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Research Article

Interactions Between Sirolimus and Anti-Inflammatory Drugs: Competitive Binding for Human Serum Albumin

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Abstract

Purpose: The aim of the present study was investigating the effects of three anti-inflammatory drugs, on Sirolimus protein biding. The binding site of Sirolimus on human serum albumin (HSA) was also determined.

Methods: Six different concentrations of Sirolimus were separately exposed to HSA at pH 7.4 and 37°C. Ultrafiltration method was used for separating free drug; then free drug concentrations were measured by HPLC. Finally, Sirolimus protein binding parameters was calculated using Scatchard plots. The same processes were conducted in the presence of NSAIDs at lower concentration of albumin and different pH conditions. To characterize the binding site of Sirolimus on albumin, the free concentration of warfarin sodium and Diazepam, site I and II specific probes, bound to albumin were measured upon the addition of increasing Sirolimus concentrations.

Results: Based on the obtained results presence of Diclofenac, Piroxicam and Naproxen, could significantly decrease the percentage of Sirolimus protein binding. The Binding reduction was the most in the presence of Piroxicam. Sirolimus-NSAIDs interactions were increased in higher pH values and also in lower albumin concentrations. Probe displacement study showed that Sirolimus may mainly bind to site I on albumin molecule.

Conclusion: More considerations in co-administration of NSAIDs and Sirolimus is recommended.

Introduction

Most of the drugs bind to tissue and plasma proteins in the body.[1] The protein binding of drugs is an important pharmacokinetic parameter affecting their biological activity, metabolism, distribution, and elimination.[2,3] The magnitude of the effect of a drug on the target organ is related to its free concentration. Human serum albumin (HSA) is the most abundant protein with a molecular weight of 65 – 69 KDa. Albumin is quantitatively the major binding protein for neutral and acidic drugs.[4,5] It can bind reversibly with different kind of ligands such as fatty acids, amino acids, steroids, metals for example calcium, copper and zinc, and numerous pharmaceuticals.[5,6] Drugs have two ligand-specific binding site namely, site- I and site-II to bind to human serum albumin.[5-7] The ligand selectivity is comparatively broader for these two sites, allowing a range of drug molecules to bind at these sites. This broad selectivity is considered to be a consequence of the significant allosteric effects in HSA and drug molecules can also interact nonspecifically with HAS.[5] HSA is responsible for maintaining the osmotic pressure of the blood and is a carrier of many molecules such as free fatty acids, bilirubin and various hormones (such as cortisone, aldosterone, thyroxin, etc.).[5,6] Since HSA has limited number of binding sites, it is expected that drugs or agents with high affinity to protein will displace others leading to higher plasma concentration and probable toxicity. This competitive displacement is more significant, when the binding occurs to the same sites on the protein. However competition ability of drugs depends on their binding affinities, relative concentration, and specificity of binding.[8-10]

Sirolimus (SRL, formerly Rapamycin: $C_{51}H_{79}NO_{13}$, CAS: 53123-88-9) is a lactone-lactam macrolide antibiotic that joined the immunosuppressant depot when it was approved by the Food and Drug Administration (FDA) for the prevention of kidney transplant rejection (Figure 1).[8,11] Sirolimus is metabolized by CYP3A4 in human liver.[12,13] The structure of this drug is related to tacrolimus but the mechanism of action of these drugs is different. It inhibits the second phase of T-cell activation.[14] In addition it binds to the FK (peptidyl-prolyl cis-trans isomerase) binding protein which modulates the activity of the mammalian Target of Rapamycin (mTOR). The mTOR inhibits Interleukin-2-mediated signal transduction, resulting in cell cycle stop in the G1-S phase and prevents cell cycle progression and

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proliferation, hence blocks the response of T- and B-cell activation by cytokines. These properties of sirolimus allow it to be a possible chemotherapeutic agent against many types of solid tumor.[12,13,15]
In the present study the potential interactions between Sirolimus and NSAIDs at the level of protein binding displacement was investigated.

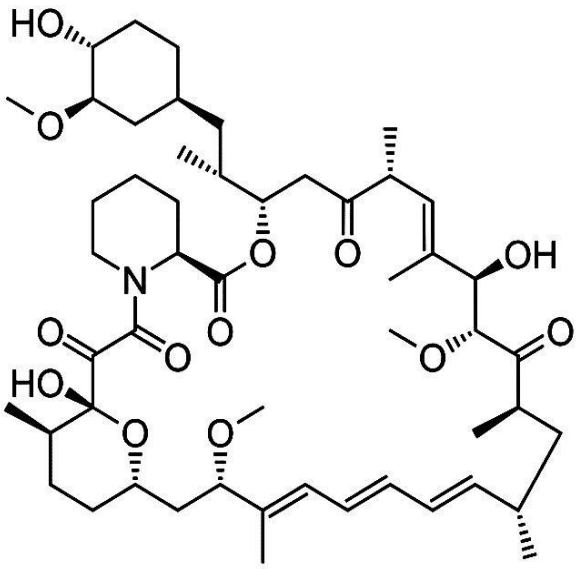

Figure1. Structure of Sirolimus (Rapamycin) molecule.

Materials and Methods

Chemicals

Sirolimus was obtained from Poli (Lazio, Italy). Diclofenac, Naproxen, Warfarin sodium, and diazepam were provided from Sigma (Louis, MO, USA). Piroxicam was provided from Cenateur (India). Monobasic potassium phosphate (KH2PO4), sodium hydroxide, ammonium acetate, sodium lauryl sulfate (SLS) were obtained from Merck (Darmstadt, Germany). Pharmaceutical-grade human albumin 20% was purchased from (CSL Behring GmbH, Germany). HPLC grade solvents, such as methanol and acetonitril (ACN) were also purchased from Merck (Darmstadt, Germany). Double-distilled water was used during the procedure.

Preparation of Stock solutions and standards

The primary stock solutions of Sirolimus, Diclofenac, Piroxicam and Naproxen with a concentration of 500 µg/ml were prepared. These stock solutions were stored in glass vials at +4°C. Finally, working standard solutions for HPLC calibrations or binding experiments were prepared by serially dilution to obtain Sirolimus concentrations of 8, 10, 12, 14, 16 and 18 µg/ml in monobasic potassium phosphate buffer solution (pH=7.4). Also, for investigating of Sirolimus complexions with NSAIDs, these drugs were added to diluted concentrations in their plasma concentrations.

Preparation of buffer solution

Buffer solution was prepared by placing 50 ml of the monobasic potassium phosphate solution in a 200 ml volumetric flask and 39.1 ml of the 0.2 M sodium hydroxide solution was added to the mixture. Then purified water was added to volume and pH was adjusted to 7.4.

Protein binding investigation by ultrafiltration

Solutions containing 8, 10, 12, 14, 16 and 18 µg/ml of Sirolimus were separately exposed to 0.04 g/ml human serum albumin in glass vials for one hour to complete protein binding in pH 7.4. For enhancing the stability of Sirolimus against the light, incubations were performed at 37°C, in glass vials, away from light, for 1 hour. After 1 hour, to produce ultrafiltrates containing unbound drug, the samples were added in the ultrafiltration system (cellophane membrane with 12,000 molecular weight cut-off). Centrifugations were performed at 37°C, for 10 min at 4000 rpm, resulting in filtrate volumes of 0.3–0.4 mL. The same process was performed in the presence of NSAIDs, Diclofenac, Piroxicam and Nproxen at the concentrations of 5, 17, and 70 µg/ml respectively for investigating of complexion of these drugs with Sirolimus.

Determination of binding site of Sirolimus on HSA at pH 7.4 and 37°C

Warfarin sodium and Diazepam were used as site I and site II specific probes respectively. HSA solutions of 6×10^{-4} molar concentration (5 ml) were taken in six test tubes. Then ten ml of 3×10^{-4} M warfarin sodium solution were added to each five tube. The sixth tube was taken as control containing only HSA (6×10^{-4} M). The final protein/warfarin ratio in tubes was 1:1. Increasing concentrations of Sirolimus solution was added to four tubes of five, so as the fifth tube contained only protein and Warfarin. The samples were mixed and to ensure the maximum binding of protein and warfarin, the mixture were allowed to stand for one hour. After that, the samples were added in the ultrafiltration system and the above mentioned process was followed. The free warfarin concentration was measured using a HPLC method developed by Yung et al with UV detection at 300 nm.[16] The same protocol was followed for diazepam as site II specific marker. A simple HPLC method developed and validated by Sruthi et al with UV detection at 230 nm was used for determination of free diazepam concentration.[17] The reverse experiment with the same protocol was also performed to verify the ability of warfarin sodium and diazepam to displace Sirolimus from its binding site.

HPLC Determination of Sirolimus

The validated RP-HPLC method was used for calculating of free Sirolimus concentration in the absence and presence of the NSAIDs after ultrafiltration.[11] Briefly C8 (10 µm, 4.6 ×150 mm) column was used as analytical column. The temperature

of column was set at 55°C. The mobile phase was a mixture of 70% ACN and 30% ammonium acetate buffer, containing 10 mM sodium lauryl sulfate (SLS). The ammonium acetate buffer was prepared by dissolving 0.8 g ammonium acetate in 1000 ml water, adjusted to pH 5.8 with NaOH 0.1M. Injection volume of 100 µl was injected into the column using a Hamilton injector syringe (Bonaduz, Switzerland). The flow rate was set at 1.5 ml/min. Sirolimus was detected by UV detector at 272 nm. Binding data were evaluated based on both the Scatchard plots.[18]

Data treatment

The fraction of unbound drug was calculated by measuring the ultrafilterate concentrations using HPLC analysis. The drug concentrations were plotted according to Scatchard where the abscissa represents the factor r (the number of molecules of drug bound per molecule of albumin), and the ordinate represents r/D_f (D_f =free drug concentration). The data of r and D_f were fitted by linear least squares regression analysis when number of binding sites and association constants were calculated. Results are the mean±SD of three replicates. Finally, all data were analyzed statistically. Difference was considered significant when p-value was < 0.05.

Results and Discussion

There are two main types of protein binding, such as strong affinity binding to a small number of sites (site I in subdomain IIA) and weak affinity binding to a large number of sites (site II within subdomain IIIA).[7] Drug interactions leading to pharmacokinetic changes, arise from effects on drug-protein binding in both plasma and tissues. Concurrent administration of drugs which are capable of binding to the same site on protein leads to competitive displacement. Noncompetitive or allosteric displacement may also occur due to drug-induced conformational change in binding sites.[19,20] Drugs can bind to albumin at site I or site II or both sides. In the present study based on the probe displacement method, Sirolimus binding site on albumin was investigated using warfarin sodium as a site-I-specific probe and diazepam as a site-II-specific probe. According the obtained results the free fraction of warfarin sodium increased from initial 100% to 410% with increasing Sirolimus concentration. On the other hand the same increase in Sirolimus concentration led to 2.1 times augmentation in free diazepam concentration (210% vs initial 100%). That means free fraction increment in the case of warfarin was more than that of diazepam. In the reverse experiment, the free concentration of Sirolimus was increased from initial 100% to 350% and 150% with increasing warfarin sodium and diazepam respectively. This confirms the results of previous experiment. Therefore data suggested that Sirolimus is mainly binds to site I (high-affinity binding) than site II (low affinity binding). In other words in low plasma concentrations, Sirolimus binds to site I while in higher concentration both sites are involved in drug binding. So concurrent administration of drugs having a high affinity to site I may cause rapid action or excretion of Sirolimus from the body. It may even cause drug toxicity at normal doses.

In the next step binding characteristics of Sirolimus was investigated in the presence of some anti-inflammatory drugs. For drug-protein binding data interpretation, different analysis models could be used. These models are based on various features of binding process. In the present study the most common graphical presentation of the binding data, the Scatchard plot, was employed.

According to the obtained data for Sirolimus and HSA binding, Scatchard plot was drawn and the binding parameters of Sirolimus were characterized (Figure 2). As it is clear, the binding of the drug at pH 7.4 and protein concentration of 0.04 g/L (4%) provided a linear plot, suggesting the presence of single class of binding sites for Sirolimus on the protein. The calculated affinity association constant (K), the number of binding sites (ν) for Sirolimus binding to HSA, and the mean protein binding percentage were found to be 3.99×10^5, 0.06, and 91.24 $\pm$0.41 respectively. Therefore a high protein binding level and affinity association constant for Sirolimus were exhibited. Figures 3, 4 and 5 show, respectively, the Scatchard plots for Sirolimus-HSA binding in the presence of Diclofenac, Naproxen, and Piroxicam. Table 1 lists the calculated results for protein binding level (%), binding constants (K_a), and the number of binding sites (υ) per albumin molecule, for three investigated systems. Each system were examined at three pHs and two HSA concentrations. As it is clear from the Table, the values of K_a were of the order of 10^5. This demonstrates that there was a strong binding force between HSA and Sirolimus. It was found that the mean protein binding level of Sirolimus decreased significantly ($p<0.05$) in the presence of Diclofenac, Naproxen, and Piroxicam as compared with that in the absence of NSAIDs. The decrease was more noticeable in lower HSA concentration. The decrease in protein binding level could be attributed to reduced number of binding sites and/or reduced binding affinity. Based on the data in Table 1, the values of K_a (binding constant) in the presence of Diclofenac and 2% HSA was decreased significantly ($p<0.05$) in all three pH values while in the case of 4% HSA, reduced affinity was observed only in pH 7.9. Similarly, the same significant reduction was found in the number of binding sites (υ) in the presence of Diclofenac and HSA 4% compared with that in the absence of any NSAIDs. However in systems containing 2% HSA the reduced number of binding sites was only seen in low pH of 6.9. The results indicated that the presence of Diclofenac decreased the binding capability of sirolimus with HSA, which was raised from the replacement of sirolimus by Diclofenac from site I on HSA. Based on the obtained results, in most cases, both reduced

binding constant and number of binding sites were responsible for the replacement. Therefore Diclofenac could change free sirolimus concentration in plasma. Considering the data obtained in systems containing Naproxen and Piroxicam, it seems that in most cases, reduction in number of binding sites is the predominant reason for reduced protein binding of Sirolimus. The non-steroidal anti-inflammatory drugs selected in the present study, Naproxen (pKa 4.38), Diclofenac (pKa 4.08) and Piroxicam (pKa 5.1), are in their anionic form in the investigated pH range of 6.9-7.9. Since HSA has the strongest affinities for anionic drugs, therefore displacement of Sirolimus by these NSAIDs were expected. Our results are in consistence with the observations of Jose´ Pe´rez-Urizar that demonstrated experimental hypoalbuminemia could increase free plasma tolmetin concentration by not only changing the number of available sites.[21] In a study by Rahman et al it was suggested that NSAIDs with a carboxyl group (like Diclofenac and Naproxen) and without a carboxyl group (like Piroxicam) are able to displace probes from site II and I on albumin molecule.[22] Our results demonstrated relatively higher displacement of sirolimus (site I binding drug) by Piroxicam compared with two other NSAIDs confirming the above mentioned finding.[22] Other processes like alterations in drug affinity to its binding site could also be considered. These changes may be a consequence of conformational changes in the albumin molecule. However in the body, pathophysiological conditions in hypoalbuminema and the probable generated metabolites could give further explanation for the observation. This is in agreement with our finding that the level of protein binding in systems containing 2% HSA, resembling hypoalbuminemia, were lower compared with those containing 4% HSA. Sirolimus is essentially unionized at all investigated pH values. On the other hand, as mentioned earlier, according to the results of the present study, pH changes resulted in alteration of Sirolimus-HSA binding. Since there is no known changes in physicochemical properties of Sirolimus in the pH Ranges of 6.9-7.9, the observed pH-dependent alterations must be due to conformational changes in HSA molecule.[23]

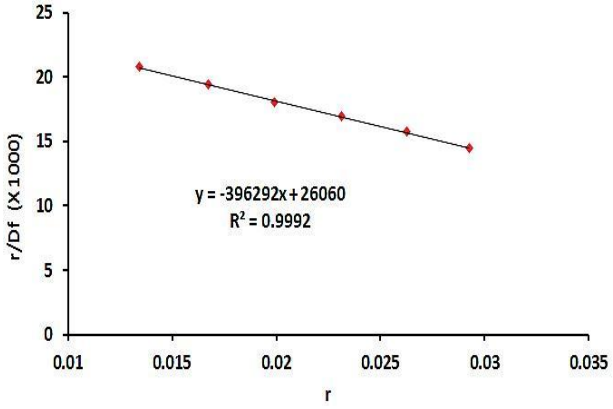

Figure 2. The Scatchard plot for Sirolimus-HSA binding (pH=7.4, HSA 4%). The abscissa represents the factor r (the number of molecules of drug bound per molecule of albumin), and the ordinate represents r/D_f (D_f =free drug concentration)

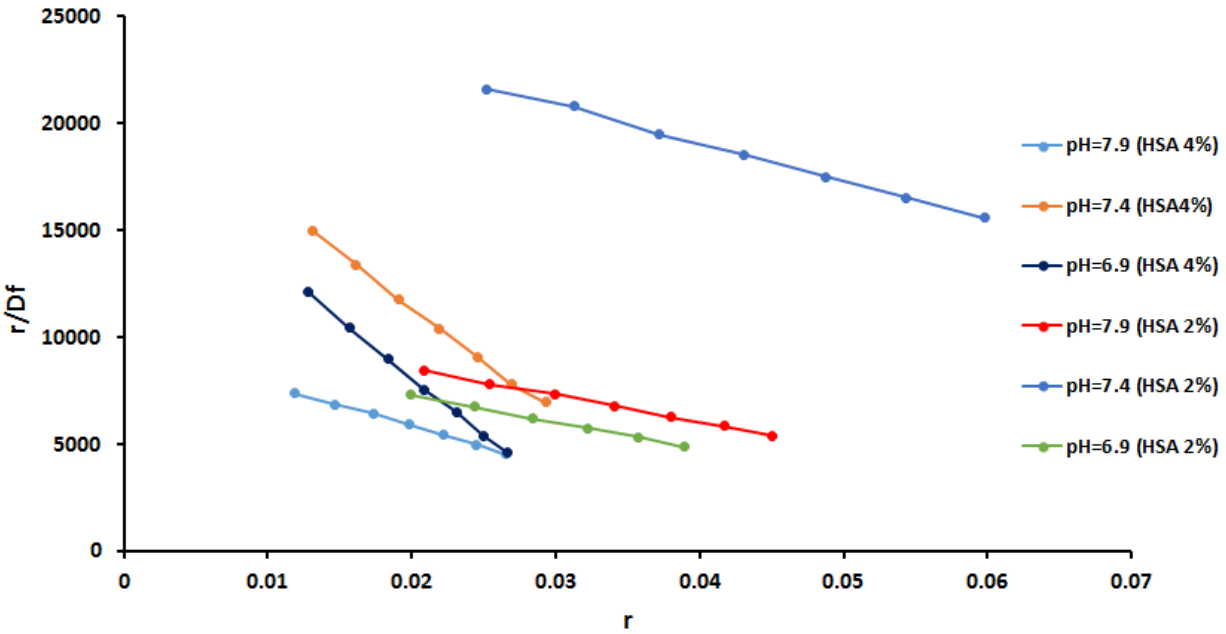

Figure 3. Scatchard plots for Sirolimus binding in the presence of Diclofenac at three different pHs (6.9, 7.4, 7.9) and two different HSA concentration (2% and 4%). Diclofenac concentration was 5 µg/ml. The abscissa represents the factor r (the number of molecules of drug bound per molecule of albumin), and the ordinate represents r/D_f (D_f =free drug concentration)

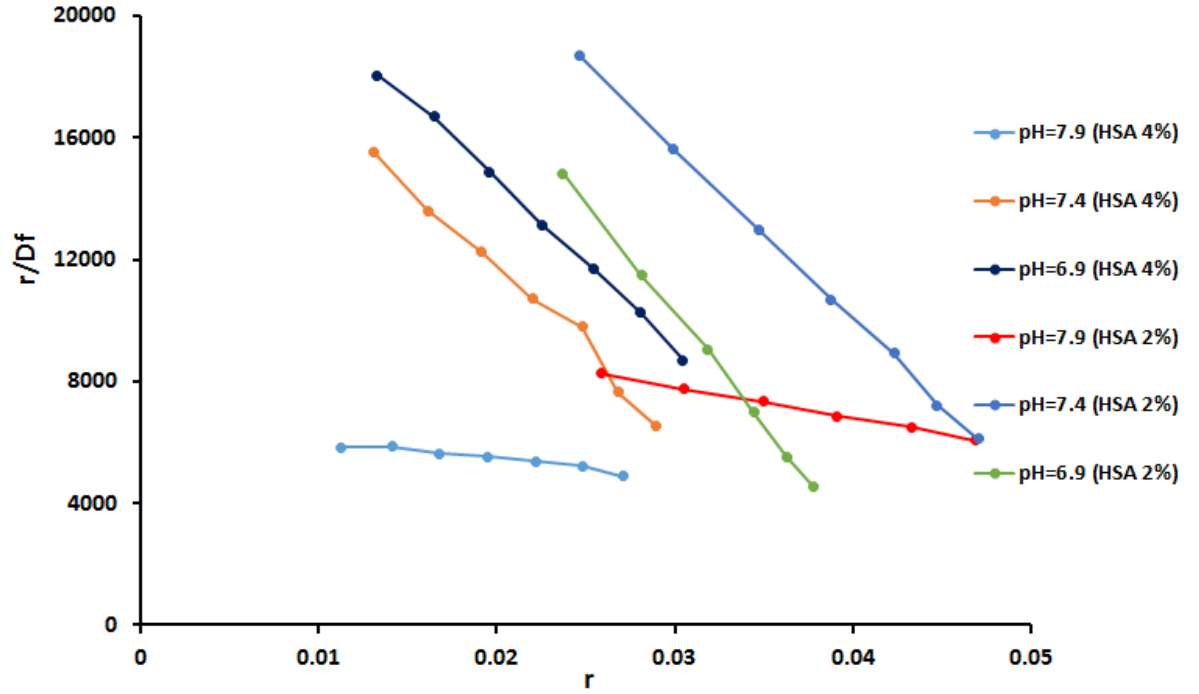

Figure 4. Scatchard plots for Sirolimus binding in the presence of Naproxen at three different pHs (6.9, 7.4, 7.9) and two different HSA concentration (2% and 4%). Naproxen concentration was 70 µg/ml. The abscissa represents the factor r (the number of molecules of drug bound per molecule of albumin), and the ordinate represents r/D_f (D_f =free drug concentration)

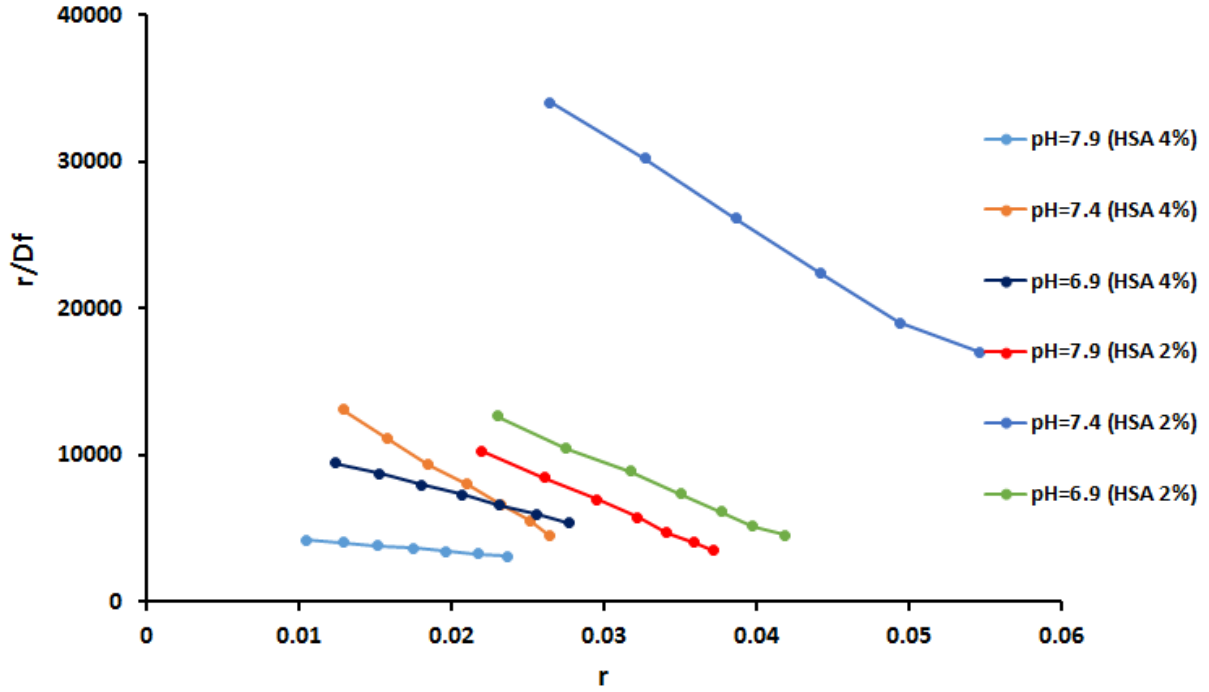

Figure 5. Scatchard plots for Sirolimus binding in the presence of Piroxicam at three different pHs (6.9, 7.4, 7.9) and two different HSA concentration (2% and 4%). Piroxicam concentration was 17 µg/ml. The abscissa represents the factor r (the number of molecules of drug bound per molecule of albumin), and the ordinate represents r/D_f (D_f =free drug concentration)

Conclusion

According to the results obtained presence of anti-inflammatory drugs Diclofenac, Piroxicam and Naproxen, could significantly decrease the percentage of sirolimus protein binding. This may lead to a higher apparent clearance and shorter half-life of drug. The Binding reduction was the most in the presence of Piroxicam. Therefore in patients under sirolimus regimen, concurrent administration of these NSAIDs (especially Piroxicam) is not recommended. Moreover Sirolimus-NSAIDs interactions were increased in higher pH values while in lower pH conditions, such as

acidosis, less interactions were observed. It was also concluded that sirolimus-NSAIDs interactions were more considerable in low albumin concentrations and more considerations in co-administration of NSAIDs and sirolimus for patients with hypoalbumiemia is recommended. Therefore, extrapolating our results to the clinical situation, a higher level of sirolimus displacement could be expected in patients administering NSAIDs concomitantly and also in diseases involving hypoalbuminemia. However, further in vivo studies are needed to explore the biological significance of the above mentioned interaction.

Table 1. Mean of protein binding level (PB%), association constant (K), and number of equivalent binding sites (ν) of Sirolimus-HSA complex at three different pHs (6.9, 7.4, 7.9) and two different HSA concentration (2% and 4%)

Systems	HSA Concentration (%)	pH	Mean PB% ± SD	K	ν
HSA-Sirolimus + Diclofenac	4%	7.9	78.60±0.61	1.94×10^5	0.05
		7.4	86.67±0.11	5.01×10^5	0.04
		6.9	82.83±0.13	5.40×10^5	0.03
	2%	7.9	67.91±0.82	1.25×10^5	0.08
		7.4	85.09±0.12	1.77×10^5	0.14
		6.9	64.31±0.71	1.25×10^5	0.07
HSA-Sirolimus + Naproxen	4%	7.9	77.05±0.22	5.78×10^4	0.11
		7.4	86.97±0.11	5.51×10^5	0.04
		6.9	68.16±0.20	5.46×10^5	0.04
	2%	7.9	69.79±1.27	1.03×10^5	0.10
		7.4	77.46±0.74	5.62×10^5	0.05
		6.9	71.36±2.77	7.34×10^5	0.04
HSA-Sirolimus + Piroxicam	4%	7.9	69.28±0.37	8.38×10^4	0.06
		7.4	83.43±0.25	6.23×10^5	0.03
		6.9	81.94±0.20	2.67×10^5	0.04
	2%	7.9	65.44±0.73	4.50×10^5	0.04
		7.4	78.66±3.06	6.24×10^5	0.03
		6.9	70.51±0.55	4.34×10^5	0.05

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Ethical Issues
Not applicable.

Conflict of Interest
The Authors report no conflict of interests in the present study.

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