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Pharmacokinetic and pharmacodynamic profiles of the antitussive principles of *Glycyrrhizae radix* (licorice), a main component of the Kampo preparation Bakumondo-to (Mai-men-dong-tang)

Junzo Kamei[a,*], Akiyoshi Saitoh[a], Takayuki Asano[b], Rie Nakamura[c], Hiroyuki Ichiki[c], Akira Iiduka[d], Masayoshi Kubo[d]

[a]*Department of Pathophysiology and Therapeutics, School of Pharmacy and Pharmaceutical Sciences, Hoshi University, 4-41, Ebara 2-chome, Shinagawa-ku, Tokyo 142-8501, Japan*

[b]*Medicinal Evaluation Laboratory, Tsumura and Co., Ibaraki, 300-1192, Japan*

[c]*Pharmacognosy and Medicinal Resources Laboratory, Tsumura and Co., Ibaraki, 300-1192, Japan*

[d]*Botanical Drug Development Dept., China Division, Tsumura and Co., Tokyo, 102-8422, Japan*

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Abstract

We examined the pharmacokinetic and pharmacodynamic properties of liquiritin apioside, a main antitussive component of *Glycyrrhizae radix* (licorice), with regard to its antitussive effect in guinea pigs. The peak plasma concentration of the unchanged compound was observed 15 min after the administration of liquiritin apiosaide. The plasma concentration then gradually decreased and was almost undetectable 4 h after administration. Liquiritigenin, a des-glycoside of liquiritin apioside, appeared in the plasma 2 h after the administration of liquiritin apioside and remained for more than 6 h after administration. The plasma concentration of unchanged liquiritigenin was observed 15 min after administration and then gradually increased for more than 6 h after administration. When the antitussive effects of liquiritin apioside, liquiritin and liquiritigenin, at respective doses of 30 mg/kg, p.o., were examined 1 h after administration, liquiritin apioside and liquiritigenin caused a significant reduction in the number of capsaicin-induced coughs. However, at the same dose, liquiritin had no significant effect on the number of capsaicin-induced coughs. On the other hand, when the antitussive effects of liquiritin apioside, liquiritin and liquiritigenin, at doses of 30 mg/kg, p.o., were examined 4 h after administration, each caused a more than 40% reduction in the number of capsaicin-induced coughs. The present results suggest that *G. radix* (licorice) may produce a persistent antitussive effect, and that liquiritin apioside plays an important role in the earlier phase, while liquiritigenin, which is a metabolite of liquiritin apioside and liquiritin, plays an important role in the late phase.

Keywords: *Glycyrrhizae radix*; Bakumondo-to (Mai-men-dong-tang); Antitussive effect; Liquiritin apioside; Liquiritin; Liquiritigenin

1. Introduction

Bakumondo-to (TJ-29: Mai-men-dong-tang) has been used to treat severe dry cough in patients with bronchitis and pharyngitis (Asano et al., 1993; Takahama and Miyata, 1995). *Glycyrrhizae radix* (licorice) is a main component of Bakumondo-to. It has been used since ancient Egyptian times as a drug for the respiratory organs and has long been used as a flavoring and sweetening agent as well as an analgesic and expectorant in Europe. Furthermore, *G. radix* has been used as a very important crude drug in many Kampo preparations (traditional Chinese medicine) and acts as an antispasmodic, carminative and antidote, and is also taken for bronchial problems, coughs, mucous congestion, stomach problems, such as peptic ulcers, and for bladder and kidney ailments.

* Corresponding author. Tel.: +81 3 5498 5030; fax: +81 3 5498 5029.
E-mail address: kamei@hoshi.ac.jp (J. Kamei).

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Recently, we demonstrated that *G. radix* contained a potent antitussive compound: liquiritin apioside (Kamei et al., 2003). However, liquiritin apioside is hydrolyzed under weakly acidic conditions and is converted into liquiritin (Homma et al., 1997). In turn, liquiritin is metabolized to liquiritigenin, a des-glycoside of liquiritin, and to davidigenin, a hydrogenated metabolite of liquiritigenin (Homma et al., 1992, 1997). Furthermore, it has been reported that the urinary secretion of liquiritigenin and davidigenin in patients with bronchial asthma who have been effectively treated with Bakumondo-to was higher than those in subjects in whom this drug had no effect (Tamaki and Niitsuma, 1999). Based on these findings, they suggested that none of the pharmacological effects of Bakumondo-to are due to the direct action of its constituent components, like liquiritin apioside, while its metabolic products are thought to play a role. Identification of the biologically active components that provide satisfactory properties with respect to pharmacokinetic and pharmacodynamic behavior might help to clarify the effect of *G. radix*, a crude drug.

Thus, in the present study, we examined the pharmacokinetic and pharmacodynamic properties of liquiritin apioside with regard to its antitussive effects in guinea pigs.

2. Materials and methods

2.1. Animals

Male Hartley guinea pigs (Tokyo Animal Laboratory, Tokyo, Japan), weighing about 300–350 g, were used. The animals were housed in groups of four per cage under a 12-h light–dark cycle with food and water available continuously. This study was carried out in accordance with the guide for the care and use of laboratory animals as adopted by the committee on the care and use of laboratory animals of Hoshi University, which is accredited by the Ministry of Education, Science, Sports and Culture.

2.2. Antitussive assay

The cough reflex was induced as described previously (Kamei et al., 1989; Kamei and Kasuya, 1992). Briefly, animals were exposed to a nebulized solution of capsaicin (30 μM) under conscious and identical conditions using a body-plethysmograph. Capsaicin was dissolved to a concentration of 30 mg/ml in a 10% ethanol and 10% Tween 80 saline solution. The solution was diluted with saline. The animals were exposed for 7 min to capsaicin 60 min before the injection of drugs to determine the frequency of control coughs. The animals were also exposed for 7 min to capsaicin 60 or 240 min after the injection of drugs. The number of coughs produced after antitussive drug injection was compared with the number of control coughs. The antitussive effect was expressed as the % inhibition of the number of control coughs [(number of control coughs-number of coughs produced after antitussive drug injection)/number of control coughs×100].

2.3. Isolation of compounds from G. radix

The air-dried root of *Glycyrrhiza uralensis* (5.0 kg) was crushed and extracted twice with H_2O under reflux. The water extract was suspended in 30 l of water and chromatographed on Diaion HP20 (Mitsubishi Kasei, Tokyo, Japan), eluting with 20 l of water (235 g), 20 l of 50% methanol (138 g) and then 20 l of 100%methanol (115 g).

The 50% methanol extract was further chromatographed on silica gel (eluted with $CHCl_3/CH_3OH/H_20$=10:3:1) to give three fractions as checked by TLC, and then subjected to C_{18} column chromatography (500 g, Cosmosil 140 C_{18}-OPN, Nakarai Chemicals, Japan), eluting with H_2O/CH_3CN (4:1) to obtain liquiritin (3.9 g) and liquiritin apioside (726 mg). The 100% methanol extract was further chromatographed on a silica gel column with gradient elution with $CHCl_3/CH_3OH$(4:1?1:1) and then subjected to reverse-phase column chromatography (TSK gel ODS-80Ts, TOSOH), eluting with H_2O/CH_3CN(4:1) to obtain liquiritigenin (250 mg). Liquiritin, liquiritin apioside and liquiritigenin were identified by comparison with the spectral data reported previously (Nakanishi et al., 1985; Aida et al., 1989; Kobayashi et al., 1995).

2.4. Blood sample collection

Blood samples were collected from polyethylene tubes cannulated in the carotid artery of guinea pigs at 0 (predosing), 0.25, 0.5, 1, 2, 4 and 6 h. Plasma samples were prepared by centrifugation at 3500 rpm for 10 min and stored below 20 °C until analysis.

2.5. Enzyme hydrolysis

Concentrations of liquiritigenin and davidigenin were determined in enzyme-hydrolyzed plasma samples obtained at 1 and 4 h after administration. An aliquot of plasma sample (100 μl) was mixed with 100 μl of enzyme solution containing 10 mg of β-glucuronidase Type-H1 (Sigma) in 1.5 ml of 200 mmol/l sodium acetate buffer (pH 5.0). The mixture was incubated at 37 °C for 5 h.

2.6. Analytical method

The concentration of each compound in a plasma sample was determined by high-performance liquid chromatography (HPLC) equipped with a multichannel UV-detector. 7-Ethoxycoumarin (for liquiritigenin and davidigenin) and 4-hydroxymethyl-7-methoxycoumarin (for liquiritin and liquiritin apioside) were used as internal standards. The calibration curves for HPLC analyses were prepared daily using 50, 100, 500, 1000 and 5000 ng/ml solutions of each compound in blank guinea pig plasma.

One hundred and 1000 ng/ml solutions were used as quality-control samples.

One ml of CH_3OH, 10 μl of acetic acid and internal standards solution were added to 100 μl of plasma samples. This mixture was vortexed and centrifuged. The supernatant was dried under a N_2 stream. The precipitate was dissolved in 150 μl of HPLC mobile phase and filtered by Ultrafree® C3-LH (0.5 μm, Millipore). The HPLC system used in this study consisted of a solvent delivery pump (LC10A, Shimadzu, Kyoto, Japan), a column oven (CTO10A, Shimadzu), a UV detector (SPD10A, Shimadzu) and an analytical column (Waters Symmetry C18, 4.6 mm I.D.×150 mm, Waters). The mobile phase was a mixture of 0.05 mol/l ammonium acetate buffer (pH 3.6) and acetonitrile at a flow rate of 1 ml/min with a column temperature of 40 °C. The mobile phase mixtures used for the isolation of liquiritigenin and davidigenin were 0.05 mol/l ammonium acetate buffer (pH 3.6) and acetonitrile (7:3 at 20 min, 3:7 at 25 min or 7:3 at 30 min, v/v). The mobile phase mixtures used for isolation of liquiritin apioside and liquiritin were 0.05 mol/l ammonium acetate buffer (pH 3.6) and acetonitrile (82:18 at 15 min, 3:7 at 20 min or 82:18 at 250 min, v/v). The detection wave was set at 277 nm for the isolation of liquiritigenin and davidigenin, and 312 nm for the isolation of liquiritin apioside and liquiritin.

2.7. Drugs

Liquiritin apioside, liquiritin, liquiritigenin and davidigenin were suspended in 0.5% carboxyl methylcellulose solution. A feeding probe was used when drugs and their vehicle were given p.o. Dosing solution (30 mg/kg/10 ml) was orally administered to guinea pigs that had been fasted for 12 h prior to administration.

2.8. Pharmacokinetic analysis and statistical analysis

Data are expressed as the means±S.E.M. Pharmacokinetic parameters of each compound were estimated by WinNonlin® (Pharsight). The statistical significance of differences was assessed by the Mann–Whitney *U*-test to evaluate the antitussive effect. A level of probability of 0.05 or less was considered significant.

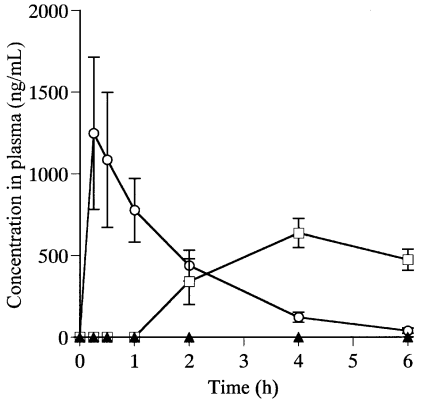

Fig. 1. Plasma concentration–time curves of liquiritin apioside (○), liquiritigenin (□) and davidigenin (▲) after the oral administration of liquiritin apioside (30 mg/kg) in guinea pigs. Each point represents the mean with S.E. for five animals in each group.

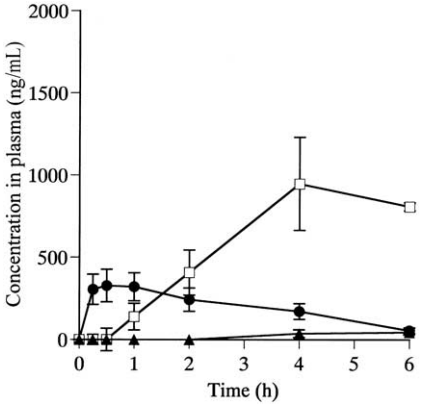

Fig. 2. Plasma concentration–time curves of liquiritin (●), liquiritigenin (□) and davidigenin (▲) after the oral administration of liquiritin (30 mg/kg) in guinea pigs. Each point represents the mean with S.E. for five animals in each group.

3. Results

3.1. Plasma concentration–time curves after the oral administration of liquiritin apioside, liquiritin and liquiritigenin

The plasma liquiritin apioside, liquiritigenin and davidigenin concentration–time curves after the oral administration of liquiritin apioside are shown in Fig. 1. The peak plasma concentration of unchanged compound was observed 15 min after administration of liquiritin apiosaide. The plasma concentration then gradually decreased and was almost undetectable at 4 h after administration. Liquiritigenin, a des-glycoside of liquiritin apioside, appeared in the plasma 2

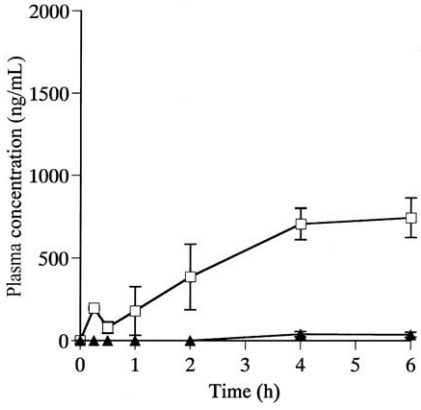

Fig. 3. Plasma concentration–time curves of liquiritigenin (□) and davidigenin (▲) after the oral administration of liquiritigenin (30 mg/kg) in guinea pigs. Each point represents the mean with S.E. for five animals in each group.

Table 1
Pharmacokinetic parameters of liquiritin, liquiritin apioside and liquiritigenin in guinea pigs after oral administration of liquiritigenin and its glycosides

Administered compounds	Observed compounds	t_{max} (h)	C_{max} (ng/ml)	AUC (ng · h/ml)	$T_{1/2}$ (h)
Liquiritin	Liquiritin	1.3±1.8	403.1±142.1	1169.7±546.2	2.2±1.6
Liquiritin apioside	Liquiritin apioside	0.5±0.3	1442.8±877.6	2179.3±789.4	1.5±0.6
Liquiritin	Liquiritigenin	5.5±1.0	1037.8±498.9	3449.3±667.3	N.C.
Liquiritin apioside	Liquiritigenin	3.1±2.3	373.8±347.7	2302.6±925.1	8.3±4.7
Liquiritigenin	Liquiritigenin	4.8±1.1	825.4±253.0	2949.7±1357.8	11.4±1.8

Data represent the mean with S.D. for five animals in each group. N.C.—Not calculable. AUC was calculated by plasma concentrations higher than lower limit of quantitation until 6 h after administration.

h after the administration of liquiritin apioside and remained for more than 6 h after administration. The plasma concentration of a presumed metabolite of liquiritin apioside, davidigenin, was lower than the limit of detection (50 ng/ml).

The plasma liquiritigenin and davidigenin concentration–time curves after the oral administration of liquiritin are shown in Fig. 2. The peak plasma concentration of unchanged compound was observed 15 min after the administration of liquiritin. The plasma concentration then gradually decreased and was almost undetectable 4 h after administration. Liquiritigenin, a des-glycoside of liquiritin, appeared in the plasma 1 h after the administration of liquiritin and remained for more than 6 h after administration. The time vs. plasma concentration curve of liquiritigenin is similar to that after the administration of liquiritin apioside. The concentration of davidigenin in plasma was lower than the limit of detection (50 ng/ml).

As shown in Fig. 3, unchanged liquiritigenin was first noted in plasma at 15 min after the administration of liquiritigenin, and the concentration then gradually

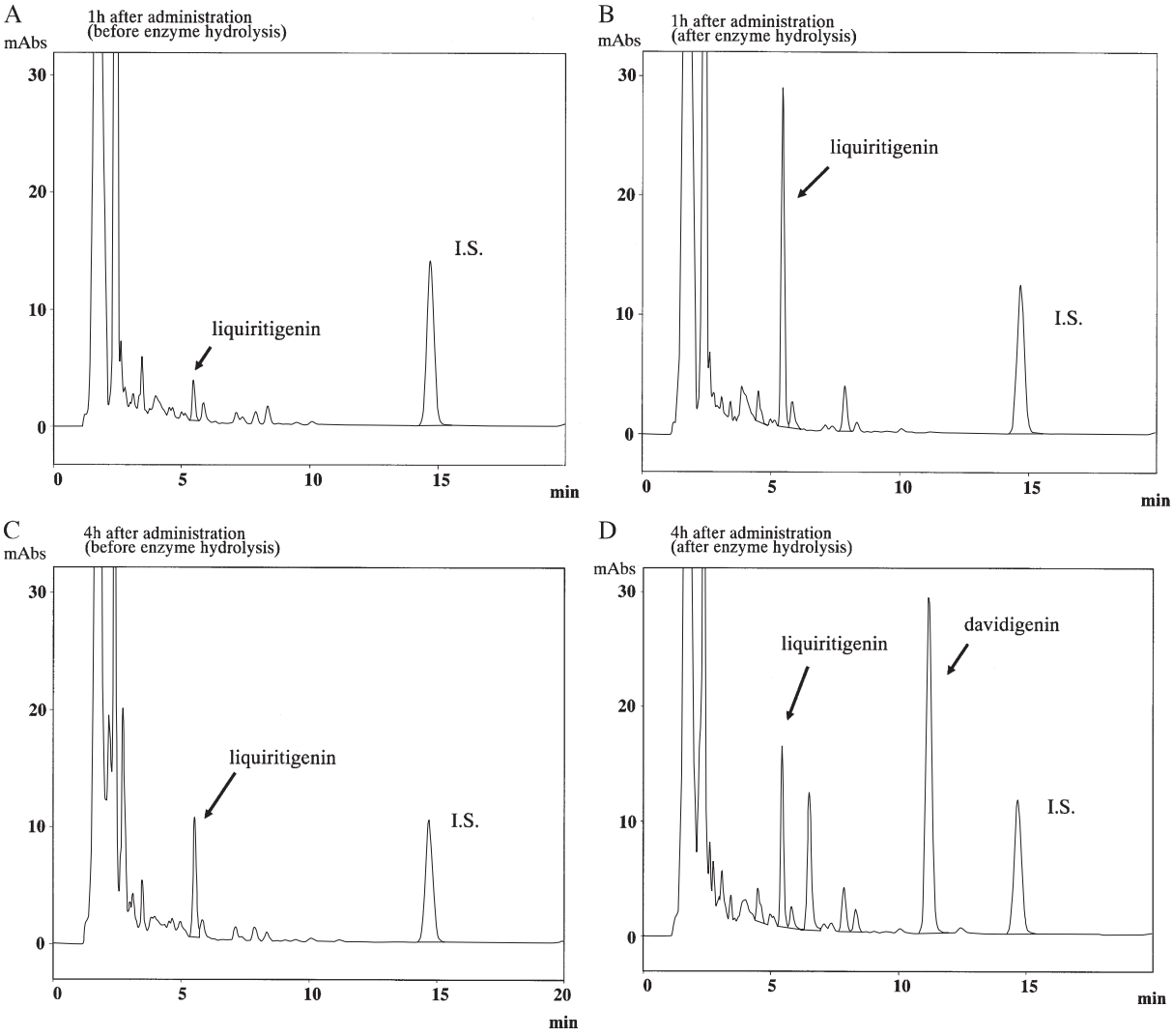

Fig. 4. HPLC chromatograms of before and after enzyme hydrolysis of plasma samples obtained at 1 and 4 h after the administration of liquiritigenin.

increased for more than 6 h after administration. The plasma concentration–time curve profile of liquiritigenin after the administration of liquiritigenin was similar to that after the administration of liquiritin.

Pharmacokinetic parameters of liquiritin, liquiritin apioside and liquiritigenin in guinea pigs after oral administration of liquiritigenin and its glycosides are shown in Table 1.

Fig. 4 shows the HPLC chromatograms of before and after the enzyme hydrolysis of plasma samples at 1 and 4 h after the administration of liquiritigenin. The peak area of liquiritigenin in the hydrolyzed plasma was 9.4 times larger than that in intact plasma at 1 h after administration. The fold-increase in the peak area of liquiritigenin with hydrolysis was only 1.3 in plasma at 4 h after administration. The peak area of liquiritigenin in the hydrolyzed plasma at 1 h after administration was 1.7 times larger than that in hydrolyzed plasma at 4 h after administration. However, in hydrolyzed plasma, the peak area of davidigenin, which was not detected in intact plasma, was 3.5 times larger than that of liquiritigenin at 4 h after administration.

3.2. Antitussive effects of liquiritin apioside, liquiritin and liquiritigenin on capsaicin-induced coughs in guinea pigs

The number of control coughs induced by capsaicin was 21.8±2.3 coughs/7 min (n=50). The effects of liquiritin apioside, liquiritin and liquiritigenin on capsaicin-induced coughs 1 h after oral administration of each compound are shown in Fig. 5A. Liquiritin apioside, at a dose of 30 mg/kg, p.o., caused a more than 60% reduction in the number of capsaicin-induced coughs. Furthermore, liquiritigenin, at a dose of 30 mg/kg, p.o., caused a more than 40% reduction in the number of capsaicin-induced coughs. However, at the same dose, liquiritin had no significant effect on the number of capsaicin-induced coughs.

As shown in Fig. 5B, when the antitussive effects of liquiritin apioside, liquiritin and liquiritigenin, at respective doses of 30 mg/kg, p.o., were examined 4 h after administration, liquiritin apioside, liquiritin and liquiritigenin each caused a more than 40% reduction in the number of capsaicin-induced coughs.

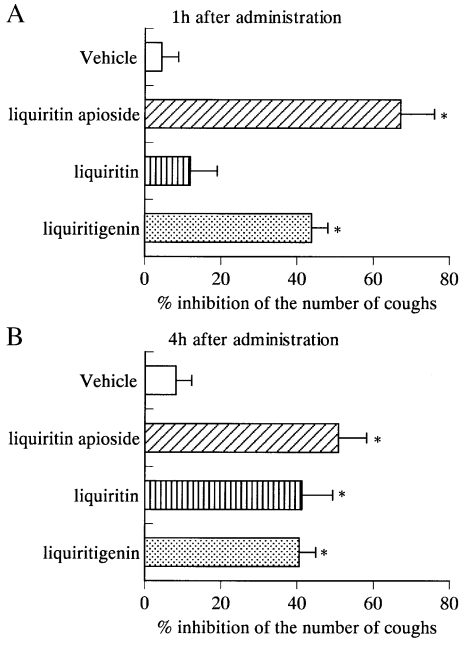

Fig. 5. Antitussive effects of liquiritin apioside, liquiritin and liquiritigenin. The antitussive effects of liquiritin apioside, liquiritin and liquiritigenin were assessed 1 (A) or 4 h (B) after p.o. administration of each drug. The effects of liquiritin apioside, liquiritin and liquiritigenin on the number of capsaicin-induced coughs were determined. Each column represents the mean with S.E. (n=5–7). *P<0.05 vs. vehicle-treated group.

4. Discussion

It is well recognized that glycosides in plants can be absorbed from the intestine after hydrolysis by glycosidase from intestinal flora (Kobashi and Akao, 1997; Akao et al., 1998). Indeed, it has been reported that liquiritin is transformed to liquiritigenin by intestinal flora (Zuo et al., 2002). However, in our study, liquiritin apioside and liquiritin appeared in plasma in an unchanged form at t_{max}; 0.5–1.3 h after oral administration of the corresponding compounds. Thus, hydrolysis was not thought to be essential for the absorption of either compound in guinea pigs soon after dosing. On the other hand, liquiritigenin, an aglycone of these two glycosides, appeared in plasma at t_{max}; 3.1–5.5 h. The hydrolysis of these glycosides to aglycone was also confirmed at the later time after dosing. On the other hand, the plasma concentration of unchanged liquiritigenin showed two peaks in its profile at approximately 15 min and 4 h after oral administration. However, the profiles of the plasma concentration curves of liquiritigenin after the administration of liquiritigenin itself, liquiritin and liquiritin apioside were similar to each other. These results suggest that deglycosidation by intestinal flora might be more rapid than absorption of the corresponding glycosides. Moreover, in plasma samples at 1 and 4 h after the administration of liquiritigenin, the HPLC peak of liquiritigenin increased after enzyme hydrolysis, indicating the presence of glucuronic acid and/or sulfuric conjugates. Since the concentration of total parent compound (unconjugate+conjugate) in plasma samples at 1 h was higher than that in plasma samples at 4 h after the administration of liquiritigenin, we propose the following hypotheses. The absorption of liquiritigenin from the gastrointestinal tract in guinea pigs is rapid. However, the absorbed liquiritigenin is immediately conjugated to the glucuronide or sulfate. Moreover, davidigenin was also observed in the hydrolyzed plasma sample at 4 h after the administration of liquiritigenin, and the conjugate of liquiritigenin was observed in earlier (1 h) plasma, whereas the conjugate of davidigenin was not detectable in earlier plasma but appeared in later (4 h) plasma. Li et al. (1998) reported that the conversion of liquiritigenin to davidigenin

in human feces may be attributable to reductive reaction by intestinal flora. These findings suggest that the conversion of liquiritigenin into davidigenin also occurs in the lower gastrointestinal system in guinea pigs.

In the present study, we examined the antitussive effects of liquiritin apioside, liquiritin and liquiritigenin on capsaicin-induced coughs 1 and 4 h after oral administration. Liquiritin apioside produced a significant antitussive effect at both 1 and 4 h after administration. Furthermore, liquiritin produced significant antitussive effect at 4 but not 1 h after administration. The peak plasma concentration of unchanged compound was observed within 30 min after the administration of either liquiritin apioside or liquiritin, and each unchanged compound was almost undetectable at 4 h after administration. These results indicate that liquiritin apioside and liquiritin by themselves do not contribute to their antitussive effects at 4 h after their administration. On the other hand, significant antitussive effects were observed 1 and 4 h after the administration of liquiritigenin. Since the unchanged form of liquiritigenin was observed in plasma at least 15 min after administration and its concentration then gradually increased for more than 6 h after administration, the antitussive effect observed at 1 and 4 h after the administration of liquiritigenin may be due to the action of liquiritigenin itself. As described above, we observed liquiritigenin in the serum for up to 6 h after the oral administration of either liquiritin apioside or liquiritin. Liquiritin apioside and liquiritin each produced a more than 40% inhibition of the number of coughs when the antitussive effect was examined 4 h after administration. Based on these pharmacokinetic and pharmacodynamic results, we hypothesize that the antitussive effects observed 4 h after the administration of either liquiritin apioside or liquiritin may be induced by the action of their desglycoside metabolite, liquiritigenin.

We previously reported that the antitussive effect of liquiritin apioside may depend on both peripheral (modulation of ATP-sensitive K^+ channels) and central mechanisms (modulation of serotonergic systems; Kamei et al., 2003). Although there are no data concerning the permeability of liquiritigenin into the brain based on its molecular weight and lipid solubility, it is reasonable to speculate that liquiritigenin cross the blood brain barrier. Therefore, it is possible that the antitussive effect of liquiritigenin might depend on both peripheral and central mechanisms. However, further studies are necessary before this possibility can be established with greater certainly.

In conclusion, the present results suggest that *G. radix* (licorice) may produce a persistent antitussive effect and suggest that liquiritin apioside plays an important role in the earlier phase, while liquiritigenin, which is a metabolite of liquiritin apioside and liquiritin, plays an important role in the late phase.

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