs can be speculated and for reversible drug-protein binding, binding forces like hydrogen bonds, vander Waals forces, hydrophobic and electrostatic forces are involved[7-8]. Protein binding of a drug is not a phenomenon particular to the plasma. It deals with the pharmacokinetic and pharmacodynamic behaviors of a drug so we have chosen this topic to estimate protein-binding parameters of Chloramphenicol.

MATERIALS AND METHODS

Equilibrium dialysis method[9] was used to study protein binding of Chloramphenicol. Association constants (k_1 and k_2) and number of binding sites (n_1 and n_2) on plasma protein molecule for drug are determined by the Scatchard plot. To determine the association constant of Chloramphenicol at pH 7.4 and 25°C the following steps were pursued:

i)   Ten clean and dried test tubes were taken and 3 mL of previously prepared 2×10^{-5}M BSA solution at pH 7.4 was taken in each of them. Chloramphenicol was added in nine out of 10 test tubes to have the following concentrations: 0.5×10^{-6}, 1×10^{-6}, 2×10^{-6}, 4×10^{-6}, 6×10^{-6}, 8×10^{-6}, 10×10^{-6},[6] 12×10^{-6} [6]and 14×10^{-6}M. The tenth test tube containing only BSA solution at pH 7.4 was marked as "control".

ii) After mixing 2 mL from each test tube was pipette out into previously prepared semi-permeable membranes tubes and clipped properly so that there was no leakage.

iii) The membrane tubes containing the drug-protein mixture were immersed in ten 50 mL flasks containing 30 mL of phosphate buffer solution of pH 7.4.

iv) The conical flasks were then placed in a metabolic shaker for dialysis for 10 hours at 25°C and 20 rpm and buffer samples were collected from each flask to take the absorbance of Chloramphenicol by a UV spectrophotometer at a wavelength of 278 nm.

Thermodynamic parameters of Chloramphenicol was measured by using the Van't Hoff plots at different temperatures. Drugs and Probes used in the experiment: Warfarin Sodium (Site-I specific probe; Incepta Pharmaceuticals Ltd.), Diazepam (Site-II specific probe; Square Pharmaceuticals Ltd.) and Chloramphenicol

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(IBN Sina Pharmaceuticals Ltd.). Reagents used: Disodium hydrogen phosphate (Na_2HPO_4), Potassium dihydrogen phosphate (KH_2PO_4), Borax ($NaB_4O_7.H_2O$) (Analytical grade, Glaxo), Cellulose Membrane (Medical International Limited. Liverpool Road, London; mol. wt. 1200 Daltons), Bovine Serum Albumin (BSA) (fatty acid free, fraction V, 96-98%, mol. wt. 66500 and purchased from the Sigma Chemical Co. USA). Instruments used: pH meter (HANNA Microprocessor pH Meter, Portugal), SP8-400 UV/VIS Spectrophotometer (Thermospectronic, England), Metabolic Shaking Incubator (Clifton Shaking Bath, Nickel Electro Ltd., England) and Micro syringe (Well. Liang, Jin.Yang., q.I., China).

RESULTS AND DISCUSSION

Sufficient information about binding parameters is required for proper explanation of serum protein binding. It is an essential tool for the rational understanding of serum albumin binding of drugs during various physiological conditions and concurrent administrations. So the mechanism of drug-albumin binding is of vital importance.

Estimation of binding parameters: The binding parameters both association constants (k_a) and number of binding sites (n) of Chloramphenicol were determined by equilibrium dialysis (ED) and Scatchard plot method.

The results obtained by this analysis suggest that Chloramphenicol has two types of association constants: high affinity association constant (k_1) with low capacity (n_1) and low affinity association constant (k_2) with high capacity (n_2). The high affinity association constant (k_1) to BSA for Chloramphenicol is $16.9 \pm 0.01 \times 10^5 \ M^{-1}$ at pH 7.4 and 25°C. While the low affinity association constant (k_2) is nearly 4 times lower [$4.23 \pm 0.04 \times 10^5 \ M^{-1}$] than that of higher affinity constant (k_1). For this drug the number of high affinity and low affinity binding sites are 1.8 ± 0.3 and $4.6 \pm 0.1 M^{-1}$, respectively. Similar results were observed at 30 and 40°C also (Table 1).

It is meant from the study that Chloramphenicol binds with less number of binding sites with higher affinity which can not easily release free drug in the blood but for lower affinity there are more binding sites and can easily release free drug in the blood.

Estimation of binding mode: The binding mode of Chloramphenicol has been determined on the basis of thermodynamic data. When both enthalpy (ΔH) and entropy (ΔS) are positive, the interactions involve in drug-protein binding is hydrophobic[10].

If ΔH and ΔS both are negative, vander Waals forces and hydrogen binding are involved in drug-protein binding. For electrostatic interactions, ΔH should be negative and very small or almost zero. If free energy change, (ΔG) is negative, the binding is always considered to be spontaneous[7,8].

Thus the following comments can be made from the obtained data (Table 2). For Chloramphenicol ΔH is $-13.27 \ cal^{-1} \ M^{-1}$, the negative value implies that the binding reaction of this drug to BSA is exothermic. In both case the free energy change (ΔG) is $-8.49 \ kcal \ M^{-1}$ and the negative value indicates that the spontaneous reactions are involved in the binding of Chloramphenicol to BSA. Entropy (ΔS) is 28.45 cal/mole/°k. This positive value of ΔS for Chloramphenicol indicates the unfolding of albumin molecule during drug-albumin binding process. But this unfolding requires the breaking or bending of some of the bonds on albumin molecules. Thus the reaction would be endothermic. But negative value of (ΔH) indicates it is exothermic which stands against the possibility of unfolding the protein molecule at the time of the drug's binding to albumin. The value of ΔH for Chloramphenicol is low and negative, which suggest that electrostatic interaction of this drug with binding site may be involved. The negative of ΔH for Chloramphenicol gives the clue that vander Waals force and hydrogen bonding may also play a role in protein binding of the drug. Again the negative value of ΔS indicates the presence of hydrophobic binding in drug-protein interaction.

Thus it can be concluded that there are the rational involvement of the forces as mentioned above in binding of Chloramphenicol to BSA with high affinity association constant at the high affinity binding sites and low affinity association constant at low affinity binding sites.

Effect of pH on association constants: Association constants of Chloramphenicol bound to BSA at different pH and at 25°C were studied. In the binding of Chloramphenicol to BSA the value for high affinity association constant (k_1) was found to decrease whereas the value for low affinity constant (k_2) increase with the increase of pH (Table 1).

When physiological pH is changed, BSA undergoes conformational alteration, which is generally termed as N-B transition[11,5]. Thus the high affinity and low affinity binding of Chloramphenicol is affected by pH changes.

These differences in effect of pH may be due to the structural modification of protein molecule and for this reason at a specific pH the binding site for Chloramphenicol is more suitable or properly accommodated and at other pH values the binding sites

Table 1: Binding parameters of Chloramphenicol bound to BSA at different pH and 25°C temperature

	Association constant		Numbers of binding sites	
pH	k_1 (High affinity) x 10^5 M $^{-1}$	k_2 (Low affinity) x 10^5 M $^{-1}$	n_1 (High affinity)	n_2 (Low affinity)
6.4	20.7±0.60	2.64±0.70	1.4±0.7	5.1±0.9
7.4	16.9±0.01	4.23±0.04	1.8±0.3	4.6±0.1
8.4	12.5±0.30	4.53±0.40	2.1±0.7	4.3±o.4

Table 2: Thermodynamic parameters of Chloramphenicol bound to BSA

Parameters	Values
Enthalpy (ΔH)	-13.27 cal /mole
Entropy (Δ S)	28.45 cal/mole/°k
Free energy change (ΔG)	-8.49 Kcal/mole

Table 3: Binding parameters of Chloramphenicol bound to BSA at pH 7.4 and different temperatures

	Association constant		Numbers of binding sites	
Tem	k_1 (High affinity) x 10^5 M $^{-1}$	k_2 (Low affinity) x 10^5 M $^{-1}$	n_1 (High affinity)	n_2 (Low affinity)
25°C	16.9±0.01	4.23±0.04	1.80±0.3	4.61±0.1
30°C	8.9±0.02	3.20±0.30	3.04±0.2	4.70±0.9
40°C	8.8±0.70	2.72±0.80	2.50±0.3	4.60±0.4

Effect of Temperature on the binding of Chloramphenicol at BSA binding sites
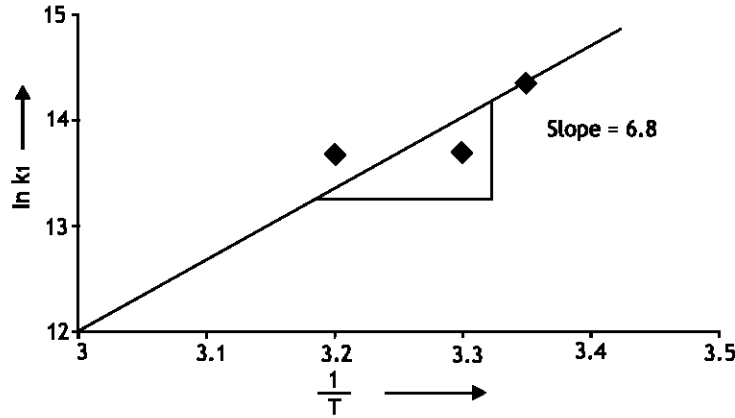

Fig. 1: Effect of temperature on high affinity association constant of Chloramphenicol bound to BSA at pH 7.4

become less convenient and less accommodating to the drugs in concern.

Further we can say, as there is rational involvement of hydrogen bonds, vander Waals force and electrostatic attraction in the binding to higher affinity sites. These binding forces and favorable condition may change due to change of pH.

Effect of pH and temperature on binding sites: Different values of binding sites at different pH and at temperature 25°C were observed in the study. Number of high affinity binding sites (n_1) and low affinity binding sites (n_2) increased and decreased with the increase of pH, respectively. It has been found that, the number of binding sites for higher affinity binding sites increases from 1.4 to 2.1 that for lower affinity binding sites decreases from 5.1 to 4.3 over the change of pH from 6.4 to 8.4 (Table 1). Again number of high affinity binding sites (n_1) and low affinity binding sites (n_2) increased

(1.80 to 2.50) and remained almost constant with the increase of temperature from 25 to 40°C at pH 7.4, respectively (Table 3). These effects may be due to the conformational change of binding sites of specific affinity for the change of pH and temperature.

Effect of temperature on the association constants: It is determined the association constants of Chloramphenicol at normal physiological pH 7.4 as a function of temperature from 25 to 40°C (Table 3). It has been observed that the high affinity association constant k_1 for Chloramphenicol decreased from 16.9± 0.01x10^5 to 8.8±0.7 x10^5 M^{-1} with the increase of temperature from 25 to 40°C. Whereas the association constants of lower affinity also decrease from 4.23±0.04x10^5 to 2.72±0.8 x10^5 M^{-1} with the increase of temperatures from 25 to 40°C (Fig. 1 and Table 3).

According to Rahman *et al*.[12] the affinity of binding is inversely related to the temperature within the range 10

to 40°C. The results obtained in the experiment also coincide with that of previously established results and thus finally it can be inferred that the binding of Chloramphenicol to high affinity binding sites on the protein molecule takes place more firmly at low temperature than at high temperature.

The results of the present study with the current advances in the binding of Chloramphenicol might be helpful in the rational understanding of overall binding behaviors of this drug with HAS.

ACKNOWLEDGMENTS

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