



American Journal of
**Drug Discovery
and Development**

ISSN 2150-427X



Academic
Journals Inc.

www.academicjournals.com

***In vitro* Binding Chemistry of Amlodipine Besylate (Calcium Channel Blocker) and Atorvastatin Calcium (HMG-CoA Reductase Inhibitor) to Serum Albumin and their Mutual Effect to Displace Each Other from the Binding Site**

¹K.D. Alam, ²M.K. Hosain, ²S. Kabir, ²R.M.A.A. Chowdhury, ³S. Mahjabeen, ⁴M.S. Mondal, ⁴S.M. Abuzar and ¹M.F. Rahman

¹Department of Clinical Pharmacy and Pharmacology, Faculty of Pharmacy, University of Dhaka, Bangladesh

²Department of Pharmaceutical Chemistry, University of Dhaka, Bangladesh

³Department of Pharmaceutical Technology, University of Dhaka, Bangladesh

⁴Department of Pharmacy, University of Asia Pacific, Bangladesh

Corresponding Author: Khondoker Dedarul Alam, Department of Clinical Pharmacy and Pharmacology, Faculty of Pharmacy, University of Dhaka, Bangladesh

ABSTRACT

Combination therapy is now very common for the effective management of cardiovascular problems. The aim of the present study was to evaluate how clinically two important drugs, Amlodipine Besylate and Atorvastatin Calcium, bind with serum protein and the effect of drug-protein binding when they administered concomitantly. In this study the binding chemistry of Amlodipine and Atorvastatin to Bovine Serum Albumin (BSA) was evaluated by Equilibrium dialysis method utilizing Warfarin Sodium (site-I specific probe) and Diazepam (site-II specific probe). Association constant and number of binding sites of the experimental drugs were carried out at pH values of 6.4 and 7.4. The non-linear curve of the plot suggests the presence of at least two classes of binding site (low affinity binding site and high affinity binding site) of experimental drugs to BSA. In both cases of high affinity binding site and low affinity binding, value of association constants of experimental drugs were found higher at pH 7.4. Both of the experimental drugs found to bind site-I preferentially as both of drugs displaced Warfarin Sodium more from the binding site on BSA than that of Diazepam. During concurrent administration of Amlodipine Besylate and Atorvastatin Calcium in presence or absence of diazepam, it was found that the ability of Atorvastatin Calcium to displace Amlodipine Besylate is more than the ability of Amlodipine Besylate to displace Atorvastatin Calcium from the binding site on BSA. The ability of experimental drugs to displace each other is found more in presence of Diazepam. As both drugs compete for the same binding site care should be given during concurrent administration of these two drugs.

Key words: Amlodipine besylate, atorvastatin calcium, serum albumin, equilibrium dialysis, binding chemistry

INTRODUCTION

Efficacy as well as toxic profile of drug depends largely on its ability to bind with serum protein. The more binding of drug indicates the less free drug in the blood. It is generally assumed that free

drug concentration in the blood is responsible for its action on biological system. Given that as most of the cases only a little percentage of drug remains in free form, small displacement of drug can result many fold increment in its activity in the biological system (Alam *et al.*, 2008).

Detection of binding protein and binding site of drug on serum protein is important to assume the probable interaction between two drugs (Rahman *et al.*, 2005). One of the factors upon which pharmacokinetic properties like plasma clearance, elimination half-life, apparent volume of distribution, area under the curve of a drug depend upon binding of that drug in plasma protein. Proper knowledge on composition, size and location of the binding site is very important to explain such pharmacokinetics properties of drug (Mahbubul Alam *et al.*, 2004). That's why study on protein binding of drug and the effect of one drug on the binding of another drug is getting importance day by day.

From different studies, it is known that there are mainly three high affinity drug binding sites on human serum albumin which are generally termed as warfarin, benzodiazepine and digoxin binding sites, respectively (Uddin *et al.*, 2004). When two or more drugs are administered in any biological system they compete for each other to bind with these binding sites. Problems create when the concomitantly administered drugs show their affinity to the same binding site (Rahman and Sharker, 2009). If such instances, patient may experience mild to severe alteration in action of drugs than that of individual when administered in different time.

Importance of drug-protein binding study is not only to evaluate pharmacokinetic properties of related drugs but also as a part of the drug development process. Such study also helps in the evaluation of drug delivery phenomenon and knowledge of the structural details of small molecule-albumin interactions (Basken *et al.*, 2009).

Site-to-site displacement should be considered when studying drug-protein interactions as can result significant differences in the free concentrations of a displaced drug. It is also possible that the displaced drug from one site can rebinds with the other site. Such incidence can result conformational changes and can favor one molecule to bind preferentially (Yamasaki *et al.*, 2000).

The purpose of this study was to investigate the displacement effect from serum protein, to identify the major binding protein, the binding sites upon concurrent administration of Amlodipine and Atorvastatine.

MATERIALS AND METHODS

The present investigation was carried out from April 2010 to March 2011 at Department of Clinical Pharmacy and Pharmacology, Faculty of Pharmacy, University of Dhaka, Bangladesh.

Drugs and reagents: Experimental drugs Amlodipine besylate and Atorvastatin Calcium were purchased from Eskayef Bangladesh Ltd. Warfarin Sodium and Diazepam, used as probe, were purchased from Incepta Pharmaceuticals Ltd. Bangladesh. Vehicle Methanol, Ethanol and Acetone used in this experiment all were of analytical grade and purchased from BDH, UK. Buffer chemicals Disodium hydrogen phosphate (Na_2HPO_4) and Potassium dihydrogen phosphate (KH_2PO_4) were purchased from GSK, UK. Cellulose Membrane (molecular weight cut off at 3500 Daltons) used in the experiment was purchased from Medical International Limited in England. Bovine serum albumin (fatty acid free, fraction V, 96-98%) was purchased from the Sigma Chemical CO. USA. The molecular weight of the protein was approximately 66,210 Da with 581 amino acids and 4.7 of isoelectric point (Bogusz, 2000).

Instruments: The following instruments were used: pH Meter (HANNA Microprocessor pH Meter, Portugal), SP8-400 UV/VIS Spectrophotometer (Thermospectronic, England), Metabolic Shaking Incubator (Nical electro Ltd., England), Micro syringe (Hamilton, Switzerland).

Preparation of membrane: The supplied membrane was cut into small pieces (9 cm in length) and taken in 500 mL beaker containing de-ionized water. The membranes were immersed beneath the de-ionized water and heated for more than 8 h in order to remove sulfur as sulfur may interfere in the overall binding process. The temperature was controlled between 65-70°C and hot water was replaced by fresh de-ionized water at every one hour interval for efficient removal of sulfur. The prepared pieces of membrane were kept in a beaker containing fresh de-ionized water and preserved in a refrigerator (chilling chamber) until use.

Preparation of Bovine Serum Albumin (BSA) Solution: In this study, Bovine Serum Albumin (BSA) was used in stead of Human serum albumin (HAS) due to structural similarity, good stability in various media, reproducibility, availability and cost effectiveness (Brown, 1976; Simion *et al.*, 2009). To prepare 100 mL of 2×10^{-5} M solution of Bovine serum albumin (BSA) 0.133 of protein was accurately measured and dissolved in a 100 mL volumetric flask with the previously prepared phosphate buffer solution of 6.4. This was done carefully and gently so that it did not form foam. The protein solution was kept in a refrigerator until use.

Same procedure was followed for the preparation of 100 mL of 2×10^{-5} M protein solutions in phosphate buffer of pH 7.4.

Preparing standard curve

Amlodipine besylate: In each of seven test tubes Phosphate buffer solution of pH 6.4 was taken. Amlodipine Besylate solution at pH 6.4 and conc. of 1×10^{-5} M was added to make the concentration 0.5×10^{-5} M, 0.8×10^{-5} M, 1×10^{-5} M, 2×10^{-5} M, 3×10^{-5} M, 4×10^{-5} M and 5×10^{-5} M, respectively. The same procedure is followed for preparing standard curve of Amlodipine Besylate at pH 7.4.

The absorbance values of the solutions were determined by a UV spectrophotometer at λ_{\max} 361 nm. As a control or reference sample, phosphate buffer solution of pH 6.4 and pH 7.4 were used, respectively. The standard curve was obtained by plotting the absorbance values against the corresponding concentration.

Atorvastatin calcium: Phosphate buffer solution of pH 6.4 was taken in each of seven test tubes. Atorvastatin Calcium solution at pH 6.4 and conc. of 1×10^{-5} M was added in different volume to seven test tubes to have the following concentration: 0.1×10^{-5} M, 0.3×10^{-5} M, 0.5×10^{-5} M, 0.6×10^{-5} M, 0.8×10^{-5} M, 1×10^{-5} M and 2×10^{-5} M. The same procedure is followed for preparing standard curve of Atorvastatin Calcium at pH 7.4.

The absorbance values of the solutions were determined by a UV spectrophotometer at λ_{\max} 247 nm. As a control or reference sample, phosphate buffer solution of pH 6.4 and pH 7.4 were used, respectively. The standard curve was obtained by plotting the absorbance values against the corresponding concentration.

Warfarin sodium and diazepam: Phosphate buffer solution of pH 7.4 was taken in each of seven test tubes. Warfarin Sodium stock solution at pH 7.4 and concentration of 1×10^{-5} M was added in

different concentrations to the seven test tubes, to have the following concentrations: 0.5×10^{-5} M, 0.8×10^{-5} M, 1×10^{-5} M, 2×10^{-5} M, 3×10^{-5} M, 4×10^{-5} M and 5×10^{-5} M.

The absorbance values of the solutions were determined by a UV spectrophotometer at λ_{\max} 306 nm. As a control or reference sample, phosphate buffer solution of pH 7.4 was used. Same procedure is followed for Diazepam. The absorbance values of the solutions were determined by a UV spectrophotometer at λ_{\max} 235 nm. The standard curve of Warfarin Sodium and Diazepam were obtained by plotting the absorbance values against the corresponding concentrations.

Experimental design

Estimation of association constant: The value of association constant (k_a) and the number of corresponding binding sites (n) of Amlodipine Besylate and Atorvastatin Calcium bound to BSA were determined by Scatchard plot utilizing equilibrium dialysis technique (Singlas, 1987a, b). In this method, a curve was obtained by plotting r/D_f versus r values where D_f stands for the molar concentration of free drug and r describes the ratio of the molar concentration of bound drug to the molar concentration of protein, i.e.,:

$$r = \frac{[D_B]}{[P_t]}$$

The extrapolation of the plot gives an intercept on Y-axis representing nK_a values and the intercept thus obtained on X-axis represents n ; the slope of the line being K_a .

Estimating association constant of amlodipine besylate bound to BSA at pH 6.4 and pH 7.4 at temperature 28°C: 5 mL of 2×10^{-5} M BSA solution at pH 6.4 was taken in each of 9 test tubes and Amlodipine Besylate was added to make the concentrations 1.5×10^{-5} M, 1.75×10^{-5} M, 2×10^{-5} M, 4×10^{-5} M, 6×10^{-5} M, 10×10^{-5} M, 14×10^{-5} M, 16×10^{-5} M and 20×10^{-5} M, respectively. Another test tube containing only BSA solution at pH 6.4 was marked as control. The solutions were then properly mixed and allowed to stand for 30 min in order to ensure maximum binding of Amlodipine Besylate to BSA.

From each of the test tube, 3.5 mL of solution was pipetted out and poured into 10 tubes containing semipermeable membrane. Both ends of the membrane tubes were clipped so that there was no leakage. The membrane tubes were then immersed in ten separate 50 mL conical flasks containing 20 mL of phosphate buffer solution of pH 6.4. The mouths of the conical flasks were covered by foil paper. The conical flasks were then placed in a metabolic shaker for dialysis for 12 h at 28°C and 20 rpm. Same procedure is followed for pH 7.4.

After completion of dialysis samples were collected from each flask and free concentrations of Amlodipine Besylate were measured by a UV spectrophotometer at a wavelength of 361 nm.

Estimating association constant of atorvastatin calcium bound to bsa at ph 6.4 and pH 7.4 at temperature 28°C: 5 mL of 2×10^{-5} M BSA solution at pH 6.4 was taken in each of 9 test tubes and Atorvastatin Calcium was added to make the concentrations 0.1×10^{-5} M, 0.2×10^{-5} M, 0.3×10^{-5} M, 0.5×10^{-5} M, 0.8×10^{-5} M, 1×10^{-5} M, 1.5×10^{-5} M, 1.8×10^{-5} M and 2×10^{-5} M, respectively. The rest of the procedure is same for estimating association constant of Amlodipine Besylate.

After completion of dialysis samples were collected from each flask and free concentrations of Atorvastatin Calcium were measured by a UV spectrophotometer at a wavelength of 247 nm.

Identification and characterization of binding site of amlodipine besylate and atorvastatin calcium by site specific probe method: Site-specific probes were used here to enhance our understanding of the drug-BSA interaction and thereby characterization of binding sites of the drugs used in the study on the BSA molecule.

The present investigation was carried out by utilizing Equilibrium Dialysis. Warfarin Sodium (Site-I specific) and Diazepam (Site-II specific) were used as probe to determine the binding site of Amlodipine Besylate and Atorvastatin Calcium on Bovine Serum Albumin (BSA).

In the presence of the mixture of the probe (Warfarin Sodium and Diazepam) and BSA at a constant ratio (1:1, 2×10^{-5} M: 2×10^{-5} M), different concentrations of drugs (Amlodipine Besylate and Atorvastatin Calcium) were added. Free concentrations of the probe were then determined by the equilibrium dialysis method to see whether there was any change in the free concentrations of the probe by the addition of the drugs.

Drug-drug displacement study between amlodipine besylate and atorvastatin calcium in the presence and absence of diazepam: Here in drug-drug displacement study was carried out in the presence and absence of diazepam (a site-II specific probe). The reason for using diazepam is because site-II binding site is more specific than site-I (Kabir *et al.*, 2010).

Mutual effect of Atorvastatin Calcium and Amlodipine Besylate to displace each other
Effect of amlodipine besylate on atorvastatin calcium: To the mixture of Atorvastatin Calcium and BSA (1:1, 2×10^{-5} M: 2×10^{-5} M) different concentrations of Amlodipine Besylate were added both in the absence and presence of diazepam. The changes in the free concentration of Atorvastatin Calcium were measured by a UV spectrophotometer at a wavelength of 247 nm.

Here the previously described procedure for estimating of association constant was followed to observe the effect.

Effect of atorvastatin calcium on amlodipine besylate: To the mixture of Amlodipine Besylate and BSA (1:1, 2×10^{-5} M: 2×10^{-5} M) different concentrations of Atorvastatin Calcium were added both in the absence and presence of diazepam. The changes in the free concentration of Amlodipine Besylate were measured by a UV spectrophotometer at a wavelength of 361 nm.

Here the previously described procedure of estimating of association constant was followed to observe the effect.

RESULT AND DISCUSSION

Regression equations for standard curves are presented in Table 1. Regression equations are utilized to calculate the value of free drug concentration by putting the absorbance value.

Determining association constant and number of binding sites: Drug-protein binding are mainly of two types-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 plasma albumin concentration (Rahman and Sharker, 2009).

In this present investigation, the binding parameters of Amlodipine Besylate and Atorvastatin Calcium have been characterized by Scatchard plot. The non-linear curve of the plot suggests the presence of at least two classes of binding site of experimental drugs to BSA (Kabir *et al.*, 2010).

By plotting data from Table 2 Scatchard plot analysis revealed that at pH 6.4 association constant and no. of binding site of Amlodipine Besylate is about $3.75 \times 10^5 M^{-1}$ and 1.2, respectively at high affinity binding site. On the other hand, at low affinity binding site, association constant and no. of binding site is found about $0.08410^{-5} M^{-1}$ and 13, respectively (Fig. 1). At pH 7.4, association constant and no. of binding site of Amlodipine Besylate at high affinity binding site is about $6.84 \times 10^5 M^{-1}$ and 1.9, respectively. At low affinity binding site, association constant and no. of binding site is found about $0.273 \times 10^5 M^{-1}$ and 12.8, respectively (Fig. 2).

By plotting data from Table 3 Scatchard plot analysis revealed that at pH 6.4 association constant and no. of binding site of Atorvastatin Calcium at high affinity binding site is about $35.7 \times 10^5 M^{-1}$ and 0.14, respectively. Association constant and no. of binding site at low affinity binding site is found about $0.977 \times 10^5 M^{-1}$ and 1.33, respectively (Fig. 3). At pH 7.4 association constant and no. of binding site of Atorvastatin Calcium at high affinity binding site is about $142.1 \times 10^5 M^{-1}$ and 0.19, respectively. At low affinity binding site, association constant and no. of binding site is found about $5.45 \times 10^5 M^{-1}$ and 1.1, respectively (Fig. 4).

So, it is seen that, association constants of Amlodipine Besylate and Atorvastatin Calcium in both high and low affinities were found to be greater in pH 7.4 than that of in pH 6.4. The difference in association constants at different pH is not fully understood. It may be due to the structural modification of protein molecule at a given pH value.

Determining binding sites: Binding site of Amlodipine Besylate and Atorvastatin Calcium are determined by calculating the free concentration of Warfarin Sodium and Diazepam. The basic principle is that-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 (Alam *et al.*, 2009).

From Table 4, it is seen that addition of Amlodipine Besylate displace Warfarin Sodium more (as % of initial, from 100 to 198%) than that of Diazepam (as % of initial, from 100 to 144%). As the increment of free concentration of warfarin Sodium is greater than that of diazepam it can be concluded that Amlodipine Besylate preferentially bind to Site I. Alam *et al.* (2008) also found that the same result.

Accordingly, as Atorvastatin Calcium displace Warfarin Sodium more (as % of initial, from 100 to 194%) than that of Diazepam (as % of initial, from 100 to 150%) it can be concluded that Atorvastatin Calcium also preferentially bind to Site I (Table 5).

Table 1: Regression equation of standard curve of Amlodipine Besylate, Atorvastatin Calcium, Warfarin and Dizepam

pH	Regression equation			
	Amlodipine	Amlodipine	Warfarin	Diazepam
6.4	$y = 0.0567 \times -0.0112$	$y = 0.386 \times +0.013$	-	-
7.4	$y = 0.0718 \times -0.0046$	$y = 0.3636 \times +0.0317$	$y = 0.125 \times +0.006$	$y = 0.253 \times +0.066$

However, as the displacement of diazepam is quite pronounced in both cases of Amlodipine Besylate and Atorvastatin Calcium, it can be also assumed that the investigated drugs Amlodipine Besylate and Atorvastatin Calcium preferentially bind with site-I. In addition to Site I, they also bind with Site II on the BSA molecule but to a lower extent.

As both of the drugs compete for site-I preferentially concurrent administration of these two drugs will alter the therapeutic efficacy of each other. This finding will be helpful to understand the poor clinical result of these two drugs incase of some individuals when used simultaneously.

Table 2: Data for association constant of Amlodipine Besylate bound to BSA at pH 6.4 and pH 7.4 at temperature 28°C

Total drug conc. $D_T \times [10^{-5} \text{ M}]$	pH 6.4				pH 7.4			
	Absorbance	Free drug conc. $D_f \times [10^{-5} \text{ M}]$	Total free drug conc. $2 \times D_f \times [10^{-5} \text{ M}]$	Bound drug conc. $D_B \times [10^{-5} \text{ M}]$	Absorbance	Free drug conc. $D_f \times [10^{-5} \text{ M}]$	Total free drug conc. $2 \times D_f \times [10^{-5} \text{ M}]$	Bound drug conc. $D_B \times [10^{-5} \text{ M}]$
1.5	0.001	0.215	0.430	1.070	0.001	0.078	0.156	1.344
1.75	0.004	0.268	0.536	1.214	0.003	0.106	0.212	1.538
2	0.008	0.339	0.677	1.323	0.005	0.134	0.267	1.733
4	0.037	0.850	1.700	2.300	0.025	0.412	0.825	3.175
6	0.071	1.450	2.899	3.101	0.045	0.691	1.382	4.618
10	0.135	2.578	5.157	4.843	0.099	1.443	2.886	7.114
14	0.198	3.690	7.379	6.621	0.147	2.111	4.223	9.777
16	0.234	4.325	8.649	7.351	0.181	2.585	5.170	10.830
20	0.305	5.577	11.153	8.847	0.246	3.490	6.981	13.019

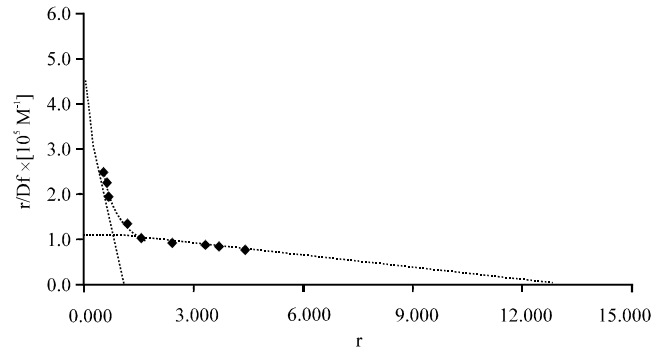


Fig. 1: Scatchard plot for the binding of Amlodipine Besylate to BSA at pH 6.4

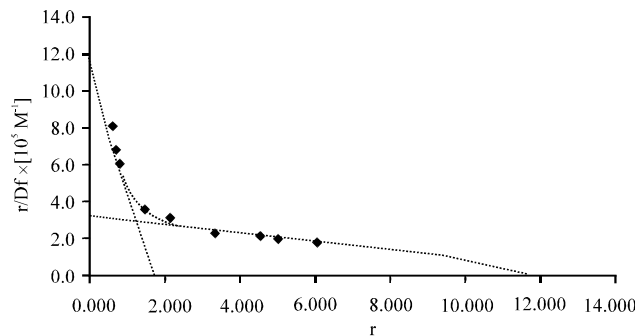


Fig. 2: Scatchard plot for the binding of Amlodipine Besylate to BSA at pH 7.4

Mutual effect of amlodipine besylate and atorvastatin calcium on the binding to bsa in the absence and presence of diazepam: The interactions at binding sites on BSA were measured between Amlodipine Besylate and Atorvastatin Calcium in the absence or in presence of site specific probes diazepam. In absence of diazepam, the free fraction of Atorvastatin Calcium

Table 3: Data for association constant of Atorvastatin Calcium bound to BSA at pH 6.4 and pH 7.4 at temperature 28°C

pH 6.4		pH 7.4						
Total drug conc. $D_T \times [10^{-5} M]$	Absorbance	Free drug conc. $D_f \times [10^{-5} M]$	Total Free Drug conc. $2 \times D_f \times [10^{-5} M]$	Bound drug conc. $D_b \times [10^{-5} M]$	Absorbance	Free drug conc. $D_f \times [10^{-5} M]$	Total free Drug conc. $2 \times D_f \times [10^{-5} M]$	Bound drug conc. $D_b \times [10^{-5} M]$
0.1	0.017	0.010	0.021	0.079	0.017	0.010	0.021	0.079
0.2	0.025	0.031	0.062	0.138	0.025	0.031	0.062	0.138
0.3	0.036	0.060	0.119	0.181	0.036	0.060	0.119	0.181
0.5	0.056	0.111	0.223	0.277	0.056	0.111	0.223	0.277
0.8	0.086	0.189	0.378	0.422	0.086	0.189	0.378	0.422
1	0.107	0.243	0.487	0.513	0.107	0.243	0.487	0.513
1.5	0.161	0.383	0.767	0.733	0.161	0.383	0.767	0.733
1.8	0.197	0.477	0.953	0.847	0.197	0.477	0.953	0.847
2	0.221	0.539	1.077	0.923	0.221	0.539	1.077	0.923

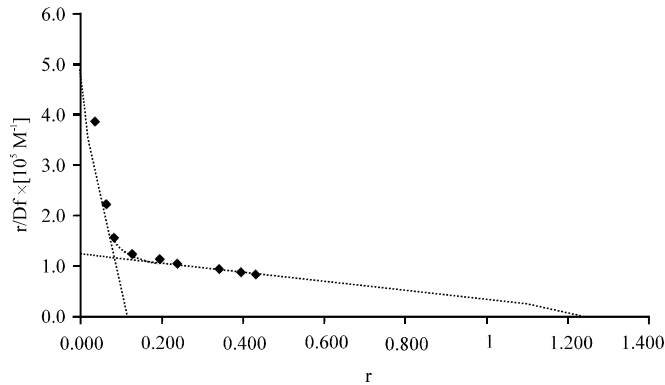


Fig. 3: Scatchard plot for the binding of Atorvastatin Calcium to BSA at pH 6.4

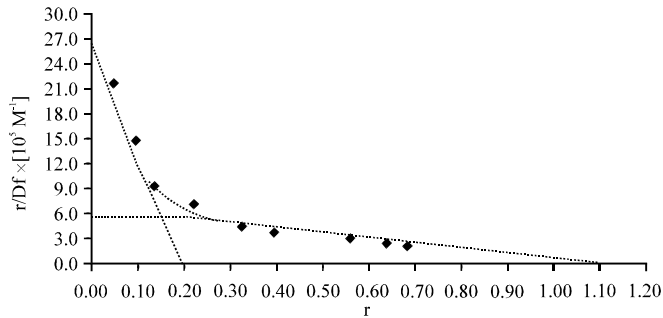


Fig. 4: Scatchard plot for the binding of Atorvastatin Calcium to BSA at pH 7.4

increased from 20 to 40% with the addition of Amlodipine Besylate from 0×10^{-5} M to 10×10^{-5} M. Whereas, in presence of diazepam, Amlodipine Besylate at the same concentration, increased the free fraction of Atorvastatin Calcium little more (from 25 to 53%) than that in the absence of diazepam (Table 6).

From Table 7, it can be assumed that, the ability of Atorvastatin Calcium to displace Amlodipine Besylate is more than the ability of Amlodipine Besylate to displace Atorvastatin Calcium from the

Table 4: Determination of binding site of Amlodipine Besylate at pH 7.4 and 28°C using Warfarin Sodium as site-I specific probe

Conc. of Amlodipine (M)	When using warfarin sodium as site-I specific probe			When using diazepam as site-I specific probe		
	Absorbance	Free conc. of warfarin	As % of Initial	Absorbance	Free conc. of diazepam	As % of Initial
0×10^{-5}	0.128	0.976	100	0.178	0.443	100
2×10^{-5}	0.197	1.528	157	0.195	0.510	115
4×10^{-5}	0.220	1.712	175	0.204	0.545	123
6.5×10^{-5}	0.232	1.808	185	0.214	0.585	132
8×10^{-5}	0.238	1.856	190	0.220	0.609	138
10×10^{-5}	0.244	1.904	195	0.222	0.617	139
12×10^{-5}	0.247	1.928	198	0.227	0.636	144

BSA: Warfarin (1:1) (2×10^{-5} M: 2×10^{-5} M) when using Warfarin Sodium as site-I specific probe, BSA: Diazepam (1:1) (2×10^{-5} M: 2×10^{-5} M) when using Diazepam as site-II specific probe

Table 5: Determination of binding site of atorvastatin calcium at pH 7.4 and 28°C using warfarin sodium as site-I specific probe

Conc. of Atorvastatin (M)	When using warfarin sodium as site-I specific probe			When using diazepam as site-II specific probe		
	Absorbance	Free conc. of warfarin	As % of Initial	Absorbance	Free conc. of diazepam	As % of Initial
0×10^{-5}	0.131	1.000	100	0.086	0.079	100
0.5×10^{-5}	0.167	1.285	129	0.088	0.088	110
1×10^{-5}	0.189	1.468	147	0.090	0.096	120
1.5×10^{-5}	0.211	1.642	164	0.092	0.101	130
2×10^{-5}	0.228	1.777	178	0.094	0.110	140
2.5×10^{-5}	0.241	1.880	188	0.095	0.114	145
3×10^{-5}	0.249	1.944	194	0.096	0.118	150

BSA: Warfarin (1:1) (2×10^{-5} M: 2×10^{-5} M) when using warfarin sodium as site-I specific probe, BSA: Diazepam (1:1) (2×10^{-5} M: 2×10^{-5} M) when using diazepam as site-II specific probe

Table 6: Effect of Amlodipine Besylate on Atorvastatin Calcium binding to BSA in the absence and presence of Diazepam

Added conc. of amlodipine (M)	In absence of diazepam			In presence of diazepam		
	Absorbance of free Atorvastatin (M)	Free conc. of Atorvastatin (M)	(%) of free fraction Atorvastatin (M)	Absorbance of free Atorvastatin	Free conc. of Atorvastatin (M)	(%) of free fraction of Atorvastatin
0×10^{-5}	0.175	0.39×10^{-5}	20	0.213	0.50×10^{-5}	25
0.5×10^{-5}	0.21	0.49×10^{-5}	25	0.267	0.65×10^{-5}	32
1×10^{-5}	0.24	0.57×10^{-5}	29	0.311	0.77×10^{-5}	38
1.5×10^{-5}	0.272	0.66×10^{-5}	33	0.343	0.86×10^{-5}	43
2×10^{-5}	0.3	0.74×10^{-5}	37	0.379	0.96×10^{-5}	48
2.5×10^{-5}	0.32	0.79×10^{-5}	40	0.416	1.06×10^{-5}	53

In the absence of Diazepam, concentration of Atorvastatin:BSA (1:1) (2×10^{-5} M: 2×10^{-5} M), In the presence of Diazepam, concentration of Atorvastatin:BSA:Diazepam (1:1: 2) (2×10^{-5} M: 2×10^{-5} M: 4×10^{-5} M)

Table 7: Effect of Atorvastatin Calcium on Amlodipine Besylate binding to BSA in the absence and presence of Diazepam

Added Conc. of Atorvastatin (M)	In absence of diazepam			In presence of diazepam		
	Absorbance of free Amlodipine	Free conc. of Amlodipine (M)	(%) of free fraction of Amlodipine	Absorbance of free Amlodipine	Free conc. of Amlodipine (M)	(%) of free fraction of Amlodipine
0×10^{-5}	0.038	0.60×10^{-5}	30	0.043	0.67×10^{-5}	34
0.5×10^{-5}	0.049	0.75×10^{-5}	38	0.061	0.92×10^{-5}	46
1×10^{-5}	0.061	0.92×10^{-5}	46	0.073	1.09×10^{-5}	55
1.5×10^{-5}	0.068	1.02×10^{-5}	51	0.086	1.28×10^{-5}	64
2×10^{-5}	0.077	1.15×10^{-5}	57	0.098	1.45×10^{-5}	72
2.5×10^{-5}	0.085	1.26×10^{-5}	63	0.112	1.64×10^{-5}	82

In absence of Diazepam, concentration of Amlodipine:BSA (1:1) (2×10^{-5} M: 2×10^{-5} M), In presence of Diazepam, concentration of Amlodipine:BSA:Diazepam (1:1: 2) (2×10^{-5} M: 2×10^{-5} M: 4×10^{-5} M)

binding site on BSA. In absence diazepam, the free fraction of Amlodipine Besylate increased from 30 to 63% with the addition of Atorvastatin Calcium from 0×10^{-5} M to 2.5×10^{-5} M. Whereas, in presence of diazepam, Atorvastatin Calcium at the same concentration, increased the free fraction of Amlodipine Besylate significantly more (from 34 to 82%) than that in the absence of diazepam.

CONCLUSION

During concurrent administration of Amlodipine and Atorvastatine, both the drugs compete for the same binding site on the albumin molecule. This results in notable increase in the free concentration of one drug by another on the basis of direct competitive displacement. So, care should be exercised if it is necessary to administer both of these drugs.

ACKNOWLEDGMENT

The authors gratefully acknowledge the contribution of Department of Clinical Pharmacy and Pharmacology and Department of Pharmaceutical Chemistry, University of Dhaka, Bangladesh to give necessary support along with different instruments and platform to continue the research work. The authors also grateful to Prof. Nazmul Qais (Chairman, Department of Clinical Pharmacy and Pharmacology) and Prof. ABM Faroque (Dean, Faculty of Pharmacy) for giving their consent to conduct the research work.

REFERENCES

- Alam, M.A., M.A. Awal, N. Subhan, M.H. Sikder, S.M.M. Alam, M. Mostofa and S.J. Uddin, 2008. Studies of *in-vitro* amlodipine and arsenic displacement interaction at binding sites of bovine serum albumin. *Iran. J. Pharmacol. Therapeutics*, 7: 1-8.
- Alam, M.A., M.A. Awal, N. Subhan and M. Mostofa, 2009. *In vitro* relationship between protein-binding and free drug concentrations of a water-soluble selective β -adrenoreceptor antagonist (Atenolol) and its interaction with arsenic. *J. Health Popul. Nutr.*, 27: 20-30.
- Basken, N.E., C.J. Mathias and M.A. Green, 2009. Elucidation of the Human Serum Albumin (HSA) binding site for the Cu-PTSM and Cu-ATSM radiopharmaceuticals. *J. Pharm. Sci.*, 98: 2170-2179.
- Bogusz, M.J., 2000. Enantioselective Liquid Chromatographic Analysis of Drugs in Forensic Science. In: *Forensic Science*, Smith, R.M. (Ed.). Elsevier, Netherland, pp: 319-338.
- Brown, J.R., 1976. Structural origins of mammalian albumin (Abstract). *Fed. Proc.*, 35: 2141-2144.

- Kabir, A.F., K.N. Uddin, A.F.M.N. Sadat, H. Mahbood and A. Mazid, 2010. Interaction of palmitic acid with losartan potassium at the binding sites of bovine serum albumin. *Ars. Pharm.*, 51: 28-36.
- Mahbubul Alam, S.M., M.J. Akter, M.N. Reza, I. Fatima, A. Firoj and J.A. Shilpi, 2004. Drug-drug interactions between ciprofloxacin and captopril at binding sites of bovine serum albumin. *Pak. J. Biol. Sci.*, 7: 79-81.
- Rahman, A.A. and S.M. Sharkar, 2009. Determination of the binding sites of propranolol HCl on bovine serum albumin by direct and reverse procedures. *Saudi Pharm. J.*, 17: 249-253.
- Rahman, M.M., M.H. Rahman and N.N. Rahman, 2005. Competitive binding of ibuprofen and naproxen to bovine serum albumin: Modified form of drug-drug displacement interaction at the binding site. *Pak. J. Pharm. Sci.*, 18: 43-47.
- Simion, M., L. Ruta, I. Kleps, D. Stan and A. Bragaru *et al.*, 2009. Porous/gold surface preparation for protein microarray applications. *Rom. J. Inform. Sci. Tech.*, 12: 385-393.
- Singlas, E., 1987a. Protein Binding of Drugs: Definition, Modalities, Effects, Changes. 2nd Edn., F. Hoffmann-La Roche and Co. Ltd., Basle, Switzerland, pp: 20-22.
- Singlas, E., 1987b. Determination of Protein Binding and its Characteristics: Protein Binding of Drug. 2nd Edn., Hoffmann La Roche and Co. Ltd., Basle, Switzerland, pp: 25-31.
- Uddin, S.J., J.A. Shilpi, G.M.M. Murshid, A.A. Rahman, M.M. Sarder and M.A. Alam, 2004. Determination of the binding sites of arsenic on bovine serum albumin using warfarin (Site-I Specific Probe) and diazepam (Site-II Specific Probe). *J. Biol. Sci.*, 4: 609-612.
- Yamasaki, K., M.H. Rahman, Y. Tsutsumi, T. Maruyama, S. Ahmed, K.H. and M. Otagiri, 2000. Circular dichroism simulation shows a site-II-to-site-I displacement of human serum albumin-bound diclofenac by ibuprofen. *AAPS PharmSciTech.*, 1: 45-54.