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A thermodynamic study on the binding of theophylline with human serum albumin

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Abstract The thermodynamic parameters of interaction between theophylline and Human Serum Albumin (HSA) in buffer solution (30 mM) of pH = 7 at 27 °C was investigated by isothermal titration calorimetry (ITC). The thermodynamic quantities of the binding mechanism, the number of binding sites (g), the dissociation binding constant (K_d), the molar enthalpy of binding (ΔH) and other thermodynamic parameters can be obtained by the extended solvation theory.

Keywords Human serum albumin · Isothermal titration calorimetry · Theophylline · Binding sites

Introduction

Isothermal Titration Calorimetry (ITC) is an attractive approach for the study of biomolecular interactions such as the interaction of drugs with proteins [1]. ITC sensitively measures changes in enthalpy during a titration experiment in which ligand is added to a protein solution in a calorimeter cell held under isothermal conditions [1]. On the other hand, an ITC experiment is a titration of a biomacromolecule solution by a solution containing a reactant (ligand) at constant temperature to obtain the exchanged heat of the reaction [2].

There are many reports on data analysis for ITC to find the number of binding sites (g), the equilibrium constant, the Gibbs free energy of binding process (ΔG), the enthalpy of binding (ΔH), and the entropy of binding (ΔS) [2, 3]. The method of ITC is now widely used to obtain thermodynamics information about biochemical binding processes [2–9].

Human Serum Albumin (HSA) is the most abundant protein in blood plasma [10–18]. Several different transport proteins exist in blood plasma but albumin only is able to bind a wide diversity of ligands reversibly with high affinity. It serves as a depot and transport protein for numerous endogenous and exogenous compounds. The two heart “lobes” contains HSA’s two binding sites, while the outside of the molecule contains most of the polar groups [16]. Its principal function is to transport fatty acids, a great variety of metabolites and drugs such as anti-coagulants, tranquilizers and general anaesthetics [16, 19].

Its primary structure has 585 amino acids and is characterized by a low content of tryptophan, a high content of cystine stabilizing a series of nine loops, and charged amino acids, such as aspartic and glutamic acids, lysine and arginine for an overall molecular weight of 66411 g/mol [19]. Scientists found that HSA is well characterized 65 KDa proteins and it is now clear that there are two major drug-binding sites [20–22]. It is important to study the interaction between drugs and serum proteins when determining the overall distribution, activity, and

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metabolism of drugs within the body [23, 24]. Drug molecules usually occur in plasma in two forms, free and reversibly bound to protein. Only the unbound fraction of a drug in plasma/biophase is pharmacologically active [23, 25].

HSA binding sites were classified into groups S1, S2, and S3 [26]. Group S1 included high-affinity binding sites for site II-bound drugs, as ibuprofen, flufenamic acid, and short or medium-length alkyl chain fatty acids. Group S2 included low-affinity binding sites of site II-bound drugs, and long-length alkyl chain fatty acid. Group S3 contained the high-affinity binding sites for site I-bound drugs, such as phenylbutazone, and long-length alkyl chain fatty acids [26].

Theophylline is used to prevent and treat wheezing, shortness of breath and difficulty breathing caused by asthma chronic bronchitis, emphysema, and other lung diseases. It relaxes and opens air passages in the lung, making it easier to breath. On the other hand, theophylline (1,3-dimethylxanthine) is widely used to treat asthma, apnea, and obstructive lung diseases [27].

To be effective, theophylline concentration in serum must be within the narrow therapeutic range of 10–20 mg/L [27–32]. A plasma concentration in the range of 55–110 μM^{-1} has been recommended for optimal therapeutic effect. In HSA solutions, the binding ratio of theophylline was significantly related to the albumin concentration and at the albumin concentration seen in the 24 normal subjects, the percentage of drug unbound was almost identical [33].

Considering that theophylline is a weak acid with a $pKa = 8.7$, the observation that the binding of theophylline in serum is pH-dependent, is not surprising [25]. Binding of theophylline in serum was pH-dependent with 25–30% bound at pH = 7.0 and 58–60% bound at pH = 8.1–8.3. Binding was significantly correlated to the fraction of ionized theophylline. So control of pH is necessary to obtain physiologically relevant data on drug binding in serum [25]. The binding of theophylline to HSA has been studied by equilibrium dialysis at 22 °C, pH = 7.4. Binding of theophylline in serum was 34–38% determined by equilibrium dialysis and 41–45% determined by ultrafiltration at pH = 7.4–7.5 [25]. The values of binding parameters obtained for HSA ($g_1 = 1.8$, $K_1 = 2.6 \times 10^3$ L/mol) ($g_2 = 6$, $K_2 = 7 \times 10^2$ L/mol) [34].

Materials and method

Human serum albumin (HSA), theophylline (1,3-dimethylxanthine), and Tris-buffer were obtained from Sigma Chemical Company. The buffer solution is (Tris-buffer), 30 mM concentration and at pH = 7.0. The isothermal titration microcalorimetry experiments were performed with a 4-channel commercial microcalorimetric system. Thermal Activity Monitor 2277, Thermodynamic, Sweden. Each channel is a twin heat-conduction colorimeter in which the heat-flow sensor is a semiconducting thermopile (multi-junction thermocouple plates) positioned between vessel holders and the surrounding heat sink. The insertion vessel was made from stainless steel. HSA solution was placed in the 1.001 mL sample cell of the calorimeter. Theophylline (1,3-dimethylxanthine) was loaded into the injection Hamilton syringe and it was injected into the calorimetric titration vessel, which contained 1.8 mL HSA. Theophylline was titrated into the sample cell as a sequence of 30 injections. The volume of each injection was 0.02 mL, and the interval between injections was 30 min to allow correct equilibration. The calorimetric signal was measured by a digital voltmeter which formed part of a computerized recording system. The heat of each injection was calculated by the "Thermometric Digitam 3" software program. For correcting the dilution effect by the injection of theophylline solution, two controls were obtained: titration of the HSA solution by the buffer for HSA dilution and titration of the buffer solution by theophylline solution for dilution of the corresponding theophylline. The heat of dilution was, therefore, subtracted from experimental titrations, although results in very small heats. The microcalorimeter was frequently calibrated electrically during the course of the study (Table 1).

Results and discussion

We have shown previously [6, 9, 11, 12, 35–39] that the heats of the macromolecules + ligands interactions in the aqueous solvent systems can be reproduced by the following equation:

$$q = q_{\max} x'_B - \delta^\theta_A (x'_A L_A + x'_B L_B) - (\delta^\theta_B - \delta^\theta_A)(x'_A L_A + x'_B L_B) x'_B \quad (1)$$

The parameters δ^θ_A and δ^θ_B reflect to the net effect of theophylline on the HSA stability in the low and high theophylline concentrations, respectively. The positive values for δ^θ_A or δ^θ_B indicate that theophylline stabilized the HSA structure and vice versa. Cooperative binding requires that the macromolecule have more than one binding site, because the interactions are between the sites that bind to the same ligand. If the binding of ligand at one site increases the affinity for ligand at another site, the macromolecule exhibits positive cooperativity. If the ligand binds at each site independently, the binding is non-cooperative. x'_B can be expressed as follows:

$$x'_B = \frac{p x_B}{x_A + p x_B} \quad (2)$$

$p > 1$ or $p < 1$ indicate positive or negative cooperativity of macromolecule for binding with ligand, respectively;

Table 1 The heats of HAS + theophylline interactions at 300 K in 30 mM Tris buffer solution of pH = 7

[HSA]t/μM	[Theo]t/mM	q/μJ	q_{dilut}/μJ
14.835	0.110	−125.7	−26.8
14.674	0.217	−218.4	−45.4
14.516	0.3231	−290.6	−59.8
14.362	0.426	−349.0	−71.5
14.211	0.526	−397.7	−81.2
14.062	0.625	−439.0	−89.5
13.917	0.722	−474.6	−96.6
13.776	0.816	−505.7	−102.8
13.636	0.909	−533.1	−108.3
13.500	1.000	−557.5	−113.3
13.366	1.089	−579.4	−117.6
13.235	1.176	−599.1	−121.5
13.107	1.262	−617.0	−125.1
12.981	1.346	−633.3	−128.4
12.857	1.429	−648.2	−131.4
12.736	1.887	−715.7	−144.8
12.617	2.336	−764.5	−154.6
12.500	2.778	−801.4	−161.9
12.385	3.211	−830.6	−167.8
12.273	3.636	−854.0	−172.5
12.162	4.054	−873.3	−176.5
12.053	4.464	−889.5	−179.6
11.945	4.867	−903.2	−182.3
11.842	5.263	−915.1	−184.7
11.739	5.652	−925.4	−186.7
11.638	6.034	−934.4	−188.6
11.538	6.410	−942.4	−190.2
11.441	6.780	−949.5	−191.6
11.344	7.143	−955.9	−192.8
11.250	7.500	−961.7	−194.2

$p = 1$ indicates that the binding is non-cooperative. x'_B is the fraction of the theophylline needed for saturation of the binding sites, and $x'_B = 1 - x'_B$ is the fraction of unbounded theophylline. We can express x_B fractions, as the total theophylline concentrations divided by the maximum concentration of the theophylline upon saturation of all HSA as follows:

$$x_B = \frac{[\mathrm{Theo}]_t}{[\mathrm{Theo}]_{max}} \quad x_A = 1 - x_B \tag{3}$$

$[\mathrm{Theo}]_t$ is the total concentration of theophylline and $[\mathrm{Theo}]_{max}$ is the maximum consternation of theophylline. L_A and L_B can be calculated from heats of dilution of theophylline in water, q_{dilut}, as follows:

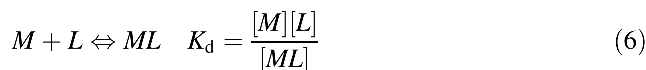
$$L_A = q_{dilut} + x_B\left(\frac{\partial q_{dilut}}{\partial x_B}\right), \quad L_B = q_{dilut} - x_A\left(\frac{\partial q_{dilut}}{\partial x_B}\right) \tag{4}$$

The heats of HSA–theophylline interactions were fitted to Eq. 1 over the entire theophylline concentrations. In the procedure, the only adjustable parameter (p) was changed until the best agreement between the experimental and calculated data was approached. δ_A^θ and δ_B^θ parameters have been also optimized to fit the data. The small relative standard coefficient errors and the high r^2 values (0.9999) support the method. The binding parameters for HSA–theophylline interactions recovered from Eq. 1 were listed in Table 2. The results are estimated. The agreement between the calculated and experimental results (Fig. 1) is excellent, and gives considerable support to the use of Eq. 1. δ_A^θ and δ_B^θ values for HSA + theophylline interaction are positive, indicating that in the low and high concentrations of the theophylline, the HSA structure was stabilized, resulting in an increase in its biological activity.

If the multiple binding sites on a biomacromolecule are identical and independent, the binding parameters can also be reproduced by the following equation:

$$\frac{\Delta q}{q_{max}} M_0 = \left(\frac{\Delta q}{q}\right) L_0 \frac{1}{g} - \frac{K_d}{g} \tag{5}$$

where $\Delta q = q_{max} - q$. q represents the heat value at a certain ligand and biomolecule concentration. M_0 is the total biomacromolecule and q_{max} represents the heat value upon saturation of all biomacromolecule. K_d is the dissociation equilibrium constant for the equilibrium:



$$M + L \Leftrightarrow ML \quad K_d = \frac{[M][L]}{[ML]} \tag{6}$$

Table 2 Binding parameters for HAS + theophylline interaction recovered from Eqs. 1 and 7

Parameters	First binding sites	Second binding sites
p	1	1
g_i	2	6
K_d/mM	2.8	0.7
ΔH/kJ mol^{-1}	−6.8	−4.5
δ_A^θ	4.89	
δ_B^θ		4.15

$p = 1$ indicates that the binding is non-cooperative in two groups of binding sites. The positive values of δ_A^θ or δ_B^θ indicate that the stability of HSA has been increased as a result of its interaction with theophylline

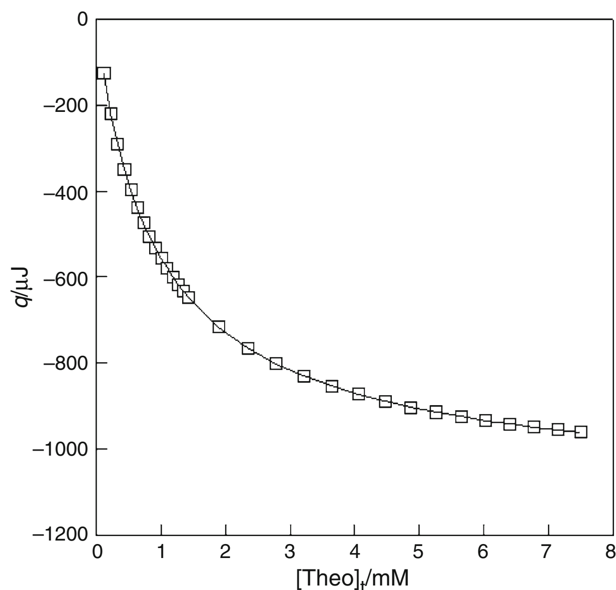


Fig. 1 Comparison between the experimental (*square*) and calculated (*line*) heats for HAS + theophylline interaction recovered from Eq. 1 versus total concentration of theophylline

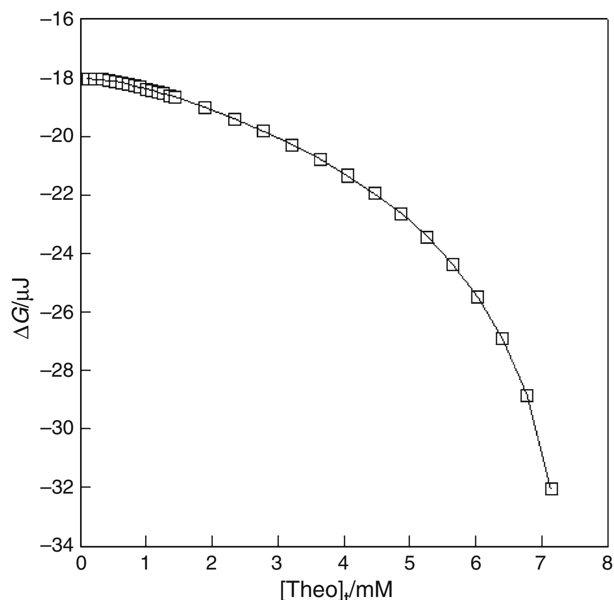


Fig. 2 Comparisons between the experimental Gibbs energy values (*square*) for HSA–theophylline interaction and calculated data (*line*). $[\mathrm{Theo}]_t$ is total concentration of theophylline. Gibbs free energy curve is roughly linear, indicating that the structural changes of HSA compensate each other in the heats of HAS + theophylline interaction (Fig. 1) and $T\Delta S$ (Fig. 3), which is another support for Eqs. 1 and 9

If q_{max} is calculated per mole of biomacromolecule then the molar enthalpy of binding for each binding site will be $\Delta H = \frac{q_{max}}{g}$.

As there are two groups of binding sites on HSA, we have introduced an empirical equation which is the best approach for fitting in such a complicated system as follows:

$$\frac{(q_{max1} - q)}{q_{max1}} M_0 + \frac{(q_{max2} - q)}{(q_{max2} - q_{max1})} M_0 = \frac{(q_{max1} - q)}{q} L_0 \frac{1}{g_1} - \frac{k_1}{g_1} + \frac{(q_{max2} - q_{max1} - q)}{F(q_{max2} - q_{max1}) - q} L_0 \frac{1}{g_2} - \frac{k_2}{g_2} \quad (7)$$

where F parameter can be defined as follows:

$$F = \frac{q}{q_{max1} + q_{max2}} \quad (8)$$

A non-linear least squares computer program has been developed to fit data in Eq. 7. The best correlation coefficient ($R^2 \approx 1$) and the least standard deviations (SD $\approx 10^{-6}$ or better) are good support for the use of Eq. 7. The binding parameters recovered from Eq. 7 (K_1, K_2, g_1, and $g_{2)}$ were listed in Table 2.

The standard Gibbs free energies as a function of theophylline concentrations can be obtained as follows:

$$\Delta G^{o} = -RT \ln K_a \quad (9)$$

where K_a is the appearance association equilibrium constant as a function of theophylline concentrations. The standard Gibbs energies (ΔG^{o}), calculated from Eq. 9 have been shown graphically in Fig. 2. The $T\Delta S$ values have been shown in Fig. 3.

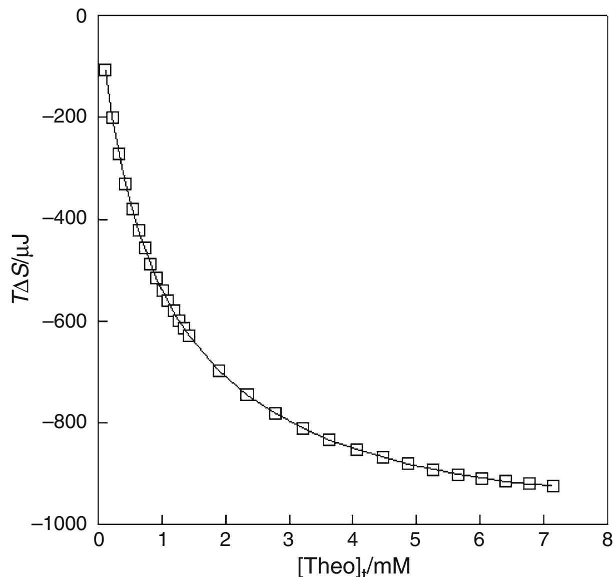


Fig. 3 Comparison between the experimental $T\Delta S$ (*square*) for HAS + theophylline interaction and calculated data (*line*). $[\mathrm{Theo}]_t$ is total concentration of theophylline

Conclusions

It has been confirmed that the extended coordination model, via Eq. 1 will satisfactorily reproduce the heats of HSA + theophylline interaction. Analysis of these in terms of the new extended coordination model confirms the model's ability. Prediction of number of binding sites on HAS molecule, structural changes, determining the binding enthalpies and associated binding constants for such a complicated system accurately, make this theory the most powerful one.

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