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Full-length article

Influence of dosage forms on pharmacokinetics of daidzein and its main metabolite daidzein-7-*O*-glucuronide in rats[1]

Feng QIU[2], Xiao-yan CHEN[2], Bo SONG[2], Da-fang ZHONG[3], Chang-xiao LIU[4]

[2]*Laboratory of Drug Metabolism and Pharmacokinetics, Shenyang Pharmaceutical University, Shenyang 110016, China*

Key words

daidzein; daidzein-7-*O*-glucuronide; influence; dosage forms; pharmacokinetics; rats

[1] Project supported by the National Natural Science Foundation of China (No 30271525).
[3] Correspondence to Prof Da-fang ZHONG. Phn/Fax 86-24-2390-2539.
E-mail zhongdf@china.com
[4] Now in *Tianjin Key Laboratory of Drug Metabolism and Pharmacokinetics, Tianjin Institute of Pharmaceutical Research, Tianjin 300193, China*.

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Abstract

Aim: To investigate the influence of dosage forms on the pharmacokinetics of daidzein and its main metabolite daidzein-7-*O*-glucuronide in Wistar rats. **Methods:** After administration of two typical dosage forms (daidzein solution and suspension), the concentrations of daidzein and daidzein-7-*O*-glucuronide were determined by an LC-MS-MS method. The pharmacokinetic parameters were calculated and analyzed statistically using the Student's *t*-test. **Results:** Absorption of daidzein after administration of daidzein solution (t_{max}=0.46 h) was more rapid than that of the suspension (t_{max}=5.00 h). The peak plasma concentrations of daidzein after administration of daidzein solution and suspension were 601.1 μg/L and 127.3 μg/L, respectively, and those of daidzein-7-*O*-glucuronide were 3000 μg/L and 192.6 μg/L, respectively. The absolute bioavailabilities of free daidzein in rats after administration of daidzein solution and suspension were 12.8% and 6.1%, respectively, which were calculated to be 47.0% and 12.2%, respectively, in the form of total daidzein (free plus conjugated daidzein). **Conclusion:** Absorption of daidzein solution was better than absorption of suspension (P<0.05).

Introduction

Daidzein [7-hydroxy-3-(4-hydroxyphenyl)-4*H*-1-benzopyran-4-one, CAS 486-66-8, Figure 1] is one of the naturally occurring isoflavones present mainly in leguminous plants, especially in soybeans, soy foods and *Pueraria lobata* Ohwi (Leguminosae). Several epidemiological studies in humans have suggested that daidzein intake is inversely associated with the incidence of hormone-dependent diseases, especially breast and prostate cancer[1]. In addition to its putative anticarcinogenic effects, daidzein has also been investigated as an antihyperlipidemic agent and a therapeutic substance to combat osteoporosis[2,3].

After oral administration, daidzein is subject to glucuronidation at the 7-hydroxyl position, and daidzein-7-*O*-glucuronide is its main metabolite in human[4] and rat[5] plasma and urine. The pharmacokinetics of daidzein in humans has been the subject of several studies[6–10], but there have been no reports about the pharmacokinetics of its main metabolite daidzein-7-*O*-glucuronide. Due to its poor hydrophilicity and lipophilicity, the pharmacokinetics of daidzein may be influenced by the dosage form. However, the influence of dosage forms on the pharmacokinetics of daidzein

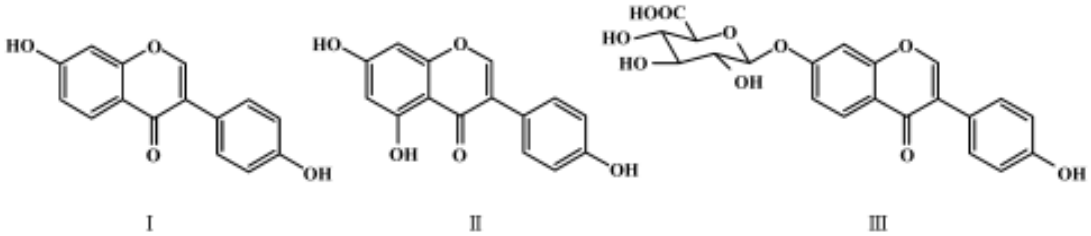

Figure 1. Chemical structures of daidzein (I), genistein (II, internal standard) and daidzein-7-*O*-glucuronide (III).

and its main metabolite daidzein-7-*O*-glucuronide have not been investigated previously. Furthermore, the extent of the influence of dosage forms remains unknown.

In the present study, the influence of two typical dosage forms (solution and suspension) on the pharmacokinetics of daidzein and its main metabolite daidzein-7-*O*-glucuronide was investigated after oral administration of 20 mg/kg purified daidzein to rats.

Materials and methods

Chemicals and reagents Daidzein and genistein (internal standard) were purchased from Huike Botanical (Xi'an, Shaanxi, China). The purities of these 2 compounds were 99.3% and 98.9%, respectively, which was verified using high-performance liquid chromatography (HPLC) methods. β-Glucuronidase (EC 3.2.2.21, 542 200 units/g of solid) was purchased from Sigma (St Louis, MO, USA). CMC-Na was purchased from Shenyang Chemical Factory (Shenyang, Liaoning, China). Acetonitrile and methanol (Yuwang Chemical Factory, Shandong, China) were of HPLC grade. Other chemicals were of analytical grade. Distilled water, prepared from demineralized water, was used throughout the study.

Preparations of dosage forms A transparent solution of daidzein was prepared by dissolving an appropriate amount of daidzein in 0.9% NaCl solution (water was used as solvent) and adjusting the pH to 7.0 by the addition of 1 mol/L NaOH. A daidzein suspension with good physical stability was achieved by dispersing grinded daidzein in 0.5% CMC-Na solution (water was used as solvent and CMC-Na was used as co-suspension reagent). The concentrations of daidzein in both dosage forms were determined to be 2.0 g/L by a validated HPLC-UV method. The 2 dosage forms were prepared freshly for animal administration.

Instrumentation A Finnigan TSQ API II tandem mass spectrometer equipped with an atmospheric pressure chemical ionization (APCI) source (San Jose, CA, USA), an Agilent 1100 autosampler (Agilent, Wilminton, DE, USA) and a Shimadzu LC-10AD pump (Kyoto, Japan) were used for LC-MS-MS analyses. Analytical data were acquired using Xcalibur 1.1 software (Finnigan) and quantitative processing was carried out using LCQuan software (Finnigan).

LC-MS-MS conditions The LC-MS-MS method used for the determination of daidzein and daidzein-7-*O*-glucuronide was a slightly modified version of one described previously[11]. The LC separation was carried out using a Diamonsil C_{18} column (200 mm×4.6 mm ID, 5 mm; Dikma, Beijing, China) and a SecurityGuard C_{18} guard column (4 mm×3.0 mm ID; Phenomenex, Torrance, CA, USA). The isocratic mobile phase consisted of acetonitrile-water-formic acid (80:20:1, *v*:*v*:*v*) at a flow rate of 0.75 mL/min. The column temperature was maintained at 20 ºC.

The mass spectrometer was operated in positive APCI mode with the corona discharge current set at 4.00 μA. Nitrogen was used as the sheath gas (0.6 MPa) and auxiliary gas (3 L/min) for nebulization. The heated capillary and vaporizer temperatures were set to 280 ºC and 450 ºC, respectively. For collision-induced dissociation (CID), argon was used as the collision gas at a pressure of 0.19 Pa. Quantification was carried out using selected reaction monitoring (SRM) of the transitions *m/z* 255→199 for daidzein and *m/z* 271→153 for genistein, with a scan time of 0.3 s/transition. The optimized collision energies of 30 eV and 35 eV were chosen for daidzein and genistein, respectively.

Sample preparation To determine free (unconjugated) daidzein, 50 μL internal standard solution (genistein, 50 mg/L in methanol) and 50 μL water were added to 50 μL of each rat plasma sample. $NH_4H_2PO_4$ buffer (pH 5.0; 200 μL of 0.05 mol/L) was added. The mixture was vortexed for 10 s and extracted with 2 mL *n*-hexane-diethyl ether (1 : 4, *v*/*v*) by shaking for 10 min. After centrifugation at 2000×*g* for 10 min, the organic phase was transferred into another tube and evaporated to dryness at 40 ºC under a stream of nitrogen. The residue was reconstituted in 100 μL of the mobile phase and vortexed for 1 min. A 20-μL aliquot of the solution was injected onto the LC-MS-MS system.

To determine total daidzein (free plus conjugated daidzein), 100 μL β-glucuronidase enzyme solution (1084 U/mL in 0.05 mol/L $NH_4H_2PO_4$ buffer, pH 5.0) was added to a 50 μL aliquot of rat plasma. The mixture was incubated in a water bath at 37 ºC for 16 h. After enzymatic hydrolysis, 50 μL internal standard and 50 μL water were added. The mixture was treated as described above.

Those plasma samples whose concentrations were higher than the highest calibration point were diluted appropriately with blank rat plasma in order to make the concentration within the range of the standard curve before sample preparation.

The concentration of daidzein-7-*O*-glucuronide was calculated using the following formula:

$$C_{DG} = (C_t - C_f) \times 430/254,$$

where C_{DG} was the mass concentration of daidzein-7-*O*-glucuronide (conjugated daidzein), C_t was the total mass concentration of daidzein, C_f was the mass concentration of free (unconjugated) daidzein, and 254 and 430 were the molecular weights of daidzein and daidzein-7-*O*-glucuronide, respectively.

Study design Eighteen Wistar rats (Grade II, Certificate

No 042; 9 males and 9 females; Laboratory Animal Center of Shenyang Pharmaceutical University, Shenyang, China) weighing 200 g–250 g were divided into 3 groups at random in the studies. Each group contained 3 male and 3 female rats. All experimental procedures were carried out in accordance with the guidelines of the Experimental Animal Care and Use Committee of Shenyang Pharmaceutical University. The rats were housed under standard conditions and had *ad libitum* access to water and a standard laboratory diet (isoflavone free). All rats were dosed following an overnight fast; food was returned 0.5 h after dosing. Water was available *ad libitum* throughout the experiments.

Polyethylene cannulas were implanted in the femoral vein 2 d before the experiment while the rats were anesthetized with pentobarbital (50 mg/kg, ip). The cannulas were externalized at the back of the neck and filled with heparinized saline (20 000 U/L). One group of 6 rats weighing 224±13 g were dosed orally with daidzein solution at 20 mg/kg (10 mL/kg, 2 g/L), the second group of 6 rats weighing 230±16 g were dosed orally with daidzein suspension at 20 mg/kg (10 mL/kg, 2 g/L), and the third group of 6 rats weighing 227±10 g were dosed intravenously with daidzein solution at 20 mg/kg (10 mL/kg, 2 g/L). Serial blood samples (0.25 mL) were collected at 0 h, 5 min, 10 min, 30 min, 1 h, 3 h, 5 h, 8 h, 12 h, 24 h and 48 h post dose. Plasma was separated by centrifugation at 2 000×*g* for 10 min and stored frozen at -20 ºC until analysis.

Data analysis Plasma concentrations were subjected to an appropriate pharmacokinetic analysis on mean data points. Values below the quantification limit were considered to be zero. The peak concentration (C_{max}) and the corresponding peak times (t_{max}) were determined by visual inspection of the mean data. The elimination half-life ($t_{1/2}$) was calculated using the non-compartmental model of the TOPFIT program on a personal computer. The area under the plasma concentration-time curve (AUC) from time zero to the last measurable plasma concentration point (t=48 h) ($AUC_{0-48\,h}$) was calculated using the linear trapezoidal rule. Extrapolation to time infinity ($AUC_{0-\infty}$) was calculated as follows:

$$AUC_{0-\infty}=AUC_{0-48\,h}+C_{48\,h}/k_e,$$

where $C_{48\,h}$ was the last measurable plasma concentration and k_e was the elimination rate constant. The bioavailability (F) of free daidzein was calculated as follows:

$$F=AUC_{0-\infty,\,po}/AUC_{0-\infty,\,iv},$$

where $AUC_{0-\infty,\,po}$ and $AUC_{0-\infty,\,iv}$ were the AUC values of free daidzein after oral and intravenous administration of daidzein. The bioavailability (F) of total daidzein (free plus conjugated daidzein) was calculated as follows:

$$F= AUC_{0-\infty,\,po}/AUC_{0-\infty,\,iv},$$

where $AUC_{0-\infty,\,po}$ and $AUC_{0-\infty,\,iv}$ were the AUC values of total daidzein after oral and intravenous administration of daidzein.

The main pharmacokinetic parameters, including $t_{1/2}$, k_e, t_{max}, C_{max}, $AUC_{0\text{-}48\,h}$, $AUC_{0-\infty}$, and F, were analyzed using the Student's t-test. A probability level of $P<0.05$ was defined as being statistically significant.

Results

Mass spectrometry Using the positive APCI mode, the analyte and internal standard formed predominately protonated molecules $[M+H]^+$ in full-scan spectra. To determine daidzein using the SRM mode, full-scan and product-ion spectra of daidzein and internal standard were investigated under the present HPLC conditions. Figure 2 shows the product ion spectra of $[M+H]^+$ ions of daidzein and genistein. Several fragment ions were observed in the product-ion spectra. The major fragment ions at *m/z* 199 and 153 were chosen in the SRM acquisition for daidzein and genistein, respectively.

Method validation Selectivity was assessed by comparing the chromatograms of 6 different batches of blank rat plasma with the corresponding spiked plasma. Figure 3

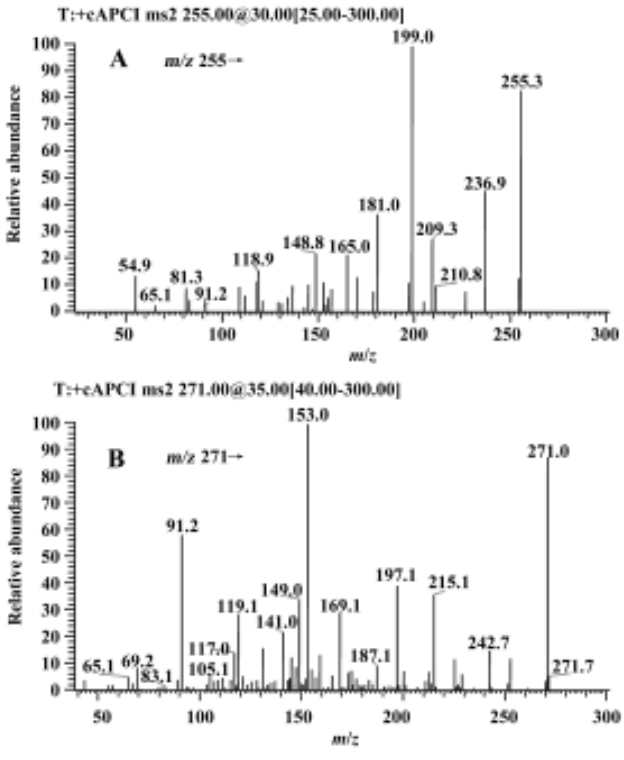

Figure 2. Full-scan product-ion spectra of $[M + H]^+$ of (A) daidzein and (B) genistein.

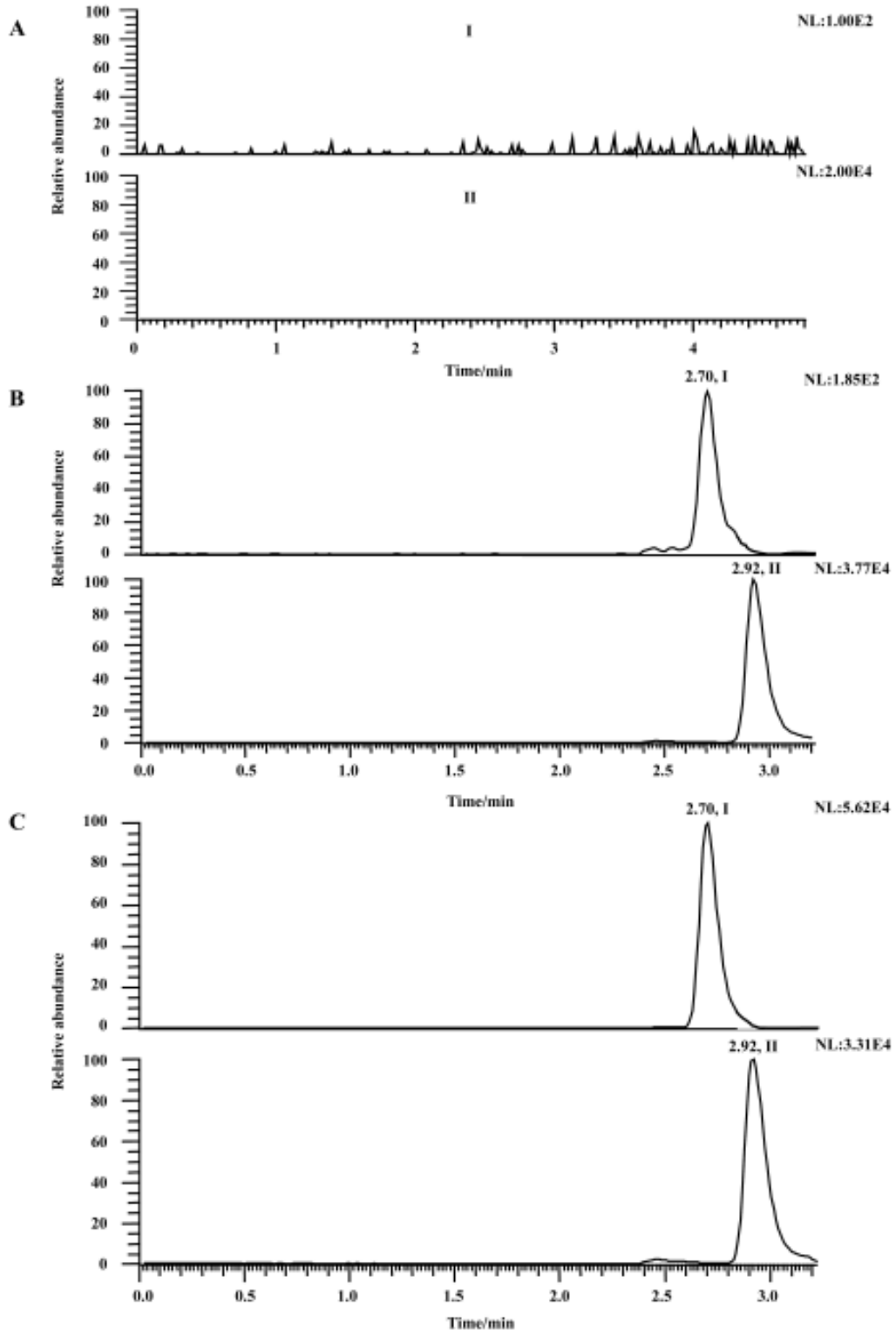

Figure 3. Representative selected reaction monitoring chromatograms of daidzein plasma samples determined using the LC-MS-MS method. (A) Blank plasma sample; (B) Blank plasma sample spiked with daidzein at the LLOQ of 0.24 μg/L and genistein (IS, 50 mg/L); (C) Rat plasma sample collected at 30 min after oral administration of daidzein (20 mg/kg). Peak I, daidzein; peak II, genistein.

shows the typical chromatograms of a blank plasma sample, a blank plasma sample spiked with daidzein at the LLOQ and genistein, and a plasma sample from a Wistar rat 30 min after oral administration. No significant interference from endogenous substances with analyte or genistein were detected. The typical retention times for daidzein and genistein were 2.7 min and 2.9 min, respectively.

Calibration standards were prepared by spiking 50 μL of the appropriate standard solutions of daidzein to 50 μL of blank rat plasma. Plasma concentrations were 0.24 μg/L, 0.50 μg/L, 1.5 μg/L, 20 μg/L, 100 μg/L, 500 μg/L and 1000 μg/L for daidzein. The linear regression of the peak area ratios versus concentrations was fitted over the concentration range of 0.24 μg/L-1000 μg/L in rat plasma. A typical equation of the calibration curve was as follows:

$$y=3.888\times10^{-4}+6.030\times10^{-4}\ x\ (r=0.9981),$$

where y is the peak area ratio of daidzein to genistein, and x is the concentration of daidzein. The present assay method had an LLOQ of 0.24 μg/L with an accuracy of 14.3% and a precision of 12.7% (n=5), which was sufficient for monitoring daidzein plasma levels over a period of 48 h after a single oral administration.

Table 1 summarizes the intra-day and inter-day precision and accuracy for daidzein from QC samples. In this assay, the intra-day and inter-day precisions ranged from 3.4% to 7.1% and from 6.3% to 13.2% for each QC level. The accuracy ranged from -0.5% to 2.4%. The results, calculated using a one-way ANOVA, indicated that the values were within the acceptable range and that the method was accurate and precise[12].

Table 1. Precision and accuracy of the LC-MS-MS method in determining daidzein concentrations in rat plasma.

Concentration/μg·L^{-1}		RSD/%		RE/%
Added	Found	Intra-day	Inter-day	
0.50	0.51	7.1	10.7	2.0
50.0	51.2	6.5	13.2	2.4
800.0	796.0	3.4	6.3	-0.5

RE, relative error. RSD, relative standard deviation.

The extraction recovery of daidzein, determined at 3 concentrations (0.50 μg/L, 50 μg/L, 800 μg/L), were 73.8%, 75.1% and 76.3% (n=6), respectively. The extraction recovery of genistein was also investigated as 64.2% (n=6).

Daidzein in the plasma was shown to be stable for at least 30 d stored at -20 ºC. The relative error (RE%) of daidzein between the initial concentrations and the concentrations of the following 3 freeze–thaw cycles ranged from -6.7% to 2.4%, which indicated the stability of daidzein during freeze–thaw. Processed samples were also found to be stable in the reconstituted solution of acetonitrile-water-formic acid (80:20:1, *v*:*v*:*v*) for at least 24 h at room temperature. These data are summarized in Table 2.

Table 2. Stability of daidzein in rat plasma (n=6).

Stability	Daidzein concentration/μg·L^{-1}		
	0.50	50.0	800.0
Storage (≤–20 ºC, relative error, %)			
0 d	2.6	-1.1	2.9
30 d	-4.7	-5.8	3.5
Freeze-thaw (relative error, %)			
0 cycles	-0.4	2.4	-2.1
3 cycles	-2.7	-5.6	-6.7
Processed plasma samples at room temperature (relative error, %)			
0 h	0.7	-3.2	0.3
24 h	-2.6	1.9	-3.8

Pharmacokinetics The mean plasma concentration versus time curves of daidzein and daidzein-7-*O*-glucuronide after oral administration of 2 different dosage forms (daidzein solution and suspension) are given in Figure 4. The mean plasma concentration versus time curves of daidzein and daidzein-7-*O*-glucuronide after intravenous administration of daidzein solution are given in Figure 5. Table 3 summarizes the pharmacokinetic parameters of daidzein and daidzein-7-*O*-glucuronide after administration of 2 different dosage forms. The pharmacokinetic parameters of daidzein and daidzein-7-*O*-glucuronide after intravenous administration of daidzein solution are given in Table 4.

Pharmacokinetics of daidzein After oral administration of 2 different dosage forms, the absorption of daidzein after administration of daidzein solution (t_{max}=0.46 h) was more rapid than that of suspension (t_{max}=5.00 h). The peak plasma concentration after administration of daidzein solution was 601.1 μg/L, which was approximately 4 times higher than that of suspension (127.3 μg/L). The absolute bioavailability of daidzein in rats after administration of daidzein solution was 12.8%, but the absolute bioavailability of daidzein after administration of daidzein suspension was only 6.1%.

There are significant differences of daidzein parameters in the values of t_{max} and C_{max} (P<0.05) between daidzein solution and suspension.

Pharmacokinetics of daidzein-7-*O*-glucuronide After

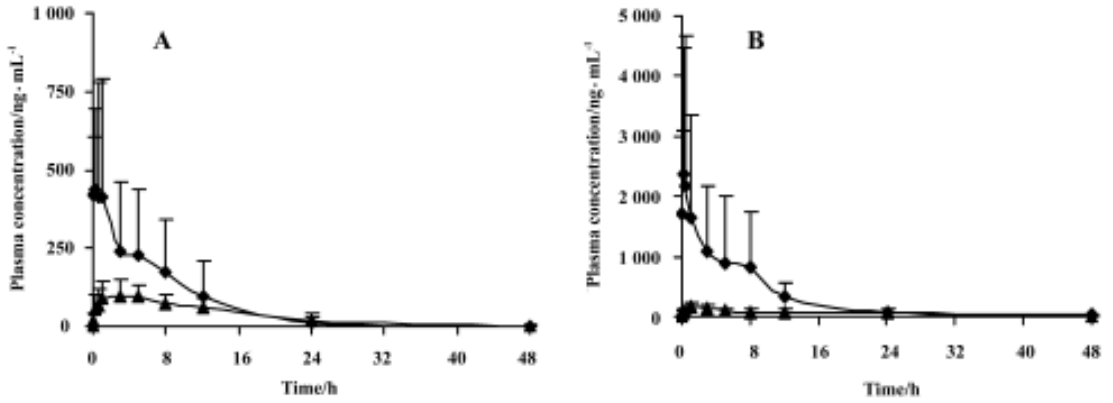

Figure 4. Mean plasma concentration–time curves of (A) daidzein and (B) daidzein-7-*O*-glucuronide following oral administration of 20 mg/kg daidzein solution (◆) and suspension (▲) to Wistar rats. *n*=6. Mean±SD.

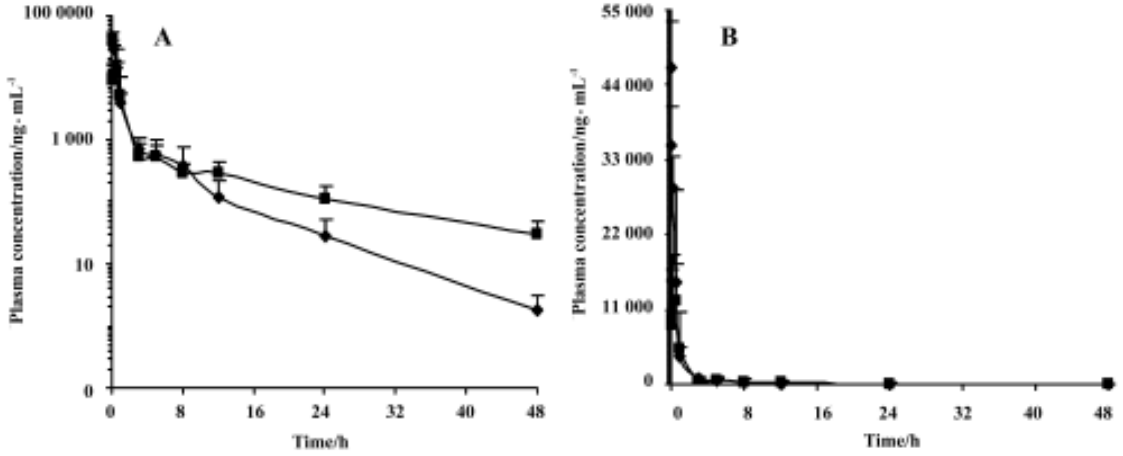

Figure 5. Mean plasma concentration–time curves of daidzein (◆) and daidzein-7-*O*-glucuronide (■) following intravenous administration of 20 mg/kg daidzein solution to Wistar rats. A: Semi-logarithmic coordinate profiles; B: Normal coordinate profiles. *n*=6. Mean±SD.

Table 3. Pharmacokinetic parameters of daidzein and daidzein-7-*O*-glucuronide after oral administration of 2 different dosage forms to rats (20 mg/kg, *n*=6). Each value represents the mean±SD for 6 individual values. [b]*P*<0.05 *vs* solution.

Parameter	Unit	Daidzein		Daidzein-7-*O*-glucuronide	
		Solution	Suspension	Solution	Suspension
$t_{1/2}$	h	3.38±1.88	4.61±1.69	10.8±4.97	10.3±2.56
k_e	h^{-1}	0.26±0.12	0.17±0.06	0.07±0.02	0.07±0.02
t_{max}	h	0.46±0.45	5.00±4.10[b]	0.40±0.34	3.67±4.18
C_{max}	$\mu g \cdot L^{-1}$	601.1±301.3	127.3±49.0[b]	3 000±2 476	192.6±54.6[b]
$AUC_{0-48\,h}$	$\mu g \cdot h \cdot L^{-1}$	3 379±2 982	1 610±819.2	15 154±13 434	2 653±1 624[b]
$AUC_{0-\infty}$	$\mu g \cdot h \cdot L^{-1}$	3 380±2981	1 615±827.7	15 343±13 318	2 734±1 719[b]
F	%	12.8±11.3	6.1±3.1	47.0±42.4[1)]	12.2±9.9[b,1)]

[1)] Calculated with total daidzein (free plus conjugated daidzein).

oral administration of 2 different dosage forms, absorption of daidzein after administration of daidzein solution (t_{max}= 0.40 h) was more rapid than that of suspension (t_{max}=3.67 h). The peak plasma concentration after administration of

Table 4. Pharmacokinetic parameters of daidzein and daidzein-7-*O*-glucuronide after intravenous administration of daidzein solution to rats (20 mg/kg, n=6). Each value represents the mean±SD for 6 individual values.

Parameter	Unit	Daidzein	Daidzein-7-*O*-glucuronide
$t_{1/2}$	h	6.40±2.45	11.4±3.93
k_e	h^{-1}	0.13±0.07	0.07±0.02
$AUC_{0-48\,h}$	$mg \cdot h \cdot L^{-1}$	26.5±7.20	20.8±12.8
$AUC_{0-\infty}$	$mg \cdot h \cdot L^{-1}$	26.5±7.20	21.4±13.2
CL_{TOT}	$mL \cdot min^{-1} \cdot kg^{-1}$	13.4±3.63	18.8±6.46
V_Z	$L \cdot kg^{-1}$	7.65±4.64	17.5±6.37
MRT	h	2.00±1.00	8.09±3.41
V_{ss}	$L \cdot kg^{-1}$	1.45±0.47	9.58±5.66

daidzein solution was 3000 μg/L, which was approximately 14 times higher than that of suspension (192.6 μg/L). The absolute bioavailability of total daidzein (free plus conjugated daidzein) in rats after administration of daidzein solution was 47.0%, but the absolute bioavailability of total daidzein (free plus conjugated daidzein) after administration of daidzein suspension was only 12.2%.

In general, there are significant differences between the main pharmacokinetic parameters of daidzein and daidzein-7-*O*-glucuronide after administration of 2 different dosage forms (P<0.05). There is better absorption following administration of daidzein solution than after administration of suspension.

Discussion

Daidzein shows poor hydrophilicity and lipophilicity due to the typical plane structure of the isoflavones. However, it can be dissolved in weak alkaline solution and form a sodium salt at the hydroxyl groups; as a result its water solubility is greatly increased to 2 g/L. Daidzein solution was found to be stable during a period of at least 24 h (data not shown).

In present study, we choose solution and suspension as the 2 representative dosage forms. Daidzein suspension has similar *in vivo* processes to solid preparations, while daidzein solution is a typical liquid preparation. As a compound with poor solubility, the form of administration may have a great influence on its absorption and pharmacokinetics.

After oral administration, daidzein is subject to glucuronidation at its 7-hydroxyl group, and glucuronide conjugate is its main metabolite and the form in which it mainly exists *in vivo*. The pharmacological effects of daidzein-7-*O*-glucuronide have not been reported in the literature. Daidzein can transform into daidzein-7-*O*-glucuronide as a substrate of glucurotransferases, and daidzein-7-*O*-glucuronide can transform back into daidzein by the action of a hydrolase. As a result, it is necessary to determine the concentrations of free daidzein and daidzein-7-*O*-glucuronide in order to study its absorption and pharmaco-kinetics.

Previous studies, which focused on the pharmacokinetics after consumption of known amounts of soy foods or limited purified isoflavones (solid preparations), found that daidzein was absorbed poorly[6–10]. In our investigations, there are significant differences in pharmacokinetic parameters of daidzein and daidzein-7-*O*-glucuronide between solution and suspension. After administration of daidzein solution, daidzein is absorbed well and is mostly metabolized into daidzein-7-*O*-glucuronide. The reason for the significant differences was the poor solubility of daidzein.

Wojcicki *et al*[13] reported that there were no statistically significant differences in the pharmacokinetics and bioavailability of flavonoid glycosides of *Ginkgo biloba* (quercetin, kaempferol and isorhamnetin) after a single oral administration of 3 formulations to healthy volunteers, which seems to contradict the results of the present study. However, the 3 formulations adopted in that report were capsules, drops and tablets, which were all solid formula-tions, while in present study, solution and suspension (solid) formulations were prepared to investigate their pharma-cokinetics. In fact, there does exist significant differences in the pharmacokinetic parameters of solid and liquid formula-tions.

Pharmacokinetic parameters varied a great deal among individuals, indicating great inter-individual variability of daidzein disposition *in vivo*, which is in accordance with the results found in the literature[9]. There were more factors to affect the pharmacokinetic behavior of daidzein and daidzein-7-*O*-glucuronide after oral administration compared with using an intravenous dose, and this may be the reason that larger RSD values of $AUC_{0-48\,h}$ were achieved after oral administration (88.2% for oral solution and 50.9% for oral suspension; 27.3% for intravenous solution). Furthermore, different distribution amounts of β-glucuronidase *in vivo* and strong hepatic–intestinal cycles may also account for the large differences in the amounts of daidzein and its main metabolite daidzein-7-*O*-glucuronide *in vivo*.

In conclusion, dosage forms have a great influence on the bioavailability of daidzein. Solution preparations are more bioavailable than solid preparations. As a result, the solution preparations of daidzein are recommended for development in order to improve its oral bioavailability.

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