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Effect of Glycyrrhizic Acid on Protein Binding of Diltiazem, Verapamil, and Nifedipine

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The effects of glycyrrhizic acid (GLZ) on protein binding of diltiazem, verapamil, and nifedipine were investigated. Protein binding studies (human serum, human serum albumin (HSA) and α_1-acid glycoprotein (AAG)) were conducted using the equilibrium dialysis method with and without addition of GLZ. The binding parameters, such as the number of moles of bound drug per mole of protein, the number of binding sites per protein molecule, and the association constant, were estimated using the Scatchard plot. The serum binding of nifedipine, verapamil, and diltiazem was displaced with addition of GLZ, and the decreases of Ks for serum were observed. GLZ decreased the association constants of three drugs for HSA and AAG, while the binding capacity remained similar with addition of GLZ. Although the characteristics of interaction were not clear, GLZ seemed to mainly affect HSA binding of nifedipine rather than AAG binding, while GLZ seemed to affect both AAG- and HSA-bindings of verapamil and diltiazem resulting in a serum binding displacement.

Key words: Protein binding, Displacement, Glycyrrhizic acid, Diltiazem, Verapamil, Nifedipine

INTRODUCTION

Protein binding plays an important role in the pharmacokinetics and pharmacodynamics of a drug. The extent of protein binding in the plasma or tissue controls the volume of distribution and affects both hepatic and renal clearance (Shargel and Yu, 1999; Pacifici and Viani, 1992). In many cases, the free drug concentration, rather than the total concentration in plasma, is correlated with the pharmacological effect of the specific drug (Schuhmacher *et al.*, 2000). Drug displacement from the drug-protein complex can occur through direct competition of two drugs for the same binding site or through the modulation of protein structure conformation by a second drug. Recently, Benet and Hoener showed that changes in plasma protein binding by drug-drug interactions or disease-drug interactions usually does not influence clinical exposure, such as AUC of a patient to a drug (Benet and Hoener, 2002). However, the drug displacement can lead to an unexpected toxicity for drugs that are highly bound (>95%) and have a narrow therapeutic index. Therefore, both drug protein binding and drug-drug interaction studies are still required to discover, develop, and assess the safety of drugs.

Calcium channel blockers have been used to treat various cardiovascular diseases including hypotension, arterial arrhythmia, and angina pectoris (van Breemen *et al.*, 1981; Hess *et al.*, 1984; Bean, 1984). Because cardiovascular diseases usually require life-long treatment to minimize the risk of stroke and heart attack, calcium channel blockers are often co-administered with other medications. In Asian countries like China, Korea, and Japan, oriental herbal medicines have been used for years to treat illnesses. However, in terms of metabolism and protein binding, their interactions with western medications have been overlooked.

Generally oriental herbal medicines are comprised of several kinds of herbs, and licorice root (*Glycyrrhiza glabra, G. inflata, or G. uralensis*) is one of the oldest and most frequently used herbs. Licorice root is believed to be beneficial for spleen, stomach, liver, and kidney (Kiso *et al.*, 1984; Wang and Nixon, 2001; Kumada, 2002), and its constituents are triterpenoids, such as glycyrrhizin and its aglycone glycyrrhizic acid, various polyphenols, and polysaccharides (Wang and Nixon, 2001).

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The current study investigated the drug-protein binding interaction between calcium channel blockers (diltiazem, verapamil and nifedipine) and GLZ, the main active ingredient of licorice root. The drugs that bind to human serum, and its two major proteins, serum albumin and á$_1$-acid glycoprotein, were investigated with and without GLZ.

MATERIALS AND METHODS

Materials

A shaking incubator (EYELA, LTI 600-SD) and a refrigerated centrifuge (Hanil, Korea) were used for the protein binding study. Equilibrium dialysis cells were obtained from Sankyo Plastic (Fukuoka, Japan), and cellulose membranes (MWCO 10K) were obtained from Viskase Sales Corp. (Willowbrook, IL, USA). Diltiazem, verapamil and nifedipine, GLZ, human serum albumin (HSA, MW 66,500), and α_1-acid glycoprotein (AAG, MW 44,000) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Human serum was prepared from healthy male volunteers and was kept at 20°C until use. All other reagents were of analytical grade.

Protein bindings

The protein binding study was carried out by using two chamber equilibrium dialysis cells. Prior to use, the cellulose membrane was boiled in distilled water for 2 h to remove preservatives. Then, it was kept in isotonic phosphate buffer (PBS, pH 7.4) and was stored at 4-8°C. Diltiazem, verapamil, and nifedipine were added to human serum, HSA (40 μM) or AAG (10 μM) solutions and drug concentrations were ranged from 1 to 200 μM. Drug-protein solutions were added to one side chamber (donor) of the dialysis cell (1 mL), and the other side (receiver) was filled with 1mL of PBS. In order to study the drug-GLZ protein binding interaction, GLZ (10 μM) was added to the drug-protein solutions as a competitor. The mutual HSA-binding interaction of verapamil and nifedipine with GLZ was studied with one ligand having the concentration range of 5 to 30 μM and the other ligand having a fixed concentration (GLZ, 10 μM, and verapamil and nifedipine, 5 μM), and vice versa.

A preliminary study showed that the equilibrium of the test drugs and GLZ between two chambers was achieved at 10 h in a shaking incubator (37°C), and no significant non-specific drug binding to the dialysis membrane was observed. Thus, the dialysis experiment was carried out for 12 to 15 h. Samples were taken from both donor and receiver cells, and the equivalent volume of human serum, HSA, AAG, or PBS was combined with the receiver or donor samples to match the sample matrix. Drugs were extracted with two volumes of acetonitrile. The drug concentrations were determined by HPLC.

HPLC analysis

Aliquots of samples were analyzed using high-performance liquid chromatography techniques (HPLC) to assess the concentration of the samples. The HPLC unit consisted of a 600E pump controller, a 600 pump, and a 486 UV/VIS spectrophotometer (Waters, Millford, MA, USA). The mobile phases for diltiazem and verapamil consisted of acetonitrile and phosphate buffer (pH 2 and pH 3, respectively) (60:40). The mobile phase for nifedipine consisted of methanol, water, and acetonitrile (36:55:9), and the mobile phase for GLZ, 2% acetic acid water and acetonitrile (60:40). Reverse phase columns (μ-Bondapak C_{18}, 100 mm×3.9 mm i.d. or 300 mm×3.9 mm i.d., Waters, MA) were used.

Data analysis

The binding parameters were estimated by using the following equations (Scatchard, 1949):

$$r = \sum_{i=1}^{n} \frac{n_i K_i D_F}{1 + K_i D_F} \quad \text{Eq. 1}$$

$$\frac{D_B}{D_F} = \sum_{i=1}^{n} (n_i K_i P_T - K_i D_B) \quad \text{Eq. 2}$$

where r is the number of moles of bound drug per mole of protein, and n_i and K_i are the number of binding sites per protein molecule and the association constant of the ith class of binding sites, respectively. P_T is the total protein concentration, and D_F and D_B are the free and protein bound drug concentrations, respectively.

The following equation was used to explain the drug-GLZ interaction for the primary binding site (Yamasaki et al., 1999):

$$r_{AD} = \frac{K_A D_{FA}(1 + \chi K_D D_{FD})}{K_A D_{FA}(1 + \chi K_D D_{FD}) + (1 + K_D D_{FD})} \quad \text{Eq. 3}$$

where K_A and D_{FA}, respectively, are the association constant and free drug concentration of drug A, and K_D and D_{FD}, respectively, stands for the association constant and free drug concentration of the displacer D. r_{AD} represents the number of moles of bound drug per mole of protein, and χ represents a coupling constant of drug A in the presence of displacer D. The coupling constant is equal to zero for the competitive interaction, while it is equal to 1 for independent binding.

RESULTS AND DISCUSSION

A specific ligand-protein binding is characterized by a high affinity and a small binding capacity, whereas a nonspecific ligand binding is characterized by a low

affinity and an unlimited binding capacity (Meyer and Guttman, 1970; Terasaki *et al*, 1992). Competition for the same binding site or alteration of the protein structure by a second drug can cause drug-protein binding displacement, and this, in turn, causes the decrease in the binding affinity for proteins. However, no changes in the number of binding sites, and thus, decreases in K values and a lack of change in n values, relate mostly to a competitive binding (Bowmer *et al.*, 1985). In contrast, the alteration of the protein conformation by a second drug causes significant changes in both K and n values (Bertucci *et al.*, 2001).

Serum protein binding experiments were conducted with and without addition of GLZ (10 μM) in order to investigate the effect of GLZ on serum protein binding of diltiazem, verapamil, and nifedipine. The ratios of bound drug concentration to free drug concentration (D_B/D_F) were plotted against the bound drug concentration (D_B) according to Eq. 2 with and without addition of GLZ (Fig. 1). The plots were depicted as curvilinear linear lines, which indicate the existence of two class binding. The association constants (Ks) were calculated from the slopes. The Ks for serum were in the order of nifedipine > verapamil > diltiazem, and the Ks of verapamil and nifedipine decreased significantly with addition of GLZ (Table I). The exact concentrations of each protein in human serum were not determined, so the number of binding sites per protein molecule (n) was not calculated. As an alternative, nP_T was calculated from the intercepts (products of the number of binding sites, protein concentration, and association constant) and slopes (association constants), and according to these calculations. It was observed that the addition of GLZ did not significantly change the nP_T values (Table I).

It was reported that the binding sites of GLZ on HSA were located mainly in ibuprofen binding sites (Site II) and partially in warfarin binding sites (Site I) (Ishida *et al.*, 1992). The measured K and n values of GLZ for HSA were 4.27×10^5 M^{-1} and 1.84, respectively. Although they do not bind exclusively, weak acidic drugs bind mainly to HSA, and basic drugs bind to AAG (Alfonso R Gennaro *et al.*, 2000). Since diltiazem, verapamil, and nifedipine are basic drugs, the effects of GLZ on both HSA- and AAG-bindings were examined. The Scatchard plots of diltiazem-, verapamil-, and nifedipine-HSA binding with and without addition of 10 μM GLZ were drawn according to Eq. 1 (Fig. 2). The Scatchard plots were depicted as curvilinear lines, which indicates the presence of two class binding, high (class I, K_1) and low (class II, K_2) affinity bindings. The K_1s for HSA were in the order of nifedipine > verapamil ≈ diltiazem without addition of GLZ. The changes in both K_1 and K_2 for HSA were observed with addition of GLZ (Table II). GLZ caused a significant decrease in the K values of nifedipine and verapamil, and only a minimal

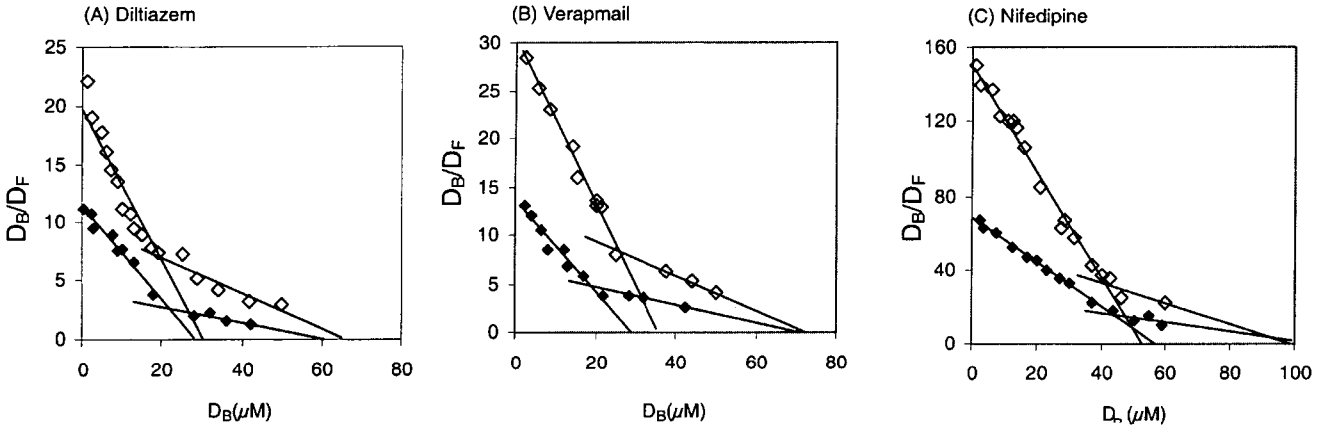

Fig. 1. The Rosenthal plots of serum protein binding. (A) Diltiazem, (B) verapamil, and (C) nifedipine with (■) and without (□) addition of GLZ. D_F and D_B respectively are the free and protein bound drug concentrations.

Table I. The association constants and nP_T values of diltiazem, verapamil, and nifedipine for human serum with and without addition of GLZ

		Diltiazem		Verapamil		Nifedipine	
		K ($\times10^5$ M^{-1})	nP_T ($\times10^{-6}$ M)	K ($\times10^5$ M^{-1})	nP_T ($\times s10^{-6}$ M)	K ($\times10^5$ M^{-1})	nP_T ($\times10^{-6}$ M)
Class I	Drug alone	5.8	33.4	8.6	35.3	28.5	52.9
	Drug + GLZ	4	28	4.7	29	12	56.7
Class II	Drug alone	1.5	65.8	1.8	72.6	5.6	98.5
	Drug + GLZ	1	77.6	0.9	69.8	2.5	105.5

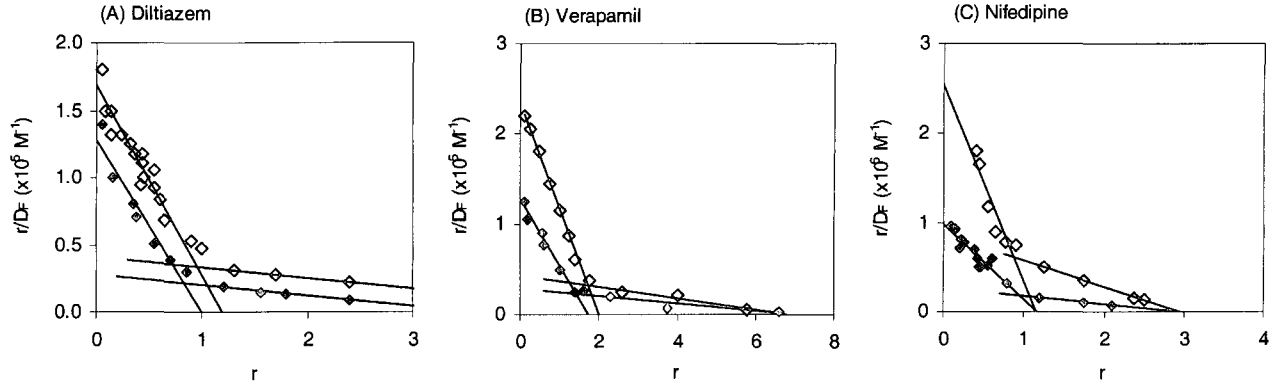

Fig. 2. The Scatchard plots of human serum albumin (HSA) binding. (A) Diltiazem, (B) verapamil, and (C) nifedipine with (■) and without (□) addition of GLZ. r represents the number of moles of bound drug per mole of protein.

Table II. The association constants and n values of diltiazem, verapamil, and nifedipine for HSA and AAG with and without addition of GLZ.

			Diltiazem		Verapamil		Nifedipine	
			K ($\times 10^5$ M^{-1})	n	K ($\times 10^5$ M^{-1})	n	K ($\times 10^5$ M^{-1})	n
HSA	Class I	Drug alone	1.42	1.19	1.16	2	22.0	1.15
		Drug + GLZ	1.16	1.15	0.74	1.71	8.8	1.13
	Class II	Drug alone	0.08	5.11	0.06	6.84	2.93	2.93
		Drug + GLZ	0.08	3.48	0.04	6.83	0.89	2.88
AAG		Drug alone	5.07	1.28	3.5	0.65	1.1	1.2
		Drug + GLZ	3.91	0.91	1.47	0.9	0.8	0.85

change in the K values of diltiazem. There was little variation in the n values for HSA with and without addition of GLZ.

The Scatchard plots of drug-AAG binding were shown as straight lines (graphs not shown), indicating one class binding. Table II listed the K values of diltiazem, verapamil, and nifedipine with and without addition of GLZ. GLZ decreased the K values of these drugs for AAG in the order of verapamil > nifedipine ≈ diltiazem. It has been known that GLZ binds to HSA predominantly (Ishida *et al.*, 1989), and thus, the decrease in the K values for AAG suggested that GLZ can possibly affect AAG-binding as well as HSA-binding.

Drug-drug binding interaction with GLZ was examined with a fixed concentration of one ligand while varying the concentrations of the other ligand in order to test for competitive displacement. Fig. 3 presents verapamil-HSA binding (A) with and without the addition of GLZ (10 μM), and also illustrates GLZ-HSA binding (B) with and without addition of verapamil (5 μM). The theoretical binding curve for verapamil and GLZ was obtained by using Eq. 3, assuming competition of the ligands for the high-affinity binding site. As shown, the experimental binding curve did not follow either the theoretical (competitive) binding curve or the independent binding curve. This suggested that verapamil and GLZ do not compete for the same high affinity binding site on HAS. However, both verapamil-

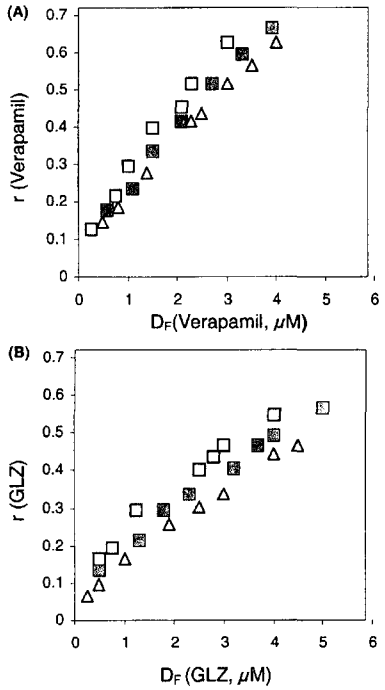

Fig. 3. (A)Verapamil-HSA binding with (■) and without (□) addition of GLZ (10 μM). The open triangles (△) represent the theoretical competitive binding curve of verapamil and GLZ. (B) GLZ-HSA binding with (■) and without (□) addition of verapamil (5 μM). The open triangles (△) represent the theoretical competitive binding curve of verapamil and GLZ.

and GLZ-HSA bindings were affected by the presence of the other ligand. In the same way, Fig. 4 presents nifedipine-HSA binding (A) with and without addition of GLZ (10 μM), and presents GLZ-HSA binding (B) with and without addition of nifedipine (5 μM). Both experimental binding results were in step with the competitive binding curve, which indicates that nifedipine and GLZ compete for the same high affinity binding site on HSA.

The serum binding of nifedipine, verapamil, and diltiazem was displaced with addition of GLZ, and the decreases in the K values for the serum were observed. Although GLZ predominantly binds to HSA, GLZ decreased the association constants of the three drugs for HSA and AAG, while the binding capacities remained similar with addition of GLZ (Table II). GLZ seems to mainly affect HSA binding of nifedipine rather than AAG binding, leading to the serum binding displacement (Table II and Fig. 4). On the other hand, GLZ seemed to affect both AAG- and HSA-bindings of verapamil and diltiazem resulting in the serum binding displacement, even though the characteristics of interaction were not revealed clearly.

Protein binding changes are clinically relevant for drugs with a high extraction ratio after intravenous administration, and are unlikely for drugs with low extraction ratio after intravenous and oral administration. Nifedipine is a low to intermediate extraction ratio drug and is given orally, so it would not see a meaningful change in pharmacokinetics in the presence of GLZ. Verapamil and diltiazem are high extraction ratio drugs, and thus, protein binding changes of these would be clinically relevant after non-oral administration. However, the adverse effects of diltiazem and verapamil due to the protein binding displacement in the presence of GLZ are expected to be marginal.

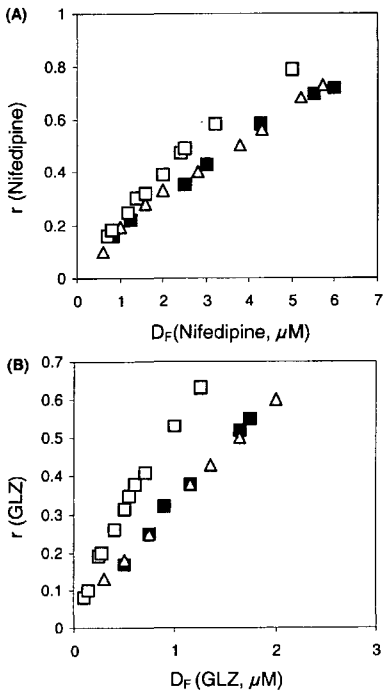

Fig. 4. (A) Nifedipine-HSA binding with (■) and without (□) addition of GLZ (10 μM). The open triangles (△) represent the theoretical competitive binding curve of nifedipine and GLZ. (B) GLZ-HSA binding with (■) and without (□) addition of nifedipine (5 μM). The open triangles (△) represent the theoretical competitive binding curve of nifedipine and GLZ.

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