Disposition of Glycyrrhizin in the Perfused Liver of Rats

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Received November 5, 1993; accepted April 6, 1994

The disposition of glycyrrhizin (GLZ) in the perfused liver of rats after dosing in the range of 0.5–30.0 mg was investigated and a pharmacokinetic model was devised to interpret the results. The uptake rate of GLZ into the liver with respect to the unbound GLZ concentration (C_f) in the perfusate followed a Michaelis–Menten type equation with a $K_{m,up}$ of 1.17 $\mu\text{g/ml}$ and $V_{max,up}$ of 13.9 $\mu\text{g/min/g}$ of liver. The efflux clearance (0.044 ml/min/g of liver) from the liver was independent of the C_f in the liver. The biliary excretion rate at a steady-state C_f level in the liver followed a Michaelis–Menten type equation with a substrate inhibition constant ($K_{i,B}$) of 42.3 $\mu\text{g/ml}$, $K_{m,B}$ of 1.68 $\mu\text{g/ml}$, and $V_{max,B}$ of 3.11 $\mu\text{g/min/g}$ of liver. The proposed model, with the holding time fitted to biliary excretion at each dose, accurately described both the perfusate concentration–time profile and the cumulative biliary excretion profile.

Keywords glycyrrhizin; perfused liver; rat; pharmacokinetic model

Glycyrrhizin (GLZ), which is used to treat chronic hepatitis,^{1–4)} is predominantly excreted in the bile (ca. 80%) after i.v. administration of 80 mg/kg to patients with liver failure.⁵⁾ Previously we showed that the biliary excretion of GLZ is also the major route in rats (ca. 86–98%) after i.v. administration in the dose range of 5–50 mg/kg, and the dose-dependency in the total body clearance is attributed to that in the biliary clearance.⁶⁾ We also showed that the uptake of GLZ into isolated rat hepatocytes is a carrier-mediated process.⁷⁾

The hepatic transport of xenobiotics has been studied by using several experimental systems.^{8–12)} However, the use of perfused rat liver is attractive for hepatic uptake studies of drugs because of the intact architecture of the organ, the possibility of controlling blood flow, and the smaller number of interactions with endogenous compounds. It is also possible to use various concentrations of drug, to vary the composition of the perfusion medium, and to collect a large number of perfusate samples within a short time in comparison with the use of intact animals.

In the present study, the disposition of GLZ in the perfused liver of rats after dosing in the range of 0.5–30.0 mg was elucidated and a pharmacokinetic model was developed to describe the results.

MATERIALS AND METHODS

Chemicals Monoammonium glycyrrhizinate (GLZ-NH₄) was kindly supplied by Minophagen Pharmaceutical Co. (Tokyo, Japan). Bovine serum albumin (Fraction V) (BSA) was purchased from Sigma Chemical Co. (St. Louis, MO). All other reagents were commercial products of analytical grade.

Animals Male Wistar rats weighing 240–260 g, fed on standard chow, were used throughout. Rats were lightly anesthetized with ether for all surgical procedures.

Binding Study (1) Binding of GLZ to BSA was determined by an ultrafiltration technique. One milliliter of perfusate containing 10 mg of BSA and GLZ-NH₄ (5–300 μg as GLZ) was applied to a filtration membrane (Amicon Micropartition System, MPS-1, Danvers, MA) after incubation at 37 °C for 5 min. Ultrafiltration was

accomplished by centrifugation at 37 °C in a centrifugal separator (KB/200B, Kubota Co., Tokyo, Japan) for a period (about 10 min) sufficient to afford an ultrafiltrate volume of approximately 20% of the initial sample. (2) Binding of GLZ to 16.7 and 33.3% liver homogenates was determined by the above method. Blood-free liver was obtained by the same method as described previously.¹³⁾ The liver was homogenized with two or five volumes of 0.05 M Tris-HCl buffer solution (pH 7.4) containing 0.25 M sucrose in an ice-bath using a Teflon glass homogenizer. One milliliter of 16.7 or 33.3% homogenate containing 2–160 or 4–340 μg (as GLZ) of GLZ-NH₄, respectively, was applied to the filtration membrane after incubation at 4 °C for 10 min. The filtrate was obtained by centrifugation at 4 °C for 40 min. (3) Binding of GLZ to biliary micelles was also determined by this method. Bile samples were obtained from ten rats by the same method as in the perfused liver study without drug administration. The bile (0.5 ml) containing GLZ-NH₄ (0.05–2.5 mg as GLZ) was applied to a filtration membrane after incubation at 37 °C for 5 min. The filtrate was harvested immediately at 37 °C. The adsorption of the drug on the membrane and the leakage of macromolecular components (BSA and liver protein) into the filtrate were negligible.

Perfused Liver Study The bile duct was cannulated with PE-10 polyethylene tubing and the portal vein was rapidly catheterized with PE-120 polyethylene tubing, which was attached to the perfusion system. The portal circulation was interrupted within 10 s. Then, PE-120 polyethylene tubing was inserted through the right atrium into the upper portion of the inferior vena cava and tied above the renal vein. The abdominal cavity of the animal was covered by warm saline-wetted cotton gauze. The liver was perfused in a temperature-controlled cabinet (Natsume Seisakusho Co., Tokyo, Japan) at 37 °C. The perfusate (100 ml) in a 120-ml glass beaker was recirculated at the rate of 40 ml/min using a peristaltic roller pump (model 1500N, Taitec Co., Kosigaya, Japan), stirred and passed over a multibulb oxygenator with a countercurrent exchange of O₂:CO₂ (95:5) before delivery to the liver. The perfusate (100 ml) consisted of Krebs–Ringer bi-

carbonatebuffer (pH 7.4) containing 0.1 g of glucose and 1.0 g of BSA. The solution was equilibrated with O₂:CO₂ (95:5) for 20 min. After a stabilization period of 20 min, GLZ-NH₄ (0.5, 2.5, 5.0, 10.0, 20.0, or 30.0 mg as GLZ) was added to the perfusate. Effluent perfusate samples (200 µl each) were withdrawn from the reservoir at 5, 10, 15, 20, 30, 40, 60, 80, 100, and 120 min and immediately replaced with the same volume of fresh perfusate. Bile was simultaneously collected over 20 min intervals for 120 min in a tared polyethylene tube. At the end of the experiment, the liver was excised, blotted dry, and weighed. The liver and bile volumes were estimated gravimetrically, assuming a density of 1.0. All the samples were stored at -20 °C until assay.

Biliary Excretion Study The biliary excretion of GLZ at a steady-state hepatic concentration (C_H^{ss}) was studied. The perfusion method was the same as that described above. After a 20 min initial perfusion, GLZ-NH₄ (0.2, 0.5, 1.5, 4.0, 7.0, or 15.0 mg as GLZ) was added to the perfusate and immediately the drug was infused into the reservoir at the rate of 2, 10, 20, 40, 60, or 100 µg/min, respectively, using a constant-rate infusion pump (model KN-201, Natsume Seisakusho Co., Tokyo, Japan). As the steady-state level of GLZ (*ca.* 1, 5, 15, 50, 70, or 150 µg/ml) in the effluent perfusate was obtained within 60 min after the initiation of perfusion at each dose, the bile samples were collected from 70 to 90 min and the perfusate sample was taken at 80 min. Then, the liver was excised, rinsed with cold saline, blotted dry, and weighed. The liver was homogenized with two volumes of physiological saline in an ice-bath using a Teflon glass homogenizer before analysis. All the samples were stored at -20 °C until assay.

Wash-Out Study The efflux of GLZ from the liver into the perfusate was studied by the wash-out method.¹⁴⁾ The liver was perfused for 60 min with a perfusate containing GLZ-NH₄ (5, 10, 20, or 30 mg as GLZ). Bile was collected during the 60-min perfusion and the effluent perfusate (200 µl) was sampled at the end of the perfusion. Immediately, the efflux study was initiated by switching to a single-pass perfusion with freshly oxygenated perfusate without GLZ. The outflowing perfusate was collected over 0.5 min intervals for 10 min. At the end of the experiment, the liver was treated by the same method as in the perfused liver study. All the samples were stored at -20 °C until assay. The viability of the liver was evaluated in terms of the level of glutamic pyruvic transaminase (GPT) in the perfusate at the end of the experiment in all cases. GPT was determined by using a GPT assay kit (Transaminase CII-test Wako, Wako Chemical Co., Tokyo, Japan). The GPT level was less than 7 Karmen units in all cases.

Analytical Method Samples of 100 µl of the perfusate, liver homogenate, and filtrate, and 20 µl of the bile were used for GLZ determination. The extraction procedure and high-performance liquid chromatographic (HPLC) method for GLZ in each sample were the same as those described previously.¹⁵⁾ The total bile acids in a bile sample were determined by using a total bile acids assay kit (Total bile acids test Wako, Wako Chemical Co., Tokyo, Japan).

Data Analysis The binding data of GLZ to 1% BSA in the perfusate were subjected to curve fitting based on

Eq. 1 by using a nonlinear least-squares regression analysis program, MULTI,¹⁶⁾ on a digital computer (NEC PC-9801 RA).

$$C_{b,p} = \frac{N_1 \cdot C_{f,p}}{K_{d1} + C_{f,p}} + \frac{N_2 \cdot C_{f,p}}{K_{d2} + C_{f,p}} \quad (1)$$

Here, $C_{b,p}$ and $C_{f,p}$ are the bound and unbound concentrations of GLZ in the perfusate, respectively; K_{d1} and K_{d2} are the dissociation constants corresponding to N_1 and N_2 , which are presumed to be the primary and secondary binding capacities, respectively. $C_{f,p}$ was calculated from a cubic equation (Eq. 2) by using the Hitchcock-Bairstow program¹⁷⁾ on a digital computer.

$$C_p = C_{f,p} + \frac{N_1 \cdot C_{f,p}}{K_{d1} + C_{f,p}} + \frac{N_2 \cdot C_{f,p}}{K_{d2} + C_{f,p}} \quad (2)$$

Here, C_p is the total GLZ concentration in the perfusate. The binding data of GLZ to liver homogenates (16.7 and 33.3%) were subjected to curve fitting based on Eq. 3 by using a nonlinear least-squares regression analysis program¹⁶⁾ on a digital computer.

$$C_{b,LH} = \frac{N_s \cdot C_{f,LH}}{K_{ds} + C_{f,LH}} + K_n \cdot C_{f,LH} \quad (3)$$

Here, $C_{b,LH}$ and $C_{f,LH}$ are the bound and unbound GLZ concentrations in the homogenate; K_{ds} is the dissociation constant corresponding to N_s , which is presumed to be the binding capacity of the specific binding sites; K_n is the linear binding coefficient of nonspecific binding sites. The values of N_s and K_n for whole liver were estimated by multiplying the observed N_s or K_n by the dilution factor (3 or 6). By using the mean values of N_s , K_n , and K_{ds} , the unbound GLZ concentration ($C_{f,H}$) for whole liver was estimated according to Eq. 4 (derived from Eq. 3).

$$C_{f,H} = \frac{a + \sqrt{a^2 + 4b}}{2(1 + K_n)} \quad (4)$$

$$a = C_H - N_s - K_{ds}(1 + K_n)$$

$$b = (1 + K_n)K_{ds} \cdot C_H$$

Here, C_H is the total concentration of GLZ in liver. Unbound fractions in the perfusate (f_p) and liver (f_H) were estimated from $C_{f,p}/C_p$ and $C_{f,H}/C_H$, respectively. Since a plot of the unbound ($C_{f,B}$) versus bound ($C_{b,B}$) concentrations of GLZ in bile showed a straight line, the linear binding coefficient ($K_{n,B}$) was calculated from Eq. 5 using linear regression analysis.

$$C_{b,B} = K_{n,B} \cdot C_{f,B} \quad (5)$$

The steady-state bound ($C_{b,B}^{ss}$) and unbound ($C_{f,B}^{ss}$) concentrations of GLZ in bile were calculated from $C_B^{ss} \cdot K_{n,B}/(K_{n,B} + 1)$ and $C_B^{ss}/(K_{n,B} + 1)$ derived from Eq. 5, respectively. Here, C_B^{ss} is the steady-state total concentration of GLZ in bile.

The hepatic uptake rate (R_{up}) was calculated as follows. Since the GLZ amount in the perfusates after dosing declined linearly from 5 until 20 min at each dose, the R_{up} at each dose was taken to be the slope of the linear regression line. The initial concentration of GLZ in the perfusate ($C_{0,p}$) at each dose was inferred from the

y -intercept at zero time, obtained by extrapolating the linear regression line of C_p at the time of 5–20 min. The R_{up} data were fitted to a Michaelis–Menten type equation (Eq. 6) by using a nonlinear least-squares regression analysis program¹⁶⁾ on a digital computer.

$$R_{up} = \frac{V_{max,up} \cdot C_{f,p}}{K_{m,up} + C_{f,p}} \quad (6)$$

Here, $V_{max,up}$ and $K_{m,up}$ are the maximum uptake rate and Michaelis constant, respectively, and $C_{f,p}$ is the unbound GLZ concentration at $C_{0,p}$.

The hepatic clearance (CL_H) of GLZ was calculated from dose/ AUC . Here, AUC is the area under the perfusate concentration *versus* time curve from time zero until the last sampling time, which was estimated using the trapezoidal method. The hepatic extraction ratio (E) of GLZ is given by CL_H /hepatic perfusate flow (Q_H).

The biliary excretion rate (R_B) data at C_H^{ss} were fitted to Eq. 7 or 8 by using a nonlinear least-squares analysis program¹⁶⁾ on a digital computer.

$$R_B = \frac{V_{max,B} \cdot C_{f,H}^{ss}}{K_{m,B} + C_{f,H}^{ss}} \quad (7)$$

$$R_B = \frac{V_{max,B} \cdot C_{f,H}^{ss}}{K_{m,B} + C_{f,H}^{ss}(1 + C_{f,H}^{ss}/K_{i,B})} \quad (8)$$

Here, $V_{max,B}$, $K_{m,B}$, and $K_{i,B}$ are the maximum excretion rate, Michaelis constant, and the substrate inhibition constant for the biliary excretion, respectively, and $C_{f,H}^{ss}$ is the unbound GLZ concentration in the liver at C_H^{ss} .

The efflux clearance (CL_{eff}) was estimated as follows. By using the data of perfusate GLZ concentration ($C_{p,out}$) and perfusate volume ($V_{p,out}$) effused from the liver for 0.5 min (sampling time interval), $\ln(C_{p,out} \times V_{p,out}/0.5)$ was plotted at the midpoint of each time interval. As the plot declined linearly with time at each dose, the efflux rate (R_{eff}) was inferred from the y -intercept at zero time, obtained by extrapolating the linear regression line. Because R_{eff} increased linearly with increasing $C_{f,H}$, passing through the origin, CL_{eff} was taken to be the slope of the linear regression line. The amount of GLZ in the liver at the end of preloading was estimated from the loading dose $-(Am_p + Am_B)$, where Am_p and Am_B are the amount of GLZ in the perfusate at the end of preloading and that in the bile excreted up to that point, respectively.

Physiological Constants The physiological constants were based on a 250 g rat. The liver volume was determined experimentally from the wet liver weight by assuming a density of 1.0. The liver was divided into three fluid compartments: capillary bed (CA), interstitial fluid (IS), and intracellular fluid (IC). The volumes of CA (V_{CA}) and IS (V_{IS}) used were taken from the literature,¹⁸⁾ and the volume of IC (V_{IC}) was estimated as the liver volume minus the sum of V_{CA} and V_{IS} .

Model Development The pharmacokinetic model for the GLZ disposition in perfused rat liver is shown in Fig. 1. The liver was assumed to consist of CA, IS, and IC fluid compartments. In formulating the kinetics of GLZ in the perfused liver, the following assumptions, based on the experimental evidence described in previous and present sections, were made: (a) a well-mixed state exists

Fig. 1. Pharmacokinetic Model for GLZ Disposition in Perfused Rat Liver

For abbreviations, see Glossary.

in the three fluid compartments; (b) GLZ entering with the perfusate mixes with GLZ in the capillary bed; (c) only a free drug in the capillary bed can diffuse rapidly in the interstitial fluid; (d) only a free drug in the interstitial fluid is transported into the intracellular space, at the rate given by the Michaelis–Menten equation (Eq. 6); (e) only a free drug in the intracellular space is effused into the interstitial fluid at a first-order rate; (f) only a free drug is metabolized in liver during the perfusion (the metabolic clearance (CL_M) value was taken from our previous report¹³⁾); (g) the drug in liver is excreted in the bile at the rate R'_B , with the holding time H , which was calculated by fitting the cumulative biliary excretion-time profile at each dose to certain equations (Eqs. 9–13), using a nonlinear least-squares regression program, MULTI-RUNGE,¹⁹⁾ with a digital computer. The principles of mass balance are expressed as follows:

Perfusate:

$$V_P \frac{dC_P}{dt} = Q_H(C_{Cl} - C_P) \quad (9)$$

where C_{Cl} is the GLZ concentration in CA or IS.

Capillary bed and interstitial fluid:

$$(V_{CA} + V_{IS}) \frac{dC_{Cl}}{dt} = Q_H(C_P - C_{Cl}) - \frac{V_{max,up} \cdot C_{f,Cl}}{K_{m,up} + C_{f,Cl}} + CL_{eff} C_{f,H} \quad (10)$$

Intracellular fluid:

$$V_{IC} \frac{dC_{IC}}{dt} = \frac{V_{max,up} \cdot C_{f,Cl}}{K_{m,up} + C_{f,Cl}} - (CL_{eff} + CL_M) C_{f,H} - R'_B \quad (11)$$

where C_{IC} is the GLZ concentration in IC.

Biliary excretion:

$$H \frac{dR'_B}{dt} = \frac{V_{\max,B} \cdot C_{f,H}}{K_{m,B} + C_{f,H}(1 + C_{f,H}/K_{i,B})} - R'_B \quad (12)$$

$$\frac{dAm_B}{dt} = R'_B \quad (13)$$

C_{Cl} and C_{IC} are expressed by

$$C_{Cl} = C_{f,Cl} + C_{b,Cl} = C_{f,Cl} + \frac{N_1 \cdot C_{f,Cl}}{K_{d1} + C_{f,Cl}} + \frac{N_2 \cdot C_{f,Cl}}{K_{d2} + C_{f,Cl}} \quad (14)$$

$$C_{IC} = C_{f,H} + C_{b,H} = C_{f,H} + \frac{N_s \cdot C_{f,H}}{K_{ds} + C_{f,H}} + K_n C_{f,H} \quad (15)$$

The following equations hold:

$$\frac{dC_{Cl}}{dt} = \frac{dC_{Cl}}{dC_{f,Cl}} \times \frac{dC_{f,Cl}}{dt} \quad (16)$$

$$\frac{dC_{IC}}{dt} = \frac{dC_{IC}}{dC_{f,H}} \times \frac{dC_{f,H}}{dt} \quad (17)$$

Substituting Eqs. 14 and 15 into Eqs. 16 and 17 gives Eqs. 18 and 19, respectively:

$$\frac{dC_{Cl}}{dt} = \left(1 + \frac{N_1 \cdot K_{d1}}{(K_{d1} + C_{f,Cl})^2} + \frac{N_2 \cdot K_{d2}}{(K_{d2} + C_{f,Cl})^2} \right) \frac{dC_{f,Cl}}{dt} \quad (18)$$

$$\frac{dC_{IC}}{dt} = \left(1 + \frac{N_s K_{ds}}{(K_{ds} + C_{f,H})^2} + K_n \right) \frac{dC_{f,H}}{dt} \quad (19)$$

Substituting Eqs. 18 and 19 into Eqs. 10 and 11 gives Eqs. 20 and 21, respectively:

$$(V_{CA} + V_{IS}) \frac{dC_{f,Cl}}{dt} = \frac{Q_H(C_P - C_{Cl}) - \frac{V_{\max,up} \cdot C_{f,Cl}}{K_{m,up} + C_{f,Cl}} + CL_{eff} \cdot C_{f,H}}{1 + \frac{N_1 \cdot K_{d1}}{(K_{d1} + C_{f,Cl})^2} + \frac{N_2 \cdot K_{d2}}{(K_{d2} + C_{f,Cl})^2}} \quad (20)$$

$$V_{IC} \frac{dC_{f,L}}{dt} = \frac{\frac{V_{\max,up} \cdot C_{f,Cl}}{K_{m,up} + C_{f,Cl}} - (CL_{eff} + CL_M)C_{f,H} - R'_B}{1 + \frac{N_s \cdot K_{ds}}{(K_{ds} + C_{f,H})^2} + K_n} \quad (21)$$

The complete set of differential equations was solved

numerically by the Runge-Kutta-Gill method using a digital computer, and physiological constants (Table I) and pharmacokinetic parameters (Tables II and III) were used for the simulation.

RESULTS

Binding Study The binding of GLZ to 1% BSA and to 16.7 and 33.3% liver homogenates was determined by means of an ultrafiltration technique. Rosenthal plots of the binding data to BSA and liver homogenates are shown in Figs. 2A and B, respectively. In both cases, the binding parameters were calculated using a nonlinear iterative least-squares method without parameter constraints.¹⁶⁾ Fitting of the BSA data with a general Langmuir-type equation (Eq. 22, $m=2$ or 3) gave converged parameters in the case of $m=2$. Therefore, Eq. 1 was used for curve fitting to the BSA data. The binding data to liver protein were

$$C_{b,P} = \sum_{i=1}^m \frac{N_i \cdot C_{f,P}}{K_{di} + C_{f,P}} \quad (22)$$

fitted with Eq. 3, considering that the curves at $C_{b,LH}$ higher than approximately 50 $\mu\text{g/ml}$ run almost parallel to the $x(C_{b,LH})$ -axis. Converged parameters were obtained for the data in the two diluted homogenates. From the parameters obtained, those for whole liver were estimated using the extrapolation method. All the obtained binding parameters are listed in Table II. As shown in Fig. 3, a plot

TABLE I. Physiological Parameters for Perfused Liver in a 250 g Rat

Volume (ml)	
Total liver (V_H)	10.0 $\pm$ 0.2 ^{a)}
Capillary bed (V_{CA})	1.0 ^{b)}
Interstitial fluid (V_{IS})	1.3 ^{b)}
Intracellular fluid (V_{IC})	7.7 ^{c)}
Perfusate (V_P)	100.0
Hepatic perfusate flow (Q_H) (ml/min)	40.0

a) Determined experimentally from the wet liver weight by assuming a density of 1.0. The value is presented as the mean $\pm$ S.E. of 24 rats. b) Obtained from ref. 18. c) $V_H - V_{CA} - V_{IS}$.

Fig. 2. Rosenthal Plots of GLZ Binding to BSA and Liver Homogenate

A: GLZ binding to 1% BSA in perfusate. $C_{b,P}$ and $C_{f,P}$, bound and unbound concentrations of drug in the perfusate, respectively. B: GLZ binding to 16.7 (Δ) and 33.3 ($\blacktriangle$) % liver homogenates in 0.25 M sucrose-0.05 M Tris-HCl buffer (pH 7.4). $C_{b,LH}$ and $C_{f,LH}$, bound and unbound concentrations of drug in the liver homogenates. The points represent the experimental values. The solid lines in A and B were generated from Eqs. 1 and 3, respectively, using the MULTI-fitted parameters listed in Table II.

of $C_{f,B}$ versus $C_{b,B}$ yielded a straight line. Therefore, $K_{n,B}$ was taken to be the slope of the line. The $K_{n,B}$ value estimated from Eq. 5 was 0.13 ± 0.001 ($n=3$). This suggests that about 90% of GLZ exists in an unbound form in the bile.

Perfused Liver Study The GLZ amount in the effluent perfusate after loading in the dose range of 0.5–30.0 mg declined linearly from 5 until 20 min at each dose (Fig. 4A). Therefore, R_{up} was taken to be the slope of the linear

Fig. 3. Relationship between Bound ($C_{b,B}$) and Unbound ($C_{f,B}$) Concentrations of GLZ in Bile

The points represent the experimental values. The solid line ($y=0.13x-0.004$, correlation coefficient $r=0.97$) was drawn by linear regression analysis.

regression line. As can be seen in Fig. 4B, the R_{up} analyzed as a function of $C_{f,P}$ seemed to be saturable by increasing $C_{f,P}$. Therefore, the R_{up} data were analyzed according to a Michaelis-Menten type equation (Eq. 6), and $V_{max,up}$ and $K_{m,up}$ were estimated by a nonlinear least-squares method,¹⁶⁾ since the analysis based on Eq. 6 with a linear R_{up} term failed to give converged parameters. The obtained parameters are listed in Table III.

As shown in Table IV, the CL_H and E values decreased with increasing dose. This indicates a dose-dependency in the CL_H and E . The E value at each dose was less than 5%, suggesting that CL_H is relatively insensitive to changes in hepatic blood flow but is sensitive to the degree of unbound drug in the perfusate.²⁰⁾

As can be seen in Fig. 5, GLZ caused an immediate dose-dependent inhibition of bile flow. The maximal inhibition occurred at high doses (2.5–30.0 mg) and at 40 min. Recovery of the bile flow was observed at the lower doses of 0.5–5.0 mg, but it did not recover at the higher doses of 10.0–30.0 mg.

Biliary Excretion Study As shown in Fig. 6, the R_B with respect to $C_{f,H}^{ss}$ exhibited a curve having a peak at approximately $10 \mu\text{g/ml}$, and it showed a gradual decrease with increasing $C_{f,H}^{ss}$. The profile is similar to that of a Michaelis-Menten plot of substrate inhibition in enzyme kinetics.^{21–23)} The R_B data were analyzed according to a Michaelis-Menten type equation with or without $K_{i,B}$ (Eq.

TABLE II. Binding Parameters of GLZ to BSA and Liver Homogenate

Binding site	1% BSA	Binding site	Liver homogenate		100% liver ^{d)}
			16.7%	33.3%	
Primary		Specific			
$N_1^a)$ ($\mu\text{g/ml}$)	26.9 ± 4.2	$N_s^a)$ ($\mu\text{g/ml}$)	13.3 ± 3.0	24.7 ± 3.9	76.3 ± 9.6
$K_{d1}^b)$ ($\mu\text{g/ml}$)	0.40 ± 0.06	$K_{ds}^b)$ ($\mu\text{g/ml}$)	6.70 ± 1.96	7.57 ± 1.33	7.14 ± 1.08
Secondary		Nonspecific			
$N_2^a)$ ($\mu\text{g/ml}$)	386 ± 11	$K_n^c)$	0.44 ± 0.05	1.07 ± 0.18	2.92 ± 0.31
$K_{d2}^b)$ ($\mu\text{g/ml}$)	8.67 ± 0.70				

The parameters for BSA and liver homogenates were calculated according to Eqs. 1 and 3, respectively, using the least-squares method.¹⁶⁾ Results are given as the mean $\pm$ S.E. of 3 experiments. a) The binding capacity. b) The dissociation constant. c) The linear binding coefficient. d) N_s or $K_n \times 3$ or 6 (dilution factor). K_{ds} is the mean value of K_{ds} in the homogenates.

Fig. 4. Perfusate Disappearance Curves of GLZ (A) and the Uptake Rate (R_{up}) of GLZ as a Function of the Initial Unbound GLZ Concentration ($C_{f,P}$) in the Perfusate (B) after Administration at Various Doses in Perfused Rat Liver

○, 0.5; ●, 2.5; △, 5.0; ▲, 10.0; □, 20.0; ■, 30.0 mg. For the calculation of R_{up} and $C_{f,P}$, see the text. Each point and vertical bar represents the mean and S.E. of 4 experiments. The data points without a vertical bar include the S.E. within the point. B: The solid line was generated from Eq. 6 using the MULTI-fitted parameters listed in Table III.

TABLE III. Pharmacokinetic Parameters of GLZ in Perfused Rat Liver

$V_{\max, \text{up}}^a$ ($\mu\text{g}/\text{min}/\text{g}$ of liver)	13.9 ± 0.8
$K_{\text{m, up}}^b$ ($\mu\text{g}/\text{ml}$)	1.17 ± 0.19
$V_{\max, \text{B}}^c$ ($\mu\text{g}/\text{min}/\text{g}$ of liver)	3.11 ± 0.78
$K_{\text{m, B}}^d$ ($\mu\text{g}/\text{ml}$)	1.68 ± 0.94
$K_{\text{i, B}}^e$ ($\mu\text{g}/\text{ml}$)	42.3 ± 22.3
CL_{eff}^f ($\text{ml}/\text{min}/\text{g}$ of liver)	0.044
CL_{M}^g ($\text{ml}/\text{min}/\text{g}$ of liver)	0.11
H^h (min/g of liver)	0.5 mg 1.95 ± 0.50
	2.5 mg 4.37 ± 1.04
	5.0 mg 5.10 ± 1.53
	10.0 mg 6.05 ± 1.45
	20.0 mg 7.75 ± 1.08
	30.0 mg 8.88 ± 0.88

The values ($\pm$ computer-calculated S.D.) for *a*) and *b*), and *c*), *d*), and *e*) were calculated according to Eqs. 6 and 8, respectively, using the least-squares method.¹⁶⁾ *a*, *b*) Maximum rate and Michaelis constant for the hepatic uptake, respectively. *c*—*e*) Maximum rate, Michaelis constant, and inhibition constant for the biliary excretion, respectively. *f*) Efflux clearance. *g*) Metabolic clearance cited from ref. 13. *h*) Holding time. Results are given as the mean $\pm$ S.E. of 4 experiments. For the calculation of *H*, see the text.

TABLE IV. Hepatic Clearance (CL_H) and Extraction Ratio (E) of GLZ after Administration at Various Doses in Perfused Rat Liver

Dose (mg)	CL_H ($\text{ml}/\text{min}/\text{g}$ of liver)	E
0.5	0.195 ± 0.018	0.049 ± 0.004
2.5	0.151 ± 0.074	0.038 ± 0.002
5.0	0.143 ± 0.060	0.035 ± 0.002
10.0	0.138 ± 0.007	0.034 ± 0.002
20.0	0.128 ± 0.007	0.032 ± 0.002
30.0	0.109 ± 0.001	0.027 ± 0.0002

Results are given as the mean $\pm$ S.E. of 4 experiments. For the calculation of CL_H and E , see the text.

Fig. 5. Time Course of Bile Flow after Administration of GLZ at Various Doses in Perfused Rat Liver

Each point and vertical bar represents the mean and S.E. of 4 experiments. The data points without a vertical bar include the S.E. within the point. $\blacklozenge$, control (without drug administration). For other symbols, see Fig. 4.

7 or 8), and the best-fit equation was evaluated from the Akaike's information criteria (AIC) value.²⁴⁾ The AIC value was smaller in the case with $K_{\text{i, B}}$ (53) than in that without $K_{\text{i, B}}$ (88). The parameters in the case with $K_{\text{i, B}}$ are listed in Table III. The simulated line coincided well with the experimental points, suggesting that GLZ itself inhibits its biliary excretion.

Figure 7 shows plots of $C_{\text{f, H}}^{\text{ss}}/C_{\text{f, P}}^{\text{ss}}$ (the steady-state unbound GLZ concentration in the perfusate) versus $C_{\text{f, P}}^{\text{ss}}$,

Fig. 6. Biliary Excretion Rate (R_B) at Steady-State Hepatic Unbound Concentrations ($C_{\text{f, H}}^{\text{ss}}$) of GLZ in Perfused Rat Liver

The points represent the experimental values. The solid line was generated from Eq. 8 using the MULTI-fitted parameters listed in Table III.

Fig. 7. $C_{\text{f, H}}^{\text{ss}}/C_{\text{f, P}}^{\text{ss}}$ versus $C_{\text{f, P}}^{\text{ss}}$ and $C_{\text{f, B}}^{\text{ss}}/C_{\text{f, H}}^{\text{ss}}$ versus $C_{\text{f, H}}^{\text{ss}}$

$C_{\text{f, B}}^{\text{ss}}$: The biliary unbound concentration of GLZ at steady-state unbound perfusate concentration ($C_{\text{f, P}}^{\text{ss}}$). For $C_{\text{f, H}}^{\text{ss}}$, see Fig. 6. For the calculation of $C_{\text{f, H}}^{\text{ss}}$, $C_{\text{f, P}}^{\text{ss}}$ and $C_{\text{f, B}}^{\text{ss}}$ see the text.

and of $C_{\text{f, B}}^{\text{ss}}/C_{\text{f, H}}^{\text{ss}}$ versus $C_{\text{f, H}}^{\text{ss}}$. The $C_{\text{f, H}}^{\text{ss}}$ was 15–40 times higher than $C_{\text{f, P}}^{\text{ss}}$, suggesting that the hepatic uptake of GLZ involves active transport. The $C_{\text{f, B}}^{\text{ss}}$ was 2–130 times higher than $C_{\text{f, H}}^{\text{ss}}$ and decreased with increasing $C_{\text{f, H}}^{\text{ss}}$, suggesting the active transport of GLZ via the bile canalicular membrane.

It has been reported that the bile flow in rats can be divided into a bile salt-dependent and a bile salt-independent fraction.^{25,26)} Since the dependent fraction is derived from the active transport of bile acids into the canalicular lumen,²⁷⁾ the influence of GLZ on the bile flow and biliary excretion of total bile acids was examined. As shown in Fig. 8, the decrease of bile flow increased saturably with increasing $C_{\text{f, H}}^{\text{ss}}$, while the biliary excretion rate of total bile acids was almost constant despite the increase in $C_{\text{f, H}}^{\text{ss}}$. The results suggest that total bile acids do not participate in the GLZ concentration-dependent decrease of bile flow in the liver.

Wash-Out Study As shown in Fig. 9, the R_{eff} increased linearly with increasing $C_{\text{f, H}}^{\text{ss}}$. As the line passed through the origin, CL_{eff} was taken to be the slope of the linear regression line. The obtained CL_{eff} value is included in

Fig. 8. The Decrease in Bile Flow (A) and the Biliary Excretion Rate of Total Bile Acids (B) as a Function of $C_{f,H}^{ss}$

The decrease in bile flow was calculated by subtracting the bile flow at each $C_{f,H}^{ss}$ from the mean bile flow ($0.93 \pm 0.06 \mu\text{l/min/g}$ of liver, $n=4$) of the control (bile was collected from 70 to 90 min after the initiation of perfusion without the infusion of drug). The points represent the experimental values. $\circ$, the control value.

Fig. 9. The Efflux Rate (R_{eff}) of GLZ as a Function of the Unbound GLZ Concentration ($C_{f,H}$) in Liver

For the calculation of R_{eff} and $C_{f,H}$, see the text. The solid line ($y=0.044x+0.034$, $r=0.78$) was drawn by linear regression analysis.

Table III.

Simulation with the Model The perfusate concentration-time profile and the cumulative biliary excretion profile of GLZ were simulated using the pharmacokinetic model illustrated in Fig. 1. As shown in Fig. 10, fairly good agreement was observed between the simulated and observed profiles in both cases. This indicates that GLZ disposition in the perfused liver is well described by the model. When the model without holding time H was used, the perfusate simulation curves at each dose also agreed with the observed values, but the simulated cumulative biliary excretion curves rose markedly above the observed values at all doses. So, the H value that fitted the biliary excretion curve at each dose (Table III) was used for simulation. The obtained H values tended to be saturable with increasing dose (Fig. 11A), and increased linearly with a decrease in bile flow, passing through the origin (Fig. 11B).

DISCUSSION

BSA has generally been used as the protein in the

perfusate for studies on the uptake of a drug by isolated perfused liver and *in situ* liver of rats.¹⁰⁻¹² In this study, GLZ bound to primary and secondary binding sites in BSA with a high binding ratio ($>94\%$ in the concentration range of 1–300 $\mu\text{g/ml}$). In our previous study, GLZ in rat plasma was also shown to bind mainly to primary and secondary binding sites on albumin with a high binding ratio ($>80\%$ at below 2 mM GLZ).¹⁵ Further, GLZ bound to specific and nonspecific binding sites of liver protein, and the binding ratio was high ($>75\%$ at below 1 mg/ml GLZ).

The uptake of GLZ into perfused liver may be an active, carrier-mediated process, because the uptake of GLZ by isolated rat hepatocytes was shown to be carrier-mediated in our previous study,⁷ in which the uptake rate below 0.2 mM (165 $\mu\text{g/ml}$) followed a Michaelis-Menten type equation, with a K_m of 9.29 $\mu\text{g/ml}$ and V_{max} of 92.2 ng/min/ 10^6 cells.⁷ Assuming 1.14×10^8 cells/g of liver,²⁸ the V_{max} gives a predicted value of 10.5 $\mu\text{g/min/g}$ of liver. In a comparison of these values with the $K_{m,\text{up}}$ and $V_{\text{max},\text{up}}$ values (Table III) obtained here at below 16 $\mu\text{g/ml}$ (Fig. 4B) respectively, the former shows an about an 8-fold difference and the latter is almost identical. The difference in K_m may result from the difference in the following condition. Although the quality of liver cell isolation as judged by dye exclusion was quite good according to generally accepted criteria for cell viability,⁷ changes in membrane conformation did not detect morphological and biochemical parameters of the hepatocytes. Groothuis *et al.* showed that the isolated rat hepatocytes rapidly lose their membrane specialization morphologically and functionally with respect to the localization of alkaline phosphatase, ATPase, and 5'nucleotidase.²⁸ Therefore, the isolation of hepatocytes might change the micro-environment of the carrier protein molecules and their affinity ($K_{m,\text{up}}$) to GLZ.

Bile acids are present in bile as mixed micelles.²⁹ M. H. Bickel and R. Minder reported that the incorporation of two lipophilic drugs, imipramine and desmethylinipramine, into biliary micelles contributes to the concentrative excretion of the drugs in the bile of rats. Furthermore, R.

Fig. 10. Simulated (Lines) and Observed (Points) log Perfusate Concentrations (A) and Cumulative Biliary Excretion (B) of GLZ after Administration at Various Doses in Perfused Rat Liver

Each point and vertical bar represents the mean and S.E. of 4 experiments. The data points without a vertical bar include the S.E. within the point. —, 0.5 mg; ---, 2.5 mg; - - - -, 5.0 mg; ·····, 10.0 mg; — · —, 20.0 mg; - - - - - , 30.0 mg. For other symbols, see Fig. 3.

Fig. 11. Holding Time (H) for Biliary Excretion (A) and the Relationship between H and Decrease in Mean Bile Flow (B) after Administration of GLZ at Various Doses in Perfused Rat Liver

Each point and vertical bar represents the mean and S.E. of 4 experiments. For the calculation of H , see the text. The decrease in mean bile flow at each dose was calculated by subtracting the mean bile flow from that of the control. The mean bile flow was estimated from the data in Fig. 5. A: The solid line is a visually fitted curve. B: The regression line is $y = 11.3x - 0.064$, $r = 0.94$.

Fig. 12. Uptake (CL_{up}) and Efflux (CL_{eff}) Clearances versus $C_{f,P}$, and Biliary Clearance (CL_B) versus $C_{f,H}$

a) CL_{up} : calculated from $V_{max,up}/(K_{m,up} + C_{f,P})$ using the parameters listed in Table III, b) CL_{eff} : the value (0.044 ml/min/g of liver) listed in Table III, and c) CL_B : calculated from $V_{max,B}/(K_{m,B} + C_{f,H} (1 + C_{f,H}/K_{i,B}))$ using the parameters listed in Table III.

J. Vonk *et al.* showed that 65% of dibromosulphthalein (a cholephylic organic anion) is associated with biliary micelles in rats.³⁰⁾ Therefore, we examined the actual concentrative transfer of GLZ from liver to bile by determining the binding of GLZ to biliary micelles. As can be seen in Fig. 3, the binding of GLZ to biliary micelles was constant over the range of the biliary concentrations (0.1–5.0 mg/ml) in the perfused liver study (Fig. 10B). The $K_{n,B}$ value (0.13) indicates that GLZ binds only very slightly to biliary micelles. Consequently, the $C_{f,B}^{ss}/C_{f,H}^{ss}$ ratios of 2–130 (Fig. 7) suggest the actual active transport of GLZ from liver into bile.

In an *in vivo* study,⁶⁾ the biliary excretion rate of GLZ at steady-state rat plasma unbound levels ($C_{f,plasma}^{ss}$) (0.2–4.0 μg/ml) followed a Michaelis–Menten type equation with a K'_m of 1.83 μg/ml and V'_{max} of 98.8 μg/min/250 g rat. The $V_{max,B}$ value (Table III) was about 3 times smaller than the V'_{max} value. The small V_{max} value might result from the decrease in bile flow (Figs. 5 and 8A), which may participate in the decrease of the

biliary excretion rate of GLZ. The substrate inhibition by GLZ of its own biliary excretion seems to appear as a result of study in the range of $C_{f,p}$ (0.1—74.3 $\mu\text{g/ml}$) higher than the $C_{f,plasma}^{ss}$ levels. The biliary excretion rate with $K_{i,B}$ is supported by the good agreement of the simulation curve of R_B versus $C_{f,H}^{ss}$ with the observed points (Fig. 6). Also, the $K_{m,B}$ value was close to the $K_{m,up}$ value (Table III), suggesting almost identical affinity of GLZ to the carrier on liver sinusoidal and bile canalicular membranes.

As described already, the bile flow was decreased by GLZ (Figs. 5 and 8A), which is cholephilic organic anion.⁶⁾ It has been reported that other cholephilic organic anions such as indocyanine green, rose bengal, bromosulfophthalein, and bromocresol green are cholestatic in rats^{31,32)} and the cholestatic effects may be due to an inhibition of mitochondria respiration,^{33,34)} or Mg- and Na,K-ATPase activities.^{34,35)} Since GLZ has an inhibitory effect on Na,K-ATPase activity in the cell membrane,³⁶⁾ the cholestatic effect of GLZ may be due to an inhibition of this enzyme activity.

In our previous study,¹³⁾ GLZ was metabolized only in liver and small intestine homogenates of rats. Therefore, the hepatic metabolic clearance (CL_M) of GLZ was included in the model. As described already, the simulation curves for the perfusate concentration–time profiles were not affected by the holding time H , but those for the cumulative biliary excretion profiles in the model without H rose markedly above the observed values at each dose. As can be seen in Fig. 12, the simulation curve for uptake clearance (CL_{up}) was 15—252 times higher than the CL_{eff} line, and 5—20 times higher than the simulation curve for biliary clearance (CL_B), with a decrease with increasing $C_{f,p}$. This indicates that the uptake of GLZ into liver is faster than its efflux and biliary excretion from liver. Because of such rapid uptake, the influence of H should not appear in the perfusate concentration–time simulation curves, while the simulation of slow biliary excretion may be greatly affected in the case without H . As can be seen in Fig. 11, the holding time, H , increased linearly with decreasing bile flow. Such a dose-dependent H would be related to a decrease in bile flow in rats.

In conclusion, the disposition of GLZ in the perfused rat liver was well simulated by the proposed model, which may be useful for predicting the disposition of GLZ in rat plasma *in vivo*, as well as that in the liver of patients given GLZ.

Acknowledgement This work was supported in part by the Sasakawa Scientific Research Grant from The Japan Science Society.

GLOSSARY

Am :	amount of drug in the perfusate or bile
C :	total concentration of drug
C_f :	unbound concentration of drug
C_b :	bound concentration of drug
CL :	clearance
E :	hepatic extraction ratio
f :	free fraction
GLZ:	glycyrrhizin
H :	holding time
K_d :	dissociation constant
K_n :	linear binding coefficient

K_m :	Michaelis-Menten constant
K_i :	substrate inhibition constant
N :	binding capacity
Q :	flow rate
R :	uptake, biliary excretion, or efflux rate
V :	volume
V_{max} :	maximum rate
t :	time

Superscript
ss: steady state

Subscript
B: bile
CA: capillary bed
CI: capillary bed or interstitial fluid
IS: interstitial fluid
IC: intracellular fluid
H: liver
P: perfusate
LH: liver homogenate
eff: efflux
M: metabolism
up: uptake

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