[Chem. Pharm. Bull. 36(1) 440—443 (1988)]

Binding of Glycyrrhetinic Acid to Rat Plasma, Rat Serum Albumin, Human Serum, and Human Serum Albumin

SHIRO ISHIDA,* TSUTOMU ICHIKAWA, and YOKO SAKIYA

Faculty of Pharmaceutical Sciences, Tokushima Bunri University, Yamashiro-cho, Tokushima-shi 770, Japan

(Received June 1, 1987)

The binding of glycyrrhetinic acid (GLA) to rat plasma, rat serum albumin (RSA), human serum, and human serum albumin (HSA) was examined by an ultrafiltration technique. Specific and nonspecific bindings were observed in all cases. The association constant (K) for the specific binding was $1.34 \times 10^5\ \text{M}^{-1}$ in rat plasma and $1.56 \times 10^5\ \text{M}^{-1}$ in RSA. The K value for human serum ($14.91 \times 10^5\ \text{M}^{-1}$) and that for HSA ($15.09 \times 10^5\ \text{M}^{-1}$) were approximately 10-fold larger than those for rat plasma and RSA, suggesting a species difference in the specific binding affinity for GLA. The number of specific binding sites (n) was 2.49 and 1.18 for RSA and HSA, respectively. The linear binding coefficient (ψ) for the nonspecific binding was $7.11 \times 10^3\ \text{M}^{-1}$ in RSA and $6.57 \times 10^3\ \text{M}^{-1}$ in HSA.

When the rat plasma and human serum protein concentrations are respectively assumed to be 3.5% and 4.2% (equal to the measured plasma and serum albumin concentrations), n was 1.83 in rat plasma and 1.31 in human serum, which corresponded fairly well to those for RSA and HSA. Further, for ψ in rat plasma and human serum, when rat plasma and human serum protein are the measured albumin concentrations, respectively, ψ was $10.97 \times 10^3\ \text{M}^{-1}$ for rat plasma and $14.83 \times 10^3\ \text{M}^{-1}$ for human serum, which corresponded well to those for RSA and HSA.

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

Keywords——glycyrrhetinic acid; protein binding; rat plasma; rat serum albumin; human serum; human serum albumin

It has been reported that glycyrrhetinic acid (GLA) has many therapeutic effects, *e.g.*, anti-ulcer,[1] anti-inflammatory,[2-4] anti-hepatotoxic,[5-7] and interferon-inducing[8,9] actions. However, there is no report on the binding of GLA to protein. Plasma protein binding of a drug affects its pharmacokinetic behavior and can provide considerable insight into the drug dynamics.

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

Experimental

Materials——Sodium glycyrrhetinate (GLA-Na) was kindly supplied by Minophagen Pharmaceutical Co., Ltd. (Tokyo, Japan). MeOH was of liquid chromatographic reagent grade (Wako Pure Chemical Ind., Tokyo, Japan). RSA, human serum, and HSA were purchased from Sigma Chemical Co. (St. Louis, MO). Albumin B-test kit was purchased from Wako Pure Chemical Ind. Ltd. All other reagents were commercial products of analytical grade.

Animals——Male Wistar rats (Shizuoka Laboratory Animal Center, Shizuoka, Japan), weighing 240—260 g, were used.

Binding of GLA to Rat Plasma, RSA, Human Serum, and HSA——Rat plasma, RSA, human serum, and HSA bindings of GLA were determined by an ultrafiltration technique by using dialysis tubing (Spectrapor 2, Spectrum Medical Ind. Inc., Los Angeles). The dialysis tubing (15-cm lengths) was prewashed with distilled water and dried before use. Plasma was obtained by centrifugation, 30 min after i.v. injection of heparin, of fresh blood from a rat fed

NII-Electronic Library Service

a normal laboratory diet. RSA and HSA were dissolved in 0.25 M sucrose–0.05 M Tris–HCl buffer solution (pH 7.4). One milliliter of plasma, 3.5% (w/v) RSA solution, human serum, or 4.0% (w/v) HSA solution containing 0.31 to 2.10 mg of GLA-Na was placed in the dialysis tubing sealed at one end after incubation at 37 °C for 5 min. The other end of the tube was sealed and the tube was bent into a U-shape and hung in a 50-ml centrifuge tube by using a conical support (Amicon Co., Mass.).[10] Ultrafiltration of samples was accomplished by centrifugation ($1000 \times g$) at 37 °C for a period (approximately 60 min) sufficient to produce an ultrafiltrate volume of approximately 10% of the initial sample. The applied plasma, RSA, human serum, and HSA solutions, and their filtrates (50 μl each) were used for GLA determination. The adsorption of the drug on the membrane and the leakage of macromolecular components of plasma, RSA, human serum, and HSA into the filtrate were negligible.

High-Performance Liquid Chromatography (HPLC) Conditions——The apparatus used was a Hitachi liquid chromatograph, model 655, with a Zorbax ODS column (250 × 4 mm i.d.) and an ultraviolet (UV) detector operating at 254 nm. The mobile phase consisted of MeOH–0.05 M phosphate buffer solution, pH 2.1, (5 : 1, v/v). The column was maintained at room temperature and the mobile phase flow rate was 1.0 ml/min.

Analytical Method——GLA in each sample was extracted by the same method as reported previously.[11] The extracts containing GLA were dried under a nitrogen stream. The residue was dissolved in 200 μl of MeOH, and 5—10 μl of this solution was injected into the liquid chromatograph.

Determination of Albumin in Rat Plasma and Human Serum——Albumin in plasma and human serum was determined by using a commercial kit. Albumin contents in plasma and human serum were $3.5 \pm 0.1\%$ and $4.2 \pm 0.1\%$ ($n=5$, each), respectively.

Data Analysis——The rat plasma and human serum binding data were subjected to curve fitting based on Eq. 1 by using a digital computer.[12] Data were weighted with the reciprocals of the binding concentrations.

$$C_b = \frac{n(p)KC_f}{1+KC_f} + \psi(p)C_f \tag{1}$$

Here, 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 plasma and human serum, respectively; (p) is the protein concentration. The value of $n(p)$ is presumed to be the binding capacity and the value of $\psi(p)$ is the linear binding coefficient. The data of binding experiments to RSA and HSA were subjected to curve fitting based on Eq. 2 by using a digital computer.[12] Data were weighted by the same method as in the case of rat plasma and human serum.

$$r = \frac{nKC_f}{1+KC_f} + \psi C_f \tag{2}$$

Here, r is the molar ratio of the bound drug to the binding protein for RSA or HSA, assuming a molecular weight of 69000. The value of ψ is the linear binding coefficient.

Results and Discussion

Figure 1 shows Rosenthal plots of the rat plasma, RSA, human serum, and HSA binding data of GLA obtained by an ultrafiltration technique. The binding parameters were calculated by using a nonlinear iterative least-squares method without parameter constraints.[12] Fitting the binding data with a general Langmuir-type equation (Eq. 3, $m=2$ or 3) failed to give converged parameters in all cases.

$$r = \sum_{i=1}^{m} \frac{n_i K_i C_f}{1+K_i C_f} \tag{3}$$

Therefore, Eq. 1 for rat plasma and human serum data and Eq. 2 for RSA and HSA data were used for curve fitting, considering that the binding curve at higher C_b than approximately 2 mM runs almost parallel to x (C_b)-axis in all cases. In each case, converged parameters were obtained. The calculated association constants (K) in rat plasma, RSA, human serum, and HSA, the number of binding sites (n) in RSA and HSA, the binding capacity ($n(p)$) in rat plasma and human serum, and the linear binding coefficients ($\psi(p)$ in rat plasma and human serum, and ψ in RSA and HSA) are listed in Table I. Similar K values were observed between rat plasma and RSA, and between human serum and HSA. However, the K values for human serum and HSA were approximately 10-fold larger than those for rat plasma and RSA. The

NII-Electronic Library Service

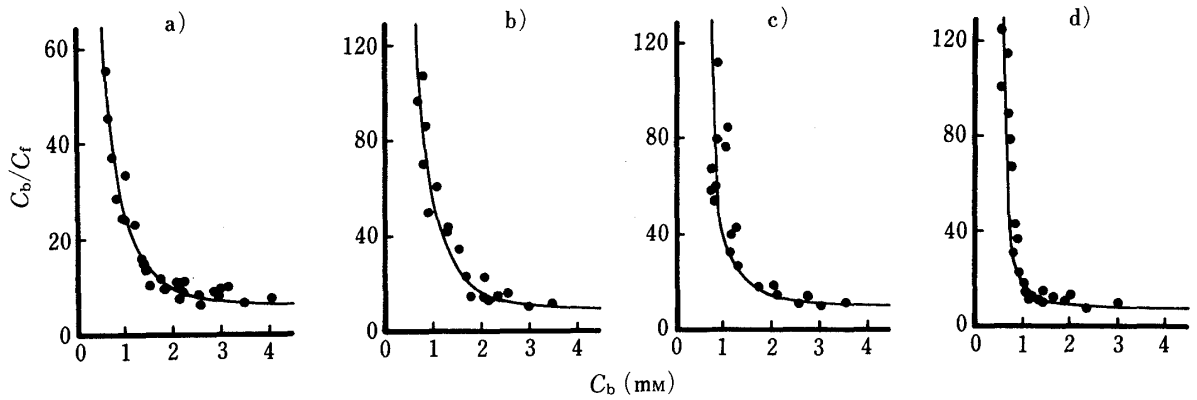

Fig. 1. Rosenthal Plots for Rat Plasma, RSA, and Human Serum, and HSA Bindings of GLA

a) GLA binding to rat plasma. b) GLA binding to RSA (3.5%) in 0.25 M sucrose–0.05 M Tris–HCl buffer (pH 7.4). c) GLA binding to human serum. d) GLA binding to HSA (4.0%) in 0.25 M sucrose–0.05 M Tris–HCl buffer (pH 7.4).
C_f, free concentration; C_b, bound concentration.
The points represent the experimental values determined by an ultrafiltration method.
The solid lines are the curves simulated by the least-squares method by using a digital computer.[11]

TABLE I. Binding Parameters of GLA to Rat Plasma, RSA, Human Serum, and HSA Determined by an Ultrafiltration Technique

	$n(p)^{a)}$ (mM)	$n^{b)}$	$K\times10^{5\,c)}$ (M^{-1})	$\psi(p)^{d)}$	$\psi\times10^{3\,d)}$ (M^{-1})
Rat plasma	0.93±0.13	—	1.34±0.71	5.56±0.45	—
RSA	—	2.49±0.24	1.56±0.37	—	7.11±0.80
Human serum	0.82±0.05	—	14.91±1.03	9.24±0.58	—
HSA	—	1.18±0.08	15.09±0.84	—	6.57±0.41

The values (±S.D.) were calculated according to Eq. 1 for rat plasma and human serum, and Eq. 2 for RSA and HSA. For details, see the text. *a*) The binding capacity. *b*) The number of binding sites. *c*) The association constant. *d*) The linear binding coefficient. For other details, see in Fig. 1.

results indicate that there is a species difference in affinity for the specific binding of GLA.

As regards $n(p)$ for rat plasma protein binding, when (p) is assumed to be 3.5% (equal to the measured albumin concentration in plasma), n is 1.83, which corresponds fairly well to the value of n, 2.49, obtained from the RSA binding experiment (Table I). In the case of $\psi(p)$, a similar calculation gave ψ of 10.97, which corresponds well to ψ of 7.11 for RSA. As regards $n(p)$ and $\psi(p)$ for human serum protein binding, when (p) is assumed to be 4.2% (equal to the measured albumin concentration in human serum), n and ψ are 1.31 and 14.83, respectively. These n and ψ values correspond well to n and ψ in HSA and RSA, respectively. Specific and nonspecific bindings have often been seen in other binding studies, *e.g.*, the binding of quinidine to rat serum protein[13] and the binding of prednisolone to rat, dog, rabbit, and human serum protein.[14]

It was concluded that the binding sites of GLA in rat plasma and human serum exist in albumin and GLA binds to specific and nonspecific binding sites at lower and higher concentrations than approximately 2 mM, respectively.

Previously, we reported the plasma disposition of GLA after i.v. dosing (60 mg/kg) in the rats with biliary fistulization, in which the biliary excretion of the drug was negligible.[11] By using the rat plasma binding parameters, we attempted to calculate the unbound concentration of GLA at the rat plasma levels (<0.5 mg/ml) obtained from that study. The unbound concentration (C_f) of GLA in rat plasma was calculated from Eq. 4:

NII-Electronic Library Service

$$C_{tot}=C_f+\frac{n(p)KC_f}{1+KC_f}+\psi(p)C_f \quad (4)$$

where C_{tot} is total plasma concentration of GLA. From the calculation, it was found that more than 96.1% of GLA binds to rat plasma protein at lower GLA concentrations than 0.5 mg/ml. When the binding parameters for RSA are used and (p) is 3.5% (equal to the measured rat plasma albumin concentration), a similar binding ratio of 98.0% was calculated.

Further, C_f for human serum concentrations of GLA, which is produced from glycyrrhizin by hydrolysis after oral administration of the drug (100 mg) to normal subjects,[15] was calculated from Eq. 4, using the binding parameters for human serum. From the calculation, it was found that more than 99.9% of GLA binds to albumin at low GLA concentrations, *e.g.*, below 5 μg/ml.

Such results are important, because it is considered that the extensive binding of GLA to RSA and HSA can affect the pharmacological activity and general pharmacokinetic behavior of the drug.

References

1) K. Takagi, S. Okabe, and R. Saziki, *Jpn. J. Pharmacol.*, **19**, 418 (1969).
2) R. S. H. Finney and G. F. Somers, *J. Pharm. Pharmacol.*, **10**, 613 (1958).
3) E. Colin-Jones, *Postgrad. Med. J.*, **36**, 678 (1960).
4) K. K. Tangri, P. K. Seth, S. S. Parmar, and K. P. Bhargava, *Biochem. Pharmacol.*, **14**, 1277 (1965).
5) Y. Kiso, M. Tohkin, and H. Hikino, *Planta Medica*, **49**, 222 (1983).
6) Y. Kiso, M. Tohkin, H. Hikino, M. Hattori, T. Sakamoto, and T. Namba, *Planta Medica*, **50**, 298 (1984).
7) H. Hikino, *Yakugaku Zasshi*, **105**, 109 (1985).
8) N. Abe, T. Ebina, and N. Ishida, *Microbiol. Immunol.*, **26**, 535 (1982).
9) N. Ishida, "Symposium on Liver and Glycyrrhizin," Minophagen Pharmaceutical Co., Ltd., Tokyo, 1983, pp. 120—124.
10) M. Hanano, K. Umemura, and T. Iga (ed.), "Applied Pharmacokinetics: Theory and Experiments," Soft Science, Inc., Tokyo, 1985, pp. 354—355.
11) T. Ichikawa, S. Ishida, Y. Sakiya, and Y. Akada, *Chem. Pharm. Bull.*, **32**, 3734 (1984).
12) T. Nakagawa, Y. Koyanagi, and H. Togawa, "SALS, a computer program for statistical analysis with least-squares fitting," Library Program of University of Tokyo Computer Center, 1978.
13) H. Harashima, Y. Sawada, Y. Sugiyama, T. Iga, and M. Hanano, *J. Pharmacokinet. Biopharm.*, **13**, 425 (1985).
14) M. L. Rocci, Jr., N. F. Johnson, and W. J. Jusko, *J. Pharm. Sci.*, **69**, 977 (1980).
15) N. Nakano, H. Kato, H. Suzuki, K. Nakao, S. Yano, and M. Kanaoka, *Proc. Symp. WAKAN-YAKU*, **14**, 97 (1981).

NII-Electronic Library Service