Turk J Pharm Sci 10 (1), 49-56, 2013

Original Article

BINDING OF TRIPROLIDINE HYDROCHLORIDE TO BOVINE SERUM ALBUMIN: ESTIMATION OF BINDING PARAMETERS & CHARACTERIZATION OF BINDING SITES

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Abstract

The binding of triprolidine hydrochloride, an H_1-receptor antagonist, to bovine serum albumin (BSA) was studied by equilibrium dialysis (ED) method at various temperatures. Scatchard method of analysis showed that the binding of triprolidine hydrochloride 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). With the increase in temperature from 10 to 25°C the value of high affinity association constant increased and again this value decreased at 30°C. Binding data suggested the presence of three high affinity binding sites with k_1 value of $2.9x10^6$ M^{-1} and ten low affinity binding sites with k_2 value of $3.9x10^5$ M^{-1} at pH 7.4 and 25°C. Site-specific probe displacement data suggested that triprolidine hydrochloride binds to site II, the benzodiazepine site, with a higher affinity, while to site I, the warfarin site, with relatively lower affinity. The binding process involved in the triprolidine hydrochloride-BSA interaction could not be concluded from the binding parameters obtained from the experiment because of the non-linear relationship between temperature and association constants.

Key words: *Triprolidine hydrochloride, Equilibrium dialysis, Bovine serum albumin.*

Triprolidin Hidroklorür'ün Sığır Serum Albumin'e Bağlanması: Bağlanma Parametrelerinin Değerlendirilmesi & Bağlanma Bölgelerinin Karakterizasyonu

Bir H_1-reseptör antagonisti triprolidin hidroklorür'ün serum sığır albumine (SSA) bağlanması çeşitli sıcaklıklarda denge diyalizi (DD) metodu ile çalışıldı. Scatchard metot analizi triprolidin hidroklorür'ün bağlanmasında iki birleşme sabitine sahip olduğunu gösterdi: düşük kapasiteli (n_1) ve yüksek afiniteli birleşme sabiti (k_1), yüksek kapasiteli (n_2) ve düşük afiniteli birleşme sabiti (k_2). 10 °C 'den 25 °C'ye sıcaklık artışı ile yüksek afiniteli birleşme sabiti değeri arttı ve 30 °C'de bu değer düştü. Bağlanma verilerine göre pH 7.4 ve 25 °C'de k_1 değeri $2.9x10^6$ M^{-1} olan yüksek afiniteli üç bağlanma bölgesi ve k_2 değeri $3.9x10^5$ M^{-1} olan düşük afiniteli on bağlanma bölgesi olduğu bildirildi. Bölge-spesifik prob değiştirme verileri triprolidin hidroklorit'in yüksek afinite ile bölge II'ye, benzodiazepin bölgesine, rölatif olarak daha düşük afinite ile bölge I'e, warfarin bölgesine bağlandığını gösterdi. Triprolidin hidroklorit-SSA etkileşmesindeki bağlanma işlemi, sıcaklık ve birleşme sabitleri arasındaki lineer olmayan ilişkiden dolayı, deneyden elde edilen bağlanma parametrelerinden sonuçlandırılamadı.

Anahtar kelimeler: *Triprolidin hidroklorür, Denge diyalizi, Sığır serum albumini.*

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INTRODUCTION

Triprolidine hydrochloride, a pyridine derivative, is a potent histamine H_1-receptor antagonist (H_1-blocker). It has a rapid onset and long duration action, almost up to 12 h. Triprolidine hydrochloride is effective for the symptomatic treatment of seasonal and perennial allergic rhinitis, vasomotor rhinitis, allergic conjunctivitis due to allergens, foods and prevention of allergic reactions to blood or plasma (1). As the drug is pharmacologically and therapeutically important, the study of protein binding phenomena will provide the basic information on the pharmacological actions, side effects, pharmacokinetic parameters, etc. Hence, the study to investigate the interactions of triprolidine hydrochloride with plasma protein was thought worthwhile.

Protein binding of drugs is not a phenomenon particular to the plasma. It is a well recognized fact that, at least for small molecules, only free, unbound drugs distribute into the extravascular space and are responsible for pharmacological activity and/or side effects (2-4). The binding of a drug to a plasma protein influences its pharmacodynamics and pharmacokinetics (5). Plasma protein binding of drugs has been shown to have significant effects on various aspects of pharmacokinetics (including hepatic metabolism rate, renal excretion, biomembrane permeation rate and steady state distribution volume) and pharmacodynamics (6-8). The binding constants of drugs to plasma protein are useful in studying the pharmacological response of drugs and design of dosage forms (9-10).

Serum albumin is the most abundant protein in blood plasma with concentrations of 3.5 to 5.0 g/dl in humans and 2.7 to 3.3 g/dl in rodents (-9). It has been shown to carry out a broad range of endogenous and exogenous ligands, including >70% of drugs (11). Crystallographic structures have provided a detailed map for several binding sites on the protein, but they do not provide information about the binding affinity and the kinetic of the binding process (12-18). Human serum albumin (HSA) has two main high affinity drug binding sites characterized as Sudlow site I and Sudlow site II (19), which bind drugs selectively. Site I, also known as the warfarin binding site, is formed by a pocket in subdomain IIA of human serum albumin (5). Warfarin is the selective probe drug for this site I (Petitpas et al., 2001). Site II is located in subdomain IIIA and is known as the benzodiazepine binding site. Ibuprofen and diazepam are selective drug probes for site II (5, 20-22).

The early work of Klotz (23) and Scatchard (24) 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 the number of binding sites on the protein molecule are essential. The binding affinity of a drug is quantified in terms of association constant. Recent reports have shown that the binding interactions of drugs are highly influenced by physicochemical parameters including the pH of the medium and temperature (25-28). The binding affinity of some drugs with serum albumin has been shown to be inversely related to the temperature within the range of 10 to 40 $^{\circ}$C (29). Study of the effect of temperature on binding affinity is important to determine the binding mode i.e., the chemical forces that are involved in the drug protein interaction.

Among the serum albumins, bovine serum albumin (BSA) and HSA have extensively been studied. However, because of availability and cost effectiveness we used BSA in our current study. BSA has been shown to have 76% homology with that of HSA (30). BSA has 582 amino acid residues in a single chain, two of which are tryptophans located within the hydrophobic pocket of domain II A at positions 134 and 214 while HSA consists of a single polypeptide chain of 585 amino acid residues in which the single tryptophan 214 residue measures the drug-binding affinity (31). In the present study we have characterized the binding affinity as well as the number of binding sites of triprolidine hydrochloride on BSA. We used BSA because of its stability, easy availability, its lack of effect in many biochemical reactions, low cost, and structural homology with HAS. A probable mechanism of triprolidine hydrochloride and BSA interaction was also postulated by determining different thermodynamic parameters.

MATERIALS AND METHODS

Triprolidine hydrochloride was obtained from Organon (Bangladesh) Ltd. Site-specific probes (warfarin sodium and diazepam) were kindly 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 triprolidine hydrochloride for BSA were studied by Scatchard method (24) of analysis using equilibrium dialysis technique (32).

Triprolidine hydrochloride solution (0.01 M) was added with increasing concentrations into 7 out of 8 test tubes containing 5 ml of previously prepared $2x10^{-5}$ M BSA solution in each so that the final concentrations of triprolidine hydrochloride were $0.8x10^{-5}$ M, $2x10^{-5}$ M, $4x10^{-5}$ M, $6x10^{-5}$ M, $9x10^{-5}$ M, $12x10^{-5}$ M and $19x10^{-5}$ 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. After proper shaking in a metabolic shaker for 10 hours at 20 rpm and at 25^0C to complete dialysis, the concentrations of free triprolidine hydrochloride were measured by an UV spectrophotometer (Spectronic, Genesys^{TM} 2, U.S.A.) at a wavelength of 226nm. In order to assess the effect of temperature on binding, the binding of triprolidine hydrochloride to BSA was also studied at various temperatures (10 and 30^0C) and at pH 7.4.

Characterization of binding site of triprolidine hydrochloride using warfarin as site I specific probe and diazepam as site II specific probe

Ten microlitres of $2x10^{-3}$ M warfarin sodium solution was added to 7 test tubes containing $2x\ 10^{-5}$ M BSA solution to have the final warfarin and protein ratio at 1:1($2x10^{-5}$ M: $2x10^{-5}$ M). Triprolidine hydrochloride 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 triprolidine hydrochloride and the protein were 0.25:1, 0.4:1, 0.5:1, 1:1, 2:1 and 3:1. Triprolidine hydrochloride was not added into the seventh test tube containing warfarin–protein mixture (1:1) and 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 7 different semipermeable membrane tubes (one end of which was previously tied with thread). The other end of membrane was then folded and tied with thread. These 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 25^0C 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 308 nm. Similar method was followed for diazepam. The concentrations of free diazepam were measured by UV spectrophotometric method at a wavelength of 235nm.

Estimation of thermodynamic parameters

Thermodynamic parameters of drug-BSA interaction are usually determined by the method of Pedersen (33) using the van't Hoff plots constructed at different experimental temperatures. The linear relationship between high affinity association constants and temperature makes it possible to calculate the values for thermodynamic parameters involved in the binding process. However, thermodynamic parameters of triprolidine hydrochloride-BSA interaction could not be determined due to lack of linear relationship between temperature and high affinity association constant.

RESULTS AND DISCUSSION

Estimation of binding parameters

Scatchard analysis of triprolidine hydrochloride at pH 7.4 and 25^{0}C is shown in Figure 1. Scatchard analysis of the ED data showed a non-linear curve, suggesting the presence of at least two classes of binding sites for the binding of triprolidine hydrochloride to BSA. As observed in Figure 1, the number of high affinity binding site (n_1) for triprolidine hydrochloride was approximately three (low capacity) and the number of low affinity binding site (n_2) was approximately ten (high capacity). The high affinity association constant (k_1) for the triprolidine hydrochloride binding to BSA at pH 7.4 is quite high ($2.9x10^6$ M^{-1}), while the low affinity association constant (k_2) for this drug to BSA is about 7 fold lower ($3.9x10^5$ M^{-1}) than that of high affinity association constant. Binding parameters of triprolidine hydrochloride to BSA at three different temperatures are shown in Table 1. A recent report showed that the binding constant value of triprolidine hydrochloride-BSA interaction determined by fluorescence quenching studies using modified Stern-Volmer equation was 4.75 $x10^3$ M^{-1} (34), but binding constant in our study was found to be a few hundred times higher. This may me because of the difference of the study method where we used equilibrium dialysis technique and UV spectrometric method and the data was analysed by Scatchard equation. A comparative study for the determination of binding constants for the same drug-protein interaction by different techniques may be required to explain the difference.

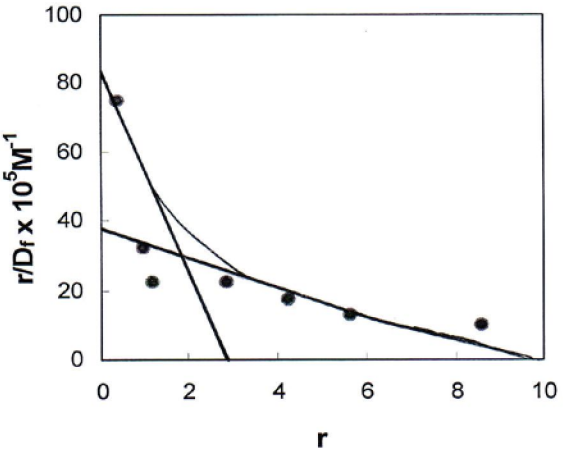

Figure 1. Scatchard plot for the binding of triprolidine hydrochloride to BSA by equilibrium dialysis at pH 7.4 and 25^{0}C.

Concentrations used: [BSA]=$2x10^{-5}$M [Triprolidine hydrochloride]=$0.8x10^{-5}$M - $19x10^{-5}$M

r : the ratio of the concentration of bound triprolidine hydrochloride to the concentration of BSA molecules

Df: the concentration of unbound (free) triprolidine hydrochloride

Table 1. Binding parameters of triprolidine hydrochloride bound to BSA at pH 7.4 and various temperatures

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)
10^{0}C	1.5±0.7	2.66±0.3	4±0.07	10.5±0.60
25^{0}C	2.9±0.6	3.9±0.04	2.8±0.06	9.8±0.08
30^{0}C	0.586±0.9	1.28±0.4	5.8±0.04	14.8±0.40

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

Determination 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. The experimental results are shown in Figure 2 that shows the change in free concentrations of warfarin and diazepam by triprolidine hydrochloride. Free concentration of warfarin bound to BSA (1:1) was increased from 100% (as % of initial) to 408% by triprolidine hydrochloride at a triprolidine hydrochloride 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 833% by triprolidine hydrochloride in the same drug protein ratio. The increment in the free concentration of diazepam by triprolidine hydrochloride was significantly higher as compared to warfarin, which suggests that triprolidine hydrochloride has got more affinity to site II on the BSA molecule than diazepam. This further suggests that triprolidine hydrochloride has also an affinity for site I. This implies the fact that at lower drug to BSA ratio, triprolidine hydrochloride binds to its high affinity binding site i.e., site II, whereas at higher ratio it not only binds to its high affinity site but also to its low affinity site i.e., site I on the BSA molecule. In the same recent report Sandhya *et al*, (34) have showed that triprolidine hydrochloride binds to site-I on BSA although there was no indication about concentration dependency during site specificity of a drug. However, in our study we found triprolidine hydrochloride to bind to BSA at lower concentration to site II whereas at higher ratio to both site I and site II.

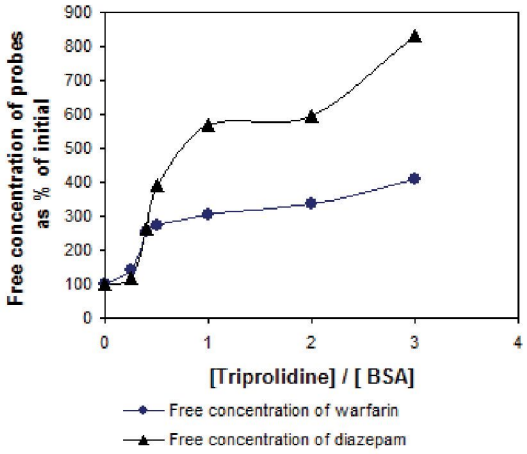

Figure 2. Free fraction of warfarin (●) or diazepam (▲) as % of initial upon the addition of triprolidine hydrochloride at 25^0C and pH 7.4.

Concentrations used:
(●), [BSA] = [warfarin]= $2x10^{-5}$ M
(▲), [BSA] = [diazepam]= $2x10^{-5}$ M
For both curves, [triprolidine hydrochloride]=0-$6x10^{-5}$ M

Effect of temperature on binding parameters

Binding parameters of triprolidine hydrochloride bound to BSA were also determined at pH 7.4 as function of temperatures 10, 25 and 30^0C (Table 1). Figure 3 shows that the high affinity association constant of triprolidine hydrochloride increases significantly as the temperature increases from 10 to 25^0C, which again decreases drastically as temperature is further increased to 30^0C. This suggests that at 25^0C binding of triprolidine hydrochloride with the protein may occur more strongly than at 10^0C and 30^0C. This might be due to the allosteric modification of the BSA molecule. Allosterically the protein molecule was probably more accommodating at 25^0C whereas at 10 and 30^0C it was less accommodating. Therefore there was always an increase in binding at 25^0C but a decrease in binding at 10 and 30^0C. Although the temperature had a significant effect on high affinity association constant of triprolidine hydrochloride when bound to BSA, no linear relationship could be derived between temperature and high affinity association constant.

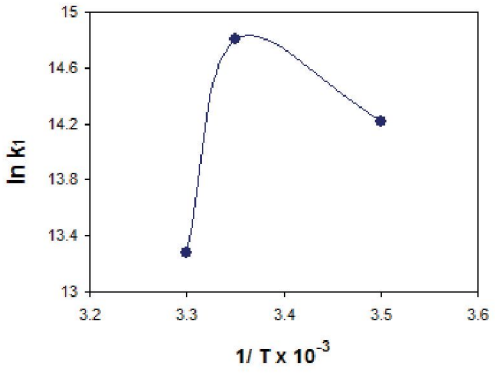

Figure 3. Effect of temperature on high affinity association constant of triprolidine hydrochloride bound to BSA at pH 7.4.

Binding mode

The binding mode of a drug to BSA is evaluated on the basis of thermodynamic data. There are essentially four types of non-covalent interactions that are involved in ligand binding to proteins. These are hydrogen bonds, van der Waals forces, hydrophobic forces and electrostatic interactions (35–36). As no linear relationship could be derived between binding affinity and temperature, the thermodynamic data (H, G, and S) required for determination of binding mode could not be obtained.

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Received: 14.07.2011
Accepted: 08.12.2011