Bioavailability of Pure Isoflavones in Healthy Humans and Analysis of Commercial Soy Isoflavone Supplements[1,2]

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ABSTRACT The pharmacokinetic behavior of naturally occurring isoflavones has been determined for the first time in healthy adults. We compared plasma kinetics of pure daidzein, genistein and their β-glycosides administered as a single-bolus dose to 19 healthy women. This study demonstrates differences in the pharmacokinetics of isoflavone glycosides compared with their respective β-glycosides. Although all isoflavones are efficiently absorbed from the intestinal tract, there are striking differences in the fate of aglycones and β-glycosides. Mean time to attain peak plasma concentrations (t_{max}) for the aglycones genistein and daidzein was 5.2 and 6.6 h, respectively, whereas for the corresponding β-glycosides, the t_{max} was delayed to 9.3 and 9.0 h, respectively, consistent with the residence time needed for hydrolytic cleavage of the glycoside moiety for bioavailability. The apparent volume of distribution of isoflavones confirms extensive tissue distribution after absorption. Plasma genistein concentrations are consistently higher than daidzein when equal amounts of the two isoflavones are administered, and this is accounted for by the more extensive distribution of daidzein (236 L) compared with genistein (161 L). The systemic bioavailability of genistein [mean AUC = 4.54 μg/(mL · h)] is much greater than that of daidzein [mean AUC = 2.94 μg/(mL · h)], and bioavailability of these isoflavones is greater when ingested as β-glycosides rather than aglycones as measured from the area under the curve of the plasma appearance and disappearance concentrations. The pharmacokinetics of methoxylated isoflavones show distinct differences depending on the position of the methoxyl group in the molecule. Glycitin, found in two phytoestrogen supplements, underwent hydrolysis of the β-glycoside moiety and little further biotransformation, leading to high plasma glycitein concentrations. Biochanin A and formononetin, two isoflavones found in one phytoestrogen supplement, were rapidly and efficiently demethylated, resulting in high plasma genistein and daidzein concentrations typically observed after the ingestion of soy-containing foods. These differences in pharmacokinetics and metabolism have implications for clinical studies because it cannot be assumed that all isoflavones are comparable in their pharmacokinetics and bioavailability. An analysis of 33 phytoestrogen supplements and extracts revealed considerable differences in the isoflavone content from that claimed by the manufacturers. Plasma concentrations of isoflavones show marked qualitative and quantitative differences depending on the type of supplement ingested. These studies indicate a need for improvement in quality assurance and standardization of such products. J. Nutr. 131: 1362S–1375S, 2001.

KEY WORDS: • *phytoestrogens* • *isoflavones* • *pharmacokinetics* • *soy foods* • *supplements* • *humans* • *blood*

Interest in soy isoflavones has exploded in the past 5 y after a wealth of scientific data showing that these phytoestrogens possess potent and wide-ranging biological activities. Much of this interest has been directed to the area of women's health and is stimulated by promising preliminary data from clinical studies showing the effectiveness of phytoestrogen-rich soy protein–containing foods in a range of hormone-dependent conditions [reviewed recently by Setchell (1998) and Setchell and Cassidy (1999)]. There is compelling evidence from studies of animal models and in vitro assay systems that isoflavones have significant direct and indirect hormonal and nonhormonal effects of relevance to human disease prevention or

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treatment (Adlercreutz 1995, Barnes 1995, Barnes et al. 1994, Barnes and Peterson 1995, Kim et al. 1998, Setchell and Adlercreutz 1988).

The discovery of very high urinary concentrations of isoflavones in adults who consume soy protein and the evidence supporting their biological potency are probably the prime reasons that the soybean has been elevated to the rank of a functional food. The recent approval by the Food and Drug Administration (November 10, 1999; No. 279) to allow a cardiovascular health claim for foods that contain at least 6.25 g of soy protein/serving will undoubtedly lead to a large increase in the sales of soy-fortified foods and the constituent isoflavones. The impact of this ruling is already evident because the food industry has seized the opportunity to label soy products with the isoflavone content, as well as the soy protein content, even though the former is not required under the food-labeling laws.

More disturbing, however, is the plethora of dietary supplements of isoflavones that have flooded the market, with wide-ranging claims and little regulation regarding their manufacture or efficacy. There is a paucity of information regarding the bioavailability, metabolism and clinical effectiveness of dietary isoflavone supplements, and in many cases, the consumer may simply be generating very expensive urine. Until proved otherwise, it cannot be assumed that a dietary isoflavone supplement will behave in the same manner as an isoflavone-rich food, and up to this point, there is a lack of information on isoflavone pharmacokinetics. In this report, we determined for the first time the pharmacokinetics of individual purified soy isoflavones in healthy subjects to assess the bioavailability of daidzein and genistein and their respective β-glycosides. We also analyzed a range of dietary isoflavone supplements for their isoflavone content using liquid chromatography and mass spectrometry techniques and compared the observed concentrations with the manufacturer's claim of content. The fate of the methoxylated isoflavones such as glycitin, a main component of many phytoestrogen supplements, and formononetin and biochanin A, the constituent isoflavones of clover supplements sold to women for the relief of menopausal symptoms, were examined. We also bioassayed the estrogenicity of one supplement that claims to enhance breast size in women through its phytoestrogen content. The findings raise serious concerns regarding some of the marketing ploys that promote isoflavone supplements and speak to the need of more rigorous policing of these products along the guidelines for pharmaceutical agents.

METHODS

Pharmacokinetics and apparent bioavailability of individual isoflavones in healthy adults: study design

Nineteen healthy premenopausal women aged ≥18 y were recruited for pharmacokinetic studies that were carried out using the resources of the National Institutes of Health–funded General Clinical Research Center at Children's Hospital Medical Center in Cincinnati, Ohio. Subjects were excluded if they had preexisting chronic renal, liver, pulmonary or cardiovascular disease; had been administered antibiotics within the preceding 3 mo; or were taking oral contraceptives. At the time of the study, the prohibitive costs and limited availability of the glycoside conjugates restricted the determination of pharmacokinetics to only a small number of women. The lack of prior quantitative data for plasma concentrations of these compounds precluded performing accurate power calculations to establish the optimal sample size for the comparison of different isoflavone forms. The number of subjects enrolled was based on the feasibility of analytically handling the large numbers of plasma samples collected; the study was therefore observational. The Human Investigations Review Board of Children's Hospital Medical Center approved the study protocol, and informed consent was obtained from each subject.

The study subjects were divided into four groups, and they consumed daidzein ($n = 6$), genistein ($n = 6$), daidzin ($n = 4$) or genistin ($n = 3$). Subjects were asked to abstain from foods containing soy protein for ≥1 wk before and during the study. Each subject fasted overnight and then consumed a standardized 50-mg dose of the isoflavone together with a drink and followed by breakfast. This dose was chosen because it was considered at the time within the expected range of isoflavone intake by persons consuming soy as a staple food and was the approximate level of intake previously shown to have endocrine effects in healthy premenopausal women (Cassidy et al. 1994).

Blood samples (5 mL) were obtained via venipuncture before (baseline) and then after 2, 4, 6, 8, 12 and 24 h in all subjects and in some, after 48 h. Blood was obtained via an indwelling catheter for the more frequent samplings and/or via Vacutainer for the later sample times depending on the choice of the individual. Blood samples were centrifuged, and the plasma was separated and immediately frozen at −20°C before analysis of isoflavone concentrations.

Pharmacokinetics of methoxylated isoflavones glycitin, formononetin and biochanin A

The pharmacokinetics of three methoxylated isoflavones were examined in one healthy man with the identical protocol. Glycitin (25 mg), the β-glycoside of glycitein, was administered orally as a single-bolus dose, and blood samples were collected. After a 2-wk washout period, a mixture of formononetin and biochanin A, contained in a tablet of the over-the-counter supplement Promensil (40 mg total isoflavones per capsule), was then consumed orally as a single dose, and blood samples were obtained according to the protocol above. Plasma concentrations of the methoxylated isoflavones and their demethylated metabolites were measured in the blood by gas chromatography-mass spectrometry (GC-MS).

Determination of isoflavones in plasma and urine by GC-MS

The concentrations of daidzein, genistein, glycitein, biochanin A, formononetin and equol were measured by GC-MS using two stable isotopically labeled internal standards, and an isoflavone homologue as a second internal standard. These internal standards were added to the plasma prior to its extraction and work-up. Total and individual isoflavones were determined after extraction and enzymatic hydrolysis of the conjugates with a combined sulfatase and glucuronidase enzyme preparation. Unconjugated isoflavones were determined separately after group separation from their respective glucuronide and sulfate conjugates on a lipophilic anion exchange gel (Axelson and Setchell 1980). After equilibration of the plasma (0.25–0.50 mL) with 100-ng amounts of the internal standards [^{13}C]daidzein, [^{13}C]genistein and 7,4′-dihydroxyflavone, 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. The solid-phase cartridge was then washed with distilled water (10 mL), and 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 acetate/L buffer (pH 4.5) and hydrolyzed at 37°C overnight with a solution of 10,000 Fishman Units of a mixed β-glucuronidase/sulfatase (*Helix pomatia*; Sigma Chemical Co., St. Louis, MO) that was prefiltered through a cartridge of C18-Bond Elut to remove naturally occurring isoflavones in the enzyme preparation. After hydrolysis, isoflavones were isolated by solid-phase extraction on a C18-Bond Elut cartridge as described earlier. 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) prepared in the [OH^-] form and packed in methanol. The phenolic compounds were

recovered by elution of the gel bed with 15 mL of methanol saturated with CO_2 (Axelson and Setchell 1980). The phenolic fraction was taken to dryness under a stream of nitrogen gas, and isoflavones were converted to the *tert*-butyldimethylsilyl (*t*-BDMS) ether derivatives for analysis by GC-MS. *t*-BDMS ethers were prepared by the addition of acetonitrile (100 μL) and *N*-methyl-*N*-*t*-butyldimethylsilyltrifluoroacetamide 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).

Determination of unconjugated isoflavones in plasma. The concentrations of individual unconjugated isoflavones were determined separately after solid-phase extraction of the plasma and omission of the hydrolysis step. Group fractionation and isolation of unconjugated nonsteroidal phenolic compounds were achieved through lipophilic anion exchange chromatography on TEAP-LH-20. In this system, the glucuronide and sulfate conjugates are retained on the gel because of their lower pK_a, and the unconjugated isoflavones are eluted with methanol saturated with CO_2 (15 mL). This fraction was then evaporated to dryness, and the *t*-BDMS ether derivatives were prepared.

GC-MS conditions. Isoflavone *t*-BDMS ethers were separated and quantified by GC-MS. Chromatographic separation was achieved on a DB-1 fused silica capillary column (30 m × 0.25 mm i.d., 0.25-μm film thickness; J & W Scientific, Folsom, CA) using helium as the carrier gas (flow rate, ~2 mL/min) and with a temperature program that increased from 260° to 310°C in increments of 10°C/min. Selected ion monitoring GC-MS of specific and characteristic ions in the electron ionization (70 eV) spectra of the *t*-BDMS ether derivatives of each isoflavone permitted highly sensitive and specific quantification. The following ions were monitored: *m/z* 425 (daidzein and 7,4′-dihydroxyflavone internal standard), *m/z* 426 ([^{13}C]daidzein), *m/z* 470 (equol), *m/z* 555 (genistein), *m/z* 556 ([^{13}C]genistein), *m/z* 455 (glycitein), *m/z* 382 (formononetin) and *m/z* 455 (biochanin A). The individual isoflavones were quantified by comparing the peak area in the specific ion channels at the correct retention time determined from authentic compounds with the peak area response for the internal standard. This area ratio was then interpolated against calibration curves constructed for known amounts (0–200 ng) of the individual isoflavones. Concentrations were expressed in ng/mL for individual plasma isoflavones.

The within day reproducibility for repeat analysis of the same plasma sample was 0.5% for daidzein and 1.0% for genistein. The mean between-batch reproducibility determined over a 18-month period of 19 separate runs where duplicate plasma samples were assayed was 5% (range 1.0–11.9%) for daidzein and 7% (range 1–17%) for genistein at concentrations of 100–200 ng/mL. The precision of equol measurements was 10% at a concentration of 5–7 ng/mL.

Determination of plasma isoflavone pharmacokinetics. A noncompartmental approach was used for the pharmacokinetic analysis with WinNonlin 1.5 (Pharsight Corporation, Mountain View, CA) computer software. This approach uses the trapezoidal rule for the determination of area under the plasma concentration-time curve (AUC). The total AUC ($AUC_{0\rightarrow\infty}$, or AUC_{inf}) is calculated in a two-step process. In the first step, AUC from time point 0 to any time point t on the log-linear region of the terminal part of the curve is determined. The remaining area from t to infinity is determined as C_t/λ_z, where C_t is the plasma concentration of the isoflavone at time t and the rate constant is calculated from the slope of the terminal phase of disposition. At least three 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 $1/y^2$, where y represents the observed concentration, were used to improve the goodness-of-fit of the data. Other parameters that were determined included peak plasma isoflavone levels (C_{max}), time required to achieve the peak levels (t_{max}), systemic clearance (normalized to the bioavailable fraction) and volume of distribution normalized to the bioavailability fraction (F) Vd/F. AUC_{inf}, t_{max}, $t_{1/2}$ of elimination and Vd/F reflect the systemic exposure to the isoflavone, rate of absorption, rate of elimination and the extent of isoflavone distribution in the body, respectively.[4] While the exact bioavailable fraction could not be determined in our experiments, comparison of AUCs, Vd/F and CL/F of the isoflavones facilitated a comparative evaluation of the apparent bioavailability (or systemic exposure), extent of distribution and the rate of elimination, respectively.

Analysis of soy isoflavone supplements by HPLC and electrospray-mass spectrometry

Qualitative and quantitative analyses of 33 commercially available isoflavone supplements or extracts were performed with HPLC and electrospray ionization LC-MS (ESI-MS). These supplements were obtained from various sources in the United States and elsewhere, and the following products were analyzed: #1, Carlson Easy Soy and #2, Carlson Easy Soy Gold, (J. R. Carlson Laboratories, Inc. Arlington Hts., IL); #3, Erdic-Busting Out (Cerdic B.V. 6718TBEde, The Netherlands); #4, Estroven, (AMERIFIT, Bloomfield, CT); #5, Genistein, Soy Isoflavone Extract (Solgar Laboratories, Leonia, NJ); #6, Kudzu Root Extract (Solaray, Inc., Park City, UT); #7, Healthy Woman, (Personal Products Co., Skillman, NJ); #8, One a Day Menopause Health, (Bayer Corp., Consumer Care Division, Morristown, NJ); #9, PhytoEstrin (USANA, Inc., Salt Lake City, UT); #10, Phyto Soya, (Arkopharma, Coulsdon, Surrey. UK); #11, Soy Extract, (Enzymatic Therapy®, Green Bay, Wisconsin); #12, Nature's Herbs, Phytoestrogen Power, (Alvita® a TWINLAB® Division, Quality Drive, American Fork, UT); #13, Promensil, (Novogen, Inc., Stamford, CT); #14, PhytoEstrogen, Solaray, (Nutraceutical Corp. for Solaray, Inc., Park City, UT); #15, Soya Isoflavones, Holland and Barrett Ltd., Nuneaton, England; #16, Soyamax (USANA, Inc., Salt Lake City, UT); #17, Soy Care, S.C.P.I., Waltham, MA; #18, Nature's Resources Soy Isoflavones, Mission Hills, CA; #19, Soy Plus—Dr. Art Ulene's Phyto-protein formula, Feeling Fine Co., Marina del Rey, CA; #20, Naturally Preferred Soy Germ, Inter-American Products, Ohio; #21, Trinovin, Novogen Inc., Stamford, CT.; #22, Basic's Soy Isoflavones, Basic Drugs. Inc. Vandalia, OH; #23, Flash Fighters, Nature's Bounty, Bohemia, NY; #24, Menopause Balance, Walgreens, Deerfield, IL; #25, Novasoy, Archer Daniel Midland, Decatur. IL; #26, New Phase–Sunsource, Chatham Inc., Chattanooga, TN; #27, Spring Valley Phytoestrogen Complex, NaturPharma, American Fork, UT; #28, Sundown–Soy Isoflavones, Sundown Vitamins, Boca Raton, FL; #29, Phytosoy, Nature's Sunshine, Spanish Fork, UT; #30, Soy Choice, Vitanica, Sherwood, OR; #31, Revival, Physician's Laboratory, Walkertown, NC; #32, Nutrisoy Flour, Archer Daniel Midland, Decatur. IL; #33, Soy Life 25, Schouten USA Inc., Minneapolis, MN.

Four tablets or capsules of each supplement were analyzed separately. The supplements were ground to a very fine powder using a small coffee grinder, and the isoflavones were extracted from an accurately weighed portion of each by refluxing the sample in 80% methanol (50 mL) for 1 h. After being filtered through Whatman No. 1 filter paper, the aqueous methanolic phase was made up to a fixed volume of 100 mL, and the internal standard equilenin (60 μg) was added to 1.0 mL (1/100th) of this extract. This was then used for direct HPLC and ESI-MS analyses.

Extracts of the soy supplements were analyzed on a Waters Alliance 2690 HPLC system. The sample size injected was 10 μL, at a flow rate of 1.0 mL/min, and UV absorbance was monitored at 260 nm using a Waters 2487 UV detector. Separation of the individual isoflavones was accomplished on a 250 × 4.6-mm ODS (C18) reversed phase HPLC column (Keystone Scientific, Bellefone, PA). The column was eluted with a water/acetonitrile gradient. The mobile phase was 100% of 10 mmol ammonium acetate/L [0.1% trifluoroacetic acid (TFA)] held isocratic for the first 2 min and then decreased to 50% in a constant gradient from 2 to 24 min and then finally held isocratic period with 50% acetonitrile and 50% 10 mmol ammonium acetate/L (0.1% TFA) for 5 min, before being returned to

[4] Abbreviations: AUC, area under the plasma concentration-time curve; C_{max}, peak plasma isoflavone level; ESI, electrospray-mass spectrometry; GC, gas chromatography; MS, mass spectrometry; t_{max}, time required to achieve the peak levels; TFA, trifluoroacetic acid; V_d, volume of distribution normalized to the bioavailability.

the original composition of 100% 10 mmol ammonium acetate/L (0.1% TFA).

ESI-MS was performed on a Micromass Quattro LC/MS. The HPLC effluent to the ESI probe was split 10:1. The desolvation temperature was 300°C, and the source temperature was 100°C. The sampling cone was held at 50 V, and the extractor was held at 2 V. Data were collected in the positive ion mode. **Table 1** lists the $[M + H]^+$ ions that were monitored for the phytoestrogens and their conjugates.

Only the isoflavones that could be positively identified with ESI-MS were quantified. Isoflavone content was established by comparing the isoflavone peak area ratio with the internal standard equilinen and through interpolation against calibration plots constructed of known quantities of the pure compounds. Reproducibility of the method was established from intrabatch replicate analyses of 12 samples of one supplement (#7) and found to be 2.7% for the total concentration (coefficient of variation). Interbatch precision when two different soy extracts were used as quality control samples was 5–6% (coefficient of variation).

Bioassay for estrogenicity in the phytoestrogen supplement Erdic

A classic in vivo bioassay for estrogenicity was performed on one of the supplements (Erdic, also sold under the name "Busting Out" in the United States) to test for the presence of biologically active phytoestrogens that might not have been detected on HPLC analysis. This supplement was chosen for analysis because of our failure to confirm the manufacturer's claims that it contains "natural phytoestrogens" that help to enhance a woman's breast size. Four 70-d-old female and two young adult male Sprague-Dawley rats were obtained from Charles Rivers Laboratory, placed on AIN-93G diet [a soy-free diet designed for pregnant or lactating dams and growing pups; Reeves (1993)] and bred in-house. After breeding, the females were housed individually and maintained on the AIN-93G diet until the pups were weaned. On parturition, litters were reduced to 10 pups per dam. On d 20 after parturition, the pups were weaned, and the female pups from each litter were randomly distributed to one of three different treatment groups, for a total of six weanlings per group. The groups were fed one of the following diets:

Food supplement. Erdic tablets and AIN-93G pellets were reduced to powder, and the Erdic powder was added to pellet powder at a ratio of 20% of the test supplement (by weight) to 80% of the AIN-93G. The two components were mixed by rotation for a minimum of 3 h and supplied in a feeding jar to six 20-d-old weanlings on d 20–23.

Control group A. The positive control group of six weanling rats were fed powered AIN-93 to which 160 μg estradiol 3-benzoate (Sigma Aldrich, St. Louis, MO) had been added per 100 g of feed (Odum et al. 1997). This was mixed by rotation as described and supplied ad libitum to the weanling rat pups on d 20–23.

Control group B. This control group consumed powered AIN-93G only, supplied ad libitum on d 20–23.

Beginning on d 19, before the pups were removed from the dam, they were weighed and assigned to their test group. On d 20, they were weaned and housed two to four per cage; daily weights were taken throughout the experiment. On d 23, the pups were killed by CO_2 inhalation, the uterus was removed and a wet weight was obtained. The uteri were then dried at 70°C overnight to constant weight, and the dry weight was recorded.

TABLE 1

$[M + H]^+$ ions monitored for the phytoestrogens and their conjugates

$[M + H]^+$ ions	Aglycone	β-Glycoside	Acetyl β-glycoside	Malonyl β-glycoside
Daidzein	255.07	417.12	459.13	503.12
Formononetin	269.08	431.13	473.14	517.13
Genistein	271.06	433.11	475.12	519.11
Biochanin A	285.07	447.13	489.14	533.13
Glycitein	285.07	447.13	489.14	533.13

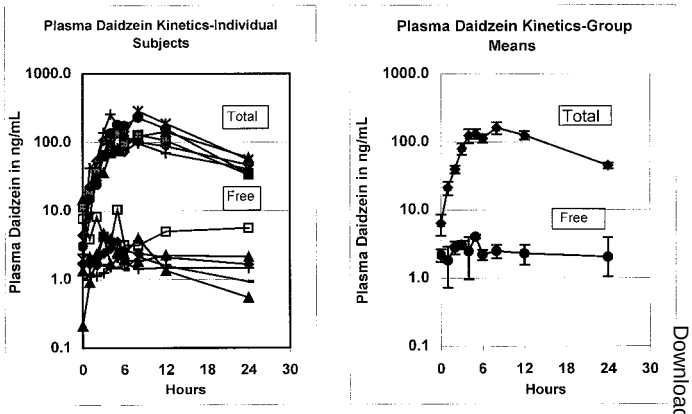

FIGURE 1 Individual (*left*) and group mean (*right*) plasma appearance and disappearance curves for total and free (unconjugated) daidzein in six healthy premenopausal women after oral administration of 50 mg daidzein.

RESULTS

Plasma kinetics of isoflavones

Daidzein. The individual and group mean plasma daidzein appearance and disappearance curves for women administered a single bolus dose of 50 mg of daidzein are shown in **Fig. 1**. The profiles were similar for all women. The curves were characterized by a rapid increase in plasma isoflavone concentrations followed after ~2 h by a slight decline and then a second rise that attained a mean maximum plasma concentration at time (t_{max}) 6.6 ± 1.36 h. At this time, the mean maximum plasma daidzein concentration (C_{max}) was 194 ± 30.6 ng/mL (0.76 ± 0.12 μmol/L). Pharmacokinetic analysis of the plasma curves showed the half-life of elimination to be 9.34 ± 1.3 h for daidzein. The AUC was 2.94 ± 0.22 μg/(mL · h), and the mean plasma clearance was 17.5 ± 1.4 L/h. The average volume of distribution normalized to apparent bioavailable fraction Vd/F; where F is the bioavailability fraction (V_d, Vd/F) for daidzein was large at 236.4 ± 35.9 L. Daidzein predominantly circulated in plasma in the conjugated form. Unconjugated daidzein concentrations were relatively low after the administration of a 50-mg dose of the aglycone. In the first 2 h, the proportions of unconjugated daidzein increased and accounted for 8.4 ± 0.9% of the total daidzein, but once steady state was established, the unconjugated daidzein fraction constituted an average of 2.7 ± 03%. A true elimination phase for both aglycones was not detected because of the extensive persistence and steady-state levels of the unconjugated daidzein in plasma, which made it difficult to accurately determine pharmacokinetic parameters, including $t_{1/2}$ of elimination.

Genistein. The individual and group mean plasma genistein appearance and disappearance curves for women administered a single-bolus dose of 50 mg of genistein are shown in **Fig. 2**. The plasma profiles were similar for all of the women. The early kink in the absorptive phase of the individual plasma curves seen for daidzein was less evident for genistein. The mean t_{max} for peak plasma concentration occurred 9.33 ± 1.33 h after administration of the single-bolus oral dose, with a mean C_{max} for genistein of 341 ± 74 ng/mL

(1.26 ± 0.27 μmol/L). Pharmacokinetic analysis of the disappearance curves showed the half-life of elimination of genistein to be 6.78 ± 0.84 h. The AUC of the plasma concentrations was 4.54 ± 1.41 μg/(mL · h). The mean plasma clearance normalized to the bioavailable fraction (CL/F) of genistein was 18.3 ± 5.7 L/h, and the average V_d was 161.1 ± 44.1 L. As seen for daidzein kinetics, the unconjugated genistein concentrations in plasma were very low, accounting for only 3.7 ± 1.1% in the first 2 h and 1.6 ± 0.1% once steady state had been established. Although there was an increase in unconjugated genistein after administration of the aglycone, the steady-state and persistent low levels in the elimination phase precluded the calculation of pharmacokinetics.

Glycosides daidzin and genistin. **Figure 3** shows the plasma appearance and disappearance curves for daidzein and genistein measured in premenopausal women after they received a single-bolus dose of the glycoside conjugate of either daidzin ($n = 4$) or genistin ($n = 3$). These curves displayed characteristics similar to those of the corresponding aglycones. However, the time to attain maximum plasma daidzein and genistein concentrations (t_{max}) was longer at 9.0 ± 1.0 and 9.3 ± 1.3 h, respectively, after the glycosides were ingested. The C_{max} for daidzein after administration of its glycoside conjugate was 394 ± 61 ng/mL (1.55 ± 0.24 μmol/L), whereas the C_{max} for genistein after genistin was ingested was 341 ± 127 ng/mL (1.22 ± 0.47 μmol/L). For daidzin pharmacokinetics, the $t_{1/2}$ was 4.59 ± 0.5 h, whereas the corresponding value for genistin administration was 7.0 ± 0.76 h. The Vd/F for genistin was 112.3 ± 34.5 L, and its clearance was 10.8 ± 2.2 L/h. When daidzin was administered, AUC was 4.52 ± 0.49 μg/(mL · h). The CL/F for daidzin was 11.6 ± 1.6 L/h, and its Vd/F was 77 ± 14 L. The apparent bioavailability for genistin calculated AUC of genistin was 4.95 ± 1.03 μg/(mL · h). These values closely parallel the corresponding values for aglycone administration. Unconjugated daidzein and genistein in plasma accounted for 1.7 ± 0.4% and 1.5 ± 0.2% of the total isoflavones during the first 2 h, respectively, and for 1.1 ± 0.2% and 1.1 ± 0.45 once steady-state had been attained, indicating extremely efficient first-pass conjugation after intestinal hydrolysis of the glycoside moiety. Pharmacokinetics were not determined for the plasma unconjugated fractions.

Formation of metabolites-equol production

Equol, an important intestinal bacterial metabolite of daidzein, was expectedly not detected in significant amounts in the plasma of women who consumed genistein (or genistin, because it is a specific metabolite of daidzein). Surprisingly, it was not found in the plasma of the women who ingested daidzein, but it did appear in the plasma of two of the four women who consumed daidzin. The plasma profiles (**Fig. 4**) show that there is a time lag in its appearance and that after a single-bolus ingestion, it takes at least 6–8 h before equol appears in substantial amounts in plasma. This observation is consistent with a distal or colonic origin for its formation. At the time of this study, the metabolites of genistein, were unavailable as a standard and consequently not measured in the plasma samples from this study.

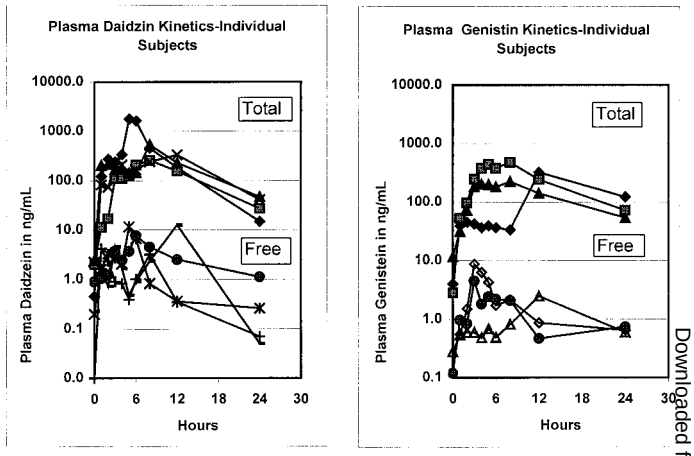

FIGURE 3 Plasma appearance and disappearance curves plotted on a log-linear scale for total and free (unconjugated) daidzein (*left*) and genistein (*right*) in healthy premenopausal women after oral administration of 50 mg of their respective glycosides daidzin and genistin.

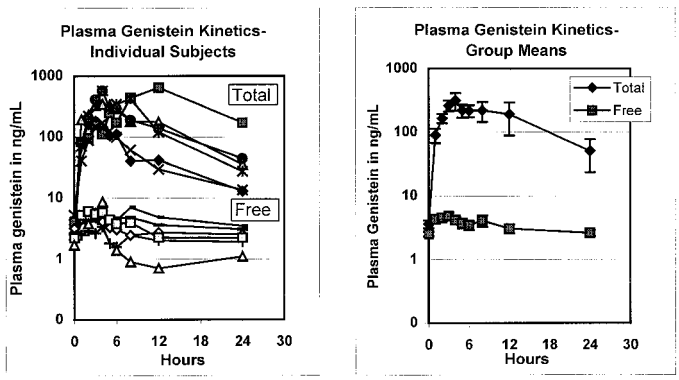

FIGURE 2 Individual (*left*) and group mean (*right*) plasma appearance and disappearance curves for total and free (unconjugated) genistein in six healthy premenopausal women after oral administration of 50 mg genistein.

Pharmacokinetics of methoxylated isoflavones glycitin, formononetin and biochanin A

The pharmacokinetics of glycitin (7,4′′-dihydroxy-6-methoxyisoflavone-7-D-glucoside) was examined in one healthy man according to the same protocol as that described for daidzein and genistein. Glycitein appeared rapidly in plasma after the administration of a single-bolus dose of 25 mg of its β-glycoside. Peak plasma concentration was reached 4 h after oral ingestion, and thereafter plasma concentrations declined, with an elimination $t_{1/2}$ of 8.9 h. The CL/F for glycitein was 32.1 L/h, and its AUC was 0.7 μg/(mL · h).

The Vd/F for glycitein was relatively high at 415 L. There was a small rise in the plasma concentration of daidzein after the administration of glycitin, but overall the plasma profiles indicated there was negligible biotransformation of glycitin other than initial hydrolysis of the glycosidic group. Demethoxylation to daidzein was clearly a minor biotransformation pathway. (**Fig. 5**).

The behavior of a supplement containing methoxylated isoflavones from clover was examined in the same healthy adult by oral administration of a single tablet of the commercially available supplement Promensil (Novogen). HPLC analysis of this supplement (**Fig. 6**) showed that it contained predominantly formononetin and biochanin A in the aglycone form, but there were minor amounts of the glycosides of these methoxylated isoflavones, as well as daidzein and genistein. Overall, our analysis showed excellent agreement with the manufacturer's claim for composition. The oral administration of one tablet of Promensil led to a rapid increase in the plasma concentrations of daidzein and genistein (Fig. 5). These were

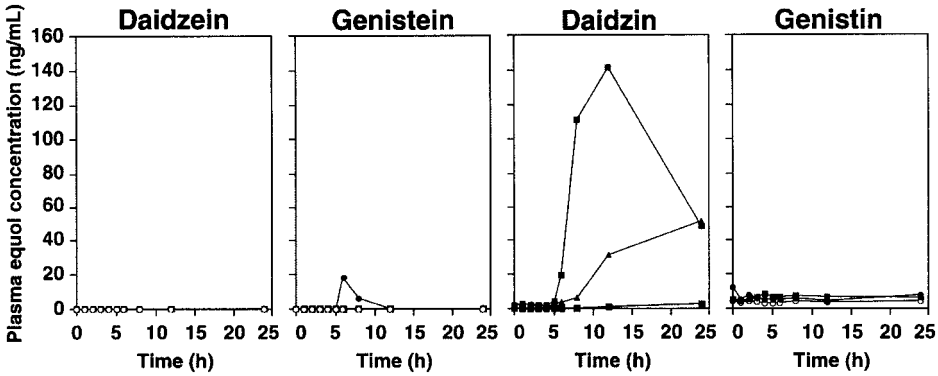

FIGURE 4 Plasma equol concentrations (ng/mL) in 17 healthy women after administration as a single-bolus dose of 50 mg of the pure isoflavones daidzein (n = 5), genistein (n = 5), daidzin (n = 4) or genistin (n = 3).

the major isoflavones appearing in plasma, and they accounted for >95% of the total isoflavones measured. Although increases in the plasma concentrations of formononetin and biochanin A were observed, these were minor compared with the plasma appearance of the demethylated metabolites, daidzein and genistein. Due to the complexity of the mixture administered and because values were for just one subject, the pharmacokinetics were not determined, but overall it was evident that in contrast to glycitin, very little of the methoxylated isoflavones survived intestinal bacterial demethylation.

Qualitative and quantitative analyses of commercially available phytoestrogen supplements

Thirty-three commercially available phytoestrogen supplements or extracts were analyzed for isoflavone content by HPLC and ESI-MS. Only peaks that could be positively confirmed from their mass spectra were quantified. Individual concentrations of the aglycones and the various glycosides are summarized in **Table 2**. These are expressed as mg/g and are not corrected for the aglycone equivalency because this appears to be the manner in which most manufacturers are labeling these products. There was a large variability in the composition of these supplements as demonstrated from just four of the HPLC profiles shown in Fig. 6 and **Fig. 7.** Each of the 33 supplements revealed a different profile, and many contained an abundance of peaks of unknown origin and chemical structure. Overall, it was evident that these supplements could be broadly grouped into three main categories: *1*) those in which the amounts of glycoside and aglycone forms of daidzein plus glycitein exceeded genistein by >2.0 fold and *2*) those in which this ratio was <1.50 and only small proportions of glycitein and its glycosides were present. Supplements that contained high levels of glycitin and glycitein were apparently manufactured from extracts of the soy germ, as this is a major isoflavone of the soybean hypocotyl, 3) those made from extracts of clover and containing mainly methoxylated isoflavone (Fig. 7).

The methoxylated clover isoflavones were major components of several of the supplements that we analyzed (Promensil). Tea made from red clover is available in health food stores but on analysis (data not shown) was found to contain very low levels of isoflavones. The Chinese plant kudzu is also available as an extract, and this was shown to contain high levels of the isoflavone glycoside puerarin but only low levels of daidzein and genistein. Because puerarin glycoside was not available to us as a pure standard, its concentration in the kudzu extract was not accurately determined, and therefore our measurement of isoflavone content of products containing kudzu is underestimated. The concentrations of isoflavones expressed on a per-gram basis of the product are summarized in Table 2. The isoflavone content per capsule or tablet as judged

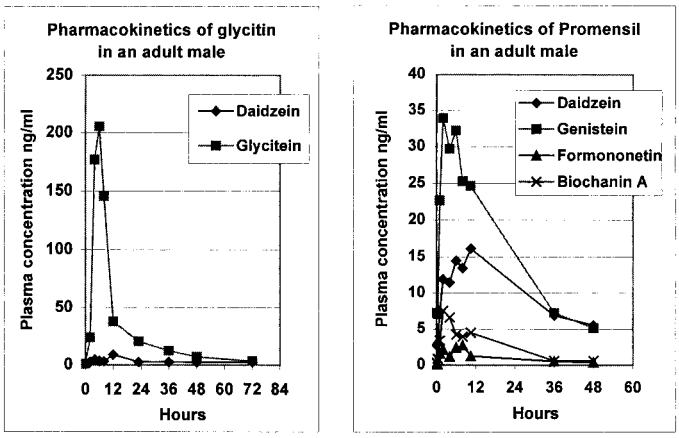

FIGURE 5 Plasma appearance and disappearance curves for daidzein, genistein, glycitein, formononetin and biochanin A after single-bolus administration of 25 mg of glycitin (*left*) or 40 mg of Promensil (*right*) to the same healthy adult man.

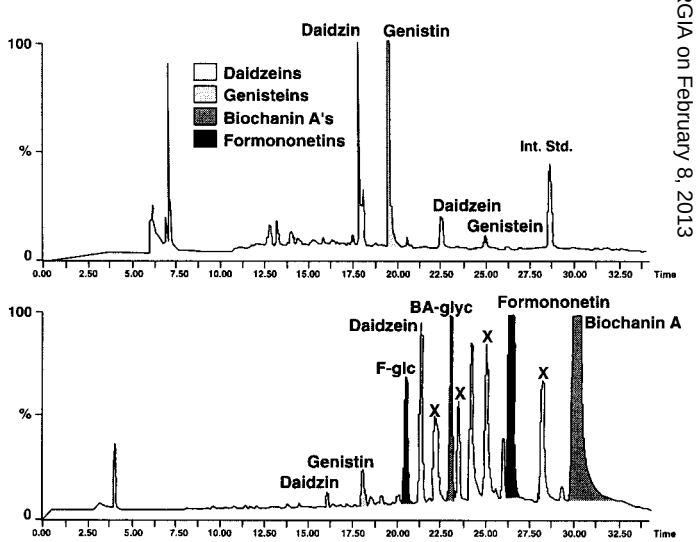

FIGURE 6 High performance liquid chromatogram showing the composition of two different isoflavone supplements: one made from a soybean extract (top) and the other from clover (bottom).

by our analytical methods compared with the respective claim made by the manufacturer is given in **Table 3**. It was evident that many of the commercial supplements contained levels of isoflavones that, based on our analysis, are not in accord with the manufacturer's claim. One of these (Erdic) was found to contain insignificant amounts of phytoestrogens.

Plasma isoflavone concentrations resulting from food and supplement sources of isoflavones

Two supplements selected at random and a soy-containing food (So Good soy milk; Sanitarium Health Food Company, Berkeley Vale, New South Wales, Australia) were each consumed by six healthy adults (three men and three women) during a 5-d period with a washout of 5 d between successive intakes of the products. Fasting blood samples were obtained for the determination of plasma daidzein and genistein concentrations. The results show the contrasting effect on plasma isoflavone concentrations of different supplements (see Fig. 8). One of the supplements (labeled B), containing 21 mg isoflavones, resulted in very high plasma daidzein concentrations and a relatively low genistein level. The mean ratio of plasma daidzein to genistein during steady state was 5.08. Administration of the other supplement (labeled A) led to a reversal of this ratio (0.65), so genistein was the major isoflavone in plasma. The plasma profile of supplement A was qualitatively similar to that obtained when most soy-containing foods are consumed. Soy-containing foods typically lead to higher plasma genistein concentrations (**Fig. 8**).

In vivo estrogenicity of the breast-enhancing supplement Erdic

In an attempt to determine whether there was any estrogenic activity in one of the supplements that was found by HPLC to contain insignificant levels of isoflavones yet claimed to have phytoestrogens to enhance the breast size of women, the supplement was tested for its estrogenicity using an in vivo bioassay. Body weight gains were similar for all rats in the three diet groups: the control diet (AIN-93G), the estradiol-spiked AIN-93G diet and the AIN-93G diet containing 20% Erdic supplement (**Fig. 9**). There was no significant difference in the weights of the rats assigned to the three different groups on d 19 or 20 of life. Between d 20 and 21, when the pups were weaned and adjusting to the new diets, mean body weight increased from 52 to 53 g only in the pups fed the powdered AIN-93G diet. The mean body weight of the rats fed the AIN-93G with added estradiol remained constant (52 g), whereas those fed the AIN-93G with added Erdic supplement declined significantly ($P < 0.05$) from 53 to 50 g during this short period. Within 24 h (by d 22), the mean body weight of all three groups increased and continued to do so through d 23. There were no significant differences among the final mean body weights of the rats in the three groups (Fig. 9). When uterus weight was measured and expressed in terms of wet weight and dry weight, no significant differences in mean uterine weight were observed between the AIN-93G controls Erdic fed rats. However, the wet and dry uterine weights of positive controls fed AIN-93G containing estradiol increased significantly ($P < 001$) compared with the controls (AIN-93G) and those fed AIN-93G with 20% Erdic added (Fig. 9).

DISCUSSION

The hormonal potency of dietary isoflavones first became apparent in the 1940s when their abundance in species of clover (*Trifolium* spp.) caused a devastating infertility syndrome in sheep grazing in pastures in which this clover was prevalent (Bennetts et al. 1946). Interestingly, isoflavones from extracts of clover constitute one commercially available phytoestrogen supplement targeted at postmenopausal women for the relief of menopausal symptoms. The majority of the phytoestrogen supplements, however, are derived from soybeans, and to a lesser extent from extracts of the Chinese vine Kudzu (Keung 1993, Keung and Vallee 1998). All of the supplements we analyzed are commercially available as over-the-counter products, and presently, there is limited regulation of the supplement industry because these products fall under the scrutiny of the Dietary Supplement Health and Education Act of 1994. More disturbing is the fact that there are virtually no data on the pharmacokinetics, pharmacology or safety of phytoestrogen supplements, and many of the health claims that drive supplements sales are based on clinical and nutritional data from phytoestrogen-rich foods, such as soy, rather than from supplements.

It has been known for >60 y that soybeans contain very high concentrations of isoflavones (Walz 1931). However, the full impact of these compounds in human nutrition did not become apparent until the chance observation that persons consuming soy-containing foods excreted isoflavones in the urine at levels far exceeding endogenous estrogen concentrations (Axelson et al. 1984, Setchell et al. 1984). This intriguing observation led to the hypothesis that soy isoflavones would have beneficial effects in protecting against many hormone-dependent conditions because of their partial estrogen antagonist and agonist properties (Setchell et al. 1984). Animal studies, supported by in vitro data, have demonstrated the anticancer properties of isoflavones (Adlercreutz 1995, Barnes 1998, Barnes et al. 1990, Fournier et al. 1998, Messina et al. 1994), and there is vast literature to confirm that isoflavones have a number of nonhormonal properties of relevance to disease prevention (Kim et al. 1998, Setchell 1988). The animal data have been increasingly supported by clinical trials that established the physiological effects of isoflavones in areas such as cardiovascular health, menopause and cancer (reviewed recently in Setchell 1998 and Setchell and Cassidy 1999). The recent Food and Drug Administration approval of a cardiovascular health claim for soy protein through its ability to lower blood cholesterol concentrations will inevitably bring isoflavones into mainstream consumer circles (November 10, 1999, No. 279). Not unexpectedly, the consumer can find a plethora of supplements being marketed in different forms and compositions and with various health claims. It is evident from our study that for a high proportion of these products, the consumer should have little confidence in what they are purchasing. Regrettably, there is a paucity of data to confirm that isoflavone supplements are as nutritionally effective as isoflavone-rich foods, and several recent studies have shown that isoflavones in the form of supplements are ineffective in lowering serum cholesterol concentrations (Hodgson et al. 1998, Nestel et al. 1997 and 1999).

Establishing knowledge of the bioavailability and safety of isoflavones should be, in our opinion, mandatory given that these compounds are bioactive and that very high doses can be ingested from supplements. There should be less concern regarding natural foods as sources of isoflavones because there is a long history of isoflavone consumption from foods and because the presence of the food matrix may limit their bioavailability (Setchell 2000). As part of an ongoing program designed to assess the bioavailability of isoflavones from various sources, we examined in detail the pharmacokinetics of the pure isoflavones daidzein and genistein and their respective

TABLE 2

Isoflavone composition of thirty-three commercially available phytoestrogen supplements or extracts

	Daidzin	Glycitin	Genistin	*Daidzein	Glycitein	Genistein	Formono.	Biochan. A	Mal-glu daidzein	Mal-glu glycitein	Mal-glu genistein	Ac-glu daidzein	Ac-glu glycitein	*Ac-glu genistein	Totals (μg/g)
#1.	5,291 ± 140	3,744 ± 87	1,223 ± 35	917 ± 25	245 ± 5	trace	0	63 ± 5	558 ± 21	437 ± 13	243 ± 9	2,929 ± 98	1,827 ± 68	0	17,478 ± 384
#2.	10,951 ± 50	7,713 ± 76	12,002 ± 154	5,631 ± 102	346 ± 8	1,021 ± 22	0	0	1,493 ± 53	382 ± 28	2,353 ± 83	4,688 ± 136	333 ± 234	0	46,913 ± 690
#3.	trace	trace	trace	trace	0	trace	0	0	0	0	0	0	0	0	—
#4.	3,992 ± 3	530 ± 6	462 ± 4	713 ± 3	112 ± 1	trace	0	681 ± 1	235 ± 16	99 ± 7	303 ± 2	353 ± 25	119 ± 1	713 ± 3	7,598 ± 40
#5.	2,335 ± 121	496 ± 25	3,026 ± 128	0	0	84 ± 5	0	trace	trace	159 ± 12	trace	0	128 ± 8	259 ± 14	6,484 ± 320
#6.	16,347 ± 101	4,406 ± 93	3,396 ± 27	3,735 ± 16	540 ± 2	161 ± 4	238 ± 3	0	3,052 ± 39	0	1,134 ± 14	1,785 ± 25	1,102 ± 18	0	35,895 ± 172
#7.	23,767 ± 234	4,760 ± 51	27,385 ± 156	5,155 ± 68	513 ± 20	773 ± 12	0	0	667 ± 9	863 ± 13	0	3,088 ± 37	796 ± 19	0	67,767 ± 523
#8.	3,964 ± 88	776 ± 30	3,797 ± 59	0	trace	88 ± 3	0	0	135 ± 5	0	235 ± 10	346 ± 13	0	560 ± 22	9,900 ± 216
#9.	6,715 ± 117	1,443 ± 57	7,269 ± 50	736 ± 13	54 ± 18	140 ± 4	0	0	0	0	0	386 ± 7	89 ± 7	0	16,831 ± 259
#10.	10,278 ± 181	6,901 ± 118	2,095 ± 40	0	516 ± 13	86 ± 11	0	trace	0	0	0		3,895 ± 52	1,812 ± 31	31,948 ± 543
#11.	14,152 ± 322	3,431 ± 125	8,102 ± 363	3,239 ± 118	101 ± 2	565 ± 25	0	0	163 ± 5	0	212 ± 9	2,040 ± 75	312 ± 11	0	32,318 ± 1,046
#12.	2,940 ± 33	2,423 ± 22	717 ± 8	0	233 ± 12	72 ± 1	0	trace	499 ± 10	385 ± 6	223 ± 3	1,064 ± 14	858 ± 9	609 ± 7	10,022 ± 94
#13.**	0	0	112 ± 60	1,532 ± 163	2,547 ± 235	2,900 ± 65	26,726 ± 540	44,330 ± 1,072	0	0	0	0	0	0	78,147 ± 1,605
#14.	5,254 ± 430	4,269 ± 60	1,310 ± 21	0	319 ± 5	87 ± 6	0	0	795 ± 28	515 ± 28	306 ± 18	2,621 ± 26	1,776 ± 22	934 ± 8	18,197 ± 567
#15.	5,121 ± 102	6,000 ± 123	1,460 ± 29	0	358 ± 7	73 ± 4	0	trace	754 ± 29	639 ± 23	268 ± 13	2,446 ± 48	2,630 ± 58	1,047 ± 19	20,796 ± 443
#16.	481 ± 3	100 ± 2	859 ± 6	114 ± 1	0	66 ± 1	0	0	100 ± 6	0	240 ± 1	0	0	0	1,961 ± 11
#17.	29,915 ± 2,639	5,773 ± 153	25,031 ± 2,540	2,287 ± 121	114 ± 66	317 ± 4	0	0	0	0	0	1,161 ± 98	971 ± 38	0	66,017 ± 5,071
#18.	36,832 ± 248	10,631 ± 92	39,839 ± 541	4,127 ± 54	700 ± 40	872 ± 6	0	0	0	0	0	2,350 ± 17	856 ± 7	0	96,206 ± 943
#19.	14,684 ± 509	4,249 ± 71	15,171 ± 1,454	1,416 ± 42	264 ± 11	263 ± 6	0	0	0	0	0	850 ± 25	315 ± 27	0	37,211 ± 1,825
#20.	10,768 ± 312	5,142 ± 159	2,542 ± 67	1,044 ± 45	320 ± 5	trace	0	0	0	0	0	2,836 ± 191	1,367 ± 82	0	24,035 ± 507
#21.**	0	0	180 ± 2	967 ± 17	1,770 ± 33	1,556 ± 33	23,965 ± 506	42,104 ± 671	0	0	0	0	0	0	73,587 ± 1,327
#22.	10,954 ± 157	4,047 ± 69	9,078 ± 144	1,522 ± 22	321 ± 11	389 ± 3	trace	81 ± 42	0	208 ± 7	trace	763 ± 12	302 ± 5	0	27,666 ± 305
#23.	3,558 ± 60	1,622 ± 29	2,103 ± 39	785 ± 14	153 ± 3	53 ± 3	155 ± 2	0	66 ± 15	76 ± 7	93 ± 5	1,994 ± 34	938 ± 17	0	11,596 ± 217
#24.	0	0	255 ± 13	0	0	88 ± 1	1,203 ± 15	994 ± 15	0	0	0	0	0	0	2,540 ± 34
#25.	27,116 ± 1176	5,969 ± 253	28,131 ± 745	2,464 ± 117	569 ± 81	377 ± 15	0	0	0	0	0	1,553 ± 112	626 ± 66	0	66,805 ± 2,434
#26.	4,136 ± 98	366 ± 12	706 ± 23	1,621 ± 25	0	0	203 ± 4	0	0	0	0	0	0	0	7,032 ± 154
#27.	6,929 ± 287	6,308 ± 260	1,842 ± 84	1,482 ± 165	985 ± 104	0	0	0	0	0	0	3,644 ± 170	3,079 ± 154	0	24,269 ± 1,126
#28.	35,853 ± 457	8,172 ± 128	31,437 ± 388	3,331 ± 114	581 ± 11	619 ± 19	0	0	0	0	0	2,069 ± 60	711 ± 137	0	82,773 ± 1,034
#29.	1,665 ± 28	0	3,722 ± 122	1,693 ± 53	637 ± 55	2,491 ± 95	0	0	0	0	0	0	0	0	10,207 ± 349
#30.	23,376 ± 433	12,823 ± 244	5,574 ± 105	4,654 ± 106	1,332 ± 53	252 ± 17	0	0	0	0	0	14,706 ± 244	7,338 ± 128	0	70,055 ± 1,235
#31.	475 ± 6	243 ± 3	307 ± 4	132 ± 2	trace	trace	0	0	trace	0	195 ± 4	241 ± 3	135 ± 1	0	1,729 ± 24
#32.	639 ± 3	186 ± 1	778 ± 2	173 ± 1	0	57 ± 0.4	0	0	340 ± 1	129 ± 1	459 ± 1	83 ± 0.4	0	0	2,844 ± 6
#33.	4,765 ± 228	4,958 ± 211	1,439 ± 54	0	286 ± 3	54 ± 1	0	0	532 ± 64	504 ± 66	210 ± 18	3,283 ± 82	2,948 ± 50	1,244 ± 39	20,222 ± 314

Values shown are means ± SEM of 4 analysis.

* The acetyl glycoside of genistein co-elutes with daidzein, making it difficult to discern the relative proportions of these two isoflavones by HPLC alone. Therefore assignment was based on the prodominant ion in the mass spectrum.

** Values for Formononetin and Biochanin A include the glycoside conjugates, but the aglycones were the main component.

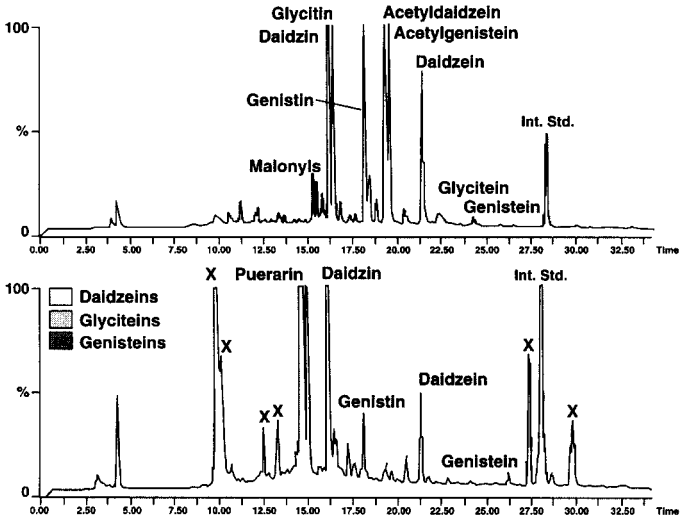

FIGURE 7 High performance liquid chromatogram showing the composition of two different isoflavone supplements, one contained soy germ (top), and the other contained kudzu extract (bottom).

β-glycosides, which are the major isoflavones of most soy-containing foods. These purified isoflavones were administered individually as a single-bolus dose to healthy women. We made limited observational studies on the absorption and clearance of several methoxylated isoflavones that are contained in many over-the-counter phytoestrogen supplements. The results from these pharmacokinetic studies confirm that healthy adults absorb isoflavones rapidly and efficiently. The fates of daidzein, genistein and their respective β-glycosides are similar. In most adults, the time it takes to attain peak plasma concentrations after ingesting the aglycones is 4–7 h, whereas when the β-glycosides are ingested, the t_{max} is shifted to 8–11 h, indicating that the rate-limiting step for absorption is initial hydrolysis of the sugar moiety. Our pharmacokinetic studies show that isoflavones have relatively long half-lives of elimination that are fairly consistent among adults. The $t_{1/2}$ for daidzein and genistein was computed to be 9.34 and 7.13 h, respectively. These values for pure compounds are longer than what has been previously reported when soy-containing foods were consumed by adults (King and Bursill 1998, Watanabe et al. 1998). Whether this is a function of differences in the matrix of foods compared with the pure compounds or supplements remains to be determined. Some comment is appropriate regarding methods for measuring isoflavones in plasma, because it is essential to use methods that have high sensitivity and precision to accurately determine the low plasma concentrations that occur in the terminal elimination phase. Methods based on HPLC with uv detection, which are frequently used, do not have sufficient sensitivity to determine low concentrations of isoflavones in plasma. Consequently, the shape of the plasma elimination curve will be skewed or artificially distorted, giving the appearance of a faster $t_{1/2}$ than is actually the case. It is also important to sample multiple time points during the elimination phase and after attaining steady-state to be able to compute the pharmacokinetics. This seems to have been disregarded in a few studies that were performed with foods (Tew et al. 1996, Xu et al. 1994, Izumi et al. 2000) and conclusions have been drawn based on just two time points in the elimination phase. This may account for the evidently erroneously reported conclusion that daidzein is more bioavailable than genistein (Xu et al. 1994). Our data with pure compounds, and more recently with stable isotopically labeled isoflavones, clearly show that genistein is more bioavailable than daidzein. The anomalous data we obtained for the pharmacokinetics of daidzin in our studies probably result from having too few time points to accurately compute the elimination phase rate given the delay in the t_{max} for the glycoside, and it would have been advantageous to have sampled blood over at least an additional 24 h. The shapes of the plasma appearance and disappearance curves revealed an early peak before the C_{max}; this is typical of compounds that undergo enterohepatic recycling and is a phenomenon that is apparent in studies using soy-containing foods (King and Bursill 1998, Setchell 1998, Watanabe et al. 1998). Isoflavones, like estrogens, have been shown to undergo enterohepatic recycling, and when administered orally, they soon appear in bile (Axelson and Setchell 1981, King et al. 1996, Sfakianos et al. 1997). The early rapid increase in plasma isoflavone concentration could also be explained by some initial absorption of aglycones in the stomach, duodenum and proximal jejunum. However, the kink in the plasma curve was more obvious when the glycosides were ingested, suggesting there may be some initial limited hydrolysis in the proximal gastrointestinal tract.

When the β-glycosides are ingested, daidzein or genistein appear rapidly in plasma, and thereafter the plasma profiles show similarities to those observed after the ingestion of the corresponding aglycones. The systemic bioavailability as determined by comparing dose-normalized AUCs was found to be greater for the β-glycosides than for the corresponding aglycones. Similar pharmacokinetics have been described for flavonoids. Quercetin glycoside, for example, shows higher bioavailability than its aglycone as measured from the appearance of quercitin in the plasma (Hollman et al. 1995 and 1996). These observations led to the suggestion that intact flavonoid glycosides can be absorbed, even though the glycoside was not measured in plasma. However, there are abundant β-glucosidases along the entire length of the human intestine sufficient to hydrolyze isoflavone glycosides. β-Glucosidase activity shows a pattern of developmental expression (Mykkanen et al. 1997) being present early in life and facilitating the absorption of isoflavones contained in soy infant formula, resulting in very high plasma concentrations in infants (Setchell et al. 1997). Although β-glucosidase (EC 3.2.1.21) has been isolated from human feces, there also are a number of membrane-bound β-glucosidases (Ioku et al. 1998, McMahon et al. 1997), and the activity of these enzymes toward flavonoids is highly expressed in the jejunum. The intestinal β-glucosidases also show a high affinity for isoflavones, especially when the glucose residue is at position 7 of the molecule, as is the case for most dietary isoflavones (Day et al. 1998). It is difficult to conceive of β-glycosides surviving hydrolysis during their passage along the gastrointestinal tract, and uptake as intact conjugates would require some form of active transport, which to our knowledge has not been demonstrated. Rather, the pK_a of isoflavone aglycones is favorable for non-ionic passive diffusion from the jejunum, and we suggest that this is the most likely mechanism of absorption. Recent studies by Liu et al. (2000) using Caco-2 TC7 monolayers have demonstrated that genistin does not readily penetrate the enterocyte, whereas its aglycone, genistein, is readily permeable. Therefore, the weight of the evidence to date indicates the bioavailability of isoflavones is contingent on hydrolysis of the glycosidic moiety. The greater bioavailability of isoflavones determined from the AUC when the isoflavone glycosides are ingested is, we believe, explained by the glycoside moiety acting as a protecting group to prevent biodegradation of the isoflavone structure. Isoflavones undergo significant

intestinal metabolism in humans to produce a number of metabolites (Axelson et al. 1982, Bannwart et al. 1984, Joannou et al. 1995, Kelly et al. 1993). The concept of attaching protecting groups to molecules is common practice in pharmacology and is generally done to prevent biotransformation of the parent drug, thereby improving its absorption. We speculate that this happens with isoflavones. Most of the soy-containing foods consumed in the Western world tend to be made from soy proteins that contain mainly isoflavone glycosides and as such can be expected to be more bioavailable than if they were consumed in fermented soy-containing foods with mostly aglycones (Coward et al. 1993). A recent study showed that isoflavone aglycones in fermented foods were absorbed faster than the glycosides and attained a higher Omax (Izumi et al. 2000) which is in accord with our observations on the pharmacokinetics of isoflavones, however since single plasma concentrations were measured conclusions cannot be drawn regarding relative bioavailabilities.

A conspicuous feature of pharmacokinetics is the relatively small proportion of the aglycone that appears in plasma, even after relatively high doses were ingested. Although there was an increase in unconjugated daidzein and genistein in plasma, the aglycones accounted for 8.42 ± 0.89% and 3.71 ± 1.06%, respectively, of the total isoflavones in the first 2 h after isoflavone administration. However, under steady-state conditions, unconjugated isoflavones accounted for a relatively constant 2.74 ± 0.33% and 1.59 ± 0.07%, respectively, of the total isoflavones in plasma. The observed plasma profiles for unconjugated daidzein are similar to those previously reported for three adults who consumed 125 g soybeans when a specific radioimmunoassay was used for daidzein, although peak concentrations were higher due to the larger dietary intake of isoflavones from the food (Lapcik et al. 1997). Our finding of predominantly conjugated isoflavones in plasma is in accord with older and recent studies that show glucuronides to be the major circulating form of all phytoestrogens (Adlercreutz et al. 1995, Axelson and Setchell 1980, Coward et al. 1996, Huggett et al. 1997). When the phytoestrogens were first discovered in plasma, it was observed that portal venous blood of rats contained almost exclusively glucuronide conjugates (Axelson

TABLE 3

Summary of the isoflavone composition of various commercially available phytoestrogen supplements

Product	Total Isoflavones mg/g	Ration of (Daidzein + Glycitein) derived/Genistein	% Aglycones	Total Isoflavones per capsule, tablet or serving (mg)	Claimed* Isoflavone content per capsule (mg)
#1. Carlson Easy Soy	17.52 ± 0.38	10.59 ± 0.14	7.3%	10.2	12.5
#2. Carlson Easy Soy Gold	46.91 ± 0.69	2.05 ± 0.01	14.9%	36.2	50.0
#3. Erdic (Busting Out)[1]	—	—	—	—	not stated
#4. Estroven[2]	7.63 ± 0.04	3.61 ± 0.06	10.8%	7.8	50.0
#5. Solgar	6.56 ± 0.32	0.93 ± 0.02	1.4%	9.4	15.0
#6. Kudzu Root Extract[2]	35.89 ± 0.17	6.65 ± 0.04	13.0%	11.5	3.0
#7. Healthy Woman	67.77 ± 0.52	1.41 ± 0.01	9.5%	48.8	55.0
#8. One a Day	9.94 ± 0.22	1.12 ± 0.01	1.2%	12.8	42 mg soy std. ext.
#9. PhytoEstrin	16.83 ± 0.26	1.27 ± 0.02	5.5%	10.3	14.0
#10. Phyto Soya	31.97 ± 0.54	7.01 ± 0.01	2.0%	12.5	17.5
#11. Soy Extract	32.32 ± 1.05	2.65 ± 0.05	12.1%	11.3	13.0
#12. Phyto Estrogen-Power	10.03 ± 0.09	5.19 ± 0.01	3.1%	7.3	5.3
#13. Promensil[3]	78.15 ± 1.61	1.36 ± 0.07	99.9%	41.7	40.0
#14. PhytoEstrogen Solaray	18.20 ± 0.57	5.9 ± 0.14	2.2%	10.6	10.0
#15. H & B Soya Isoflavones	20.81 ± 0.44	6.31 ± 0.02	2.1%	16.2	16.7
#16. Soyamax[4]	1.96 ± 0.01	0.68 ± 0.01	9.2%	58.0	60 mg/29 g
#17. Soy Care	66.02 ± 5.07	1.63 ± 0.09	4.2%	23.2	25.0
#18. N Resources Soy Isoflavones	96.21 ± 0.94	1.36 ± 0.01	5.9%	43.4	50.0
#19. Soy Plus	37.21 ± 1.82	1.45 ± 0.13	5.3%	18.1	20.0
#20. Naturally Preferred Soy Germ	24.03 ± 0.51	8.41 ± 0.13	5.7%	12.3	10.0
#21. Trinovin[3]	73.59 ± 1.33	1.58 ± 0.01	95.6%	36.9	40.0
#22. Basic Soy Isoflavones	27.74 ± 0.3	1.92 ± 0.01	8.5%	16.6	25.0
#23. Nature's Bounty Flash Fighters	11.6 ± 0.22	4.16 ± 0.01	9.9%	16.8	21.7
#24. Herbal Blends Menopause Balance	3.13 ± 0.03	0	86.0%	2.3	8.0
#25. NovaSoy	66.80 ± 2.43	1.34 ± 0.03	5.1%	40.8	50.0
#26. New Phase-Sunsource	7.03 ± 0.15	8.69 ± 0.12	26.0%	8.6	80.0
#27. Spring Valley	24.27 ± 1.13	12.17 ± 0.10	10.1%	12.7	7.0
#28. Sundown	82.77 ± 1.03	1.58 ± 0.01	5.5%	39.2	40.0
#29. Phytosoy	10.21 ± 0.35	0.64 ± 0.00	47.2%	3.4	4.0
#30. Soy Choice Vitanica	70.05 ± 1.23	11.03 ± 0.03	8.9%	25.8	56.0
#31. Revival[4]	1.78 ± 0.02	2.41 ± 0.01	9.6%	8.9	13.8
#32. Nutri Soy[5]	2.84 ± 0.01	1.20 ± 0.00	8.1%	2.8	not stated
#33. Soy Life 25[6]	20.22 ± 0.31	5.86 ± 0.10	1.7%	20.2	25.0

* A number of manufacturers indicate a range for isoflavone content, in which case the minimum amount was selected. Values shown are means ± SEM of four analyses

[1] Questionable peaks detected by mass spectrometry, but too low for reliable quantification.

[2] Measurement does not include puerarin glycosides, due to lack of pure standards for quantification.

[3] Supplement contains mainly methoxylated isoflavones from clover as its aglycones.

[4] Powdered supplement, values expressed per serving.

[5] Toasted soy flour ingredient.

[6] Soy germ extract used as an ingredient.

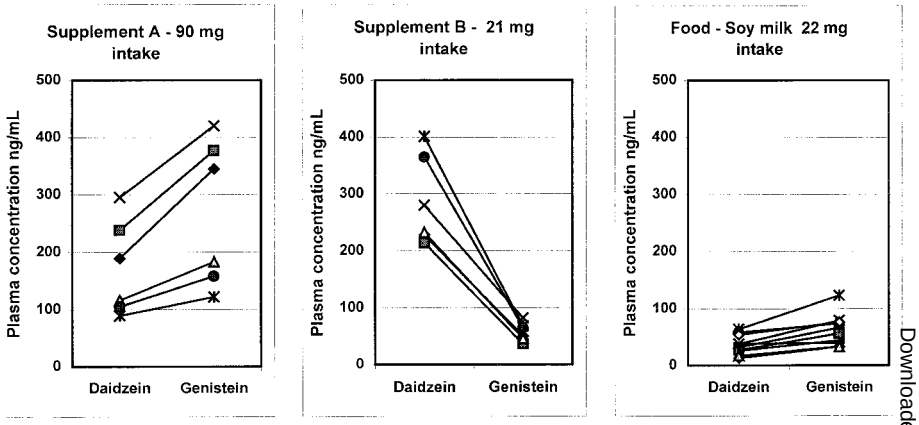

FIGURE 8 Comparison of the plasma concentrations of daidzein and genistein in the same six healthy adults 5 d after the daily ingestion of two different phytoestrogen supplements (designated A and B) and a soy food containing isoflavones.

and Setchell 1981), suggestive of conjugation during first-pass uptake by the enterocyte. This has since been confirmed in the rat using everted intestinal sacs (Sfakianos et al. 1997) and, more recently, from studies of Caco-2 TC monolayers (Liu et al. 2000). Indeed, it appears that the major site for glucuronidation of isoflavones is probably the intestine, and not the liver, as is the case for most steroid hormones. The specific UDP-glucuronyl transferase catalyzing conjugation (Mackenzie et al. 1992) has to our knowledge not been characterized. Despite the circulation of daidzein and genistein in plasma mainly as glucuronides and, to a lesser extent, sulfates, this does not render them biologically inactive. Moreover, it serves to retain them within the enterohepatic circulation, because hydrolysis by intestinal glucuronidases then provides a constant source to account for the persistent levels of unconjugated isoflavones in plasma seen in these studies. Estrogen shows a similar pattern of metabolism, and endogenous estrogens also circulate mainly as sulfate and glucuronide conjugates.

The Vd/F was large for both daidzein and genistein, indicating extensive tissue distribution. Isoflavones have been found to be concentrated in the human breast, appearing in nipple aspirates (Petrakis et al. 1996), whereas studies with rats have shown they rapidly partition into the brain (Lephart et al. 2000, Setchell 1998). Daidzein exhibited a much higher Vd/F than genistein (236 L for daidzein versus 161 L for genistein), and this explains why genistein levels in plasma always exceed daidzein concentrations when equivalent amounts of the two isoflavones are ingested. For most foods, genistein and its glycosides tend to be present in higher levels than daidzein and its glycosides, and therefore clinical feeding studies should always reveal a higher plasma genistein concentration than daidzein. This has been our experience to date.

With regard to metabolism, we measured equol, the bacterially derived metabolite of daidzein (Axelson et al. 1982, Setchell et al. 1984), in the plasma but did not measure desmethylangolensin, or dihydrodaidzein (Bannwart et al. 1984, Kelly et al. 1993) because these studies predated interest in these metabolites and standards were not available to us. Equol was the first isoflavone to be identified in human urine and blood, and its discovery led to the identification of soy as a rich source of isoflavones (Axelson et al. 1982, Setchell et al. 1984). In the early studies, it was found that two thirds of adults who consumed soy-containing foods converted daidzein to equol (Setchell et al. 1984). This ability to convert daidzein into equol led to use of the term "converters" to describe persons who have the necessary bacterial enzymes or intestinal conditions to make this biotransformation. Over time, the proportion of "converters" in a population seems to have decreased for some unexplained reason, and several groups have reported that only one third of the population are converters (Cassidy et al. 1994, Kelly et al. 1993, Sathyamoorthy and Wang 1997). Methods for measuring isoflavones have evolved since their initial discovery in urine, and the question of whether equol is being lost or missed in the work-up procedures is a valid one. The GC-MS techniques we use consistently find equol as the major circulating isoflavone of rats and mice (Brown and Setchell 2001); hence, the methodology used in our laboratory is excluded as an explanation for the differences. Whether the proportion of converters shows geographical differences is unknown; the early studies were of adults living in Britain (Setchell et al. 1984), whereas those reported here are of U.S. adults. The makeup of the diet can alter the metabolism of isoflavones in the intestine. In experiments using an in vitro colonic fermentation system, it was found that under a high carbohydrate environment, fermentation was stimulated, and this increased the rate of conversion of daidzein to equol (Cassidy 1991, Setchell 1998, Setchell and Cassidy 1999). Virtually no conversion occurred when a low carbohydrate milieu was mimicked in vitro. This was also shown to be the case in a human study where greater equol production was seen with a diet that was higher in carbohydrates (Lampe et al. 1998). This suggests that the overall composition of the diet may have to be taken into consideration in clinical studies investigating the potential efficacy of isoflavones. In the "acute" studies described here, we found equol in the plasma of only three of nine adults fed its

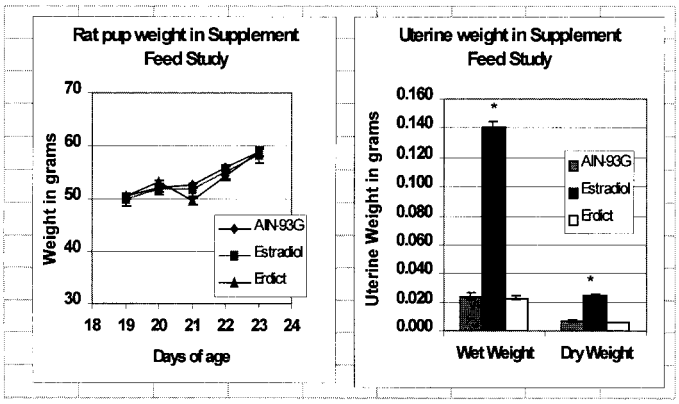

FIGURE 9 Changes in body weight and uterus wet and dry weights in immature rats fed an AIN-93G phytoestrogen-free diet and diets containing 20% Erdic supplement or estradiol.

precursors, daidzin or daidzein. None of the subjects fed the aglycone showed equol in the plasma, and this was somewhat surprising. It is possible that this is explained by the aglycone being absorbed passively in the proximal small intestine, whereas the glycoside would not be taken up by the enterocyte, thus becoming delivered to the distal small intestine and colon for metabolism by bacteria. The observed time delay of at least 6–8 h before any equol appears in the plasma of those converters would be consistent with the bacterial enzymes being of colonic origin. Because the binding affinity of equol for the estrogen receptor is an order of magnitude greater than that of its precursor, daidzein (Shutt 1976, Shutt and Cox 1972) and because its protein binding to serum SHBG and albumin is much lower (Nagel et al. 1998), equol has greater estrogenic potency than daidzein. There may therefore be advantages to improve the intestinal conversion of daidzein to equol.

Although these studies define for the first time the plasma pharmacokinetics of pure isoflavones in healthy adults, similar data on isoflavone supplements are lacking, and there is little incentive under the Dietary Supplement Health and Education Act of 1994 for commercial manufacturers of these supplements to obtain this type of information. Our studies of a selection of commercially available over-the-counter phytoestrogen supplements show that there is a wide variation in composition and that no two supplements appear to be the same. This poses some difficulties for the consumer as to what supplement is "best" to purchase. All vary in the amount of isoflavone that is "packaged" in the capsule, tablet or powdered extract, and there is no consensus on standardization of an appropriate or optimal dose. It is evident that the qualitative compositions of the isoflavone supplements are broadly divided into those that use soy germ as an ingredient and those that use isolate, or some other plant source, such as clover or kudzu. Some are clearly blends of several ingredients. Many of the supplements we analyzed had high levels of glycitin, a 6-O-methoxylated isoflavone glycoside. Little is known about its biological properties or bioavailability relative to other isoflavones, but based on its chemical structure, it is predicted to be less estrogenic than daidzein or genistein. Feeding studies have been carried out in humans with soy germ, which has a high glycitin content, and glycitein was identified in plasma up to 24 h later (Zhang et al. 1999). The pharmacokinetics of glycitin and glycitein are unknown. When pure glycitin was ingested as a single-bolus dose by one healthy man, its aglycone glycitein, rapidly appeared in the plasma with peak plasma concentrations occurring 4 h after ingestion. Other than hydrolysis of the sugar moiety, glycitin appears to undergo limited further biotransformation. Plasma daidzein levels do show a small increase 12 h after the ingestion of glycitin, indicating minor demethylation that undoubtedly occurs in the colon. Steric hindrance of the 6-methyoxyl group by the 7-hydroxyl in the structure of glycitein is presumed to account for the lack of intestinal bacterial conversion to daidzein. By contrast, the B-ring methoxylated isoflavones of clover, formononetin and biochanin A, structurally exhibit no steric hindrance, so these are rapidly and efficiently demethylated, giving rise to daidzein and genistein, respectively, in humans. Less than 5% of the methoxylated isoflavones remain intact, and this product effectively makes bioavailable the isoflavones that characterize soy proteins. The fate of isoflavones in clover extracts such as Promensil is consistent with the known metabolism of formononetin and biochanin A in sheep and several other animal species (Lindsay and Kelly 1970, Lundh 1995, Lundh et al. 1988). Formononetin and biochanin A show extremely poor affinity for the estrogen receptor (Shutt 1976, Shutt and Cox 1972). Molecular modeling studies show the 4′-hydroxyl on the B-ring of isoflavones to be the binding site for the estrogen receptor (Brzozowski et al. 1997, Pike et al. 1999), and therefore the presence of a methyl group markedly reduces estrogenic potency. The conversion of clover isoflavones to isoflavones typical of soy that clearly occurs when this supplement is ingested can be considered advantageous because it is difficult to understand the physiological advantages of the consumption of methoxylated isoflavones other than using these as a form of prodrug (precursor) for the delivery of genistein and daidzein.

The starting materials used to manufacture phytoestrogen supplements will have a significant effect on the ultimate bioavailability and characteristics of the circulating isoflavone levels. We demonstrated that two different supplements yield quite different plasma isoflavone profiles (Fig. 8). Those that incorporate the soy germ as a starting material result in plasma that is enriched in daidzein and low in genistein. By contrast, when the supplement is made from an extract of soy protein, the plasma is enriched to a greater extent in genistein. Plasma concentrations of genistein are typically higher than those of daidzein when soy protein foods are consumed, as is evident when these subjects consumed a soy milk drink (Fig. 8). Such differences make it impossible to compare data from clinical studies where sources of isoflavones are from foods or supplements unless plasma isoflavones are monitored. This is rarely done. Thus, depending on the clinical effect being sought, the type of supplement may strongly influence the end point. For cancer prevention, higher plasma genistein concentrations may be advantageous, whereas for cardiovascular protection, the maintenance of higher antioxidant activity by sustained high concentrations of daidzein may be preferable. These are the types of issues and discrepancies that need to be resolved for the design of clinical studies in which phytoestrogens are investigated.

We found, based on our methodology using HPLC with the addition of an internal standard for quantification and ESI-MS for positive identification of each component, that approximately half (16/31) of the supplements had isoflavone levels that deviated by more than 10% from the claimed value. This is not surprising, because discrepancies in the contents of other herbal supplements have been described. In the case of one phytoestrogen product, Erdic (Busting Out), we found virtually no detectable isoflavones in the tablets, yet this product is marketed to women to enhance breast size, and its product literature and Internet Web site, claimed it was a good alternative to silicone implants for breast augmentation. At the time of these studies, it was being sold for $1485 for a 6-mo supply with the statement that, "Compared to the cost of implants ($4,000 and up) and the safety factor, this is a great product." Women were instructed to take "10 tablets a day or more." The product information under the section "How Does It Work?" indicated that the mechanism of breast enhancement occurred because "The Erdic product has natural estrogenic properties from plant sources (phytoestrogens) which promote tissue development". We failed to identify significant levels of phytoestrogens of the isoflavone class or for that matter any other similarly related compounds. The possibility that there may be other phytoestrogens present was considered, especially since one of the ingredients of the product is hops. Endocrine disruption has been noted in female hop workers and attributed to 8-prenylnaringenin, which binds to estrogen receptors with greater affinity than isoflavones (Milligan et al. 1999). Therefore, we subjected this supplement to bioassay for estrogenicity. When fed to immature rats at a level of 20% of the diet, there was no detectable increase in uterine

weight consistent with a lack of significant estrogenicity. In the absence of clinical data to the contrary, there must be serious doubt that this product will achieve the effects claimed for it. This specific example illustrates the sort of problems in an industry that is poorly regulated.

So far, there is little evidence that isoflavone supplements have the same clinical effects as phytoestrogen-rich foods, and whether this relates to differences in their bioavailability or metabolism remains unknown. Three recent studies have found that isoflavones have no effects on lowering serum cholesterol (Hodgson et al. 1998, Nestel et al. 1997 and 1999), yet a comprehensive clinical study of the effects of soy protein containing different levels of isoflavones has shown a dose-dependent effect of isoflavones on reducing LDL cholesterol concentrations (Crouse et al. 1999). In connection with lipid-lowering, it appears that the presence of a protein matrix is necessary for the effectiveness of isoflavones. Much work is needed to better understand the mechanism behind such a phenomenon. Isoflavone supplements are in large part being targeted to postmenopausal women for the relief of hot flushes. Clinical studies show that they have a modest effect on hot flushes that exceeds the placebo response, but they are not as effective as hormone replacement therapy. (NAMS Consensus Opinion 2000)

Finally, the safety of phytoestrogen supplements should be addressed. The misconception that it is safe if it is "natural" is a widely held belief. Many of the supplements analyzed contained numerous compounds that we could not identify, and nothing is known about these components of these mixtures. With supplementation, the dangers of overdosing becomes a reality. Although diets rich in phytoestrogens have been consumed by millions of humans for millennia, the amounts ingested daily, estimated at 15–50 mg (Chen et al. 1999, Nagata et al. 1999, Wakai et al. 1999) are below the dose promoted for supplementation, and in some cases, fortified functional foods. It is known that deleterious effects can result from high levels of isoflavones fed to animals (Bennetts et al. 1946, Leopold et al. 1976, Setchell et al. 1987), and there is little reason to believe that adverse effects could not occur in humans as a result of excessive intakes.

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