Research Article

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Urine Accurately Reflects Circulating Isoflavonoids and Ascertains Compliance During Soy Intervention

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Abstract

Background: Isoflavonoids (IFL) may protect against chronic diseases, including cancer. IFL exposure is traditionally measured from plasma (PL), but the reliability of urine is uncertain. We assessed whether IFL excretion in overnight urine (OU) or spot urine (SU) reliably reflects IFLs in PL and the usefulness of the three matrices to determine soy intake compliance.

Methods: In a randomized, double-blind, placebo-controlled soy intervention trial with 350 postmenopausal women, IFLs (daidzein, genistein, glycitein, equol, *O*-desmethylangolensin, dihydrodaidzein, dihydrogenistein) were analyzed by liquid chromatography/mass spectrometry in OU, SU, and PL collected at baseline and every 6 months over 2.5 years.

Results: High between-subject intraclass correlations between all three matrices (median, 0.94) and high between-subject Pearson correlations (median $r_{OU\text{-}PL}$ = 0.80; median $r_{SU\text{-}PL}$ = 0.80; median $r_{OU\text{-}SU}$ = 0.92) allowed the development of equations to predict IFL values from any of the three matrices. Equations developed from a randomly selected 87% of all available data were valid because high correlations were found on the residual 13% of data between equation-generated and measured IFL values (median $r_{OU\text{-}PL}$ = 0.86; median $r_{SU\text{-}PL}$ = 0.78; median $r_{OU\text{-}SU}$ = 0.84); median absolute IFL differences for OU-PL, SU-PL, and OU-SU were 8.8 nmol/L, 10.3 nmol/L, and 0.28 nmol/mg, respectively. All three matrices showed highly significant IFL differences between the placebo and soy intervention group at study end ($P < 0.0001$) and highly significant correlations between IFL values and counted soy doses in the intervention group.

Conclusions: OU and SU IFL excretion reflect circulating PL IFL levels in healthy postmenopausal women accurately.

Impact: Noninvasively-collected urine can be used to reliably determine systemic IFL exposure and soy intake compliance. *Cancer Epidemiol Biomarkers Prev; 19(7); 1775–83. ©2010 AACR.*

Introduction

Isoflavonoids (IFL) exposure occurs predominantly through consumption of soy products that typically contain 0.01% to 0.3% IFLs composed mainly of glycosides of genistein, daidzein, and glycitein, which are associated with the protein fraction of soy foods (1, 2). IFLs are suggested to protect against many chronic diseases, including cancer, osteoporosis, and cardiovascular disorders, as well as ameliorate menopausal symptoms (1, 2). The protective effect against breast cancer is particularly strong when soy consumption occurs at an early age (3-6). IFLs might play a role in this context because they have been found to be negatively correlated with breast (7-9) and prostate (10) cancer and can block pathways during carcinogenesis.

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Several studies have found urinary or plasma (PL) IFLs to be reliable biomarkers of soy consumption (1, 2). In biological fluids, IFLs occur >80% as glucuronide and sulfate conjugates and their concentrations change markedly over time (11). Although urinary IFLs have been reported in a limited number of studies comprised of a few participants, they seem to accurately reflect circulating PL levels when timing of urine collection is considered accurately (12-15).

Given the invasiveness of venipuncture, our goal was to conclusively assess whether IFL excretion of overnight urine (OU) or spot urine (SU) reliably reflects IFLs in PL using repeated specimen collections in a large randomized, double-blind, placebo-controlled soy intervention trial with 350 postmenopausal women. We also evaluated the usefulness of IFLs from SU and OU versus PL as compliance markers for soy consumption.

Materials and Methods

Study products

Study products consisted of 18.3-g beverage powder packets (providing 12.5 g of soy or 12.8 g of milk protein)

and 66-g bars (providing 13.6 g of soy or 14.6 g of milk protein). The soy products were made with isolated soy protein (partially hydrolyzed) that was selected and processed to maintain a high level of the naturally occurring isoflavones. The soy protein beverage powder and protein bar, respectively, contained the following isoflavone concentrations in milligram per gram soy protein, including aglycons, glycosides, and glycoside esters (expressed as aglycon equivalents; aglycons relative to total respective isoflavone based on weight in aglycon units): genistein, 3.43 and 3.28 (2.01 and 1.97; 11% for both); daidzein, 2.48 and 2.22 (1.44 and 1.33; 19% and 18%); glycitein, 0.24 and 0.17 (0.14 and 0.11; 14% for both); and total, 6.15 and 5.67 (3.59 and 3.41; 14% for both). This isoflavone profile is very similar to soy foods habitually consumed by Asians (16). The placebo products were made with milk protein isolate (powder) or a combination of calcium sodium caseinate and whey protein concentrate (bars) and contained no isoflavones. Other macronutrients, minerals, and vitamins were also kept at similar levels in all soy and milk protein products. Protein, isoflavone (soy products), and selected vitamin and mineral content was determined by analytic testing in all production lots before release to assure that the products met the target levels for macronutrients and micronutrients. Products additionally met microbiological and sensory specifications during release testing.

Study population

Eligible subjects were postmenopausal women with serum estradiol levels <20 pg/mL participating in a single-center, randomized, double-blind, placebo-controlled trial (Women's Isoflavone Soy Health, WISH). One hundred seventy-five subjects were randomized to the soy protein and 175 to the milk protein matched placebo group (Table 1). One beverage or one bar was consumed in the morning and also in the evening daily during the 30-month study period.

Subject follow-up and sample collection

Clinic visits occurred in the morning, and OU collection began the previous night. Subjects emptied their bladders immediately before retiring and recorded the time of void but did not collect the urine. Urine was collected and refrigerated throughout the night. Upon arising, the first-morning urine was collected, and the time was recorded. SU was collected at the clinic visit at the time of the blood collection. The SU was typically the next urinary void following the first-morning urine collection. All specimens, obtained during a fasting state, were immediately processed and stored at −80°C.

Follow-up clinical evaluations were conducted every month for the first 6 months following randomization and then every other month thereafter for a period of 2.5 years. At every clinic visit, data about dietary intake, product compliance, nonstudy and nutritional medications, and clinical adverse events, as well as vital signs, were ascertained. Number of consumed soy protein packs and soy bars were counted at each visit when specimens were collected. EDTA blood and urine samples were collected every 6 months for 2.5 to 3 years.

Product compliance

Number of protein powder packets and protein bars consumed was calculated at each visit by subtracting the numbers of packets and bars returned from the number distributed at the previous visit. Percent compliance at each visit was calculated as the numbers of packets and bars consumed since the last visit divided by the number that should have been consumed.

Laboratory assays

Daidzein (DE), genistein (DHGE), glycitein (GLYE), equol (EQ), dihydrodaidzein (DHDE), dihydrogenistein (DHGE), and *O*-desmethylangolensin (DMA) were analyzed from PL and urine by HPLC with isotope dilution electrospray ionization (negative mode) tandem mass spectrometry as described in detail (1, 2). Samples from each individual were run in one batch to limit variability. Between-day coefficients of variation ranged 4% to 18% for all analytes, whereas intraday variation was half or less of that. All urinary IFL concentrations were adjusted for urinary creatinine concentrations and expressed in nanomole per milligram creatinine units. Urinary creatinine concentrations were measured with a Roche-Cobas MiraPlus chemistry autoanalyzer using a kit from Randox Laboratories that is based on a kinetic modification of the Jaffé reaction with a limit of quantitation of <15 μmol/L (1.7 mg/L) and mean interassay coefficient variation (CV) of 0.8% at 187 μmol/L (21.1 mg/L). Creatinine-based urinary excretion was converted to time-based units using established conversion factors taking into account gender, body weight, and age (17-19).

Statistical analyses

All analyses used SAS 9.2 software (SAS Institute). Because of nonnormal distributions, all urine and PL IFL values were log transformed. We assumed that the distributions of the three bivariate pairs (OU-PL, SU-PL, and OU-SU) were bivariate log normal. Linear relations (reliabilities) of log-transformed OU and SU IFL values were assessed by correlating them with PL measures using between-subject (20) Pearson correlations (association between two variables) and between-subject intraclass correlations (estimate of reliability across three or more variables) using means across time for each person (21). IFL values at baseline (before randomization) and end of the study in each matrix were also compared between active soy and placebo groups using Wilcoxon rank sum tests; this nonparametric approach was chosen for these comparisons because of their more conservative nature and their standard application for reporting of clinical trial

Table 1. Baseline demographics by treatment; WISH trial

Variable	Placebo (n = 175)	Soy (n = 175)	P*
Age			
40-54	32 (18)	35 (20)	0.95
55-59	47 (27)	41 (23)	
60-64	44 (25)	44 (25)	
65-69	32 (18)	35 (20)	
≥70	20 (12)	20 (12)	
Age, y	60.9 ± 6.8	61.0 ± 7.4	0.93
Race			
White (non-Hispanic)	118 (67)	105 (60)	0.60
Black (non-Hispanic)	9 (5)	12 (7)	
Hispanic	24 (14)	31 (18)	
Asian	19 (11)	19 (11)	
Other	5 (3)	8 (5)	
Education			
≤High school	5 (3)	14 (8)	0.03
>High school	170 (97)	161 (92)	
Smoking history			
Current	5 (3)	3 (2)	0.51
Former	63 (36)	72 (41)	
Never smoked	107 (61)	100 (57)	
Years smoked			
Among current or former smokers (n)	16.7 ± 12.2	16.9 ± 11.9	0.85
Weight, lbs	153 ± 32	152 ± 30	0.82
Body mass index, kg/m^2	26.7 ± 5.4	26.5 ± 5.0	0.76
Pulse rate, beats/min	66 ± 7	66 ± 7	0.38
Systolic blood pressure, mm Hg	119 ± 14	117 ± 14	0.45
Diastolic blood pressure, mm Hg	75 ± 9	75 ± 9	0.55
Cholesterol, mg/dL	222 ± 31	219 ± 29	0.31
Triglycerides, mg/dL	109 ± 56	112 ± 64	0.89
HDL cholesterol, mg/dL	63 ± 17	62 ± 16	0.57
LDL cholesterol, mg/dL	137 ± 30	134 ± 26	0.32

NOTE: Mean ± SD or n (%).

*t Test was used for all comparisons, except for years smoked, triglycerides, and PL isoflavone, in which Wilcoxon rank sum test was used.

data. To assess the association of IFL levels with product compliance, IFL values in each matrix were correlated with measures of percentage product compliance; these between-subject correlations were computed in each treatment group separately.

We developed equations for predicting PL from urine values using log transformed data and linear regression analysis with the PL measure regressed onto a urine measure. Development of these models used a random 87% of available data. After back transformation, these equations were of the form PL (nmol/L) = a * urine (nmol/mg)^b (Table 2), in which a is slope and b is exponent. Validity of the equations was evaluated by correlating and computing mean differences in estimated versus measured values of the residual 13% of data.

Results

Demographic characteristics of the WISH cohort are summarized in Table 1. Collections from all three matrices totaled 5,223 samples (1,677 OU, 1,763 SU, 1,783 PL) providing 1,677 OU-PL, 1,763 SU-PL, and 1,661 OU-SU and OU-SU-PL matched sets, which were used for between-subject correlation calculations after adjustment for within-person variation. Of 350 participants, 302 provided two or more samples.

Results for the overall log-transformed data showed excellent reliability between IFL measurements of the three matrices because most Pearson correlations were >0.8 (range of correlations, 0.60-0.94; Table 2). The intraclass correlations, as reliability estimates across all three

Table 2. IFL correlations and equation parameters for converting PL, OU, and SU values

	ICC*	OU vs PL			
		$r^{\dagger}$ (MAD‡)	$a^{\S}$ (95% CI)	$b^{\S}$ (95% CI)	$r_{test}^{\|}$
DE	0.95	0.83 (16.4)	9.33 (8.62-10.10)	1.08 (1.04-1.12)	0.87
GE	0.97	0.91 (27.8)	9.49 (8.94-10.08)	1.51 (1.47-1.56)	0.93
GLYE	0.86	0.60 (2.6)	4.85 (4.67-5.05)	0.97 (0.90-1.05)	0.66
EQ	0.94	0.80 (4.4)	5.97 (5.66-6.30)	1.21 (1.16-1.26)	0.86
DMA	0.95	0.83 (6.3)	5.96 (5.53-6.42)	1.31 (1.26-1.36)	0.86
DHDE	0.93	0.76 (3.2)	5.81 (5.41-6.23)	1.07 (1.01-1.12)	0.82
DHGE	0.93	0.78 (0.8)	2.6 (2.47-2.73)	1.26 (1.20-1.31)	0.79

(*Continued on the following page*)

measures (OU, SU, and PL), were even higher for all IFLs with a maximum observed for genistein (intraclass correlation, 0.97) and genistein or *O*-desmethylangolensin (intraclass correlation, 0.95) and a minimum intraclass correlation for glycitein (intraclass correlation, 0.86) using all data or a random 87-fraction thereof (Table 2). This indicates that all three matrices (OU, SU, PL) can be used to assess the comparative IFL level. In comparison with all data combined, the soy group showed very similar values, which was true also for the placebo group; however, the latter showed somewhat smaller (on average, 0.18) PL-OU and PL-SU Pearson correlations, but all relationships remained to be highly significant.

Because of these strong correlations, we determined equations for directly converting between any of the three matrices applying nanomole per milligram creatinine units for urine and nanomole per liter values for PL using the log-transformed data. The equation ($y = ax^b$) developed for the conversions used a randomly selected 87% of all available data (1,459 OU-PL, 1,536 SU-PL, 1,444 OU-SU pairs). Testing this model used the residual 13% of the data (Table 2). Overall results from the 87% data set (median intraclass correlation, 0.93; median $r_{OU\text{-}PL} = 0.78$; median $r_{SU\text{-}PL} = 0.76$) were almost unchanged relative to all data (Fig. 1). Equations showed excellent validity because correlations between equation-based and measured values using the residual 13% of data showed high correlations (median $r_{OU\text{-}PL} = 0.86$; median $r_{SU\text{-}PL} = 0.78$). All correlations were highly significant ($P < 0.00001$), and on average, median absolute IFL differences between predicted and measured values were small (OU-PL = 8.8 nmol/L, SU-PL = 10.3 nmol/L, OU-SU = 0.28 nmol/mg; Table 2).

Creatinine-based urine excretion (nanomole per milligram) was converted into a time-based unit (nanomole per hour) by adjusting for body weight and age according to established procedures (17-19). The overall results provided similar and highly significant correlations (for example for genistein $r_{OU\text{-}PL} = 0.80$ and $r_{SU\text{-}PL} = 0.72$; $P < 0.00001$).

As further tests of reliability, we stratified the data by visit and found again very similar reliability indices (daidzein and genistein showed SDs for intraclass correlations, $r_{OU\text{-}PL}$, and $r_{SU\text{-}PL}$ between 0.02 and 0.06 when each 6-month visit was evaluated; other results were similar but are not shown), suggesting that the time of observation did not have a substantial impact on these correlations.

The difference in IFL measurements between the placebo and soy intervention group at study end was highly significant ($P < 0.001$) in all three matrices, except for equol measured in OU (Table 3). Median IFLs were 5- to 9-fold higher in the intervention compared with the placebo group when determined in OU, SU, or PL, except for equol (13-19 fold higher) and glycitein (3-4 fold higher). Medians of 93% and 94% of the instructed amounts were consumed in the placebo and intervention group, respectively, according to dose counts (see Methods). After adjustment for within-person variation between-subject correlations between log IFL values and percentage of study product doses ideally consumed as determined from each specimen collection visit were again similar in all three matrices (Table 3). Correlation between IFL values and percentage of study product doses were of small to moderate magnitude and statistically significant for the intervention group but were not correlated in the placebo group.

Discussion

Because of the predominant occurrence of IFLs in biological fluids as conjugates (11), we determined the total of conjugated and unconjugated isoflavone levels in all matrices after enzymatic hydrolysis. In this sample of postmenopausal women, we found remarkably high intraclass correlations (0.86-0.97; Table 2) among isoflavones measured across the three matrices. In addition, very high Pearson *r*'s (0.60-0.91; Table 2) between urine and PL values were observed, whereas the correlations between OU and SU were as expected, even higher an

Table 2. IFL correlations and equation parameters for converting PL, OU, and SU values (Cont'd)

SU vs PL				OU vs SU			
$r^{\dagger}$ (MAD‡)	$a^{\S}$ (95% CI)	$b^{\S}$ (95% CI)	$r_{test}^{\|}$	$r^{\dagger}$ (MAD‡)	$a^{\P}$ (95% CI)	$b^{\P}$ (95% CI)	$r_{test}^{\|}$
0.83 (17.7)	11.43 (10.56-12.37)	1.15 (1.11-1.20)	0.78	0.93 (0.81)	0.97 (0.91-1.02)	0.82 (0.79-0.84)	0.84
0.89 (4.4)	12.14 (11.33-13.03)	1.54 (1.49-1.60)	0.82	0.92 (0.39)	0.93 (0.89-0.97)	0.83 (0.80-0.85)	0.81
0.61 (2.0)	6.44 (6.25-6.64)	1.11 (1.03-1.20)	0.67	0.82 (0.11)	0.65 (0.62-0.67)	0.79 (0.76-0.82)	0.70
0.79 (4.4)	5.62 (5.29-5.96)	1.16 (1.11-1.22)	0.85	0.94 (0.04)	1.02 (0.99-1.05)	0.94 (0.92-0.97)	0.93
0.81 (8.1)	6.63 (6.17-7.12)	1.25 (1.20-1.31)	0.82	0.93 (0.36)	1.12 (1.07-1.17)	0.88 (0.85-0.90)	0.90
0.80 (3.5)	5.81 (5.42-6.23)	1.12 (1.07-1.17)	0.78	0.92 (0.23)	1.03 (0.98-1.08)	0.85 (0.82-0.87)	0.85
0.79 (1.0)	2.69 (2.56-2.84)	1.24 (1.18-1.30)	0.77	0.90 (0.05)	0.92 (0.88-0.95)	0.85 (0.82-0.88)	0.77

NOTE: Based on log-transformed data comparing between subjects; except for r_{test}, all values are based on 87% of randomly selected data (1,459 OU-PL, 1,536 SU-PL, 1,444 OU-SU pairs). Units are as follows: OU and SU, nanomole per milligram creatinine, and PL, nanomole.

Abbreviations: DE, daidzein; GE, genistein; GLYE, glycitein; EQ, equol; DMA, *O*-desmethylangolensin; DHDE, dihydrodaidzein; DHGE, dihydrogenistein; 95% CI, 95% confidence interval; ICC, intraclass correlation.

*Intraclass correlation as reliability estimate across OU, SU, and PL (between subjects).

†Pearson *r*'s between OU, SU, and PL pairs (between subjects).

‡Median absolute difference (OU-PL and SU-PL in nanomole per liter; OU-SU in nanomole per milligram) of predicted versus measured IFL levels.

§Parameters are for the equation to convert urine values (nanomole per milligram) into PL levels (nanomole per liter) using 87% of available data; $PL = a * urine^{b}$.

‖r_{test} = *Pearson r*'s of residual 13% of available data between predicted PL levels obtained from converting urine values with developed equations and measured PL levels.

¶Parameters are for the equation to interconvert urine values (nanomole per milligram) using 87% of available data; $SU = a * OU^{b}$.

indication for urine values reliably reflecting circulating IFL levels. An equation developed from 87% of all data after log transformation allowed prediction of PL values from urine values (Table 2). Resulting equations shown in Table 2 ($y = ax^{b}$) proved to be valid because the residual 13% of data showed excellent correlations between equation-based and measured values (mean $r_{OU\text{-}PL} = 0.83$, mean $r_{SU\text{-}PL} = 0.78$, mean $r_{OU\text{-}SU} = 0.83$) and low absolute differences (<10.3 nmol/L). The selection of 87% and 13% of all data seemed appropriate to yield sufficient data for determining the algorithms and their evaluation, respectively. All correlations were highly significant ($P < 0.00001$) and all comparable outcome measures similar to those from the entire data set.

Genistein is more bioavailable and more concentrated in the circulation at a given urinary excretion than the other IFLs: by a factor of 3 versus daidzein, equol, and *O*-desmethylangolensin in contrast to factors of 5 versus dihydrodaidzein and 7 versus dihydrogenistein and glycitein applying the developed algorithms. This is in excellent agreement with previous pharmacokinetic findings (2), as well as previous experimental results when one individual was followed over many periods with repeated and strictly timed urine and blood collections (12).

Although SU was collected closer to the time of venipuncture, OU correlated on average, as well with PL, as SU (mean difference in correlations, 0.01). Variabilities could have been caused by an inconsistent urine collection period, which is the period of time used to collect the entire bladder content starting with an empty bladder or by varying time intervals before or after blood collection, although this seems to have played a minor role in this study because of the collection of SU never >90 to 120 minutes before or after the blood draw. These variabilities would lead to inconsistent reflections of IFL exposure relative to the PL based determination. OU was collected over a relatively consistent period (circa 7 hours) and a relatively consistent period of time between last urine collection and blood draw but did not lead to better OU-PL than SU-PL correlations. Food intake between the urine and blood collections could not have been a factor in this study because all specimens were collected consecutively and during a fasting state.

After converting creatinine-based excretion to time-based excretion, the preferred unit for urine, by adjusting for body weight and age (17-19), we did not find significant changes in our outcome measures. This could be due to the rather homogenous study population or to chance.

Because each IFL acts so differently, particularly genistein versus the other IFLs (see above; Table 2), and with daidzein being quantitatively the predominant IFL in urine, thereby masking effects of other IFLs, combining

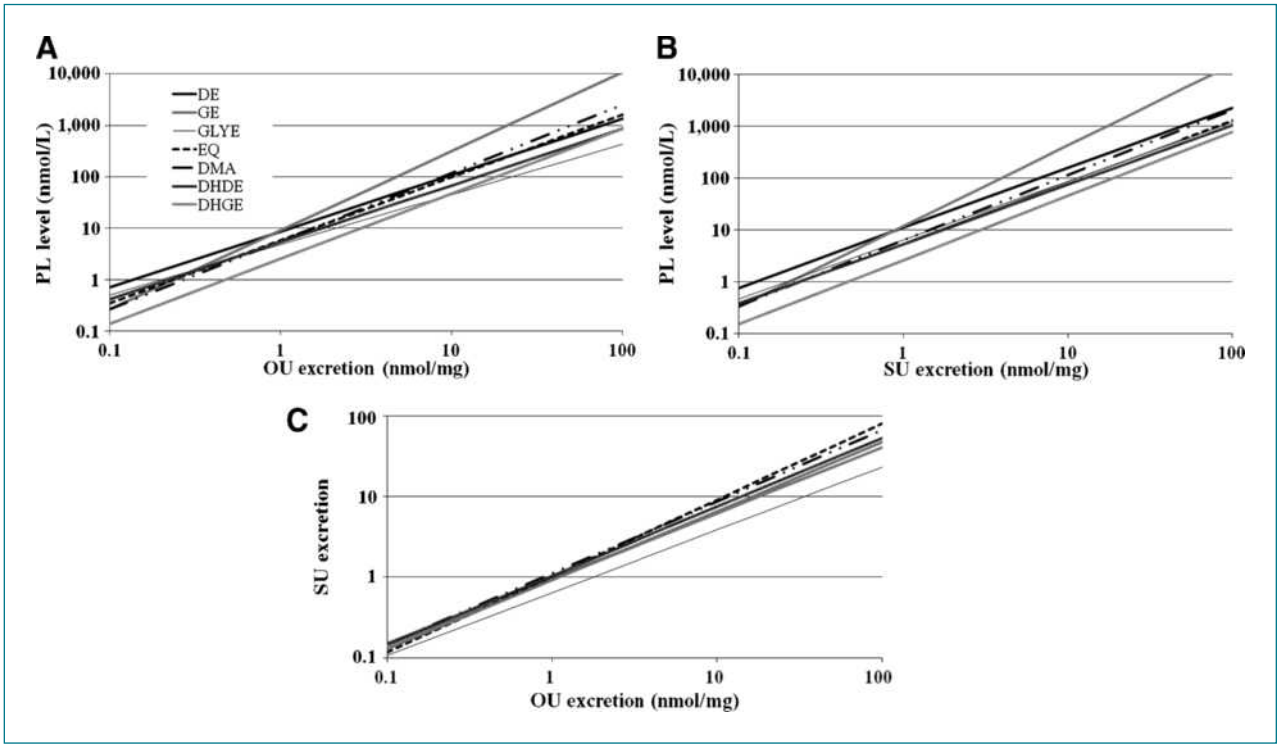

Figure 1. Relationship of urine excretion rates and plasma levels of isoflavonoids obtained from all logged data. The established conversions are based on exponential equations $y = a^{*}$ urine^{b} using all available data. A, values for *a* and *b* converting overnight urine (nmol/mg creatinine) into plasma level (nmol/L) are for DE 8.67 and 1.097, GE 9.10 and 1.540, GLYE 4.73 and 0.980, EQ 5.81 and 1.224, DMA 5.57 and 1.331, DHDE 5.24 and 1.108, and DHGE 2.55 and 1.268, respectively. B, values for *a* and *b* converting spot urine (nmol/mg creatinine) into plasma level (nmol/L) are for DE 10.86 and 1.158, GE 11.74 and 1.557, GLYE 6.27 and 1.137, EQ 5.46 and 1.183, DMA 6.21 and 1.261, DHDE 5.30 and 1.148, and DHGE 2.63 and 1.236, respectively. C, values for *a* and *b* converting overnight urine (nmol/mg creatinine) into spot urine (nmol/mg creatinine) are for DE 0.98 and 0.810, GE 0.94 and 0.819, GLYE 0.64 and 0.780, EQ 1.02 and 0.947, DMA 1.12 and 0.887, DHDE 1.04 and 0.852, and DHGE 0.92 and 0.853, respectively.

all individual IFLs for a total IFL value is relatively meaningless and was omitted here.

Numerous previous studies have investigated the association between urine and PL IFLs, but most of these studies had a limited number subjects or repeat collections and often with little time control. It is important to keep in mind that specimen timing needs to be kept consistent to obtain valid results because the urine collection period can change urinary values distinctly. Assuming an IFL elimination half-life of 8 hours (reviewed in ref. 2), the urinary excretion rate (nanomole per hour) decreases by ~4% per hour. Accordingly, excretion rates of urine collected over longer urine collection periods will be markedly smaller versus shorter urine collection periods. Because SU is lacking the urine collection period information, considerable variability is introduced. Similarly, the period between completion of urine collection and blood draw introduces and error of the same magnitude. Not surprisingly, strong PL-urine associations were therefore observed only in those previous studies that considered timing accurately. A soy intervention among 12 female and 2 male subjects with time-controlled specimen collections reported a PL 24-hour urine correlation of $r_{GE} = 0.99$ (13), similar to $r_{GE} = 0.91$ from our strictly time-controlled, multi–time point intervention with one male subject (12) or our intervention among 8 male and 4 female health professionals ($r = 0.93$; $P < 0.001$) using area under curve values that integrate over the time domain always connected with urine collections (19). We also found $r_{\text{Total IFLs}} = 0.95$ ($P < 0.001$) between PL and SU in an intervention with one female participant whose nine blood and SU collections were consistently timed (22). Again, excellent correlations between PL and urine IFL values were reported when timing was considered appropriately (23). In contrast, two Japanese cross-sectional studies ($n = 90$ men and women; $n = 106$ women) with only one time point showed weak (albeit significant) correlations ranging from 0.22 to 0.45 for daidzein and from 0.32 to 0.50 for genistein (24, 25). These lower correlations were likely due to PL and urine being collected at widely varying points in time (24), the ambiguous timing (absolute time of day and duration) of urine collections, and interval between urine and PL collections (25). A British case-control study examining the relationship between serum and SU adjusted for urinary creatinine concentration reported significant correlations

(P = <0.001), with coefficients ranging from 0.63 for glycitein to 0.88 for daidzein (23). Interestingly, these correlations were based on single untimed samples from 284 subjects with low IFL exposure (3% of the population consumed soy foods, and the average daily dietary intake for all subjects was 437 μg). In 66 subjects from the National Health and Nutrition Survey III study (1988-1994), highly significant correlations of phytoestrogen levels in urine and serum samples collected at the same time were observed for daidzein (r = 0.72), genistein (r = 0.79), and *O*-desmethylangolensin (r = 0.41), although urine concentrations were not adjusted for volume using creatinine or other means (26). However, it is important to note that these urine-PL correlations do not apply to renal failure patients whose main IFL excretion pathway through the kidney is partially or entirely blocked (27, 28).

We found OU, SU, and PL all to be equally suitable as a compliance marker matrix in this large soy intervention trial. All IFLs measured in the three matrices showed very similar quantitative differences: a 3- to 19-fold difference between placebo and soy group was found at the end of the study, and significant correlations between IFL values and percent compliance to the ideal soy doses were found in the soy but, as expected, not in the placebo group (Table 3). All differences in IFL measurements at

Table 3. IFLs in PL and urine in the placebo and intervention groups and correlations with compliance to study product

	Isoflavone values						Isoflavone values vs study product doses*			
	Placebo		Soy		P†		Placebo		Soy	
	Baseline	End	Baseline	End	Baseline	End	*r*	*P*	*r*	*P*
PL‡										
DE	62 ± 131	79 ± 249	52 ± 113	400 ± 510	0.78	<0.0001	0.11	0.21	0.40	<0.0001
GE	63 ± 161	64 ± 151	53 ± 103	517 ± 577	0.11	<0.0001	0.04	0.62	0.44	<0.0001
GLYE	5.2 ± 12	8.4 ± 32	5.8 ± 11	16 ± 29	0.93	<0.0001	0.15	0.07	0.37	<0.0001
EQ	13 ± 55	25 ± 72	9.8 ± 24	129 ± 232	0.25	0.0008	0.03	0.70	0.17	0.03
DMA	22 ± 59	31 ± 64	23 ± 70	214 ± 283	0.70	<0.0001	0.13	0.11	0.31	0.0001
DHDE	23 ± 75	38 ± 184	18 ± 43	120 ± 224	0.76	<0.0001	0.10	0.23	0.30	0.0001
DHGE	6.8 ± 30	14 ± 100	5.8 ± 16	27 ± 88	0.71	<0.0001	0.08	0.31	0.21	0.009
OU§										
DE	4.2 ± 9.6	3.8 ± 9.2	3.7 ± 7.7	25 ± 25	0.45	<0.0001	−0.03	0.74	0.46	<0.0001
GE	1.6 ± 5.7	1.5 ± 5.2	1.4 ± 3.0	13 ± 14	0.22	<0.0001	0.01	0.94	0.47	<0.0001
GLYE	0.6 ± 1.5	0.5 ± 1.2	0.5 ± 1.0	2.3 ± 2.6	0.34	<0.0001	−0.04	0.64	0.45	<0.0001
EQ	0.9 ± 5.1	1.4 ± 6.0	0.5 ± 1.7	9.6 ± 18	0.77	0.21	−0.12	0.13	0.16	0.05
DMA	1.4 ± 3.0	1.1 ± 3.0	1.4 ± 3.4	12 ± 13	0.57	<0.0001	0.00	0.96	0.34	<0.0001
DHDE	2.3 ± 6.9	1.7 ± 4.9	1.8 ± 4.0	11 ± 14	0.69	<0.0001	0.00	0.97	0.32	0.0001
DHGE	0.9 ± 4.6	0.6 ± 2.4	0.8 ± 2.8	3.9 ± 9.3	0.10	<0.0001	0.02	0.81	0.23	0.004
SU‖										
DE	3.3 ± 9.4	5 ± 15	3.0 ± 6.7	16 ± 20	0.36	<0.0001	−0.10	0.22	0.47	<0.0001
GE	1.3 ± 3.8	2.7 ± 13	1.2 ± 3.0	8.0 ± 9.7	0.23	<0.0001	−0.06	0.45	0.45	<0.0001
GLYE	0.4 ± 1.3	0.4 ± 1.0	0.4 ± 1.5	1.2 ± 1.6	0.51	<0.0001	−0.05	0.52	0.40	<0.0001
EQ	0.6 ± 3.9	1.3 ± 6.3	0.6 ± 2.4	10 ± 21	0.25	0.02	−0.11	0.19	0.17	0.03
DMA	1.5 ± 3.8	2.3 ± 6.9	1.7 ± 4.0	11 ± 12	0.61	<0.0001	−0.03	0.69	0.35	<0.0001
DHDE	1.8 ± 4.9	3.2 ± 13	2.1 ± 4.8	9.7 ± 14	0.40	<0.0001	−0.05	0.52	0.35	<0.0001
DHGE	0.6 ± 2.2	3.2 ± 22	0.9 ± 3.1	3.6 ± 12	0.10	0.001	−0.06	0.50	0.24	0.002

*Comparing interindividually logged isoflavone values with percentage of ideally consumed soy protein doses (bars or protein packages) determined at each specimen collection visit; *r, Pearson* correlations*; P, t* test value; 653 PL, 618 OU, and 643 SU data pairs in the placebo group from 146 subjects; 721 PL, 673 OU, and 714 SU data pairs in the soy group from 154 subjects.

†Wilcoxon rank sum test comparing placebo to soy group.

‡PL mean ± SD in nanomole per liter; baseline n = 175 placebo + 175 soy subjects; study end n = 136 placebo + 143 soy subjects.

§OU mean ± SD in nanomole per milligram creatinine; baseline n = 172 placebo + 170 soy subjects; study end n = 120 placebo + 126 soy subjects.

‖SU mean ± SD in nanomole per milligram creatinine; baseline n = 174 placebo, 175 active; study end n = 135 placebo, 143 soy subjects.

the end of the study were highly significant in all matrices except equol in OU ($P = 0.05$) despite a 19-fold higher amount found in the soy versus placebo group. The lack of significance for this difference might be due to the variable equol production interindividually and intraindividually, leading to large SDs resulting in large P values.

Strengths of this study are foremost the very large number of participants, the long period of the study, the frequent sample collection from the same individuals, the state-of-the-art methodology to measure IFLs, and the ability to examine the relationship of PL, OU, and SU because all specimens were collected at the same time. However, this study was limited by observations restricted to postmenopausal women. This precludes generalization to other age groups and to men. On the other hand, this led to a very homogenous cohort avoiding many confounders.

Our findings indicate that IFLs in urine reflect circulating IFL levels. Although we investigated in our study exclusively postmenopausal women, we believe that the presented PL-urine relationships holds true also for other gender and age groups after adjustment of creatinine concentrations (19) because of the good agreement of our results with previous studies that included men and younger participants. Although the determination of blood levels is required to obtain detailed pharmacokinetic parameters, urine is superior to PL for compliance measurement purposes during soy intervention studies because of easier collection and handling than PL at equal or superior functionality. Benefits of using urine include its noninvasiveness (particularly important for research in children) compared with venipuncture, as well as the ability to collect a concentrated matrix in large amounts leading to low quantitation limits. Urine can be self-obtained by participants without medical supervision, in privacy without the expense of a trained phlebotomist, and with minimal time, effort, and biological hazard for participants and study staff. Most importantly, urine can be accumulated over many hours (even days), reflecting exposures over much longer periods compared with data from blood, which only reflects one given point in time per collection or requires repeated invasive venipunctures. In addition, the accuracy of urine collection can be examined by comparing the measured urine creatinine amount with established body-weight dependent data for each gender and age group (19). Based on these benefits and our outcome results, we believe that urine, including SU, because of the logistic ease and overall effortlessness of collection, is a superior alternative to blood, especially as a matrix for compliance testing in soy intervention studies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

1. Franke AA, Halm BM, Kakazu K, Li X. Metabolism, bioavailability, and analysis of dietary isoflavones. In: Fraga C, editor. Plant phenolics and human health: biochemistry, nutrition, and pharmacology. Wiley & Sons; 2009.
2. Franke AA, Halm BM, Kakazu K, Li X, Custer L. Phytoestrogenic isoflavonoids in epidemiologic and clinical research. Drug Testing and Analysis 2009;1:14–21.
3. Thanos J, Cotterchio M, Boucher BA, Kreiger N, Thompson LU. Adolescent dietary phytoestrogen intake and breast cancer risk (Canada). Cancer Causes Control 2006;17:1253–61.
4. Korde LA, Wu AH, Fears T, et al. Childhood soy intake and breast cancer risk in Asian American women. Cancer Epidemiol Biomarkers Prev 2009;18:1050–9.
5. Shu XO, Jin F, Dai Q, et al. Soyfood intake during adolescence and subsequent risk of breast cancer among Chinese women. Cancer Epidemiol Biomarkers Prev 2001;10:483–8.
6. Wu AH, Wan P, Hankin J, Tseng CC, Yu MC, Pike MC. Adolescent and adult soy intake and risk of breast cancer in Asian-Americans. Carcinogenesis 2002;23:1491–6.
7. Ingram D, Sanders K, Kolybaba M, Lopez D. Case-control study of phyto-oestrogens and breast cancer. Lancet 1997;350:990–7.
8. Zheng W, Dai Q, Custer LJ, et al. Urinary excretion of isoflavones and the risk of breast cancer. Cancer Epidemiol Biomarkers Prev 1999;8:35–40.
9. Dai Q, Franke AA, Jin F, et al. Urinary excretion of phytoestrogens and risk of breast cancer among Chinese women in Shanghai. Cancer Epidemiol Biomarkers Prev 2002;11:815–21.
10. Park SY, Wilkens LR, Franke AA, et al. Urinary phytoestrogen excretion and prostate cancer risk: a nested case-control study in the multiethnic cohort. Br J Cancer 2009;101:185–91.
11. Gu L, House SE, Prior RL, et al. Metabolic phenotype of isoflavones differ among female rats, pigs, monkeys, and women. J Nutr 2006;136:1215–21.
12. Franke AA, Custer LJ, Hundahl SA. Determinants for urinary and plasma isoflavones in humans after soy intake. Nutr Cancer 2004;50:141–54.
13. Ritchie MR, Morton MS, Deighton N, Blake A, Cummings JH. Plasma and urinary phyto-oestrogens as biomarkers of intake: validation by duplicate diet analysis. Br J Nutr 2004;91:447–57.
14. Franke AA, Halm BM, Custer LJ, Tatsumura Y, Hebshi S. Isoflavones in breastfed infants after mothers consume soy. Am J Clin Nutr 2006;84:406–13.

15. Franke AA, Halm BM, Ashburn LA. Urinary isoflavones are increased in adults, but decreased in children, consuming soy when on oral antibiotic therapy. Nutr Cancer 2008;60:627–35.
16. Franke AA, Hankin JH, Yu MC, Maskarinec G, Low SH, Custer LJ. Isoflavone levels in soy foods consumed by multiethnic populations in Singapore and Hawaii. J Agric Food Chem 1999;47:977–86.
17. Fomon SJ. Nutrition of normal infants. St. Louis: Mosby; 1993.
18. Remer T, Neubert A, Maser-Gluth C. Anthropometry-based reference values for 24-h urinary creatinine excretion during growth and their use in endocrine and nutritional research. Am J Clin Nutr 2002;75:561–9.
19. Franke AA, Halm B, Ashburn L. Isoflavones in children and adults consuming soy. Arch Biochem Biophys 2008;476:161–70.
20. Bland JM, Altman DG. Calculating correlation coefficients with repeated observations: part 2—correlation between subjects. BMJ 1995;310:633.
21. Shrout PE, Fleiss JL. Intraclass correlations: uses in assessing rater reliability. Psychol Bull 1979;86:420–8.
22. Maskarinec G, Hebshi S, Custer L, Franke AA. The relation of soy intake and isoflavone levels in nipple aspirate fluid. Eur J Cancer Prev 2008;17:67–70.
23. Grace PB, Taylor JI, Low YL, et al. Phytoestrogen concentrations in serum and spot urine as biomarkers for dietary phytoestrogen intake and their relation to breast cancer risk in European prospective investigation of cancer and nutrition—Norfolk. Cancer Epidemiol Biomarkers Prev 2004;13:698–708.
24. Yamamoto S, Sobue T, Sasaki S, et al. Validity and reproducibility of a self-administered food-frequency questionnaire to assess the isoflavone intake in a Japanese population in comparison with dietary records and blood and urine isoflavones. J Nutr 2001;131:2741–7.
25. Arai Y, Uehara M, Sato Y, et al. Comparison of isoflavones among dietary intake, plasma concentration and urinary excretion for accurate estimation of phytoestrogen intake. J Epidemiol 2000;10:127–35.
26. Valentin-Blasini L, Blount BC, Caudill SP, Needham LL. Urinary and serum concentrations of seven phytoestrogens in a human reference population subset. J Expo Anal Environ Epidemiol 2003;13:276–82.
27. Fanti P, Sawaya PB, Custer LJ, Franke AA. Serum levels and metabolic clearance of the isoflavones genistein and daidzein in hemodialysis patients. J Am Soc Nephrol 1999;40:382–9.
28. Fanti P, Stephenson TJ, Kaariainen IM, et al. Soy food intake and serum levels of soy isoflavones in Japanese, Thai and North American end-stage renal failure patients on chronic hemodialyis therapy. Nephrol Dial Transplant 2003;18:1862–8.

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