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In vitro Model for Studying Interactions between Ketorolac and Omeprazole with Bovine Serum Albumin by UV-Spectroscopic Method

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Abstract

The binding of Ketorolac and Omeprazole to bovine serum albumin (BSA) was studied by equilibrium dialysis method followed by UV spectroscopy. Warfarin and Diazepam were used as site-I and site-II specific probe, respectively. The binding of Ketorolac and Omeprazole was characterized by two sets of association constant: high affinity association constant (K_1) with low capacity binding site (n_1) and low affinity association constant (K_2) with high capacity binding site (n_2). In this study, n_1 and n_2 values were found to be 0.25 ± 0.006 and 1.8 ± 0.025 for Ketorolac and 0.22 ± 0.030 and 1.3±0.035 for Omeprazole at pH 7.4 and 37°C, respectively. At the same condition, the values of K_1 and K_2 for Ketorolac were found to be 0.624 ± 0.033 μM^{-1} and 0.133 ± 0.023 μM^{-1} and that of Omeprazole were 0.51 ± 0.001 μM^{-1} and 0.28 ± 0.005 μM^{-1}, respectively. Site specific probe displacement studies implied that both Ketorolac and Omeprazole bind predominantly to site-II, the Diazepam site. In the present study, both Ketorolac and Omeprazole increased the free fraction of each other when they simultaneously bound to BSA. They compete for a common binding site on the albumin molecule, thereby free fraction of both the drugs was increased as compared to the level obtained when the drugs were given individually. We, thus, conclude that during concurrent administration of Ketorolac and Omeprazole adequate precautions should be taken. However, further studies are needed on *in-vivo* model to substantiate the findings from *in-vitro* experiments.

Key words: Ketorolac Tromethamine, Omeprazole, Bovine serum albumin (BSA), Protein binding, Dialysis membrane.

Introduction

In recent years, the interactions between biomacromolecules like serum albumins and medicines have been widely studied with great interest. The most important property of this group of proteins is that they serve as a depot protein and transport protein for many drugs and other bioactive small molecules (Elena *et al*., 2007; Hu *et al*., 2006; Neelam *et al*., 2006). The interactions between drug molecule and protein may result in the formation of stable and reversible drug-protein complex having important effect on the distribution, metabolism and excretion of drugs. To know the effect of drug in metabolic process in human body as well as its clinical and toxic effects, it is essential to know the interactions of biomacromolecules with drugs (Chen *et al*., 2008).

Ketorolac is a non-steroidal anti-inflammatory drug (NSAID) that exhibits analgesic activity. Patients taking NSAID including Ketorolac may have symptoms of peptic ulceration or gastrointestinal bleeding due to inhibition of production of protective mucous in gastric mucosa. Omeprazole is used to prevent NSAIDs induced gastritis. For these actions, Ketorolac is prescribed along with Omeprazole to reduce the incidence of hyperacidity.

There are two main types of protein bindings, viz-strong affinity binding to a small number of sites and weak affinity binding to a large number of sites. The protein binding of some drugs depends on the plasma albumin concentration (Jiunn *et al*., 1987). Keeping these considerations in mind, a NSAID, Ketorolac and a PPI, Omeprazole have been investigated to determine the respective parameters.

In this study, BSA was selected as our protein model because bovine serum albumins and human serum albumins display approximately 76% homology, and the 3D structure of BSA is believed to be similar to that of HSA. And also for its medical importance, low cost, ready availability, and the results of all studies are consistent with the fact that bovine and human serum albumins are homologous proteins (He *et al*., 1992).

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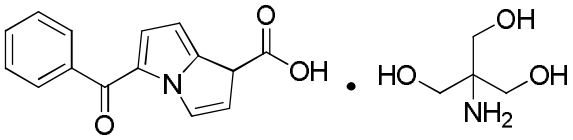
The type and nature of protein binding depends on the physiochemical properties of drug molecules, their concentration, pH of the medium and also on concentration and number of available binding sites on plasma protein. Protein binding of a drug is a limiting factor for drug effect. Simultaneous administration of two or more drugs into the systemic circulation can modify the affinity of the drug to bind with plasma protein and thus percentage of protein binding. Due to this modification, the combined therapy can change the volume of distribution, renal and hepatic clearance and hence drug effect (Singlass *et al*., 1987; Cadwallader *et al*., 1985).

The ability of one drug to inhibit the binding of another molecule is a function of their relative concentrations, binding affinities and specificity of binding (Koch-Weser *et al*., 1976). Since only a small fraction of the drug would ordinarily be available in the free from, the displacement of even small percentage of the amount that is bound to proteins could produce considerable increase in activity. Thus, when studying the drug-drug displacement, the possibility of occurrence of site to site displacement should be considered as there will be a difference between the free concentration of a displaced drug with or without site to site displacement (Kabir *et al*., 1999).

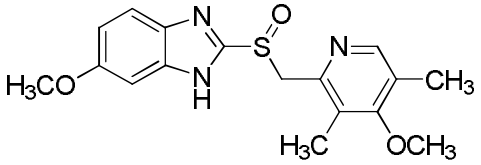
The purpose of our work was to see the mechanism of binding of Ketorolac and Omeprazole to BSA and to observe the effect on the free concentration of Ketorolac by Omeprazole and *vice versa* when used concurrently.

Materials and Methods

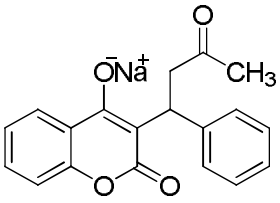
Drugs and Chemicals: Ketorolac tromethamine, Omeprazole and Warfarin sodium were kind gifts from Incepta Pharmaceutical Ltd., Dhaka, Bangladesh and gift sample of Diazepam was from Square Pharmaceuticals Ltd., Dhaka, Bangladesh. Bovine serum albumin (Fraction V, 96-98%, Sigma Aldrich Co. USA.) and semi permeable membrane (Mediceil, England) were purchased from BDH (England). Phosphate buffers were prepared from di-sodium hydrogen phosphate and potassium di-hydrogen phosphate. All other reagents were of analytical grade and purchased from local suppliers.



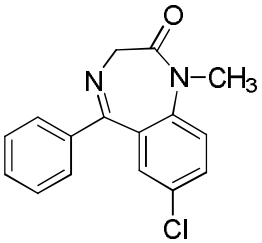
Ketorolac Tromethamine



Omeprazole



Warfarin sodium



Diazepam

Figure 1. Chemical structures of drugs studied

Apparatus: A digital pH meter (Hanna instrument & Co., USA) was used to adjust the pH of the buffer solutions. A recording UV/VIS spectrophotometer (UV-1800, Shimadzu, Japan) was used for the measurement of absorbance of the unbound drugs present in the buffer compartment. A metabolic shaking incubator (Fisher Scientific, UK) was used to shake the plasma-drug mixtures for the attainment of equilibrium.

Equilibrium dialysis: In this experiment, the semipermeable membrane were cut into small pieces and

was boiled for 8 hours at 65-70 °C in de-ionized water to remove sulfur. Activated membrane bags (9 cm long, 3.5 mL capacity) were filled with solutions of serum bovine albumin with different concentration of the drugs and their mixtures. The membrane bags were immersed in a fixed amount (20 mL) of phosphate buffer and the system was shaken gently for 12 hours in a metabolic shaking incubator at 37±1ºC. After completion of dialysis the absorbance of the buffer (outside the membrane bags) were measured at corresponding λ_{max} and the concentrations of the bound and unbound drugs were determined by using a standard curve.

Determination of λ_{max}: Stock solutions of drugs (Ketorolac, Omeprazole, Warfarin and Diazepam) were prepared in small volume of solvent and then working solutions were prepared by diluting with buffer of pH 7.4 in all cases. Thus, different concentrations of drugs were prepared and their absorbances were measured to find out the λ_{max} of these drugs. It was observed that for Ketorolac maximum absorption occurred at 278 nm and that of Omeprazole, Warfarin and Diazepam occurred at 293 nm, 306 nm and 235 nm, respectively.

Preparation of standard curve: For the preparation of standard curves of Ketorolac, Omeprazole, Warfarin and Diazepam, solutions of different concentrations of these drugs were prepared in phosphate buffer of pH 7.4 by taking absorbance values at λ_{max} of 278 nm, 293 nm, 306 nm and 235 nm, respectively. Standard curves were obtained by plotting the absorbance values against the corresponding concentrations.

Estimation of association constants: To determine the association constant of Ketorolac and Omeprazole, different concentrations (5 µM, 10 µM, 15 µM, 20 µM, 40 µM, 60 µM, 80 µM, 100µM and 140 µM) of Ketorolac solutions and different concentrations (3 µM, 5 µM, 10 µM, 15 µM, 20 µM, 40 µM, 60 µM, 80µM and 100 µM) of Omeprazole solutions were mixed with prepared BSA solution (20 µM in phosphate buffer, pH 7.4) to get a final volume of 5 mL of each. These solutions were allowed to stand for few minutes for the maximum binding of Ketorolac and Omeprazole to BSA. From each mixture, 3.5 mL of solution was withdrawn, filled into previously prepared semipermeable membrane tubes and both sides of the membranes were sealed properly. The membrane tubes containing drug-protein mixture were immersed in conical flasks containing 20 mL of phosphate buffer (pH 7.4) and were placed in a metabolic shaker for dialysis for 12 hours (37°C, 40 rpm). Buffer samples were collected from each conical flask after dialysis and fraction of free Ketorolac and Omeprazole were measured by UV spectrophotometer.

Determination of binding site of Ketorolac and Omeprazole using Warfarin as site-I specific probe: To determine the binding sites of Ketorolac and Omeprazole on BSA, the concentrations of BSA and probe (Warfarin Sodium as site-I specific probe) were maintained in 1:1 ratio (20 µM: 20 µM) and Ketorolac and Omeprazole were added in increased concentration (0 to 16 µM). So, the final ratio of BSA:probe:test drugs were 1:1:0, 1:1:1, 1:1:2, 1:1:4, 1:1:5, 1:1:6 and 1:1:8. The dialysis was carried out as described above and free fraction of Warfarin was measured at 306 nm.

Determination of binding site of Ketorolac and Omeprazole using Diazepam as site-II specific probe: The binding sites of Ketorolac and Omeprazole on BSA using Diazepam as site-II specific probe were determined by employing the same methods as described above. Briefly the final ratio of BSA: Diazepam: test drugs were 1:1:0, 1:1:1, 1:1:2, 1:1:4, 1:1:5, 1:1:6 and 1:1:8. The dialysis was carried out as described above and free concentration of Diazepam was measured at 235 nm.

Effect of Omeprazole on Ketorolac bound to BSA: The effect of Omeprazole on Ketorolac, when bound to BSA was estimated in absence and presence of Warfarin as site-I specific probe. In absence of Warfarin, the BSA and Ketorolac were mixed at 1:1 ratio (20 µM: 20 µM) and then Omeprazole was added in increasing concentration (0 to 16 µM) to make final ratio of BSA: Ketorolac: Omeprazole in each experiment as 1:1:0, 1:1:1, 1:1:2, 1:1:4, 1:1:6 and 1:1:8. While in presence of Warfarin, BSA, Warfarin and Ketorolac were mixed at ratio of 1:2:1 and Omeprazole was added in increasing concentration to make the final ratio of protein, probe, Ketorolac and Omeprazole as 1:2:1:0, 1:2:1:1, 1:2:1:2, 1:2:1:4, 1:2:1:6 and 1:2:1:8. Dialysis was carried out and the amount of free Ketorolac was measured in absence and in presence of Warfarin as described above.

Effect of Ketorolac on Omeprazole binding to BSA: The effect of Ketorolac on Omeprazole, when bound to BSA was estimated in the presence and absence of

Warfarin as site-I specific probe. In absence of Warfarin, the BSA and Omeprazole was mixed at 1:1 ratio (20 µM: 20 µM) and then Ketorolac was added in increasing concentration (0 to 16 µM) to make final ratio of BSA, Omeprazole and Ketorolac in each experiment as 1:1:0, 1:1:1, 1:1:2, 1:1:4, 1:1:6 and 1:1:8. While in presence of Warfarin, BSA, Warfarin and Omeprazole were mixed at ratio of 1:2:1 and Ketorolac was added in increasing concentration to make the final ratio of BSA, Warfarin, Omeprazole and Ketorolac as 1:2:1:0, 1:2:1:1, 1:2:1:2, 1:2:1:4, 1:2:1:6 and 1:2:1:8. Dialysis was carried out and the amount of free Omeprazole was measured in absence and in presence of Warfarin as described above.

Results and Discussion

Estimation of binding parameters: The Scatchard analysis of the equilibrium dialysis data showed a non-linear curve, suggesting the presence of at least two classes of binding sites (n_1 and n_2) for the binding of Ketorolac and Omeprazole with BSA as shown in Figure 2. Both high affinity association constant (K_1) and low affinity association constant (K_2) of Omeprazole and Ketorolac were determined by the Scatchard plot. As shown in Figure 2, the number of high affinity binding site with low capacity (n_1) for Ketorolac was 0.25±0.006 and the number of low affinity binding site with high capacity (n_2) was 1.8±0.025. The high affinity association constant (K_1) for the Ketorolac binding to BSA at pH 7.4 is quite high 0.624±0.033 μM^{-1}, while the low affinity association constant (K_2) for this drug is about 0.133±0.023 μM^{-1}. As shown in Figure 2, the number of high affinity binding site (n_1) for Omeprazole was 0.22±0.030 (low capacity) and the number of low affinity binding site (n_2) was 1.3±0.035 (high capacity). The high affinity association constant (K_1) for the Omeprazole binding to BSA at pH 7.4 is quite high 0.51±0.001 μM^{-1}, while the low affinity association constant (K_2) for this drug to BSA is about 0.28±0.005 μM^{-1}.

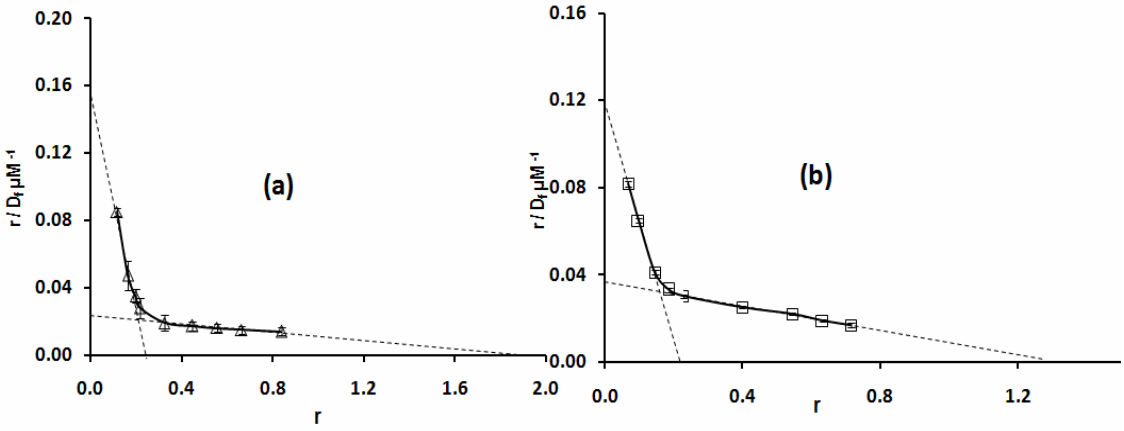


Figure 2. Scatchard plot for the binding of Ketorolac **(a)** and Omeprazole **(b)** bound to BSA at pH 7.4 and 37ºC. Ketorolac (5-140 µM) was added to 20 µM of BSA and Omeprazole (3-100 µM) was added to 20 µM of BSA. SD values from three measurements are shown as error bars.

Table 1. Association constant of Ketorolac and Omeprazole bound to BSA at pH 7.4 and 37°C.

Drug	K_1 (μM^{-1})	n_1	K_2 (μM^{-1})	n_2
Ketorolac	0.624 ± 0.033	0.25 ± 0.006	0.133 ± 0.023	1.8 ± 0.025
Omeprazole	0.51 ± 0.001	0.22 ± 0.030	0.28 ± 0.005	1.3 ± 0.035

Each value represents the mean ± SD, where, n=3

Interaction of Ketorolac and Omeprazole with site specific probes: The effects of Ketorolac on the binding of site specific probes were examined to determine whether or not Ketorolac binds preferentially with site-I or site-II on BSA. The results showed that Ketorolac cause the increment of free fraction of Warfarin and Diazepam from 100% (as % of initial) to 148.9% and 100% (as % of initial) to 170.2%, respectively (Figure 3).

The effect of Omeprazole was measured to know whether or not it can release the Warfarin and Diazepam from their binding sites. It was found that Omeprazole increased the free fraction of Warfarin from 100% (as % of initial) to 124% and that of Diazepam from 100% (as % of initial) to 177.7% (Figure 3).

Interaction of Omeprazole and Ketorolac at the binding sites on BSA: In absence of Warfarin, the free fraction of Ketorolac bound to BSA (1:1) was increased from 40.5% to 87.8% by Omeprazole. Whereas, in presence of Warfarin, this increment was from 51.4% to 98.6% (Figure 4). Free fraction of Omeprazole was increased by Ketorolac from 75.3% to 88% in the absence of Warfarin, and in presence of Warfarin, Ketorolac in the same concentration ratio increased the free Omeprazole from 79.1% to 99.4% (Figure 4).

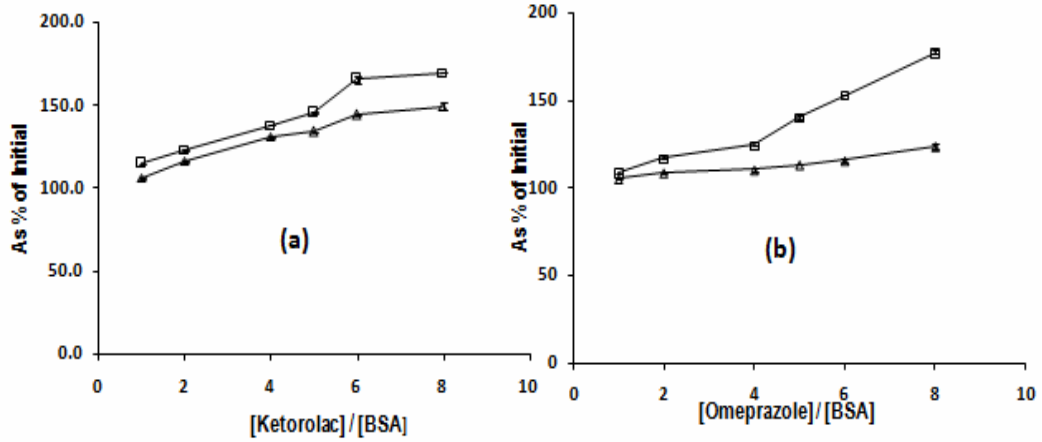


Figure 3. Free fraction of Warfarin (△) and Diazepam (☐) to BSA (1:1) upon the addition of Ketorolac (a) and Omeprazole (b) at pH 7.4 and 37°C. For both curve concentration of BSA, Diazepam and Warfarin was 20 µM. For curve (a) Ketorolac concentrations were (0-160 µM) and for curve (b) Omeprazole concentrations were (0-160 µM). SD values from three similar measurements are shown as error bars.

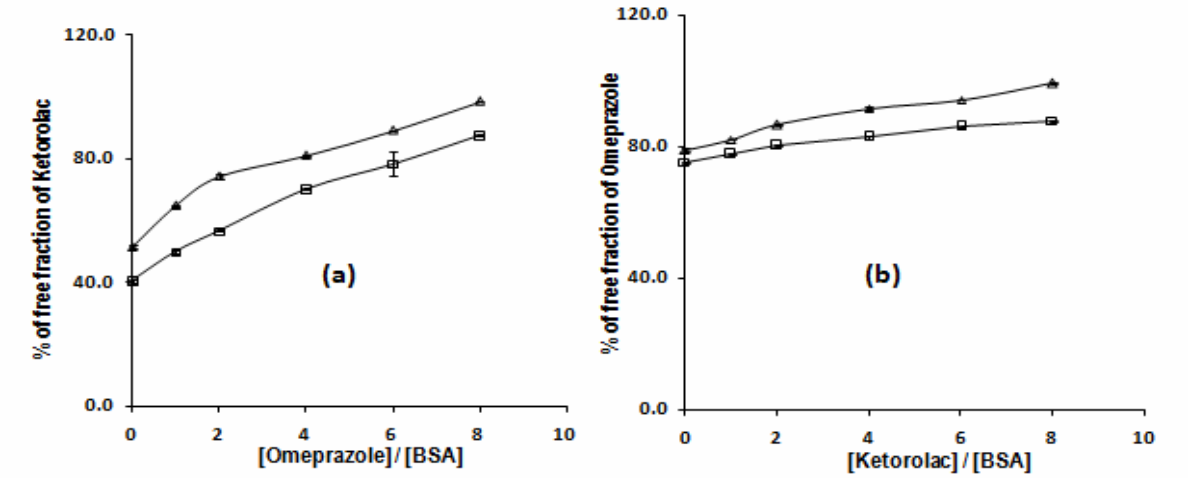


Figure 4. Free fraction of Ketorolac **(a)** bound to BSA (1:1) upon the addition of Omeprazole and Free fraction of Omeprazole **(b)** bound to BSA (1: 1) upon the addition of Ketorolac at pH 7.4 and 37°C in the absence (☐) and presence (△) of Warfarin as site I specific probe. For curve **(a)** the molar concentrations of [BSA]:[Warfarin]:[Omeprazole] = 1:2:1; and [Ketorolac] = 0-160 µM and for curve **(b)** molar concentrations of [BSA]:[Warfarin]:[Ketorolac] = 1:2:1; and [Omeprazole] = 0-160 µM. SD values from three measurements are shown as error bars.

Binding of drugs were determined by studying its ability to displace the site specific probes. In this study, Warfarin and Diazepam were used as site-I and site-II specific probes, respectively. The association constants as shown in Table 1 indicate that both Ketorolac and Omeprazole are highly bound to BSA.

Figure 3 shows the change in free concentration of Warfarin and Diazepam from 100% (as % of initial) to 148.9% and 100% to 170.2%, respectively by Ketorolac. From this observation it can be said that Ketorolac at higher concentration displaced Diazepam to a greater extent as compared to Warfarin. Therefore Ketorolac has greater affinity for site II than for site I on the BSA molecule.

It was found that Omeprazole at a higher concentration increased the free fraction Warfarin from 100% to 124% and that of Diazepam from 100% to 177.7%, respectively. Therefore, Omeprazole has also greater affinity for site II than for site I on the BSA molecule (Figure 3). It implies the fact that at a lower ratio of drug to BSA, both drugs binds to its high affinity 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 or the warfarin site on the BSA molecule.

All these results suggest that in presence of Omeprazole, Ketorolac is displaced from the high affinity binding sites and then a significant portion of the displaced drug rebinds to its low affinity site (site-I) on the BSA molecule. When site-I is sufficiently blocked by site-I specific probe (Warfarin), the free concentration of Ketorolac was increased further by the addition of Omeprazole. The same pattern of change was seen in case of Omeprazole. Omeprazole was displaced from its high affinity binding site (site-II) and also from low affinity binding site (site-I) in the presence of Ketorolac and Warfarin.

In absence of Warfarin, the free fraction of Ketorolac bound to BSA (1:1) was increased from 40.5% to 87.8% by Omeprazole, whereas, in presence of Warfarin, this increment was from 51.4% to 98.6% (Figure 4). In the absence of Warfarin, the free fraction of Omeprazole was increased by Ketorolac from 75.3% to 88%. In presence of Warfarin, Ketorolac in the same concentration ratio increased the free Omeprazole from 79.1% to 99.4% (Figure 4). This suggests that Omeprazole is displaced to a greater extent by Ketorolac in the presence of Warfarin.

Conclusions

The pharmacologic effects of a drug are determined by the intrinsic activity of the product. Sometimes the pharmacologic activity of a drug can also be related to its protein binding which is an important pharmacokinetic parameter. Because of alteration in protein binding if a drug shows less affinity for albumin, the pharmacologic effect of the drug may significantly be enhanced. But this is not always true, as the protein binding of a drug is not always indicative of its tissue distribution, its elimination or its activity. If two drugs have similar physicochemical, metabolic and pharmacologic properties, it is legitimate to assume that the one with the lowest plasma protein binding will have a large distribution, higher active concentrations and a greater effect. In our study, both Ketorolac and Omeprazole increased the free fraction of one another, when they simultaneously bound to BSA. They compete for a common binding site on the albumin molecule, thereby free fraction of both the drugs was increased compared to the level obtained when the drugs were given individually.

The concurrent administration of Ketorolac and Omeprazole is a common practice. From the pharmacological point of view, this combination brings more beneficial effects. They compete for the common binding site on the albumin molecules. Therefore the free concentration of both drugs increased to a significant level as compared to the level obtained when the drugs are given individually. As a result, during chronic to acute pain management, particularly patients who need to control both pain and NSAIDs induced gastritis or to prevent peptic or duodenal ulcer combination of Ketorolac, an NSAID and Omeprazole, a proton pump inhibitor, will help to reduce pain and eradicate associated gastric acidosis. However, from our limited data it is too early to draw such conclusion about the pharmacokinetic or pharmacological properties of the drug. It deserves a more detailed study using *in vivo* experimental model.

Acknowledgements

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