Uptake of Glycyrrhizin by Isolated Rat Hepatocytes

Shiro Ishida,* Yoko Sakiya, Tsutomu Ichikawa, and Zenei Taira

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

Received July 22, 1992

The mechanism of uptake of glycyrrhizin (GLZ) by isolated rat liver cells was studied. The uptake rate was dependent on the unbound GLZ concentration. The initial uptake rate with respect to the unbound GLZ concentration reflected the operation of both saturable and nonsaturable processes, which followed Michaelis–Menten type kinetics; the process involves a $K_m$ of 11.3 $\mu M$, $V_{max}$ of 0.112 mmol/min/10^6 cells, and a first-order rate constant ($K_a$) of 0.195 mmol/min/10^6 cells/mM. GLZ adsorption on the cell membrane occurs at two types of binding sites with a linear adsorption $K_a = 2.81$ mmol/10^6 cells/mM and a dissociation constant $= 18.3 \mu M$ and its adsorption capacity $= 0.12$ mmol/10^6 cells describing specific adsorption. GLZ uptake did not require the presence of Na’ in the incubation medium and was not significantly inhibited by ouabain. The Arrenhius plot of uptake of 10 $\mu M$ GLZ presented a single straight line in the range of 4—37°C, with an activation energy of 15.9 kcal/mol. An energy requirement was also demonstrated, as all metabolic inhibitors studied (rotenone, antimycin A, 2,4-dinitrophenol, and KCN) significantly reduced the uptake of 10 $\mu M$ GLZ ($p<0.01$). The uptake was competitively inhibited by glycyrrhetinic acid (GLA), taurocholate (TCA), and probenecid (PBC) with inhibition constants, $K_i$, of 13.7, 48.5, and 115.9 $\mu M$, respectively, and it was noncompetitively inhibited by bromosulfophthalein ($K_i$, 9.2 $\mu M$) and indocyanine green ($K_i$, 13.5 $\mu M$) only at low GLZ concentrations (5 and 10 $\mu M$).

It was concluded that a carrier-mediated transport system participates in the uptake of GLZ into isolated rat hepatocytes and the affinity site of the transport carrier commonly binds GLA, TCA, and PBC.

Keywords: glycyrrhizin; hepatic uptake; isolated rat hepatocyte; carrier-mediated uptake

Introduction

Glycyrrhizin (GLZ) is an anionic drug used frequently in the treatment of chronic hepatitis.1 Yamamura et al.3 showed that GLZ is predominantly excreted in the bile (ca. 80%) after 80 mg/kg i.v. administration to patients with liver failure. We also showed that the biliary excretion of GLZ is the major route in rats (ca. 86—98%) after an i.v. administration at the dose range of 5—50 mg/kg.3 We established that the biliary excretion of the drug in rats follows Michaelis–Menten kinetics ($K_{m,b}$ 1.83 $\mu M/ml$ and $V_{max,b}$ 98.77 $\mu M/min$) and the dose dependency of biliary clearance determines that of the total body clearance.3 Such observations emphasize the importance of investigating the mechanism of hepatic uptake of GLZ.

The mechanisms of hepatic uptake of anionic drugs such as bromosulfophthalein (BSP), indocyanine green (ICG), and bile acids have been studied using isolated rat hepatocytes.4—10 isolated perfused rat liver,11—13 and whole animals.13—15 These studies suggested that these organic anions are taken up by a carrier-mediated system. Isolated liver cells are particularly advantageous in the study of the hepatic uptake process, as the uptake can be studied independently of unspecific binding to plasma proteins, different distributional compartments, and hemo-dynamic factors.

The purpose of the present study is to elucidate the mechanism of hepatic uptake of GLZ by using isolated rat liver cells, and to determine whether the transport system is shared by other organic anions such as BSP, ICG, and bile acid.

Experimental

Chemicals

GLZ monooammonium salt and glycyrrhetinate (GLA) sodium salt were kindly supplied by Minophagen Pharmaceutical Co. (Tokyo, Japan). BSP sodium salt, probenecid (PBC), taurocholate (TCA) sodium salt, p-aminohippurate (PAH) sodium salt, antimycin A, ouabain, rotenone, bovine serum albumin (fraction V) (BSA), fluorescein iso-thiocyanate dextran (avg. mol. wt. 64,200), and collagenase (type II) were purchased from Sigma Chemical Co. (St. Louis, MO). Silicone oil (d = 1.02) and KCN, LiCl, and trypan blue were purchased from Aldrich Chemical Company Co. (Milwaukee, WI) and Wako Pure Chemical Ind., Ltd. (Osaka, Japan), respectively. ICG was obtained from Daiichi Pharmaceutical Co. (Tokyo, Japan). All other reagents were commercial products of analytical grade.

Preparation of Isolated Hepatocytes

Isolated liver cells from male Wistar rats weighing 250—300 g, fed on standard chow, were prepared according to the method of Lin et al.,16 which was slightly modified from that of Baur et al.17 Cell viability was estimated by trypan blue exclusion. All experiments were carried out on cell suspensions with at least 90% viability.

Uptake Studies

The centrifugal filtration method was used to measure the uptake of GLZ by isolated hepatocytes.17 Hepatocytes were suspended in the incubation medium containing 131 mM NaCl, 5.2 mM KCl, 0.9 mM MgSO_4, 0.12 mM CaCl_2, 3 mM Na_2HPO_4, 5 mM glucose, and 15 mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES), pH 7.4. After preincubation of 5.0 ml of the cell suspension containing 8 $\times$ 10^6 cells/ml for 5 min at 37°C, the reaction was started by the addition of 20 $\mu l$ of the incubation medium containing GLZ preincubated for 5 min at 37°C. After incubation for a suitable time at 37°C, 200 $\mu l$ of the reaction mixture was placed on top of an upper layer of silicone oil (50 $\mu l$) with an under layer (50 $\mu l$) of 3 M KOH solution, which was added in order to dissolve the cells, in a polyethylene tube, and the whole was centrifuged for 5 s in a Beckman microfuge (Beckman Instrument Inc., CA) to stop the reaction by separating the cells from the incubation medium. To 50 $\mu l$ of the KOH layer was added 40 $\mu l$ of 20% HClO_4 solution to bring the medium pH to approximately 7, and then 300 $\mu l$ of MeOH was added. The solution was centrifuged and the obtained supernatant solution was used for the determination of GLZ taken up by the cells. Upon centrifugation in a Beckman microfuge, the cells together with a small amount of adherent medium passed through the silicone oil. The volume of adherent medium was determined by the method of Mizuma et al.,18 using impermeable fluorescein isothiocyanate dextran (final concentration: 5 $\mu M$). The volume of adherent fluid obtained was 1.03 ± 0.02 $\mu l$ of 10^6 cells (mean ± S.E., n = 7). All the uptake data were corrected for the amount of GLZ in this volume of adherent fluid.

Measurement of Unbound GLZ Concentration

The unbound GLZ concentration in the cell suspension was determined by an ultrafiltration technique using an ultrafiltration membrane (Amicon Micropartition System, MFS-1, Danvers, MA). GLZ in the incubation medium preincubated for 5 min at 37°C was added to the stirred cell suspension preincubated similarly, and the mixture was immediately centrifuged for
3s at 16000 rpm. The obtained supernatant solution was applied to the determination of GLZ.

**Determination of GLZ.** The supernatant solution and filtrate (50 µl each) were each directly injected into a high performance liquid chromatograph (HPLC), Hitachi type L, with a Wakosil II SC18-HG column (250 x 4.6 mm i.d.) and an ultraviolet detector operating at 254 nm. Other conditions were the same as those described previously.\(^{16}\)

**Data Analysis.** Uptake of GLZ was measured at 1, 2, 3, 4, and 5 min. Efflux was assumed to be negligible at these sampling points. Since the uptake at various GLZ concentrations proceeded linearly for 5 min in all cases, the initial uptake rate was taken to be the slope of the linear regression line. The amount of GLZ adsorbed on the cell membrane was inferred from the y-intercept at time zero, obtained by extrapolating the linear regression line. The kinetic parameters of uptake were estimated by a least-squares regression analysis program (MULTI\(^{19}\)) using a digital computer (NEC PC-9801 RA).

**Results**

**Uptake of GLZ by Isolated Hepatocytes**. The time course of uptake of GLZ into isolated hepatocytes is shown in Fig. 1 at various GLZ concentrations ranging from 5.0 µM to 1.0 mM. The uptake proceeded linearly over the 5-min incubation period. GLZ uptake in the presence of 2% BSA was also linear over the 5-min incubation period at various GLZ concentrations ranging from 0.1 to 2.4 mM (results not shown). Therefore, the initial uptake rate was taken to be the slope of the regression line in both cases. The initial uptake rate in the absence (A) and presence (B) of 2% BSA was analyzed as a function of the unbound GLZ concentration. Figure 2 shows the results. The results suggest that GLZ uptake consists of two components, i.e., the saturable and nonsaturable processes at lower and higher unbound GLZ concentrations than 0.1 mM, respectively. Thus, the initial uptake rate \((V_0)\) can be related to the unbound GLZ concentration \((C_t)\) as follows:

\[
V_0 = \frac{V_{\text{max}}C_t}{K_m + C_t} + K_dC_t
\]

where \(V_{\text{max}}\) is the maximum uptake rate for a carrier-mediated process, \(K_m\) is the Michaelis constant, and \(K_d\) is the first-order uptake rate constant. Table I lists the kinetic parameters estimated from Eq. 1 by MULTI analysis.\(^{19}\) None of the parameters was significantly changed in the presence of BSA.

The adsorption data of GLZ on the cells were analyzed by means of a Scatchard plot (Fig. 3). From the steep part of the curve, a dissociation constant of \(18.3 \pm 7.3 \mu M\) was obtained. The corresponding adsorption capacity was \(12.0 \pm 0.02 \text{ mmol} / \text{10}^6 \text{ cells}\). The linear part of the curve had a linear adsorption coefficient of 2.81 \pm 0.05 \text{ mmol} / 10^6 \text{ cells/mm}.

**Effect of Na\(^+\) on Initial Uptake Rate** To study the Na\(^+\) dependency of the GLZ uptake process, NaCl in the medium was replaced by LiCl (131 mM), so that 99.8% of Na\(^+\) was substituted. The replacement did not significantly change the initial uptake rate of 10 µM GLZ (102.5 \pm 4.8% control, \(n = 4\)). Also, the Na\(^+\), K\(^+\)-dependent ATPase

**Table I.** Kinetic Parameters of GLZ Uptake by Isolated Rat Hepatocytes in the Absence and Presence of 2% BSA

<table>
<thead>
<tr>
<th></th>
<th>(K_m) (µM)</th>
<th>(V_{\text{max}}) (nmol/min/10^6 cells)</th>
<th>(K_d) (nmol/min/10^6 cells/mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absence</td>
<td>11.3 \pm 2.3</td>
<td>0.112 \pm 0.013</td>
<td>0.195 \pm 0.032</td>
</tr>
<tr>
<td>Presence</td>
<td>15.4 \pm 2.3</td>
<td>0.124 \pm 0.016</td>
<td>0.188 \pm 0.049</td>
</tr>
</tbody>
</table>

Data are presented as the mean \pm S.E. of seven or nine independent experiments.

a) The Michaelis constant.  b) The maximum uptake rate.  c) The first-order uptake rate constant.

**Fig. 1.** Time Course of Uptake of GLZ by Isolated Rat Hepatocytes in the Absence of BSA at 37°C

**Fig. 2.** The Initial Uptake Rate of GLZ by Isolated Rat Hepatocytes in the Absence (A) and Presence (B) of 2% BSA at 37°C as a Function of the Unbound GLZ Concentration

Each point is the mean \pm S.E. of seven or nine independent experiments. The solid lines were generated from Eq. 1 using the MULTI-fitted parameters listed in Table I. For the determination of the unbound GLZ concentration, see the text.
inhibitor ouabain (1 mM) did not significantly alter the uptake rate (86.0 ± 4.3% of control, n = 4).

**Temperature Dependence of Initial Uptake Rate** Uptake of 10 μM GLZ into isolated hepatocytes was determined at four different temperatures, 4, 15, 25, and 37°C. Figure 4 shows an Arrhenius plot for the initial uptake rate. The plot exhibited a single straight line corresponding to an activation energy of 15.9 kcal/mol (66.5 kJ).

**Effects of Metabolic Inhibitors on Initial Uptake Rate** The respiratory chain inhibitors rotenone (50 μM), antimycin A (10 μM), and KCN (1 mM), and the uncoupler 2,4-dinitrophenol (1 mM), added 10 min prior to GLZ to the cells, significantly (p < 0.01) diminished the initial uptake rate of 10 μM GLZ by about 44, 58, 60, and 57%, respectively (Fig. 5).

**Inhibition of Initial Uptake Rate by Other Organic Anions** To determine the specificity of the GLZ uptake process, the influence of several organic anions on the initial uptake rate was studied. BSP, GLA, ICG, TCA, and PBC (50 μM each), added 10 s prior to GLZ to the cells, significantly (p < 0.01) reduced the initial uptake rate of 10 μM GLZ by about 11, 25, 34, 63, and 73%, respectively, but PAH (50 μM) was ineffective (Fig. 6). A Dixon plot of GLZ showing inhibition by organic anions is presented in Fig. 7. As can be seen in Figs. 7A–C, the straight lines
converged to a point above the abscissa, showing competitive inhibition by GLA, TCA, and PBC. In Fig. 7D, the straight lines converged to a point on the abscissa, showing noncompetitive inhibition by BSP. The non-competitive inhibition by ICG was observed only for low GLZ concentrations (5 and 10 μM) and the straight line at high GLZ concentration (20 μM) did not pass through the convergence point (Fig. 7E). The inhibition type was evaluated from the Akaike’s information criteria values listed in Table II, together with the inhibition constant (K_i) values.

Discussion

The hepatic uptake of organic anions (e.g., BSP, TCA, and rose bengal) by albumin-mediated transport has been reported by several groups. In this study, the binding of GLZ to BSA was observed, because the sum of GLZ binding to BSA and GLZ adsorption on the cells in the incubation medium with 2% BSA was 53–98%, and GLZ adsorption on the cells in the medium without BSA was 2.8–7.6%. Therefore, the effect of BSA on GLZ uptake was examined. The total GLZ concentration in the medium with BSA was chosen such that the unbound GLZ concentration was similar to that in the case without BSA (Fig. 2). In both cases, the initial uptake rate with respect to the unbound GLZ concentration reflected the operation of both saturable and nonsaturable uptake processes (Fig. 2), and none of the parameters was significantly altered in the presence of BSA (Table I). This suggests that albumin does not participate to GLZ uptake, but that the unbound GLZ concentration determines the uptake rate.

The evidence of a saturable process suggests that the uptake of GLZ into hepatocytes is carrier-mediated. The nonsaturable process could be explained by passive diffusion with the participation of a very low affinity system. The unbound GLZ concentration at the rat plasma levels (<1.1 mM) after 50 mg/kg i.v. dose is estimated to be smaller than 30 μM by using the unbound plasma fraction (0.006–0.026). Further, from the kinetic parameters (Table I), the V_max / (K_m + C_i) value obtained is 17 times or more greater than the K_d · C_i value. It is suggested that a carrier-mediated transport system is the dominant process for the hepatic uptake process of GLZ at plasma levels lower than 1.1 mM. Assuming 1.1 × 10^9 cells/g of liver, the V_max in Table I gives a predicted value of 12.8 nmol/min/g of liver. This value is close to the V_max value 12.0 nmol/min/g of liver, suggesting that the saturable uptake of GLZ into liver is rate-limiting in biliary excretion. However, the K_m,b value of 2.2 μM is about 5 times smaller than the K_m value (11.3 μM). Such a low affinity of GLZ to carrier on the isolated cells might be due to changes in membrane composition by the isolation. The Scatchard plot of the adsorption data reveals the existence of two types of binding sites, of which the one with specific affinity for GLZ has a dissociation constant (18.3 μM) of the same order of magnitude as the K_m value for the uptake process. As shown in Fig. 4, the reduction of the incubation temperature from 37 to 4°C decreased the initial uptake rate, with an activation energy of 15.9 kcal/mol, a value which is in the normal range for carrier-mediated transport. An energy requirement for the transport of GLZ was also apparent from the significant decrease of the initial uptake rate by all metabolic inhibitors examined (rotenone, antimycin A, 2,4-dinitrophenol, and KCN) (Fig. 5).

As shown in Fig. 6, GLA, BSP, ICG, TCA, PBC, and PAH used as inhibitors for GLZ uptake have following characteristics. GLA is a major metabolite of GLZ and after an i.p. dose of ^3H-GLA (25 mg/kg) to rats, all the
drug is excreted in the bile as metabolites. The uptake of BSP, ICG, and TCA into isolated rat hepatocytes is a carrier-mediated process, as described already. PBC is taken up into rat liver slices via the organic acid transport mechanism. PAH is transported by a carrier-mediated transport system through the renal basolateral membrane and is actively secreted into bile, but GLZ uptake was not significantly inhibited by PAH. Kinetic analysis demonstrated a fully competitive inhibition of GLZ uptake by GLA, TCA, and PBC (Figs. 7A–C). This suggests a common carrier-mediated transport system for GLZ and these three drugs. Anwer and Hegner showed that TCA is transported into isolated rat liver cells by both Na+-independent and Na+-dependent systems. The \( K_m \) value (57 \( \mu M \)) for the Na+-dependent transport process was of the same order of magnitude as the \( K_m \) value for TCA (Table II), suggesting that GLZ and TCA have a common Na+-independent transport system. The Na+-independent process for GLZ uptake was also indicated from the results that the uptake did not require the presence of Na+ in the incubation medium and was not significantly inhibited by 1 mM ouabain. Boyer and Reno showed that 1 mM ouabain in isolated rat liver cells completely blocks Na+, K+-ATPase activity. The affinity to the putative site was in the order of GLZ>GLA>TCA>PBC, as the values of \( K_m \) for GLZ and \( K_i \) for GLA, TCA, and PBC were 11.3, 13.7, 48.5, and 115.9 \( \mu M \), respectively. Noncompetitive inhibition by BSP (Fig. 7D) suggests that the affinity site of the transport carrier for GLZ is different from that for BSP. It has been reported that the Dixon plot for the uptake of 5 \( \mu M \) BSP inhibited by ICG shows a straight line that deviated from those of 1 and 2 \( \mu M \) but converged at a point above the abscissa. Such a pattern is similar to that of Fig. 7 E. It was found that the different inhibitions by ICG at high and low GLZ concentrations are similar to the patterns in the case of BSP uptake.

In conclusion, a carrier-mediated transport system participates in the uptake of GLZ into isolated rat hepatocytes and requires metabolic energy. The GLZ affinity site of the transport carrier in hepatocytes is common to GLA, TCA, and PBC, which act as competitive inhibitors of GLZ uptake. Further, it is suggested that the saturable biliary excretion rate may be attributed to a saturable transport of GLZ from plasma into liver.

References and Notes