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BINDING OF ESTRIOL TO BOVINE SERUM ALBUMIN: DETERMINATION OF BINDING PARAMETERS AND CHARACTERIZATION OF BINDING SITES

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Abstract: The binding of estriol, a female reproductive hormone, to bovine serum albumin (BSA) was studied by equilibrium dialysis (ED) method at 25°C and pH 7.4. Scatchard method of analysis showed that the binding of estriol has two sets of association constants: the high affinity association constant (k_1) with low capacity (n_1) and low affinity association constant (k_2) with high capacity (n_2). Binding data of ED method suggested the presence of two high affinity binding sites with k_1 value of 1.54×10^6 M^{-1} and ten low affinity binding sites with k_2 value of 1.66×10^5 M^{-1} at 25°C and pH 7.4. Site-specific probe displacement data suggested that estriol binds to site I, the warfarin site, with a higher affinity, while to site II, the benzodiazepine site, with relatively lower affinity.

Key words: Estriol; Equilibrium dialysis; Bovine serum albumin

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 (Jiunn *et al.*, 1987). The early work of Klotz *et al.* (1949) and Scatchard (1949) 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. The pH of the medium and temperature are two important factors that affect association constant (Foster, 1977; Harmsen *et al.*, 1971; Peters, 1985 and Rahman *et al.*, 1993). Physical characteristics and chemical structures of a drug mainly dictate drug protein binding. Several types of bonding may be involved in reversible drug protein binding such as hydrogen bonds, hydrophobic forces electrostatic interactions and van der Waals forces (Klotz, 1973 and Timaseff, 1972).

He and Carter (1992) have studied the binding property of albumin. According to their findings albumin can bind reversibly with large variety of ligands and this ability of binding is considered as the most intriguing property of albumin. On the basis of probe displacement method, it has been detected that there exist at least three relatively high affinity-binding sites on human serum albumin (HSA). 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 (Fehske *et al.*, 1981; Sudlow *et al.*, 1975 and 1976). Binding affinity of a drug in terms of association constant and the number of binding sites are significant phenomena both with respect to pharmacokinetics and pharmacology. Estriol, a female reproductive hormone, is used in the treatment of menopausal disorders. The binding parameters of this drug when it binds to plasma protein have not been studied adequately. In the present study Scatchard analysis has been used to estimate the high affinity and low affinity association constants, number of corresponding binding sites of estriol. Effort has also been made to characterize the binding sites of estriol on BSA molecule using probe displacement method.

Materials and Methods

Estriol was supplied by Organon (Bangladesh) Ltd., site specific probes (warfarin sodium and diazepam) were supplied by Gaco Pharmaceuticals Ltd., Bangladesh. Dialysis membrane was purchased from Medicell International Ltd., 239 Liverpool Road, London and BSA from the Sigma Chemical Co. Ltd.

Estimation of binding parameters: The association constants and the number of corresponding binding sites of estriol for BSA were studied by Scatchard method (Scatchard, 1949) of analysis using equilibrium dialysis technique (Singlas, 1987). Estriol solution (0.01 M) was added with increasing concentrations into 7 out of 8 test tubes containing 5 ml of previously prepared 2×10^{-5} M BSA solution in each so that the final concentrations of estriol were 0.8×10^{-5} M, 2×10^{-5} M, 4×10^{-5} M, 6×10^{-5} M, 9×10^{-5} M, 12×10^{-5} M and 19×10^{-5}

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M. The eighth test tube containing only BSA solution was taken as 'control'. After proper mixing of drug with BSA, 2.0 ml of solution was taken from each test tube and poured into 8 different semipermeable membrane tubes (one end of which was previously tied with thread). The other end of membrane was then folded and also tied with thread. The tubes were then immersed in eight separate 50-ml conical flasks containing 30 ml of phosphate buffer solution (pH 7.4) in each. Then the flasks were shaken in a metabolic shaker for 10 hours at 20 rpm and at 25^{0}C to complete the dialysis. The concentrations of free estriol outside the membrane were measured by an UV spectrophotometer (Spectronic, Genesys^{TM} 2, U.S.A.) at a wavelength of 280nm. The shaking time was set 10 hrs, which was determined experimentally by trial and error method. This time was found to be adequate to complete the dialysis of estriol under the same experimental condition. Estriol was taken into the membrane and allowed to undergo dialysis. After shaking for 10 hours, the free drug concentration on either side of the membrane was found to be identical i.e. dialysis was complete.

Characterization of binding site of estriol using warfarin sodium as site I specific probe and diazepam as site II specific probe: Ten microlitre of 2x 10^{-3} M warfarin sodium solution was added to 7 test tubes containing 2x10^{-5} M BSA solution to have the final warfarin and protein ratio at 1:1 (2x10^{-5} M: 2x10^{-5} M). Estriol solution (0.01 M) was then added with increasing concentrations into six out of seven test tubes containing protein and warfarin (1:1) so that the final ratios of estriol and the protein were 0.25:1, 0.4:1, 0.5:1, 1:1, 2:1 and 3:1. Estriol was not added into the seventh test tube containing warfarin–protein mixture (1:1) that was marked as 'control'. After proper mixing of drug with BSA, 2.0 ml of solution was taken from each test tube and poured into 8 different semipermeable membrane tubes (one end of which was previously tied with thread). The other end of membrane was then folded and also tied with thread. The tubes were then immersed in seven separate 50-ml conical flasks containing 30 ml of phosphate buffer solution (pH 7.4) in each. After proper shaking in a metabolic shaker for 10 hours at 20 rpm and at 25^{0}C to complete dialysis the concentrations of free warfarin were measured by an UV spectrophotometer (Spectronic, Genesys^{TM} 2, U.S.A.) at a wavelength of 308nm.

Similar method was followed for diazepam. The concentrations of free diazepam were measured by UV spectrophotometric method at a wavelength of 235nm.

Results and Discussion

Estimation of binding parameters: Scatchard analysis of estriol binding to BSA at pH 7.4 and at 25°C is shown in Figure 1. Scatchard analysis of the ED data for the binding of estriol to BSA showed a non-linear curve, suggesting the presence of at least two classes of binding sites on BSA for the binding of estriol to BSA. The steeper portion of the curve represents the high affinity binding of the drug and the less steep portion for the low affinity binding on BSA molecule.

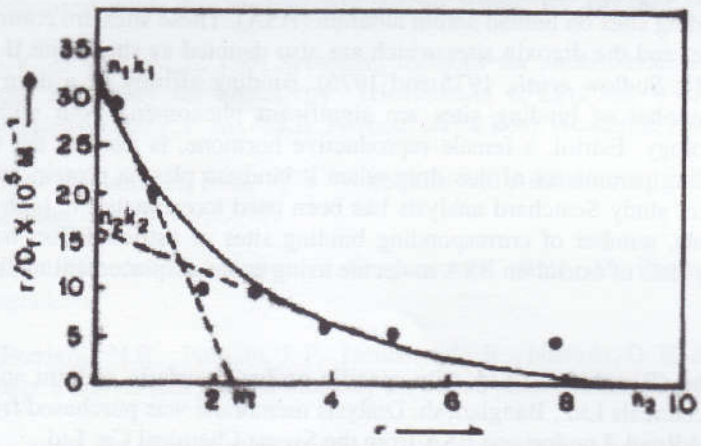

Fig.1. Scatchard plot for the binding of estriol to BSA by equilibrium dialysis at 25^{0}C and pH 7.4. Concentrations used:[BSA]=2x10^{-5}M; [estriol]=0.8x10^{-5}M - 19x10^{-5}M.

As can be seen in Fig. 1 that the extrapolation of the steeper section of the Scatchard plot up to the X-axis showed that the number of high affinity binding site (n_1) for estriol was approximately two (low capacity). Whereas, the extrapolation of the less steep section up to the same axis showed that the number of low affinity binding site (n_2) was approximately ten (high capacity). The high affinity association constant (k_1) for the estriol binding to BSA at pH 7.4 is quite high (1.54x10^6 M^{-1}), while the low affinity association

constant (k_2) for this drug to BSA is about 9 fold lower ($1.66x10^5 M^{-1}$) than that of high affinity association constant. Binding parameters of estriol to BSA at pH 7.4 and 25°C are shown in Table-1.

Table 1. Binding parameters of estriol bound to BSA at pH 7.4 and 25°C.

Temperature	*Association constants*		*Number of binding sites*	
	k_1 (high affinity) $x10^6 M^{-1}$	k_2 (low affinity) $x10^5 M^{-1}$	n_1 (high affinity)	n_2 (low affinity)
25°C	1.54±0.6	1.66±0.04	2.1±0.06	9.6±0.08

Each value represents the average value ± SD (standard deviation) from three experiments

Characterization of binding sites: Binding sites of drugs are determined by studying its ability to displace the site-specific probes. In this study warfarin sodium and diazepam were used as site I and site II specific probes, respectively.

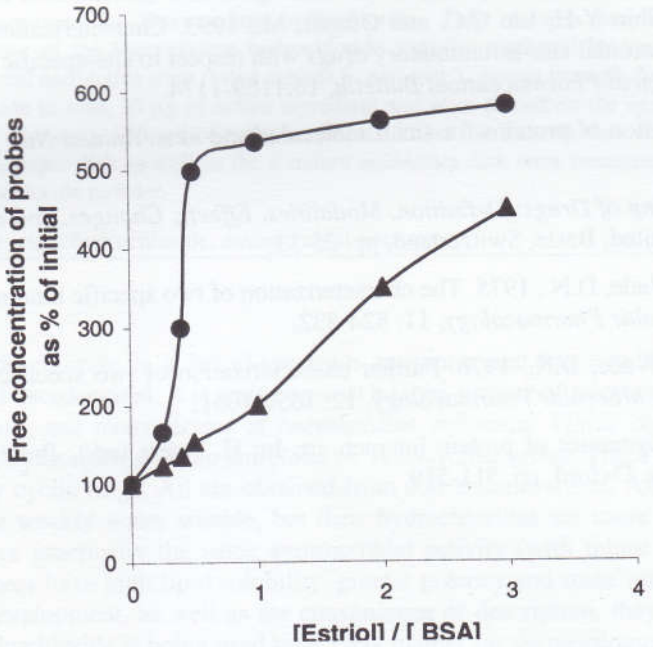

Fig.2. Free fraction of warfarin (•) or diazepam (▲) as % of initial upon the addition of estriol at 25°C and pH 7.4. Concentrations used: (•), [BSA] = [warfarin]= $2x10^{-5}$ M; (▲), [BSA] = [diazepam]= $2x10^{-5}$ M; For both curves, [estriol] = $0\text{-}6x10^{-5}$ M

The experimental results are shown in Figure 2, which shows the change in free concentrations of warfarin and diazepam by estriol. It is evident from the figure that free concentration of warfarin bound to BSA (1:1) was increased from 100% (as % of initial) to 583% by estriol at a estriol to protein ratio of 3:1, while the free concentration of diazepam bound to BSA (1:1) was increased from 100% (as % of initial) to 449% by estriol in the same drug protein ratio. The increment in the free concentration of warfarin by estriol was significantly higher as compared to diazepam suggesting that estriol binds strongly to site I on the BSA molecule. This further suggests that estriol has also an affinity for site II. This implies the fact that at lower drug to BSA ratio, estriol binds to its high affinity binding site i.e., site I, whereas at higher ratio it not only binds to its high affinity site but also to its low affinity site i.e., site II on the BSA molecule.

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