Thermodynamics study of binding of oxazepam and flurazepam to Human Serum Albumin (HSA) by spectrophotometery

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Abstract

Objective: HSA is the highly water-soluble plasma protein, which is the smallest and most abundant plasma protein in the human body. Oxazepam (O) and Flurazepam (F) include the most frequently prescribed sedative-hypnotic agents. (F) and (O) bind to human serum proteins more than 95%. Investigations show that HSA has an important role as a carrier for diazepins. The interaction of drugs with HSA, which may have important pharmacokinetics implications, has been extensively studied by several workers.

Materials and Methods: The binding of two diazepins [Oxazepam (O) and Flurazepam (F)] to HSA was investigated by means of spectrophotometry. The binding isotherms for interaction of (F) and (O) with HSA at 25 ˚C shows the variation of ν, the average of bound (O) and (F) per HSA molecule, versus log [D]. The corresponding Scatchard plots for these isotherms were driven. They coincide with usual shapes of Scatchard plots and can represent the existence of one binded set. The binding parameters, binding constant and binding capacity of these medicines were obtained from Hill equation.

Results: The binding constants of Flurazepam (F) and Oxazepam (O) were determined $0.6 \pm 0.1(x10^5)$ and 1.4 ± 0.3 ($x10^5$) respectively. The binding capacity of (F) and (O) were computed 1 ± 0.1 and 1.3 ± 0.1, and the Hill constant (n_H) was obtained 4.076 and 2.44 respectively. The results of this study show, spectrophotometry can be a simple and fast technique to determine the binding constant for some ligands.

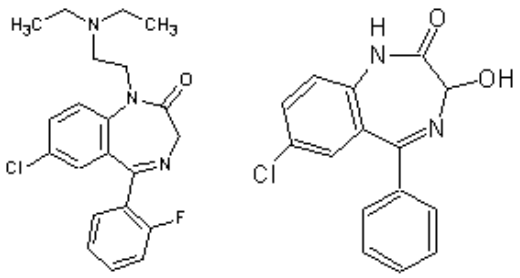
Conclusion: These values show the binding affinity of (O) is more than (F), on the other hand, the cooperativity of (F); is higher than (O). With regards to the amount of K, binding affinity of (O) to HSA is more than (F). These results can be justified by the amounts of partition coefficient of (O) and (F).

Keywords: HSA, Drug Binding, Cooperativity, Flurazepam, Oxazepam, Spectrophotometry.

Introduction

HSA is the highly water-soluble plasma protein, which is the smallest and most abundant plasma protein in the human body accounting for 55% of the total protein in blood plasma (6). Its ionizable groups include 110 total acidic groups (98 carboxyl and 18 phenolic –OH) and 100 total basic groups (60 amino, 16 imidazolyl, 24 guanidyl). The absolute molecular weight of 66436 D was calculated from the numbers and molar masses of the consistent amino acid residues. HSA contains 16 disulfide bridges, one free tiol (Cys 34) and a single tryptophan typically bind 1-2 fatty acids per protein (21), which effectively reduces the isoelectric point in 0.15 M NaCl, the pI for lipid-bound HSA is 4.7, while pI= 5.7 for defatted HSA (14). At pH 7.4 the shape of Native HSA in solution is thought to be a prolate ellipsoid of revolution with major and minor axes, respectively, 12.0 and 2.7 nm or 14.1 and 4.1 nm (20), linking there homologous, globular domains in series.

(O) and (F) include the most frequently prescribed sedative-hypnotic agents (4). (F) and (O) bind to human serum proteins more than 95%. The volume of distribution of (F) and (O) are 22 ± 7 and 0.6 ± 2 (L/Kg) and their half life ($t_{1/2}$) are 74 ± 24 and 6.8 ± 1.3 (hr) respectively (17). (F) and (O) bind to HSA strongly (7,11). Investigations show that HSA has an important role as a carrier for diazepins (16).



Flurazepam Oxazepam

The interaction of drugs with HSA, which may have important pharmacokinetics implications, has been extensively studied by several workers (8, 15, 24, 28, 29). HSA possesses at least three sites or areas for high affinity binding of drugs (11, 30, 31). Most investigations on the binding mechanism of drugs to HSA have implicated hydrophobic interaction, on the basis of quantitative structure-activity relationship (QSAR) studies (5, 22, 25, 26), Spectroscopic studies (10, 18, 19), ulterafiltration method (9) and analysis of thermodynamic parameters (1, 2, 3, 23).

In the present work, with regards to Beer-Lambert equation in spectrophotometery, we developed a simple, fast and accurate technique to determination of the binding parameters.

Materials and Methods

Human Serum Albumin (HSA; lot no. 36F-9333) was purchased from Sigma Chemical Co. The molecular weight of HSA was assumed to be 66500 D. (F) and (O) were donated by Lorestan Pharmaceutical Co. (Khoramabad-Iran).

All other materials were of reagent grade, and all solutions were prepared in dionized and distilled water. All protein and drug solutions were prepared in 0.05 M phosphate buffer (pH=7.4). Spectrophotometery measurements were made using a Shimatzu-UV-160 spectrophotometer (Tokyo-Japan). All solutions were scanned from a wave-length at which no absorption was observed.

Visking tubing, as the semipermeable membrane, was boiled three times, each time for 15 min in ethylenediaminetetraacetic acid (EDTA) and sodium bicarbonate and then washed several times with distilled water and stored in (0.2 C_2H_5OH + 0.8 H_2O). The HSA solution was dialyzed against buffer (0.05 M Phosphate buffer, in pH=7.4). The extinction coefficient was used to calculate the concentration of the native protein at pH=7.4. If the initial concentration and volume of protein solution are $[P]_0$ and V_0, respectively, and the stock ligand concentration is $[L]_0$, then the total concentration of protein $[P]_t$ and Ligand $[L]_t$ can be obtained by accounting for the total volume of the aliquot (V_c) added during the titration experiment (29):

$$[P]_t = [P]_0V_0/(V_0 + V_c),\ [L] = [L]_0V_0/(V_0 + V_c)$$

Aliquots of diazepins were injected into the HSA solution (and viceversa) at 5-min intervals to allow for equilibration; each experiment was repeated three times.

Determination of binding parameters of (O) and (F) to HSA was proceeded as follow:

Determination of molar extinction coefficient of (O) and (F): Molar extinction coefficient of (O) and (F) were determined in phosphate buffer 0.05 M (pH=7.4), separately.

Determination of molar extinction coefficient of (O) and (F) binded to HSA: For this purpose, the absorbance of solutions with constant concentrations of drugs (O & F) and different concentrations of HSA against reference cell, which contains similar concentration of HAS, were measured at 250 and 260 nm, respectively.

Determination of concentration of free drugs in present of HSA: The absorbance of solutions contain different concentrations of drugs (O & F) in presence of a definite concentration of HSA (0.1%) versus

reference cells contain similar concentration of HSA were measured at 250 and 260 nm, respectively.

Data treatment: According to Beer-Lambert equation, absorbance (A) corresponds to concentration (C) as follow:

(eq. 1) $A = \varepsilon . b . C$

If C is expressed in (mol/lit), ε (absorptivity) will be experssed in molar extinction coefficent, b is the thickness of the absorber (cell) in centimeter which usually equals to unit.

Equation (1) for multi-compound systems is as follow:

(eq. 2) $A = \Sigma_i \varepsilon_i . b . C_i$

Since, there are different compounds in solutions containing HSA and drug, like: free-drug, free-protein and different complexes of protein and drugs, accounting of molar extinction coefficient of each compound is necessary to estimate of concentration of free-drug and binded-drug. We suppose the molar extinction coefficient of binded-drug for all drug-HSA complexes are similar.

Then:

(eq. 3) $A = \varepsilon_d^f b[D] + \varepsilon_p^f b[P] + \varepsilon_d^b b[D]_b$

Where, ε_d^f, ε_p^f and ε_d^b are molar absorption for free-drug, free-HSA and binded-drug respectively. The amount of ε_d^f, was calculated from the plot of (A) versus (C) for each drug (O & F), separately.

If there is drug in sample cell and just buffer in reference cell, the absorbance is derived only from free-drug and binding drug while there is HAS in both cells with the same concentration. It means:

(eq. 4) $A = A_f + A_b$

Where, A_f and A_c are absorbance of free-drug and binded drug, respectively.

Adding of HSA will increase the concentration of binded-drug gradually. We supposed that in present of very high concentration of HSA, the concentration of free-drug becomes negligible:

(eq. 5) $A = \varepsilon_d^b b [D]_b$

ε_d^b was calculated from the plot of function 1/A versus $1/[P]_t$. Where $[P]_t$ is total concentration of HSA. The amount of $1/A_\infty$ was estimated by extrapolation of plot to vertical axis. A_∞ is absorbance of initial drug [D] when it wholly binded to HSA:

(eq. 6) $A_\infty = \varepsilon_d^b b [D]$

To determination of free-drug concentration in present of HSA, a definite concentration of protein (0.1%) was added to sample and reference cells. The drug was added to sample cell gradually. Absorbance in this situation is the sum of absorbance of free-drug and binded-drug:

(eq. 7) $A = \varepsilon_d^b b [D]_b + \varepsilon_d^f b [D]$

We suppose that b=1, then:

(eq. 8) $A = \varepsilon_d^b [D]_b + \varepsilon_d^f [D]$

On the other hand we have:

(eq. 9) $[D] + [D]_b = [D]_t$

Where, $[D]_t$ is total concentration of drug.

(eq. 10) $[D]_b = [D]_t - [D]$

Then:

(eq. 11) $A = \varepsilon_d^b ([D]_t - [D]) - \varepsilon_d^f [D]$

(eq. 12) $= \varepsilon_d^b [D]_t + [D](\varepsilon_d^f - \varepsilon_d^b)$

In the equation 12, [D] is only unknown. So, the amount of ν, $([D]_b/[P]_t)$ could be estimated. The Scachard plot for determination of type of drug-protein binding (Fig. 3) shows permeability of cooperativety

of the reaction. In this case we cannot have any estimation on binding constant and binding capacity, then using of Hill equation is necessary:

(eq. 13) $\log(\nu/g-\nu) = n_H \log K + n_H \log [D]$

the number of binding sets , g, was estimated by replacement of some number from 1 to 2.5 instead of n, until regression coefficient (r) in equation 13 become more than 0.99. The slope of the plot of log (ν/g-ν) versus [D] was purposed as Hill coefficient, n_H.

Results and discussion

The molar extinction coefficient of (O) and (F) in phosphate buffer was determined 17298 $M^{-1}.cm^{-1}$and 9526 $M^{-1}.cm^{-1}$ respectively. The molar extinction coefficient of binded drug to HSA (ε_d^b) at 250 and 260 nm were determined for (O) and (F), 618.4 and 417.5 $M^{-1}.cm^{-1}$ respectively (figure 1).

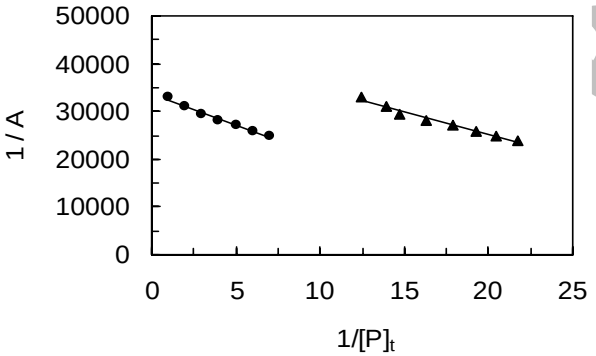


Figure 1: plot of 1/A against $1/[p]_t$, to determination of molar extinction coefficient of binded Oxazepam (•) and Flurazepam (▲) to HSA in phosphate buffer solution (0.05M, pH=7.4) at 250 and 260 nm respectively.

The amounts of free-drug ([D]) and binded-drug ($[D]_b$) were determined from the plot of absorbance (A) against $[D]_t$ for each drugs (O & F), based on equation 12.

Figure 2 is the binding isotherms for interaction of (F) and (O) with HSA at 25 ˚C and shows the variation of ν, the average of bound (O) and (F) per HSA molecule, versus log [D]. The corresponding Scatchard plots for these isotherms are shown in figure 3.

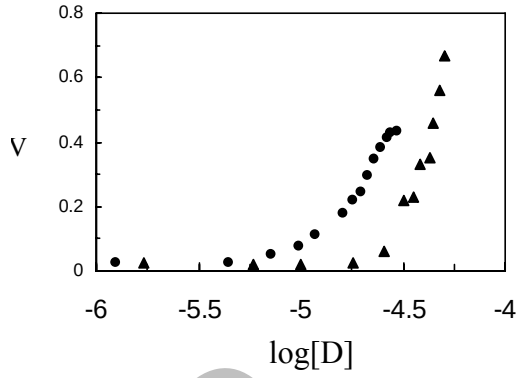


Figure 2: the binding isotherms for interaction of Oxazepam (•) and Flurazepam (▲) with HSA (0.1%) at 25°C in phosphate buffer solution (0.05 M, pH=7.4).

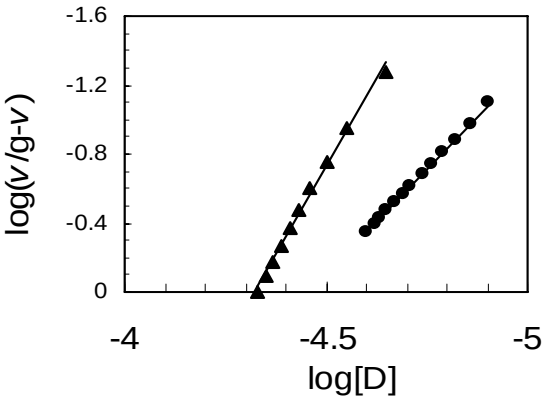


Figure 3: the Scachard plots for interaction of Oxazepam (•) and Flurazepam (▲) with HSA in 0.05 M phosphate buffer (pH=7.4).

These are coincidence with usual shapes of Scatchard plots and can be represent the existence of one binded set (14).

The Hill plot for binding of (O) and (F) to HSA has been driven (figure 4).

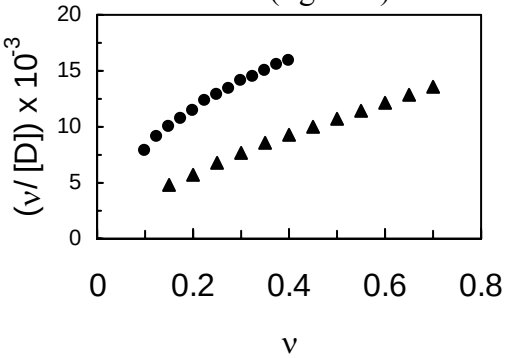


Figure 4: the Hill plots for binding of Oxazepam (•) and Flurazepam (▲) with HSA in 0.05 M phosphate buffer (pH=7.4).

In Hill Plot, the best line (r>0.99) has been obtained when the numbers 1.3 and 1 is replaced instead of (n) in Hill equation for (O) and (F) respectively. The slope of diagrams, n_H (Hill coefficient) were calculated 2.44 and 4.076 for (O) and (F) respectively. The amounts of K (binding constant) have been calculated from the intercepts of graphs, 0.6 ± 0.1 ($x10^5$) and 1.4 ± 0.3 ($x10^5$) for (F) and (O) respectively. The results of this study are similar to the results of Maruyane et al. (1991) for Oxazepam, in their study the Fluorescence method has been used to determination of binding parameters of Diazepines, (table 1). The results of the present study show, spectrophotometery can be simple, fast and accurate technique to determination of the binding constant for some ligands.

Table 1: Binding Parameters for the Oxazepam at pH 7.4 obtained from Fluorescence (Maruyane et al.) and spectrophotometery (UV) methods.

Method	n_H	K ($\times 10^5$)
Fluoresence	1.2 ± 0.1	1.3 ± 0.2
Specterophotometery	1.3 ± 0.1	1.4 ± 0.3

The Hill coefficient (n_H) for Flurazepam is more than Oxazepam (i.e. 4.076 and 2.44). Meaning to binding of Flurazepam is more cooperative than Oxazepam. Table 2 shows some physical parameters of (F) and (O).

Tablet 2: Physical Parameter of Oxazepam and Flurazepam.

Diazepine	log P*	MW	pK_a
Oxazepam	2.2	286.7	1.7
Flurazepam	2.3	387.9	1.9

The partition coefficient (The octanol-water partition coefficient is the ratio of the concentration of a chemical in octanol and in water at equilibrium and at a specified temperature. Octanol is an organic solvent that is used as a surrogate for natural organic matter. This parameter is used in many environmental studies to help determine the fate of chemicals in the environment) of Flurazepam is more than Oxazepam, it means that Oxazepam is more polar than Flurazepam and we suppose that it binds to more polare binding site. When one molecule of Flurazepam binds to HSA, it will be accessible to receive next molecules easier. Oxazepam is more polar than Flurazepam and binds to the HSA, but the its cooperativity is less than Flurazepam.

Acknowledgement

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