# Urinary Pharmacokinetics of the Glucuronide and Sulfate Conjugates of Genistein and Daidzein[1]

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## Abstract

**Consumption of soybean-rich diets is thought to provide significant health benefits such as prevention of cancer, primarily because of the high contents of factors such as the isoflavones genistein and daidzein. Isoflavones circulate and are excreted into the urine mainly as glucuronide and sulfate conjugates. This study was conducted to determine the urinary pharmacokinetics of sulfate and glucuronide conjugates of genistein and daidzein. Twelve volunteers consumed a soy beverage providing 1 and 0.6 mg/kg body weight of genistein and daidzein equivalents, respectively. Urine was collected at various times during the 48 h after soy consumption and was digested with either glucuronidase or sulfatase, and the liberated aglycones were extracted and analyzed by liquid chromatography-mass spectrometry. Urinary isoflavone sulfate levels were determined by two methods: (*a*) assessment of aglycone after sulfatase hydrolysis (measured); or (*b*) calculated by subtracting the aglycone + glucuronide levels from the total urinary isoflavone levels. The apparent terminal half-life for daidzein sulfate (3.9 ± 0.5 h) that was determined from sulfatase-treated urine was 32% shorter ($P \leq 0.02$) than that of the calculated daidzein sulfate (5.7 ± 0.08 h). A similar trend was obtained for genistein sulfate (4.5 ± 0.7 *versus* 6.8 ± 0.1 h). The apparent terminal half-lives for genistein and daidzein glucuronides were 6.0 ± 0.4 and 3.8 ± 0.4 h, respectively. These data suggest that the measured urinary isoflavone sulfate values provide a better understanding of the pharmacokinetics than the calculated values. Additional studies are needed to determine whether the apparent terminal half-lives can be attributed to elimination or absorption processes.**

## Introduction

The isoflavone phytoestrogens, genistein and daidzein, found in soybeans, are currently being studied for prevention of breast cancer (1–4) and colon cancer (5). Evidence for chemopreventative effects of soy consumption has come from epidemiological studies, which show that the incidence of breast cancer is reduced in populations consuming high amounts of soy (3) and from animal studies, which show that diets high in soy protein and/or isoflavones protect against chemically induced breast cancer (6, 7) and colon cancer (5).

Several mechanisms for the chemopreventative properties of genistein have been proposed. *In vitro*, genistein binds estrogen receptors (ER$\alpha$ and ER$\beta$) and acts as either an agonist or antagonist, depending on the concentration. Genistein has a variety of other effects, such as: (*a*) inhibition of protein tyrosine kinases; (*b*) inhibition of DNA topoisomerase II activity; (*c*) modification of cell differentiation; and (*d*) inhibition of reactive oxygen species production (for review, see Ref. 8).

To design appropriate studies of the mechanisms and efficacy of soy isoflavones for cancer prevention, a thorough knowledge of the concentrations of biologically active forms in biological fluids and of their pharmacokinetics is needed. Although genistein and daidzein aglycones are associated with biological activity, in humans the majority of isoflavones circulate and are excreted as the glucuronide conjugates, with lower percentages existing as the sulfate conjugates. Little attention has been given to isoflavone sulfate conjugates in the literature. However, these sulfated conjugates may have biological activity. For example, daidzein-7,4′-di-*O*- sulfate has been reported to competitively inhibit sterol sulfatase in hamster liver microsomal fractions, whereas daidzein does not (9). Similarly, sulfate conjugates of endogenous steroids are thought to possess biological activity and to be an important source of free cellular steroids on sulfatase hydrolysis (10). It is possible, therefore, that sulfated isoflavones are active *in vivo* or are a primary source of free cellular aglycones after enzymatic hydrolysis in target tissues. Genistein glucuronides may also be active *in vivo* inasmuch as they have been demonstrated to have weak estrogenic activity and can activate human natural killer cells *in vitro* (11). It is also possible that the glucuronide conjugates of isoflavones are a source of free cellular aglycones, because the deglucuronidation of estradiol- or estrone-3$\beta$-D-glucuronide has been demonstrated in Syrian hamster kidney and liver lysosomes and microsomes (12).

Several pharmacokinetic studies of isoflavones in humans after soy consumption have been conducted (13–22). However, these studies have used either measurements of "total" isoflavones (after digestion of sample with sulfatase plus glucuronidase) or measurements of glucuronide conjugates alone for their analysis. In the present study, we report the urinary estimates of pharmacokinetic parameters for genistein and daidzein sulfate and glucuronide conjugates after soy protein ingestion.

## Materials and Methods

**Subjects.** Twelve healthy adults (six males and six females) participated in the soy isoflavone pharmacokinetic study. This

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study was approved by the Human Research Advisory Committee of the University of Arkansas for Medical Sciences. All of the subjects gave their written consent. None of the subjects were taking hormone replacement therapy or oral contraceptives, were pregnant, or had taken antibiotics in the last 4 months. All of them were considered generally healthy. Their average weight was 86.0 ± 6.4 and 60.2 ± 0.8 kg for male and female subjects, respectively.

The subjects were given a list of phytoestrogen-containing foods and asked to avoid them for 1 week before and during the study. Subjects recorded all of the food and drink ingested during this time period, and this information was used to help verify compliance. A baseline level of phytoestrogens was determined in a 24-h urine sample collected the day before the study started. On the first day of the study, the subjects fasted overnight and then consumed a soy beverage prepared to provide a dose of 1.0 mg/kg genistein aglycone equivalents and 0.6 mg/kg daidzein aglycone equivalents. Doses were calculated based on the concentrations of isoflavones in the soy protein as determined by the manufacturer, Protein Technologies International (St. Louis, MO). Each gram of soy protein contained 0.72 mg of genistein equivalents, 0.39 mg of daidzein equivalents, and 0.07 mg of glycitein equivalents. The soy beverage was prepared with soy protein and a banana and was diluted with equal parts of pineapple and orange juice (except for one subject who requested no banana and another subject who consumed the soy protein isolate dissolved in orange juice only). The amounts of genistein, daidzein, and glycitein in bananas and pineapple and orange juice were assumed to be negligible.

Subjects were presented a nutritious balanced meal program (formulated by our dieticians to avoid isoflavone-containing foods) and were allowed *ad libitum* access to these foods for the duration of the study. All of the urine produced after soy ingestion was collected at 1, 2, 4, 6, 8, 12, 16, 20, 24, 28, 32, and 48 h after ingestion, in containers with ascorbic acid and sodium azide added as preservatives (0.1% concentration of each). Samples were stored at −20°C until analysis.

**Materials.** Genistein (5,7,4′-trihydroxyisoflavone) and daidzein (7, 4′-dihydroxyisoflavone) were purchased from Indofine Chemical Company, Inc. (Belle Mead, NJ). Glycitein (7,4′dihydroxy-6-methoxyisoflavone), DHD[3] (7,4′-dihydroxyisoflavanone), O-DMA [1-(2,4-dihydroxyphenyl)-2–2(4-hydroxyphenol)-propan-1-one], and DHG (5,7,4′-trihydroxyisoflavanone) were purchased from Plantech (Reading, England). β-glucuronidase (type B-1 from bovine liver), sulfatase type V (aryl-sulfate sulfohydrolase from *Helix pomatia*; reported sulfatase activity of 15–40 units/mg and glucuronidase activity of 400–600 units/mg; referred to as sulfatase/glucuronidase in this paper), sulfatase type VIII (from abalone entrails; reported sulfatase activity of 20–40 units/mg and glucuronidase activity less than 3 units/mg; referred to as sulfatase in this paper), were purchased from Sigma Chemical Company (St. Louis, MO). Soy protein (Take Care high protein food ingredient powder) was a kind gift from Protein Technologies International.

**Methods.** A LC method was developed to separate isoflavones and metabolites (23). A Supelco Discovery RPamide-C16 high-performance LC column (25 cm × 4.6 mm × 5 μm; Supelco, Bellafonte, PA) was used, and isoflavones were eluted with a mobile phase of Solvent A [25% methanol containing 10 mM ammonium acetate and 71 mM triethylamine (pH 4.5)] and Solvent B [95% methanol containing 10 mM ammonium acetate and 71 mM triethylamine (pH 4.5)] at a flow rate of 1.0 ml per min. Isoflavones were separated using a linear gradient from 65% Solvent B to 95% Solvent B over 10 min. Solvent B was held for 1 min at 95% before returning to initial conditions. The column was equilibrated for 5 min before any subsequent injections. Excellent separation was obtained with this procedure. Detection sensitivity of carbonyl-containing isoflavones was increased by the infusion of 15% ammonium hydroxide at 0.14 ml per min to the eluate (24). Isoflavones were detected using a PE Sciex API 100 Mass Spectrometer by negative single-ion monitoring using a heated nebulizer atmospheric pressure-chemical ionization interface.

Standard stock solutions were prepared by dissolving 2–5 mg of genistein, daidzein, DHD, O-DMA, DHG, and biochanin A in 5 ml of methanol and 2–5 mg of glycitein in 125 ml of methanol. Final solutions were prepared from these stocks in Solvent A to generate calibration curves from 1 to 80 ng. Peak area ratios plotted against the ratios of analyte:internal standard (biochanin A at 20 ng) for genistein, daidzein, DHD, O-DMA, glycitein, and DHG and equol showed a high degree of linearity ($r > 0.99$).

Urinary isoflavones were extracted with diethyl ether or digested with specific enzymes and extracted (23). To measure the sulfate or glucuronide conjugates, or total isoflavones, ammonium acetate containing sulfatase (100 units), or β-glucuronidase (1000 units), or sulfatase/glucuronidase (100/1000 units), respectively, was added to the urine and incubated at 37°C for 3 h. The enzymes used are highly specific when used under the conditions of our assay (23). All of the samples were extracted twice with 5 ml of diethyl ether, and the organic layers were evaporated to dryness at 55°C with nitrogen. Dried extracts were reconstituted in 0.5–1 ml of Solvent A containing a known amount of biochanin A and were injected into the LC-MS system. All of the samples were analyzed in triplicate, and the results were expressed as nmol/liter of urine after normalization with biochanin A.

Extraction recoveries for genistein and daidzein were obtained from selected urine samples analyzed before and after the addition of known amounts of these analytes. The mean extraction recovery ± SE for all of the aglycones was 90.8 ± 2.5%. Intra- and interassay variabilities were determined using

*Table 1* Baseline excretion rates[a]

| Subject | Total Gen[b] | Total Daid | Total DHG | Total DHD | Total O-DMA | Total Glycitein |
|---|---|---|---|---|---|---|
| 1 | 1 | 1 | 1 | 8 | 8 | 4 |
| 2 | 4 | 12 | 15 | 20 | 16 | 4 |
| 3 | 0 | 0 | 14 | 73 | 22 | 5 |
| 4 | 15 | 28 | 7 | 5 | 9 | 3 |
| 5 | 5 | 18 | 8 | 10 | 15 | 2 |
| 6 | 3 | 3 | 5 | 5 | 8 | 2 |
| 7 | 2 | 2 | 0 | 2 | 5 | 0 |
| 8 | 0 | 14 | 3 | 11 | 1 | 8 |
| 9 | 0 | 3 | 3 | 6 | 6 | 0 |
| 10 | 0 | 0 | 0 | 0 | 6 | 0 |
| 11 | 0 | 5 | 8 | 5 | 26 | 0 |
| 12 | 0 | 5 | 5 | 11 | 0 | 0 |
| Mean±SE | 3 ± 2 | 9 ± 3 | 4 ± 3 | 6 ± 1 | 8 ± 3 | 2 ± 1 |

[a] nmol/h.
[b] Gen, genistein; Daid, daidzein.

[3] The abbreviations used are: DHD, dihydrodaidzein; O-DMA, *O*-desmethylangolensin; DHG, dihydrogenistein; LC, liquid chromatography; LC-MS, LC-mass spectrometry; CV, coefficient(s) of variation.

*Table 2* Apparent half-lives of isoflavone metabolites[a]

| Subject | Subject gender | Subject weight (kg) | Genistein | | Daidzein | |
|---|---|---|---|---|---|---|
| | | | Glucuronides $t_{1/2}$ | Sulfates $t_{1/2}$[b] | Glucuronides $t_{1/2}$ | Sulfates$t_{1/2}$ |
| 1 | M | 101 | 5.3 | N.D.[c] | 2.3 | 5.7 |
| 2 | M | 76 | 5.6 | 6.3 | 3.2 | 4.6 |
| 3 | M | 114 | 10.0 | 6.3 | 4.7 | 8.8 |
| 4 | M | 73 | 4.0 | 5.3 | 3.0 | 4.4 |
| 5 | M | 80 | 5.7 | 6.5 | 3.3 | 4.4 |
| 6 | M | 73 | 6.1 | 8.0 | 4.3 | 6.0 |
| 7 | F | 61 | 5.9 | 7.2 | 6.2 | 7.8 |
| 8 | F | 56 | 4.7 | 4.3 | 3.2 | 4.9 |
| 9 | F | 61 | 5.0 | N.D. | N.D. | 6.0 |
| 10 | F | 60 | 6.2 | 4.0 | 2.9 | 3.2 |
| 11 | F | 63 | 6.7 | 5.9 | 3.3 | 5.2 |
| 12 | F | 60 | 7.4 | 6.5 | 5.2 | 5.2 |
| Mean±SE | | 73.1 ± 5.1 | 6.0[d] ± 0.4 | 6.0[d] ± 0.04 | 3.8[d] ± 0.4 | 5.5[d] ± 0.04 |

[a] Hours.
[b] Calculated sulfates: [total − (glucuronides + aglycones)].
[c] N.D., not determined.
[d] Harmonic means are: genistein glucuronides, 5.7; genistein calculated sulfates, 5.8; daidzein glucuronides, 3.5; and daidzein calculated sulfates, 5.2.

*Table 3* Genistein and daidzein sulfate half-lives

| Subject | Genistein sulfates $t_{1/2}$[a] | | Daidzein sulfates $t_{1/2}$[a] | |
|---|---|---|---|---|
| | Calculated | Measured | Calculated | Measured |
| 4 | 5.3 | 3.6 | 4.4 | 2.9 |
| 5 | 6.5 | 6.2 | 4.4 | 3.2 |
| 6 | 8.0 | 5.2 | 6.0 | 4.2 |
| 7 | 7.2 | 3.1 | 7.8 | 5.2 |
| Mean ± SE | 6.8 ± 0.1 | 4.5 ± 0.7 | 5.7 ± 0.8 | 3.9 ± 0.5 |

[a] Hours.

a control human urine sample containing known amounts of genistein and daidzein. Interassay CV for the glucuronide-associated or total aglycones ranged from 10–17%, except for glycitein, which had a CV of 29.4%. Intra-assay variability was assessed by analyzing triplicates of samples containing known amounts of analytes, and the CV were <10%.

Noncompartmental pharmacokinetic analysis of data were conducted using WinNonlin (Pharsight, Mountain View, CA). Data were visually selected for the terminal slope calculation. Linear regression was conducted using uniform weighting. A best-fit line was calculated after assessment of the residuals, evaluation of the goodness of fit statistic, and visual inspection of the line.

**Statistical Analysis.** Data are presented as the mean ± SE. Statistical analysis was conducted using SigmaStat version 2.0 (Jandel Scientific, San Rafael, CA). Student's *t* tests were used to compare groups unless the normality test failed, and then a Mann-Whitney rank-sum test was used.

## Results

The following definitions are used for terms in this paper. Unconjugated isoflavones recovered in urine after extraction only (*i.e.*, no enzymatic digestion) are referred to as "aglycones." Isoflavones recovered from sulfatase/glucuronidase-treated urine are referred to as total isoflavones (aglycones plus the aglycones released from sulfate conjugates and glucuronide conjugates). Isoflavone sulfate or glucuronide conjugate levels were determined by subtracting the aglycone level from the aglycone released after sulfatase or glucuronidase digestion, respectively. An isoflavone "calculated sulfate" level was determined by subtracting the aglycone level plus the glucuronide-associated aglycone level (*i.e.*, the aglycone after glucuronidase digestion) from the total aglycone level (*i.e.*, the total aglycone in urine after glucuronidase plus sulfatase digestion).

Baseline concentrations of isoflavones were determined in the 24-h urine sample obtained from all of the subjects after the 1 week of non-isoflavone-containing diets and immediately prior to consuming the soy protein meal. The baseline excretion rates were low or undetectable in all of the subjects (see Table 1 for baseline excretion rates of total isoflavones).

Table 2 shows subject weight and gender and the apparent terminal half-lives determined for genistein and daidzein glucuronides and the calculated genistein and daidzein sulfates. No gender differences were detected with the relatively small number of subjects. The apparent terminal half-lives of genistein and daidzein free aglycones could not be determined because free aglycone levels were detectable only in a few urine samples. Whereas calculated sulfate values were determined in all of the subjects, sulfate conjugates were assessed in four subjects that had high calculated sulfate concentrations (presented in Table 3). The results demonstrate that apparent terminal half-lives for both genistein and daidzein sulfates were approximately 4 h (Table 3). The apparent terminal half-lives of the sulfate metabolites were shorter than those of the calculated sulfates (by 31.9 ± 10.8% for genistein and 30.9 ± 1.8% for daidzein). This difference was statistically significant for daidzein ($P = 0.01$), but failed to attain statistical significance for genistein ($P = 0.07$). Similarly, the time of peak urinary excretion for the sulfate metabolites was earlier than that of the calculated sulfate (by 27.1 ± 10.3% for genistein and 19.6 ± 12.2% for daidzein). The measured genistein sulfates accounted for 35 ± 8% of the calculated genistein sulfates value at the peak urinary excretion rate, whereas measured daidzein sulfates accounted for 60 ± 5% of the calculated daidzein sulfates value at the peak urinary excretion rate. Mixed sulfoglucuronide conjugates were not measured; these may comprise a large percentage of the calculated sulfate values and, thus, may be responsible for the differences in pharmacokinetics between measured and calculated sulfate conjugates.

*Table 4* Peak excretion rates and times of peak excretion[a]

| | Total genistein[b,c] | Genistein glucuronides[d] | Genistein sulfates[e] | Genistein calculated sulfates | Genistein |
|---|---|---|---|---|---|
| Peak excretion rate (nmol/kg/h) | 32.0 ± 5.3 (11) | 28.5 ± 4.7 (11) | 2.4 ± 0.8 (4) | 5.7 ± 1.3 (10) | 0.2 ± 0.0 (6) |
| $T_{max}$ (h)[f] | 4.6 ± 0.5 (12) | 4.3 ± 0.5 (12) | 5.4 ± 1.1 (4) | 5.6 ± 0.7 (10) | 3.1 ± 0.8 (6) |
| | Total daidzein[b] | Daidzein glucuronides | Daidzein sulfates[e] | Daidzein calculated sulfates | Daidzein |
| Peak excretion rate (nmol/kg/h) | 67.9 ± 5.4 (11) | 57.3 ± 4.2 (11) | 7.3 ± 1.7 (4) | 12.0 ± 2.1 (11) | 0.4 ± 0.1 (8) |
| $T_{max}$ (h)[f] | 5.3 ± 0.3 (12) | 5.2 ± 0.4 (12) | 5.4 ± 1.1 (4) | 6.2 ± 0.3 (12) | 4.9 ± 1.9 (8) |
| | Total DHD[b] | DHD glucuronides | DHD sulfates[e] | Total O-DMA[b] | O-DMA glucuronides |
| Peak excretion rate (nmol/kg/h) | 18.9 ± 5.0 (10) | 17.8 ± 4.6 (9) | 3.7 ± 1.3 (4) | 17.4 ± 3.9 (10) | 17.2 ± 3.9 (10) |
| $T_{max}$ (h)[f] | 10.1 ± 0.9 (11) | 10.2 ± 1.2 (10) | 9.6 ± 1.7 (4) | 15.1 ± 2.2 (11) | 14.4 ± 1.9 (11) |

| | Total DHG[b] | DHG glucuronides | DHG sulfates[e] | Total glycitein[b] | Glycitein glucuronides | Glycitein sulfates[c,f] |
|---|---|---|---|---|---|---|
| Peak excretion rate (nmol/kg/h) | 11.9 ± 7.9 (8) | 10.9 ± 7.2 (8) | 1.0 ± 0.6 (4) | 9.5 ± 0.06 (11) | 8.3 ± 0.6 (11) | 0.9 ± 0.1 (4) |
| $T_{max}$ (h)[f] | 7.1 ± 1.1 (9) | 6.8 ± 1.1 (9) | 7.1 ± 0.1 (4) | 5.0 ± 0.4 (12) | 4.8 ± 0.4 (12) | 5.9 ± 1.1 (4) |

[a] The number of subjects is listed in parentheses below the mean ± SE. In some cases, peak rates were below the limits of detection or could not be determined because of variability in the data. Subject 1 did not consume all of the soy; therefore, peak excretion rates were not used for that subject.
[b] Total isoflavones = isoflavones measured after sulfatase/glucuronidase digestion.
[c] For males: 3125 ± 602 ($n$ = 5); for females: 1543 ± 328 ($n$ = 6); $P < 0.05$.
[d] For males: 2820 ± 544 ($n$ = 5); for females: 1365 ± 271 ($n$ = 6); $P < 0.05$.
[e] Sulfates were measured in only 4 of the 12 subjects.
[f] $T_{max}$ is the time of peak excretion rate.

The peak urinary excretion rates (corrected for body weight because dosing was based on body weight) and times of peak excretion rate are presented in Table 4. The urinary excretion rates and the best-fit regression lines for genistein and daidzein sulfate, calculated sulfate and glucuronide metabolites, respectively, for four subjects are presented in Fig. 1 and 2. Genistein and daidzein aglycones are included for two subjects, but the levels were low and variable; therefore, pharmacokinetic analysis could not be done. The peak excretion rate for daidzein was nearly twice that of genistein, and this same difference was also reflected in the peak excretion rate for the respective metabolites. No gender differences were detected with the relatively small number of subjects. Peak excretion rates for each compound were highly variable across subjects.

The total amounts of isoflavones and metabolites excreted in the urine were expressed as a percent of the dose of the respective isoflavone administered and are shown in Table 5. The "% of Gen Dose Excreted" column shows the sum of the percentages of the genistein dose recovered in urine as genistein plus its sulfate and glucuronide conjugates (total genistein) plus the genistein metabolite DHG and its sulfate and glucuronide conjugates (total DHG). The "% of Daid Dose Excreted" column shows the sum of the percentages of the daidzein dose recovered in urine as daidzein plus its sulfate and glucuronide conjugates (total daidzein) plus the daidzein metabolites DHD and O-DMA and their sulfate and glucuronide conjugates (total DHG and total O-DMA, respectively). Only a small percentage (approximately 10%) of the genistein ingested was accounted for in the urine. Approximately 5-fold greater percentage urinary recovery was observed for daidzein equivalents.

The percent of the genistein dose excreted in urine as the free aglycone, glucuronides, and calculated sulfates was 0.015 ± 0.005, 5.7 ± 1.0, and 1.1 ± 0.3, respectively. The percent of the daidzein dose excreted as the free aglycone, glucuronides, and calculated sulfates in urine was 0.11 ± 0.03, 21.2 ± 1.5, and 5.5 ± 0.8, respectively. The percent of the dose excreted as the sulfate conjugates could not be determined because not all of the urine samples were analyzed for sulfate conjugates.

## Discussion

Lower incidences of certain cancers occur in areas of the world in which soybean consumption is high. Although there are many factors other than soybeans that could account for these effects, the isoflavones present in soybeans have been demonstrated to affect cellular processes important in these cancers.

The study of isoflavone action requires a good understanding of the circulating levels and pharmacokinetics of the parent compounds and their metabolites. In the present study, we determined urinary estimates of pharmacokinetic parameters for genistein, daidzein, and their metabolites after soy ingestion. Because both the sulfate and glucuronide conjugates may be biologically active and may have differential activity, it is important to understand the pharmacokinetics of each of these metabolites. To our knowledge, this is the first paper to describe the pharmacokinetics of genistein and daidzein sulfates.

In this study, we compared the calculated genistein and daidzein sulfate pharmacokinetics with the pharmacokinetics of the measured sulfates. The peak excretion rates and terminal half-lives were considerably less for the measured sulfates than for the calculated sulfates. Thus, measured sulfate conjugate values must be used to determine pharmacokinetics, as reported in this paper. The differences between calculated and measured sulfate conjugates could be attributable to several factors, including the presence of mixed sulfoglucuronide conjugates that are not measured by our analytical method. These mixed sulfoglucuronide conjugates have one of the two possible sites of the aglycone conjugated with a sulfate group and one conjugated with a glucuronide group, as noted by Adlercreutz *et al.* (25). With our digestion method using either sulfatase or β-glucuronidase coupled with diethyl ether extraction, mixed sulfoglucuronide conjugates would be expected to be only partially digested and the remaining monoconjugate (either

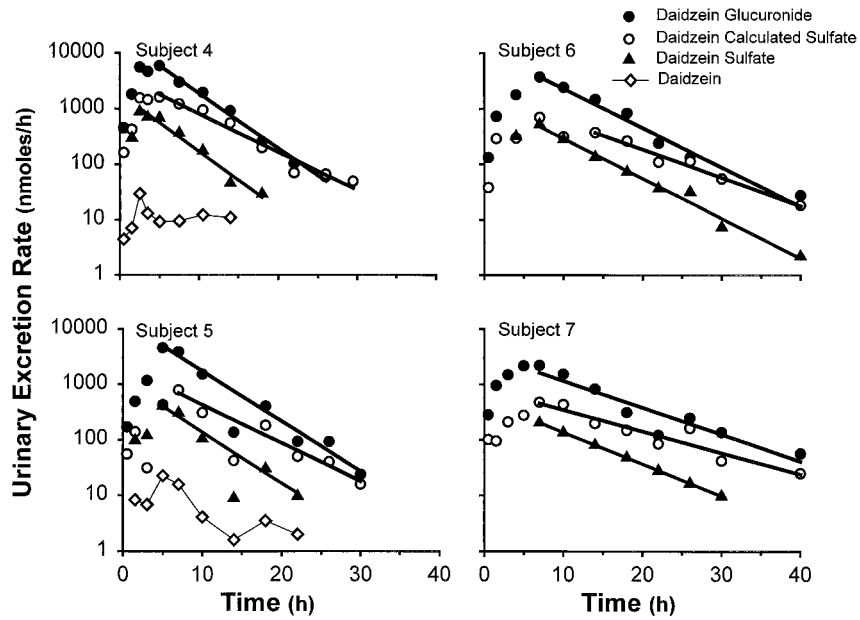


*Fig. 1.* The urinary excretion rates over time and the best-fit nonlinear regression lines for daidzein sulfate, calculated sulfate, and glucuronide are shown for four subjects. The excretion rates of daidzein (aglycone) are included on the plots for the subjects that had detectable levels, but the levels were so low and variable that pharmacokinetic analysis was not conducted. Soy protein was consumed to give a 1 mg/kg dose of genistein and 0.5 mg/kg dose of daidzein. Time points on the plots are the midpoint of the urine collection interval.

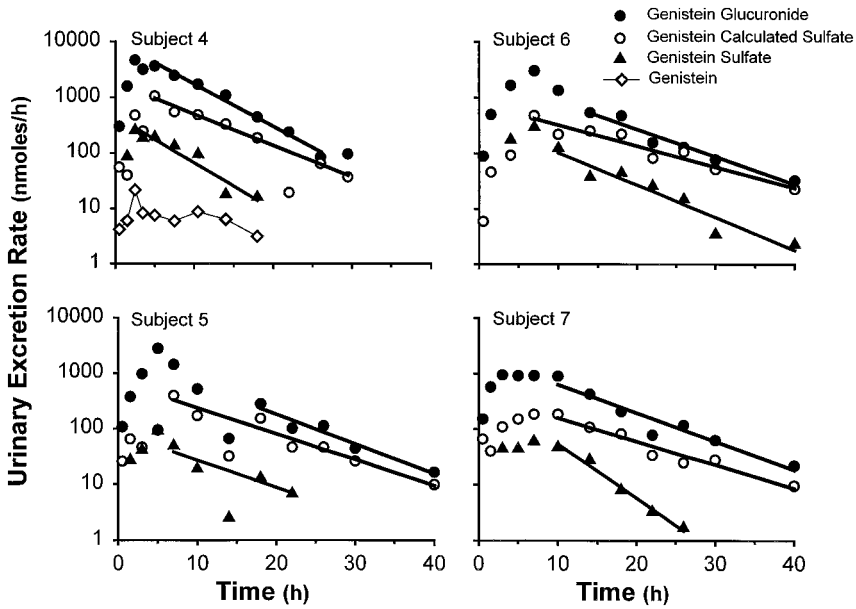


*Fig. 2.* The urinary excretion rates over time and the best-fit nonlinear regression lines for genistein sulfate, calculated sulfate, and glucuronide are presented for four subjects. See Fig. 1 legend for details.

glucuronide or sulfate, respectively) would be retained in the aqueous phase and not extracted for analysis. However, these mixed conjugates would be digested by the sulfatase/glucuronidase used to determine total isoflavones; and total isoflavones are then used in the equation to determine calculated sulfates. Thus, the percentage of isoflavones that are metabolized to mixed sulfate and glucuronide conjugates may account for the discrepancy between calculated and measured sulfate values in the human.

It should be noted that in addition to mixed sulfoglucuronide conjugates, genistein and daidzein form both mono- and disulfate and mono- and diglucuronide conjugates (25). Adlercreutz *et al.* reports that 2.5 ± 1.9% of genistein is present in human female urine as the monosulfate, compared with 5.7 ± 2.5 for disulfates. For daidzein, the percent is 2.8 ± 1.6 compared with 2.0 ± 1.1, respectively. The pharmacokinetic parameters determined in this paper are hybrid values for the mono- and diconjugates.

Our results show that the apparent terminal half-lives of genistein and daidzein sulfate conjugates are approximately 4 h each. However, caution is needed in describing the apparent terminal half-life as an elimination half-life. Because the appearance of aglycone conjugates in the urine is dependent on both the absorption and metabolism of the aglycone as well as the elimination of the conjugate, the terminal half-life calculated from an excretion-rate plot will represent the most rate-limiting process (26). Thus, apparent terminal half-lives of conjugates after oral ingestion of isoflavones will represent

*Table 5* Percent of dose excreted in urine[a,b]

| Subject | % of Gen dose excreted[c] | % of Daid dose excreted[d] | % of glycitein dose excreted[e] | % of Gen dose excreted as total gen | % of Daid dose excreted as total daid | % of Gen dose excreted as total DHG | % of Daid dose excreted as total DHD | % of Daid dose excreted as total O-DMA |
|---|---|---|---|---|---|---|---|---|
| 2 | 5.8 | 52.4 | 15.1 | 5.1 | 24.6 | 0.6 | 8.0 | 19.8 |
| 3 | 6.1 | 46.1 | 13.5 | 5.4 | 20.1 | 0.7 | 16.4 | 9.6 |
| 4 | 36.6 | 65.2 | 26.0 | 17.3 | 43.3 | 19.2 | 18.9 | 3.0 |
| 5 | 7.7 | 53.6 | 20.5 | 6.4 | 21.9 | 1.3 | 19.7 | 12.0 |
| 6 | 13.0 | 49.1 | 17.3 | 11.4 | 31.6 | 1.6 | 8.0 | 9.6 |
| 7 | 7.6 | 51.5 | 26.4 | 7.5 | 28.1 | 0.1 | 0.3 | 23.1 |
| 8 | 2.2 | 55.8 | 17.3 | 2.2 | 23.7 | 0.0 | 2.6 | 29.4 |
| 9 | 6.6 | 19.6 | 20.1 | 5.4 | 16.4 | 1.2 | 1.8 | 1.4 |
| 10 | 8.8 | 56.2 | 16.3 | 7.5 | 33.1 | 1.3 | 14.8 | 8.3 |
| 11 | 3.2 | 56.2 | 19.3 | 3.1 | 24.7 | 0.1 | 1.9 | 29.6 |
| 12 | 4.5 | 51.0 | 16.8 | 3.7 | 23.9 | 0.8 | 15.6 | 11.6 |
| Mean±SE | 10.0 ± 3.5 | 50.9 ± 4.2 | 20.0 ± 1.3 | 7.2 ± 1.6 | 27.4 ± 2.6 | 2.9 ± 2.1 | 9.3 ± 2.7 | 14.2 ± 3.5 |

[a] Gen, genistein; Daid, daidzein.
[b] $n = 11$; one subject did not consume all of the soy.
[c] Total Gen + total DHG.
[d] Total Daid + total DHD + total O-DMA.
[e] Total glycitein.

elimination half-lives of the conjugates only if the aglycone absorption and elimination half-lives are shorter than the elimination half-lives of the conjugates.

There is little information known about the elimination of aglycones. The terminal half-lives of genistein and daidzein (aglycones) could not be determined in this study because of the low levels present in urine. It also may be difficult to determine pharmacokinetics of the aglycones in plasma inasmuch as levels less than 3% of the total isoflavone level have been reported at single time points after soy ingestion (19). But because the vast majority of genistein and daidzein exist as conjugates in the blood and urine, it may be valid to assume that the elimination half-lives of genistein and daidzein are short because of rapid metabolism and that the half-lives of the conjugates are longer. Metabolites with longer half-lives than the parent compound, although unusual, have been reported (26).

If the elimination half-lives of genistein and daidzein are shorter than the sulfate and glucuronide metabolites, the terminal half-life could represent the absorption half-life of the aglycone, or the elimination half-life of the conjugates, depending on which is the rate-limiting step. Little is known about isoflavone absorption as well, although it seems to be a slow process. Data from an excellent study of genistein aglycone pharmacokinetics in mice (27) support this for genistein. In this study, the i.v. half-life of genistein is extremely short (7.1 min in the phase before a secondary plasma peak attributed to enterohepatic recycling), and the authors suggest that the longer terminal half-life (4.8 h) of genistein seen after oral dosing in mice in that same study is due to a slow absorption process. A slow absorption phase is consistent with the isoflavone conjugates found in soy foods being cleaved by intestinal bacteria to aglycones prior to absorption as proposed by Setchell *et al.* (28).

The terminal half-life of a compound is often used in combination with the frequency of ingestion to estimate the accumulation of the compound in the body over time. For example, the 4-h terminal half-life of the sulfate metabolites of genistein and daidzein found in this study could indicate that soy needs to be eaten frequently to have sustained blood levels of this metabolite. However, if the terminal half-life represents absorption, predictions are more variable because absorption half-lives are variable both between and within subjects and can be greatly influenced by many factors including the presence or absence of other foods and the type of the soy food (29).

The times of peak urinary excretion for total genistein and daidzein in our study were 4.6 ± 0.5 and 5.3 ± 0.3 h, respectively, which are similar to what has been found by Watanabe *et al.* [6 h for both total genistein and daidzein (14)], and Lu *et al.* [6.7 and 7.1 h for total genistein and daidzein, respectively (15)]. These values could represent a slow absorption process as well as the time needed to form these metabolites.

The percent of the dose excreted in urine (a sum of all of the metabolites detected, expressed as a percent of isoflavone dose) represents the minimum bioavailability for the isoflavones. In this study, 9.3 ± 2.9% of the dose of genistein was recovered (total genistein + total DHG), 50.6 ± 3.4% of the dose of daidzein was recovered (total daidzein + total DHD + total O-DMA) and 19.0 ± 1.2% of the dose of glycitein (total glycitein) was recovered. We did not measure equol [a metabolite of daidzein produced in some (but not all) individuals] in our urine samples. Other researchers have reported the percent of the genistein and daidzein dose excreted in urine as 17.6 and 35.3% (14), 22 and 62% (13), and 14.6 and 46.9% (15), respectively. Some of the variability is due to the fact that the metabolites selected for measurement in each study were not the same. Variable absorption processes of the different soy foods used could also affect the minimum bioavailability. In addition, it seems likely that there are other metabolites of genistein and daidzein that are not known at this time (30), and identification and measurement of these metabolites could show that the minimum bioavailability is much higher.

In summary, urinary isoflavone pharmacokinetics were studied in adult male and female subjects after a single meal rich in soy protein. The terminal half-life of daidzein sulfate that was determined by assessing aglycone release from the conjugate after specific sulfatase hydrolysis was significantly shorter than the calculated daidzein sulfate value. A similar trend was obtained with measurement of genistein sulfate. The shorter terminal half-life of the measured sulfate conjugate would suggest that soy would have to be eaten more frequently to achieve sustained blood levels than what would be expected based on the half-life determined for the calculated sulfate conjugate. Although plasma isoflavone pharmacokinetic studies are required before any stronger conclusion can be made

about the relationship between meal frequency and the circulating dynamics of these possibly bioactive compounds, our data provide evidence that more direct analysis of the isoflavone sulfates will provide a better understanding than calculated methods.

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