Comparing the pharmacokinetics of daidzein and genistein with the use of $^{13}$C-labeled tracers in premenopausal women$^{1-3}$

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ABSTRACT

Background: Despite significant interest in the risks and benefits of phytoestrogens to human health, few data exist on their pharmacokinetics in humans.

Objective: We investigated the pharmacokinetics of the $^{13}$C isoflavones daidzein and genistein in healthy humans, specifically assessing intraindividual variability, effect of increasing intake, and influence of prolonged exposure to a soy food diet.

Design: Premenopausal women ($n = 16$) were administered 0.4 mg $[^{13}$C]daidzein or $[^{13}$C]genistein/kg body wt orally on 3 occasions, including once after eating soy foods for 7 d. On a further occasion the dose was doubled. Plasma and urinary $[^{13}$C]isoflavone concentrations were measured by mass spectrometry.

Results: Serum concentrations of $[^{13}$C]genistein and $[^{13}$C]daidzein peaked after 5.5 and 7.4 h, respectively. The systemic bioavailability and maximum serum concentration of $[^{13}$C]genistein were significantly greater than those of $[^{13}$C]daidzein. The bioavailability of both isoflavones did not increase linearly when the dietary intake was doubled. The mean volume of distribution normalized to bioavailability ($V_d/F$), clearance rate, and half-life of $[^{13}$C]daidzein were 336.25 L, 30.09 L/h, and 7.75 h, respectively; the corresponding values for $[^{13}$C]genistein were 258.76 L, 21.85 L/h, and 7.77 h. The average recovery of $[^{13}$C]daidzein and $[^{13}$C]genistein in urine was 30.1% and 9.0% of the dose ingested, respectively.

Conclusions: The serum pharmacokinetics of $[^{13}$C]daidzein and $[^{13}$C]genistein were reproducible among healthy women, and genistein was more bioavailable than was daidzein. Pharmacokinetics were unaffected by chronic exposure to soy foods. Urinary isoflavone concentrations correlated poorly with maximal serum concentrations, indicating the limitations of urine measurements as a predictor of systemic bioavailability. The bioavailability of both isoflavones was nonlinear at higher intakes, suggesting that uptake is rate-limiting and saturable. Am J Clin Nutr 2003;77:411–9.

KEY WORDS Isoflavones, pharmacokinetics, stable isotopes, bioavailability, absorption, metabolism, women, phytoestrogens, daidzein, genistein, soy foods

INTRODUCTION

Dietary phytoestrogens have a wide range of biological effects, and over the past decade interest in their potential health benefits has escalated (1). However, information on the absorption, metabolic handling, and pharmacokinetics of soy isoflavones in humans has lagged far behind advances in our understanding of their potential health benefits and mechanisms of action (1–3). Only a limited number of studies in humans have provided insight into the extent to which soy isoflavones are absorbed or how bioavailability varies among individuals (4–9). The effect of differing dietary intakes on bioavailability and the ultimate systemic isoflavone concentration remains to be clarified. Such fundamental information on pharmacokinetics is crucial to evaluating safety and understanding efficacy. Furthermore, a better understanding of the pharmacokinetics of isoflavones will greatly facilitate the design of clinical trials, in which up until now dietary intakes have largely been empirically derived.

Several previous studies alluded to a large interindividual variability in the metabolism and recovery of isoflavones in urine (4, 5, 10–13), and there is some discrepancy in the literature regarding the relative bioavailability of the 2 major soy isoflavones, daidzein and genistein (4, 6, 9). The recent availability of stable-isotope-labeled analogues of daidzein and genistein (14–16) now means we have the tools to accurately obtain fundamental data on their pharmacokinetics in humans independent of the presence of naturally occurring isoflavones from the diet. These labeled tracers circumvent the need to use radioisotopic tracers, and importantly are synthesized with $[^{13}$C] atoms that are both chemically and metabolically inert, unlike previously available deuterium-labeled isoflavones (17, 18) that are less suitable for human metabolism studies. We now report for the first time the pharmacokinetics of $[^{13}$C]daidzein and $[^{13}$C]genistein in healthy adults. By repeated administration of these isotopes, we
determined the intra- and interindividual variability among subjects, performed a limited dose-response study, and determined whether prolonged ingestion of soy isoflavones from foods leads to any changes in the characteristics of the pharmacokinetics of the [13C]isoflavones administered.

SUBJECTS AND METHODS

Isotopically labeled isoflavone tracers

The isotopically labeled isoflavones, [4-13C]daidzein and [4-13C]genistein (Figure 1), were synthesized by methods described previously by Whalley et al (15) and were 99% isotopically pure. These compounds were accurately weighed and packaged into gelatin capsules for oral administration.

Subjects

Classical single-bolus, oral pharmacokinetic studies were carried out on 17 healthy premenopausal women recruited from the staff and student population at the University of Surrey, United Kingdom. Premenopausal women only were studied to minimize variables and maintain a more homogeneous study population (10, 19). All subjects reported a history of regular menstrual cycles and were not using oral contraceptives. Women were excluded if they were vegetarian, were regular soy food consumers, or had taken antibiotics in the previous 6 mo. After the protocol was explained to the subjects, they provided informed consent in writing. All subjects were then instructed to adhere to a diet deficient in soy foods for 1 mo before and for the duration of the study. The study protocol was approved by the Ethics Committee of the University of Surrey.

Study design

After the subjects had fasted overnight, they were assigned to receive either [13C]daidzein (n = 8) or [13C]genistein (n = 9). These stable-isotope-labeled isoflavones were administered orally as a single-bolus dose on 4 different occasions, separated by a minimum 2-wk washout period. On 2 of the visits, a dose of 0.4 mg/kg body wt, defined as the “low dose” and the “low dose repeat,” was used, with the objective of examining the reproducibility of the pharmacokinetics of [13C]daidzein and [13C]genistein. On a third occasion, each subject was again given the same dose (0.4 mg/kg body wt) but 1 wk after adhering to daily consumption of 500 mL soymilk [2 × 250-mL glasses (So Good soymilk; Sanitarium Health Food Company, Berkeley Vale, Australia)] containing 50 mg natural isoflavones (20). The subjects consumed the soymilk in divided doses for 7 d up to the evening before the administration of the isotope. This group was defined as the “low dose after food” group, and this was an attempt to determine whether changes in the pharmacokinetics of isoflavones would occur with habitual consumption of soy isoflavones. On another visit, each subject consumed 0.8 mg/kg body wt, defined as the “moderate dose,” to establish whether doubling the oral intake of [13C]daidzein and [13C]genistein altered the pharmacokinetics. The order in which the various doses were given was randomized.

The clinical component of the study was carried out in the Clinical Investigation Unit at the University of Surrey under medical supervision. At baseline, each subject was weighed and asked to provide a 24-h pooled urine collection and a 10-mL blood sample. After they ingested the 13C-labeled isoflavone, all subjects consumed a standardized breakfast meal, and blood samples were obtained 2, 4, 6, 8, 9, 10, 11, 12, 24, 36, 48, and 72 h after dosing. Blood was obtained via an indwelling catheter for the more frequent sampling and by use of evacuated tubes for the later sampling times. Consecutive, pooled 24-h urine collections were obtained over the next 4 d. To assess completeness of the urine collections, the recovery of a marker substance, p-aminobenzoic acid (administered each day in a dose of 240 mg; Laboratory for Applied Biology Ltd, London), was measured (21). Subjects consumed their habitual diets, taking care to avoid inclusion of soy-protein-containing foods. At each visit, breakfast was taken 15 min after the ingestion of the 13C-labeled compounds, and thereafter the subjects were allowed to consume their habitual diets.

Blood samples were centrifuged at 3000 × g for 10 min at 4°C, and the serum was removed and stored in 1.5-mL aliquots. The volume of each 24-h pooled urine collection was measured, and 30-mL aliquots were retained for analysis. Blood and urine samples were stored at −80°C until analyzed.

Analytic methods

Measurement of isoflavones in serum by gas chromatography–mass spectrometry

The concentrations of [13C]daidzein and [13C]genistein were measured in the serum samples by gas chromatography–mass spectrometry (GC-MS). An isoflavone homologue, dihydroflavone (DHF), was used as the internal standard and was added to the serum sample before extraction and work-up. After equilibration of the serum (0.25–0.50 mL) with 100-ng amounts of the internal standard, the sample was diluted with 10 volumes of 0.5 mol triethylamine sulfate/L (pH 5.0) and heated to 64°C before passage through a wetted solid-phase C18 Bond Elut cartridge (Varian, Harbor City, CA). The solid-phase cartridge was then washed with distilled water (10 mL), and the isoflavones and their conjugates were recovered by elution with methanol (5 mL). The methanol extract was evaporated to dryness under nitrogen, reconstituted with 0.5 mol acetic acid buffer/L (pH 4.5), and hydrolyzed at 37°C overnight with 0.02 mL of helix pomatia digestive juice, a mixed β-glucuronidase-sulfatase preparation (product code G0876; 105 000 U glucuronidase/mL and 4300 U sulfatase/mL; Sigma Chemical Co, St Louis) that had been prefiltered through a C18 Bond Elut cartridge to remove naturally occurring isoflavones present in the enzyme preparation. After hydrolysis, isoflavones were isolated by solid-phase extraction on a C18 Bond Elut cartridge as described above. The phenolic isoflavones were separated from neutral compounds and purified by passage of the sample through a small column bed (7 × 0.4 cm) of triethylaminohydroxypropyl Sephadex LH-20 (TEAP-LH-20;
Pharmacia, Piscataway, NJ) prepared in the [OH]− form and packed in methanol. The phenolic compounds were recovered by elution of the gel bed with 15 mL methanol saturated with carbon dioxide (22). The phenolic fraction was taken to dryness under a stream of nitrogen gas, and isoflavones were converted to the terti-butyldimethylsilyl (tBDMS) ether derivatives for analysis by GC-MS. tBDMS ethers were prepared by addition of acetonitrile (100 μL) and N-methyl-N-t-butyldimethylsilyl trifluoroacetic acid in 1% t-butyldimethylchlorosilane (100 μL), and the sample was heated at 65 °C for 2 h. The reagents were removed by evaporation in a stream of nitrogen and the derivatives were dissolved in hexane (100 μL).

Isoflavone tBDMS ethers were separated and quantified by GC-MS. Chromatographic separation was achieved on a DB-1 fused silica capillary column (30 m x 0.25 mm internal diameter, 0.25-μm film thickness; J & W Scientific Inc., Folsom, CA) with helium as the carrier gas (flow rate of ∼2 mL/min) and with a temperature program from 260 to 310 °C with increments of 10 °C/min. Selected ion monitoring GC-MS of specific and characteristic ions in the electron ionization (70 eV) spectra of the tBDMS ether derivatives of each isoflavone permitted highly sensitive and specific quantification. The following ions were monitored: mass-to-charge ratio (m/z) 425 (daidzein and DHP internal standard), m/z 426 ([13C]daidzein), m/z 470 (equol), m/z 471 ([13C]equol), m/z 555 (genistein), and m/z 556 ([13C]genistein). Metabolites such as dihydrodaidzein or desmethylangolensin were not quantified because of the lack of availability of stable-isotope-labeled reference compounds. The individual isoflavones were quantified by comparing the peak area in the specific ion channels at the retention time corresponding to authentic compounds, with the peak area response for the internal standard constructed for known amounts (0–200 ng) of the individual isoflavones. Concentrations were expressed as μmol/L for the individual plasma isoflavones.

The method was validated by repeated analysis of a pooled sample of serum obtained from healthy adults who had consumed soy foods. Duplicate quality-control samples were included with each analytic run, and one study sample selected at random was repeated within each analytic batch. The within-day instrument reproducibility, expressed as CV%, was 0.5% for daidzein and 1.0% for genistein. The mean between-batch reproducibility (on the basis of 19 runs performed over a 1.5-y period) was 5.0% (range: 1.0–11.9%) for daidzein and 7% (range: 1.0–17.0%) for genistein. The within-day instrument reproducibility was 3% for daidzein and 4% for genistein. Between-batch reproducibility (based on 12 runs over a 6-mo period) was 8% for daidzein and 10% for genistein at concentrations between 200 and 300 ng/mL.

**Determination of serum isoflavone pharmacokinetics and statistical analysis**

A noncompartmental approach was used for the pharmacokinetic analysis with WINNONLIN 1.5 (Pharsight Corporation, CA) computer software. This technique uses the trapezoidal rule to determine the area under the serum concentration-versus-time curve (AUC). The total AUC (AUC_{t-last} or AUC_{0-t}) was calculated in a 2-step process. First, the AUC from time point 0 to any time point t on the log-linear region of the terminal part of the curve was determined. Second, the remaining area from t to infinity was determined as C_{t/λ_z}/λ_z; where C_t is the serum concentration of the isoflavone at time t and the rate constant (λ_z) was calculated from the slope of the terminal phase of disposition. At least 3 points were included for the purpose of λ_z determination. The number of points to be included was based on the correlation coefficient and residual analysis. Appropriate weighting schemes, with weights of 1/y or 1/y^2, were used to improve the goodness-of-fit of the data. Other parameters determined included C_{max}, the peak plasma isoflavone concentration; t_{max}, the time required to attain the peak concentration; CL, systemic clearance (normalized to bioavailable fraction); and V/F, the volume of distribution normalized to bioavailability.

**Statistical analysis** was carried with SPSS 10.0 (SPSS Inc, Chicago). All data are expressed as means ± SEMs. The effect of dose regimen on urinary and serum variables was assessed separately in each cohort (daidzein or genistein) by using 2-factor analysis of variance (ANOVA), the main effects being dose regimen and subject. A Tukey’s multiple-comparison adjustment was then made when significant dose effects were found to investigate which doses were contributing to the significance. To compare the variables between the cohorts, a three-factor ANOVA was used (the additional factor being the cohort, with the subject factor now...
Daidzein cohort (n = 8)

<table>
<thead>
<tr>
<th>Dose regimen</th>
<th>Low dose</th>
<th>Low dose repeat</th>
<th>Mean of low dose and low dose repeat</th>
<th>Low dose after food</th>
<th>Moderate dose</th>
<th>Mean for visits</th>
</tr>
</thead>
<tbody>
<tr>
<td>( C_{\text{max}} ) (µmol/L)</td>
<td>0.31 ± 0.07 (^{3})</td>
<td>0.45 ± 0.12</td>
<td>0.38 ± 0.10 (^{5})</td>
<td>0.31 ± 0.07</td>
<td>0.71 ± 0.15</td>
<td>0.36 ± 0.05 (^{4})</td>
</tr>
<tr>
<td>( t_{1/2} ) (h)</td>
<td>8.17 ± 0.81</td>
<td>7.67 ± 0.75</td>
<td>7.92 ± 0.78</td>
<td>7.99 ± 0.89</td>
<td>7.18 ± 0.49</td>
<td>7.75 ± 0.36</td>
</tr>
<tr>
<td>( \text{AUC}_{\text{inf}} ) (µmol-h/L)</td>
<td>3.96 ± 0.71 (^{3})</td>
<td>6.3 ± 1.6</td>
<td>5.1 ± 1.16 (^{3})</td>
<td>4.78 ± 0.86</td>
<td>8.70 ± 1.61</td>
<td>5.02 ± 0.67 (^{4})</td>
</tr>
<tr>
<td>( V_{\text{f}} ) (observed)/F (L)</td>
<td>30.46 ± 7.82</td>
<td>230.99 ± 42.55</td>
<td>205.73 ± 60.37</td>
<td>334.35 ± 97.23</td>
<td>399.21 ± 148.59</td>
<td>336.25 ± 48.63</td>
</tr>
<tr>
<td>( \text{CL}_{\text{observed}}/F ) (L/h)</td>
<td>32.00 ± 4.37</td>
<td>21.46 ± 3.84</td>
<td>26.73 ± 4.29</td>
<td>30.24 ± 9.38</td>
<td>36.69 ± 12.51</td>
<td>30.09 ± 4.08 (^{6})</td>
</tr>
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</table>

Genistein cohort (n = 8)

<table>
<thead>
<tr>
<th>Dose regimen</th>
<th>Low dose</th>
<th>Low dose repeat</th>
<th>Mean of low dose and low dose repeat</th>
<th>Low dose after food</th>
<th>Moderate dose</th>
<th>Mean for visits</th>
</tr>
</thead>
<tbody>
<tr>
<td>( C_{\text{max}} ) (µmol/L)</td>
<td>0.55 ± 0.09</td>
<td>0.41 ± 0.06 (^{7})</td>
<td>0.48 ± 0.08 (^{5})</td>
<td>0.43 ± 0.07 (^{7})</td>
<td>0.87 ± 0.14</td>
<td>0.46 ± 0.04</td>
</tr>
<tr>
<td>( t_{1/2} ) (h)</td>
<td>7.52 ± 0.38</td>
<td>7.84 ± 0.30</td>
<td>7.68 ± 0.34</td>
<td>8.31 ± 0.80</td>
<td>7.41 ± 0.39</td>
<td>7.77 ± 0.25</td>
</tr>
<tr>
<td>( \text{AUC}_{\text{inf}} ) (µmol-h/L)</td>
<td>6.75 ± 1.29</td>
<td>5.90 ± 1.22 (^{5})</td>
<td>6.33 ± 1.26 (^{5})</td>
<td>5.35 ± 0.85 (^{5})</td>
<td>9.77 ± 1.32</td>
<td>6.01 ± 0.63</td>
</tr>
<tr>
<td>( V_{\text{f}} ) (observed)/F (L)</td>
<td>198.02 ± 32.49</td>
<td>250.10 ± 49.07</td>
<td>224.06 ± 40.78</td>
<td>343.86 ± 150.02</td>
<td>243.06 ± 37.97</td>
<td>258.76 ± 40.47</td>
</tr>
<tr>
<td>( \text{CL}_{\text{observed}}/F ) (L/h)</td>
<td>17.67 ± 2.41</td>
<td>22.66 ± 4.59</td>
<td>20.17 ± 3.50</td>
<td>24.68 ± 6.82</td>
<td>22.39 ± 2.56</td>
<td>21.85 ± 2.17</td>
</tr>
</tbody>
</table>

\(^{1,2}\) SEM. The low dose was 0.4 mg/kg body wt, and the moderately dose was 0.8 mg/kg body wt. The low dose after food was administered after the women had consumed a soy-rich diet (containing 50 mg isoflavone/d) for 7 d. \( C_{\text{max}} \), peak plasma isoflavone concentration; \( t_{1/2} \), time to peak concentration; \( \text{AUC}_{\text{inf}} \), total area under the serum concentration-versus-time curve; \( V_{\text{f}}/F \), volume of distribution normalized to bioavailability; \( \text{CL}_{\text{observed}}/F \), systemic clearance normalized to bioavailable fraction.

\(^{3}\) The mean of all 4 dose regimens, except for \( C_{\text{max}} \) and \( \text{AUC}_{\text{inf}} \), which represent a mean of all 3 low-dose regimens.

\(^{4,5}\) Significantly different from moderate dose (two-factor ANOVA with Tukey’s post hoc test): \( p \leq 0.01, 3 p \leq 0.05. \)

\(^{6,7}\) Significantly different from genistein (three-factor ANOVA): \( 4 p \leq 0.01, 5 p \leq 0.05. \)
on 18 April 2018


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FIGURE 3. Mean (±SEM) serum [13C]genistein concentrations after the administration of a single oral bolus of [13C]genistein on 4 separate occasions to 8 premenopausal women. The low dose was 0.4 mg/kg body wt, and the moderate dose was 0.8 mg/kg body wt. The low dose after food was administered after the women had consumed a soy-rich diet (containing 50 mg isoflavones/d) for 7 d.

visits. No significant difference in the pharmacokinetics of [13C]daidzein was observed among the visits at which the subjects consumed the 0.4-mg/kg body wt dose (low dose, low dose repeat, and low dose after food), showing good reproducibility within individuals in the handling of this isotope. However, the mean value for $C_{\text{max}}$ and $\text{AUC}_{\text{inf}}$ for [13C]daidzein was significantly greater ($P < 0.01$ in both cases) when 0.8 mg/kg body wt (moderate dose) was administered, as expected. Bioavailability, however, as measured from the $\text{AUC}_{\text{inf}}$ of the serum profiles, did not double with the higher dose [5.15 (mean for the low dose and low dose repeat) compared with 8.70 (moderate dose) μmol·h/L]. Prolonged exposure to soy isoflavones from a soy food diet consumed for 7 d (low dose after food group) did not significantly change the pharmacokinetics of [13C]daidzein.

[13C]Genistein kinetics

The appearance and disappearance curves for [13C]genistein in serum for each of the 4 study visits are compared in Figure 3. After ingestion of the [13C]genistein, serum concentrations rapidly increased in all individuals. $C_{\text{max}}$ was attained at 5.5 ± 2.7 h ($t_{\text{max}}$) after oral administration of the isotope, and the $t_{\text{max}}$ was not significantly different among the 4 dosing regimens. Serum concentrations returned close to baseline values 2 d after ingestion of the isotope.

Shown in Table 1 are the values for $C_{\text{max}}$, $t_{1/2}$, $\text{AUC}_{\text{inf}}$, $V_d$, and CL/F computed from the plasma appearance and disappearance curves of the serum concentrations for each of the visits. No significant differences in the pharmacokinetics of [13C]genistein were observed among the 3 visits at which the subjects consumed the 0.4-mg/kg body wt dose, showing good reproducibility within individuals in the handling of this isotope. The value for $\text{AUC}_{\text{inf}}$ for [13C]genistein was significantly greater ($P < 0.05$) when the isoflavone dose was increased to 0.8 mg/kg body wt. Like [13C]daidzein, however, the mean values for the bioavailability of [13C]genistein did not double with a doubling of the intake of isotope [6.33 (moderate dose) compared with 9.77 (mean for the low dose and low dose repeat) μmol·h/L]. $C_{\text{max}}$ also significantly increased in 2 of the low-dose regimens compared with the moderate dose ($P < 0.05$). The values for $C_{\text{max}}$ and $\text{AUC}_{\text{inf}}$ were unaffected by prior exposure to isoflavones (low dose after food). For $\text{AUC}_{\text{inf}}$, the mean was slightly lower, but the difference was not significantly different from the values for other low-dose regimens. The calculated $t_{1/2}$, CL, and $V_d/F$ were not significantly different among the 4 dosing regimens.

Monitoring of natural isoflavone concentrations

At baseline ($t = 0$ h), the group mean serum concentration of the naturally occurring, nonisotopic isoflavones daidzein, genistein, and equol in women administered 0.4 mg [13C]daidzein/kg body wt was 41.0 ± 4.4 nmol/L; this was not significantly different from the baseline concentration of naturally occurring isoflavones in the low dose [13C]daidzein repeat group. The corresponding baseline isoflavone concentration in the same women fed soymilk for 1 wk was 445.6 ± 73.3 nmol/L. The profiles for baseline serum concentrations of the naturally occurring isoflavones were not significantly different in the corresponding [13C]genistein cohorts (data not shown). Irrespective of the dose of isoflavone given, the concentrations of the naturally occurring isoflavones were low and consistent with abstinence from soy foods. When women were given soy foods daily for 7 d and blood was collected on the 8th day, the women still had elevated concentrations of daidzein and genistein in serum consistent with the terminal elimination phase of their pharmacokinetics, and the concentration continued to decline exponentially throughout the day. Although the pharmacokinetics of the natural isoflavones were not computed, on the basis of the slope of the terminal elimination curve, their pharmacokinetics appear similar to that of the [13C]isotopes. Measurement of the natural isoflavones served to confirm compliance with the study protocol.

Urinary isoflavone pharmacokinetics

The daily urinary excretion of [13C]daidzein and [13C]genistein at baseline and on the 4 d after administration of the isotopically labeled isoflavones are shown in Figure 4. Most of the recovered isoflavones were excreted in the first 24 h after their administration. By days 3 and 4, very little appeared in the urine.

The cumulative recovery of isotope over the 4 dosing regimens averaged 30.1 ± 2.39% and 9.0 ± 0.78% for the administered dose of [13C]daidzein and [13C]genistein, respectively, for all groups. Concentrations of [13C]daidzein in urine were ~3-fold and significantly higher than concentrations of [13C]genistein; the inverse of the relation observed in serum. The urinary excretion rates for [13C]daidzein and [13C]genistein were not significantly different among the 3 visits when the 0.4-mg/kg body wt dose was administered. When this dose was doubled for both [13C]isoflavones, the cumulative percentage recovery of [13C]daidzein and [13C]genistein did not increase linearly, resulting in a decreased fractional absorption (Table 2).

Shown in Figure 5 are the correlations between the serum $C_{\text{max}}$ values and the excretion of [13C]daidzein and [13C]genistein in urine, expressed as total urinary output (μmol) and urinary isoflavone concentration (μmol/L) in the first 24-h collection, when most of the isotope was excreted. There was no significant correlation between serum daidzein concentrations and urinary daidzein excretion; however, correlations between serum genis-
Metabolism of $^{13}$C-daidezine to $^{13}$C-equol

Though there was considerable scatter in the data ($P < 0.01$, respectively).

**DISCUSSION**

The current study, to our knowledge, is the first detailed pharmacokinetic investigation of the stable isotopes of daidezine and genistein in healthy adults. This study was possible because of the recent synthesis of chemically and metabolically stable labeled $^{13}$C analogues of daidezine and genistein (14–16) in quantities sufficient for making comparisons with usual dietary intakes from soy foods. Although several stable isotopes of daidezine and genistein were previously synthesized incorporating deuterium (17, 18), these analogues are unsuitable for use as tracers in human studies because of the instability of the deuterium label and its propensity to undergo hydrogen-deuterium exchange. The isotopes used in this study were both isotopically pure (99%) and chemically synthesized with $^{13}$C labeled in the heterocyclic ring at position C-4 (15) and as such were chemically inert. The use of mass spectrometry allowed us to differentiate the stable-isotope-labeled tracer from the unlabeled compound. This enabled us to study the pharmacokinetics in a background of natural isoflavones that might be present from the diet, even though in 3 phases of this study the subjects did not consume soy foods. The high specificity and sensitivity of GC-MS for measuring plasma $^{13}$C-daidezine and $^{13}$C-genistein concentrations afforded accurate and precise measurement of the terminal elimination phase, an important criterion in establishing reliable pharmacokinetic data.

In this study, classic pharmacokinetic studies based on single-bolus oral administration of the isotopes were performed on 3 different occasions on which the same low dose (0.4 mg/kg body wt) of isofate was administered and on 1 occasion on which this dose was doubled. The amount of isotopically labeled isoflavones administered was in the range reported for the daily isoflavone intake of persons living in Asian countries who regularly consume soy foods (23–25). This approach established a reproducibility in the pharmacokinetics of isoflavones among individuals over time.

**TABLE 2**

Urinary $^{13}$C-daidezine and $^{13}$C-genistein recovery expressed as the amount recovered and as fractional absorption.

<table>
<thead>
<tr>
<th>Dose regimen</th>
<th>Dose recovered (µmol excreted)</th>
<th>Fractional absorption (% dose)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Daidzein cohort (n=8)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low dose</td>
<td>31.70 ± 5.72$^1$</td>
<td>29.50 ± 4.78</td>
</tr>
<tr>
<td>Low dose repeat</td>
<td>37.60 ± 6.65</td>
<td>34.49 ± 5.86</td>
</tr>
<tr>
<td>Mean of low dose and low dose repeat</td>
<td>34.65 ± 6.19$^2$</td>
<td>26.58 ± 3.10</td>
</tr>
<tr>
<td>Low dose after food</td>
<td>28.38 ± 4.91</td>
<td>25.62 ± 2.69</td>
</tr>
<tr>
<td>Moderate dose</td>
<td>45.42 ± 4.90</td>
<td></td>
</tr>
<tr>
<td><strong>Genistein cohort (n=8)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dose recovered (µmol excreted)</td>
<td>7.67 ± 1.49</td>
<td>8.89 ± 1.39</td>
</tr>
<tr>
<td>Fractional absorption (% dose)</td>
<td>7.69 ± 1.10$^1$</td>
<td>8.86 ± 1.87</td>
</tr>
<tr>
<td>Low dose after food</td>
<td>7.68 ± 1.30$^1$</td>
<td>8.88 ± 1.63</td>
</tr>
<tr>
<td>Moderate dose</td>
<td>7.93 ± 1.29</td>
<td>9.27 ± 1.86</td>
</tr>
<tr>
<td></td>
<td>15.32 ± 1.92</td>
<td>8.25 ± 1.16</td>
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$^1$× ± SEM. The low dose was 0.4 mg/kg body wt, and the moderate dose was 0.8 mg/kg body wt. The low dose after food was administered after the women had consumed a soy-rich diet (containing 50 mg isoflavones/d) for 7 d.

$^2$Significantly different from moderate dose (two-factor ANOVA with Tukey’s post hoc test): $^1P < 0.01$, $^2P < 0.05$. 

**Figure 4.** Mean (± SEM) urinary excretion of $^{13}$C-daidezine and $^{13}$C-genistein compounds during 4 dosing regimens in which premenopausal women ingested a single oral bolus of either $^{13}$C-daidezine (n=8) or $^{13}$C-genistein (n=8). The low dose was 0.4 mg/kg body wt, and the moderate dose was 0.8 mg/kg body wt. The low dose after food was administered after the women had consumed a soy-rich diet (containing 50 mg isoflavones/d) for 7 d. B, baseline.
and showed that prolonged intake of isoflavones (50 mg/d over 7 consecutive days) from a diet containing soy foods does not alter bioavailability or pharmacokinetics. After oral administration, \([^{13}\text{C}]\)daidzein and \([^{13}\text{C}]\)genistein rapidly appeared in serum, reaching maximal concentrations between 2 and 8 h after ingestion, and the isotopes were slowly eliminated according to first-order kinetics. The half-lives, based on the combined data for all women at all visits, were 7.75 ± 0.36 and 7.72 ± 0.25 h for \([^{13}\text{C}]\)daidzein and \([^{13}\text{C}]\)genistein, respectively. These values are almost identical to the previously reported half-lives of the naturally occurring pure compounds (9) and are similar to values reported by others, allowing for some limitations in the methods used (5, 7).

On the basis of the principles of pharmacology and the elimination half-life of daidzein and genistein, it can be predicted that steady state plasma concentrations would be more readily maintained by repeated ingestion of isoflavones throughout the day than by ingestion just once a day. This is an important consideration when clinical studies are being designed to examine the health benefits of soy isoflavones. In such studies, a portfolio of different soy foods would probably ensure maintenance of constant blood concentrations and improve compliance.

Our studies found serum concentrations of \([^{13}\text{C}]\)genistein to be consistently higher than those of \([^{13}\text{C}]\)daidzein, consistent with previous pharmacokinetics when the natural compounds were similarly administered (9). This holds true for all healthy adults who consume soy products containing approximately equal amounts of daidzein and genistein. However, it is worth noting that in patients with kidney disease, the reverse relation has been reported in serum (26). This indicates that the kidneys play a key role in eliminating these compounds from the body. Fecal excretion of isoflavones is a relatively minor route of elimination for these compounds (4, 7); although we collected fecal samples, only traces of the isotopes were identified in feces. The clearance rates of \([^{13}\text{C}]\)daidzein coupled with its high Vd/F contribute to consistently lower serum concentrations of daidzein than of genistein (P < 0.05), a consistent finding in most studies (5, 7, 9, 27, 28).

The bioavailability of \([^{13}\text{C}]\)genistein determined from the AUCinf of the serum isoflavone profiles was significantly greater (P = 0.01) than that of \([^{13}\text{C}]\)daidzein (Table 1). These data agree with a previous finding that genistein is more bioavailable than daidzein (9) but are at variance with one previous report (4). In the latter study, Xu et al (4) judged bioavailability on the basis of plasma concentrations at only 2 time points (6.5 and 24 h)—an impossibility because the AUC cannot be computed from such a study design. Furthermore, the early sampling point can be disregarded because it is unlikely that a steady state was attained by 6.5 h postingestion. Nevertheless, the authors did show that plasma concentrations of genistein were much higher than those of daidzein at the 24-h time point for all doses of isoflavones ingested. This is what one would anticipate with genistein being more bioavailable than daidzein and is what we have shown here and elsewhere (7, 9). The flaw in the study by Xu et al (4) is that bioavailability cannot be determined with just 2 time points, and reliance of urinary excretion is misleading unless the compound is 100% bioavailable, which is not the case for isoflavones.

Linear regression analysis comparing the serum Cmax values and urinary isoflavone outputs or concentrations in the first 24 h (Figure 5) failed to show a strong correlation for \([^{13}\text{C}]\)daidzein, although a weak relation for \([^{13}\text{C}]\)genistein was seen. There was considerable scatter in the data, indicating that for any individual the urinary isoflavone concentration gives only a crude indication of the dietary isoflavone intake. Indeed, noteworthy was the decreased fractional absorption of isoflavones with an increase (in this case a doubling) in intake. In healthy individuals, urine consistently has higher concentrations of daidzein than of genistein when equimolar amounts of the 2 compounds are ingested. This would be expected on the basis of the greater plasma clearance rate of daidzein (Table 1), and it is perhaps this consistently higher urinary output that led to the perception that daidzein is more bioavailable than genistein (4). However, the current data confirm that bioavailability cannot be assessed from urine excretion.
Only 2 studies have examined the dose-response effects of isoflavone intake, and these examined urinary isoflavone excretion exclusively, with no attempt made at assessing systemic bioavailability (4, 13). In our study, we examined the effect of doubling the dose of \(^{13}\text{C}\)daidzein and \(^{13}\text{C}\)genistein on the serum pharmacokinetics. Some variability existed between the low-dose regimens. However, when the mean AUC of the 3 low-dose regimens was compared with that of the moderate dose, it increased but not in a linear manner, even though a positive correlation between isoflavone intake and peak concentration of \(^{13}\text{C}\)daidzein (\(r^2 = 0.314, P < 0.001;\) linear regression) and \(^{13}\text{C}\)genistein (\(r^2 = 0.266, P < 0.01;\) linear regression) was observed. This nonlinear increase was also noted in our other studies in which different isoflavone intakes were studied by using soy foods (29). Overall, these findings show that there may be a limited advantage to consuming high amounts of isoflavones in single doses because absorption appears to be rate-limiting.

The nonlinear relation between bioavailability and dietary intake was also evident from the urinary data, which showed that the total recovery of \(^{13}\text{C}\)daidzein and \(^{13}\text{C}\)genistein in the urine over a 4-d period did not double with the doubling of the dose. The fractional absorption consequently decreased (see Table 2) with increased intake. Two previous dose-response studies reported urinary excretion of isoflavones after soy-protein consumption (4, 13). In one, the percentage recovery of daidzein and genistein was not significantly different over a range of isoflavone intakes equivalent to 0.7–2.0 mg/kg body wt, somewhat higher than the doses used in our study (4). The percentage fractional absorption of daidzein and genistein in urine at that study was similar to the values reported here. In a second study in which subjects consumed 0–15 mg isoflavones (we estimate on the basis of an average body weight of 70 kg that this is equivalent to 0.2 mg/kg body wt), approximately one-half the amount used in our study, a linear relation was observed in absolute urinary recovery.

Whether the food matrix can alter the pharmacokinetics and bioavailability of isoflavones remains a matter of conjecture. It is interesting that in subsequent studies we have performed using soy nuts and soymilk, we observed a similar curvilinear relation between AUC\(_{\text{inf}}\) and dose (29; M Faughnan, KDR Setchell, and A Cassidy, unpublished observations, 2003) at higher isoflavone intakes. Overall, these findings raise questions regarding the clinical advantage of recommending extremely high intakes of dietary isoflavones, as was the case in several clinical studies (30–33). It is possible that higher intakes may be necessary in the treatment of disease, but that low intakes, more in tune with exposure in Asia, may be valuable for disease prevention. These issues remain to be investigated.

Izumi et al. (28) showed that very high concentrations of isoflavones can be attained in blood after the ingestion of pharmacologic doses. Pharmacologic doses may override any rate-limiting step, a common phenomena, as was shown for vitamin E intake (34). Although the doses required to deliver specific health effects to humans remain largely to be determined, the safety issues involved in consuming pharmacologic doses is presently unknown. There are sufficient examples of deleterious effects of isoflavones in animals consuming high intakes (35–37).

Most of the stable-isotope-labeled isoflavones were excreted in the first 24 h after administration, but a significant proportion of the administered dose must have been metabolized to compounds that we could neither detect nor measure. This has been a consistent enigma in all metabolic studies of isoflavone excretion (4–7, 10, 18, 38). It is probable that there is extensive biodegradation in the intestine to metabolites that have yet to be identified. The metabolism of isoflavones has been extensively studied in many animal species (35–42) and a wide range of metabolites have been identified, including dihydroadzein, equol, O-desmethylanglenesin, dihydrogenistean, and p-ethylphenol. However, stable-isotope-labeled forms of these compounds are not yet commercially available as reference standards for quantification, save \(^{13}\text{C}\)equol, which we recently synthesized. For this reason, we obtained quantitative data on the conversion of \(^{13}\text{C}\)daidzein to \(^{13}\text{C}\)equol only.

Equol was shown to be the specific metabolite of daidzein in humans (43). In the 8 subjects who were administered \(^{13}\text{C}\)daidzein, only 3 (37.5%) could convert this into equol, and \(^{13}\text{C}\)equol did not immediately appear in serum or urine, which is consistent with the colonic origins of its formation (40). There is significant interest in equol given its higher affinity for the estrogen receptor and its greater antioxidant potential than its precursor daidzein. The factors governing the production of equol in adults remain unknown. It was evident from our study that women producing \(^{13}\text{C}\)equol on their first visit continued to do so on all subsequent visits when challenged with \(^{13}\text{C}\)daidzein. This suggests that, “once an equol producer, always an equol producer”; the question of whether a person who is unable to synthesize equol will ever be able to do so remains unclear.

The characterization of the pharmacokinetics of \(^{13}\text{C}\)daidzein and \(^{13}\text{C}\)genistein provides fundamental data to further our understanding of the bioavailability of isoflavones. However, the limitations of interpreting the findings relate to whether data from administration of pure compounds can really be translated to isoflavones contained within the soy food matrix. This is always a limitation in studies of dietary constituents administered as single compounds. The data we present here provide the basis of a long-term program in which we are examining the pharmacokinetics of isoflavones in foods; these data will be reported in the future. Initial comparisons appear to indicate that, overall, the pharmacokinetics of isoflavones in foods are similar and that there is saturable uptake with higher intakes. Differences have been observed with regard to the shape of the plasma appearance and disappearance curves depending on the food matrix.

In conclusion, our findings indicate that the serum pharmacokinetics of isoflavones is consistent among healthy adult women and that genistein is more bioavailable than daidzein. The fundamental rules of pharmacokinetics suggest that the rule of diminishing returns applies for high intakes: there is a curvilinear relation between bioavailability and dose ingested. Although it has yet to be proven, we suggest on the basis of these pharmacokinetics and by analogy to the characteristics of many drugs that there are probable advantages to consuming modest amounts of isoflavones throughout the day to sustain more constant steady state plasma concentrations. These findings provide fundamental data on which to make comparisons with the behavior of isoflavones in soy foods and subsequently to afford a more rigorous optimization of intakes to be used in clinical and dietary studies designed to study efficacy.

REFERENCES