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Binding of Glycyrrhizin to Rat Plasma and Rat Serum Albumin

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The binding of glycyrrhizin to rat plasma and rat serum albumin (RSA) was examined by using an ultrafiltration technique. Two classes of binding sites, primary and secondary, were observed in both plasma and RSA. It was concluded that the binding of glycyrrhizin to the primary binding sites mainly determines the plasma protein binding of the drug in rats.

Keywords——glycyrrhizin; protein binding; rat plasma; rat serum albumin

In the previous paper,[1] we reported the small distribution volume of glycyrrhizin in rats (only approximately 1.5 times the whole blood volume[2]). Since drug plasma protein binding has an important role in drug distribution to organs and tissues, the small distribution volume may be attributable to plasma protein binding of glycyrrhizin. However, there is no report on glycyrrhizin binding to protein. The purpose of this study was therefore to investigate glycyrrhizin binding to rat plasma protein and rat serum albumin (RSA).

Experimental

Materials——Glycyrrhizin (Minophagen Pharmaceutical Co., Ltd., Tokyo, Japan) was used as supplied. RSA and albumin B-test kit were purchased from Sigma Chemical Co. and Wako Pure Chemical Ind. Ltd., respectively. MeOH was of liquid chromatographic reagent grade (Wako Pure Chemical Ind. Ltd.). All other reagents were commercial products of special grade.

Animal——Male Wistar rats weighing 260—280 g were used.

Unbound Fraction of Glycyrrhizin in Rat Plasma and RSA Solution——Plasma protein and RSA bindings of glycyrrhizin were determined by an ultrafiltration technique using a membrane cone (Amicon Centriflo ultrafiltration membrane filter cone, type CF-25). (1) Plasma was obtained by centrifuging, 30 min after *i.v.* injection of heparin, the fresh blood of a rat fed a normal laboratory diet. (2) RSA was dissolved in isotonic phosphate buffer (pH 7.4). (3) Two ml of plasma or 4% (w/v) RSA solution containing 4.0 to 24.0 mg of glycyrrhizin was applied to the membrane cone after incubation at 37 °C for 10 min. The applied plasma and RSA solution, and their filtrates (100 μl each), were analyzed for glycyrrhizin. The absorption of glycyrrhizin on the membrane and the leakage of macromolecular components of plasma and RSA into the filtrate were negligible.

High Performance Liquid Chromatography (HPLC) Conditions——The apparatus used was a Hitachi liquid chromatograph, model 655, with Zorbax ODS column (250 × 4 mm i.d.) and an ultraviolet (UV) detector operating at 252 nm. The mobile phase consisted of MeOH–0.05 M phosphate buffer (pH 2.1) solution (2 : 1, v/v). The column was maintained at room temperature and the mobile phase flow rate was 1.0 ml/min. For quantitative calculation, a reporting integrator (model 3390 A, Yokogawa-Hewlett Packard Co.) was employed.

Analytical Method——The extraction procedure and determination method for glycyrrhizin in each sample were the same as those described in a previous paper.[1]

Determination of Albumin in Rat Plasma——RSA was determined by using a commercial kit. Albumin content in plasma was $3.5 \pm 0.1\%$ ($n = 5$).

Data Analysis——The results of binding experiments to RSA (presented in Fig. 1) were subjected to curve fitting based on Eq. 1 by using a digital computer[3]:

$$r=\frac{n_1K_1C_f}{1+K_1C_f}+\frac{n_2K_2C_f}{1+K_2C_f} \quad (1)$$

where K_1 and K_2 are the association constants corresponding to n_1 and n_2, the numbers of primary and secondary binding sites; C_f is the free drug concentration, and r is the molar ratio of the bound drug to the binding protein assuming a molecular weight of 69000. The plasma binding data presented in Fig. 1 were subjected to curve fitting based on Eq. 2 by using a digital computer.[3] Data were weighted with the reciprocals of the binding concentrations:

$$C_b=\frac{n_1(p)K_1C_f}{1+K_1C_f}+\frac{n_2(p)K_2C_f}{1+K_2C_f} \quad (2)$$

where (p) is the protein concentration and C_b is the bound drug concentration in plasma. The value of $n_1(p)$ or $n_2(p)$ is presumed to be the binding capacity.

Results and Discussion

Figure 1 shows glycyrrhizin binding to RSA and rat plasma protein determined by an ultrafiltration method. As shown in Fig. 1, the patterns of the plots for 4% RSA solution and

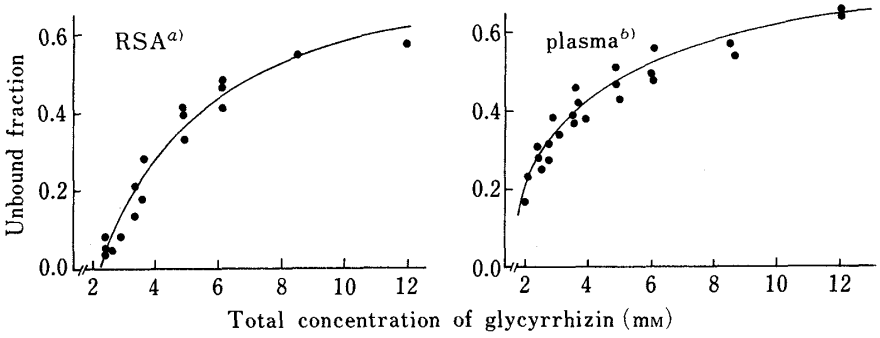

Fig. 1. Binding of Glycyrrhizin to RSA and Rat Plasma

a) Glycyrrhizin binding to RSA (4%) in an isotonic phosphate buffer (pH 7.4).
b) Glycyrrhizin binding to rat plasma.
The points represent the experimental values determined by an ultrafiltration method.
The smooth lines are the simulated curves calculated from the data by using a digital computer[3] (see the text).

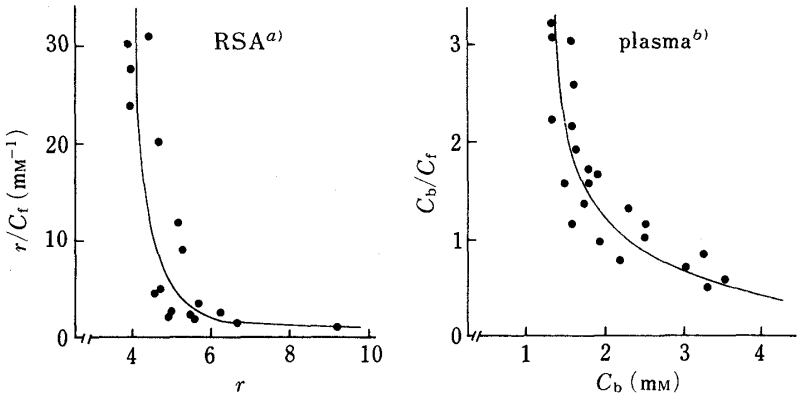

Fig. 2. Scatchard Plot for RSA Binding and Rosenthal Plot for Rat Plasma Binding of Glycyrrhizin

For *a*) and *b*), see Fig. 1.
r; the molar ratio of the bound drug to the binding albumin.
C_f; free concentration.
C_b; binding concentration.
The points represent the experimental values.
The smooth curves are the best fit to the data.

TABLE I. Binding Parameters of Glycyrrhizin to RSA and Rat Plasma Determined by an Ultrafiltration Technique

	n_1[a]	$n_1(p)$[b]	K_1[c]	n_2[a]	$n_2(p)$[b]	K_2[c]
RSA	4.32	—	9.31×10^4	48.24	—	11.57
Plasma	—	1.35	12.42×10^4	—	6.83	94.77

a) The number of binding sites. *b*) The binding capacity (mM). *c*) The association constant (M^{-1}). For other details, see Fig. 1.

plasma were similar. The plasma curve suggests that more than 80% of glycyrrhizin binds to plasma protein at low glycyrrhizin concentrations, *e.g.*, below 2 mM. A Scatchard plot for the RSA data and a Rosenthal plot for the plasma data are shown in Fig. 2. In both cases, there was evidence for the existence of more than one class of binding site. The binding parameters were calculated by a nonlinear iterative least-squares method without parameter constraints. The data fitted the case of two binding sites reasonably well. The calculated association constants (K) in RSA and plasma, the number of binding sites (n) of RSA, and the binding capacity ($n(p)$) of plasma are listed in Table I. Similar K_1 values were observed for RSA and plasma. Furthermore, as regards $n_1(p)$ for plasma protein binding, when (p) is taken as the albumin concentration (3.5%) in plasma, n_1 is 2.66, which corresponds fairly well to n_1, 4.32, obtained from the RSA binding experiments. In the case of $n_2(p)$, a similar calculation gave n_2 of 13.47, which is smaller than the n_2 value for RSA, although K_2 for plasma was larger than that for RSA, as shown in Table I. These results suggest that two classes of binding sites for glycyrrhizin exist in albumin, and that the plasma protein binding of the drug is mainly determined by the binding to the primary binding sites. The binding of glycyrrhizin to albumin is probably an important cause of the small distribution volume of the drug, since the rat plasma glycyrrhizin levels following *i.v.* administration (100 mg/kg) were below 2 mM, as reported already.[1]

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