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Glycyrrhizin and Licorice Significantly Affect the Pharmacokinetics of Methotrexate in Rats

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Glycyrrhizin (GZ) and licorice (root of *Glycyrrhiza uralensis*) are worldwide food additives and important oriental phytomedicines. This study investigated the biological fate of GZ by orally giving GZ and licorice decoction (LD) to rats. The serum concentrations of GZ and glycyrrhetic acid (GA) were determined by high performance liquid chromatography. The results showed that GZ was not detected and GA was present in serum until 3 days postdosing of GZ and LD. To evaluate the effects of GZ and licorice on the pharmacokinetics of methotrexate (MTX), an important immunosuppressant with a narrow therapeutic window, rats were orally given MTX with and without GZ and LD in different dosage regimens. The serum MTX concentration was determined by fluorescence polarization immunoassay. The results revealed that the AUC and MRT of MTX were significantly increased by GZ and LD. In conclusion, the concurrent use of GZ or licorice with MTX should be with caution.

KEYWORDS: Licorice; glycyrrhizin; glycyrrhetic acid; methotrexate pharmacokinetics; interaction

INTRODUCTION

Licorice (roots of *Glycyrrhiza uralensis* FISCH) and the major constituent—glycyrrhizin (GZ, **Figure 1**) have been widely used as flavoring, sweetening, and foaming agents in foods, beverages, candies, tobacco, and dietary supplements in the U.S., Europe, the Middle East, and Russia. In the United States, the use of licorice extract and GZ as food additives was affirmed by the U.S. Food and Drug Administration (21 CFR 184.1408). More importantly, licorice and GZ are widely used for clinical therapy in China, Japan, Korea, India, and Taiwan. Licorice has been reported to show various beneficial effects, including anti-inflammation (*1*), antivirus (*2*), and liver function improvement (*3*). In addition, GZ, a triterpenoid saponin, has been recognized to be responsible for the anti-inflammation (*4*) and antivirus effects of licorice (*5*, *6*). However, many previous findings were obtained from in vitro studies (*2*, *5*, *6*). Whether those in vitro activities and mechanisms of GZ and licorice can be extended to in vivo situation remains to be clarified by pharmacokinetic study.

Methotrexate (MTX, **Figure 1**), an antifolate agent, anticancer agent, and immunosuppressant, is commonly used for anticancer chemotherapy (*7*), rheumatoid arthritis (*8*), and severe psoriasis (*9*), but with a narrow therapeutic window. The adverse reactions of MTX include nausea, vomiting, diarrhea, and hepatotoxicity (*10*, *11*). Critical life-threatening adverse interactions of MTX with nonsteroidal anti-inflammatory drugs (NSAIDs) including ketoprofen and naproxen, which are substrates of multidrug resistance proteins (MRPs), have been reported (*12*, *13*).

In recent years, there were reports indicating that the resistance and drug interaction of MTX were associated with MRPs, breast cancer resistance protein (BCRP), and organic anion transporters (OATs) (*14*−*16*). On the other hand, GZ has been reported as an inhibitor of MRP2 (*17*), which might result in competitive inhibition on MTX elimination. Nevertheless, there remains unsettled controversy concerning the biological fate of GZ in the literature (*18*−*21*). Therefore, whether the in vitro modulation of MRP2 by GZ could predict the in vivo interaction with MTX requires animal study for clarification. This study first investigated the metabolism and pharmacokinetics of GZ after oral administrations of GZ and licorice decoction (LD) to rats.

Based on preliminary pharmacokinetic studies of GZ and LD, no trace of GZ was detected in rat serum, whereas glycyrrhetic acid (GA, **Figure 1**) was found present until 3 days after oral administration. Because GA was recently reported as an inhibitor of BCRP and MRP2 (*22*), we thus hypothesized that GA might inhibit the elimination of MTX in vivo and possibly enhance its toxicity (*23*, *24*). Therefore, this study investigated the effects of coadministrations of GZ and LD on MTX pharmacokinetics in rats.

MATERIALS AND METHODS

Chemicals. GZ and propylparaben were purchased from Sigma (St. Louis, MO). GA and 2-methylanthraquinone were obtained from Aldrich (Milwaukee, WI). MTX (25 mg/mL) was obtained from Wyeth (Wolfratshausen, Germany). Ethyl acetate, acetonitrile, and methyl

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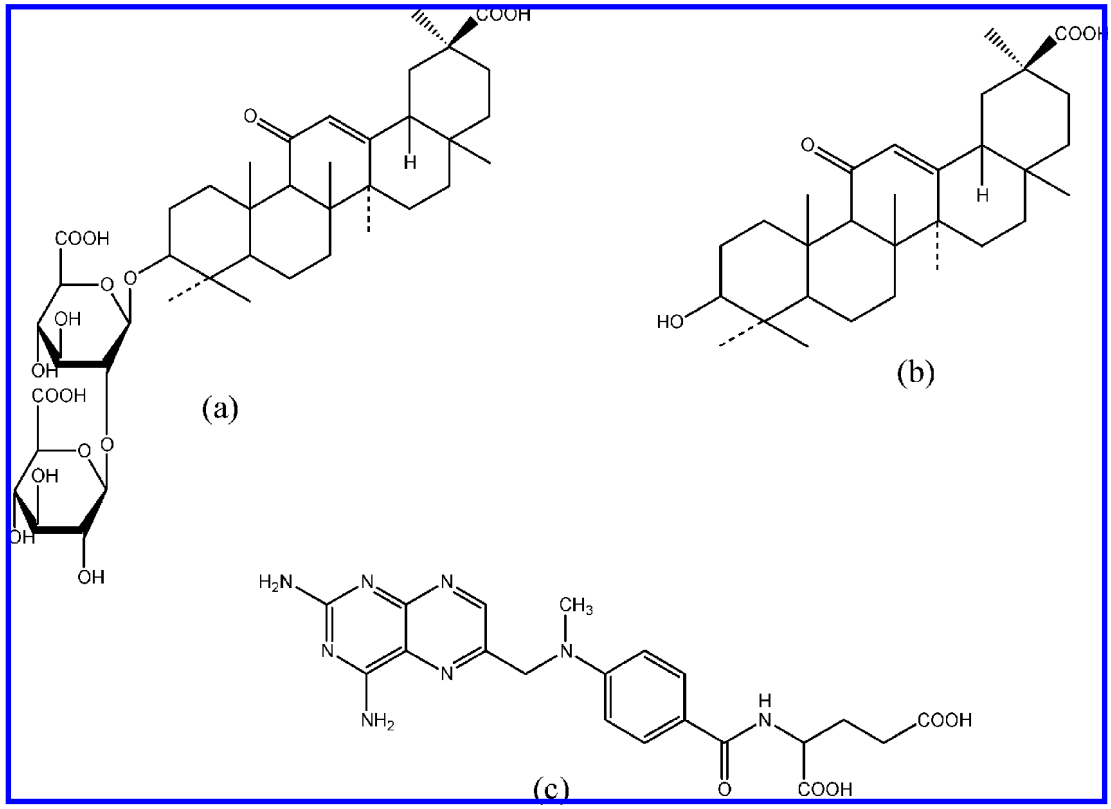

Figure 1. Chemical structures of glycyrrhizin (a), glycyrrhetic acid (b), and methotrexate (c).

alcohol were purchased from Mallinckrodt Baker, Inc. (Phillipsburg, NJ). Sodium acetate was obtained from Kohusan Chemical Works, Ltd. (Tokyo, Japan). L-(+)-Ascorbic acid, and sodium hydroxide solution (1 mol/L) were purchased from Riedel-deHaen AG (Seelze, Germany). Diclofenac was purchased from Yung Shin Pharmaceutical Ind. Co., Ltd. (Taichung, R.O.C.). Milli-Q plus water (Millipore, Bedford, MA) was used for all processes.

Instrumentation. The high-performance liquid chromatography (HPLC) apparatus included a pump (LC-6AD, Shimadzu, Japan), an UV spectrophotometric detector (SPD-6A, Shimadzu, Japan), a chromatopac (C-R6A, Shimadzu, Japan), and an autosampler (Series 200, Perkin-Elmer, U.S.A.). A RP-18e column (LiChrospher, 250 mm × 4.0 mm) was employed with a prefilter (Isolation Technologies, U.S.A.).

Preparation and Quantitation of GZ in LD. Licorice was purchased from a Chinese drugstore in Taichung, Taiwan. Sixty grams of licorice were soaked in 2.4 L of distilled water for 30 min and then heated to boiling on a gas stove. The mild boiling was maintained to concentrate the volume until less than 100 mL remained. The marc was filtered, and the filtrate was added with water to make 100 mL and stored at −20 °C for later use. The filtrate (300 μL) was mixed with 700 μL of methanol, and then the mixture was vortexed and centrifuged at 10,000*g* for 15 min. The supernatant (200 μL) was diluted with 1.0 mL of methanol and then mixed with an equal volume of an internal standard solution (100 μg/mL of propylparaben in methanol). A volume of 20 μL was subjected to HPLC analysis. The mobile phase was acetonitrile/1% acetic acid (30:70) and run isocratically. The flow rate was 1.0 mL/min with the detection wavelength set at 248 nm.

Animals and Drug Administration. All animal experiments adhered to "The Guidebook for the Care and Use of Laboratory Animals (2002)" (Published by the Chinese Society of Animal Science, Taiwan, R.O.C.). Male Sprague−Dawley rats (350 − 450 g) were supplied by the National Laboratory Animal Center (Taipei, Taiwan) and kept at least 1 week under a conditioned environment with free access to food and water. Before experiments, rats were fasted overnight, but drinking water was allowed ad libitum. Food was supplied 3 h after dosing. In pharmacokinetic studies, rats were orally administered with 150 mg/6 mL/kg of GZ ($n = 6$) and 12.5 mL/kg of LD containing 150 mg/kg of GZ ($n = 5$) by gastric gavage. In drug interaction studies, the dosage regimens of GZ and LD in 1−7 groups are listed in **Table 1**. Of the seven groups, rats were given MTX (5 mg/kg) orally after the administration of a single dose or after the seventh dose of GZ and LD. For the seven-dose pretreatment, GZ and LD were orally administered to rats twice daily. The five rats in the eighth group were given diclofenac (as a positive control) before dosing with MTX (5 mg/kg).

Table 1. Dosage Regimens of GZ and LD before Methotrexate Dosing[a]

treatment groups	dose of GZ or LD	no. of dosings
1	0	0
2	75 mg/kg of GZ	1
3	150 mg/kg of GZ	1
4	LD containing 75 mg/kg of GZ	1
5	LD containing 150 mg/kg of GZ	1
6	150 mg/kg of GZ	7
7	LD containing 150 mg/kg of GZ	7

[a] Six rats in group 1 were given 5 mg/kg of MTX with water as the control. Each treatment group contained six rats.

Serum Sample Collection. Rats were anesthetized with ether, and the blood was withdrawn via cardiac puncture. In pharmacokinetic studies, the blood samples (1.0 mL) were collected from each rat at 1, 3, 6, 8, 9, 12, 24, 32, 48, 56, 72, and 80 h after administration of GZ and LD. During the experiment, rats were supplemented with 3 mL of water at 5 and 10 h via gastric gavage to avoid hypovolumic shock. For drug interaction studies, blood samples (0.4 mL) were withdrawn from each rat at 15, 30, 60, 120, 240, 480, 720, 1440, 1980, 2880, 3420, and 4320 min after administration of MTX. The sera were collected by centrifuging at 10,000*g* for 15 min to obtain serum and stored at −30 °C until analysis.

Determination of GZ and GA in Serum. Analysis of GZ and GA in serum followed the methods reported in a previous study (*20*). For the determination of GZ, to 200 μL of serum was added 800 μL of methanol containing 0.12 μg/mL of propylparaben as the internal standard. The mixture was centrifuged, and the supernatant was evaporated to dryness by blowing nitrogen. The residue was reconstituted with 50 μL of mobile phase (acetonitrile/1% acetic acid = 30:70), of which 20 μL was subject to HPLC analysis. For the analysis of

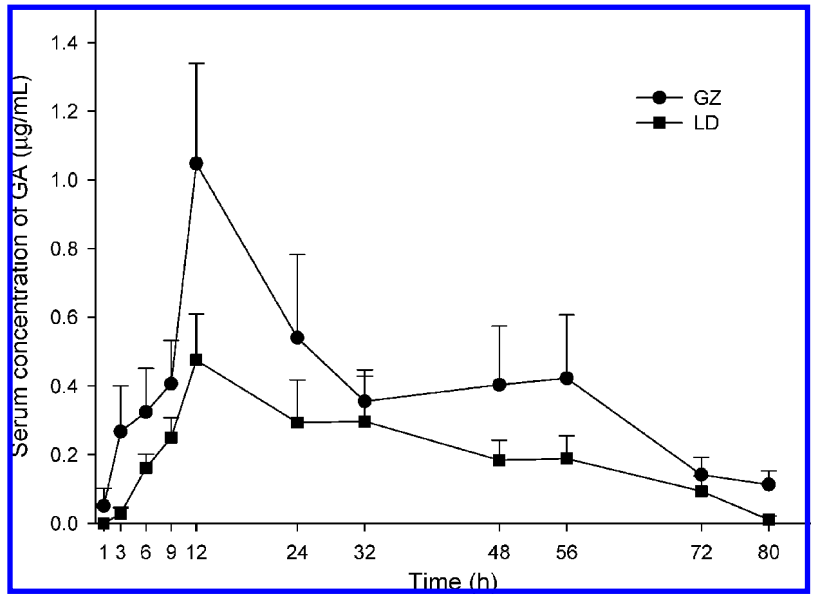

Figure 2. Mean (±S.E.) serum concentration—time profiles of glycyrrhetic acid after oral administration of glycyrrhizin (GZ, 150 mg/kg) and licorice decoction (LD, containing 150 mg/kg of GZ) to rats.

GA, 300 μL of serum and 100 μL of 0.1N HCl was mixed and then partitioned with 400 μL of ethyl acetate containing 0.1 μg/mL of 2-methylanthraquinone as the internal standard. After centrifugation, the supernatant was evaporated to dryness by blowing nitrogen. The residue was reconstituted with 50 μL of mobile phase (acetonitrile/1% acetic acid = 60:40), of which 20 μL was subject to HPLC analysis. For both HPLC methods, the detection wavelength was set at 248 nm and the flow rate was 1.0 mL/min and was run isocratically.

Validation of Assay Methods. The precision and accuracy of the assay methods were evaluated by intraday and interday analysis of triplicates at concentrations of 125, 250, 500, 1000, 2000, and 4000 μg/mL of GZ, and 0.25, 0.31, 0.50, 0.63, 1.00, 1.25, and 2.50 μg/mL of GA in serum over a period of 3 days. A lower limit of quantitation (LLOQ) represents the lowest concentration of analyte in a sample that can be determined with acceptable precision and accuracy, whereas the limit of detection (LOD) represents the lowest concentration of analyte in a sample that can be detected (with S/N > 3).

Determination of MTX Concentration in Serum. The serum concentration of MTX was analyzed by a fluorescence polarization immunoassay (Abbott, Abbott Park, IL). All the procedures, calibration curves, and validations followed those described in the kit. The calibration range was 0−1.0 μmol/L, and the LOQ was 0.02 μmol/L.

Calculation of Pharmacokinetic Parameters. Pharmacokinetic parameters were calculated by the noncompartment model of WinNonlin (version 1.1 SCI software, Statistical Consulting, Inc., Apex, NC). The peak serum concentration (C_{max}) and the time to peak concentration (T_{max}) were calculated based on experimental measurement. The areas under the curves (AUC_{0-t}) from time zero to last were calculated by the trapezoidal rule. Unpaired Student's *t*-tests and one-way ANOVA were used for statistical comparisons for pharmacokinetic and the interaction studies, respectively.

RESULTS

The calibration curve of GZ showed good linearity (r > 0.99) in the range 125−4000 μg/mL. The precision evaluation showed that all coefficients of variation were below 7.1%, and the accuracy analysis showed that the relative errors to the true concentration were below 6.3%. The quantitation results showed that the concentration of GZ in LD was 12 mg/mL, and only a trace of GA was present, but below the LLOQ.

In the quantitation of GA in serum, the calibration curve showed good linearity (r > O0.99) in the range 0.25−2.5 μg/mL. The precision evaluation showed that all coefficients of variation were below 14.9%, and the accuracy analysis showed that the relative errors to the true concentrations were below 12.0%. The LOD of GA was 0.125 μg/mL. After giving GZ and LD, no trace of GZ was detected in serum and GA was found. The mean serum concentration—time profiles of GA after administration of GZ and LD are shown in **Figure 2**, and the pharmacokinetic parameters are listed in **Table 2**. The results indicated that GA emerged gradually following administration of GZ and LD, and the T_{max} values of GA were 20.7 and 15.4 h, respectively. The C_{max} and AUC_{0-t} of GA after giving GZ were significantly higher than those after giving LD containing an equivalent amount of GZ by 124% and 86%, respectively.

Table 2. Pharmacokinetic Parameters of Glycyrrhetic Acid after Oral Administrations of Licorice Decoction (Containing 150 mg/kg of Glycyrrhizin) and Pure Compound Glycyrrhizin (150 mg/kg)[a]

	treatment		
parameters	LD	GZ	difference (%)
T_{max}	15.4 ± 4.2	20.7 ± 7.4	34
C_{max}	0.6 ± 0.1	1.3 ± 0.2[b]	124
AUC_{0-t}	17.4 ± 4.4	32.4 ± 4.3[b]	86
MRT	28.4 ± 2.7	28.6 ± 3.9	0.6

[a] Data expressed as mean ± S.E. LD: licorice decoction. GZ: glycyrrhizin. T_{max} (h): time to peak concentration. C_{max} (μg/mL): peak serum concentration. AUC_{0-t} (μg·h/mL): areas under the curves from time zero to the last point. MRT (h): mean residence time. [b] $p < 0.05$.

In drug interaction studies, the mean serum concentration—time profiles of MTX after giving MTX alone and coadministered with diclofenac as well as various combination treatments with GZ and LD are shown in **Figures 3** and **4**. MTX was absorbed rapidly, and the mean T_{max} was within the first hour. In the control group, the MTX concentration fell below the LLOQ after 12 h postdosing. In contrast, when MTX was coadministered with diclofenac, GZ, or LD, the serum MTX concentration could be quantitated up to 33 h. The pharmacokinetic parameters of MTX after giving MTX alone and various combination treatments are listed in **Table 3**.

When MTX was coadministered with diclofenac, the C_{max}, AUC_{0-t}, and MRT of MTX were significantly enhanced by 64%, 324%, and 185%, respectively. Following coadministrations of 75 and 150 mg/kg of GZ, the AUC_{0-t} of MTX were significantly increased by 161% and 207%, respectively, whereas the MRT

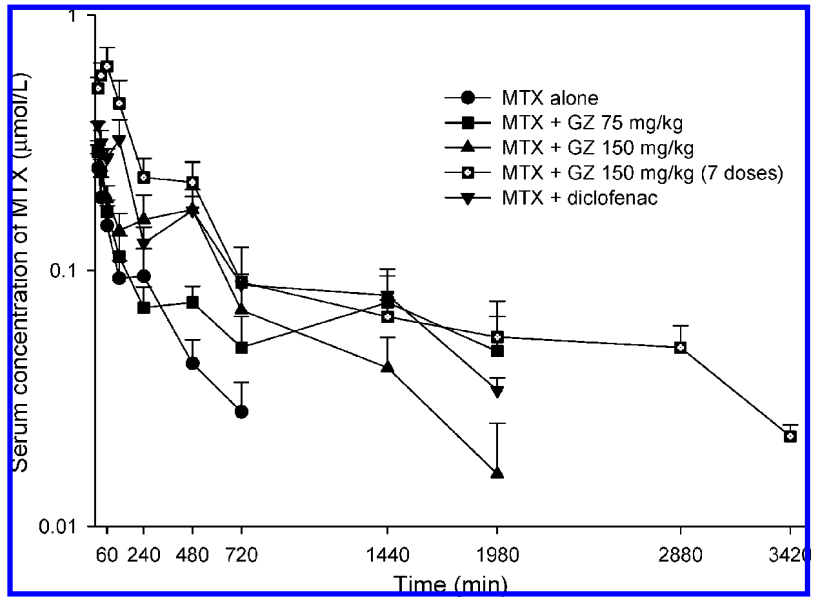

Figure 3. Semilog serum concentration−time profiles of MTX (mean ± S.E.) after oral administration of MTX (5 mg/kg) (●) alone and coadministration with a single dose of 75 mg/kg (■) or 150 mg/kg (▲) of GZ or seven doses of 150 mg/kg GZ (□) and 25 mg/kg of diclofenac (▼).

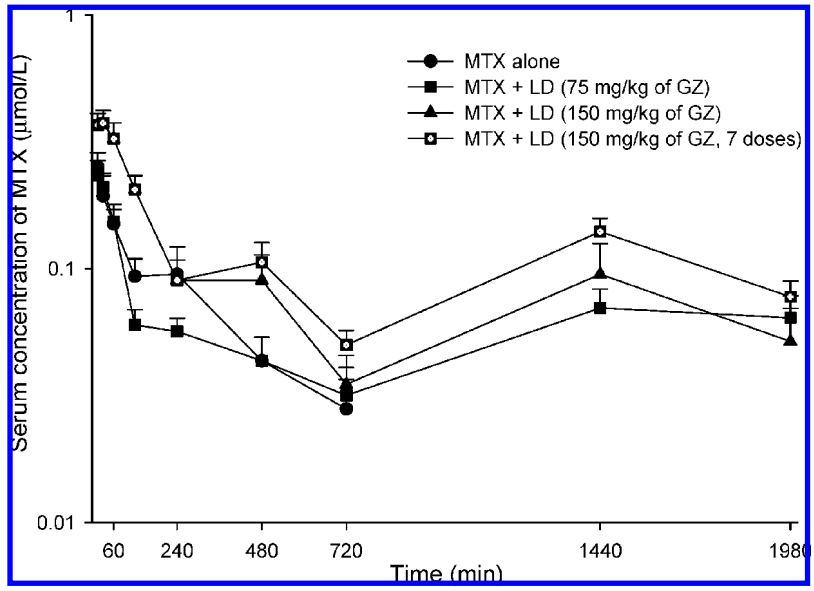

Figure 4. Semilog serum concentration−time profiles of MTX (mean ± S.E.) after oral administration of MTX (5 mg/kg) (●) alone and coadministration with a single dose of LD containing 75 mg/kg (■) or 150 mg/kg (▲) of GZ and seven doses of LD (□, containing 150 mg/kg of GZ).

was significantly prolonged by 177% at the dose of 75 mg/kg of GZ. Following coadministration with LD containing 75 and 150 mg/kg of GZ, the AUC_{0-t} of MTX were significantly increased by 264% at the higher dose, whereas the MRT were significantly prolonged by 283% and 172%, respectively.

After pretreatment with seven doses of GZ (150 mg/kg), the C_{max}, AUC_{0-t}, and MRT of MTX were significantly increased by 176%, 480%, and 193%, respectively. The magnitude of the increase of C_{max} and AUC_{0-t} after a seven-dose pretreatment of GZ was more pronounced than that caused by a single dose. In regard to the seven-dose pretreatment of LD containing 150 mg/kg of GZ, the AUC_{0-t} and MRT of MTX were significantly increased by 399% and 280%, respectively. In comparison to the effect caused by single- dose coadministration of LD, the magnitude of the interaction showed no significant difference between two treatment groups.

DISCUSSION

In this study, when rats were orally administered with a single dose of GZ and LD, GA was present for more than 3 days in the circulation, whereas no trace of GZ was detected in the blood. This finding is consistent with a previous report of rats (*19*); nonetheless, it contradicts an earlier study which found both GZ and GA in rat blood after giving GZ orally (*18*). However, a pharmacokinetic study of GZ in humans indicated that GZ was hydrolyzed by the intestinal bacteria and then absorbed into blood only in the form of GA (*25*). On the other hand, our present result does not conform to what we had found in rabbits (*20*, *21*), revealing that both GZ and GA were present in the serum after giving GZ and LD. This discrepancy may be due to animal species differences. Accordingly, the biological fate of GZ in humans was consistent with our findings in rats rather than rabbits.

Table 3. Pharmacokinetic Parameters of Methotrexate after Oral Administration of Methotrexate (5 mg/kg) Alone and Combined Treatments with Glycyrrhizin, Licorice Decoction, and Diclofenac

	parameters		
treatment	C_{max}	AUC_{0-t}	MRT
methotrexate alone	$0.3 \pm 0.0_3^a$	52.0 ± 17.6^a	240.2 ± 025.4^a
+ GZ (75 mg/kg)	$0.3 \pm 0.0_3^a$	135.7 ± 31.3^b	664.9 ± 181.1^b
+ GZ (150 mg/kg)	$0.3 \pm 0.0_4^a$	159.5 ± 20.8^b	554.8 ± 071.5^{ab}
+ LD (75 mg/kg of GZ)	$0.2 \pm 0.0_3^a$	118.3 ± 11.2^{ab}	920.5 ± 079.7^b
+ LD (150 mg/kg of GZ)	$0.4 \pm 0.0_4^b$	189.2 ± 41.0^b	654.2 ± 152.3^b
+ GZ (150 mg/kg)	$0.3 \pm 0.0_4^a$	159.5 ± 20.8^a	554.8 ± 071.5^{ab}
+ GZ (7 doses, 150 mg/kg)	0.7 ± 0.1^b	301.6 ± 54.2^b	703.5 ± 148.2^b
+ LD (150 mg/kg of GZ)	$0.4 \pm 0.0_4^b$	189.2 ± 41.0^b	654.2 ± 152.3^b
+ LD (7 doses, 150 mg/kg of GZ)	$0.3 \pm 0.0_4^{ab}$	259.3 ± 25.0^b	912.7 ± 094.3^b
+ diclofenac (25 mg/kg)	$0.4 \pm 0.0_3^b$	220.6 ± 25.7^b	648.5 ± 027.1^b

LD: licorice decoction. GZ: glycyrrhizin. C_{max} (μmol/L): peak serum concentration. AUC_{0-t} (μmol·min/L): areas under the curves from time zero to the last point. MRT (min): mean residence time. Groups in each section were statistically compared with the control group given methotrexate alone. Values are means ± S.E. Means in a column without a common superscript differ, $p < 0.05$.

Therefore, rats appear to serve as a proper animal model for studies related to GZ and LD.

After ingestion of GZ and LD, GA gradually emerged in blood. The T_{max} of GA was later than 15 h, and the MRT was 28 h. This delayed absorption of GZ was rather different from the biological fates of dietary flavonoid glycosides (*26*), which were generally absorbed rapidly and present in the circulation as glucuronides and sulfates of their aglycones (*27*). This discrepancy might stem from the differences of the sugar linkage to the aglycone. GZ, possessing diglucuronic acid on a secondary alcohol group, might be less easily hydrolyzed in vivo to form the absorbable aglycone GA than flavonoids. Comparison of the C_{max} and AUC_{0-t} of GA listed in **Table 2** clearly indicated that the bioavailability of GA from pure compound GZ was higher than that of LD, which could be accounted for by the fact that more GA had been produced by enterobacteria from pure compound GZ than GZ in LD (*21*). Based on our finding that GZ was not present in blood, we suggest that the in vitro activities of GZ and licorice make it difficult to predict their in vivo effects and mechanisms (*2, 5, 6, 28*). Instead, GA is the right target for in vitro study to explore the in vivo activities of GZ and licorice.

Our interaction study revealed that coadministration of a single dose of 75 and 150 mg/kg of GZ significantly enhanced the systemic exposure and mean residence time of MTX. The magnitudes of interaction were comparable between two doses. In contrast, coadministrations of single doses of LD containing 75 and 150 mg/kg of GZ significantly increased the systemic exposure and mean residence time of MTX. The magnitudes of interaction were also comparable between two doses. These facts imply that the interaction mechanism was saturated at the lower dose of GZ and LD.

In order to reach the steady state, seven doses of GZ and LD were given before MTX dosing. The results showed that a seven-dose pretreatment of GZ increased the systemic exposure and mean residence time of MTX more drastically than single-dose coadministration. However, seven-dose pretreatment of LD exerted comparable increases of systemic exposure and mean residence time with single dose coadministration of LD. Moreover, seven-dose pretreatment of GZ appeared to exert a higher magnitude of interaction with MTX than similar pretreatment of LD. This difference between GZ and LD can be explained by the fact that GZ resulted in higher GA blood levels than LD.

Despite the in vitro inhibition of MRP2 by GZ (*17*), based on pharmacokinetic studies of GZ in rats and humans (*19, 25*), it is evident that the causative agent to alter MTX pharmacokinetics was not GZ. In light of recent findings that GA inhibited the transport mediated by BCRP and MRP2 (*22*), we speculate that the delayed elimination of MTX may be caused by GA, which will be confirmed by a transport study using a cell model in the near future.

Upon observing the profiles shown in **Figures 3** and **4**, the curves of MTX following coadministration with GZ and LD started to diverge from the control profile after 480 min, indicating that the inhibited elimination of MTX appeared in good correlation with the increasing serum level of GA, as shown in **Figure 2**. In comparison to the diclofenac−MTX interaction, the influences on MTX pharmacokinetics by GZ and LD were comparable to that caused by diclofenac, a positive control which modulated the transport of MTX by inhibiting MRPs (*16*). In addition to MRPs, OATs have been reported to be associated with the influx of MTX (*14, 16*) and could be modulated by anionic chemicals (*29*). The pK_a of GA is 5.56 (*30*), and it largely dissociated to anion in the physiological environment. Therefore, GA may also compete with MTX for the transport mediated by OATs.

When rats were pretreated with seven doses of GZ and LD, the serum levels of MTX were not only elevated in the elimination phase but also markedly enhanced in the absorption phase. This phenomenon may result from the accumulated concentration of GA in rats post-seven-dose pretreatment of GZ or LD, which might inhibit MTX efflux transport mediated by BCRP and MRPs to gut lumen (*15, 16*). The C_{max} of MTX after seven-dose pretreatment of GZ was higher than that following seven-dose pretreatment of LD by 128%. This difference could be explained by the higher serum level of GA after intake of GZ, which might lead to greater inhibition on MTX efflux to the intestine lumen.

In addition to the pharmacokinetic aspect, GA was reported to inhibit 11 β-hydroxysteroid dehydrogenase type 2, an enzyme transforming cortisol to inactive cortisone (*31*). This inhibition would lead to sodium retention and antidiuresis, which in turn might result in reduced excretion of MTX.

Based on the finding of our interaction study, when patients are given low doses of MTX for treating rheumatoid arthritis or psoriasis, concurrent intake of GZ or licorice may lead to increased systemic exposure and longer mean residence time of MTX, which might result in higher efficacy or toxicity. Besides, the pharmacokinetics of other acidic pharmaceuticals which were putative substrates of MRPs and BCRP may also be affected by concurrent use of GZ or licorice. However, on the other hand, for patients developing drug resistance after receiving chemotherapy with MTX, the combined use of GZ or licorice may be beneficial for the reversal of drug resistance.

In conclusion, the systemic exposure and mean residence time of MTX were significantly increased by concurrent intake of GZ and LD. The concurrent use of GZ or licorice with acidic drugs such as MTX should be with caution.

ABBREVIATIONS

GZ, glycyrrhizin; MTX, methotrexate; LD, licorice decoction; GA, glycyrrhetic acid; NSAIDs, nonsteroidal anti-inflammatory drugs; HPLC, high-performance liquid chromatography; BCRP,

breast cancer resistance protein; MRPs, multidrug resistance proteins; OATs, organic anion transporters; LLOQ, lower limit of quantitation; LOD, limit of detection; C_{max}, the peak serum concentration; t_{max}, the time to peak concentration; AUCs, the areas under the curves; MRT, mean residence time.

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