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Dose-Dependent Pharmacokinetics of Glycyrrhizin in Rats

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The dose-dependent pharmacokinetics of glycyrrhizin (GLZ) was investigated by measuring drug disappearance from plasma and biliary excretion in rats. The decline in plasma concentration was biexponential after an i.v. dose of 5, 10, 20, or 50 mg/kg. Dosage, however, had a marked effect on the pharmacokinetics, with a greater-than-proportional increase in area under the plasma concentration curve (AUC) at doses of 20 and 50 mg/kg, even though the increase was proportional at doses of 5 and 10 mg/kg. There was also a significant increase of the steady-state distribution volume (Vd_{ss}), as well as significant decreases in total body (CL_{tot}) and biliary (CL_B) clearances, at 20 and 50 mg/kg from those at 5—10 and 5—20 mg/kg, respectively. The AUC, Vd_{ss}, and renal clearance (CL_R) at a given dose showed no significant difference between rats with and without bile fistulas. The plasma unbound fraction (f_p) (0.006—0.026) increased with increasing plasma GLZ concentration over the observed range (2—900 μg/ml). No significant change in Vd_{ss} for unbound GLZ was observed between the doses, indicating that the distribution of GLZ into tissues is not changed by an increase in dose. On the other hand, a dose dependency in CL_{tot} for unbound GLZ was observed and confirmed to be attributed to dose dependency in CL_B for unbound GLZ since there was no significant difference in CL_R or metabolic clearance for unbound GLZ between the doses. The biliary excretion rates (V_B) at steady-state plasma unbound levels fitted reasonably well a Michaelis-Menten type equation with a maximum V_B of 98.77 μg/min and a Michaelis constant of 1.83 μg/ml. It was suggested that the saturable V_B may result in the dose dependence of CL_B. Thus, the pharmacokinetics of GLZ in rats is dose-dependent.

Keywords glycyrrhizin; dose dependency; rat; biliary clearance; saturable biliary excretion rate; nonlinear pharmacokinetics

Introduction

Glycyrrhizin (GLZ) has frequently been used in the treatment of chronic hepatitis,[1] allergic disorders,[2-4] inflammation,[2,5] and gastric ulcers.[6] Recently GLZ has been reported to have produced some improvement in immune function[7] and an objective clinical improvement in patients with human immunodeficiency virus infection.[8] However, GLZ produces the adverse effect of aldosteronism when given in massive doses.[9-12] It is desirable, therefore, to elucidate the influence of dose on the pharmacokinetics of GLZ in order to use it safely in clinical therapeutics. Previously we reported the existence of enterohepatic cycling of GLZ following predominantly biliary excretion (*ca.* 80%) after a 100 mg/kg i.v. dose in rats,[13] but we did not characterize the pharmacokinetics at any dose.

In this study, we examined whether GLZ shows dose-dependent pharmacokinetics in rats by measuring the plasma disappearance after an i.v. administration of 5, 10, 20, or 50 mg/kg and the biliary excretion.

Experimental

Chemicals Monoammonium glycyrrhizinate (GLZ-NH₄) was kindly supplied by Minophagen Pharmaceutical Co. (Tokyo, Japan). All other reagents were commercial products of analytical grade.

Animals Male Wistar rats (weighing 240—260 g) that had been starved for 20 to 24 h prior to the experiments were used throughout. Rats were lightly anesthetized with ether for all surgical procedures. The right femoral vein and left femoral artery were cannulated with PE-50 polyethylene tubing for i.v. injection and infusion of the drug and for blood sampling, respectively. A bile fistula cannula (PE-10 polyethylene tubing) and a urinary bladder cannula (PE-60 polyethylene tubing) were used to collect samples of bile and urine, respectively. The cannulated rats were kept in restraining cages with free access to water under normal housing conditions prior to the experiments. The rats were allowed to recover from anesthesia prior to the drug administration (approximately 1 h). The body temperature was kept at 37°C throughout the experiments by using a heat lamp.

Plasma Disposition GLZ-NH₄ dissolved in a 5% glucose solution (5, 10, 20, or 50 mg/kg as GLZ) (*ca.* 1.0 ml each) was given to rats with and without biliary fistulization, followed by 0.5 ml of 5% glucose solution. After dosing, blood samples (0.3 ml each) were collected in heparinized polyethylene centrifuge tubes at 1, 5, 10, 30, 60, and 120 min. Further

samples were taken at 3 h after the 10 mg/kg dose, at 3 and 5 h after 20 mg/kg, and at 3, 5, 8 and 12 h after 50 mg/kg. Rats were given fresh blood (1.5 ml), obtained from other rats, through the left femoral artery cannula immediately after sampling at 60 min. Plasma was harvested immediately and frozen at −20°C until analysis.

Biliary and Urinary Excretion In bile fistula rats, bile samples were obtained at 0—0.5, 0.5—1, 1—2, 2—4, 4—8, 8—12, 12—24, and 24—48 h after i.v. administration of GLZ (5, 10, 20, or 50 mg/kg), and urine samples were obtained at 0—12, 12—24, and 24—48 h. Urine samples were similarly collected from rats without bile fistulas after an i.v. administration of GLZ.

Biliary Excretion Rate (V_B) at Steady-State Plasma Level (C_{ss}) After a loading dose of 1.1, 2.2, 4.4, 14.0, or 18.0 mg/kg GLZ, the drug was infused through the cannula at the rate of 3.7, 7.4, 14.9, 30.0, or 35.0 mg/min/kg, respectively, with a constant-rate infusion pump (model KN-201, Natsume Seisakusho Co., Tokyo, Japan). The C_{ss} (*ca.* 10—500 μg/ml) was obtained within 45 min after the initiation of infusion in each case. A point 60 min after the initiation of infusion was taken as 0 time, and bile was collected over 15 min intervals for 60 min through the cannula. The volume of each bile sample was measured and samples were combined after GLZ determination. All the samples were stored at −20°C until required.

Determination Method Samples of plasma and bile (20—100 μl) were used for GLZ determination by the method described previously, *i.e.*, by high-performance liquid chromatography (HPLC)[14] after extraction with MeOH.[15]

Data Analysis The area under the plasma concentration curve (AUC) and the mean residence time (MRT) were calculated by trapezoidal integration with extrapolation to infinite time.[16] The steady-state distribution volume (Vd_{ss}) and total body clearance (CL_{tot}) were calculated from dose · MRT/AUC and dose/AUC, respectively. The biliary (CL_B) and renal (CL_R) clearances were calculated from the total biliary and urinary excreted amounts divided by AUC, respectively. Metabolic clearance (CL_M) in the rats with a bile fistula was estimated from $CL_{tot} - (CL_B + CL_R)$. The V_B at C_{ss} was estimated from $B_{vol} \times B_{con}/60$ min, where B_{vol} and B_{con} are the total volume of four bile samples collected at C_{ss} and the mean GLZ concentration in those samples, respectively. The V_B data were analyzed according to Eq. 1, and the maximum V_B ($V_{B,max}$) and Michaelis constant (K_m) were calculated by a nonlinear least-squares method[17] using a digital computer (NEC PC-9801 RA).

$$V_B = \frac{V_{B,max} \cdot C_{ss,f}}{K_m + C_{ss,f}} \qquad (1)$$

Where $C_{ss,f}$ is the steady-state plasma unbound concentration. Unbound plasma concentration (C_f) of GLZ was calculated from the equation defined

in the previous report[14] as follows:

$$C_{\mathrm{tot}} = C_{\mathrm{f}} + \frac{n_1(p)K_1 C_{\mathrm{f}}}{1 + K_1 C_f} + \frac{n_2(p)K_2 C_{\mathrm{f}}}{1 + K_2 C_f} \qquad (2)$$

where C_{tot} is the total plasma concentration of GLZ, and K_1 and K_2 are the association constants corresponding to $n_1(p)$ and $n_2(p)$, which are the primary and secondary binding capacities, respectively. By using the values given in the previous paper[14] for $n_1(p)$ (1.35 mM), $n_2(p)$ (6.83 mM), K_1 (124.2 mM^{-1}), and K_2 (0.095 mM^{-1}), and C_{tot}, C_{f} was calculated from a cubic equation by using the Hitchcock-Bairstow program[18] on a digital computer. Plasma unbound fraction (f_{p}) was estimated from $C_{\mathrm{f}}/C_{\mathrm{tot}}$. All means are presented with their standard error (mean ± S.E.). Student's t test was utilized to determine the significance of differences between doses and between the rat groups with and without bile fistulas.

Results

Pharmacokinetic Aspects The plasma disposition of GLZ after an i.v. administration of 5, 10, 20, or 50 mg/kg in rats with and without biliary fistulization is plotted in Fig. 1. The decline in mean plasma concentration was biphasic in all cases, but with a much slower terminal disposition at the two high doses than at the two low doses. Table I shows the pharmacokinetic parameters for the rats

without bile fistulas. The AUC value increased proportionally to the administered dose at doses of 5 and 10 mg/kg, but the increases at 20 and 50 mg/kg were greater than the proportional increase of dose. The MRT and Vd_{ss} values at 20 and 50 mg/kg were significantly larger ($p < 0.05$) than those at 5—10 and 5—20 mg/kg, respectively. The CL_{tot} values at 20 and at 50 mg/kg were significantly smaller ($p < 0.05$) than those at 5—10 and 5—20 kg/kg, respectively, while no significant difference in the CL_{R} values was observed between the doses. As shown in Table II, in the rats with bile fistulas, the AUC also showed linear and nonlinear increases in dose ranges of 5 to 10 mg/kg and 20 to 50 mg/kg, respectively. Statistically significant differences in the MRT, Vd_{ss}, and CL_{tot} values between the doses were similar to those in the rats without bile fistulas. The CL_{B} values at 20 and 50 mg/kg were significantly smaller ($p < 0.05$) than those at 5—10 and 5—20 mg/kg, respectively, while no significant difference in the CL_{R} and CL_{M} values was obtained between the doses. Neither AUC, MRT, Vd_{ss}, CL_{tot}, or CL_{R} at a given dose showed any significant difference between the two groups. The f_{p} (0.006—0.026)
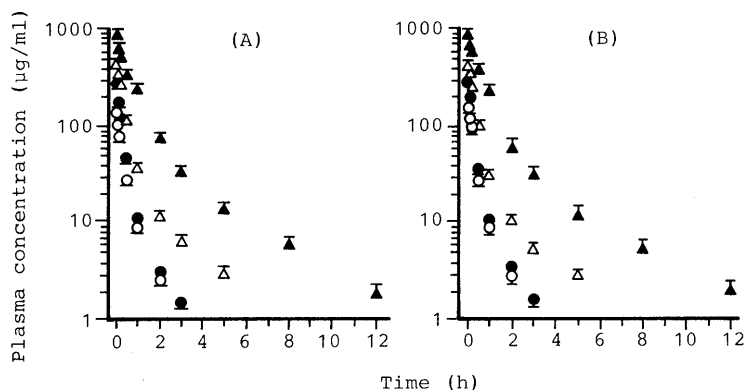

Fig. 1. Plasma Disposition Curves of GLZ after i.v. Administration of 5, 10, 20, or 50 mg/kg to Rats

Each point represents the mean ± S.E. of four to six rats without (A) and with (B) bile fistulas. ○, 5 mg/kg; ●, 10 mg/kg; △, 20 mg/kg; ▲, 50 mg/kg.

TABLE I. Pharmacokinetic Parameters of GLZ in Rats without Biliary Fistulization

Parameter	5 mg/kg	10 mg/kg	20 mg/kg	50 mg/kg
$AUC^{a)}$ (min·mg/ml)	2.62 ± 0.10	5.04 ± 0.12	13.0 ± 1.2	41.9 ± 1.3
$MRT^{b)}$ (min)	30.7 ± 1.9	30.2 ± 1.4	51.7 ± 2.8$^{f)}$	96.1 ± 12.9$^{g)}$
$Vd_{\mathrm{ss}}^{c)}$ (ml/kg)	58.2 ± 2.3	59.8 ± 3.2	86.4 ± 6.1$^{f)}$	115.0 ± 16.2$^{g)}$
$CL_{\mathrm{tot}}^{d)}$ (ml/min/kg)	1.91 ± 0.06	1.98 ± 0.09	1.54 ± 0.08$^{f)}$	1.20 ± 0.04$^{g)}$
$CL_{\mathrm{R}}^{e)}$ (ml/min/kg)	0.02 ± 0.01	0.04 ± 0.02	0.04 ± 0.01	0.06 ± 0.02

Results are given as the mean ± S.E. of four to six rats. *a*) The area under the plasma concentration curve. *b*) The mean residence time. *c*) The steady-state distribution volume. *d*) and *e*) The total body and renal clearances, respectively. *f*) Significant difference ($p < 0.05$) from 5 and 10 mg/kg. *g*) Significant difference ($p < 0.05$) from 5, 10 and 20 mg/kg. For the calculations, see the text.

TABLE II. Pharmacokinetic Parameters of GLZ in Rats with Biliary Fistulization

Parameter	5 mg/kg	10 mg/kg	20 mg/kg	50 mg/kg
AUC (min·mg/ml)	2.58 ± 0.23	5.10 ± 0.37	13.5 ± 1.3	43.1 ± 3.4
MRT (min)	30.1 ± 2.6	31.4 ± 2.3	52.4 ± 5.0$^{c)}$	97.2 ± 7.2$^{d)}$
Vd_{ss} (ml/kg)	58.3 ± 5.2	60.5 ± 4.5	77.6 ± 6.5$^{c)}$	112.8 ± 10.7$^{d)}$
CL_{tot} (ml/min/kg)	1.94 ± 0.17	1.96 ± 0.14	1.48 ± 0.14$^{c)}$	1.16 ± 0.08$^{d)}$
$CL_{\mathrm{B}}^{a)}$ (ml/min/kg)	1.85 ± 0.14	1.82 ± 0.12	1.33 ± 0.11$^{c)}$	1.00 ± 0.07$^{d)}$
CL_{R} (ml/min/kg)	0.02 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.05 ± 0.02
$CL_{\mathrm{M}}^{b)}$ (ml/min/kg)	0.07 ± 0.03	0.11 ± 0.02	0.11 ± 0.02	0.10 ± 0.03

Results are given as the mean ± S.E. of four to six rats. *a*) and *b*) The biliary and metabolic clearances, respectively. *c*) Significant difference ($p < 0.05$) from 5 and 10 mg/kg. *d*) Significant difference ($p < 0.05$) from 5, 10 and 20 mg/kg. For other keys and the calculations, see Table I and the text, respectively.
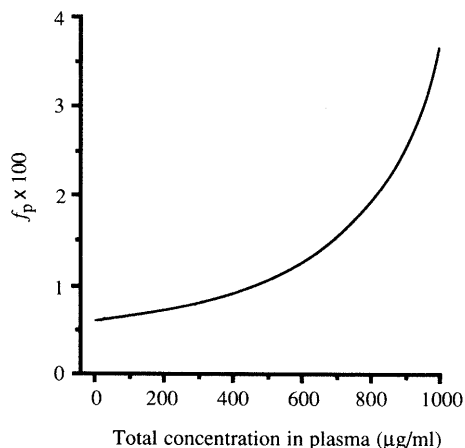
Fig. 2. Simulation Curve for Unbound Fraction (f_p) *versus* Total Concentration of GLZ in Rat Plasma

For the simulation, see the text.

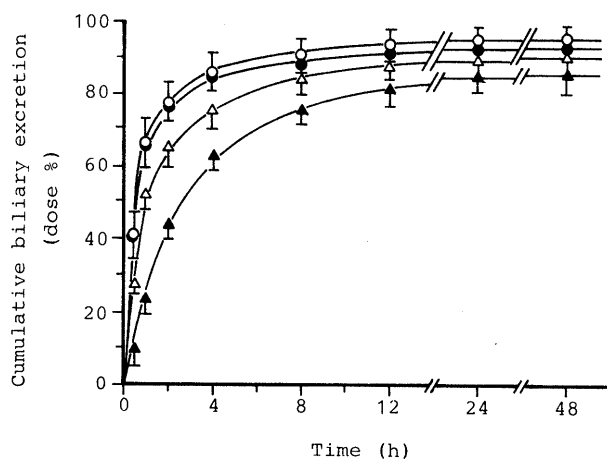
Fig. 3. Cumulative Biliary Excretion Profile of GLZ Following i.v. Administration of 5, 10, 20, or 50 mg/kg to Rats

Each point represents the mean ± S.E. of four to six rats. ○, 5 mg/kg; ●, 10 mg/kg; △, 20 mg/kg; ▲, 50 mg/kg.
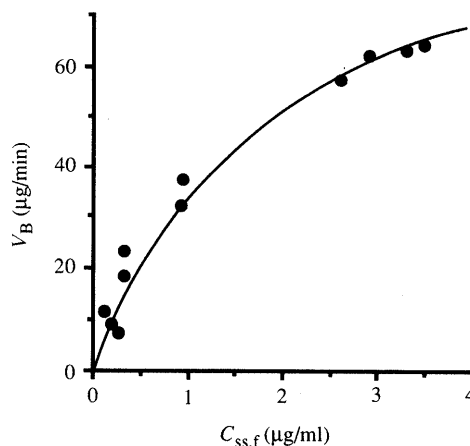

Fig. 4. Biliary Excretion Rate (V_B) at Steady-State Plasma Unbound Levels ($C_{ss,f}$) of GLZ in Rats

$C_{ss,f}$ was calculated from Eq. 2. The points represent the experimental values. The solid line represents computer-fitted curve taken from Eq. 1. For the obtained maximum V_B and Michaelis constant values, see the text.
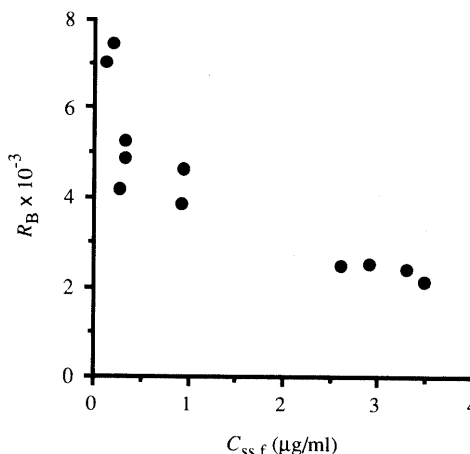

Fig. 5. Bile-to-Plasma Unbound Concentration Ratios (R_B) at Steady-State Plasma Levels of GLZ in Rats

The points represent the experimental values.

increased gradually with increasing plasma concentration over the observed range (2—900 μg/ml) (Fig. 2), suggesting that plasma protein binding may be involved in changes in pharmacokinetic parameters.

Biliary Excretion Figure 3 shows the cumulative biliary excretion profile of GLZ following an i.v. dose of 5, 10, 20, or 50 mg/kg. The excretion was no longer detectable at 48 h after any dose. Biliary excretion tended to slow with increasing dose until 8 h, but the total excretion showed no significant difference between the doses (95.7 ± 2.9, 92.2 ± 2.2, 90.2 ± 4.5, and 85.9 ± 6.0% of the dose at 5, 10, 20, and 50 mg/kg, respectively). As can be seen in Fig. 4, the V_B seemed to be saturable with increasing $C_{ss,f}$. Therefore, the V_B data were analyzed according to a Michaelis-Menten type equation (Eq. 1), and $V_{B,max}$ (98.77 μg/min) and K_m (1.83 μg/ml) were estimated by a nonlinear least-squares method,[17] as the analysis by Eq. 1 with a term of linear biliary excretion failed to give converged parameters. The simulated line follows well the experimental points. This suggests that the saturable V_B might account for the dose dependence of CL_B. Figure 5 shows the ratio (R_B) of B_{con}-to-$C_{ss,f}$. The R_B was

approximately 2100—7400 times higher than the range of $C_{ss,f}$, and showed a gradual decrease with increasing $C_{ss,f}$.

Discussion

Previously we confirmed the existence of enterohepatic cycling of GLZ following i.v. administration of 100 mg/kg to rats[13] and the absorption of GLZ from rat intestinal tract.[13,19] In the present study, GLZ was also found to be excreted predominantly in bile. Nevertheless, although enterohepatic cycling may have occurred, it showed no apparent influence on the *AUC* value at any dose, as described already. This may be because the total biliary excretion amount at 5—50 mg/kg is less than that at 100 mg/kg (the excretion ratio is almost constant at *ca.* 80—96% at these doses) and the rat intestinal absorption clearance (CL_{abs}) value is small (0.310 ± 0.021 ml/min).[19] For comparison, the CL_{abs} of salicylic acid in rats is 1.10 ml/min (CL_{abs} 13.6 μl/min/cm[20] × total small intestinal length 80.8 cm[21]), determined by the same method as in the case of GLZ. Also, GLZ might almost be metabolized by rat intestinal bacteria, as the drug is metabolized by human intestinal bacteria.[22] Since the participation of
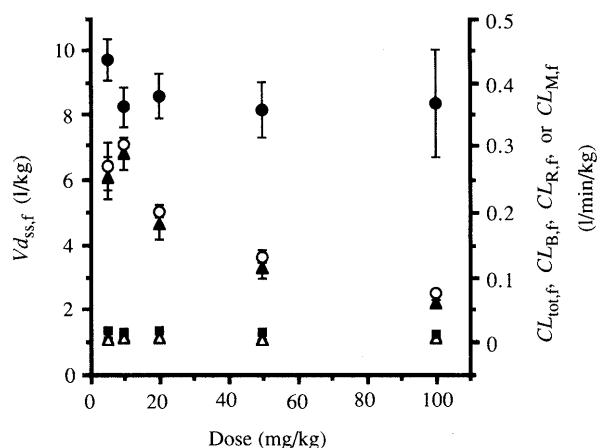
Fig. 6. Pharmacokinetic Parameters for Plasma Unbound Concentration of GLZ at Various i.v. Doses in Rats

Each point represents the mean$\pm$S.E. of four to six rats. ● $Vd_{ss,f}$, steady-state distribution volume; ○ $CL_{tot,f}$, total body clearance; ▲ $CL_{B,f}$, biliary clearance; △ $CL_{R,f}$, renal clearance; ■ $CL_{M,f}$, metabolic clearance.

plasma protein binding in changes in pharmacokinetic parameters was suggested, the nonlinearity in Vd_{ss}, CL_{tot}, and CL_B owing to the increase of f_p was examined by calculating the steady-state distribution volume ($Vd_{ss,f}$) and total body ($CL_{tot,f}$) and biliary ($CL_{B,f}$) clearances for C_f in rats with bile fistulas, as well as renal ($CL_{R,f}$) and metabolic ($CL_{M,f}$) clearances for C_f. The calculated values are shown in Fig. 6, together with those at 100 mg/kg GLZ, which were estimated by using f_p (0.006—0.197) at the observed range of plasma concentrations (2—1600 μg/ml) in rats with bile fistulas.[13] The $Vd_{ss,f}$ values were almost constant at various dose levels. It was found that the distribution of GLZ into tissues is not changed by the increase in dose. Whereas, the $CL_{tot,f}$ and $CL_{B,f}$ decreased gradually with increasing dose. The values of $CL_{B,f}$ were approximately 5—90 times larger than those of $CL_{R,f}$ and $CL_{M,f}$, which showed no significant difference between any doses. Thus, the dose dependency in CL_{tot} was confirmed to be ascribed to that in CL_B. In the case of the 100 mg/kg dose, the mean CL_B value (0.72 ml/min/kg) was smaller than that at 50 mg/kg and the mean CL_R (0.04 ml/min/kg) and CL_M (0.18 ml/min/kg) values were similar to those at 5—50 mg/kg (Table II), respectively. This further supports the idea that the dose dependency in CL_{tot} is due to that in CL_B. The dose dependence of CL_B might be attributed to the saturable V_B (Fig. 4), as described already. Further, it was suggested that an active transport process may be involved in biliary excretion, as R_B was 2100—7400 times higher than the range of $C_{ss,f}$ (Fig. 5).

It has been reported that the total biliary excretion of GLZ was approximately 80% of the i.v. dose (80 mg/man) in a cholangiocarcinomatous patient with liver failure, and the AUC value increased proportionally with i.v. dose (40, 80, and 120 mg/man) in three normal humans.[23] From the data in our previous report, human serum clearances of 0.25—0.36 ml/min/kg in three normal subjects[19] and 0.16—0.45 ml/min/kg in five patients with chronic hepatitis[24] after GLZ i.v. dosing (80 and 200 mg/man, respectively) are calculated on the assumption of 70 kg body

weight. These values are approximately 4—12 times smaller than the mean CL_{tot} value at 5 mg/kg in rats (Table I). This might suggest dose-dependent CL_{tot} at a massive dose (e.g., 200 mg/man) and upon consecutive administrations of GLZ to humans. But dose-dependent Vd_{ss} may not be observed, because GLZ showed uniformly high binding of 99.6% over the observed range of human serum levels (2—60 μg/ml) after i.v. administration (80 and 200 mg/man[25,26]).

In summary, the pharmacokinetics of GLZ in rats is dose-dependent. Such pharmacokinetic properties are likely to be an important factor in the establishment of suitable dosage regimens.

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