Binding of Glycyrrhizin to Human Serum and Human Serum Albumin

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The binding of glycyrrhizin (GLZ) to human serum and human serum albumin (HSA) was examined by an ultrafiltration technique. Specific and nonspecific bindings were observed in both human serum and HSA. The association constants (K) for the specific bindings were very similar: 1.31×10^5 M^{-1} in human serum and 3.87×10^5 M^{-1} in HSA. The number of binding sites (n) and the linear binding coefficient (ϕ) in HSA were 1.95 and 3.09×10^3 M^{-1}, respectively.

When the human serum protein concentration was assumed to be 4.2% (equal to the measured serum albumin concentration), n in human serum was 3.09, which is similar to the n value in HSA, and ϕ in human serum was 0.71×10^3 M^{-1}, which is reasonably close to that for HSA. The binding pattern of GLZ with human serum protein on Sephadex G-200 column chromatography showed that GLZ binds to only the albumin fraction.

It was concluded that the GLZ-binding sites in human serum exist mainly on albumin and GLZ binds to specific and nonspecific binding sites at lower and higher concentrations than approximately 2 mM, respectively.

Keywords glycyrrhizin; protein binding; human serum; human serum albumin; specific binding; nonspecific binding

Introduction

We previously reported that more than 80% of glycyrrhizin (GLZ) binds to rat plasma over the plasma concentration range of 0.07—1.6 μg/ml following i.v. dosing (100 mg/kg) and we suggested that the extensive GLZ plasma binding is an important cause of the small distribution volume of the drug in rat (approximately 1.5 times the whole blood volume).[1] The steady-state distribution volume of GLZ in humans is also comparatively small (approximately 2.2 times the whole blood volume in a 70 kg man[2]).[3] Such a small steady-state distribution volume may be attributable to the drug serum protein binding, because drug serum protein binding has an important role in drug distribution to organs and tissues. Further, as drug serum protein binding affects pharmacological activity and general pharmacokinetic behavior, a binding study is important. However, there is no report on the binding of GLZ to human serum protein.

The purpose of this study was, therefore, to investigate the binding of GLZ to human serum and human serum albumin (HSA).

Experimental

Materials GLZ was kindly supplied by Minophagen Pharmaceutical Co. (Tokyo, Japan). Human serum, HSA, and human globulin (Cohn fraction IV-4) were purchased from Sigma Chemical Co. (St. Louis, MO). Albumin B-test kit was purchased from Wako Pure Chemical Ind., Ltd. (Osaka, Japan). All other reagents were commercial products of analytical grade.

Binding of GLZ to Human Serum and HSA Human serum and HSA bindings of GLZ were determined by an ultrafiltration technique using an ultrafiltration membrane (Amicon Micropartition system, MPS-1, Danvers, MA). HSA was dissolved in isotonic phosphate buffer solution (pH 7.4). One milliliter of human serum containing 0.8 to 10 mg of GLZ or 4.2% HSA solution containing 0.5 to 5 mg of GLZ was applied to the filtration membrane after incubation at 37 °C for 5 min. Ultrafiltration of samples was accomplished by centrifugation (1000 g) at 37 °C for a period (approximately 10 min) sufficient to produce an ultrafiltrate volume of approximately 20% of the initial sample. The applied human serum and HSA solution (50 μl each), and their filtrates (200 μl each) were used for GLZ determination. The adsorption of the drug on the membrane and the leakage of macromolecular components of HSA and human serum into the filtrate were negligible.

Column Chromatography of GLZ with Human Serum GLZ (0.4 mg) was added to an aliquot of 0.5 ml human serum. After 30 min at room temperature, the mixture was applied to a descending column system (1.6 × 70 cm) packed with Sephadex G-200 (Pharmacia Fine Chemical Co., Ltd., Sweden).[4] Elution was performed with 0.01 M phosphate buffer solution, pH 7.4, at the flow rate of 6.0 ml/h at 4 °C, and fractions (3 ml) were collected. Further, 0.5 ml of the buffer solution containing HSA (21.0 mg) and human globulin (5.0 mg) was eluted by the same method. One hundred and 200 μl aliquots of the effluent were used for the determination of protein and GLZ, respectively.

Analytical Method The extraction procedure and high-performance liquid chromatographic (HPLC) method for GLZ in each sample were the same as those described in the previous paper.[1] The protein concentration was determined by the method of Lowry *et al.*[5] using HSA as a standard.

Determination of Albumin in Human Serum Albumin in human serum was determined by using a commercial kit. Albumin content in human serum was $4.2 \pm 0.1\%$ ($n = 5$).

Data Analysis The human serum binding data were subjected to curve fitting based on Eq. 1 by using a digital computer.[6] Data were weighted with the reciprocals of the binding concentrations:

$$C_b = \frac{n(p)KC_f}{1+KC_f} + \phi(p)C_f \quad (1)$$

where K is the association constant corresponding to n, the specific binding sites; C_f and C_b are the free and bound drug concentrations in human serum, respectively; ϕ is the linear binding coefficient; (p) is the protein concentration; $n(p)$ and $\phi(p)$ are presumed to be composite parameters. The data of binding experiments with HSA were subjected to curve fitting based on Eq. 2 by using a digital computer.[6] Data were weighted by the same method as the case of human serum.

$$r = \frac{nKC_f}{1+KC_f} + \phi C_f \quad (2)$$

where r is the molar ratio of the bound drug to the binding protein, HSA, assuming a molecular weight of 69000. The binding parameters were calculated by using a nonlinear iterative least-squares method without parameter constraints.[6] The Rosenthal plots from the binding data were resolved into two linear segments to obtain initial estimates of the binding parameters.[7]

Results and Discussion

The binding of GLZ to human serum and HSA was examined by an ultrafiltration technique at higher GLZ concentrations than 0.5 mg/ml, as C_f was under the detection limit (2 μg/ml) at less than 0.5 mg/ml. Figure 1 shows Rosenthal plots of the human serum and HSA

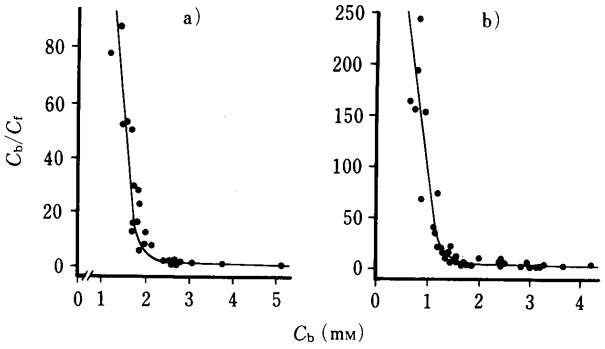

Fig. 1. Rosenthal Plots for Human Serum and HSA Bindings of GLZ

a) GLZ binding to human serum. b) GLZ binding to HSA (4.2%) in isotonic phosphate buffer (pH 7.4). C_f, free concentration; C_b, bound concentration. The points represent the experimental values determined by an ultrafiltration technique. The solid lines are the curves simulated by the least-squares method by using a digital computer.[6]

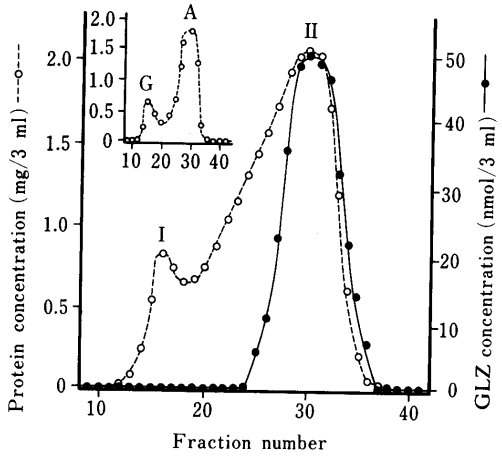

Fig. 2. Binding Pattern of GLZ with Human Serum Protein on Sephadex G-200 Column Chromatography

The inset shows the elution pattern of human serum globulin (G) and albumin (A). For details, see the text.

TABLE I. Binding Parameters of GLZ to Human Serum and HSA Determined by an Ultrafiltration Technique

	$n(p)^{a)}$ (mM)	$n^{b)}$	$K\times10^{5\,c)}$ (M^{-1})	$\phi(p)^{a)}$	$\phi\times10^{3\,d)}$ (M^{-1})
Human serum	1.88±0.06	3.09[e)]	1.31±0.26	0.43±0.03	0.71[f)]
HSA	—	1.95±0.11	3.87±0.94	—	3.09±0.24

The values (±S.D.) were calculated according to Eq. 1 for human serum and Eq. 2 for HSA. For details, see the text. *a*) Composite parameter. *b*) The number of binding sites. *c*) The association constant. *d*) The linear binding coefficient. *e*) and *f*) were calculated from the $n(p)$ and $\phi(p)$ values by assuming that (p) is equal to the albumin concentration in human serum (4.2%) measured in this study, respectively.

binding data of GLZ. Fitting the binding data with a general Langmuir-type equation failed to give converged parameters in both human serum and HSA. Therefore, Eq. 1 for human serum data and Eq. 2 for HSA data were used for curve fitting, considering that the binding curve at higher C_b than approximately 2 mM runs almost parallel to the $x(C_b)$-axis in both cases. In each case, converged parameters were obtained. The calculated association constants (K) in human serum and HSA, the number of binding sites (n) and the linear binding coefficient (ϕ) in HSA, and the composite parameters ($n(p)$ and $\phi(p)$) in human serum are listed in Table I. Similar K values were observed with human serum and HSA.

As regards $\phi(p)$ for human serum protein binding, when (p) is assumed to be 4.2% (equal to the measured albumin concentration in human serum), ϕ is $0.71\times10^3\,M^{-1}$, which is somewhat smaller than the value of ϕ, $3.09\times10^3\,M^{-1}$, for HSA (Table I). In the case of $n(p)$, a similar calculation gave n of 3.09, which close to the value of n, 1.95, obtained from the HSA binding experiment (Table I).

As shown in Fig. 2, the chromatographic pattern of human serum protein on Sephadex G-200 showed two peaks, I and II. From the elution pattern of human globulin and HSA (the inset in Fig. 2), it was clear that peaks I and II contain mainly globulin and albumin, respectively, though the two peaks did not show a clear-cut separation. GLZ bound only to peak II, namely the albumin fraction. Thus, it was found that the binding sites of GLZ in human serum exist mainly on albumin and GLZ binds to specific and nonspecific binding sites at lower and higher concentrations than approximately 2 mM, respectively.

It has been reported that GLZ human serum levels are in the ranges of 2—16 and 2—60 μg/ml following i.v. administration of 80 mg/man to normal subjects ($n=3$)[3] and 200 mg/man to patients with chronic hepatitis ($n=5$),[8] respectively. We attempted to calculate C_f at the drug human serum levels described above. C_f was calculated by using Eq. 3, derived from Eq. 1.

$$C_f=\frac{-X+\sqrt{X^2+4YC_{tot}}}{2Y} \tag{3}$$

where:

$$X=\phi(p)+1+(n(p)-C_{tot})K$$

$$Y=(\phi(p)+1)K$$

C_{tot} = total human serum concentration of GLZ

C_f was in the ranges of 0.008—0.064 and 0.008—0.24 μg/ml in the normal subjects and patients, respectively. Thus, GLZ showed a uniformly high binding of 99.6% in both groups. When the binding parameters for HSA are used and (p) is assumed to be 4.2% (the measured albumin concentration), a similar binding ratio, 99.8%, was calculated.

Previously we reported that GLZ binds to the primary and secondary binding sites in rat plasma and rat serum albumin.[1] This shows that the binding profile of GLZ is different between human serum and rat plasma, indicating that there is a species difference. However, GLZ-binding sites existed mainly on albumin in both human and rat.

In conclusion, the binding of GLZ to human serum protein is mainly determined by the binding to albumin. The extensive binding of GLZ to human serum protein is probably an important cause of the small distribution volume of GLZ in humans. Further, it is considered that such extensive binding can affect the pharmacological activity and pharmacokinetic behavior of the drug in humans.

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