

## Research Article



## Characterization of the Effect of Drug-Drug Interaction on Protein Binding in Concurrent Administration of Sulfamethoxazole and Diclofenac Sodium Using Bovine Serum Albumin

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**Abstract**

**Purpose:** This project was aimed to determine the effect of concurrent administration of sulfamethoxazole and diclofenac sodium.

**Methods:** Equilibrium dialysis method was adopted to study different protein binding aspects of sulfamethoxazole and diclofenac sodium.

**Results:** Sulfamethoxazole showed two types of association constants; high affinity constant  $29.0 \pm 0.20 \times 10^6 \text{ M}^{-1}$  with lower number of binding sites of  $0.7 \pm 1$  and low affinity constant  $1.13 \pm 0.20 \times 10^6 \text{ M}^{-1}$  with higher number of binding sites of  $3.45 \pm 1$  at pH 7.4 and 40 °C temperature. Diclofenac sodium showed high affinity constant  $33.66 \pm 0.20 \times 10^6 \text{ M}^{-1}$  with lower number of binding sites of  $1.01 \pm 1$  and low affinity constant  $1.72 \pm 0.20 \times 10^6 \text{ M}^{-1}$  with higher number of binding sites of  $6.40 \pm 1$  at the same condition. Site specific probe displacement data implied that site-I, warfarin sodium site, was the high affinity site, while site-II, diazepam site, was the low affinity site for these drugs. During concurrent administration, sulfamethoxazole increased the free concentration of diclofenac sodium from  $17.5 \pm 0.14\%$  to  $70.0 \pm 0.014\%$  in absence and from  $22.5 \pm 0.07\%$  to  $83.0 \pm 0.014\%$  in presence of site-I specific probe. Diclofenac sodium also increased the free concentration of sulfamethoxazole from  $2.8 \pm 0.07\%$  to  $52.0 \pm 0.14\%$  and from  $8.5 \pm 0.014\%$  to  $64.4 \pm 0.07\%$  in absence and presence of site-I specific probe respectively.

**Conclusions:** The study revealed that the concurrent administration of sulfamethoxazole and diclofenac sodium may result drug concentration alteration in blood.

**Introduction**

Drug-protein binding means the formation of plasma protein complex with drug after reaching the blood. It is one of the pharmacokinetic parameters of a drug. The amino acids that compose the protein chain have hydroxyl, carboxyl or other sites available for reversible drug interactions. Drug may bind to albumin, alpha acid glycoprotein, lipoproteins and immunoglobulins.<sup>1</sup> According to probe displacement method there are at least three relatively high specific drug-binding sites on the human serum albumin (HSA) molecule. These sites are generally called the warfarin binding site, the benzodiazepine-binding site and the digoxin binding site which are also denoted as site-I, site-II and site-III, respectively. Site-II is more independent binding site and more specific than Site-I and Site-III.<sup>2,3</sup> As free fraction of drug available in plasma is responsible for the pharmacological response, the drug displacement may cause excessive toxicity of the displaced drug due

to increase in free drug concentration in blood. Drug-protein deals with the pharmacokinetic and pharmacodynamic behavior of a drug. Understanding the location of drug binding sites on HSA as well as their extent and composition is essential for proper realization and prediction of drug-drug interaction, explanation of any change in HSA binding of drug under various disease conditions and clarification of various pharmacokinetic parameters.<sup>4</sup> There are two main types of protein binding; strong affinity binding to a small number of sites and weak affinity binding to a large number of sites. Since binding is almost exclusively to albumin and the number of sites available is limited, the protein binding of some drugs depends on the plasma albumin concentration. Plasma protein binding properties are primary determinants of the pharmacokinetic properties of most of the drug such

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as plasma clearance, elimination half-life, apparent volume of the distribution, and area under the curve.<sup>5</sup>

In this study, the binding sites, association constants, number of binding sites of sulfamethoxazole and diclofenac sodium and their interaction with each other both in presence and absence of site specific probe were determined in concurrent administration. Because, the information resource regarding the binding of drugs (sulfamethoxazole and diclofenac sodium) to HSA is extensive, but the mechanism of drug binding to HSA is still a subject of speculation and controversy.

This study is performed to estimate the effect of sulfamethoxazole, an effective antibacterial agent and competitive antagonist of para-aminobenzoic acid (PABA) on diclofenac sodium,<sup>6</sup> a non-steroidal anti-inflammatory drugs (NSAIDs), commonly used for the therapy of chronic forms of arthritis and mild-to-moderate acute pain, fever and inflammation and can be used without prescription.<sup>7</sup> Sulfamethoxazole combined with trimethoprim as cotrimoxazole is used in case of infection and infection often causes pain. So, concurrent or successive administration of diclofenac Na and sulfamethoxazole may be required. For instance, cotrimoxazole and diclofenac co-administration was reported to produce complete stone expulsion rate in patients with urethral colic due to juxtavesical stones.<sup>8</sup> Successive administration of diclofenac sodium and sulfamethoxazole was reported to be significantly beneficial in Whipple's disease with normal duodenal histology and ankylosing spondylitis.<sup>9</sup> Moreover, co-administration of warfarin with sulfamethoxazole, investigated in this study is reported to have clinically significant drug interactions.<sup>10</sup> So, understanding the drug interaction between these two drugs is important which has not yet done. The aim of this project was to understand protein binding parameters of concurrently used sulfamethoxazole and diclofenac sodium on bovine serum albumin (BSA) adopting equilibrium dialysis.<sup>11,12</sup>

### Materials and Methods

Equilibrium dialysis method was employed in this study according to the method of Uddin et al.<sup>12</sup> and Singlas.<sup>13</sup>

### Drugs and chemicals used

Sulfamethoxazole, diclofenac sodium, warfarin sodium (site-I probe) and diazepam (site-II probe) were purchased from different Bangladeshi pharmaceutical companies namely Beximco pharmaceuticals Ltd., ACI pharmaceuticals Ltd., Incepta pharmaceuticals Ltd. and Navana pharmaceuticals Ltd. respectively. Buffering agent disodium hydrogen phosphate ( $\text{Na}_2\text{HPO}_4$ ) and potassium dihydrogen phosphate ( $\text{KH}_2\text{PO}_4$ ) were manufactured by Glaxo; U.K. Dialysis tubing cellulose membrane used in the experiment was purchased from Medicell international Ltd., U.K. which has a molecular weight cut off at 1200 daltons. Bovine serum albumin (fatty acid free, fraction V, 96-98%) purchased from the

Sigma chemical Co. USA was used as a protein. The molecular weight of the protein was assumed to be 66500. De-ionized water, distilled water, methanol supplied by Laboratory Patterson Scientific, U.K. were used as solvent and chemicals.

### Instruments and equipment

Double beam Analykjena UV-visible spectrophotometer (Model 205, Germany) was used to measure the absorbance of drug solution. pH meter (Serial no.- 453088, Hanna, Portugal), electronic balance (Serial no.- 1508, OHAUS, Germany), metabolic shaking incubator (Serial no.- 490639525, New Brunswick Scientific, USA), micro syringe (Serial no.- HN42250, Jencons, UK), hot plate (Serial no.- SWT.550010W, Gallenkamp, England) were used in this study.

### Preparation of membrane

Cellulose membrane was cut into small pieces of- 2 inches length and taken in 1000 ml beaker containing de-ionized water. The pieces of membrane were immersed beneath the de-ionized water and heated for more than 8 hours in order to remove sulfur. The temperature was maintained between 65-70 °C and hot water was replaced by fresh de-ionized water at every 1 hour interval.

### Preparation of buffer, protein (BSA) and stock (sulfamethoxazole and diclofenac sodium, warfarin sodium, diazepam) solutions

Phosphate buffer of pH 7.4 was prepared by the method as described by Perrin and Dempsey.<sup>14</sup> A protein solution of  $2 \times 10^{-5}$  M BSA at pH 7.4 was prepared carefully and gently to avoid foam formation. The protein solution was kept in a refrigerator until use. Stock solutions of  $1 \times 10^{-3}$  M of sulfamethoxazole, diclofenac sodium, warfarin sodium and diazepam were prepared.

### Preparation of standard curve of sulfamethoxazole, diclofenac sodium, warfarin sodium and diazepam

The concentrations of  $0.5 \times 10^{-6}$ ,  $1 \times 10^{-6}$ ,  $2 \times 10^{-6}$ ,  $4 \times 10^{-6}$ ,  $6 \times 10^{-6}$ ,  $8 \times 10^{-6}$ ,  $10 \times 10^{-6}$ ,  $12 \times 10^{-6}$  and  $14 \times 10^{-6}$  M for sulfamethoxazole, diclofenac sodium, warfarin sodium and diazepam were prepared first at pH 7.4. The solutions were then properly mixed. The absorbance of the solutions were determined according to British Pharmacopoeia at  $\lambda_{\text{max}}$  265, 254, 308 and 235 nm for sulfamethoxazole, diclofenac sodium, warfarin sodium, and diazepam respectively by a UV spectrophotometer. As a control or reference sample, phosphate buffer solution of only BSA at pH 7.4 was used. The standard curve was obtained by plotting the absorbance against the corresponding concentrations.

### **Estimation of association constant of sulfamethoxazole and diclofenac sodium**

Association constant and corresponding binding site number of sulfamethoxazole and diclofenac sodium were estimated using Scatchard's method of analysis. To determine the association constant of sulfamethoxazole and diclofenac sodium at pH 7.4, 8 clean and dried test tubes were taken and 3 ml of previously prepared  $2 \times 10^{-5}$  M BSA solution was taken in each of them. Drug stock solutions ( $1 \times 10^{-2}$  and  $1 \times 10^{-3}$  M) was added in different volumes to 7 out of 8 test tubes to have the following concentrations:  $0.5 \times 10^{-6}$ ,  $1 \times 10^{-6}$ ,  $2 \times 10^{-6}$ ,  $4 \times 10^{-6}$ ,  $6 \times 10^{-6}$ ,  $8 \times 10^{-6}$  and  $10 \times 10^{-6}$  M. The eighth test tube containing only BSA solution was marked as "control". After mixing the solutions they were allowed to stand for 10 minutes for maximum binding of drug to BSA.<sup>15,16</sup>

2 ml from each test tube was pipetted out and poured into previously prepared semi-permeable membrane tubes and finally both sides of the tubes were clipped properly to prevent leakage. The membrane tubes containing the drug-protein mixture were immersed in 50 ml flasks containing 30 ml of phosphate buffer solution of pH 7.4. The conical flasks were sealed by foil paper and placed in a metabolic shaker for dialysis for 10 hours at 40 °C and 20 rpm. Buffer samples were collected from each flask after complete dialysis. Absorbance of free concentrations of sulfamethoxazole and diclofenac sodium were measured by a UV-visible spectrometer.

### **Determination of binding site of sulfamethoxazole and diclofenac sodium using warfarin sodium as a site-I specific probe and diazepam as a site-II specific probe**

To determine the binding site of sulfamethoxazole and diclofenac sodium using warfarin sodium and diazepam, 3 ml of previously prepared  $2 \times 10^{-5}$  M BSA solution was taken to each of the 8 clean and dried test tubes.<sup>15,17</sup>  $1 \times 10^{-3}$  M warfarin sodium and diazepam solution were added to the 7 out of 8 test tubes so that the final ratio of protein and probe (warfarin sodium and diazepam) was 1:1 ( $2 \times 10^{-5}$  M:  $2 \times 10^{-5}$  M) in each of these 7 test tubes. The eighth test tube containing only BSA solution was marked as "blank" or "control". These mixtures were allowed to stand for 10 minutes to allow binding of the warfarin sodium and diazepam to their particular binding sites.

Sulfamethoxazole and diclofenac sodium solutions (either  $2 \times 10^{-2}$  M or  $2 \times 10^{-3}$  M) were added with increasing concentrations into 6 out of 7 test tubes containing 1:1 mixture of protein-warfarin sodium. The final ratios of protein:probe (warfarin sodium or diazepam):drug (sulfamethoxazole or diclofenac sodium) were 1:1:0, 1:1:2, 1:1:4, 1:1:6, 1:1:8, 1:1:10 and 1:1:12.

After pipetting, the solution was properly mixed and allowed to stand for 10 minutes to ensure maximum binding of drug to sites and thereby displacing the

probe from sites on BSA. From each test tube 2 ml of the solution was taken into seven different semi-permeable membrane tubes. Two ends of the membrane a tube were clipped ensuring no leakage. The membrane tubes were then immersed in seven separates 50 ml conical flasks containing 30 ml of phosphate buffer solution of pH 7.4.

The conical flasks were then placed in a metabolic shaker for dialysis at 40 °C and 20 rpm and shaking was continued for 10 hours. At the end of dialysis, samples were collected from each flask. The free concentrations of warfarin sodium and diazepam were measured by a UV spectrophotometer at  $\lambda_{\max}$  308 and 235 nm.

### **Drug- drug displacement study**

Effect of sulfamethoxazole on diclofenac sodium binding to BSA was analyzed both in absence and presence of warfarin sodium (site-I specific probe). When the experiment was carried out in absence of warfarin sodium, 3 ml of  $2 \times 10^{-5}$  M BSA solution was taken in each of 8 clean and dried test tubes. 6  $\mu$ l of  $1 \times 10^{-2}$  M diclofenac sodium solution was taken to each of 7 test tubes so that the final ratio between protein and diclofenac sodium was 1:1 ( $2 \times 10^{-5}$  M:  $2 \times 10^{-5}$  M) in each of the 7 test tubes. The eighth test tube containing only BSA solution was marked as blank.<sup>15,16</sup> Sulfamethoxazole was added with an increasing concentration into 6 out of 7 test tubes containing 1:1 mixture of protein-drug to make the final ratio of protein, diclofenac sodium and sulfamethoxazole 1:1:0, 1:1:1, 1:1:2, 1:1:4, 1:1:6, 1:1:8 and 1:1:10.

In presence of warfarin sodium, 3 ml of  $2 \times 10^{-5}$  M BSA solution was taken to each of 8 clean and dried test tubes. 12  $\mu$ l of  $1 \times 10^{-2}$  M warfarin sodium solution was taken to each test tube, so that the final ratio between protein and warfarin sodium was 1:2 ( $2 \times 10^{-5}$  M:  $4 \times 10^{-5}$  M). 6  $\mu$ l of  $1 \times 10^{-2}$  M diclofenac sodium solution was taken to each of 7 clean and dried test tubes and the final ratio among protein, warfarin sodium and diclofenac sodium was 1:2:1 ( $2 \times 10^{-5}$  M:  $4 \times 10^{-5}$  M:  $2 \times 10^{-5}$  M) in each of the 7 test tubes. The eighth test tube containing only BSA and warfarin sodium solution was marked as "blank". Sulfamethoxazole was added with an increasing concentration into 6 out of 7 test tubes to make the final ratio 1:2:1:0, 1:2:1:1, 1:2:1:2, 1:2:1:4, 1:2:1:6, 1:2:1:8 and 1:2:1:10 of protein, warfarin sodium, diclofenac sodium and sulfamethoxazole.

In the both tests, all the solution mixtures were then properly mixed and allowed to stand for 15 minutes for the confirmation of maximum binding to BSA. After that the solution was pipetted out and poured into 7 different semi-permeable membrane tube and both ends of the membrane tubes were clipped to ensure no leakage. The tubes containing drug-protein mixture were immersed in 50 ml conical flask containing 30 ml of phosphate buffer solution of pH 7.4. The conical flasks were placed in a metabolic shaker at 40 °C and 20 rpm for about 10 hours uninterruptedly. After

shaking samples in the buffer solution were collected. Free concentrations of diclofenac sodium were measured by a UV-visible spectrometer.

Effect of diclofenac sodium on sulfamethoxazole binding to BSA was also measured in absence and presence of site-I specific probe warfarin sodium using the same protocol. Free concentrations of sulfamethoxazole were measured by a UV-visible spectrometer.

### Data Analysis

The fraction of unbound drug was calculated from the absorbance of UV spectrometer. Both association constant ( $k$ ) and number of binding sites ( $n$ ) of sulfamethoxazole and diclofenac sodium were determined by using scatchard plot. Scatchard plot is constructed by plotting the ratio between the molar concentration of bound drug and number of protein molecule ( $r$ ) in abscissa and  $r/D_f$  ( $D_f$  represents the free drug concentration). The  $r$  and  $D_f$  data were fitted by linear least squares regression analysis when number of binding sites and association constants were calculated.<sup>11,12,15,17</sup>

### Results and Discussion

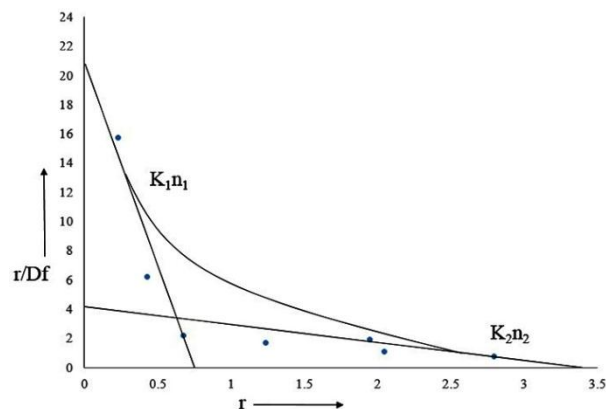
Adequate knowledge about composition, size and location of binding sites are required for a proper explanation of serum protein binding. It is important, as it is an essential tool for the rational understanding and serum albumin binding during various physiological conditions and concurrent administration. So, the mechanism of drug-albumin binding is of vital importance.<sup>2-5,18-21</sup>

#### Estimation of binding parameters

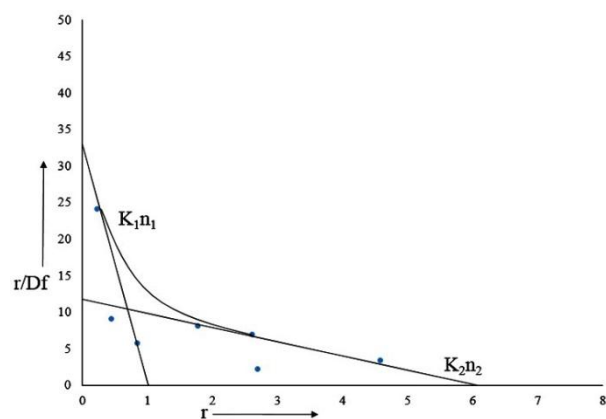
To estimate the binding parameters of sulfamethoxazole, equilibrium dialysis (ED) method was used and the subsequent non-linear shape of the Scatchard plot describes both high and low affinity binding site of the drugs on the protein molecule.<sup>11,12,15</sup>

The results obtained by this analysis suggest that both sulfamethoxazole and diclofenac sodium have two types of association constants namely high affinity association constant ( $k_1$ ) with low capacity ( $n_1$ ) and low affinity association constant ( $k_2$ ) with high capacity ( $n_2$ ). Sulfamethoxazole is characterized by a  $k_1$  to BSA and the value at pH 7.4 is  $29 \pm 0.20 \times 10^6 \text{ M}^{-1}$  while  $k_2$  is  $1.13 \pm 0.20 \times 10^6 \text{ M}^{-1}$  which is nearly 26 times lower than that of  $k_1$ . So, for sulfamethoxazole, the lower number ( $n_1$ ) and higher number ( $n_2$ ) of binding sites are found  $0.7 \pm 1$  and  $3.45 \pm 1$  respectively (Figure 1).

On the other hand, diclofenac sodium is characterized by a ( $k_1$  to BSA and the value at pH 7.4 is  $33.66 \pm 0.20 \times 10^6 \text{ M}^{-1}$ , while  $k_2$  is  $1.72 \pm 0.20 \times 10^6 \text{ M}^{-1}$  for diclofenac sodium is nearly 19.5 times lower than that of the higher affinity association constant. So, for diclofenac sodium, the lower numbers ( $n_1$ ) and higher numbers ( $n_2$ ) of binding sites are  $1.01 \pm 1$  and  $6.40 \pm 1$  respectively (Figure 2).



**Figure 1.** Scatchard plot of sulfamethoxazole at pH 7.4 and 40°C  $D_f$  = Free drug concentration,  $r$  = Ratio of molar concentration of bound drug and protein,  $K_1$ = High affinity association constant,  $n_1$ = Number of low capacity binding site per protein,  $K_2$ = Low affinity association constant,  $n_2$ = Number of high capacity binding site per protein.

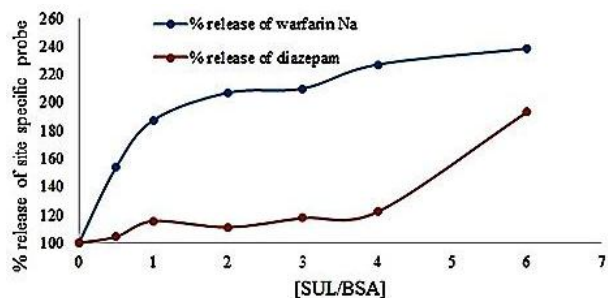


**Figure 2.** Scatchard plot of diclofenac sodium at pH 7.4 and 40 °C  $D_f$  is the free drug concentration and  $r$  is the ratio of molar concentration of bound drug and protein,  $K_1$ = High affinity association constant,  $n_1$ = Number of low capacity binding site per protein,  $K_2$ = Low affinity association constant,  $n_2$ = Number of high capacity binding site per protein.

#### Identification and characterization of binding site

Well-established probes, which are specific for particular sites on the albumin molecule, are used for identification of binding site of the drugs on the protein molecule. If a drug is able to displace a probe from its binding site, it is assumed that, the drug also binds to that particular site. Thus, the binding site as well as the specificity and relative strength of binding to albumin of sulfamethoxazole have been determined by this principle. Here, as site-I specific probe, warfarin sodium and site-II specific probe, diazepam were used.<sup>11,12,15</sup>

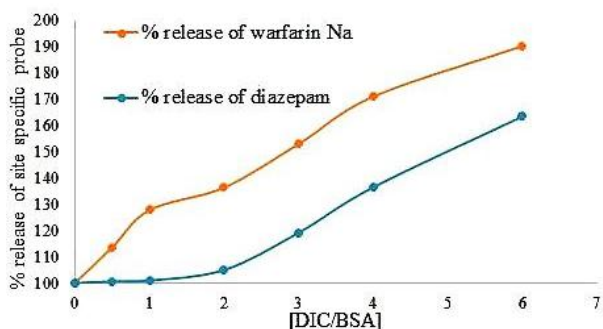
To characterize the binding site of sulfamethoxazole, the free concentration of warfarin sodium (site-I specific probe) bound to BSA was measured upon the addition of sulfamethoxazole. It was found that, the free concentration of warfarin sodium was increased from 100% (as % of initial) to 238.5% when the ratio of sulfamethoxazole to BSA was increased from 0 to 6 (Figure 3).



**Figure 3.** Data for site determination of sulfamethoxazole on BSA using warfarin sodium and diazepam  
SUL= Sulfamethoxazole, BSA= Bovine serum albumin.

In contrast, under the same experimental conditions, when in lieu of warfarin sodium, diazepam was used as site-II specific probe, the increment of the free concentration of diazepam by sulfamethoxazole was increased from 100% (as % of initial) to 193.3% when the ratio of sulfamethoxazole to BSA was increased from 0 to 6 (Figure 3).

On the other hand to characterize the binding site of diclofenac sodium, the free concentration of warfarin sodium (site-I specific probe) bound to BSA was measured upon the addition of diclofenac sodium. It was found that, the free concentration of warfarin sodium was increased from 100% (as % of initial) to 190.3% when the ratio of diclofenac sodium to BSA was increased from 0 to 6 (Figure 4).



**Figure 4.** Data for site determination of diclofenac sodium on BSA using warfarin sodium & diazepam  
DIC= Diclofenac sodium, BSA= Bovine serum albumin.

In contrast, under the same experimental conditions, when in lieu of warfarin sodium, diazepam was used as site-II specific probe, the increment of the free concentration of diazepam by diclofenac sodium was from 100% (as % of initial) to 163.5% when the ratio of diclofenac sodium to BSA was increased from 0 to 6 (Figure 4).

From the study, it was found that, sulfamethoxazole increased the free concentration of warfarin sodium from 100% to 238.5%, whereas the free concentration of diazepam from 100% to 193.3%. On the other hand, diclofenac sodium also increased the free concentration of warfarin sodium from 100% to 190.3%, while under the same experimental conditions, diclofenac sodium increased the free concentration of diazepam from 100% to 163.5%. From these data, this is evident that, the increment of free concentration of warfarin sodium is greater than that of diazepam by both sulfamethoxazole and diclofenac sodium. So it can be concluded that both drugs preferentially bind to site-I. Again, as the displacements of diazepam are quite enough, it can be also suggested that, sulfamethoxazole and diclofenac sodium in addition to the site-I also bind to site-II on the BSA molecule but to a lower extent.

**Drug-drug displacement study**

Both sulfamethoxazole and diclofenac sodium were found site-I specific drug, so warfarin sodium was chosen as the probe when only one probe was used at physiological pH. In sulfamethoxazole-diclofenac sodium interaction study, the effect of sulfamethoxazole on diclofenac sodium bound to BSA and vice versa was determined in absence and presence of site-I specific probe warfarin sodium (Table 1 and 2). Diclofenac sodium bound to BSA (1:1;  $2 \times 10^{-5}$  M:  $2 \times 10^{-5}$  M) released from  $17.5 \pm 0.14\%$  to  $70.0 \pm 0.014\%$  upon the addition of sulfamethoxazole in absence of site-I specific probe (warfarin sodium). Again, in presence of site-I specific probe free concentration of diclofenac sodium increased from  $22.5 \pm 0.07\%$  to  $83.0 \pm 0.014\%$  with the increase of sulfamethoxazole concentration from  $0 \times 10^{-5}$  M to  $10 \times 10^{-5}$  M.

**Table 1.** Effect of sulfamethoxazole on diclofenac sodium bound to BSA

Ob. No.	Added SUL conc. $\times 10^{-5}$ M	[SUL] / [BSA]	In absence of site-I specific probe warfarin Na		In presence of site-I specific probe warfarin Na	
			Free conc. of DIC $\times 10^{-5}$ M	% Displacement of DIC	Free conc. of DIC $\times 10^{-5}$ M	% Displacement of DIC
1	0	0	0.35 $\pm$ 0.07	17.5 $\pm$ 0.14	0.45 $\pm$ 0.007	22.5 $\pm$ 0.07
2	1.0	0.5	0.48 $\pm$ 0.014	24.0 $\pm$ 0.14	0.63 $\pm$ 0.001	31.5 $\pm$ 0.014
3	2.0	1	0.60 $\pm$ 0.0014	30.0 $\pm$ 0.07	0.75 $\pm$ 0.001	37.5 $\pm$ 0.014
4	4.0	2	0.76 $\pm$ 0.003	38.0 $\pm$ 0.014	0.97 $\pm$ 0.003	48.5 $\pm$ 0.07
5	6.0	3	0.93 $\pm$ 0.007	46.5 $\pm$ 0.014	1.18 $\pm$ 0.007	59.0 $\pm$ 0.14
6	8.0	4	1.12 $\pm$ 0.001	56.0 $\pm$ 0.014	1.38 $\pm$ 0.001	69.0 $\pm$ 0.028
7	10.0	5	1.40 $\pm$ 0.001	70.0 $\pm$ 0.014	1.66 $\pm$ 0.007	83.0 $\pm$ 0.014

Data are represented as mean $\pm$ SD of replicates, Ob.= Observation, conc.= Concentration, SUL= Sulfamethoxazole, BSA= bovine serum albumin, DIC=Diclofenac sodium.

**Table 2.** Effect of diclofenac sodium on sulfamethoxazole bound to BSA

Ob. No.	Added DIC conc. $\times 10^{-5}$ M	[DIC] / [BSA]	In absence of site-I specific probe warfarin Na		In presence of site-I specific probe Warfarin Na	
			Free conc. of SUL $\times 10^{-5}$ M	% Displacement of SUL	Free conc. of SUL $\times 10^{-5}$ M	% Displacement of SUL
1	0	0	0.056 $\pm$ 0.001	2.8 $\pm$ 0.07	0.170 $\pm$ 0.007	8.5 $\pm$ 0.14
2	1.0	0.5	0.16 $\pm$ 0.003	7.3 $\pm$ 0.14	0.334 $\pm$ 0.014	16.7 $\pm$ 0.14
3	4.0	2	0.392 $\pm$ 0	19.6 $\pm$ 0.14	0.620 $\pm$ 0.007	31.0 $\pm$ 0.07
4	6.0	3	0.560 $\pm$ 0.007	28.0 $\pm$ 0.28	0.800 $\pm$ 0.014	40.0 $\pm$ 0.14
5	8.0	4	0.820 $\pm$ 0.007	41.0 $\pm$ 0.07	1.090 $\pm$ 0.014	54.4 $\pm$ 0.28
6	10.0	5	1.040 $\pm$ 0.007	52.0 $\pm$ 0.14	1.288 $\pm$ 0.007	64.4 $\pm$ 0.07

Data are represented as mean $\pm$ SD of replicates, Ob.= Observation, conc.= Concentration, DIC= Diclofenac sodium, BSA= Bovine serum albumin, SUL= Sulfamethoxazole.

Sulfamethoxazole bound to BSA (1:1;  $2 \times 10^{-5}$  M:  $2 \times 10^{-5}$  M) released upon the addition of diclofenac sodium in absence of site-I specific probe (warfarin sodium) increased from 2.8 $\pm$ 0.07% to 52.0 $\pm$ 0.14%, while in presence of site-I specific probe, free concentration of sulfamethoxazole increased from 8.5 $\pm$ 0.14% to 64.4 $\pm$ 0.07% with the increase of diclofenac sodium concentration from  $0 \times 10^{-5}$  M to  $10 \times 10^{-5}$  M.

This study suggests that both sulfamethoxazole and diclofenac sodium increased the release of free drug from their binding protein both in presence and absence of site specific probe. From the binding characteristics of SUL and DIC and their interaction pattern recommend that DIC has less affinity to BSA protein than SUL which can be easily displaced by SUL and other site specific probes.

#### Drug-drug interaction

Plasma protein binding properties are considered as the primary determinants of the pharmacokinetic properties of drugs. Any physiological condition causing alteration in the protein binding of the drugs might lead to changes in the pharmacokinetic and pharmacological properties of the drugs. Drug-drug interactions thus play a vital role in the extent of plasma-protein binding and consequently the therapeutic effect of the drugs. To evaluate the probable interaction between drugs, the binding sites of the drug on the protein have to be known.<sup>22</sup>

Sulfamethoxazole is an effective antibacterial agent is used in different bacterial infections as well as is the drug of choice for treatment of many disease conditions like pneumocystosis and an acceptable oral therapy for recurrent urinary tract infections caused by susceptible bacteria.<sup>23</sup> As infectious diseases are associated with pain and treatment of pain and infection should be of equal priorities, the patients suffering from bacterial infection may require administering diclofenac during the regular use of sulfamethoxazole therapy.<sup>24</sup> In that case, if there is no alternative to sulfamethoxazole or diclofenac sodium and the drugs cannot be avoided altogether, the administration of these two drugs to be staggered over a period of time.

As, sulfamethoxazole has more affinity to plasma protein in comparison to diclofenac sodium and they have same binding site; so, sulfamethoxazole may displace diclofenac sodium slowly from its binding site resulting the increment of free diclofenac sodium concentration in blood. Dose adjustment of both drugs may be required in case of concurrent administration of sulfamethoxazole and diclofenac sodium to avoid drug toxicity.<sup>25-27</sup>

#### Conclusion

From the obtained data, it is too early to draw a concrete conclusion about the pharmacokinetic or pharmacological properties of the drug. Some recent high sensitive technologies like HPLC/UV analysis method can be adopted for quantitation and determination of the binding pattern of drugs to HSA.<sup>28</sup> A more detailed study including *in vivo* experiments is warranted in this respect. However, the results of the present study in combination with the current advances in the binding of sulfamethoxazole and diclofenac sodium, might be helpful in realizing the overall binding behavior of these two drugs with HSA.

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#### Ethical Issues

Not applicable.

#### Conflict of Interest

The authors declare no conflict of interests.

#### References

1. HKragh-Hansen U. Molecular aspects of ligand binding to serum albumin. *Pharmacol Rev* 1981;33(1):17-53.
2. Sudlow G, Birkett DJ, Wade DN. The characterization of two specific drug binding sites

- on human serum albumin. *Mol Pharmacol* 1975;11(6):824-32.
3. Sudlow G, Birkett DJ, Wade DN. Further characterization of specific drug binding sites on human serum albumin. *Mol Pharmacol* 1976;12(6):1052-61.
  4. Rahman MH, Yamasaki K, Shin YH, Lin CC, Otagiri M. Characterization of high affinity binding sites of non-steroidal anti-inflammatory drugs with respect to site-specific probes on human serum albumin. *Biol Pharm Bull* 1993;16(11):1169-74.
  5. Lin JH, Cocchetto DM, Duggan DE. Protein binding as a primary determinant of the clinical pharmacokinetic properties of non-steroidal anti-inflammatory drugs. *Clin Pharmacokinet* 1987;12(6):402-32. doi: 10.2165/00003088-198712060-00002
  6. Hardman JG, Limbird LE, Gilman AG. Antimicrobial agents, Goodman and Gilman's the pharmacological basis of therapeutics. New York: McGraw-Hill companies inc; 2001.
  7. Liver Tox: Clinical and research information on drug-induced liver injury. National institute of diabetes and kidney diseases, Diclofenac, <http://livertox.nih.gov/Diclofenac.htm>. Accessed on 17 September, 2016.
  8. Dellabella M, Milanese G, Muzzonigro G. Efficacy of tamsulosin in the medical management of juxtavesical ureteral stones. *J Urol* 2003;170(6 Pt 1):2202-5. doi: 10.1097/01.ju.0000096050.22281.a7
  9. Ahmadi-Simab K, Schnitzler P. whipple's disease with normal duodenal histology and ankylosing spondylitis. *Dtsch Med Wochenschr* 2009;134(4):127-30. doi: 10.1055/s-0028-1123969
  10. Venkatakrisnan K, von Moltke LL, Greenblatt DJ. Effects of the antifungal agents on oxidative drug metabolism: Clinical relevance. *Clin Pharmacokinet* 2000;38(2):111-80. doi: 10.2165/00003088-200038020-00002
  11. Alam SMM, Yeasmin L, Reza MN, Shilpi JA, Ahmed F, Akter MJ. Studies on binding parameters of chloramphenicol on bovine serum albumin (BSA). *J Biol Sci* 2004;4(2):203-6. doi: 10.3923/jbs.2004.203.206
  12. Uddin SJ, Shilpi JA, Murshid GMM, Rahman AA, Sarder MM, Alam MA. Determination of binding sites of arsenic on bovine serum albumin using warfarin (site-I specific probe) and diazepam (site-II specific probe). *J Biol Sci* 2004;4(5):609-12. doi: 10.3923/jbs.2004.609.612
  13. Singlas E, Determination of protein binding and its characteristics. In: Protein binding of drugs. Basel: Hoffmann-La Roche; 1987.
  14. Perrin DD, Dempsey B. Application of pH buffer. In: Buffers for pH and metal ion control. London: Chapman & Hall; 1974.
  15. Alam SMM, Rahman MM, Rahman MH, Rahman NN. Tetracycline hydrochloride binds with high affinity to warfarin site (site-I) on bovine serum albumin: temperature and pH influence the binding process. *Pak J Biol Sci* 2004;7(12):2099-104. doi: 10.3923/pjbs.2004.2099.2104
  16. Alam MA, Awal MA, Subhan N, Sikder MH, Alam SMM, Mostafa M, et al. Studies of in-vitro amlodipine and arsenic displacement interaction at binding sites of bovine serum albumin. *Iran J Pharmacol Ther* 2008;7(1):1-8.
  17. Khodaei A, Bolandnazar S, Valizadeh H, Hasani L, Zakeri-Milani P. Interactions between sirolimus and anti-inflammatory drugs: Competitive binding for human serum albumin. *Adv Pharm Bull* 2016;6(2):227-33. doi: 10.15171/apb.2016.031
  18. Birkitt DJ, Wanwimolruk S. Protein binding and drug transport, serum albumin as a specific binding protein for drugs and endogenous compounds. Stuttgart-New York: FK Schattauer Verlag; 1985.
  19. Brown JR, Shockley P. Serum albumin: Structure and characterization of its ligand binding sites. In: Jost PC, Griffith OH, editors. Lipid-protein interaction. New York: John Wiley & Sons; 1982.
  20. He XM, Carter DC. Atomic structure and chemistry of human serum albumin. *Nature* 1992;358(6383):209-15. doi: 10.1038/358209a0
  21. Peters T. Serum albumin. In: Anfinsen CB, John TE, Frederic MR, editors. Advances in protein chemistry. New York: Academic Press; 1985.
  22. Kober A, Sjöholm I. The binding sites on human serum albumin for some nonsteroidal antiinflammatory drugs. *Mol Pharmacol* 1980;18(3):421-6.
  23. Gleckman R, Alvarez S, Joubert DW. Drug therapy reviews: Trimethoprim-sulfamethoxazole. *Am J Hosp Pharm* 1979;36(7):893-906.
  24. White RJ. Wound infection-associated pain. *J Wound Care* 2009;18(6):245-9. doi: 10.12968/jowc.2009.18.6.42803
  25. Leon S, Andrew YU. Physiologic drug distribution and protein binding. In: Applied biopharmaceutics & pharmacokinetics. 4th ed. New York: Printice Hall international inc; 1999.
  26. Davies NM, Anderson KE. Clinical pharmacokinetics of diclofenac. Therapeutic insights and pitfalls. *Clin Pharmacokinet* 1997;33(3):184-213. doi: 10.2165/00003088-199733030-00003
  27. Faisal KS, Giri A, Azam KR, Rahman M, Rashid HA. Solid dispersion formulations of Pioglitazone HCl using five different polymers for enhancing dissolution profile. *Int J Pharm Life Sci* 2013;4(1):2269-75.
  28. Esmaeilzadeh S, Valizadeh H, Zakeri-Milani P. A simple, fast, low cost, hplc/uv validated method for determination of flutamide: Application to protein binding studies. *Adv Pharm Bull* 2016;6(2):251-6. doi: 10.15171/apb.2016.034