Impact of Nicotine Consumption on Hyper Acidic Patients Taking PPI: An *In-Vitro* and Computational Analysis

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Abstract

The study aims to investigate the protein binding kinetics of nicotine and a PPI (pantoprazole) with Bovine Serum Albumin (BSA) through UV spectroscopy and computational modeling. Data was obtained by using nicotine and pantoprazole and warfarin and diazepam as the two site specific probes on Bovine serum albumin (BSA). *In-vitro* and *in-silico* modeling was carried out in creating an environment that simulates the body environment. Cellulose membrane tubes were cut into 9 cm and tied tightly not to let any mixtures leak out. To determine number of binding sites, association constants by using Scatchard plot, predominant binding site of each drug and rise in % of free fraction of one by the other were analyzed using equilibrium dialysis method. Molecular docking further verifies the observations. In Scatchard plot analysis, for nicotine, n_1, n_2, k_1 and k_2 = 2.2, 7.6, 0.18 μM^{-1} and 0.02 μM^{-1} and for pantoprazole, n_1, n_2, k_1 and k_2 = 0.42, 1.2, 0.40 μM^{-1} and 0.03 μM^{-1}. Nicotine binds more to diazepam site (site-II) and pantoprazole mainly to warfarin site (site-I). In molecular docking, the binding affinity of nicotine being −5.7 kcal/mole demonstrates higher affinity for site-II than that of pantoprazole whose binding affinity is −8.0 kcal/mole. In absence and presence of warfarin, the free fraction of pantoprazole bound to BSA (1:1) was increased from 37.79% to 82.44% and 51.78% to 98.80% respectively by nicotine. On the other hand, free fraction of nicotine was raised by pantoprazole from 12.89% to 75.70% and 50.08% to 99.66% in the absence and presence of diazepam. Both the results of spectroscopic and computational molecular docking suggest that administering pantoprazole with nicotine might increase the % free fraction of pantoprazole more. Thus, nicotine consumption can be beneficial for smoker people taking PPI like pantoprazole.

1. Introduction

Nicotine, is a highly addictive alkaloid [1] found in the leaves of *Nicotianarustica*, *Nicotiana tabacum*, *Duboisia hopwoodii* and *Asclepias syriaca* and has been used as an insecticide in the past [2]. An average cigarette yields about 2 mg of absorbed nicotine, the substance acts as a stimulant in mammals, while high amounts (50 - 100 mg) can be harmful [3]. Nicotine's addictive nature includes psychoactive effects, gastric effects, drug-reinforced behavior, compulsive use, and relapse after abstinence, physical dependence and tolerance [4] which is also linked to possible birth defects [5] [6]. Pantoprazole, on the other hand, is a proton pump inhibitor that inhibits gastric acid secretion and treats gastric ulcers by irreversibly blocking the proton pump [7]. There is a lower esophageal sphincter function of chronic smokers compared with non-smokers. Cigarette smoking probably exacerbates reflux disease by directly provoking acid reflux and perhaps by a long lasting reduction of lower esophageal sphincter pressure [8]. So, smoking increases the risk of heartburn and GERD and also peptic ulcers for which smokers take omeprazole or pantoprazole for better functioning of the body.

Serum albumin is the primary extracellular protein of the circulatory system contributing to about 60% mass of plasma proteins [9]. It serves as a transport carrier for many drugs and plays a dominant role on their bioavailability [10]. The interaction of any drug with serum albumin is highly characterized by the properties of the drug, namely, its concentration, physicochemical properties, and also by number of vacant binding sites on the protein and pH of the medium [11]. Consequently, the synergy between the drug molecule and albumin exerts considerable effects on the pharmacodynamics and pharmacokinetics, *i.e.*, distribution, metabolism and excretion of the drug when stable or reversible drug-protein complexes are formed [12] [13]. Such effects are mainly altered when two or more drugs are administered simultaneously as one drug displaces the other from its binding site, increases its free fraction and hence causes a noticeable rise in activity [14] [15]. Thus, if nicotine and pantoprazole are co-administered, the pharmacokinetics of one or both might alter due to their simultaneous presence in the albumin, even though, the drugs bind on the different binding sites. For their simultaneous administration, we need to know the binding kinetics of these two drugs with serum albumin, *i.e.*, number of binding sites for each drug, their predominant binding sites, displacement of one drug from the binding pocket of albumin by the other and rise of % free fraction of one drug by the other.

Our study attempts to examine the interaction of nicotine and pantoprazole with bovine serum albumin (BSA) and the effect on % free fraction of one drug bound to BSA by the other by spectroscopic and computational tools. We chose BSA as the model protein since it bears approximately 76% of similarity with human serum albumin (HSA) [16]. Moreover, BSA has 88% of similarity in amino acid sequence with HSA and hence 3D structure of BSA is believed to be a close match to that of HSA. Furthermore, BSA was preferred over HSA owing to its wider availability at highly pure form, lower cost, high stability, and medical value. Warfarin and diazepam are used in our study as those are Site I and Site II specific probes respectively on BSA protein [10] [17] [18].

2. Methods

2.1. Study Design

The kinetics of nicotine and pantoprazole interactions with BSA were studied separately *in vitro* by equilibrium dialysis and UV spectroscopy [18]. The 3D chemical structures of nicotine, pantoprazole, warfarin sodium and diazepam were retrieved from PubChem Open Chemistry Database [19]. The 3D crystal structure of BSA with selected PDB ID (4JK4) was obtained from Protein Data Bank (PDB) database (https://www.rcsb.org/structure/4jk4).

2.2. Sample

Pantoprazole and warfarin sodium were kind gifts from Incepta Pharmaceuticals Ltd, Bangladesh. Diazepam and nicotine were received from Square Pharmaceuticals Ltd., Bangladesh and Vapes needs alltime, Dhaka, Bangladesh respectively. BSA (product number: A 5611) was purchased from Sigma-Aldrich. Spectra/PorCellulose membrane (MWCO 6 - 8 KD, Width 23 mm) was purchased from local supplier. BSA stock solution was prepared in phosphate buffer solution (PBS) (pH 7.4) at 20 µM concentration. Pantoprazole, nicotine, warfarin sodium and diazepam stock solutions were prepared in PBS (pH 7.4) at 100 µM concentration. Working concentrations were prepared by diluting in PBS (pH 7.4). Chemical structures of nicotine, pantoprazole, diazepam and warfarin are shown in Figure 1.

2.3. Measures

2.3.1. Estimation of Association Constants and Number of Binding Sites
10 nicotine-BSA solutions (0 µM, 25 µM, 30 µM, 35 µM, 40 µM, 45 µM, 50 µM, 55 µM, 60 µM and 65 µM; 5 ml each) and 10 pantoprazole-BSA solutions (0 µM, 10 µM, 20 µM, 30 µM, 40 µM, 50 µM, 60 µM, 70 µM, 80 µM and 90 µM; 5 ml each) were prepared by taking calculated amounts of nicotine (1000 µM), pantoprazole (100 µM) and BSA (20 µM) solutions. The solutions were then properly mixed and allowed to stand for 30 minutes in order to ensure maximum binding of the drugs with BSA. From each solution, 3.5 mL was drawn out and filled into previously prepared semipermeable membrane tubes (SS 1). The
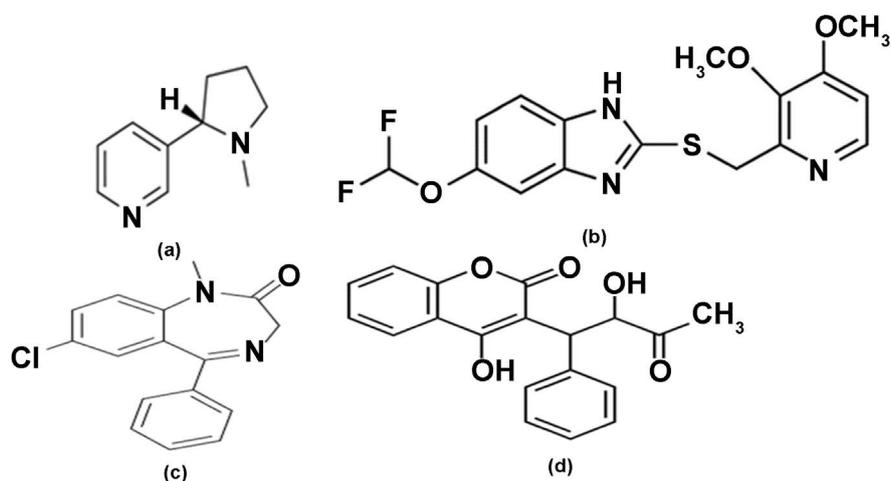
Figure 1. Chemical structure of (a) nicotine, (b) pantoprazole, (c) diazepam and (d) warfarin.

membrane tubes containing drug-protein mixture were then immersed in separate conical flasks containing 20 ml PBS (pH 7.4) and kept in a metabolic shaker (Zhicheng, China) for dialysis for 12 hours (37°C, 40 rpm).

2.3.2. Determination of Predominant Binding Site

The predominant binding site of nicotine and pantoprazole on BSA was identified *in vitro* by equilibrium dialysis and UV spectroscopy using warfarin sodium as site-I and diazepam as site-II specific probes. During all the experiments, BSA and the probes (warfarin sodium/diazepam) were taken at a ratio of 1:1 (20 μM: 20 μM) and nicotine or pantoprazole were mixed at an increasing concentration (0 to 80 μM) to get the final ratio of BSA: probe: test drugs of 1:1:0, 1:1:1.5, 1:1:2, 1:1:2.5, 1:1:3, 1:1:3.5, and 1:1:4. All the solutions were allowed for equilibrium dialysis as mentioned earlier. On completion of dialysis, samples were collected from each solution and the free fraction of warfarin sodium and diazepam were measured at 305 and 238 nm.

2.3.3. Drug Displacement Study

The effect of pantoprazole on nicotine binding to BSA and vice-versa was evaluated in absence and presence of diazepam and warfarin. At first, keeping the concentrations for nicotine and BSA constant (20 μM:20 μM), pantoprazole was added in increasing concentration (0 to 100 μM) to make final ratio of BSA: nicotine: pantoprazole in each experiment as 1:1:0, 1:1:1, 1:1:2, 1:1:3, 1:1:4 and 1:1:5. While in presence of diazepam, the final ratio of BSA: diazepam: nicotine: pantoprazole were 1:2:1:0, 1:2:1:1, 1:2:1:2, 1:2:1:3, 1:2:1:4 and 1:2:1:5. And for both the cases, dialysis was carried out and absorbance of free nicotine was measured at 260 nm and amount calculated. Secondly, the estimation for the effect of nicotine on pantoprazole, when bound to BSA was done in a same manner. Here, pantoprazole was kept constant and so the final ratio of BSA:warfarin: pantoprazole:nicotine were 1:2:1:0, 1:2:1:1, 1:2:1:2, 1:2:1:3, 1:2:1:4 and 1:2:1:5.

And again for both the cases absorbance of free pantoprazole was measured at 220.5 nm and amount calculated.

2.3.4. Quantum Chemical Calculations

This utilized density functional theory (DFT) employing Becke's (B3) exchange functional combining Lee, Yang, and Parr's (LYP) correlation functional [20] [21] and 6-311G + (d, p) basis set [22]. Molecular orbital calculations were performed using the same level of theory. Hardness (η) and softness (*S*) of the test compounds were calculated from the energies of frontier highest occupied molecular orbitals (HOMOs) and lowest unoccupied molecular orbitals (LUMOs). It considered Parr and Pearson interpretation [23] [24] of harness in DFT and Koopmans theorem [25] on the correlation of ionization potential (I) and electron affinities (E) with HOMO and LUMO energy. The following equations of hardness (*η*) and softness (*S)* were used.

$$\eta = \left(\varepsilon\text{LUMO} - \varepsilon\text{HOMO}\right)/2 \tag{1}$$

$$S = 1/\eta \tag{2i}$$

2.3.5. Molecular Docking

Docking was performed by Auto Dock Vina software (version 1.1.2) [26]. To dock the compounds against BSA, the center grid box was positioned at the center of the protein structure and was expanded in x, y and z directions until the grid box fully covered the protein structure. During docking the protein structure was kept rigid but torsional rotation was allowed for all rotatable bonds of optimized drug structures to perform flexible docking. The binding affinities of each complex were recorded from vina output. The docked pose of lowest binding free energy conformer for all ligand-BSA complexes were then investigated using Accelrys Discovery Studio 4.1 [27]. The information of non-bonding interactions of the complexes in terms of binding pocket, interface amino acids, binding residues, bond type and bond length were collected. Finally a non-bonding interaction map for each complex was generated to visualize the ligand-BSA interactions.

2.4. Analysis

The values of association constants and number of binding sites were estimated by Scatchard plot analysis [10]. For determining of binding sites, subsequent analyses were performed using standard curves (SS 2). In addition to all the above mentioned analysis with the finding of the effect of one drug with another, the unbound fraction in all the cases, from each of the flasks were collected and measured by a UV spectrophotometer (UV 1800, Shimadzu, Japan).

Gaussian 09 program package [28] was used for ligand optimization and molecular orbital calculation. Prior to docking, PyMol (version 1.3) software package program was used to erase all the hetero atoms and water molecules [29] and Swiss-Pdb Viewer software packages (version 4.1.0) helped in energy minimization [30].

3. Results

3.1. Association Constants and Binding Sites

The curvilinear Scatchard graphs of nicotine-BSA and pantoprazole-BSA bindings are presented in **Figure 2**. Drug-protein binding data reported herein that demonstrates, for each drug there are two different binding sites (n_1 and n_2) and each site is associated with its own independent association constant (k_1 and k_2). At its low (n_1 = 2.2) and high (n_2 = 7.6) capacity binding sites, the high and low affinity association constants of nicotine are found to be 0.18 μM^1 and 0.02 μM^{-1}, respectively. On the other hand, n_1, n_2, k_1 and k_2 for pantoprazole are 0.42, 1.2, 0.40 μM^{-1} and 0.03 μM^{-1}, respectively. **Figure 3** depicts the % free fraction of the probe (Pf) against the molar ratio of drug and BSA. As observed in case of nicotine that it displaces warfarin sodium (27.65% to 64.65%) way less than that of diazepam (10.5% to 99.94%) from the BSA binding pocket as the molar ratio of nicotine and BSA increases from 0 to 4. However, pantoprazole raises the % free fraction of warfarin sodium (10.65% to 99.15%) more than that of diazepam (31.96% to 60.66%) with the same amount of increase in molar ratio of pantoprazole and BSA as that of nicotine and BSA.

3.2. Drug Displacement Study

Figure 4 illustrates the effect of one drug binding with the other to BSA in presence and absence of warfarin sodium and diazepam as site-I and site-II specific probe. The experiments show that % free fraction of pantoprazole bound to BSA is increased from 37.79% to 82.44% and 51.78% to 98.80% as the molar ratio of nicotine and BSA goes up from 0 to 5 in absence and presence of warfarin sodium, respectively. On the other hand, rise in % nicotine free fraction is 12.89% to 75.70% and 50.08% to 99.66% in absence and presence of diazepam and the same amount of lifting is seen in molar ratio of pantoprazole and BSA, respectively.

3.3. Quantum Chemical Calculations and Molecular Docking

Table 1 shows the dipole moment (Debye), HOMO and LUMO energy (eV), LUMO-HOMO energy gap (eV), hardness and softness of the optimized ligands. The calculated dipole moment, and softness are found to be lower for nicotine (3.27 D, and 0.024) than that of pantoprazole (4.29 D and 0.033). On other hand, LUMO-HOMO energy gap and hardness are higher for nicotine (81.98 eV and 40.99) than that of pantoprazole (59.22 eV and 29.61). The result of molecular docking of nicotine and pantoprazole with BSA is presented in **Table 2** in terms of average binding affinity (kcal/mole) ± standard deviation, interaction type, bond distance and interacting residues. **Figure 5** and **Figure 6** portrays the docked pose of nicotine-BSA and pantoprazole-BSA complexes. The binding affinity of nicotine (−5.7 kcal/mol) is observed to be quite lesser than that of pantoprazole (−8.3 kcal/mol) when docked against BSA crystal structure (PDB ID: 4JK4).
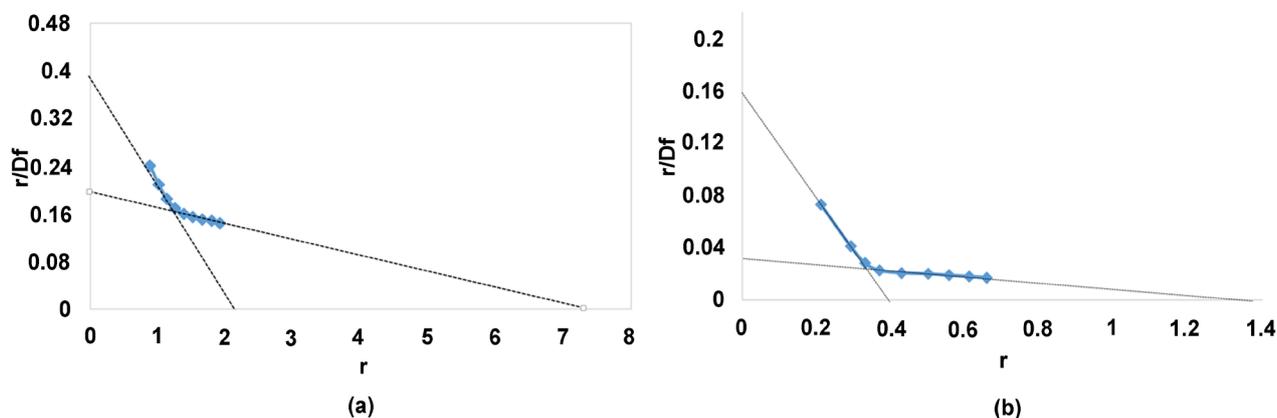
Figure 2. Scatchard plot of (a) nicotine and (b) pantoprazole bound to bovine serum albumin at p^H 7.4˚C and 37˚C. r = [bound drug]/[BSA], $[D_f]$ = free drug conc., [BSA] = 20 μM, [nicotine] = 25 - 65 μM, [pantoprazole] = 10 - 90 μM, (a) n_1, n_2, k_1 and k_2 = 0.87, 1.5, 0.69 $μM^{-1}$ and 0.133 $μM^{-1}$, respectively, (b) n_1, n_2, k_1 and k_2 = 0.42, 1.2, 0.40 $μM^{-1}$ and 0.03 $μM^{-1}$, respectively.
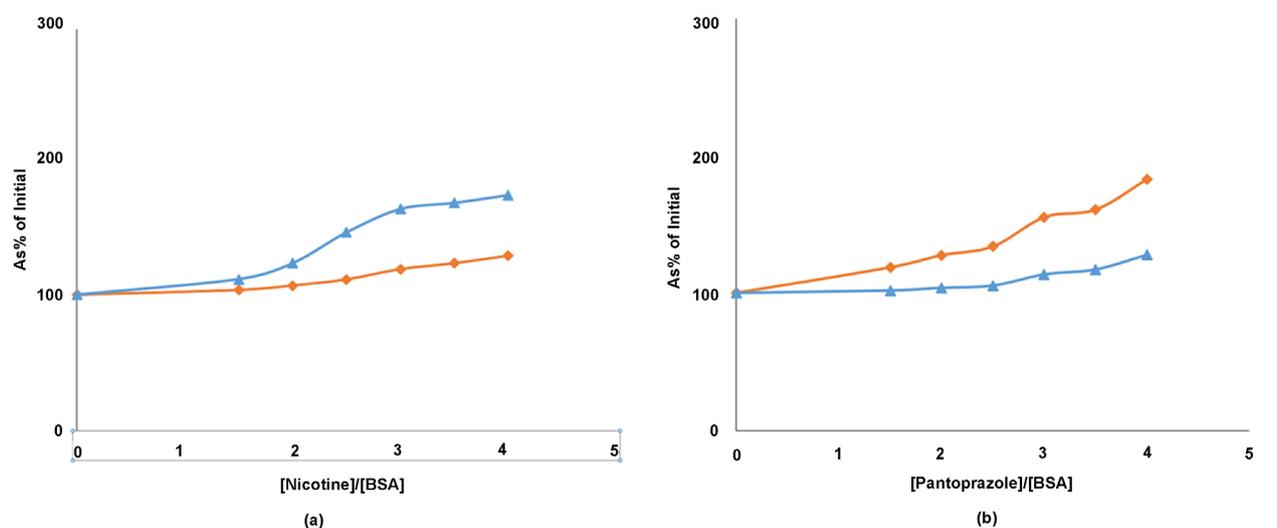

Figure 3. (a) Free fraction of Warfarin (◆) and Diazepam (▲) to BSA (1:1) upon the addition of Nicotine and (b) Free fraction of Warfarin (◆) and Diazepam (▲) to BSA (1:1) upon the addition of Pantoprazole at pH 7.4 and 37˚C. Concentration of BSA for both the curves, Diazepam and Warfarin was 20 μM. Nicotine and Pantoprazole concentrations were taken 0 - 80 μM.

4. Discussion

In the existing study, the potential of co-administration of nicotine and pantoprazole is explored in terms of protein binding kinetics taking bovine serum albumin as the model protein. For each drug, number of binding sites, independent association constants associated with each site, predominant binding site, the increase of % free fraction of one drug bound to bovine serum albumin by the other are determined by equilibrium dialysis and UV spectroscopic tools. The findings are further verified by molecular docking studies. The number of binding sites of each drug on BSA is determined by Scatchard plot analysis. The curvilinear Scatchard graphs of nicotine-BSA and pantoprazole-BSA binding (**Figure 2**) indicates that for both of them there are two different binding sites and also
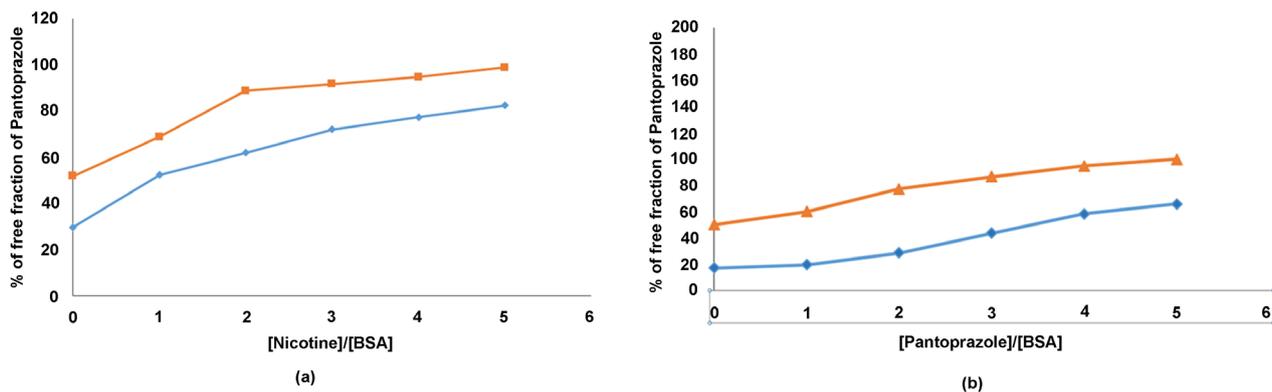
Figure 4. (a) In presence (■) and absence (◆) of Warfarin, free fraction of Pantoprazole bound to BSA (1:1) upon the addition of Nicotine and (b) in the absence (◆) and presence (▲) of Diazepam as site II specific probe, free fraction of Nicotine bound to BSA (1: 1) upon the addition of Pantoprazole both at pH 7.4 and 37°C, [BSA] = [diazepam] = [warfarin sodium] = 20 µM, (a) [nicotine] = 0, 20, 40, 80, 160 µM, (b) [pantoprazole] = 0, 20, 40, 80, 160 µM.
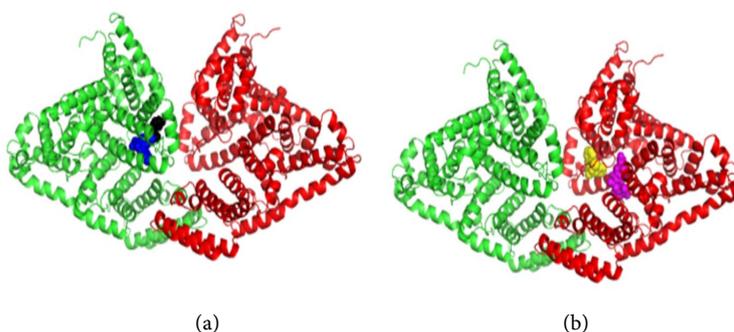

Figure 5. Nicotine (black), diazepam (blue) bound to chain (b) (green) and pantoprazole (purple), warfarin (yellow) bound to chain (a) (red) of BSA (PDB ID: 4JK4).
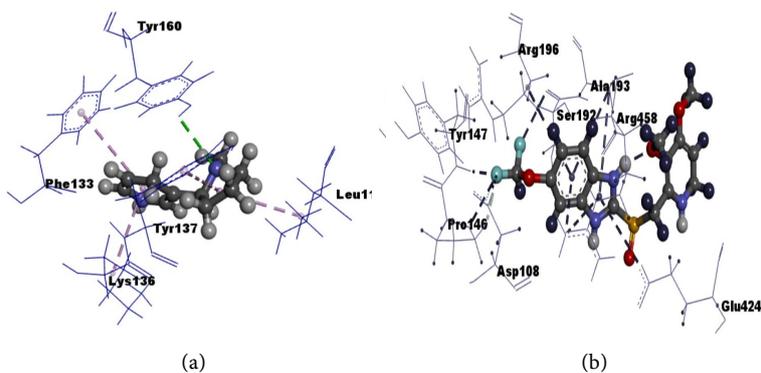
Figure 6. Nonbonding interaction maps of (a) nicotine and (b) pantoprazole bound to BSA (PDB ID: 4JK4).

two independent association constants associated with each of the site. The association constants of nicotine (0.18 µM^{-1} and 0.02 µM^{-1}) are smaller in magnitude than that of pantoprazole (0.40 µM^{-1} and 0.03 µM^{-1}) is an indication to a higher affinity of pantoprazole than that of nicotine for BSA. Moreover, one association

Table 1. Dipole moment (Debye), HOMO and LUMO energy (eV), LUMO-HOMO energy gap (eV), hardness and softness of the optimized ligands.

Ligand	μ (Dipole moment)	HOMO energy (eV)	LUMO energy (eV)	LUMO-HOMO gap	η (Hardness)	S (Softness)
Nicotine	3.27	−334.990	−253.010	81.98	40.99	0.024
Pantoprazole	4.29	−306.933	−247.713	59.22	29.61	0.033

Table 2. Nonbonding interactions of glimepiride and pantoprazole with BSA crystal structure (PDB ID: 4JK4).

Complex	Binding energy (kcal/mol)	Chain (residue in contact) [AAa--LIGa]	Bond type	Bond distance (Å)
Nicotine-BSA	−5.7	B (TYR 160) [H-H---N]	Hydrogen	3.08
		B (LEU 115) [Alkyl]	Hydrophobic	4.38
		B (LYS 136) [Alkyl]	Hydrophobic	4.26
		B (PHE 133) [Pi-Alkyl]	Hydrophobic	4.70
		B (TYR 137) [Pi-Alkyl]	Hydrophobic	5.40
Pantoprazole-BSA	−8.3	A (His 145) [HD1--O]	Conventional	2.165
		A (Pro 146) [CD--F]	Carbon	3.401
		A(Glu 186) [OE1--C]	Carbon	3.399
		A (Thr 190) [OG1--C]	Carbon	3.661
		A (Thr 190) [OG1--C]	Carbon	3.573
		A (Asp 108) [OD1--F]	Halogen (F)	3.148
		A (Arg 458) [NH1--L]	Pi-Cation	3.917
		A (Arg 458) [NH1--L]	Pi-Cation	4.422
		A (Ala 193) [Pi--A]	Pi-Alkyl	4.866
		A (Arg 196) [Pi--A]	Pi-Alkyl	5.015
		A (Arg 458) [Pi--A]	Pi-Alkyl	5.199

constant being greater than the other testifies that each of the drug predominantly binds to one site of BSA. The predominant binding sites of the test drugs are identified by the degree of displacement they confer to either of the two site specific probes (warfarin sodium as site-I and diazepam as site-II specific probes) of BSA. Here, pantoprazole increases the % free fraction of warfarin sodium more than diazepam and nicotine increases the % free fraction of diazepam more than that of warfarin sodium attesting to the fact that one primarily binds to site-I and the other one to site-II ligands of BSA. Besides, the observations in molecular docking are in close harmony with the finding of computational modelling. Pantoprazole and nicotine primarily docks with warfarin sodium and diazepam site of BSA respectively when the grid box size is maximized to cover the entire protein structure (Figure 5). Moreover, average binding af-

finity of nicotine-BSA complex (−5.7 kcal/mole) is less than that of pantoprazole-BSA (−8.0 kcal/mole) complex (Table 2).

The effect of pantoprazole on nicotine and nicotine on pantoprazole binding to BSA are investigated in presence and absence of diazepam and warfarin sodium as one drug majorly binds to warfarin sodium site and the other one mainly to diazepam site. To summarize, pantoprazole is displaced by nicotine from its BSA binding pocket at a lesser extent than that of nicotine being displacement by pantoprazole. Secondly, the degree of displacement is higher in presence of the site specific probes diazepam and warfarin sodium. The evidence that pantoprazole displaces nicotine more from its binding pocket on BSA is a clear testimony of its higher affinity. These outcomes are strongly supported by computational evidences. In quantum chemical calculations, the dipole moment of pantoprazole is found to be higher than that of nicotine. As dipole moment amplifies the polar nature of a molecule and promotes hydrogen bond and non-bonded interactions in drug protein complexes [31], it is believed to increase the association constants of the former than that of the later with BSA in spectroscopic analysis. Small HOMO-LUMO gap and hence increased softness of pantoprazole also accounts for its stronger affinity for BSA than that of nicotine (Table 1) as a low gap marks low kinetic stability of the ligand and hence higher reactivity [32] [33]. Higher affinity of pantoprazole for BSA than that of nicotine in molecular docking is evident from Table 2 which corroborates the observation that nicotine displaces BSA bound pantoprazole less than nicotine being displaced by pantoprazole from its binding pocket in BSA.

5. Conclusion

This study focuses on the protein binding kinetics of nicotine and pantoprazole with bovine serum albumin to predict their interaction *in vivo* with human serum albumin. To determine the number of binding sites, association constants associated with each site, predominant binding site, displacement of one drug bound to bovine serum albumin by the other, *in vitro* equilibrium dialysis and UV spectroscopic techniques are employed. Computational tools further verify the results. Both of the test drugs are found to bind with either of the sites of the bovine serum albumin and predominantly bind to site-I and site-II. Moreover, the affinity of pantoprazole for site-I is proved to be higher than that of nicotine for the site-II. On the other hand, pantoprazole displaces nicotine from its binding pocket in bovine serum albumin at a higher extent than nicotine displacing pantoprazole from the two pockets. So, nicotine and pantoprazole compete for the different binding sites on bovine serum albumin and one increases the % free fraction of the other. As a result, concurrent administration of pantoprazole, while smoking might be beneficial since pantoprazole will help reduce gastric acidosis and GERD more effectively, is often caused by smoking.

Authors' Note

Kanij Nahar Deepa is the principle investigator of the manuscript, carries out

primary literature review, and conceives the idea. Md. Abu Sufian did the computational analysis. Sabia Nawsheen carried out experiments, wrote the first draft of this manuscript. This experiments received no grant, or form of payment to conduct any analysis. All authors read and approved the manuscript. The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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