ORIGINAL RESEARCH ARTICLE

Protein Binding Chemistry of Amlodipine Besylate and Olmesartan Medoxomil to Bovine Serum Albumin and their Mutual Effect to Displace each other from the Binding Site: *In-Vitro* Study

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Abstract

The aim of the present study was to evaluate how clinically two important drugs, amlodipine besylate (AB) and olmesartan medoxomil (OM), bind with serum albumin protein and the effect of drug-protein binding when they administrated concomitantly. In this study the binding chemistry of amlodipine and olmesartan 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 value 7.4 and the temperature at 37°C. The nonlinear 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 the value of association constants of experimental drugs were found high at pH 7.4. One of the experimental drug (AB) found to bind Site-I and the other drug (OM) found to bind Site II preferentially. During concurrent administration of AB and OM in presence or absence of diazepam, no significant amount of drug is displaced either amlodipine or olmesartan from the binding site on BSA. Also the ability of experimental drugs to displace each other is found less significant in presence of diazepam. As both drugs do not compete for the same binding site, concurrent administration of these drugs can be the effective in the management of cardiovascular problems.

Key words: Amlodipine besylate, binding chemistry, bovine serum albumin, equilibrium dialysis, olmesartan medoxomil

INTRODUCTION

The pharmacokinetic properties of exogenous and endogenous compounds can be influenced by reversible binding to human serum albumin (HSA), which is thought to be one of the primary determinants of the pharmacokinetic properties of dugs.[1-4] Therefore, when evaluating interactions among drugs, it is important to be aware of possible identities of binding sites on the protein because of any alteration in drug-binding to HAS including binding of the antihypertensive drugs, could lead to change in pharmacokinetic properties.[5]

The more binding of drug indicates the less free drug in the blood and is generally less toxic and it is generally assumed that free drug concentration in the blood is responsible for its action on biological system. Given that as most 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.[6] It is important and can be assumed the probable interaction between two drugs by detection of binding protein and binding sites of drug on serum protein.[7] 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

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binding of that drug in plasma protein. Proper knowledge on composition, size and location of the binding site is very important to explain such pharmacokinetic properties of drug.[8] 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.

Different studies has shown that HSA has a limited number of binding sites.[4,9,10] On the basis of the probe-displacement method, there are at least three relatively-high specific drug-binding sites on the HSA molecule. These sites, commonly called warfarin, benzodiazepine, and digoxin-binding sites, are also denoted as Site I, Site II, and Site III respectively and which binds drugs selectively.[4,11,12] In this study warfarin sodium (Site I) and diazepam (Site II) were taken as selective drug specific probe (for their specific sites) as site specific probe method for determination of binding sites of the experimental drugs.[2,9] When two or more drugs are administered in any biological system they compete for each other to bind with these binding sites. Problem creates when the concomitantly administered drugs show their affinity to the same binding site.[13] If such instances, mild and severe alteration of the both pharmacodynamics and pharmacokinetic properties when administered in different time.

When studying drug-protein interactions, site to site displacement should be considered which can results in significant differences in the free concentrations of a displaced drug. And it can be possible that the displaced drug form one site can rebinds with the other site. Such incidence can result conformational changes and can favor one molecule to bind preferentially.[14]

Now-a-day, the combination of angiotensin receptor blocker (ARB) and calcium channel blocker (CCB) are being used widely for the reduction of the blood pressure. Olmesartan medoxomil (OM) (ARB) and amlodipine besylate (AB) (CCB) are investigated widely and combined as a single for the hypertension therapy. Studies have shown that combination therapy is more effective than the monotherapy treatment of these drugs.[15] The current study has been carried out for the initial understanding of the *in-vitro* displacement interaction of two drug amlodipine and olmesartan on binding site of bovine serum albumin (BSA). In this study BSA was used in lieu of HSA because of its low cost and easy availability.

MATERIALS AND METHODS

Drugs and reagents

Experimental drugs AB and OM were generous gift from Incepta Pharmaceutical Ltd., Bangladesh. Warfarin sodium and diazepam, used as probe, were also generous gift form Incepta Pharmaceutical Ltd., and Aristo Pharma Ltd., Bangladesh respectively. Vehicle methanol, ethanol, and acetone used in this experiment all were of analytical grade purchased from Merck KGaA, Gemany. Buffer chemicals disodium hydrogen phosphate and potassium dihydrogen phosphate were purchased from Merck Specialities Pvt. Ltd., India. Cellulose membrane (molecular weight cut-off at 3500 Daltons, diameter of 16 mm) used in the experiment was purchased from Medicell Membrane Ltd., UK. BSA (fatty acid free, fraction V, 96–98%) was purchased from Lobe Chemie Pvt. Ltd., India. The molecular weight of protein was approximately 66,210 Da with 581 amino acids and 4.7 of isoelectric point.[16]

Instruments

The following instruments were used: pH meter (Hanna Microprocessor pH Meter, Portugal), SP8-400 ultraviolet (UV)-visible spectrophotometer (Shimadzu, Japan), high performance liquid chromatography (HPLC) consisting of a CMB-20 Alite system controller, two LC-20AT pumps, SIL-20A auto sampler and CTO-10ASVP column oven (Shimadzu, Japan), reciprocating water bath shaker (Shanghai Zhicheng Analytical Instrument Manufacturing Co. Ltd., China), micro syringe (Wll. Liang. Jin. Yang. q.I., China).

Method used

Equilibrium dialysis was employed in the study.[17,18]

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 10 h in order to remove sulfur as sulfur may interfere in the overall binding process. The temperature was controlled between 65°C and 70°C and hot water was replaced by fresh de-ionized water at every 1 h 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 solution

In this study, BSA was used instead of HSA due to structural similarity, good stability in various media, reproducibility and cost effectiveness.[19,20] To prepare 100 mL of 2×10^{-5} M solution of BSA 0.133 of protein was accurately measured and dissolved in a 100 mL volumetric flask with the previously prepared phosphate buffer solution of 7.4. This was done carefully and gently so that it did not form foam. The protein solution was kept in a refrigerator until use.

Method validation (ultraviolet-spectrophotometer)

Precision

Precision of UV method has been determined by repeatability and recovery method. Three different drug concentrations were used for the study and percentage recovery and standard deviation (SD) were calculated.

Accuracy

Accuracy of UV method has been determined by recovery method. Placebo formulation was mixed with different drug concentrations and percentage recovery and SD were calculated.

Preparation of standard curve

Amlodipine besylate

In each of 7 test tubes phosphate buffer solution of pH 7.4 was taken. AB solution at pH 7.4 and conc. of 1×10^{-3} 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 absorbance value of solution was determined by UV spectrophotometer at λ_{max} 238 nm. As a reference sample, phosphate buffer solution of pH 7.4 was used. The standard curve was obtained by plotting the absorbance values against the corresponding concentration.

Olmesartan medoxomil

Phosphate buffer solution of pH 7.4 was taken in each of 7 test tubes. OM solution at pH 7.4 and conc. of 1×10^{-3} M was added in different volume to 7 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 value was determined by a UV spectrophotometer at λ_{max} 256 nm. As a reference sample, phosphate buffer solution of pH 7.4 was used. 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 10 in each of 7 test tubes. Warfarin sodium stock solution at pH 7.4 and concentration of 1×10^{-3} M was added in different concentrations to the 7 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 value was determined by a UV spectrophotometer at λ_{max} 306 nm. As a reference sample, phosphate buffer solution of pH 7.4 was used. Same procedure was followed for diazepam. The absorbance value was determined by a UV spectrophotometer at λ_{max} 254 nm. The standard curve was obtained by plotting the absorbance values against the corresponding concentration. The standard curve of warfarin sodium and diazepam were obtained by plotting the absorbance values against the corresponding concentrations.

Chromatographic conditions

The mobile phase consisted of a mixture of acetonitrile and buffer (60:40 v/v) which was pumped at a flow rate of 1 ml/min through the column (C18; 5 µ, 4.6 × 150 mm, waters, USA) at ambient temperature. The injection volume was 10 µl. The mobile phase was filtered through 0.45 µ membrane filter and degassed prior to use by suction pump with negative pressure filtration. Concentrations were measured at 248 nm by UV detector at a sensitivity of 0.00001. The run time was at 15 min and column temperature was maintained at 25°C. Prior to injection of analyte, the column was equilibrated for 30 min with mobile phase.

A new, affordable, cost-effective and convenient HPLC method for HPLC determination of AB and OM, diazepam and warfarin sodium was developed first. The method was validated for the parameters like system suitability, selectivity, linearity, accuracy, precision, limit of detection and limit of quantification (LOQ).

In order to assess the system suitability of the method solution containing 100% target concentration injected in six replicate and various chromatographic parameters such as retention time, peak area, tailing factor and theoretical plates (tangent) of the column were determined. Linearity of the method was determined by constructing calibration curves. Standard solutions of different concentrations level ($0.5–5 \times 10^{-5}$ M) were used for this purpose. Accuracy was determined by means of spike and recovery method. Intra-day precision (repeatability) was determined by performing repeated analysis of the standard solution on the same day and inter-day precision (intermediate precision) of the method was assessed by carrying out the analysis of standard solution on 3 different days in the same laboratory.

Ultraviolet spectrophotometric method has been used in those tests where only one drug was used. HPLC method has been used when two drugs were used simultaneously in the same tests as it is difficult to determine two drugs by UV spectrophotometric method.

Experimental design

Estimation of association constant

The value of association constant (Ka) and the number of corresponding binding sites (*n*) of AB and OM bound to BSA were determined by Scatchard plot, utilizing equilibrium technique.[17,18] 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, that is:

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

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

Estimating association constant of amlodipine besylate bound to bovine serum albumin at pH 7.4 at temperature 37°C

Five millilitre of 2×10^{-5} M BSA solution at pH 7.4 was taken in 7 test tubes and AB was added to make concentrations 2×10^{-5} M, 4×10^{-5} M, 8×10^{-5} M, 12×10^{-5} M, 16×10^{-5} M, 18×10^{-5} M respectively. Another test tube containing only BSA solution at pH 7.4 was marked as control. The solution were then mixed properly and allowed to stand for 30 min in order to ensure maximum binding of AB to BSA.

From each of the test tube, 3.5 mL of solution was pipetted out and poured into 7 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 seven separate 50 mL conical flasks containing 20 mL of phosphate buffer solution of pH 7.4. The mouths of conical flasks were covered by foil paper. The conical flasks were then placed in a reciprocating water bath shaker for dialysis for 12 h at 37°C and 20 rpm. After completion of dialysis samples were collected from each flask and free concentrations of AB were measured by a UV spectrophotometer at a wavelength of 238 nm.

Estimating association constant of olmesartan medoxomil bound to bovine serum albumin at pH 7.4 at temperature 37°C

Five millilitre of 2×10^{-5} M BSA solution at pH 7.4 was taken in 7 test tubes and OM was added to make concentrations 2×10^{-5} M, 4×10^{-5} M, 6×10^{-5} M, 8×10^{-5} M, 12×10^{-5} M, 16×10^{-5} M, 18×10^{-5} M respectively. The rest of the procedure is same for estimating association constant of AB.

After completion of dialysis samples were collected from each flask and free concentrations of OM were measured by a UV spectrophotometer at a wavelength of 256 nm.

Identification and characterization of binding site of amlodipine besylate and olmesartan medoxomil 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 study was carried out by utilizing equilibrium dialysis. Warfarin sodium (Site I specific) and diazepam (Site II specific) were used as selective drug probe (to their specific site) to determine the binding site of AB and OM on 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 (AB and OM) 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 olmesartan medoxomil 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 is more specific than Site I.[21] And in the presence of it, the Site II is sufficiently blocked so there is a displacement of experimental drugs which increases the free concentration in the medium so changes in the free concentration of experimental drugs can be observed.

Mutual effect of amlodipine besylate and olmesartan medoxomil to displace each other: Effect of amlodipine besylate on olmesartan medoxomil

To the mixture of OM and BSA (1:1, $2 \times' 10^{-5}$ M: $2 \times' 10^{-5}$ M) different concentrations of AB were added both in the absence and presence of diazepam. The changes in the free concentration of OM and AB were measured using HPLC technique.

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

Effect of olmesartan medoxomil on amlodipine besylate

To the mixture of AB and BSA (1:1, $2 \times' 10^{-5}$ M: $2 \times' 10^{-5}$ M) different concentrations of OM were added both in the absence and presence of diazepam. The changes in the free concentration of AB and OM were measured using HPLC technique.

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

RESULT AND DISCUSSION

The percentage recovery and percentage relative SD (RSD) of all the tests were within 98–102% and <2% respectively which indicates that the method is accurate, linier and

precise. In all the cases LOQ was less than 0.1×10^{-5} M which justifies the suitability of the method for the current study [Figures 1, 2 and Tables 1-3].

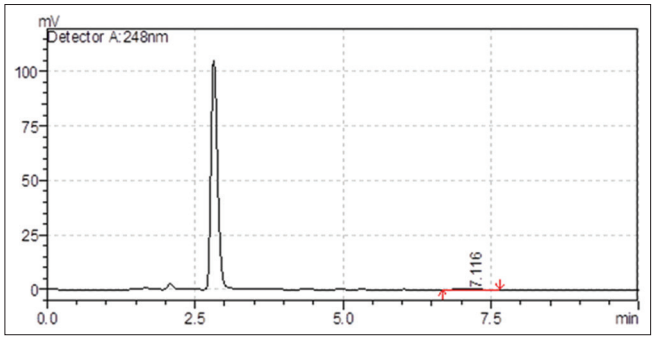

Figure 1: Chromatogram of amlodipine besylate with retention time 2.8 min

All tests are repeated 6 times and percentage RSD were calculated which was <2%.

Regression equations are utilized to calculate the value of free drug concentration by putting the absorbance value [Table 4].

Determining association constant and number of binding sites

The two types of drug-protein binding, that is, high affinity and low affinity which have a binding to small number of sites and to a large number of sites respectively. 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.[13]

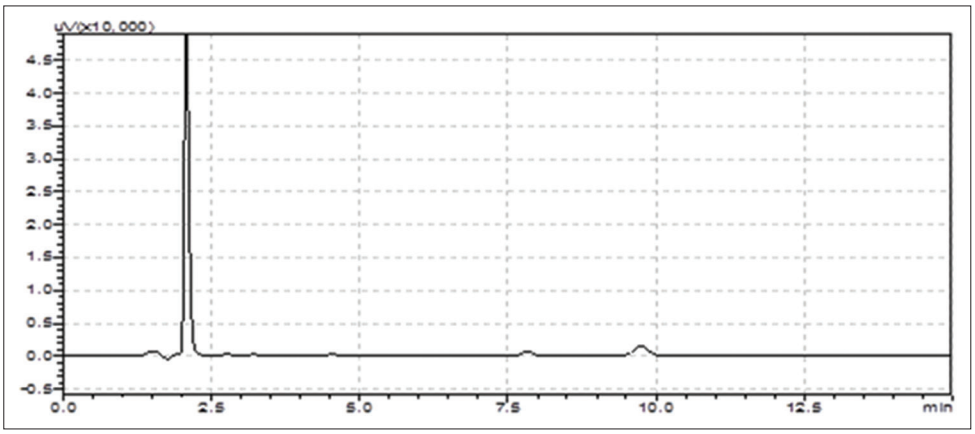

Figure 2: Chromatogram of olmesartan medoxomil with retention time 2.065 min

Table 1: Precision (time)

Time	Concentration of AB (concentration×10^{-5} M)	Measured concentration of AB (*n*=6)	Percentage recovery	Mean of percentage recovery	SD of percentage recovery
10.00 am	0.5	0.498	99.60	99.95	0.484
	2	2.01	100.50		
	5	4.987	99.74		
3.00 pm	0.5	0.492	98.40	100.40	1.833
	2	2.04	102.00		
	5	5.04	100.80		

SD: Standard deviation, AB: Amlodipine besylate

Table 2: Precision (instrument)

Instrument	Concentration of AB (concentration×10^{-5} M)	Measured concentration of AB (*n*=6)	Percentage recovery	Mean of percentage recovery	SD of percentage recovery
UV-SP8-400	0.5	0.498	99.60	99.89	0.389
	3	3.01	100.33		
	5	4.987	99.74		
UV-1200	0.5	0.491	98.20	99.56	1.251
	3	3.02	100.67		
	5	4.99	99.80		

Both percentage recovery and SD were within limit that proved that the method was precise. SD: Standard deviation, AB: Amlodipine besylate

Table 3: Accuracy recovery method

Concentration of AB (concentration×10^{-5} M)	Measured concentration of AB (*n*=6)	Percentage recovery	Mean of percentage recovery	SD of percentage recovery
0.5	0.498	99.60	99.04	1.153
0.8	0.789	98.63		
1	0.97	97.00		
2	2.01	100.50		
3	2.95	98.33		
4	3.98	99.50		
5	4.987	99.74		
Concentration of OM (concentration×10^{-5} M)	**Measured concentration of AB (*n*=6)**	**Percentage recovery**	**Mean of percentage recovery**	**SD of percentage recovery**
0.5	0.491	98.20	98.76	0.971
0.8	0.784	98.00		
1	0.98	98.00		
2	1.98	99.00		
3	3.01	100.33		
4	3.92	98.00		
5	4.99	99.80		

Both percentage recovery and SD were within limit that proved that the method was accurate. SD: Standard deviation, AB: Amlodipine besylate, OM: Olmesartan medoxomil

Table 4: Regression equation of standard curve of AB, OM, warfarin sodium and diazepam at pH 7.4

Sample	Regression equation
Experimental drugs	
AB	y=0.0718x−0.0046
OM	y=0.1562x+0.0127
Probe used	
Warfarin sodium	y=0.1263x+0.0037
Diazepam	y=0.1671x−0.0111

AB: Amlodipine besylate, OM: Olmesartan medoxomil

In this study, Scatchard plot have been used as a tool to characterize the binding parameters of AB and OM. The nonlinear curve of the plot suggests the presence of at least two classes of binding site of experimental drugs to BSA.[21]

By plotting data from Table 5 Scatchard plot analysis revealed that at pH 7.4 association constant and number of binding site of AB is about 5 × 10^5/M and 2 respectively at high affinity binding site. On the other hand, at low affinity binding site, association constant and number of binding site is found about 0.4286 × 10^5/M and 7 respectively [Figure 3].

By plotting data from Table 6 Scatchard plot analysis revealed that at pH 7.4 association constant and number of binding sites of OM is about 0.5778 × 10^5/M and 9, respectively at high affinity binding site. On the other hand, at low affinity binding site, association constant and number of binding site is found about 0.165 × 10^5/M and 20 respectively [Figure 4].

So, the nonlinear curves suggesting that the presence of at least two classes of binding sites (high affinity and low affinity) for the binding of AB and OM to BSA.

Determining binding sites

The free concentration of warfarin sodium and diazepam were calculated for the determination of binding site. 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 particulate site.[5]

From the Tables 7 and 8 [Figure 5], it is seen that addition of AB displaces warfarin sodium more (as percentage of initial, from 100% to 191.19%) than that of diazepam (percentage of initial concentration remains same, from 100% to 100%). As the increment of free concentration of warfarin sodium is significant than that of diazepam so, it can be concluded that AB preferentially bind to Site I. Alam *et al*., (2009)[5] and Alam *et al*.,(2008)[6] also found the similar result.

Similarly, OM displace warfarin sodium less (as percentage of initial from 100% to 102.098%) than that of diazepam (as percentage of initial from 100% to 109.72%), it can be concluded that OM slightly binds to Site I and preferentially bind to Site II [Tables 9, 10 and Figure 6].

However, as the displacement of warfarin sodium and diazepam is quite different in both cases of AB and OM, it can be assumed that the investigated drugs AB and OM

Table 5: Data for association constant of AB bound to BSA at pH 7.4 and 37°C

Observation number	Total drug concentration D_T (×10^{-5} M)	Absorbance	Free drug concentration D_f (×10^{-5} M)	Total free drug concentration $2×D_f$ (×10^{-5} M)	Bound drug concentration D_B (×10^{-5} M)	$r=D_B/P_tP_t=$ (2×10^{-5} M)	r/D_f×10^{-5} M^{-1}
1	2	0.006	0.148	0.295	1.705	0.852	5.8
2	4	0.032	0.510	1.019	2.981	1.490	2.9
3	8	0.098	1.429	2.858	5.142	2.571	1.8
4	12	0.165	2.362	4.724	7.276	3.638	1.5
5	16	0.252	3.574	7.148	8.852	4.426	1.2
6	18	0.31	4.382	8.763	9.237	4.618	1.1

AB: Amlodipine besylate, BSA: Bovine serum albumin

Table 6: Data for association constant of OM bound to BSA at pH 7.4 and 37°C

Observation number	Total drug concentration D_T (×10^{-5} M)	Absorbance	Free drug concentration D_f (×10^{-5} M)	Total free drug concentration $2×D_f$ (×10^{-5} M)	Bound drug concentration D_B (×10^{-5} M)	$r=D_B/P_tP_t=$ (2×10^{-5} M)	r/D_f×10^{-5} M^{-1}
1	2	0.039	0.168	0.337	1.663	0.832	4.9
2	4	0.07	0.367	0.734	3.266	1.633	4.5
3	6	0.102	0.572	1.143	4.857	2.428	4.2
4	8	0.152	0.892	1.784	6.216	3.108	3.5
5	12	0.241	1.462	2.923	9.077	4.538	3.1
6	16	0.378	2.339	4.677	11.323	5.661	2.4
7	18	0.43	2.672	5.343	12.657	6.328	2.4

BSA: Bovine serum albumin, OM: Olmesartan medoxomil

Table 7: Determination of binding site of AB at pH 7.4 and 37°C using warfarin sodium as site-I specific probe

BSA: Warfarin (1:1) (2×10^{-5} M: 2×10^{-5} M)					
Observation number	**Concentration of AB×10^{-5} M**	**Absorbance**	**Free concentration of warfarin**	**Free fraction (%) of warfarin**	**As percentage of initial**
1	0	0.132	1.018	50.91	100
2	2	0.174	1.352	67.58	132.74
3	4	0.22	1.717	85.83	168.59
4	8	0.246	1.923	96.15	188.85
5	12	0.249	1.947	97.34	191.19

BSA: Bovine serum albumin, AB: Amlodipine besylate

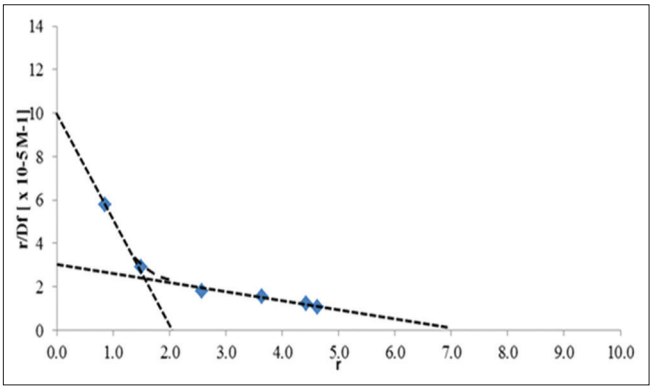

Figure 3: Scatchard plot for the binding of amlodipine besylate to bovine serum albumin by equilibrium dialysis at pH 7.4 and 37°C

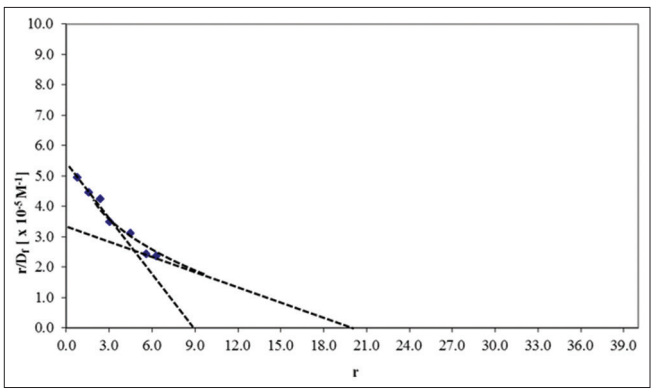

Figure 4: Scatchard plot for the binding of olmesartan medoxomil to bovine serum albumin by equilibrium dialysis at pH 7.4 and 37°C

Table 8: Determination of binding site of AB at pH 7.4 and 37°C using diazepam as Site II specific probe

BSA: Diazepam (1:1) (2×10^{-5} M: 2×10^{-5} M)

Observation number	Concentration of AB×10^{-5} M	Free concentration		Free fraction (%) of diazepam	Free fraction (%) of AB	As percentage of initial (diazepam)
		Diazepam	AB			
1	0	0.007	0	0.35	0	100
2	2	0.007	0.5453	0.35	27.265	100
3	4	0.007	1.513	0.35	37.825	100
4	8	0.007	3.9047	0.35	48.809	100

BSA: Bovine serum albumin, AB: Amlodipine besylate

Table 9: Determination of binding site of OM at pH 7.4 and 37°C using warfarin sodium as Site I specific probe

BSA: Warfarin (1:1) (2×10^{-5} M: 2×10^{-5} M)

Observation number	Concentration of OM×10^{-5} M	Absorbance	Free concentration of warfarin	Free fraction (%) of warfarin	As percentage of initial
1	0	0.146	1.135	56.746	100
2	2	0.147	1.143	57.143	100.699
3	4	0.148	1.151	57.549	101.399
4	8	0.149	1.159	57.937	102.098
5	12	0.152	1.183	59.127	104.196

OM: Olmesartan medoxomil, BSA: Bovine serum albumin

Table 10: Determination of binding site of OM at pH 7.4 and 37°C using diazepam as Site II specific probe

BSA: Diazepam (1:1) (2×10^{-5} M: 2×10^{-5} M)

Observation number	Concentration of OM×10^{-5} M	Free concentration		Free fraction (%) of diazepam	Free fraction (%) of OM	As percentage of initial (diazepam)
		Diazepam	OM			
1	0	0.0072	0	0.36	0	100
2	2	0.0076	0.46	0.38	23.00	105.55
3	4	0.0077	1.00	0.385	25.00	106.94
4	6	0.0078	2.08	0.39	34.66	108.33
5	8	0.0079	3.18	0.395	39.75	109.72

OM: Olmesartan medoxomil, BSA: Bovine serum albumin

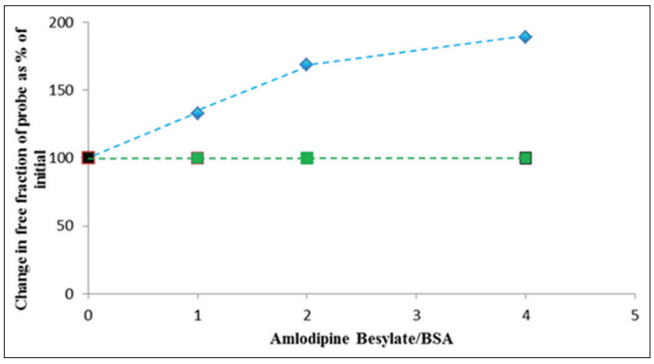

Figure 5: Free fraction of warfarin and diazepam to bovine serum albumin (BSA) (1:1) upon addition of amlodipine besylate by equilibrium dialysis at pH 7.4 and 37°C. Following concentrations were used; for curve (■), (BSA) = (diazepam) = 2×10^{-5} M, (amlodipine besylate) = 0–8×10^{-5} M; for curve (♦), (BSA) = (warfarin) = 2×10^{-5} M, (amlodipine besylate) = 0–8×10^{-5} M

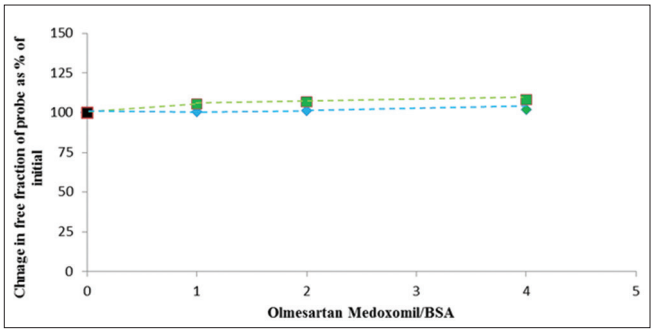

Figure 6: Free fraction of warfarin and diazepam to bovine serum albumin (BSA) (1:1) upon addition of olmesartan medoxomil by equilibrium dialysis at pH 7.4 and 37°C. Following concentrations were used; for curve (■), (BSA) = (diazepam) = 2×10^{-5} M, (olmesartan medoxomil) = 0–8×10^{-5} M; for curve (♦), (BSA) = (warfarin) = 2×10^{-5} M, (olmesartan medoxomil) = 0–8×10^{-5} M

Table 11: Data for the effect of OM on AB binding to BSA in the absence and presence of diazepam

A. In absence of diazepam; BSA used (2×10^{-5} M)

Observation number	Amlodipine: BSA ($\times10^{-5}$ M)	Added olmesartan concentration ($\times10^{-5}$ M)	Free concentration ($\times10^{-5}$ M)		Percentage of free fraction of amlodipine
			Olmesartan	Amlodipine	
1	1:1	0	0	0.195	9.75
2	1:1	2	0.7201	0.1834	9.17
3	1:1	4	1.3832	0.1919	9.595
4	1:1	8	2.6819	0.1927	9.635

B. In presence of diazepam; BSA used (2×10^{-5} M)

Observation number	Amlodipine: BSA: Diazepam ($\times10^{-5}$ M)	Added olmesartan concentration ($\times10^{-5}$ M)	Free concentration of amlodipine ($\times10^{-5}$ M)			Percentage of free fraction of amlodipine
			Diazepam	Olmesartan	Amlodipine	
1	1:1:2	0	0.034	0	0.1508	7.54
2	1:1:2	2	0.013	0.7129	0.0826	4.13
3	1:1:2	4	0.0146	1.3114	0.1015	5.075
4	1:1:2	8	0.0222	2.1046	0.1190	5.95

AB: Amlodipine besylate, OM: Olmesartan medoxomil, BSA: Bovine serum albumin

Table 12: Data for the effect of AB on OM binding to BSA in the absence and presence of diazepam

A. In absence of diazepam; BSA used (2×10^{-5} M)

Observation number	Olmesartan: BSA ($\times10^{-5}$ M)	Added amlodipine concentration ($\times10^{-5}$ M)	Free concentration ($\times10^{-5}$ M)		Percentage of free fraction of olmesartan
			Amlodipine	Olmesartan	
1	1:1	0	0	0.3170	15.85
2	1:1	2	0.2029	0.3163	15.815
3	1:1	4	0.3477	0.3165	15.825
4	1:1	8	0.8571	0.31690	15.845

B. In presence of diazepam; BSA used (2×10^{-5} M)

Observation number	Olmesartan: BSA: Diazepam ($\times10^{-5}$ M)	Added amlodipine concentration ($\times10^{-5}$ M)	Free concentration of amlodipine ($\times10^{-5}$ M)			Percentage of free fraction of olmesartan
			Diazepam	Amlodipine	Olmesartan	
1	1:1:2	0	0.0085	0	0.619	30.95
2	1:1:2	2	0.0085	0.2029	0.4343	21.715
3	1:1:2	4	0.0085	0.3477	0.4317	21.585
4	1:1:2	8	0.0085	0.8571	0.362	19.6

AB: Amlodipine besylate, OM: Olmesartan medoxomil, BSA: Bovine serum albumin

preferentially bind with Site I and Site II respectively. In addition to Site II, OM also binds with Site I on the BSA molecule but to a lower extent.

As the investigated drugs compete for different Site (Site I - AB and Site II - OM), concurrent administration of these two drugs will not alter the therapeutic efficacy of each other hence the combination pill of these two drugs will be of therapeutic value as antihypertensive drugs.

Mutual effect of amlodipine besylate and olmesartan medoxomil on the binding to bovine serum albumin in the absence and presence of diazepam

The interactions at binding sites on BSA were measured between AB and OM in the absence and in presence of site specific probe diazepam. In absence of diazepam, the free fraction of AB remains almost same (9.75–9.635%) with the

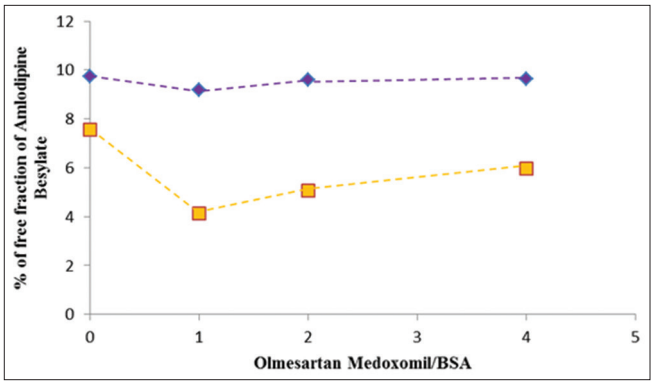

Figure 7: Free fraction of amlodipine besylate to bovine serum albumin (BSA) (1:1) upon the addition of olmesartan medoxomil at pH 7.4 and 37°C in the presence (▪) and absence (♦) of diazepam. Following concentrations were used; (BSA) = (amlodipine besylate) = 2×10^{-5} M, (diazepam) = 4×10^{-5} M, (olmesartan medoxomil) = $2 - 8 \times 10^{-5}$ M

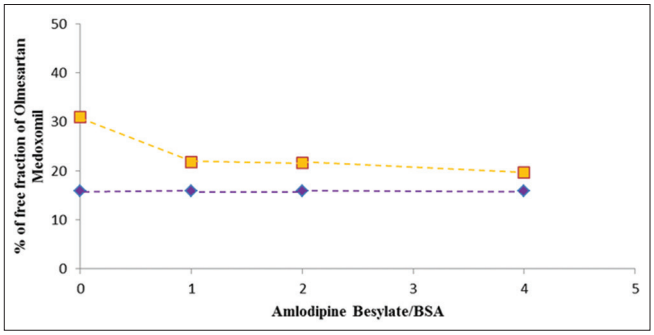

Figure 8: Free fraction of olmesartan medoxomil to bovine serum albumin (BSA) (1:1) upon the addition of amlodipine besylate at pH 7.4 and 37°C in the presence (▪) and absence (♦) of diazepam. Following concentrations were used; (BSA) = (olmesartan medoxomil) = 2 × 10–5 M, (diazepam) = 4 × 10–5 M, (amlodipine besylate) = 2 – 8 × 10–5 M

addition of OM from 0×10^{-5} M to 8×10^{-5} M. Whereas, in the presence of diazepam, OM at the same concentration, the free fraction of AB decreases but in a lower extent (7.54–5.95%) [Table 11 and Figure 7].

Similarly, from Table 12 [Figure 8] it is revealed that in the absence of diazepam, the free fraction of OM remain almost same (15.85–15.845%) with the addition of AB from 0×10^{-5} M to 8×10^{-5} M. Whereas, in the presence of diazepam, AB at the same concentration, the free fraction of OM decreases but in a lower extent (30.95–19.6%).

CONCLUSION

The resulted free fraction of one another for both AB and OM is found not so significant, when they simultaneously bind to BSA. This probably suggests that these drugs bind independently to the BSA and hence do not interfere with each other for their binding. As the investigated drugs compete for different Site (Site I - AB and Site II - OM), concurrent administration of these two drugs will not alter the therapeutic efficacy of each other hence the combination pill of these two drugs will be of therapeutic value as a single pill in cardiovascular problems.

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