Published in final edited form as:

Arch Biochem Biophys. 2014 October 1; 559: 24–28. doi:10.1016/j.abb.2014.06.007.

Absorption, distribution, metabolism, and excretion of isoflavonoids after soy intake

Adrian A. Franke*, Jennifer F. Lai, and Brunhild M. Halm

University of Hawai'i Cancer Center, Honolulu, HI, United States

Abstract

Soy is the major source of dietary exposure to isoflavonoids (IFLs). Accumulating evidence supports a role for soy and IFLs in the protection against many chronic diseases including cancer. After soy intake we found a biphasic IFL appearance pattern in plasma as well as in urine that we suggest to be due to IFL absorption in the small intestine (ca. 10%) during the first 2 h after intake and IFL absorption in the large intestine (ca. 90%) 4–6 h after intake. While each IFL disappears from the circulation at different times excellent correlations between urinary and circulating IFL values were discovered and algorithms to convert urinary excretion values into circulating levels were established. We suggest the term ‘apparent bioavailability’ when using urinary data to describe IFL exposure. The IFL bioavailability was found to be influenced by gut bacteria, oral antibiotic treatment (OABX), and an individual’s age and health status. While daidzein (DE) and genistein start to be absorbed minutes after intake, equol (EQ) appears in plasma only after a minimum of 8 h following soy intake owing to the required transit time of DE to the colon where the conversion of DE to EQ takes place by intestinal microbiota. We have also shown that the apparent IFL bioavailability is higher in children than adults, higher in healthy versus non-healthy individuals, and decreased in children but increased in adults during OABX. Finally, we propose to use a urinary EQ/DE ratio of 0.018 with a DE threshold to identify EQ producers. With this cutoff definition we observed that EQ production is inconsistent over time in 5–30% of both premenopausal and postmenopausal women.

Keywords

Soy; Isoflavonoid; Bioavailability; Daidzein; Genistein; Equol

Introduction

Isoflavonoids (IFLs) are suggested to protect against many chronic diseases (including coronary heart diseases, subclinical atherosclerosis, type 2 diabetes), to decrease the risk of certain types of cancer such as breast and prostate cancers, and to improve bone health [1–3].

IFL exposure occurs mainly through the dietary intake of soy products which typically contain 0.01–0.3% total IFL and are composed mainly of the glycosides genistein (GE), daidzein (DE) and glycitein (GLYE) [4–6] (Fig. 1). Equol (EQ) is a metabolite formed by gut bacteria during digestion through the chemical reduction of DE [7,8] and is proposed to play a major role in the health benefits of soy and IFLs. Strong evidence supports a protective effect for soy intake against breast cancer in adulthood [9] and particularly when soy is consumed at an early age [10–14]. The pharmacologic effects of soy intake are thought to be due to the structural similarity of IFLs to steroidal estrogens and the potent binding of GE to the estrogen receptor beta [15,16]. We found that children have a higher IFL bioavailability compared to adults [17] and that the cardio protective effects of IFLs are independent from lipid profiles [18]; these findings strengthen the hypothesis that IFLs play an important role in the biological effects of soy intake [19]. Understanding the basis of the health benefits derived from IFLs requires a detailed knowledge on the absorption, distribution, metabolism, elimination (ADME)¹ and bioavailability of these phytoestrogens. Here, we have summarized findings from us and others regarding ADME studies on IFLs.

Use of extinction coefficient (ϵ) to determine stock solutions of standards for external calibration of analytical instruments

To assure accuracy of analyses for compounds of interest accurate calibration of analytical instruments remains of utmost importance. The determination of a standard stock solution concentration is preferably performed through absorbance readings at the maximum wavelength ($\lambda_{\max}$) using molar extinction coefficients (ϵ) after purity is assured via HPLC. The concentration of the final standard stock solution is then calculated using the $\lambda_{\max}$ reading adjusted for the purity. This method of calibration avoids inaccuracies connected with weighing (due to hygroscopy, balance miscalibration, or other causes), allows repeated checking of existing stock solutions when necessary, and assures most of all simple inter-laboratory comparability. The estimation of a standard solution's molar concentration from its measured absorbance is easy and fast performed. ϵ represents a physical constant that does not change and measures accurately how strongly a sample absorbs light at a given wavelength. However, ϵ varies depending on the solvent, temperature and of course the selected wavelength and, therefore, these variables have to be taken into account and reported if absorbance readings are used for standard concentration determinations (Table 1). A great advantages of using absorbance readings is that it serves as a reliable reference point whereby final results can be corrected if the used ϵ values should require adjustments [20].

Elucidation of the IFL biphasic absorption pattern

Metabolic investigations by us [30,31] and others [32–34] have consistently observed a biphasic IFL appearance pattern in plasma and urine of humans after consumption of soy or purified IFL preparations with peak levels occurring 1–2 h and again 4–6 h after intake (Fig.

¹Abbreviations used: ADME, absorption, distribution, metabolism, elimination; DE, daidzein; EP, equol producer; EQ, equol; GE, genistein; GLYE, glycitein; ICC, intraclass correlations; IFL, isoflavonoid; IP, inconsistent producer; MBP, mechanical bowel preparation; NP, non-equol producer; OABX, oral antibiotic treatment; OU, overnight urine; PL, plasma; SU, spot urine; UIER, urinary isoflavonoid excretion rate.

2). We elucidated this pattern in more detail *in vivo* using a simultaneous soy challenge and oral antibiotic treatment (OABX) combined with mechanical bowel preparation (MBP), the latter being the only modality to drastically reduce intestinal bacteria [35].

Our results indicated separate and preferred locations of IFL absorption during digestion. The time at which the first IFL peak occurred (1–2 h after intake) suggested that IFL uptake occurred in the proximal small intestine. OABX + MBA did not remove peak 1; therefore, we concluded that the intestinal gut bacteria could not be responsible for the hydrolysis step necessary to convert the originally present isoflavone glycosides into the bioavailable aglycones. On the other hand, puerarin (Fig. 3), referred to as a C-glycoside of daidzein but correctly a cyclite due to the lack of a glycosidic bond, was the only DE analogue that when ingested led to the absence of peak 1 (and also absence of peak 2 but appearance of a DE peak after ca. 80 h) [35].

For that reason, we hypothesized that mucosal glucosidases such as lactate-phloridzin hydrolase [36], known to hydrolyze flavonoid beta glucosides efficiently, completed the hydrolysis. After intake, gastric absorption typically occurs within 10–20 min and possibly faster in case liquids are consumed (as in most of our experiments). Since we did not observe a peak after such a short time, we assumed that gastric absorption either played no or a minor role during IFL ingestion. In contrast, passage through the small intestine usually happens within 3 h [37], the time at which the first peak occurred. Peak 2 occurred 4–6 h post intake and suggested that IFL absorption took place in the large intestine where the majority of gut bacteria reside. A further indication of this assumption was evident by the significant delay of the second peak (Fig. 2), which occurred when solids (roasted soy beans) were consumed – approximately 6–12 h after intake, the expected time frame for solid foods to arrive in the large intestine. Additionally, the absence of the second peak after OABX + MBP provided evidence that the second peak depended on the presence of gut microbiota, which are known to occur mainly in the large intestine. As mentioned earlier, the absence of the second peak was also observed when puerarin was given. Puerarin, referred to as daidzein-8-C-glucoside (see above), is a naturally occurring compound used in Chinese medicine for the prevention and treatment of cardiovascular disorders, cancer and alcoholism (reviewed in [38,39]) and has been shown to be metabolized by the human fecal flora to DE [40]. We suggest that the late occurrence of urinary daidzein after puerarin intake (ca. 80 h) is due to bacterial degradation of the cyclite moiety, which is time consuming since many different bacteria may be involved in this non-hydrolytic process to generate DE.

Correlation between plasma and urinary IFL values and establishment of algorithms for inter-converting plasma and urine values

Several studies have investigated associations between urinary and plasma IFLs. However, many of these reports suffered from low subject numbers or lack of repeat collections and often had little control and consistency on specimen timing. The latter is an especially crucial factor to consider when obtaining valid results as the period of time in which urine is collected can distinctly alter urinary values. For example, if we assume an elimination half-life for IFLs of 8 h (reviewed in [20]), the urinary IFL excretion rate (UIER, nanomole per

hour) would decrease by ca. 8% per hour. Along these lines, the UIERs of samples collected over long periods of time after IFL intake will be substantially smaller than urine samples collected during relatively shorter collection periods after IFL intake. For this reason, considerable variability is introduced when single collection samples (such as spot urine (SU)) are collected due to the lack of collection period information. Similarly, the period between completion of urine collection and blood draw introduces an error of the same magnitude.

In a previous study [41] we showed excellent reliability between IFL measurements of SU, overnight urine (OU), and plasma (PL) with Pearson correlations >0.8 (range $r = 0.60$ – 0.94). The intraclass correlations (ICC), used as reliability estimates across all three matrices, were very high – with a maximum ICC observed for GE (0.97). These strong correlations indicated that all three matrices (SU, OU, PL) could be used to assess IFL levels and so we determined equations for inter-converting values between any of the three matrices. For urine we applied nanomole per milligram creatinine units and for PL we applied nanomole per liter values using log-transformed data. The resulting equation was $y = ax^b$ where a is the slope and b the exponent. This equation used a random 87% of available data (>1600 data pairs) and showed a median ICC = 0.93; a median $r_{OU-PL} = 0.78$, and a median $r_{SU-PL} = 0.76$ with all correlations being highly significant ($P < 0.00001$). The validity of the equations was established by correlating and computing mean differences in the estimated versus measured values of the residual 13% of data; these differences were negligible (OU–PL = 8.8 nmol/L, SU–PL = 10.3 nmol/L, OU–SU = 0.28 nmol/mg).

Apparent bioavailability of IFLs

Urinary or plasma IFLs have been shown to be reliable biomarkers of soy consumption [42–45] and, when the timing of specimen collection is considered, the urinary appearance of IFLs have been shown to accurately reflect circulating levels [35,46]. In several populations we found significant linear correlations of soy or IFL intake with urinary IFLs [31,47]; these results were confirmed by others who also found these correlations using urinary IFLs [42,48], plasma IFLs [44,49] or both [43,45,50]. The correlations were highest (up to $r = 0.6$) for recent IFL intake data (food diaries) and weaker (approximately $r = 0.2$) or non-existent for long term intake measures (food frequency questionnaires), which would be expected due to the fast elimination pattern of IFLs. Results from previous work indicated that for long-term soy intake, estimates from OU provides a better index than fasting morning serum [47] and has a high reproducibility within subjects over time [51]. Therefore, urine is favored as a reliable biomarker for soy intake when measuring IFLs.

Advantages of using urine over plasma include its higher concentration, its ability to be collected in a non-invasive manner, the option to develop protocols for self-collection by participants in private without medical supervision, be collected in large amounts (particularly OU) and over many hours thereby reflecting IFL exposure over long periods of time. In particular the higher amounts per mL result in simpler analyses compared to plasma or other body fluids. We suggest using UIERs as a reliable surrogate to determine circulating IFLs and thereby assess IFL bioavailability. Also, since bioavailability is defined

based on circulating levels, we propose to use the term ‘apparent bioavailability’ when using urinary excretion data to extrapolate to systemic exposure.

IFL bioavailability in children versus adults

We found that apparent IFL bioavailability is higher in healthy children [17], healthy infants, [46], school-aged children, [52], and pre-pubertal girls [53] than in healthy adults. In one study urinary IFL concentrations were measured from infants as young as 2–16 weeks old [54]. These infants were fed soy ($n = 4$) or dairy ($n = 25$) based infant formula. Urine from infants consuming soy formula had measurable amount of both DE and GE whereas no IFL were found in urine from the infants consuming dairy, which suggests that infants as young as 2–16 weeks old are able to absorb and excrete IFL from soy-based formulas. Additionally, based on the similarity in mean urinary recoveries of DE ($38 \pm 4\%$) and GE ($13 \pm 5\%$) to that found in adults [55,56], results from that study suggested that infants are able to absorb and excrete these IFL similarly to that of adults consuming soy products. We observed that children have significantly higher urinary excretion levels of DE, GE and GLYE, and total IFL compared to adults when consuming a body weight-adjusted dose of soy [17] and that relative to their mothers, infants have lower UIERs when they are breastfed with the opposite being true for infants who consume tofu [46]. We have also observed that pre-pubertal girls (8–14 y) excrete IFLs up to 3-times more than premenopausal women [57] despite the latter population consuming 50% more IFL per day [58]. This suggests that bioavailability is higher in girls relative to premenopausal women possibly as a result of higher IFL absorption efficiency or less degradation of IFL by the gut flora [58]. Overall, these studies suggest that children have a higher systemic IFL exposure than adults at the same body weight-adjusted soy dose, which could result in children experiencing more health benefits of soy intake than adults [10,11] and possibly explain the reduced breast cancer risk when soy exposure happens at (pre)pubertal ages [10–14].

The effects of soy compared to milk-based product intake at young ages have been evaluated in several studies with concerns regarding safety. A retrospective study reported no adverse effects in women who were exposed to soy-based products during infancy [59]. Notwithstanding slightly prolonged menstrual bleedings and greater discomfort during menstruation, no differences in growth, development, reproductive functions, and pregnancy outcomes were found between women with early exposure to soy-versus cow-milk products. Studies with infants have demonstrated normal growth and development with soy formula products [60] and no differences in weight, length, and head circumference [61,62] or hormone-sensitive reproductive organ size [63] between infants fed soy-versus cow-milk products.

Equol production is variable in pre- and post-menopausal women

EQ was shown to be produced in considerable amounts by approximately 30–35% of omnivorous and 60% of vegetarians [64–68] and Asians [69]. However, it is widely debated as to whether the ability to produce EQ is stable over time and results in favorable health outcomes [52,70,71].

Considerable attention has centered on EQ production strictly on the basis of whether individuals are able to produce EQ (EQ producer, EP) or not produce EQ (non EQ producer, NP). However, little interest has focused on individuals capable of changing EP status over time (i.e. inconsistent producers, IP). Consequently, EQ production has been viewed as a relatively stable phenomenon [11,72–74]. Several reports have even indicated that individuals are unable to change EP phenotypes [74–77] while others have reported EQ production to be stable ‘in most individuals over 1–3 years’ [73], or only ‘over 1 year’ [78,79], or in ‘85% of individuals over 1–5 years’ [72].

Findings from us and others [73,80–82], anecdotally in Halm et al. [83] and undiscussed in Lu et al. [84] seem to challenge this view. In a previous study with 350 post-menopausal women [81] we observed a relatively high proportion of individuals (up to 35%) who changed EQ status (either from NP to EP (IP+); or EP to NP (IP–)) over the course of 2.5 years. These EP status changes were unexpectedly not associated with antibiotic use (which can disrupt the ecological balance of resident gut bacterial [85] and thereby possibly decrease EQ production) except for selected groups when antibiotic use correlated with IP+. These changes were comparable with those observed in our subsequent study in premenopausal women [80] which used the same EP, NP, and IP cut-off methods to observe 6% and 11% of IP during the low-soy and high-soy diet periods, respectively.

The reason for EP status changes in our study with postmenopausal women is uncertain as unexpectedly more IP+ were observed in the placebo group exposed to soy foods from habitual diet compared to the treatment group exposed to isolated soy protein [81]. We speculate that the discrepancies in that and other studies may have been due to differences in cut-off levels defining an EP.

In many reports, the definition of an EP has been somewhat arbitrary because it has been based mostly on the limit of quantitation of the analytical method. Consequently, previous cut-off levels used to define an EP have varied markedly across studies and especially in urine, a commonly used and preferred matrix due to the reasons mentioned earlier. Previous EP cut-off points in urine using EQ concentrations of >0.182 nmol/ml [86], >0.68 nmol/ml [87], >1 mg/ml [75], >900 nmol/24 h [76], DE thresholds >10 nmol/mg creatinine [78], have made inter-study comparisons of EP difficult, if not impossible. These comparisons are further complicated when subjects are assigned arbitrary thresholds such as ‘good’ or ‘high’ EQ excretor or a ‘poor/low’ or ‘non’ EQ excretor as some individuals may inevitably fall into an intermediary position. Using various EP cut-offs have been especially problematic when defining EP status changes (e.g. a NP becoming an EP or vice versa), as EP cut-offs set very low (e.g. using the limits of the detection system) has probably and erroneously lead to a greater prevalence of IP than when EQ cut-offs are set higher.

Due to these inconsistencies, we found it necessary to standardize EP classification using urine as the matrix. Urine was deemed a more suitable matrix than blood due to its ability to integrate changes over time and, thus, obtain an accurate measure of overall IFL exposure. We found that using blood for EP classification was less informative than urine due to the fast elimination of EQ and differences in elimination half-lives between DE and EQ, as discussed previously [81].

The EP definition was fundamentally improved by using a log-transformed EQ:DE ratio of -1.75 (absolute ratio of 0.018) [88]. Applying this product:precursor ratio is the accepted procedure to evaluate enzyme activity or to classify phenotypes and is of major interest in evaluating whether the benefits of IFL exposure are due to the biological causes leading to EQ from DE [89] (reviewed in [70,90]). A product:precursor ratio is particularly superior when applied to urine as its concentrations can be used without converting to an excretion value. We suggested to further improve the EQ:DE ratio based EP definition by setting a minimal DE threshold of 2 nmol/mg creatinine (referred to as EQ:DE 2) [80,81]. In these 2 studies [80,81], we initially employed 4 cut-off methods that were derived from suggestions in the literature. Two of the cut-offs were *relative* and incorporated EQ:DE ratios of 0.018 with 2 and 5 nmol/mg creatinine (EQ:DE 2 and EQ:DE 5), and the other two cut-offs were *absolute* and consisted of EQ concentrations of either 0.5 or 1.0 nmol (EQ 0.5 and EQ 1.0); the latter two cut-off methods did not have a DE threshold. Data from these studies suggested that the addition of the DE threshold was necessary when evaluating EP status as doing so assured that only subjects with sufficient substrate exposure would be appropriately categorized. The addition of the DE threshold also avoided noise in the EQ:DE ratio caused by the high variability at low EQ and DE levels mainly due to analytical inaccuracies. At higher analyte levels (as assured by incorporating a DE threshold), variability-related errors are several-fold smaller and, consequently, the EQ:DE ratios are much more consistent. Finally, the EQ:DE ratio with a DE threshold assured sufficient substrate exposure, which minimizes misclassifications of a NP for an EP when an individual is inadvertently exposed to EQ e.g. through the diet (dairy products, etc.,) or by supplementation [81].

Conclusion

Interest in soy and IFLs continues to persist as a result of their cancer and other disease-preventing effects. This is especially true when exposure occurs at a young age. Our collection of studies on the ADMEs of IFLs have revealed that: (1) the use of molar extinction coefficients in determining stock standard solution concentrations serves as an accurate and efficient technique for external calibration of analytical instruments; (2) the biphasic absorption pattern of IFLs is due to absorption in the small intestine (1–2 h after IFL intake) where hydrolysis of IFL glycosides to the bioavailable aglycons occurs via phloridzin-lactate-hydrolase and in the large intestine (4–6 h post intake) where hydrolysis of IFL glycosides occurs via gut bacteria; (3) plasma and urinary IFL values are highly correlated and can be converted using our developed algorithms; (4) EQ status classification is most consistent when urine is used as matrix and when as cut-off an EQ/DE ratio of 0.018 with a DE threshold of 2 nmol/mg is chosen; (5) using this cut-off, EQ production is variable in ca. 10–30% of pre- and post-menopausal women; and (6) IFL bioavailability is greater in children than adults possibly explaining the benefits of soy exposure during early periods of life.

Acknowledgments

Source of funding

None.

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Fig. 1. Molecular structure of unmetabolized (A) and metabolized (B) isoflavonoids and their conjugates. Molecules shown in A occur in foods and in B in body fluids as metabolites after formation in the gut by intestinal bacteria (EQ derives from DE). In body fluids only EQ occurs predominantly as aglucon while the other aglucons occur predominantly glucuronated and sulfated.

Fig. 2. Typical biphasic IFL appearance pattern in urine and plasma after soy intake. 1 = first peak 1–2 h after soy intake; 2 = second peak 6–8 h after soy intake. From [35] with permission.

Fig. 3.
Molecular structure of puerarin. The cyclite moiety is connected non-glycosidically by a carbon-carbon bond at C-8 of the IFL moiety (daidzein).

Table 1

Molar extinction coefficients (ε) of isoflavonoids and common flavonoids as a function of wavelength and solvent.

Compound	Solvent	λ (nm)	ε (L/mol/cm)	References
Daidzein	96% EtOH	250	20893	[21]
	100% EtOH	262	24739	[22]
	n.a.	249	31563	[23]
	MeOH	248–249	27100–27200	<i>a</i> *
	80% MeOH	250	27542	[24]
Genistein	96% EtOH	263	37154	[21]
	100% EtOH	262	35842	[22]
	n.a.	263	35323	[23]
	EtOH	263	35000–38400	<i>a</i> *
	96% EtOH	262.5	37291	<i>b</i> *
	n.a.	261	33113	[25]
Glycitein	85% MeOH	261	24435	[26]
	Alcohol	256	22387	
Daidzin	n.a.	256	25388	[23]
	n.a.	249	26830	[23]
Genistin	n.a.	n.a.	23749	<i>c</i> *
	MeOH/Water	250	28561	<i>d</i> *
	MeOH/Water	262.5	39129	<i>d</i> *
Glycitin	n.a.	262.5	35323	[27]
	n.a.	262	39000–40000	<i>a</i> *
	85% aq. EtOH	262		
Acetylgenistin	n.a.	259	26713	[23]
Acetyldaidzin	n.a.	256	29007	[23]
Acetylglycitin	n.a.	261	38946	[23]
Malonyldaidzin	n.a.	260	29595	[23]
Malonylgenistin	n.a.	258	26830	[23]
Malonylglycitin	n.a.	260	29895	[23]
Dihydrodaidzen	n.a.	260	26313	[23]
Dihydrogenistin	n.a.	277	13600	<i>e</i> *
DMA	n.a.	290	18300	<i>e</i> *
Equol	n.a.	280	12023	<i>e</i> *
Formononetin	n.a.	281	6761	<i>e</i> *
Biochanin-A	n.a.	256	29512	[21]
Kaempferol	n.a.	263	27542	[21]
Myricetin	96% EtOH	273	15849	[28]
Quercetin	MeOH	378	19498	[29]
	96% EtOH	373	20892	[28]

λ (nm) = wavelength of absorbance maximum in nanometer; EtOH = ethanol; MeOH = methanol; ε = molar extinction coefficient in L/mol/cm; DMA = O-desmethyldaidzein; formononetin = 4'-O-methyl daidzein; Biochanin-A = 4'-O-methyl genistein; n.a. = not available.

* Private communications.

^a Sigma–Aldrich.

^b Merck Index 10th ed.

^c Nigel Botting 2004 (University of St Andrews, UK).

^d Purina/Nestle 2006.

^e Kristiina Wahala 1998 (University of Helsinki, Finland).

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