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Reliability of Serum and Urinary Isoflavone Estimates

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Abstract

Sporadic intake and short half lives of serum or urinary biomarkers may make serum and urinary isoflavones quite unreliable indicators of longer-term dietary soy intake.

In 26 Adventist Health Study-2 (AHS-2) participants we obtained two measures of fasting morning serum isoflavones, 1–2 years apart. In another 76 subjects we obtained an overnight urine sample, and six 24 hour dietary recalls over a period encompassing the time of the urine sample.

Intra-class correlations (ICC) values for serum isoflavones were 0.11 (log[daidzein]) and 0.28 (log[genistein]). Assuming that the correlation(true dietary intake, true urinary excretion)<0.90, it is shown that this implies an ICC for urinary estimates that exceeds 0.56. As expected, the previous day's soy intake, and its timing, influenced the next morning's serum levels.

These results suggest that fasting morning serum isoflavone estimates will provide a poor index of long-term soy intake, but that overnight urinary estimates perform much better.

Keywords

Isoflavones; Reliability; Intra-class correlations; Seventh-day Adventists

1. Introduction

There is presently much interest in the health effects of soy consumption and their isoflavone constituents(1). The major isoflavones in soy are genistein, daidzein and glycitein, usually occurring in a ratio of 1:1:0.1. A metabolite of daidzein, equol, that is formed by intestinal bacteria in approximately 30% of omnivores is probably also biologically active(2).

The challenges of accurately measuring dietary soy isoflavone intake in population studies makes the use of biological levels of isoflavones attractive additional measures of dietary and

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metabolic exposure. These will be imperfect but useful surrogates of dietary intake, and also may provide evidence about mechanisms that depend on physiological levels.

Consequently a number of epidemiologic studies have measured serum isoflavones and related these to risk of various chronic diseases (3–6). Some studies have been in Western populations where intake is generally very low, and such results are difficult to interpret.

While the low levels of intake may easily account for variable results, it is also likely that the short half lives (6–8 hours) of these isoflavones in the serum confuse things further. If, for instance, an early morning serum is used this will mainly be affected by intake of soy products the day before. Yet even relatively frequent users do so in an irregular fashion, so that a longer term average serum level may possibly be represented very poorly by the spot blood sample. That it is still an unbiased estimator of the longer-term early morning level does not remove the effects of the random errors about the desired longer term average serum levels, and these errors will bias estimated relative risks(7), perhaps seriously.

The present study uses a small data set to investigate the reliability of repeated measures of serum levels that is undoubtedly influenced by greatly varying intakes during the previous day.

2. Methods

This study was approved by the Loma Linda University Institutional Review Board. The procedures followed were in accordance with the ethical standards of this review board and with the Helsinki declaration of 1975 as revised in 1983.

2.1. Subject selection

We selected 26 subjects who were part of a diet-cancer cohort study (Adventist Health Study-2, AHS-2)(8), and who had already provided a blood sample, along with six 24-hour dietary recalls, 1–2 years previously as part of a representative calibration sub-study. These names came from a list that was initially a random sample of such subjects. Subjects lived in different parts of the U.S., as the parent study is a national study. All are Seventh-day Adventists, and the 55% who regularly eat soy products eat nearly as much soy as Chinese in China (9). This U.S. population tends not to use traditional Eastern soy-containing foods such as tofu, miso, and tempeh. Rather it is soymilk and a wide variety of commercial products that are often used as high-protein meat substitutes. These latter products are usually based on soy-protein isolate. About half of this population are vegetarian or tend strongly in that direction.

2.2 Data collection and chemical analyses

Blood was collected fasting in early to mid-morning and within 30 minutes was centrifuged and cells separated from serum. Samples were placed on wet ice and shipped to arrive at the central laboratory in Loma Linda, CA within 26 hours. These specimens were then aliquoted to 0.5 ml straws and immediately stored in liquid nitrogen. A second blood specimen was obtained late in 2006 avoiding the period around Thanksgiving and Christmas where dietary habits are atypical. The methods of collection, preparation, shipping and aliquoting were identical to those used with the first specimens. All 52 specimens (two from each subject) were sent to the University of Hawaii as one shipment on dry ice, where serum isoflavones were estimated.

Concentrations of daidzein (DE), genistein (GE), and equol (EQ) were measured from urine and plasma by high pressure liquid chromatography electrospray ionization mass spectrometry in negative mode (10–11). In brief, triply ¹³C labeled internal standards of DE, GE, and EQ, DMA (University of St. Andrews, UK) were added to 0.2 mL urine diluted with 0.2 mL triethylamine buffer (0.05 M, pH 7.0) and hydrolyzed with 5 μ L glucuronidase (isolated from

Escherichia coli 200U/mL) and 5 μ L sulfatase (5U/mL; both enzymes from Roche Applied Sciences, Indianapolis, IN) for 1 hour followed by repeated phase separation with diethyl ether (12). 0.3 mL plasma was treated accordingly with 0.1 mL buffer (0.5 M, pH 7.0) and hydrolyzed with 36 μ L of each enzyme overnight. The optimum enzyme amount and incubation conditions were determined previously (10–11). The combined ether fractions were dried under nitrogen and redissolved in a 1:1 mixture of acetonitrile/sodium acetate buffer (0.2 M, pH 5). 20 μ L of the urine extract were injected onto a HydroBond PS C18 (100 $\times$ 3.0 mm; 5 μ m) reversed phase column coupled to a HydroBond PS C18 (25 $\times$ 3.2 mm; 5 μ m) direct-connect guard column (MacMod Analytical Inc., Chadds Ford, PA), eluted with a linear gradient (water:methanol:acetonitrile=20:45:35 to 10:45:45) at 0.2 mL/min., and analyzed on a LCQ Surveyor-Advantage ion trap system (ThermoElectron Corpor., San Jose, CA) multiple reaction monitoring as described in detail previously (10,13–14). 25 μ L of the plasma extract were injected onto a Gemini C18 (150 $\times$ 2.0 mm; 5 μ m) reversed phase column coupled to a Gemini C18 (4.0 $\times$ 2.0 mm; 5 μ m) direct-connect guard column (Phenomenex, Torrance, CA), eluted with a linear gradient (water:methanol:acetonitrile=20:40:40 to 80:10:10) over 10 minutes at 0.2 mL/min. followed by adding post-column 2.5% aq. ammonia at 20 μ L/min as dopant, and analyzed on a TSQ Ultra tandem mass spectrometry system (ThermoElectron Corpor., San Jose, CA) by multiple reaction monitoring after electrospray ionization (negative mode) using transitions from m/z 253 to m/z 223, 131, 208 for daidzein, from m/z 256 to m/z 226, 129 for $^{13}\text{C}_3$ -daidzein, from m/z 241 to m/z 121, 119, 135 for equol, from m/z 244 to m/z 120 for $^{13}\text{C}_3$ -equol, from m/z 269 to m/z 159, 133, 132 for genistein, and from m/z 272 to m/z 214, 135 for $^{13}\text{C}_3$ -genistein. Details of this system were described previously (15). For urine and plasma analysis limits of quantitation were 10 nM and 1 nM respectively, and between-day coefficients of variation ranged 4–12% (DE), 5–18% (GE), and 3–14% (EQ) depending on the analyte concentrations.

Dietary intake of soy products during the day before the second serum draw was assessed very simply in 22 of the 26 subjects using a one page questionnaire that focused exclusively on soy-containing foods, divided to those consumed at breakfast, lunch and supper. The questionnaire took subjects only 2–3 minutes to complete. This questionnaire was not obtained at the time of the first serum, as it was not necessary for the original calibration purpose. At that time the reliability study was not planned. The six 24-hour dietary recalls were available to estimate usual soy intake.

Members of the AHS-2 calibration sub-study (N=950) (8) provided overnight urine samples and six 24 hour dietary recalls over a 10 month period. These recalls were obtained by telephone using NDS software (University of Minnesota)(16). Urine samples from a representative 76 subjects in this calibration study had previously been sent to the University of Hawaii for urine isoflavone estimation.

2.3 Statistical methods

The goal was to estimate intra-class correlation coefficients (ICC) that compare between-subject to total (between-person plus within-person) variances for serum and urinary values.

Hence $\text{ICC} = \sigma_b^2 / (\sigma_b^2 + \sigma_w^2)$. This provides immediate information about the effect of the nuisance within-person variance to produce errors in estimates of relative risk (7). Analysis of variance allowed these variance components to be identified in serum data (see Appendix). Confidence intervals were estimated using the BCa bootstrap method(17).

For urinary data, the ICC was estimated indirectly as shown in the Appendix. We did not have replicates of urines but it is possible to set bounds on the ICC with a sensitivity analysis.

Linear regression analyses were used to evaluate the relationship between the previous day's soy intake and the next day's isoflavone levels. The regressions had form $\log(\text{isoflavone} + 1) = a + b_1.\text{soy}_b + b_2.\text{soy}_l + b_3.\text{soy}_s + e$, where the previous day's soy intakes at breakfast, lunch and supper have subscripts b, l, and s. Confidence intervals are calculated by the bootstrap BCa method (17) as even after transformation the dependent variable was not normally distributed.

3. Results

Table 1 shows some demographic and dietary characteristics first for the subjects who provided two serum samples (Group1). As can be seen there was a fairly even gender split, and the average age was 65 years. Nearly 60% were vegetarian though about a quarter of these eat some fish. Soy protein intake was high, on average nearly at oriental levels, although 12 subjects were essentially non-users with average intakes <1 gram per day in the six 24 hour recalls. Serum levels of isoflavones reflected this relatively high average intake. Twelve subjects from the 22 assessed, reported eating soy-containing foods in the day before the blood draw, six at breakfast, seven at lunch, and five at dinner.

Descriptive data from Group 2, who provided urine samples, is generally very similar, except that there are fewer males ($p=0.13$) and somewhat more non-vegetarians ($p=0.43$). As neither soy intake or isoflavone excretion varied significantly by gender this difference is unlikely to bias the comparison between groups.

The adjusted intra-class correlation (95% confidence interval) for logarithm of serum isoflavones (genistein plus daidzein) is 0.201 (0.0 – 0.452); for $\log(\text{serum daidzein})$ it was 0.112 (0–0.520); for $\log(\text{serum genistein})$ it was 0.282 (0–0.68). Regression results where $\log(\text{morning serum isoflavone levels})$ are predicted by soy intake the previous day (meal by meal) are shown in Table 2. One would expect b_3 to be largest, as it reflects the effect of soy intake at supper the evening before the blood draw, and is thus closest in time. This is the case, and the smaller anticipated effects of soy intake at breakfast and lunch could not be demonstrated in this small sample. Intake the previous day explains about 25% of the variance in $\log(\text{serum isoflavone})$ values.

Our estimate of the correlation between $\log(\text{urinary genistein})$ and the mean soy protein intake (corrected for with-in person dietary reporting errors), $\text{Corr}(U, \mu_p)$, is 0.50. The same statistic for daidzein is 0.46. Thus Table 3 reports a sensitivity analysis for different proposed values of $\text{Corr}(\mu_u, \mu_p)$ (that equal or exceed 0.50). The estimated ICC for urinary isoflavones lies between 0.56 and 0.83. Given the vagaries of absorption and metabolism of isoflavones, it seems improbable that $\text{Corr}(\mu_u, \mu_p)$ exceeds 0.90, and this means that our best estimate from this data is that ICC of a urinary estimate exceeds 0.56. It should be pointed out that as the recalls and the urine were not separated by more than 6 months that the ICC for urinary values is over a shorter time period than that evaluated above for the serum.

4. Discussion

We find that the most likely estimate of the intra-class correlation (ICC) coefficient for serum isoflavone (daidzein + genistein) levels is 0.20, this being for a period of 1–2 years between repetitions. Similar estimates for daidzein and genistein separately are 0.11 and 0.28. Urinary isoflavones appear to have more favorable ICC results. The low estimated ICC values for serum isoflavones suggest that their use in this population to index soy intake is very inefficient and relatively uninformative. For instance, the ICC is the factor by which a regression coefficient with serum isoflavones as the exposure will be biased towards the null when used without adjustment. Yet several studies have used serum isoflavone levels in this way, not surprisingly with variable results.

It is of course possible, and preferable, to adjust the crude regression result by this factor. However, the adjustment in this case would be very large, which may detract from the face validity of the result. The adjusted regression coefficient will have a very large standard error unless the study population is very large. To estimate an ICC requires a reliability/calibration sub-study where subjects have at least two serum estimates separated by months to years, an uncommon feature.

Our results were gathered from a Western population, many of whom eat soy foods, but somewhat sporadically. It is possible that Eastern populations that eat soy every day would maintain more stable serum isoflavone levels with lower within-person variability. There appears to be little other published information about the reliability of serum isoflavone except that Zeleniuch-Jacquotte et al (18) have reported very similar results to ours from a New York population who consumed soy at low levels. There is also little published information about reliability of urinary isoflavones. Recently Horn-Ross et al (19) reported intra-class correlation coefficients between 0.41 to 0.55 for 24 hour urinary isoflavones over a 10 month period, once again similar to our findings.

It is of interest that in this population the great majority of the soy consumers had eaten soy the day preceding the blood draw, arguing for some consistency of intake. However, consumption was approximately equally split between breakfast, lunch and supper, timings that would affect blood levels differently the next morning, as at least suggested by our regression analyses. Thus subjects with the same long-term total soy consumption could appear quite different in a morning spot urine (or the long-term average of many such estimates) if they timed their usual consumption differently during the day. These differences, erroneously from our perspective, become part of the between-person variance if there is no information available about consumption patterns during the day.

5. Conclusions

Our data suggest that in this population an overnight urinary estimate is much to be preferred above the serum, although it is true that the ICC values for the serum isoflavones have rather wide confidence intervals in this small study. Further larger studies with repeated serum and urine values at the same intervals, would provide valuable additional evidence. It is probable that the performance characteristics of these measures of soy intake will vary between Eastern populations and Western populations who do and do not emphasize soy intake in their diets.

In general epidemiologists would be well advised, when considering an exposure that has a short half life but is unbiased, to carefully consider the ICC and decide whether the necessary adjustment to the crude regression coefficients can be accommodated.

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Appendix

- a. To estimate σ_b^2 and σ_w^2 for serum values, X_{il} , with L repetitions, $l = 1, 2, \dots, L$. Only the second in the series is linked to the previous day's dietary data. Define

$$S_T^2 = \sum_{i=1}^N (\bar{X}_i - \bar{X}_{..})^2 / (N - 1)$$

$$S_w^2 = \sum_{i=1}^N \sum_{l=1}^L (X_{il} - \bar{X}_i)^2 / N(L - 1)$$

Then

$$S_b^2 = S_T^2 - S_w^2 / L$$

- b.** To estimate ICC from the validity correlation coefficient between the variable of interest (urine estimate) and another variable measured without error (or in this case diet, corrected for within-person variation).

Let subscript u indicate urine and r indicate recalls. Then μ_u and μ_r are true values, and U and R are observed urine and recall levels, respectively, that include within-person error. R^- is the mean of recalls, and subscript c indicates that the correlation is corrected for within-person error of the recalls; ε_{wu} is within-person error of the urinary estimate.

$$E[Corr(U, \bar{R})_c] = Corr(U, \mu_r) = \frac{Cov(\mu_u + \varepsilon_{wu}, \mu_r)}{\sqrt{(\sigma_{\mu_u}^2 + \sigma_{\varepsilon_{wu}}^2) \sigma_{\mu_r}^2}}$$

$$\frac{Cov(\mu_u, \mu_r)}{\sigma_{\mu_u} \sigma_{\mu_r}} \sqrt{\frac{\sigma_{\mu_u}^2}{\sigma_{\mu_u}^2 + \sigma_{\varepsilon_{wu}}^2}} = Corr(\mu_u, \mu_r) \sqrt{ICC_u}$$

Then

$$ICC_u = [Corr(U, \mu_r) / Corr(\mu_u, \mu_r)]^2$$

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Table 1

Selected characteristics of the 28 study subjects (proportions or means[SD]).

Variable	Group 1 (N=26) [†]	Group 2 (N=76) [†]
Gender (% male)	42.9	27.0
Dietary group (%)		
Vegetarian *	42.9	32.8
Pesco/Semi-Vegetarian	14.9	10.5
Non-vegetarian	42.9	56.6
Soy protein intake (grams/day) **	4.68 (3.11)	4.05 (4.05)
Age (years)	65.0 (14.64)	65.1(14.45)
Plasma daidzein (nmoles/L)	143.61 (348.24)	-----
Plasma genistein (nmoles/L)	198.00 (543.33)	-----
Urinary daidzein (pmol/mg creatinine)	-----	3513(7936)
Urinary genistein (pmol/mg creatinine)	-----	1542(3825)

* Vegetarian includes lacto-ovo and vegan; Semi vegetarians eat meat<1/week; Pesco-vegetarians eat fish but other meats <1/month

** SD are between-person estimates calculated from repeated dietary recalls.

[†] Group 1 provided duplicate serum data, and Group 2 provided urine data.

Table 2

Linear Regressions of Log(morning serum isoflavone levels) on soy intake[†] at breakfast, lunch and supper the previous day.

Regression coefficients (SE) [*]			
Dependent variable	Breakfast	Lunch	Supper
Daidzein	0.21 (0.81)	-0.19 (0.61)	2.29 (0.83)
(95% confidence interval) [*]	(-0.98 to 2.19)	(-1.48 to 0.96)	(0.56 to 3.91)
Genistein	0.52 (0.92)	-0.13 (0.71)	2.12 (0.89)
(95% confidence interval)	(-0.73 to 2.81)	(-1.13 to 1.33)	(0.18 to 3.86)
Daidzein + Genistein	0.45 (0.93)	-0.21 (0.72)	2.37 (0.94)
(95% confidence interval)	(-1.09 to 2.60)	(-1.41 to 1.17)	(0.50 to 4.10)

^{*} SE calculated by bootstrap, and confidence intervals by the BCa method.

[†] Previous day meal's soy intake scored as 0 if no soy is consumed at that meal; 1 (if soy protein < 7 grams); 2 (if 7 ≤ soy protein ≤ 13 grams); 3 (if soy protein > 13 grams).

Table 3

Estimated intra-class correlation (ICC_u) of urinary soy isoflavones (daidzein + genistein): A sensitivity analysis *

Assumed Corr(μ_P, μ_U)	Estimated ICC_u
0.90	0.56
0.80	0.63
0.70	0.71
0.60	0.83

* This analysis assumes that $Cor(\mu_P, U) = 0.50$, as we observed for genistein. Urine values were log transformed. U = observed urine levels; μ_P and μ_U are long-term average soy protein intake and isoflavone excretions, respectively.