Isoflavones in human breast milk and other biological fluids[1–3]

Adrian A Franke, Laurie J Custer, and Yuichiro Tanaka

ABSTRACT We established a method for using HPLC and diode-array ultraviolet scanning to quantitate soy isoflavonoids in foods and in human plasma, urine, and breast milk. The analytes occurring as glycoside conjugates were hydrolyzed enzymatically before HPLC analysis if extracted from biological matrices or were subjected to direct HPLC analysis after extraction from foods. We monitored the isoflavones daidzein, genistein, glycitein, formononetin, and biochanin-A and their mammalian metabolites equol and *O*-desmethylangolensin in human plasma, urine, and breast milk. Analytes were identified by absorbance patterns, fluorometric and electrochemical detection, and comparison with internal and external standards. In addition, we identified analytes by using gas chromatography–mass spectrometry after trimethylsilylation. The HPLC method was also used to measure concentrations of isoflavones and their glucoside conjugates in various soy-based infant formulas. Total isoflavone concentrations varied between 155 and 281 mg/kg. After one woman received a moderate challenge with 20 g roasted soybeans (equivalent to 37 mg isoflavones), we detected mean total isoflavone concentrations of ≈2.0 μmol/L in plasma, 0.2 μmol/L in breast milk, and 3.0 μmol/h in urine. According to our measurements, with adjustment for body weight, isoflavonoid exposure is 4–6 times higher in infants fed soy-based formula than in adults eating a diet rich in soyfoods (≈30 g/d). Implications of the presented results for the potential cancer-preventing activity of isoflavones by exposing newborn infants to these phytochemicals are discussed. *Am J Clin Nutr* 1998;68(suppl):1466S–73S.

KEY WORDS Daidzein, genistein, glycitein, isoflavonoids, soy, breast milk, urine, plasma, soy-based infant formula, HPLC

INTRODUCTION

Soy consumption is suggested to contribute to the prevention of chronic diseases, including cardiovascular disorders, osteoporosis, and cancer. New findings in this regard are presented in detail in these conference proceedings. Recent animal experiments show that only 3 doses of genistein given to newborn, prepubertal, or perinatal rats can reduce the incidence and number of breast tumors (1). In addition, genistein in combination with daidzein produces a highly significant reduction in neoplastic transformation in a rodent cell model (2).

Soyfoods containing ≈0.2–0.3% daidzein and genistein (3–10) were identified as the predominant source of human exposure to isoflavones (3, 11). Because isoflavonoids are associated with the beneficial effects of soy consumption, there is great interest in rapid, accurate, and affordable methods to quantitate these analytes in foods and biological material. In the past, gas chromatography–mass spectrometry (GC-MS) was favored for the analysis of soy isoflavones and their metabolites in human plasma (12), urine (13–16), and feces (17, 18). In recent years, however, the use of HPLC for quantitating isoflavonoids in these body fluids was introduced (19–22). This method allows a variety of isoflavonoid phytoestrogens, including aglycones and conjugated analytes, to be measured in one operation. Compared with GC-MS, HPLC requires fewer steps for sample preparation and analysis, less technician time, and less expensive instrumentation. In studies of dietary intervention with soy, HPLC (19) proved to be as accurate as GC-MS (13, 15) in measuring urinary isoflavone concentrations.

To improve methods for assessment of the role of dietary soy in promoting human health, we established a technique that combines HPLC and diode-array ultraviolet scanning to quantitate soy isoflavonoids in foods and in human plasma (21), urine (19), and breast milk (23). The purpose of the present study was to quantitate glucuronide and sulfate conjugates of daidzein and genistein in human plasma, urine, and breast milk by using this HPLC method. The HPLC method was also used to monitor isoflavones and their glucoside conjugates in soy-based infant formulas. We discuss the implications of our results in relation to the potential for cancer prevention by exposure of newborn infants to isoflavones.

SUBJECTS AND METHODS

Apparatus

We performed HPLC analyses on a system Gold chromatograph with an autosampler (model 507), a dual-channel diode-array detector (model 168) (both from Beckman, Fullerton, CA), a fluorescence detector (model FD100; GTI/SpectroVision, Concord, MA), and an electrochemical 5011 coulometric cell detector (Coulochem II-5200; ESA, Bedford, MA). Absorbance readings were obtained with a spectrophotometer (DU-62, Beckman). Evap-

[1] From the Cancer Research Center of Hawaii, Honolulu.

[2] Supported by American Cancer Society Institutional Grant IRG 190 and a grant from the Hawaii Chamber of Commerce.

[3] Address reprint requests to AA Franke, Cancer Research Center of Hawaii, 1236 Lauhala Street, Honolulu, HI 96813. E-mail: adrian@crch.hawaii.edu.

Am J Clin Nutr 1998;68(suppl):1466S–73S. Printed in USA.

oration was performed with a speed vacuum (AS 160; Savant, Farmingdale, NY) at room temperature. We performed GC analysis with a mass spectrometer (model 5890; Hewlett-Packard, Wilmington, DE), using a mass selective detector (model 5971A, Hewlett-Packard) and electron impact ionization at 70 eV. Solid-phase, C_{18} reversed-phase extraction columns were obtained from PGC Scientific (Gaithersburg, MD).

Chemicals

Methanol, hydrochloric acid, acetic acid, 96% ethanol, dimethyl sulfoxide, ethyl acetate, and all solvents used for HPLC and absorbance readings were analytic grade or HPLC grade from Fisher Scientific (Fair Lawn, NJ). Butylated hydroxytoluene, sodium acetate, and genistin were purchased from Sigma Chemical Co (St Louis). Daidzein and genistein were obtained from ICN (Costa Mesa, CA), flavone was obtained from Aldrich (Milwaukee), and coumestrol was obtained from Serva (Westbury, NY). β-Glucuronidase isolated from *Escherichia coli* (200×10^3 U/L) and arylsulfatase isolated from *Helix pomatia* (1–5 U/mL) were purchased from Boehringer Mannheim (Indianapolis). Equol and *O*-desmethylangolensin were purchased from the University of Helsinki.

Subject

One woman of average height and weight who was in week 15 of the postpartum period participated in the study. She did not smoke; took no medication, hormones, or dietary supplements; had no specific dietary patterns (eg, vegetarian); and was in good health. This woman donated breast milk, blood, and urine for the present study after signing an informed consent form. During the study, the subject did not consume any alcohol and maintained her usual diet except for the intake of 5, 10, and 20 g roasted soybeans containing 0.85 mg daidzein/kg and 1.1 mg genistein/kg (3) during the dietary intervention period. The soy doses were added to the women's usual self-selected diet. All procedures of the protocol were in accordance with the ethical standards of the Helsinki Declaration of 1975 as revised in 1983.

Collection and handling of human milk, blood, and urine

Each time after the woman nursed her infant, milk was collected from the breast that was not used for breast-feeding. Collection included entire expression of the breast. We stored milk samples in plastic vials at 6 to −4°C. Isoflavones in milk were stable for ≥5 d when the milk was kept within this temperature range (23).

After the subject fasted overnight, blood samples were drawn into evacuated containers containing heparin and kept on ice until centrifuged at 850 × *g* for 30 min at 4°C. The supernatant plasma was either analyzed immediately or stored at −70°C (21).

Urine samples were stored in disposable bottles containing 0.2 g boric acid and 0.1 g ascorbic acid to control bacterial contamination and degradation of analytes (19). After we mixed each urine sample and measured the volume, we transferred the sample into a 50-mL disposable plastic tube and stored it at −4 to −20°C. Each urine volume, time of urine collection, and time of previous voiding was recorded so that values could be adjusted for urine concentration.

Extraction of isoflavones from soy-based infant formula

Soy-based infant formulas were purchased from local stores and extracted by sonicating 0.5 g dry formula powder for 10 min in 25 mL of 80% aqueous methanol containing 20 ppm flavone as an internal standard. Next, the mixture was stirred for 2 h in a sealed container at room temperature. After centrifugation at 850 × *g* for 5 min at ambient temperature, a clear aliquot of the solution was diluted 1:1 with acetate buffer (0.2 mol/L, pH 4); 20 μL of this solution, which contained the isoflavone conjugates and aglycones, was injected into the HPLC system.

Enzymatic hydrolysis and extraction of isoflavones from urine

For extraction of isoflavones from urine, we processed the urine as described previously (19, 21). In brief, 20 mL clear urine was mixed with 5.0 mL acetate buffer (0.2 mol/L, pH 4) and 200 μL flavone (60 ppm in 96% ethanol) as the internal standard and passed through a preconditioned C_{18} solid-phase extraction column. The mixture was then washed with 2 mL acetate buffer and the analytes were eluted with 100% methanol. The eluate was dried under reduced pressure at room temperature, redissolved in 1.0 mL phosphate buffer (0.2 mol/L, pH 7.0), mixed thoroughly with 10 μL β-glucuronidase (24) and 10 μL arylsulfatase, and incubated for 1 h at 37°C. Subsequently, we inactivated the enzymes of the hydrolyzed samples by adding 0.98 mL of 100% methanol. Samples were analyzed immediately or stored at −20°C and analyzed by HPLC after equilibration to room temperature, vortex mixing, and centrifugation for 5 min at 850 × *g* and 8°C. Additional concentration was achieved by partitioning the isoflavones from hydrolyzed samples into ethyl acetate, combining the organic phases, and drying under nitrogen at room temperature. We redissolved the residue in 150 μL mobile phase solvent and 50 μL acetate buffer (0.2 mol/L, pH 4) before injecting 20 μL of the solution into the HPLC system.

Enzymatic hydrolysis and extraction of isoflavones from breast milk

As reported previously (23), we mixed 2–4 mL breast milk equilibrated to room temperature with 25 μL flavone (120 ppm in 96% ethanol), 50 μL β-glucuronidase, and 50 μL arylsulfatase. The mixture was stirred for 1 h at 37°C. This sample was extracted 3 times with 2 mL ethyl acetate (certified by the American Chemical Society) and the organic phases were combined after centrifugation at 850 × *g* for 5 min at ambient temperature, followed by drying under nitrogen at room temperature. The dry extract was redissolved in 150 μL methanol by vortex mixing. Then we added 50 μL acetate buffer (0.2 mol/L, pH 4). After centrifugation for 20 min at 850 × *g* at 8°C, 20 μL of the clear sample was injected into the HPLC system.

Enzymatic hydrolysis and extraction of isoflavones from plasma

We mixed 1.0 mL plasma equilibrated to room temperature with 0.25 mL triethylamine acetate (0.5 mol/L, pH 7.0), 80 μL β-glucuronidase, 80 μL arylsulfatase, and 20 μL flavone (120 ppm in 96% ethanol). The mixture was stirred for 17 h at 37°C in a sealed container as reported previously (21). After adding 0.25 mL of 10% aqueous trichloroacetic acid, we extracted the mixture 3 times with 2 mL ethyl acetate and dried the combined organic phases under nitrogen. The residue was redissolved in 150 μL methanol by vortex mixing. Then we added 50 μL acetate buffer (0.2 mol/L, pH 4) and sonicated the mixture for 30 s. After centrifugation of 20 μL at 4°C for 5 min, the clear sam-

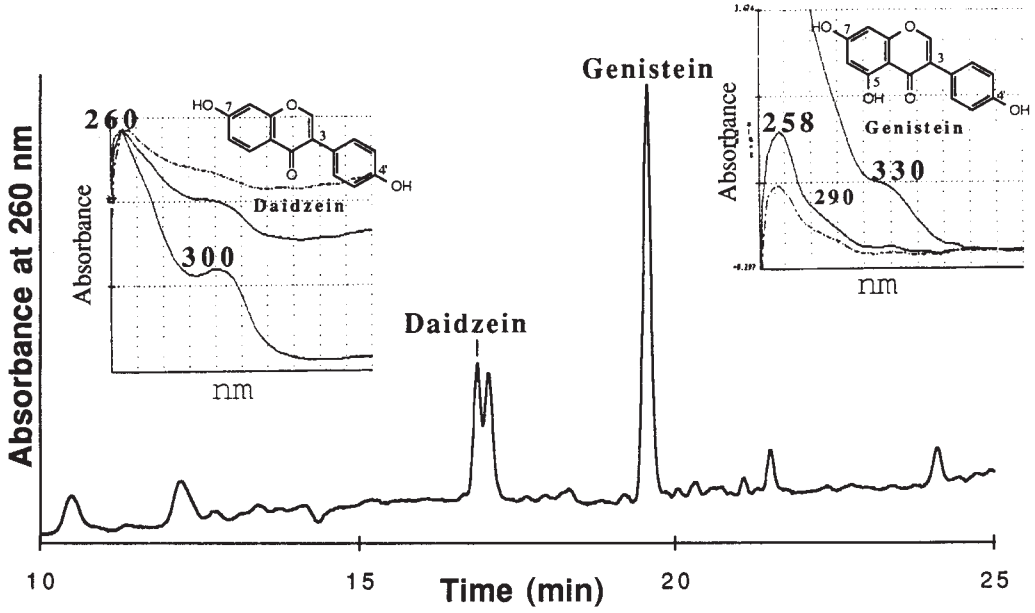

FIGURE 1. High-performance liquid chromatograms and diode-array ultraviolet scans of daidzein and genistein obtained from human breast milk. Milk was collected 18 h after a challenge with 20 g roasted soybeans and extracted as described in the Methods. Flavone was used as the internal standard and eluted after 31 min (not shown). Amounts shown correspond to 3.7 pmol daidzein and 5.4 pmol genistein. We did not detect glycitein, equol, or *O*-desmethylangolensin. Absorbance scans obtained from standards for daidzein and genistein (solid lines) are identical to those obtained from HPLC peaks (dotted lines), indicating the presence of daidzein and genistein in this milk extract.

ple was injected into the HPLC system.

Trimethylsilylation

Dry milk extracts or crystalline standards were dissolved in 0.1 mL trimethylsilyltrifluoracetamide-imidazole (100:2, vol:wt) and incubated for 15 min at 60 °C before GC-MS analysis (24).

Chromatographic conditions

All HPLC analyses were performed on a NovaPak C_{18} reversed-phase column [150 × 3.9 mm; inside diameter: 4 μm (Waters, Milford, MA)] coupled to an Adsorbosphere C_{18} direct-connect guard column [10 × 4.6 mm; inside diameter: 5 μm (Alltech, Deerfield, IL)]. We performed elution at a flow rate of 0.8 mL/min with the following linear gradient: A, acetic acid to water (10:90, by vol); B, methanol-acetonitrile-dichloromethane (10:5:1, by vol); and B in A (by vol) at 5% for 5 min, from 5% to 45% in 20 min, from 45% to 70% in 6 min, and from 70% to 5% in 3 min. Signals were scanned between 190 and 400 nm.

We performed gas chromatography by injecting 3 μL of the trimethylsilylated sample onto a Hewlett-Packard P Ultra-1 capillary column (inside diameter: 17 m × 0.2 mm; film thickness: 0.11 μm); helium was used as the carrier gas at a flow rate of 1.0 mL/min with a 1:10 flow split. The following temperature program was used: initial temperature, 180 °C; rate, 10 °C/min; and final temperature, 320 °C. Signals were registered in the selected ion monitoring (SIM) mode and the following masses were determined after analysis of standards: daidzein: mass-to-charge ratio (*m/z*) 398, 383; genistein: *m/z* 471, 399, 228; equol: *m/z* 386, 192; and *O*-desmethylangolensin: *m/z* 459, 281.

Standard solutions and calibration curves

Standard stock solutions were prepared by dissolving 1–3 mg of the crystalline compound in 20 μL dimethyl sulfoxide and adding methanol to give stock solutions of 2–5 mmol/L. We discarded compounds with <95% purity as determined by HPLC analysis. The concentration of the stock solutions was determined by absorbance readings as reported previously (19). Concentrations of analytes in milk and urine were calculated from peak areas that we determined after HPLC analyses by using the slopes of the calibration curves obtained from serial dilutions of standard solutions. Milk and plasma concentrations were expressed as nmol/L and values were adjusted for recovery of the internal standard. Urinary excretion rates were expressed as nmol/h after adjustment for the time period between the urine collection and previous voiding (in hours), urine volume (in milliliters), and recovery of the internal standard.

RESULTS

Analytes were routinely identified by retention times in various HPLC systems and by diode-array absorption patterns (**Figure 1** and **Figure 2**). The HPLC system separated completely parent and metabolized isoflavonoids such as daidzein (16.8 min), glycitein (17.9 min), genistein (20.1 min) and para-ethylphenol (22.1 min), equol (20.8 min) and *O*-desmethylangolensin (23.2 min), and mammalian lignans including enterodiol (18.9 min) and enterolactone (22.6 min). Fluorometric and coulometric detection (19, 21, 25–27) confirmed the presence of daidzein and genistein in milk, with detection limits being 2–6-fold lower than those with monitoring by ultraviolet absorbance. Finally, GC-MS-SIM analysis of trimethylsilylated milk extracts revealed GC retention times and mass fragmentation patterns identical to those for daidzein and genistein standards (18, 23), confirming the presence of these isoflavones in human milk.

The soybean intake challenge led to a rapid and dose-depen-

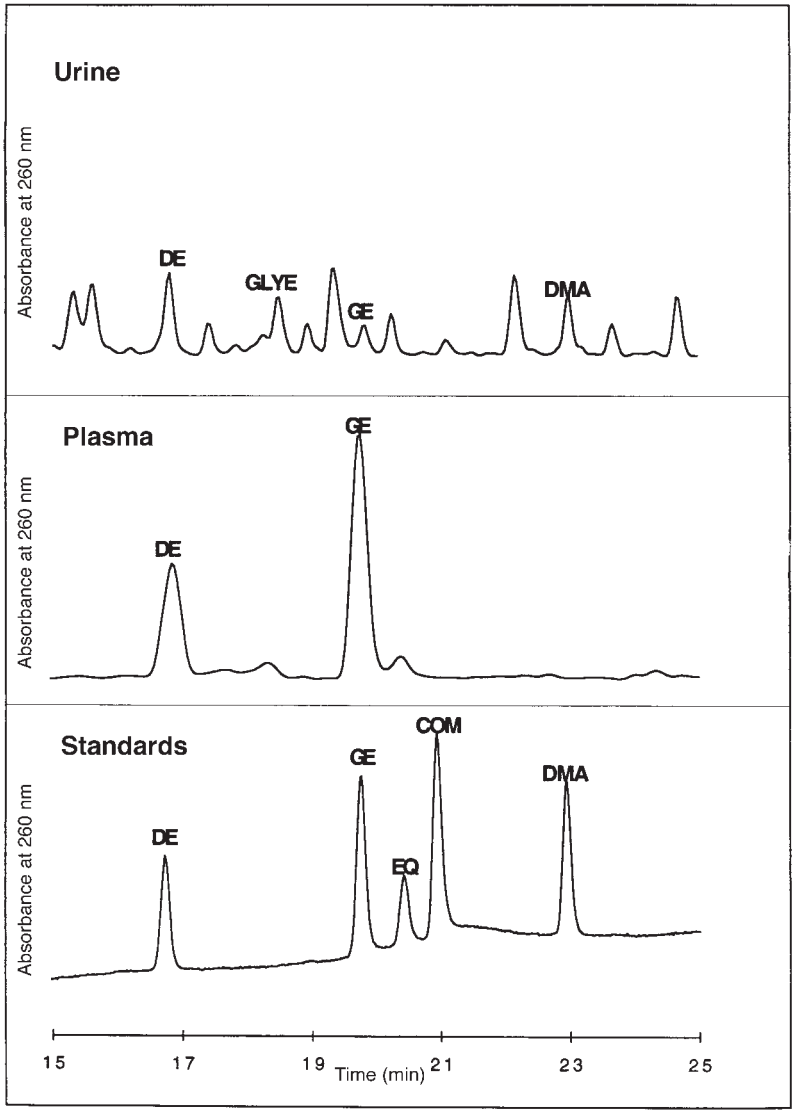

FIGURE 2. High-performance liquid chromatograms of isoflavonoids obtained from extracts of human urine and plasma and compared with that of standards for isoflavonoids. DE, daidzein; GLYE, glycitein; GE, genistein; DMA, *O*-desmethylangolensin; EQ, equol; COM, coumestrol.

dent response in isoflavone concentrations in breast milk (**Figure 3**). Our results are in excellent agreement with results reported recently by GC-MS analysis (28). Maximum milk concentrations were reached 10–14 h after soy intake and baseline concentrations were estimated to be reached 2–4 d later, depending on the dose.

Extending our previous work on the measurement of isoflavone concentrations in food items (3, 21), we analyzed concentrations of isoflavone aglycones and individual isoflavone conjugates from various soy-protein-based infant formulas. Again, HPLC peaks of extracts (**Figure 4**) were identified by comparison of diode-array absorbance patterns (5, 21). Ultraviolet scans of conjugated isoflavones showed patterns identical to those of their respective aglycones (Figure 4). This result is in excellent agreement with the finding that glycosylation at the C-7 hydroxyl position of the flavonoid molecule does not affect the flavonoid chromophore (29). Total isoflavone amounts (**Table 1**) varied between 155 and 281 μg/g depending on the brand of formula. Patterns of conjugation and isoflavone type followed those expected for soy protein isolate (6, 8, 28).

DISCUSSION

We examined the presence of isoflavonoids in breast milk to assess exposure of breast-fed infants to isoflavones, phytochemicals that may contribute to cancer prevention (23). Recovery of analytes was low for both solid-phase extraction methods and phase-separation techniques with various organic solvents. This was probably due to the high protein content and the emulsion-like nature of milk. However, phase separation with ethyl acetate resulted in selective concentration of the analytes and led to recovery that was superior to that achieved with any of the other organic solvents used. Isolation with ethyl acetate resulted in high precision (CV: 5–11%) and spiking recoveries (88–99%) of isoflavone aglycones from breast milk and did not coextract glycosidic conjugates (23). To include conjugates in the assay, we

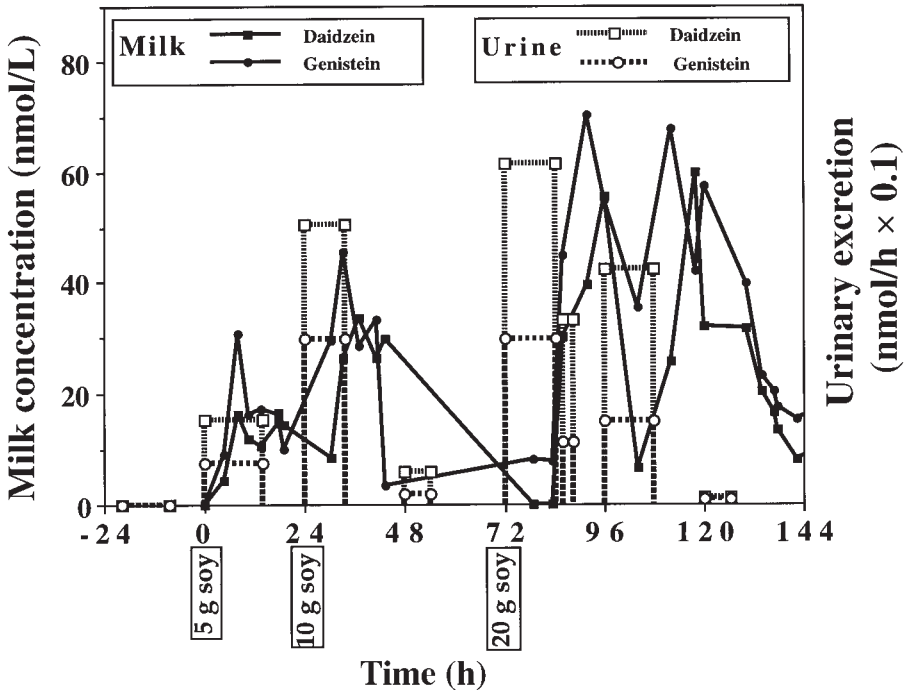

FIGURE 3. Daidzein and genistein concentrations in human breast milk and urine after a challenge with 5, 10, and 20 g roasted soybeans given at 0, 24, and 72 h. Concentrations were determined by HPLC with diode-array ultraviolet scanning. Milk samples were collected at the time of feeding. Six overnight urine samples and 2 additional samples, collected 12 h before and 87 h after the first soybean intake, were obtained to monitor urinary isoflavone excretion.

had to perform hydrolysis before extraction (24, 25). The lack of detectable isoflavones after extraction when hydrolysis was not performed suggests that all isoflavones occur as glucuronide or sulfate conjugates in human milk.

The appearance of isoflavones in milk followed their appearance in urine, with a slight delay (Figure 3). This result is in good agreement with findings for other micronutrients or drugs for which urinary excretion precedes secretion in breast milk (30). Preferential excretion of the metabolites over the parent isoflavones is suggested by the ratio of the isoflavone to its metabolite, which was previously observed to be much higher in plasma than in urine or feces (12, 18). Because milk is likely to be produced from blood by passive secretory processes (30), patterns in milk probably reflect those in plasma.

Concentrations of genistein conjugates in milk were higher than those of daidzein conjugates (Figure 3). We made a similar observation for concentrations in plasma: 15 h after the intake of 20 g soybeans, the concentration of genistein was 1039 nmol/L and that of daidzein was 481 nmol/L (data not shown). In contrast, urinary concentrations of genistein conjugates were lower than those of daidzein conjugates, which is consistent with findings in other studies (14–16, 19, 20, 31–33). The explanation may be that the higher polarity of daidzein conjugates favors urinary excretion, leading to increasing ratios of genistein to daidzein in the blood.

A distinct biphasic pattern of milk concentrations was observed, especially after the challenge with 20 g soybeans (Figure 3). This phenomenon was also reported when urinary (19) and plasma (34) isoflavone patterns were monitored in human subjects after soy dietary intervention or in animals after exposure to various flavonoids (35, 36). The findings of a study in rats suggest that the biphasic pattern of isoflavones may be due to enterohepatic circulation (37), a process that commonly occurs after exposure to flavonoid agents (38, 39). Enterohepatic circulation may also explain the biphasic pattern observed in human milk. Using mass spectrometric techniques, we detected glycitein and the isoflavone metabolites equol and *O*-desmethylangolensin in urine but not in breast milk (23). This finding suggests either that the quantities of these agents in breast milk are below detection limits or that the agents were not secreted into human milk when the woman who participated in the study consumed soybeans. The inability to detect glycitein in breast milk is most likely due to low dietary glycitein exposure resulting from the low concentration in soybeans (5–10% of total isoflavones) (4, 6, 9, 21).

Isoflavone concentrations in 4 different soy-based infant formulas varied by a factor of 2 (Table 1), in part because of the different amount of soy protein isolate in these formulas. More important, however, considering the influence of environmental conditions and genetic dispositions on the isoflavone content of soybeans (7, 9, 40), this variability was probably due to the different sources of soybeans used in these formulas. As determined by our analyses, conjugation patterns of soy isoflavones in infant formulas were not significantly different from those in traditional soyfoods (21), with malonates (32–43%) and glucosides (37–52%) dominating over acetates (6–7%) and aglycones (9–13%). As reported previously (10), soy-based infant formulas contain appreciable amounts of isoflavones. Our analyses of 4 commonly available soy-based infant formulas revealed average total concentrations of daidzein of 77 ± 25 μg/g, of glycitein of 18 ± 5 μg/g, and of genistein of 122 ± 35 μg/g. Considering that an average infant consumes 1 L liquid food and weighs 4.5 kg, by preparing the soy-based formula according to the directions on the label (8.7 g powder per 60 mL water), which we discov-

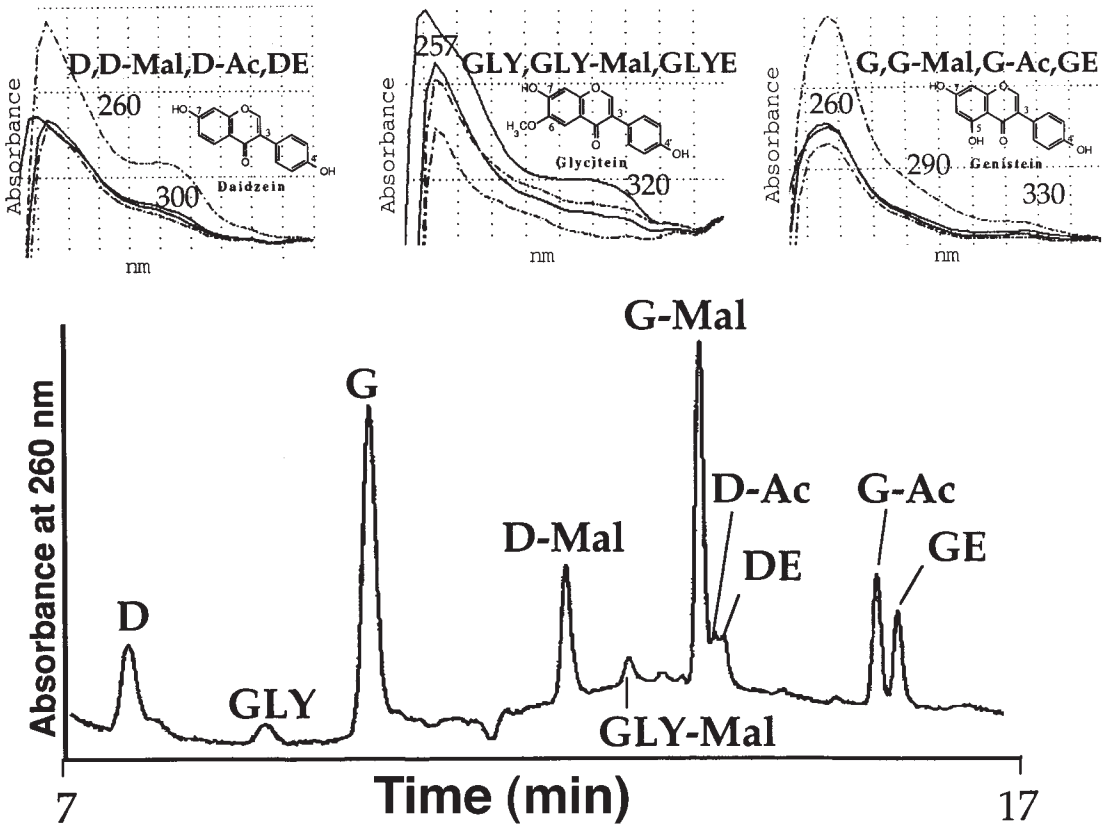

FIGURE 4. High-performance liquid chromatogram and diode-array ultraviolet scans of daidzein, glycitein, and genistein and their malonylglucoside, acetylglucoside, and glucoside conjugates obtained from a soy-based infant formula extract. D, daidzein-7-*O*-glucoside (daidzin); GLY, glycitein-7-*O*-glucoside (glycitin); G, genistein-7-*O*-glucoside (genistin); D-MAL, daidzein-7-*O*-malonyl glucoside; GLY-Mal, glycitein-7-*O*-malonyl glucoside; G-Mal, genistein-7-*O*-malonyl glucoside; D-Ac, daidzein-7-*O*-acetyl glucoside; G-Ac, genistein-7-*O*-acetyl glucoside; DE, daidzein; GE, genistein; GLYE, glycitein.

ered to contain on average 0.21 mg isoflavones/g, the daily average intake of isoflavones is ≈7 mg/kg. After adjustment for body weight, this intake is 4–6 times greater than that of an adult consuming soyfoods regularly (30 g soy protein/d). Whether this relatively high exposure results in beneficial or adverse effects in infants remains to be determined. Except for allergies (41) and reports of goiter disease (42), however, acute toxic effects or chronic adverse effects in infants fed soy-based formulas have not been observed.

Although the amount of aglycones in soy-based formulas (9–13%) is relatively low compared with that of isoflavone conjugates, the presence of aglycones is considerable in light of the effect of aglycones on the bioavailability of isoflavones in infants. In contrast with free aglycones, which are ready to be absorbed after ingestion, acylated and nonacylated isoflavone glucosides may not be absorbed as efficiently by young infants because the intestinal flora is not completely developed to perform β-glucosidic cleavage (10, 32). The impaired ability for absorption may lead to decreased systemic isoflavone concentrations. In contrast, isoflavones from breast milk may be readily absorbed because of their presence as glucuronide and sulfate conjugates that can be hydrolyzed by endogenous enzymes. Studies of the bioavailability of soy isoflavones in infants are under way as part of efforts to further explore the potential effects of these compounds in an early period of life.

In summary, our results show that the method of quantitating isoflavones by using HPLC and diode-array ultraviolet scanning is suitable for use in future epidemiologic and clinical trials to assess the role of soy or isoflavonoids in preventing chronic disease. After a healthy woman was given a moderate challenge with soy, we observed mean total isoflavone concentrations of 2.0 μmol/L in plasma, 0.2 μmol/L in breast milk, and 3.0 μmol/h in urine.

Although direct evidence of the protective effects of genistein and daidzein on cancer is derived exclusively from in vitro and recent animal studies (1, 43, 44), our findings in human milk raise an important hypothesis. In addition to protecting mothers against breast and ovarian cancer, breast-feeding is known to be beneficial to infants by contributing to the prevention of infections, diabetes mellitus, multiple sclerosis (45), and sudden infant death syndrome (46) and by inducing better intellectual development (47). Our results add another significant item to the growing list of benefits of breast-feeding. Cancer incidence and severity are significantly reduced when newborn or prepubertal animals are treated with only 3 doses of genistein (1, 44). The data presented here suggest that breast-feeding by a mother who consumes soyfoods may contribute to cancer prevention in infants because of exposure to daidzein and genistein, which are potential anticancer agents (2, 43). Although isoflavone doses used in animal models are higher than those expected after soy exposure, a cancer-protective effect of isoflavones may take place at an early and most critical developmental period and

TABLE 1
Isoflavone concentrations in 4 different soy-based infant formulas[1]

	Prosobee	Alsoy	Gerbersoy	Isomil
Protein content (%)	17	17	17	14
Malonyldaidzin (μg/g)	29.5	23.6	27.1	19.4
Malonylglycitin (μg/g)	11.1	9.3	8.3	6.1
Malonylgenistin (μg/g)	48.5	38.7	47.2	35.4
Percentage malonylglucosides (%)	32	34	32	39
Acetyldaidzin (μg/g)	ND	ND	ND	ND
Acetylglycitin (μg/g)	ND	ND	ND	ND
Acetylgenistin (μg/g)	16.2	12.3	17.3	9.9
Percentage acetylglucosides (%)	6	7	7	6
Daidzin (μg/g)	53.1	21.3	48.5	22.7
Glycitin (μg/g)	12.9	4.7	11.0	6.7
Genistin (μg/g)	76.7	36.7	74.7	41.4
Percentage glucosides (%)	51	37	52	46
Daidzein (μg/g)	19.0	13.9	17.8	10.7
Glycitein (μg/g)	ND	ND	ND	ND
Genistein (μg/g)	14.4	7.5	9.3	2.8
Percentage aglucones (%)	12	13	10	9
Total daidzein				
(μg/g)	101.6	58.7	93.4	52.7
(%)	36.1	35.0	35.8	34.0
Total glycitein				
(μg/g)	24.0	14.0	19.3	12.8
(%)	8.5	8.3	7.4	8.3
Total genistein				
(μg/g)	155.7	95.2	148.6	89.5
(%)	55.3	56.7	56.9	57.7
Total isoflavones (μg/g)	281.4	167.9	261.3	155.1

[1] Concentrations are expressed as aglycone units determined by duplicate analysis. Mean CV for intraassay variability of analytes shown: 4.1% (range: 0.1–13.2%). Prosobee, Mead Johnson Company, Evansville, IN; Alsoy, Nestlé Company, Glendale, CA; Gerbersoy, Gerber Products Company, Fremont, MI; Isomil, Ross Products Division of Abbott Laboratories, Columbus, OH. ND, not detected.

might protect an individual throughout life without requiring high doses. These findings may provide the basis for an alternative explanation of the lower cancer rates observed in Asian populations with high soya consumption. The lower cancer rates observed in these populations may be due not to isoflavone exposure by soy consumption in adulthood or childhood (48), but rather to exposure to isoflavones shortly after birth, in a critical period of life, through the mother's milk.

We thank the participant in the study for her continuous support during the intervention trial. We are also grateful for the excellent performance of GC-MS-SIM measurements by Hans Geyer, German Sports University, Cologne, Germany.

REFERENCES

1. Lamartiniere CA, Moore J, Holland M, Barnes S. Neonatal genistein chemoprevents mammary cancer. Proc Soc Exp Biol Med 1995;208:120–3.
2. Franke AA, Custer LJ, Mordan LJ, Cooney RV, Harwood PJ, Tanaka Y. Inhibition of neoplastic transformation and bioavailability of dietary phenolic agents. In: Manthey J, Buslig B, eds. Flavonoids in the living system. New York: Plenum Press, 1998.
3. Franke AA, Custer LJ, Cerna CM, Narala KK. Quantitation of phytoestrogens in legumes by HPLC. J Agric Food Chem 1994; 42:1905–13.
4. Kudou S, Fluery Y, Welti D, et al. Malonyl isoflavone glycosides in soybean seeds (glycine max MERRILL). Agric Biol Chem 1991; 55:2227–33.
5. Wang H, Murphy PA. Isoflavone composition of American and Japanese soybeans in Iowa: effects of variety, crop year, and location. J Agric Food Chem 1994;42:1674–7.
6. Wang H, Murphy PA. Isoflavone content in commercial soybean foods. J Agric Food Chem 1994;42:1666–73.
7. Tsukamoto C, Shimada S, Igita K, et al. Factors affecting isoflavone content in soybean seeds: changes in isoflavones, saponins, and composition of fatty acids at different temperatures during seed development. J Agric Food Chem 1995;43:1184–92.
8. Coward L, Barnes NC, Setchell KDR, Barnes S. The antitumor isoflavones, genistein and daidzein, in soybean foods of American and Asian diets. J Agric Food Chem 1993;41:1961–7.
9. Barnes S, Kirk M, Coward L. Isoflavones and their conjugates in soy foods: extraction conditions and analysis by HPLC–mass spectrometry. J Agric Food Chem 1994;42:2466–74.
10. Setchell KDR, Welch MB, Lim CK. HPLC analysis of phytoestrogens in soy protein preparations with ultraviolet, electrochemical, and thermospray mass spectrometric detection. J Chromatogr 1987;386:315–23.
11. Adlercreutz H, Mazur W. Phytoestrogens and Western diseases. Ann Med 1997;29:95–120.
12. Adlercreutz H, Fotsis T, Lampe J, et al. Quantitative determination of lignans and isoflavonoids in plasma of omnivorous and vegetarian women by isotope dilution gas chromatography–mass spectrometry. Scand J Clin Lab Invest 1993;53:5–18.
13. Cassidy A, Bingham S, Setchell KD. Biological effects of a diet of soy protein rich in isoflavones on the menstrual cycle of premenopausal women. Am J Clin Nutr 1994;60:333–40.
14. Adlercreutz H, Fotsis T, Bannwart C, Wahala K, Brunow G, Hase T. Isotope dilution gas chromatographic–mass spectrometric method for the determination of lignans and isoflavonoids in human urine, including identification of genistein. Clin Chim Acta 1991;199:263–78.
15. Kelly GE, Nelson C, Waring MA, Joannou GE, Reeder AY. Metabolites of dietary (soya) isoflavones in human urine. Clin Chim Acta

1993;223:9–22.
16. Hutchins AM, Slavin JL, Lampe JW. Urinary isoflavonoid phytoestrogen and lignan excretion after consumption of fermented and unfermented soy products. J Am Diet Assoc 1995;95:545–51.
17. Kurzer MS, Lampe JW, Martini MC, Adlercreutz H. Fecal lignan and isoflavonoid excretion in premenopausal women consuming flaxseed powder. Cancer Epidemiol Biomarkers Prev 1995;4:353–8.
18. Adlercreutz H, Fotsis T, Kurzer MS, Wähälä K, Mäkelä T, Hase T. Isotope dilution gas chromatographic–mass spectrometric method for the determination of unconjugated lignans and isoflavonoids in human feces, with preliminary results in omnivorous and vegetarian women. Anal Biochem 1995;225:101–8.
19. Franke AA, Custer LJ. High-performance liquid chromatography assay of isoflavonoids and coumestrol from human urine. J Chromatogr B Biomed Appl 1994;662:47–60.
20. Xu X, Wang H-J, Murphy PA, Cook L, Hendrich S. Daidzein is a more bioavailable soymilk isoflavone than is genistein in adult women. J Nutr 1994;124:825–32.
21. Franke AA, Custer LJ, Wang W, Shi SJ. HPLC analysis of isoflavonoids and other phenolic agents from foods and from human fluids. Proc Soc Exp Biol Med 1998;217:263–73.
22. Coward L, Kirk M, Albin N, Barnes S. Analysis of plasma isoflavones by reversed-phase HPLC–multiple reaction ion monitoring–mass spectrometry. Clin Chim Acta 1996;247:121–42.
23. Franke AA, Custer LJ. Daidzein and genistein concentrations in human milk after soy consumption. Clin Chem 1996;42:955–64.
24. Geyer H, Schänzer W, Mareck-Engelcke U, Donike M. Factors influencing the steroid profile. In: Donike M, Geyer H, Gotzmann G, Mareck-Engelke U, eds. Recent advances in doping analysis. Cologne, Germany: Sport und Buch Strauss Press, 1996:95–113.
25. Wang G, Kuan SS, Francis OJ, Ware GM, Carman AS. A simplified HPLC method for the determination of phytoestrogen in soybean and its processed products. J Agric Food Chem 1990;38:185–90.
26. Lundh TJ-O, Pettersson H, Kiessling KH. Liquid chromatographic determination of the estrogens daidzein, formononetin, coumestrol, and equol in bovine blood plasma and urine. J Assoc Off Anal Chem 1988;71:938–41.
27. Gamache PH, Maher TJ, Setchell KDR, Wu TH, Acworth IN. Sensitive analysis of phytoestrogens in animal tissue by HPLC with coulometric array detection. Am J Clin Nutr 1998;68(suppl):1520S (abstr).
28. Bluck LJC, Bingham SA. Isoflavone content in breast milk and soy formulas: benefits and risks. Clin Chem 1997;43:850–1.
29. Markham KR. Techniques of flavonoid identification. New York: Academic Press, 1982.
30. Lawrence RA. Breastfeeding. 4th ed. St Louis: Mosby, 1994.
31. Hutchins AM, Lampe JW, Martini MC, Campbell DR, Slavin JL. Vegetables, fruits, and legumes: effect on urinary isoflavonoid phytoestrogen and lignan excretion. J Am Diet Assoc 1995;95:769–74.
32. Cruz MLA, Wong WW, Mimouni F, et al. Effects of infant nutrition on cholesterol synthesis rates. Pediatr Res 1994;35:135–40.
33. Baird DD, Umbach DM, Lansdell L, et al. Dietary intervention study to assess estrogenicity of dietary soy among postmenopausal women. J Clin Endocrinol Metab 1995;80:1685–90.
34. Barnes S, Sfakianos J, Coward L, Kirk M. Soy isoflavonoids and cancer prevention: underlying biochemical and pharmacological issues. In: American Institute for Cancer Research, ed. Dietary phytochemicals in cancer prevention and treatment. New York: Plenum Press, 1996:87–100.
35. Marzo A, Arrigoni Martelli E, Bruno G. Assay of hydroxyfarrerol in biological fluids. J Chromatogr 1990;535:255–61.
36. Wakui Y, Yanagisawa E, Ishibashi E, et al. Determination of baicalin and baicalein in rat plasma by high-performance liquid chromatography with electrochemical detection. J Chromatogr 1992;575:131–6.
37. Sfakianos J, Coward L, Kirk M, Barnes S. Intestinal uptake and biliary excretion of the isoflavone genistein in the rat: evidence for its enterohepatic circulation. J Nutr 1997;127:1260–8.
38. Scheline RR. Handbook of mammalian metabolism of plant compounds. Boca Raton, FL: CRC Press, 1991.
39. Griffith LA. Mammalian metabolism of flavonoids. In: Harborne JB, Mabry TJ, eds. The flavonoids: advances in research. London: Chapman & Hall, 1982:681–718.
40. Eldridge AC, Kwolek WF. Soybean isoflavones: effect of environment and variety on composition. J Agric Food Chem 1983; 31:394–6.
41. Lalles JP, Peltre G. Biochemical features of grain legume allergens in humans and animals. Nutr Rev 1996;54:101–7.
42. Shepard TH, Pyne GE, Kirschvink JF, Mclean M. Soybean goiter. N Engl J Med 1960;262:1099–103.
43. Messina M, Persky V, Setchell KDR, Barnes S. Soy intake and cancer risk: a review of in vitro and in vivo data. Nutr Cancer 1994;21: 113–31.
44. Murrill WB, Brown NM, Zhang J-X, Manzolillo PA, Barnes S, Lamartiniere CA. Prepubertal genistein exposure suppresses mammary cancer and enhances gland differentiation in rats. Carcinogenesis 1996;17:1451–7.
45. Anonymous. A warm chain for breastfeeding. Lancet 1994;344: 1239–41 (editorial).
46. Savage F. Breastfeeding and SIDS. MIDIRS Midwifery Digest 1992;4:227–6.