JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Absorption and Plasma Disposition of Genistin Differ from Those of Genistein in Healthy Women

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ABSTRACT: The chemical forms in which isoflavones appear in food or supplements seem to play an important role in their absorption efficiency. However, the influence of the chemical form of isoflavones on their plasma disposition has never been reported, although the metabolites of isoflavones circulating in the blood may have biological activity themselves. The purpose of the study was to investigate the pharmacokinetic profiles of genistein (GEN) and its phase II metabolites in the plasma and urine of healthy young women after multiple doses of pure aglycone and glucoside forms of GEN. Genistein-7-glucuronide (G-7-G), 4′-glucuronide (G-4′-G), 7-sulfate (G-7-S), 4′-sulfate (G-4′-S), 4′,7-diglucuronide (G-4′,7-diG), and 7-glucuronide-4′-sulfate (G-7-G-4′-S) besides unconjugated GEN were observed in human plasma after ingestion of GEN and its glucoside. Among these metabolites, G-4′,7-diG and G-7-G-4′-S were the major ones, comprising both about 30% of the total amount of GEN in plasma. Compared with the aglycone, the amount of total GEN in vivo and those of G-4′,7-diG and G-7-G-4′-S were increased after the glucoside intake. No difference was observed in urinary excretion between the aglycone and the glucoside. Overall, the absorption and plasma disposition of GEN were affected by the glucoside form.

KEYWORDS: *genistein, phase II conjugates, pharmacokinetics, urinary excretion*

INTRODUCTION

Isoflavones, a kind of plant polyphenolic compound, mainly exist in soybeans with chemical structures similar to those of estrogens. They have been reported to exert purported health benefits in aging-related and hormone-dependent disorders as well as cardiovascular diseases.[1,2] Isoflavones, especially genistein (GEN), are thought to be responsible for the cancer prevention effects of soy and soy products.[3−5] Although the scientific world has not come to consensus on the exact mechanism of action for GEN's cancer prevention function, multiple studies suggest that GEN exerts significant direct and indirect hormonal and nonhormonal effects in preventing tumor initiation and suppressing the growth of tumor cells (reviewed by Steiner et al.[5]).

GEN is present in soybeans and most soy foods primarily as glycoside forms, including glucoside, acetylglucoside, and malonylglucoside. Fermentation processing increases the aglycone form in soy products. Isoflavone supplements contain both the aglycone and glucoside forms of GEN. After ingestion, the absorption of the glucoside forms requires hydrolysis of the sugar moiety by β-glucosidase in the intestinal microflora to release the aglycone.[6,7] The chemical forms in which GEN appears in foods or supplements seem to play an important role in its absorption efficiency,[8] although conflicting results are reported in the literature. Setchell et al. showed greater bioavailabilty of the glucoside.[9] Izumi et al. and Okbe et al. indicated greater bioavailability of the aglycone.[10,11] Two other studies found no difference between the absorption of the aglycone and that of the glucoside.[12,13]

It is generally believed that the aglycone is the biologically active compound in human. However, most of the absorbed GEN circulates in the blood as glucuronide, sulfate, and sulfoglucuronide conjugates due to extensive metabolism in the intestine and liver.[14,15] The plasma concentration of the aglycone (<0.4 μM) is lower than the IC_{50} values (10−50 μM) reported for its anticancer effect in vitro even after ingestion of large amounts of GEN-containing soy products (16 mg/kg).[5,16,17] Several investigators have reported that the conjugates may have biological activity themselves. They have shown that both GEN glucuronides and GEN sulfates have binding ability to ERs; GEN sulfates have weak estrogenic agonist activity, and the glucuronides can activate human natural killer cells.[18,19] In addition, the biological effects of GEN phase II metabolites are correlated not only to the type of conjugates but also to the conjugation position.[19] These results suggest that the bioactivities of GEN would be affected by its plasma disposition in addition to its bioavailability.

To date, whether and how the chemical form of GEN affects its plasma disposition have never been reported, although the bioavailability of the aglycone seems different from that of the glucoside. Elucidation of the influence of the chemical form of GEN on its disposition will be a great help in understanding the bioactivities of isoflavones. Therefore, in this paper, we make the first report of this influence by investigating the pharmacokinetic profiles of six conjugated metabolites of GEN in plasma and their excretion from urine after the administration of pure GEN and GIN in healthy women. The six conjugated metabolites of GEN detected in this study were genistein-7-glucuronide (G-7-G), 4′-glucuronide (G-4′-G), 7-sulfate (G-7-S), 4′-sulfate (G-4′-S), 4′,7-diglucuronide (G-4′,7-diG),

Received: October 30, 2011
Revised: December 31, 2011
Accepted: January 15, 2012
Published: January 19, 2012

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and 7-glucuronide-4′-sulfate (G-7-G-4′-S), which were the major metabolites observed in human.[20−22]

MATERIALS AND METHODS

Chemicals. GEN (>98% purity) and GIN (>98% purity) were supplied by Xi'an Shanchuan Biopharm Co. (Xi'an, China) and Shanghai Yanjing Biotech Co. Ltd. (Shanghai, China), respectively. Tolbutamide (internal standard, IS) was obtained the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). G-7-G, G-4′-G, and G-7-G-4′-S were purchased from Toronto Research Chemicals Inc. (North York, ON, Canada). G-7-S and G-4′-S were chemically synthesized in our laboratory; their structures were identified by nuclear magnetic resonance (NMR). β-Glucuronidase (type B-1, from bovine liver) was obtained from Sigma-Aldrich (St. Louis, MO). HPLC grade methanol and acetonitrile were purchased from Yuwang industrial Co. Ltd. (Shandong, China). Distilled water, prepared from demineralized water, was used throughout the study. All other chemicals and solvents were of analytical grade and used without further purification.

Chemical Synthesis of G-7-S. G-7-S was synthesized as described previously.[23] In brief, GEN (1 g, 0.0037 mol) and hexanoyl chloride (0.8 mL) were mixed in DMF (8 mL) for 24 h to form genistin-7-hexanoate. Then genistin-7-hexanoate was purified on silica gel by elution with ethyl acetate/petroleum ether (5:1, v/v). After chlorosulfonic acid (2% in 10 mL of CH_2Cl_2) was slowly added into the purified genistein-7-hexanoate (1.1 g, 0.0013 mol), which was dissolved in pyridine (10 mL) at 0 °C, the mixture was reacted at room temperature for 12 h. White precipitant was obtained by adding ether into the reaction solution. The precipitant (0.23 g, ≈98%) was identified as G-7-S by NMR [δ 6.89 (1H, d, H-8), 6.63 (1H, d, H-6), 6.82−6.85 (2H, d, H-3′ and 5′), 7.38−7.41 (2H, d, H-2′ and 6′), 8.42 (1H, s, H-2), 12.89 (1H, s, 5-OH)].[24]

Chemical Synthesis of G-4′-S. G-4′-S was synthesized as described previously.[23] Benzoyl chloride (0.7 mL) was mixed with GEN (1.5 g, 0.0056 mol, in 10 mL of pyridine) at room temperature for 24 h. Then genistein-7-benzoate was precipitated from the solution by the addition of 5% HCl. After chlorosulfonic acid (7% in 10 mL of CH_2Cl_2) was slowly added into genistein-7-benzoate (1.7 g, 0.0045 mol), which was dissolved in pyridine (10 mL) at 0 °C, the following operations were performed in the same way as described for the synthesis of G-7-S. The white precipitant (0.9 g, ≈98%) was identified as G-4′-S by NMR [δ 6.23 (1H, d, H-8), 6.39 (1H, d, H-6), 7.20−7.23 (2H, d, H-3′ and 5′), 7.44−7.47 (2H, d, H-2′ and 6′), 8.34 (1H, s, H-2), 12.89 (1H, s, 5-OH)].[24]

Subjects. Six healthy Chinese women, aged 20−27 years with BMI between 18 and 21 kg/m^2, were recruited from the staff and student population of Shenyang Pharmaceutical University. Subjects with preexisting chronic renal, liver, pulmonary, or cardiovascular disease, or who were taking oral contraceptives, were excluded. The participants were required to abstain from consuming any drugs, especially antibiotics, for at least 30 days prior to the beginning of the study and during the study. The main foods containing polyphenols, such as red fruits, red wine, chocolates, tea, or coffee, were prohibited for 10 days prior to the study and during the experimental period. This study was conducted according to the guidelines laid down in the Declaration of Helsinki (2000), and all procedures involving human subjects were approved by the Ethics Committee of the Second Affiliated Hospital of Liaoning Traditional Chinese Medicine University. Written informed consent was obtained from each subject.

Study Design. This was an open-label, two-phase crossover, multidose study with a 10-day washout period between phases. Six subjects were randomly assigned to two groups (n = 3 per group). Each group was assigned to receive pure GEN or GIN at a dose of 4.0 mg of GEN aglycone equivalent (14.8 μmol) twice daily for 7 days. GEN and GIN were accurately weighed and packaged into gelatin capsules for oral administration. Our preliminary study showed that the content of GEN (aglycone equivalent) in commercially available soy foods such as tofu and soy milk was in the range of 0.05−0.3 mg/mg isoflavones (data not shown), which was supported by previously reported data.[25,26] Dietary intake of isoflavones is about 40 mg/day in the Chinese population.[27] Therefore, the dosage was designed to 4.0 mg of GEN aglycone equivalent twice daily on the basis of on the dietary intake of GEN and its half-life (6−17 h).[8] The meals consumed throughout the pharmacokinetic study were provided at specified times and were strictly the same for all subjects.

Venous blood samples (3 mL) were collected into heparin-containing tubes before administration on days 1, 5, 6, and 7 and at 1, 2, 4, 6, 8, 10, 12, 14, and 24 h after administration on day 7. Plasma was immediately separated from blood samples by centrifugation at 3984g for 10 min and stored at −80 °C. Urine samples were collected before administration on day 1 and at 0−12, 12−24, 24−36, 36−48, 48−60, and 60−72 h after administration on day 7. All of the urine samples were stored in containers with ascorbic acid and sodium azide (0.1 wt %/vol for each) added as preservatives[28] at −80 °C until analysis.

Determination of GEN and Its Phase II Conjugates in Plasma and Urine. Free GEN, G-7-G, G-4′-G, G-7-S, G-4′-S, and G-7-G-4′-S in plasma and urine were measured by an LC-MS/MS method. G-4′, 7-diG were determined by the total amount of nonconjugated GEN that was released after treatment of the biological matrix with β-glucuronidase (type B-1) minus the sum amounts of free GEN, G-7-G, and G-4′-G.

An API 4000 triple-quadrupole tandem mass spectrometer (Applied Biosystem/MDS SCIEX, Foster City, CA) with electrospray source (ESI) was operated in negative ion mode. The quantification was performed using MRM method with the transitions m/z 269.0 → 133.1 for GEN, m/z 349.0 → 269.2 for G-7-S and G-4′-S, m/z 445.4 → 269.1 for G-7-G and G-4′-G, m/z 525.0 → 349.0 for G-7-G-4′-S, and m/z 269.9 → 171.0 for tolbutamide (IS). The main working parameters were set as follows: ionspray voltage, −4.5 kV; ion source temperature, 600 °C; gas 1, 50 psi; gas 2, 40 psi; curtain gas, 25 psi. Analyte concentrations were determined using the software Analyst 1.5.

Chromatographic separation was performed on a Hypersil BDS C_{18} column (150 mm × 4.6 mm, 5 μm, Elit Analytical Instruments, Dalian, China). The solvents 5 mM ammonium acetic acid (pH 7.8) (A) and acetonitrile (B) were used as mobile phase for elution. The gradient was controlled as follows: 0−3.0 min, 1% B; 3.0−5.0 min, 1−10% B; 5.0−10 min, 10−25% B; 10−14 min, 25−100% B; 14−16 min, 100% B; 16−18 min, 100−1% B; 18−23 min, 1% B. The flow rate was 1.0 mL/min. The outlet of the column was split, and only 0.5 mL/min portion of the column effluent was carried into the mass spectrometer.

Sample Preparation. For the analysis of free GEN, G-7-G, G-4′-G, G-7-S, G-4′-S, and G-7-G-4′-S in plasma, 20 μL of 20 μg/mL tolbutamide (IS) was added into a 200 μL aliquot of plasma. After the addition of 400 μL of methanol for protein precipitation, the sample was vortex-mixed for 1 min, followed by centrifugation at 18500g for 5 min at 4 °C. The supernatant was transferred to a new tube and evaporated to dryness under a stream of nitrogen. The residue was reconstituted in 100 μL of 20% acetonitrile aqueous solution and centrifuged at 18500g for 5 min at 4 °C. A 10 μL aliquot of the supernatant was injected for LC-MS/MS analysis. Urine samples were prepared as follows: to a 200 μL portion of each urine sample was added 20 μL of 70 μg/mL tolbutamide (IS), and the mixture was vortex-mixed for 10 s. After centrifugation at 18500g for 5 min at 4 °C, the supernatant was separated and a 10 μL aliquot was injected into the LC-MS/MS system.

To determine the amounts of G-4′,7-diG in plasma or urine, 50 μL of plasma or 50 μL of urine was treated with 0.5 mol/L acetate buffer (pH 4.5, 150 μL) containing 1000 units of β-glucuronidase at 37 °C for 3 h. Then the hydrolyzed plasma or urine samples were prepared according to the corresponding methods as described above.

Bioanalytical Method Validation. The linearity of the method was assessed by processing (in duplicate) an eight-point calibration curve on three consecutive batches. The peak area ratios of analyte/IS were plotted against the nominal analyte concentrations. Calibration curves were generated by a weighted linear least-squares regression analysis with a weighting factor of $1/x^2$ (where x is the concentration).

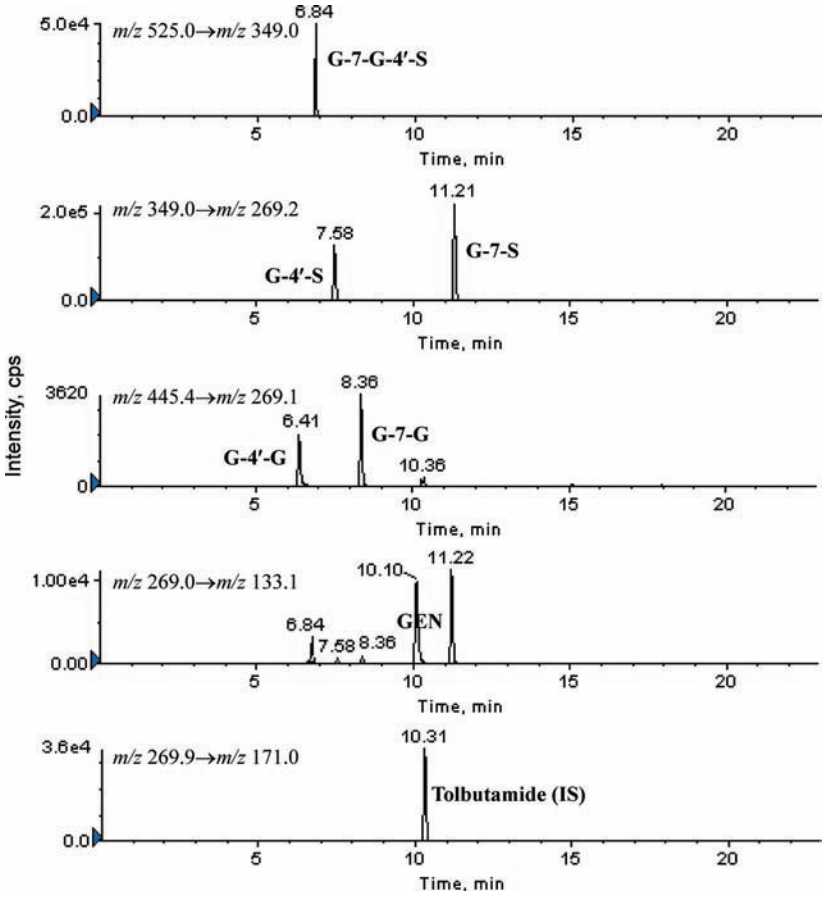

Figure 1. Representative MRM chromatograms for GEN, G-7-G-4′-S, G-7-G, G-4′-G, G-7-S, G-4′-S, and tolbutamide (IS). The retention times for GEN, G-7-G-4′-S, G-7-G, G-4′-G, G-7-S, G-4′-S, and IS were 10.1, 6.8, 8.4, 6.4, 11.2, 7.6, and 10.3 min, respectively.

The lowest concentration of the calibration curve served as the lower limit of quantification (LLOQ). The intra- and interassay precision and accuracy were evaluated by analyzing quality control samples (QCs) at three concentrations. Intra-assay precision was estimated in six replicate analyses of QCs at each concentration on the same batch. Interassay precision was assessed by the repeated analysis on three consecutive batches. Accuracy was determined by comparing the calculated concentration using calibration curves to the known concentration.

Statistical Analysis. The pharmacokinetic parameters of GEN and its phase II conjugates were calculated by a noncompartmental method using the DAS 2.0 pharmacokinetic program (Chinese Pharmacology Society). The maximum plasma concentrations (C_{max}) and the corresponding peak time (T_{max}) were observed from the individual drug plasma concentration−time profile. The minimum plasma concentrations (C_{min}) were calculated as the average of the concentrations before administration on days 5, 6, and 7. The terminal elimination rate constant (k_e) was estimated by log−linear regression of concentrations observed during the terminal phase of elimination. The elimination half-life ($t_{1/2}$) was calculated as $0.693/k_e$. The area under the plasma concentration−time curve was calculated by the linear trapezoidal rule. The mean residence time (MRT) was estimated as MRT = AUMC/AUC, where AUMC is the area under the first moment curve.

Comparison of the pharmacokinetic parameters and urinary excretions of each treatment was based on a three-factor analysis of variance (ANOVA), the main effects being treatment, phase, and subject. The between-treatment T_{max}, $t_{1/2}$, and MRT were compared using the Wilcoxon signed-rank test. All data were analyzed with SPSS software (version 11.5, SPSS Inc., Chicago, IL).

RESULTS

Method Validation. Under the validated LC-MS/MS system, optimal separation and determination of GEN and its phase II conjugates were achieved in both plasma and urine samples. Typical chromatograms of all analytes are shown in Figure 1. No significant interferences from endogenous substances with any analyte or IS were detected.

For plasma samples, the calibration curves were constructed over concentration ranges of 1.46−1500 nM for GEN, 0.49−500 nM for G-7-G and G-4′-G, and 0.39−400 nM for G-7-S, G-4′-S, and G-7-G-4′-S. For urine samples, the calibration curve was constructed over concentration ranges of 2.93−3000 nM for GEN, 4.88−5000 nM for G-7-G and G-4′-G, and 0.49−500 nM for G-7-S, G-4′-S, and G-7-G-4′-S. The correlation coefficient of determination (r) for each analyte in plasma or urine was >0.992. All of the analytes in plasma and urine were stable at room temperature on the benchtop for 2 h and at −70 °C for 30 days. Processed samples were stable for 24 h at room temperature.

Table 1 summarizes the results for intra- and interday precision and accuracy of the assay measured by QCs. The intra- and interday precisions were all below 15% with a maximum RSD of 12.7%, and a maximum bias of 8.0% for accuracy was calculated.

Plasma Profile of GEN and Its Metabolites. The levels of GEN and its conjugated metabolites were less than the LLOQ of the assay after subjects avoided soy products for 10 days, indicating that the 10-day washout periods before and between treatments were sufficient.

Table 1. Precision and Accuracy of the LC-MS/MS Method to Determine GEN and Its Phase II Conjugates in Human Plasma and Urine (on Three Consecutive Days, Six Replicates for Each Day)

	plasma					urine				
	concentration (nM)		precision (RSD%)		accuracy (%)	concentration (nM)		precision (RSD%)		accuracy (%)
	added	measured	intraday	interday		added	measured	intraday	interday	
GEN	2.34	2.34 ± 0.18	8.8	2.1	98.6−102.0	4.68	4.62 ± 0.40	9.0	6.4	96.5−101.5
	75.0	77.4 ± 4.0	4.9	6.5	100.2−105.6	150	147 ± 11	7.1	9.5	93.6−100.7
	1200	1208 ± 46	3.4	6.2	98.2−103.3	2400	2460 ± 147	5.0	10.6	98.3−107.2
G-7-G	0.78	0.78 ± 0.06	7.5	6.0	98.2−103.1	7.81	7.74 ± 0.46	6.3	0.9	98.8−99.1
	25.0	25.2 ± 2.3	9.6	5.4	98.3−102.5	250	250 ± 16	6.3	8.1	96.4−102.8
	400	399 ± 25	6.7	1.9	99.2−100.6	4000	4066 ± 282	7.2	4.8	99.5−103.5
G-4′-G	0.78	0.79 ± 0.06	7.5	11.7	95.7−104.3	7.81	7.96 ± 0.44	5.6	5.3	99.6−103.9
	25.0	24.3 ± 2.0	8.5	4.5	95.5−99.1	250	248 ± 15	6.0	6.7	96.4−101.9
	400	395 ± 20	4.1	9.6	95.6−103.0	4000	4103 ± 237	6.0	3.8	100.6−103.7
G-7-S	0.62	0.62 ± 0.04	6.3	8.2	97.9−104.5	0.78	0.79 ± 0.05	7.0	5.5	98.6−102.7
	20.0	20.1 ± 1.9	9.2	10.6	97.0−105.5	25.0	25.1 ± 2.0	8.5	5.2	98.2−102.4
	320	306 ± 25	8.6	1.0	95.4−96.1	400	401 ± 22	5.3	6.6	977−103.1
G-4′-S	0.62	0.62 ± 0.04	6.1	4.3	98.1−101.4	0.78	0.78 ± 0.06	6.7	10.4	95.3−103.5
	20.0	20.7 ± 1.9	8.8	10.6	99.2−108.0	25.0	25.8 ± 1.7	6.7	6.6	100.9−106.4
	320	323 ± 28	8.9	7.8	97.5−103.8	400	401 ± 25	5.9	8.3	97.8−104.2
G-7-G-4′-S	0.62	0.60 ± 0.03	4.4	3.2	96.2−98.7	0.78	0.80 ± 0.06	5.8	12.6	96.8−107.1
	20.0	19.7 ± 1.9	10.1	3.5	96.7−99.4	25.0	25.3 ± 2.1	7.6	12.7	96.3−106.5
	320	321 ± 24	7.6	7.4	97.4−103.4	400	402 ± 34	8.8	6.2	98.1−103.2

The plasma concentration−time curves of free GEN and GEN conjugated metabolites in six healthy women after administration of 14.8 μmol of pure GEN and GIN twice daily for 7 days are shown in Figure 2. The main pharmacokinetic parameters of GEN and its conjugated metabolites are presented in Table 2. Total GEN was the sum of free GEN and six GEN conjugated metabolites detected in this study.

According to AUC_{0-24}, G-4′,7-diG, G-7-G-4′-S, and G-7-S were the major metabolites of GEN circulating in the blood after ingestion of pure GEN and GIN. The AUC_{0-24} values of G-4′,7-diG, G-7-G-4′-S, and G-7-S as a percentage of the total GEN were 30.5, 32.2, and 26.8% for the aglycone, respectively, and were 39.9, 35.4, and 15.7% for the glucoside, respectively. Less than 1% of the absorbed GEN was circulating in the blood as free GEN. The $t_{1/2}$ and MRT values of free GEN were not calculated in the study because the plasma levels of free GEN at some time points were too low to be detected.

After ingestion of the pure aglycone, C_{max}, C_{min}, and AUC_{0-24} of the total GEN were significantly lower than the corresponding data after ingestion of the equivalent amount of the glucoside, indicating a higher bioavailability of the glucoside compared with the aglycone. The $t_{1/2}$ and MRT of the total GEN were similar between both chemical forms. Although the absorption of the glucoside was more effective than that of the aglycone, the C_{max} and AUC_{0-24} values were not increased for all of the metabolites after intake of the glucoside. No particular difference was observed in the pharmacokinetic profiles of G-7-G, G-4′-G, and free GEN between both chemical forms. The increased C_{max} and AUC_{0-24} appeared in G-4′,7-diG and G-7-G-4′-S after ingestion of the glucoside. In addition, the C_{min} values of G-7-S and G-4′-S were significantly increased for the glucoside, despite the C_{max} and AUC_{0-24} values not being affected by the chemical forms of GEN.

Urinary Excretion of GEN and Its Metabolites. The urinary excretion of GEN and its metabolites in six healthy women after multiple doses of pure GEN and GIN are shown in Figure 3. The total urinary excretion amount of each metabolite within 72 h is listed in Table 3. The excreted amount of G-7-G was greatest among the GEN metabolites, comprising 64.6% of the total excretion amount (mean, 7.17 μmol) for the aglycone and 60.9% of the total (mean, 7.69 μmol) for the glucoside. The excretion of GEN metabolites did not differ after the subjects ingested the aglycone and the glucoside except for G-4′,7-diG and free GEN. Compared with the aglycone, the excretion of G-4′,7-diG was increased, whereas that of free GEN was decreased after the glucoside intake.

DISCUSSION

The pharmacokinetics of isoflavones in humans has been well studied in single-dosage design, whereas multiple doses have not been adequately studied.[5] In the present study, a multidose protocol was performed to investigate the pharmacokinetic profiles of GEN and its glucosides in healthy young women. The mean plasma pattern of total GEN showed two peaks from 1 to 6 h after administration (Figure 2), which is the typical pattern of enterohepatic recirculation in isoflavones as has been suggested by several authors.[12,29,30] A delay in T_{max} was usually reported after the glucoside forms were ingested. We did not observe this delay in the absorption of GIN, probably due to cumulative effects caused by the multidose protocol. At least two of the studies have reported that only modest or negligible differences were detected in T_{max} between the aglycones and the glucosides after a single dose of isoflavone products.[12,13] The elimination $t_{1/2}$ of total GEN in this study was comparable to the values of 6−17 h from other studies (Table 2).[8]

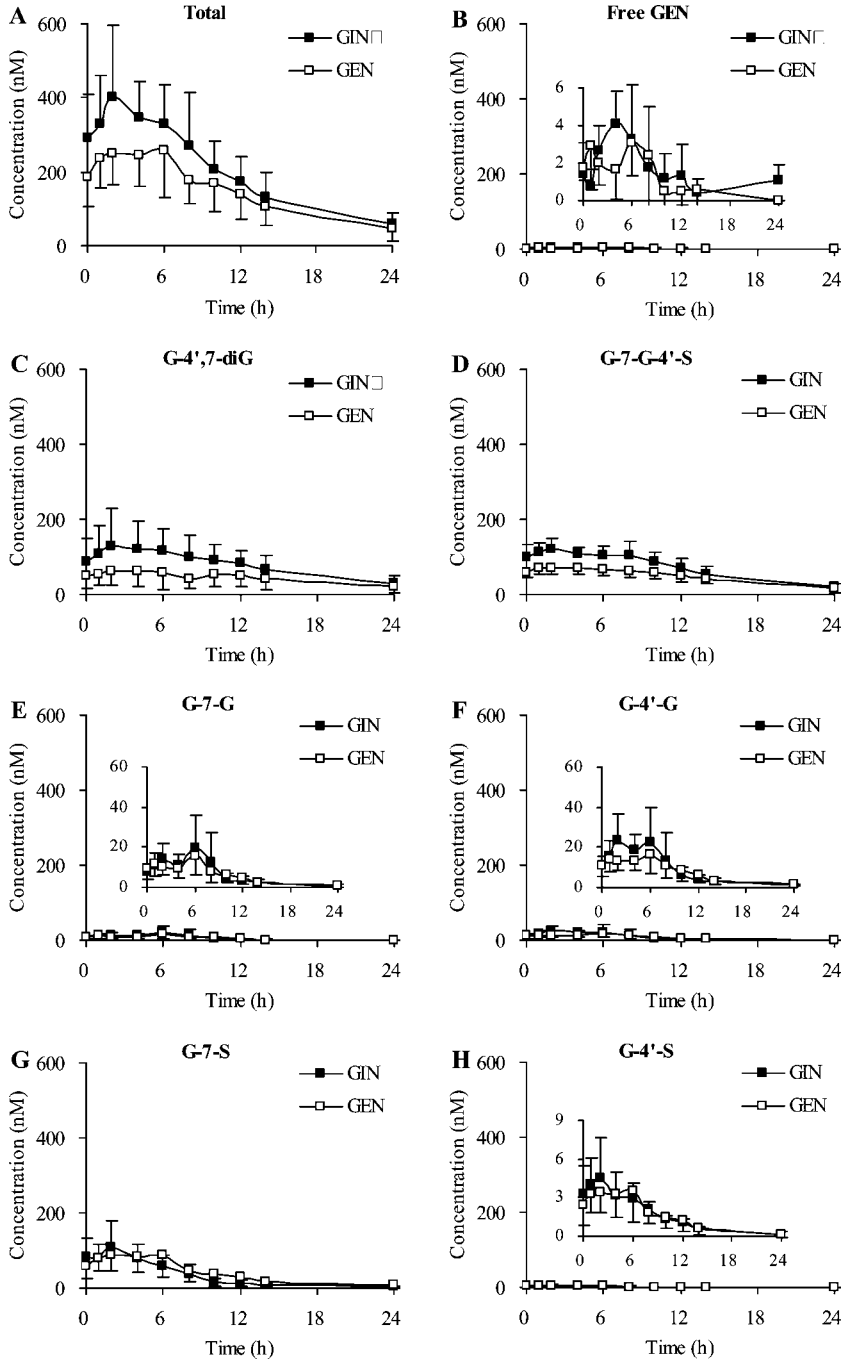

Figure 2. Mean plasma concentration−time curve of total GEN (A), free GEN (B), G-4′,7-diG (C), G-7-G-4′-S (D), G-7-G (E), G-4′-G (F), G-7-S (G), and G-4′-S (H) in six healthy women on the last day after administration of 14.8 μmol of pure GEN (□) and GIN (■) twice daily for 7 days. Total GEN indicates the total concentrations of free GEN and GEN conjugated metabolites.

The primary objective of this study was to investigate the influence of the chemical forms of isoflavones on the plasma disposition of isoflavones. Several clinical studies have been performed to compare the pharmacokinetics of the aglycones versus the glucosides of isoflavones.[9−13] However, the results were not in agreement due to differences in the food matrix, isoflavone sources, dose ingested, race, etc. Setchell et al. found higher bioavailabilty of the glucosides.[9] Izumi et al. and Okbe et al. showed greater bioavailability of the aglycones.[10,11] Two other studies reported no difference between aglycone and glucoside absorption.[12,13] In the present study, pure compounds were applied to avoid the influence of food matrix and isoflavone sources. In addition, all of the subjects consumed the defined diet at breakfast, lunch, and dinner throughout the experiment to eliminate the influence of ingested food on the absorption of GEN and GIN. Our observation agrees with the results of Setchell et al.,[9] who reported that pure isoflavones were ingested, indicating that the bioavailability of GEN is higher from the glucoside form than from the aglycone form. It was suggested by Nielsen and Williamson that the increased absorption of the glucosides may be caused by the lower solubility of the aglycones during digestion compared with the glucosides.[8] Setchell et al. speculated that the higher bioavailability of the glucosides is due to the glucoside moiety

dx.doi.org/10.1021/jf204421c | J. Agric.Food Chem. 2012, 60, 1428−1436

Table 2. Pharmacokinetic Parameters of Free GEN and GEN Phase II Metabolites in Six Healthy Women after Multiple Doses of Pure GEN and GIN[a]

		T_{max} (h)	C_{max} (nmol/L)	C_{min} (nmol/L)	AUC_{0-24} (nmol/L·h)	$t_{1/2}$ (h)	MRT (h)
free GEN	GEN	4.7 (2.9)	4.31 (1.44)	2.37 (0.72)	26.47 (11.17)		
	GIN	6.0 (3.6)	5.83 (1.64)	3.35 (1.02)	36.79 (9.20)		
G-4′,7-diG	GEN	5.5 (3.9)	67.1 (40.6)	49.3 (33.6)	1082.8 (652.3)	9.0 (3.1)	17.7 (6.7)
	GIN	4.0 (2.5)	143.1 (86.4)*	90.6 (66.7)*	1925.6 (1094.9)*	11.1 (4.0)	14.9 (4.2)
G-7-G-4′-S	GEN	3.0 (2.4)	76.2 (15.5)	59.5 (9.8)	1146.5 (290.7)	8.3 (2.6)	13.6 (3.5)
	GIN	4.3 (2.7)	130.2 (31.8)*	121.7 (30.3)*	1711.6 (443.0)*	7.7(2.4)	12.4 (2.5)
G-7-G	GEN	3.7 (2.6)	17.30 (8.64)	8.58 (5.63)	135.0 (69.6)	4.0 (1.7)	7.6 (2.2)
	GIN	4.7 (2.4)	25.02 (16.60)	10.72 (5.94)	152.4 (91.1)	13.1 (10.3)	12.9 (6.9)
G-4′-G	GEN	4.7 (2.4)	17.74 (7.76)	10.70 (5.47)	174.2 (76.3)	7.5 (5.7)	10.7 (4.6)
	GIN	4.3 (2.3)	32.79 (16.02)	13.00 (6.46)	207.9 (87.4)	10.9 (10.8)	11.1 (7.8)
G-7-S	GEN	4.2 (2.2)	101.1 (48.2)	56.47 (36.51)	954.2 (566.3)	5.9 (2.2)	8.4 (2.3)
	GIN	4.3 (2.3)	119.9 (60.8)	82.55 (51.70)*	756.2 (264.2)	9.1 (6.3)	8.8 (3.5)
G-4′-S	GEN	3.0 (2.4)	4.21 (2.07)	2.13 (1.20)	36.27 (17.52)	6.8 (3.4)	9.0 (4.0)
	GIN	3.5 (2.8)	5.03 (2.75)	3.95 (2.64)*	37.85 (15.00)	6.2 (3.4)	7.5 (1.7)
total[b]	GEN	4.0 (2.2)	276.7 (96.8)	189.6 (73.2)	3555.4 (1395.3)	8.5 (2.6)	13.1 (3.6)
	GIN	4.3 (2.3)	443.9 (171.8)*	323.6 (144.6)*	4828.4 (1718.0)*	8.1 (1.9)	12.2 (2.5)

[a]Values are presented as mean (SD). An asterisk (*) indicates statistical significance ($p < 0.05$) compared to corresponding values of pure GEN. [b]Total indicates the total concentration of free GEN and GEN conjugated metabolites.

acting as a protecting group in the molecule to prevent biodegradation of the isoflavone structure.[9]

Shelnutt et al. first suggested that there were diglucuronide, disulfate, and sulfoglucuronide conjugates of GEN besides its monoglucuronides and monosulfates in the plasma of persons who consume a soy meal.[14] Later, Hosoda et al. found that G-4′,7-diG and G-7-G-4′-S were the major metabolites of GEN in plasma after oral administration of *kinako* (baked soybean powder).[20,22] We observed that G-7-S was also the major metabolite of GEN besides G-4′,7-diG and G-7-G-4′-S after ingestion of pure GEN and GIN. This difference may result from the different doses and isoflavone sources. Our result clearly showed that in vivo amounts (AUC) of G-4′,7-diG and G-7-G-4′-S as well as the predose plasma levels (C_{min}) of G-7-S and G-4′-S were significantly increased by the glucoside form of GEN (Table 2).

Since soybean was elevated to the rank of functional food by the U.S. Food and Drug Administration (FDA) for the cholesterol-lowering properties of soy protein in 1999,[31] lots of soy-fortified foods and dietary isoflavones supplements have flooded the market. The biological effects of soy foods and isoflavone supplements have been tested in clinical trials.[32−36] Regrettably, there is not sufficient evidence to prove that isoflavone supplements are as effective as isoflavone-rich foods. For example, previous data showed that isoflavones in the form of supplements were ineffective in lowering serum cholesterol concentrations.[37−39] The bioactivities of G-4′,7-diG have not been reported yet, whereas in vitro studies have suggested that GEN monoglucuronides and GEN monosulfates have binding ability to ERs and that G-7-G-4′-S stimulates the growth of MCF-7 cells.[18,19,40] Thus, the conjugates in blood cannot be simply treated as biologically inactive metabolites. They may have biological activity themselves or may be precursors of

Table 3. Urinary Excretion Amounts of Free GEN and GEN Phase II Metabolites from Six Healthy Women after Multiple Doses of Pure GEN and GIN[a]

	excretion (μmol)	
	GEN	GIN
free GEN	0.10 (0.10)	0.04 (0.02)*
G-4′,7-diG	0.74 (0.20)	1.14 (0.44)*
G-7-G-4′-S	0.30 (0.15)	0.41 (0.29)
G-7-G	4.63 (1.98)	4.68 (2.54)
G-4′-G	0.96 (0.37)	1.09 (0.55)
G-7-S	0.29 (0.24)	0.20 (0.18)
G-4′-S	0.16 (0.10)	0.13 (0.06)
total[b]	7.17 (2.98)	7.69 (3.89)

[a]Values are presented as mean (SD). An asterisk (*) indicates statistical significance ($p < 0.05$) compared to corresponding values of pure GEN. [b]Total indicates the total amount of free GEN and GEN conjugated metabolites.

biologically active compounds circulated in vivo. Although caution is needed in translating the data from the administration of pure compounds to isoflavones contained within soy products, our results suggesting the plasma disposition of isoflavones is affected by glycosidation may help to explain some of the inconsistency of the effects of soy isoflavones on a variety of potential health outcomes.

Despite superior bioavailability of the glucoside form, the excretion amount of total GEN within 72 h did not differ significantly between both chemical forms (Table 3). The unchanged excretion amount of total GEN together with similar $t_{1/2}$ and MRT (Table 2) indicated that the increased bioavailability of the glucoside resulted from absorption, not

dx.doi.org/10.1021/jf204421c | J. Agric.Food Chem. 2012, 60, 1428−1436

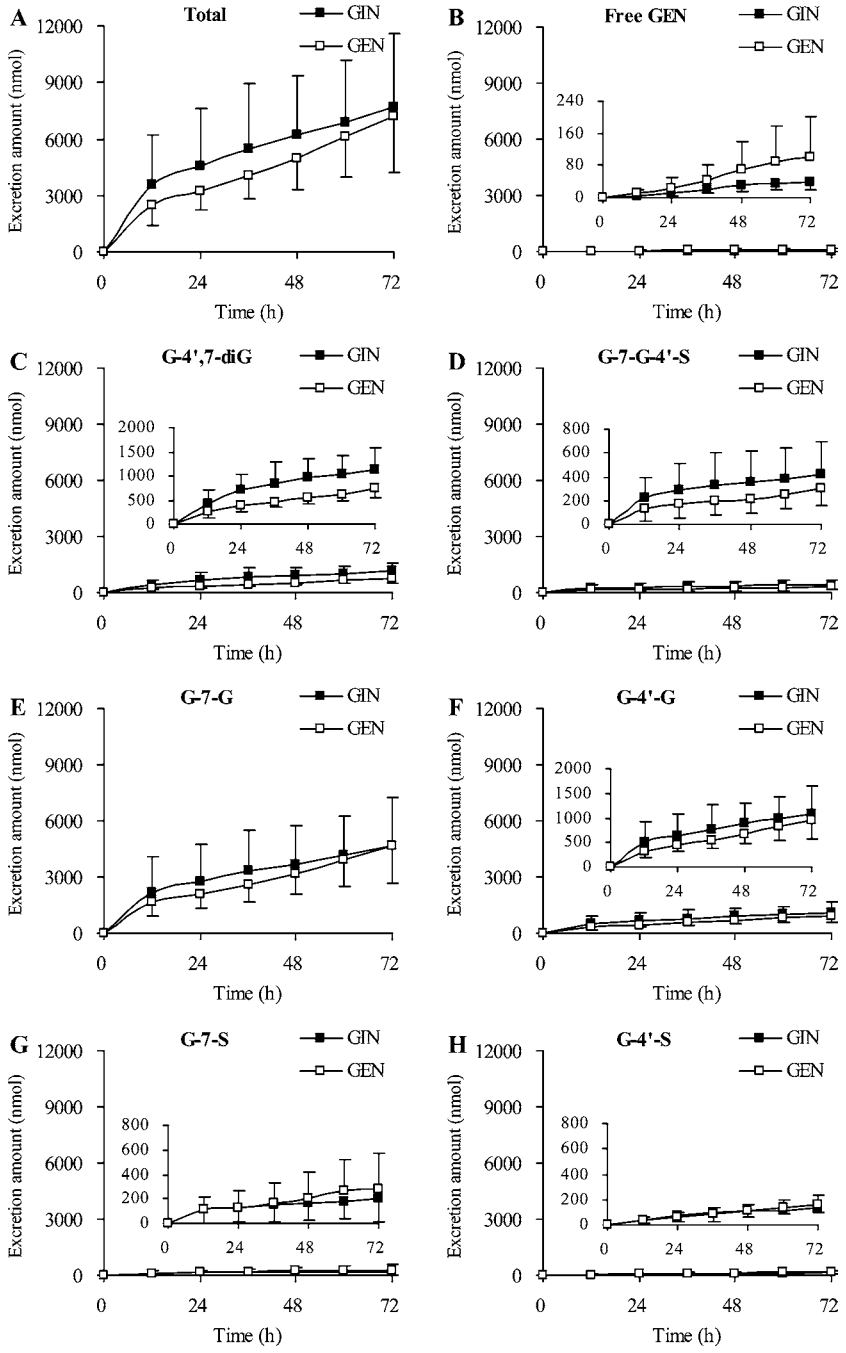

Figure 3. Mean urinary excretion curve of total GEN (A), free GEN (B), G-4′,7-diG (C), G-7-G-4′-S (D), G-7-G (E), G-4′-G (F), G-7-S (G), and G-4′-S (H) in six healthy women on the last day after administration of 14.8 μmol of pure GEN (□) and GIN (■) twice daily for 7 days. Total GEN indicates the total amount of free GEN and GEN conjugated metabolites.

from elimination. G-4′,7-diG and G-7-G-4′-S comprised both approximately 30% of the total GEN in plasma on the basis of AUC_{0-24}, making them the major metabolites in plasma, but their 72 h urinary excretion decreased to about 10 and 5% of the total urinary excretion of GEN, respectively. G-7-G comprised only ∼3% of the total GEN in plasma; however, its urinary excretion increased to >60% of the total GEN in urine. Similar results were found by Hosoda et al.,[22] who suggested that desulfation of G-7-G-4′-S and deglucuronidation of G-4′,7-diG would happen for their urinary excretion.

In conclusion, the current study, to our knowledge, is the first to investigate the influence of chemical forms of GEN on its plasma disposition. The glucoside of pure GEN appeared to be more bioavailable than the aglycone. The major metabolites of GEN in plasma, G-4′,7-diG and G-7-G-4′-S, were increased after the glucoside intake. More than 60% of the total urinary excretion of GEN was recovered as G-7-G. A similar urinary excretion was observed between the aglycone and the glucoside.

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Funding

This work was supported by Grant 30801420 of the National Natural Science Foundation of China.

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