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ABSORPTION AND URINARY EXCRETION OF DAIDZEIN AND GENISTEIN FROM SOY PROTEIN IN HUMANS

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ABSORPTION AND URINARY EXCRETION OF DAIDZEIN AND GENISTEIN FROM SOY PROTEIN IN HUMANS

BY

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ABSTRACT

ABSORPTION AND URINARY EXCRETION OF DAIDZEIN AND GENISTEIN FROM SOY PROTEIN IN HUMANS

By

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Four male and four female subjects consumed 18.8, 37.5, 50.3 and 75.0 g of soy protein (0.88 mg daidzein and 1.05 mg genistein/g protein) in a split-plot, repeated measures design. The 24 hour urinary excretion total of daidzein and genistein was 17.57 ± 5.48% and 4.36 ± 3.96% respectively of total aglycones consumed for all doses. Daidzein and genistein excretion in urine increased linearly and proportionally to isoflavone intake ($P < 0.05$). First detection in urine occurred in 1.6 hours and 90% of the 24 hour excretion total was achieved within 16 hours of soy consumption. In a separate study subjects were fed soy or no soy and 128 urine samples from 24 subjects were analyzed for daidzein and genistein to monitor compliance. Daidzein was falsely detected in some samples therefore genistein was utilized as a primary indicator of soy consumption. Genistein was putatively identified in 96% (94:98) of the samples from subjects consuming soy and 7% (2:28) of the samples from subjects consuming casein.

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CHAPTER 1.

INTRODUCTION

A number of Asian populations have lower incidences of breast and colon cancers and a high consumption of soy based products in their diets when compared to the U.S. and some European countries that have high fat and very low soy consumption (Adlercreutz 1988 & 1991; Armstrong & Doll 1975; Messina 1995). The relatively high amount of soy consumed by Asians could be responsible for the differences in cancer mortality (Adlercreutz et al. 1995). Epidemiological data suggests that consumption of a plant based diet containing phytoestrogens is related to a decreased incidence of breast cancer (Adlercreutz 1984). Soy contains a number of components that are potential anti-carcinogens: acid phenolics, isoflavones, phytates, phytosterols, protease inhibitors and saponins (Anderson and Wolf 1994; Kennedy 1995). The presence of these compounds makes soy a prime target for the investigation of chemopreventive action.

The early discovery that isoflavones elicited estrogenic effects led to the suggestion that isoflavones may be related to estrogen dependant breast cancer (Martin et al. 1978). Martin et al. (1978) showed that genistein competes with estradiol to stimulate MCF-7 cell growth. Other studies showed low-levels of isoflavone excretion by American and Finnish women who are at high risk for breast cancer compared to Japanese women at lower risks (Adlercreutz et al. 1987). Isoflavones applied to an

increasingly diverse number of different cancer cell lines (prostate, breast, colon and leukemia) used in cell culture experiments resulted in favorable findings for their use as cancer inhibitors. A review of different cancer experiments which utilized genistein and daidzein to inhibit leukemia and breast cancer cell proliferation *in vitro* (Herman et al. 1995) found that cell growth was inhibited by the isoflavones. It is not known at this time if levels used in these studies can be generated *in vivo* from dietary sources.

Evaluation of the effects of potential cancer chemopreventive agents can be done using azoxymethane (AOM)(Pereira et al. 1994). AOM is a specific inducer of colon cancer. In this study a diet containing genistein (0.075 g/kg diet) significantly reduced the formation of aberrant crypts formed in the colons of rats exposed to AOM. Isoflavone levels in human foods vary widely. Some varieties of soybeans contain up to 3.5 mg isoflavones per gram of bean. It is possible that some populations consuming soybeans as a regular part of the diet are exposed to a 0.075 g genistein/kg diet on a daily basis. Barnes et al.(1990) reported that feeding soy bean chips inhibited the growth of chemically induced mammary cancer tumors in rats. The reduction in the tumor numbers of was attributed to the presence of isoflavones. It was suggested that cancer inhibition occurred due to estrogen antagonism on the

estrogen dependent cancer by isoflavones. In this study it was not clearly determined whether genistein or if other anti-carcinogenic components in the soy were responsible for decreasing mammary cancer. Genistein has also been found to possess antioxidant activity (Wei et al. 1995) which has been linked to a decrease in the development of some cancers.

The isoflavones are present at varied levels in soy beans and soy products depending on variety, growing conditions and processing (Eldrige and Kwolek 1983; Tsukamato et al. 1995; Wang and Murphy 1994). The majority of the isoflavones are derived from the glucosides of mainly two isoflavones: genistein and daidzein (Barnes et al. 1994). When consumed, glucosides of the isoflavones are digested and metabolized with only a fraction of the original compounds accounted for in body excretions (Kelly et al. 1993). After absorption into the blood, then enzymatic conjugation in the liver, solubilized isoflavones are then excreted through the kidneys in different isomer conjugates in the urine (Adlercreutz et al. 1995). Studies in humans have attempted to relate isoflavone consumption to blood and excreted metabolite levels after isoflavone ingestion (Xu et al. 1994; Cassidy et al. 1994; Franke and Custer 1994; Kelly et al 1993; Lampe et al. 1994; Aldercreutz et al. 1991). The studies cited did not examine

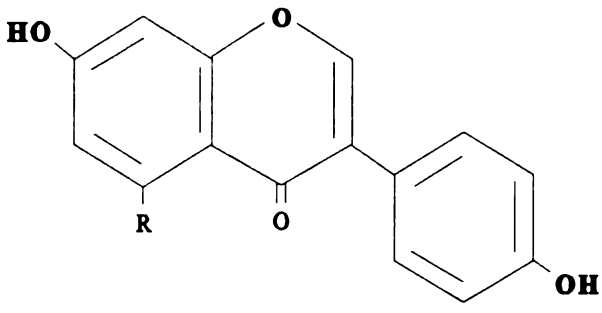
the hour to hour changes in blood or excreted levels. Evaluation of the continuous metabolism of isoflavones in the body still needs to be determined. It was found that isoflavones from a single dose remain resident in the blood and urine for approximately 72 hrs, though individuals seem to metabolize and excrete the compounds differently (Kelly et al. 1995). Monitoring of isoflavone excretions in urine can potentially be used to confirm soy consumption and to determine absorption and excretion of isoflavones by humans.

LITERATURE REVIEW

Isoflavones As Anticarcinogens:

There is great interest in isoflavones due to the possibility that they have anti-carcinogenic activity. Epidemiological data suggested a link to high levels of soy consumption with a decreased incidence of breast and colon cancer (Adlercreutz 1984). Soy foods are a unique source of phytoestrogens isoflavones. Daidzein and genistein are the two most concentrated isoflavones in soy. Daidzein and genistein have been found to be highly excreted in populations at lower risks for breast and colon cancer (Adlercreutz 1991). Further evidence of anti-carcinogenic activity of soy foods was suggested in reviews of animal studies on experimental carcinogenesis (Messina 1994). This review concluded that the majority of animal studies resulted in inhibition of cancer proliferation due to soy products.

Inhibitory effects on cancerous cell growth have also been demonstrated for the individual isoflavones: genistein and daidzein. In a number of experiments utilizing the isoflavones daidzein and genistein (Figure 1), both have been shown to inhibit breast and colon cancer cell proliferation (Herman et al. 1995; Messina 1994). An isoflavones anti-carcinogenic effect may depend upon the type of cancer (Herman et al 1995; Whitten et al. 1995).



R=H (DAIDZEIN) R=OH (GENISTEIN)

Figure 1. Isoflavone structure

Breast cancers can be estrogen dependant or independent. Estrogen dependant (estrogen receptor positive) and estrogen independent (estrogen receptor negative) breast cancer cell lines were both inhibited by genistein treatment (Peterson and Barnes 1991). The nature of genistein's inhibition is not clear but Hoffman (1995) also linked breast cancer growth inhibition to estrogenic independent activity. Overall, reviews of colon and leukemia cancer cell studies indicate inhibition by genistein while daidzein data is less conclusive (Messina et al. 1994). It has also been suggested that the anti-oxidant activity of genistein may be responsible for its anti-carcinogenic effect through the reduction of free radicals which may damage cellular genetic structure (Wei et al. 1995).

Much of the anti-carcinogen attention has been focused on genistein due to its ability to alter cellular functions. Genistein's inhibition of cancerous cell growth may be

linked to limiting tyrosine kinase activity often associated with unregulated cellular proliferation (Uckun et al. 1995; Akiyama and Ogawara 1991). These observations include: 1) genisteins specific inhibition of tyrosine kinase phosphorylation in Pseudomonas (Ogawara et al. 1989); 2) genistein limits EGF (epidermal growth factor) stimulated tyrosine kinase activity (Akiyama et al.1987); 3) genistein inhibits the phosphorylation activation cascade of protein tyrosine kinase, without inhibiting insulin auto-phosphorylation in rats (Abler et al. 1991) and 4) genistein inhibits endothelial cell proliferation and angiogenesis which may explain the cancer preventative effects of a plant based diet (Fotsis et al. 1993). Genistein's ability to directly alter cellular proliferation has made it a good chemopreventive candidate.

The many studies pointing to the inhibitory effects of genistein on cellular metabolism favors the possibility that this action might be possible *in vivo* provided the isoflavone can reach physiologically active concentrations. Monitoring of genistein levels in the urine might allow further understanding as to the nature of its specific activity as a bioactive compound. Also, the presence of isoflavones in much of the worlds human and animal food sources warrants further investigation into the nature and metabolism of these bioactive compounds.

Isoflavones

Plant isoflavones have primary roles in plant metabolism, yet they are little understood. Isolated isoflavones have been shown to act as natural fungicides (phytoaxelins), insecticides and herborvoric deterrents. Isoflavones *in vivo* function as plant regulators (hormones) and UV protectants (Harborne 1971; Tsukamoto et al. 1995). The active compounds in plants are the isoflavone glucosides while the active compounds in animals appear to be the isoflavone aglycones (Braden and Shutt, 1970).

Isoflavones In Soybeans

Isoflavones are present in soybeans in the approximate ratios of: 64% genistin, 23% daidzin and 13% glycetin (Naim et al. 1974). The glucosides genistin and daidzin make up the majority of isoflavones found in soybeans with malonyl-glucosides being the primary conjugates (Figure 2)(Tsukamoto et al. 1995). Glucoside conjugates account for approximately 98% of the isoflavones present in soybeans with the remaining 2% in the aglycone form as genistein, daidzein and glycetin (Anderson and Wolf 1995). The isoflavone content of soybean varies (<0.5 to >3.5 mg/g) depending upon: bean variety, environmental conditions (Eldrige and Kwolek 1983; Tsukamoto et al. 1995) and processing (Wang and Murphy 1994). Total isoflavone content of the bean may vary, but the relative properties of the isoflavones tend to remain

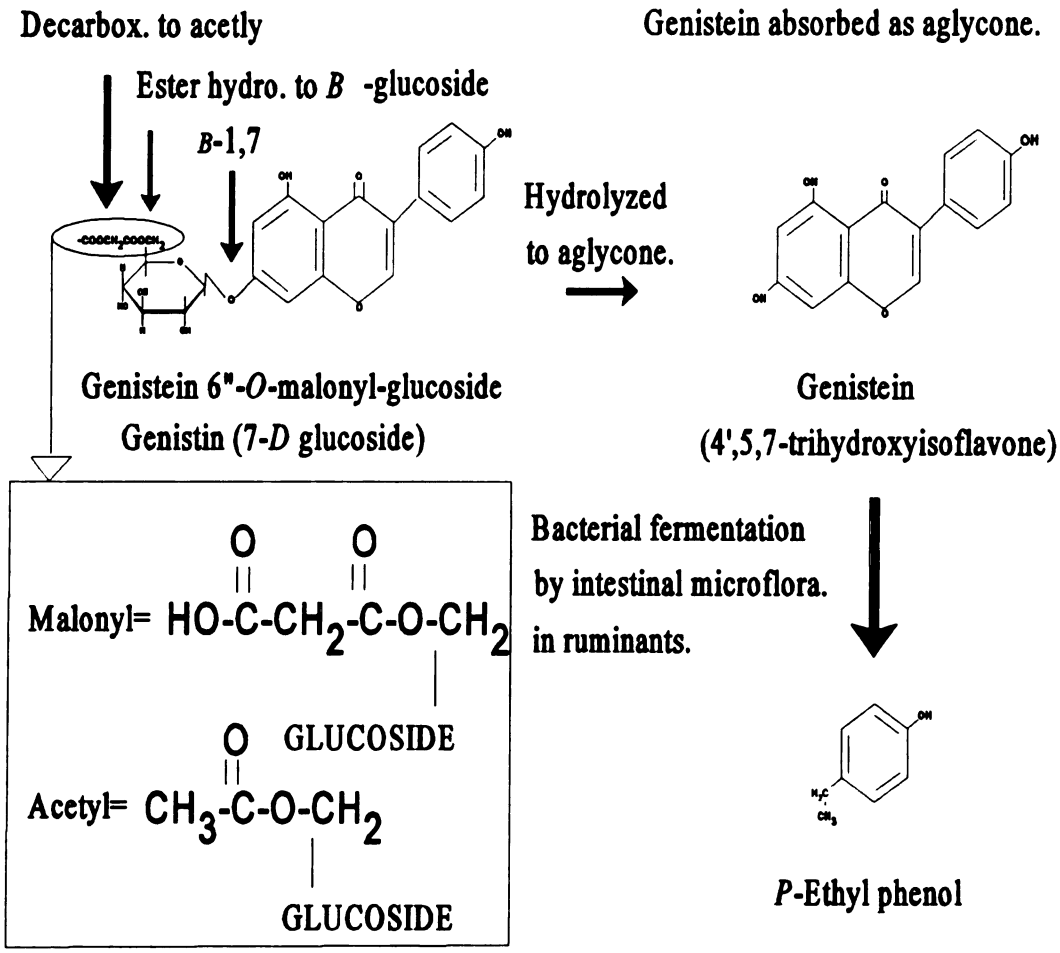


Figure 2. Proposed pathway for the microbial digestion of isoflavone glucosides.

constant (Tsukamoto et al. 1995).

Isoflavones In Foods

Food processing and preparation significantly affects isoflavone level and form (Golbitz 1995). Processed foods which are fermented and/or heated have varying ratios of conjugates to aglycones (Barnes et al. 1994). Many recipes for traditional soy foods come from the Far East where they are most widely consumed. The type of soy food, either fermented or non, usually dictates whether the isoflavones are present as aglycones or glucosides. Fermented foods such as tempeh, miso and soy sauce contain higher proportions of the aglycones and lower proportion of the glucosides (Wang and Murphy 1994). The microbial fermentation process utilized in preparation of soy foods, such as *Rhizopus oligosporus* used for production of tempeh; results in a predominance of aglycones in the fermented foods. High heat causes malonyl decarboxylation to form acetyl derivatives. Heating a liquid suspension or slurry results in loss of the malonyl or acetyl moiety leaving the glucoside.

Defatted soy flakes are precursors for soy flours, concentrates and isolates. Soy flour is ground from defatted soyflakes (Lusas and Riaz 1995). Further processing of soy flour results in ever decreasing amounts of isoflavones. Soy concentrate is produced primarily two ways: water or alcohol wash of the soy flour. The alcohol wash process for reducing

soluble carbohydrates results in a major loss of the alcohol soluble isoflavones. Isoflavone levels in soy protein isolates, produced from soy concentrate will have isoflavone levels reflective of its parent concentrate process, either alcohol or water washed (Wang and Murphy 1994).

Soy concentrates and isolates appear in many second generation foods supplying protein to infant formulas, nutritional beverages and extruded meat products. Soy protein products are also utilized for other functional properties such as flavor binders, thickening agents and fat binders (Lusas and Riaz 1995). Many other soy foods are now being introduced such as soy hot dogs and bacon, tofu burgers and yogurt, tempeh burgers as well as inclusion into pasta products like flat noodles (Wang and Murphy 1994; Anderson and Wolf 1995). All of the soy products listed have isoflavones present which vary in levels and proportions of the different isomers (aglycones, glucosides and malonyl or acetyl glucosides)(Anderson and Wolf 1995). As the consumption of soy foods increases, so has the isoflavones which are intrinsic to many of these foods.

Estrogenic Activity of Isoflavones

Much of the interest in soy isoflavones has been related to their estrogenic activity and possible relationship to estrogen dependant breast cancer.

Isoflavones are in a group of polycyclic phenols that have demonstrated interaction with human estrogen receptors *in vitro* (Miksicek, 1995; Cassidy et al. 1994) and have produced *in vivo* effects in animals (Braden and Shutt 1970; Shutt et al. 1970; Hawrylweicz et al. 1995). The phytoestrogens daidzein and genistein and the metabolite equol have varying degrees of interaction with estrogen receptors *in vivo* and *in vitro* (Miksicek 1995). Equol, an intestinally produced bacterial metabolite of daidzein, has approximately 0.061% the activity of 17-*B* estradiol demonstrated *in vitro* with human estrogen receptors (Markiewicz et al. 1993). According to Miksicek (1995) chloroamphenicol acetyl transferase (CAT) activity of genistein (@ 1 uM) was approximately 1/5000 of 17-B-estradiol (@ 5 nM) activity. Daidzeins estrogenic activity was half that of genistein (ibid). Depending on the substitution of structures at specific sites, heightened or diminished affinities for estrogen receptors were demonstrated when compared to 17-*B* estradiol (ibid). For example 4'-hydroxylated isoflavones (genistein and daidzein) have higher estrogen receptor interaction than compounds such as biochanin A and formononetin which are 4'-methoxylated compounds (Martin et al. 1978; Miksicek 1994).

Estrogenic Activity In Animals

Isoflavones were originally discovered because they

induced estrus in mares (Marrian and Haslewood 1932) and sheep (Braden et al. 1967; Shutt and Braden 1968). The compound responsible for the estrogenic activity, that of induced estrus and infertility, was found to be equol (Figure 3) the microbial breakdown product of the isoflavone daidzein (Lundh et al. 1990). Relative binding affinities to sheep uterine estrogen receptors showed genistein to be twice that of equol and equol to be four times that of daidzein. Sensitivity to phytoestrogens is species dependent (Lundh 1995). The reason why sheep are more sensitive to isoflavones compared to cows is not clear. The mechanism for the sensitivity differences of cows and sheep to isoflavones has as yet to be completed. Uterine estrogen receptor numbers in sheep are two to four times higher than in cows. The increased number of estrogen receptors may account for the varied responses of the two species to the isoflavones and their bacterial metabolites (Koligian and Stormshak 1977; Henriks and Harris 1978). However, cattle are exposed to higher levels of equol for longer periods of time than sheep but with minimal estrogenic effects. As shown by lower blood retention levels, sheep did not receive the same extended exposure to equol as cows but were subject to more severe estrogenic effects. Isoflavones in sheep dropped to 50% of maximal levels 14-16 hours after food intake while levels in cattle remained constant (Lundh 1995). Equol looks

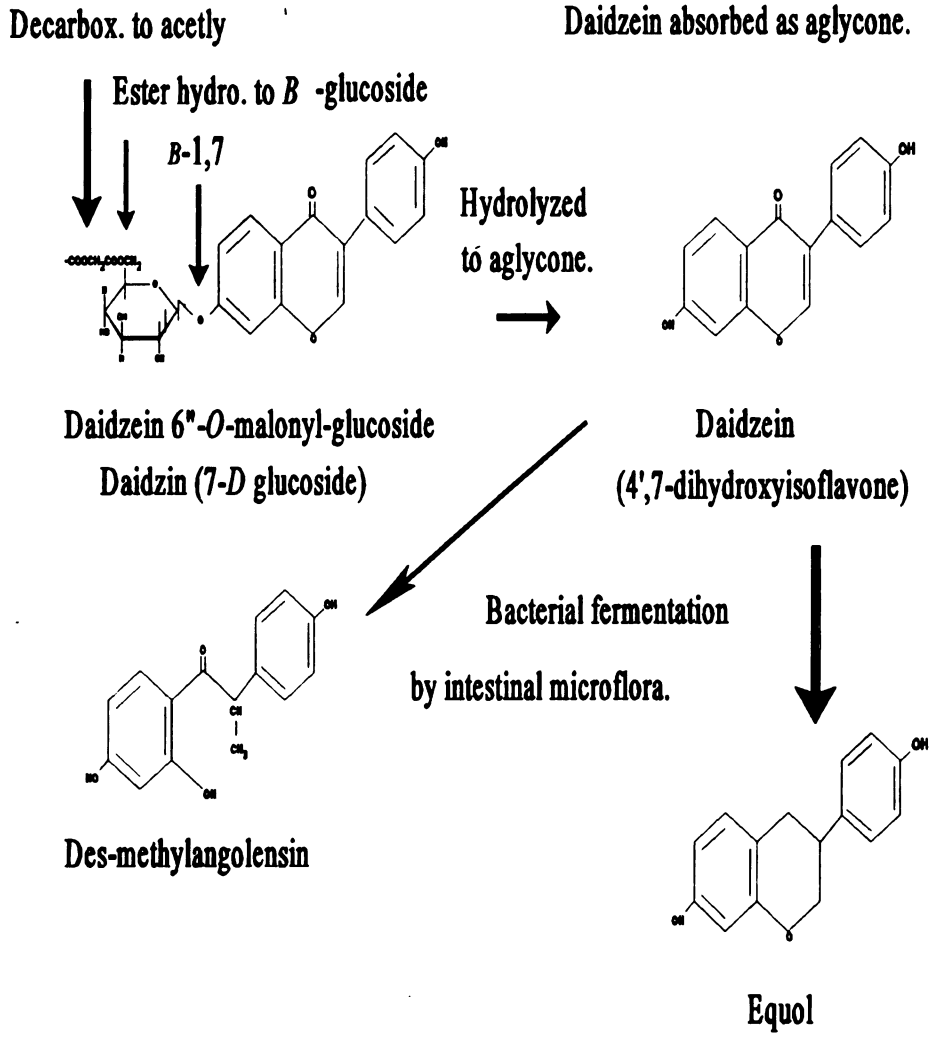


Figure 3. Microbial digestion and compound formation from daidzein.

to be cleared faster in sheep but the levels present are still high enough to cause estrogenic effects shown by reproductive dysfunction (Shutt and Braden 1968). The difference in sensitivity must be attributed to factors other than estrogen receptor concentration difference between the species.

Estrogenic Activity In Humans

Findings on the estrogenic activity of isoflavones in humans for the moment is in question. The few studies linking estrogenic activity in humans to isoflavone consumption have been inconclusive. Cassidy et al.(1994) reported that consuming 60 g/day of soy protein (25.1 mg daidzein and 19.85 mg genistein) lengthened the onset of menses by 1-5 days. Average increase of the follicular phase was 2.5 days and no apparent change in the luteal phase was observed. This effect could be due to the estrogenic effects of isoflavones present in the soy protein which was consumed. Cassidy et al. (1994) also showed a great deal of individual variability in the pattern of isoflavone and metabolite excretion. Other areas of concern in this study were the lack of a larger average menstrual cycle length evaluation before and after treatment. Pre and post treatment cycle averages were based on 1-3 month data only. Monitoring of the subjects menstrual cycles for a greater length of time might have revealed variations within the

error limits of the study. Making conclusions as to the human estrogenic effect attributed to isoflavones from this study alone would seem premature.

Isoflavones As Xenobiotics

Hepatic and epithelial cell conjugation patterns vary with species (Lundh et al. 1990; Axelson et al. 1984). Conjugation patterns could account for the many varied responses found in man (Cassidy et al. 1994) and animals (Adams 1995; Shutt and Braden 1968). The majority of Xenobiotics are metabolized by the liver which increases the molecules solubility and allows it to be expelled in the urine or bile. Isoflavones have characteristics which lend their structures to glucuronidation via UDP-glucuronosyl-transferase activity (Sipes and Gandolfi 1991). Xenobiotics, in this case isoflavones, can also be subject to sulforonyltransferases resulting in sulfate esters or ethereal sulphates via the attack action similar to hydroxy-steroid transferase attack at the -OH of the phenol groups (Sipes and Gandolfi 1991). Mono-glucuronides and sulfates make up a majority of glucuronidated isomers of digested isoflavones although there is a large diversity among individuals (Adlercreutz et al. 1995). The presence of three hydroxyls on genistein lends itself to form other conjugates not possible from daidzein. The presence of multiple

hydroxyl groups on daidzein and genistein result in different conjugated isomers among individuals (Adlercreutz et al. 1995)

Excretion of Isoflavones

Intestinal absorption and liver conjugation of isoflavones results in the excretion of isoflavone glucuronides in the urine and the possibly bile. In order for this to happen isoflavones must be absorbed through the epithelial lining of the stomach, intestine or colon. Isoflavones enter the blood where they are then transported to the liver. In the liver isoflavones are conjugated with glucuronic acid or sulfate. Conjugated isoflavones are then partitioned to the blood and eventually the urine or to biliary secretions. Molecular weight of the parent aglycone is the main determinant of whether the xenobiotic is excreted in bile or urine and there is a wide disparity in secretory partitioning and the concentrations for each species. In rats, aglycones with molecular weights of 250 or less were glucuronidated and excreted in the urine while larger molecules are secreted in bile (Sipes and Gandolfi 1991). Daidzein and genistein have molecular weights of 254.23 and 270.23 respectively. Isoflavones excreted into the small intestine may be reabsorbed (enterohepatic recirculation) or undergo microbial degradation and

eventually be excreted in the feces. The isoflavones in urine are primarily glucuronidated conjugates with lesser amounts of the sulfate conjugates (Bannwart et al. 1983). Utilization of the Helix pomatia glucuronidase-sulfatase (type H2) lyses both glucuronic acids and sulfate conjugates to produce aglycones of the original compounds (Xu et al. 1994)

Isoflavone Excretion In Animals

Animals obtain isoflavones from feeds such as clover and soy. Isoflavones can account for 0.5-2.5% of the dried weight in varieties of subterranean clover fed to sheep and cattle (Petterson and Kiessling 1984). One study determined levels of the plant estrogen daidzein and the metabolite equol in the blood of dairy cattle and sheep after consuming a subterranean clover high in isoflavones. Levels of total and free daidzein in the blood were comparable in both species (Lundh et al. 1990). Ninety-percent of isoflavones present in the blood samples of cows and sheep were conjugated and with the remaining 10% as the free aglycone form. Levels of different conjugates (sulfates and glucuronic acids) showed little variation between species (Lundh et al. 1988). Slightly different conjugation rates were found in a study utilizing cow and sheep liver homogenates (Lundh 1995). The highest levels of activity were attributed to conjugation rates with minimal or non-

detectable levels of demethlyation thus eliminating liver breakdown as a step in the conversion of daidzein to equol. Conjugation was found to occur in the epithelial lining of rumen, reticulum, omasum and small intestine. This indicates that the primary mode of detoxification in ruminants may be the epithelial cell lining of the gastrointestinal tract. Sheep however, showed two to twenty times the intestinal conjugation activity compared to cows (ibid). Highest levels of conjugation in ruminants appears to occur in the epithelial lining of the rumen (Shutt et al. 1970).

Dietary isoflavones are metabolized by microbes to equol in cows and sheep (Lundh et al. 1988). Less than 1% of the original isoflavone aglycones were shown present in urine and fecal specima of both species of rumen. Daidzein, represents up to 70% of the isoflavone conversion products that are recovered as glucuronides in urine of cows and sheep (Lundh 1995). The levels of O-desmethlyangolensin, another metabolite of daidzein; and free daidzein varied from 5-20% in the urine samples (Shutt et al. 1970).

Pigs are monogastric animals that could serve as a model for humans. Pigs fed a mixed diet of legumes and red clover, both containing isoflavones, showed maximal blood levels one hour after feed consumption (Lundh 1995). The rapid appearance of isoflavones in the blood indicates that absorption might be occurring in the stomach. The

isoflavones daidzein and genistein were found in the plasma mostly in conjugated forms with only 0.5% present as the free aglycone. However, 30-50% of the equol was present in the free form in pig blood compared to 1 and 5% in sheep and cow respectively. The total amounts of all forms of equol were 10-15 times lower in swine than cows and sheep but free levels of equol were approximately the same. Swine excreted 55% of formononetin and daidzein consumed within 8 hours after a morning feed (Lundh 1995).

Isoflavone Excretion Profiles In Humans

The excretory forms of daidzein and genistein in urine were determined with four females and two males consuming vegetarian and semi-vegetarian diets (Adlercreutz et al. 1995). Isoflavone quantities in the diet prior to consumption were not evaluated. Daidzein was excreted in the urine as mono-glucuronides (79-82%), sulfoglucuronides (6-17%) and as aglycones (1-5%). Genistein was excreted as monoglucuronides (53-76%), diglucuronides (12-26%), sulfoglucoronides (2-15%) and as the disulfates (1-4%). Daidzein was bacterially metabolized to equol and O-desmethylangolensin in ratios unique to each individual studied. Equol represented less than 1% of the total isoflavone output in urine. Equol was excreted in urine as monoglucuronides (32-93%), sulfoglucuronides (0-43%), monosulfates (0-15%) and disulfates (0-10%) while

O-desmethlyangolensin was primarily excreted as mono-glucuronides (97%) with the remaining 3% divided among the other conjugates (Adlercreutz et al. 1995). Further microbial breakdown of genistein and daidzein occurs but the identity of the compounds has not been determined (Chang and Nair 1994; Xu et al. 1995). Identification of these compounds will aid in determining if they have biological activity.

Apparent Absorption of Isoflavones By Humans

Urinary excretion of a 45 mg dose of isoflavones (56% daidzein and 44% genistein) showed that 1.8%-12.9% of the total isoflavones were excreted in urine (Cassidy et al. 1994). Dose response of individual isoflavones were accounted for in a study utilizing soy milk as the medium of isoflavone introduction (Xu et al. 1994). Soy milk contains isoflavones with 44% as genistein and 56% as daidzein, both are present as glycosides. Urine, fecal and blood samples were taken at intervals to monitor biological availability. Urinary recoveries were greater for daidzein than genistein, 21% and 9% respectively. There were no isoflavones in the urine at 24 hours. Fecal excretion of daidzein and genistein were ≤1-2% of that consumed (Xu et al. 1994). The glucuronides of the genistein and daidzein were recovered and analyzed. Microbial break down products equol and

O-desmethlyangolensin were not quantitated. Excretion was not proportional to consumption with a majority of isoflavone recovered as daidzein (Xu et al. 1994). In ruminant studies, genistein was shown to be metabolized to p-ethyl phenol (intermediates unknown). However, Kelly et al. (1995) showed that daidzein was degraded to equol in some humans. Variability of gut micro flora has been attributed to the ability of some individuals to produce equol (Xu et al. 1996).

Justification

Compliance monitoring in human studies is difficult. In clinical trials an objective measurement of adherence to the research protocol is critical for the evaluation and interpretation of results. There is little genistein or daidzein in commonly consumed foods of the diets of Western populations. Thus, measuring isoflavone excretion in urine may be a good way to measure daily intake of soy products. From the discussion above, it is apparent that we do not know if isoflavone absorption is proportional to intake. Also we know little about isoflavone clearance rates for various doses of dietary isoflavones.

The objectives of this study were: 1) to gain a better understanding of daidzein and genistein urinary excretion patterns from the consumption of a known amount and

2) to evaluate urinary excretion of daidzein and genistein as compliance markers for the consumption of soy based products.

Hypothesis

Increasing the dose of the isoflavones daidzein and genistein from soy protein will result in proportional increases in the level of both isoflavones present in the urine.

CHAPTER 2.

Monitoring Isoflavone Dose Response In Humans By the Urinary Excretion of Daidzein and Genistein.

ABSTRACT

The urinary excretions of the isoflavones daidzein and genistein were monitored in 8 subjects (4 males and 4 females) over a 24 hour period after consumption of a beverage containing soy protein. Subjects consumed 18.76, 37.52, 50.28 or 75.04 g of soy protein (1.053 mg genistein/g and 0.875 mg daidzein/g protein) as four separate randomized treatments. Treatments were consumed weekly with subjects abstaining from other sources of dietary soy between treatments. Excretion patterns for daidzein and genistein were consistent for an individual for all four doses with no significant differences between males and females($P < 0.05$). Daidzein and genistein were detectable in urine an average of 1.6 hrs after consuming a treatment. Fifty-percent of the 24 hr excretion total occurred in 7.5 hrs for daidzein and 6.5 hrs for gensitein. Ninety-percent of the same total was achieved in 16.5 for daidzein and 16 hrs for genistein. Apparent absorption was 17.57 ± 5.48% for daidzein and 4.36 ± 3.96% for genistein. Dose effect was significant and proportional to intake ($P < 0.05$).

INTRODUCTION

Soy contains a variety of phytochemical compounds including isoflavones (Tsukamoto et al. 1995). The quantity and the form of the isoflavones daidzein and genistein are present in soy products depends upon the degree of processing and the amounts of soy included as ingredients (Barnes et al. 1994, Wang and Murphy 1994). Isoflavones are of interest due to inhibitory effects on prostate, colon and breast cancer cell lines (Hoffman 1995) and their demonstrated estrogenic effects in animals (Adams 1995). Soy protein, containing isoflavones, was also found to decrease bone loss in ovariectomized rats (Bahram et al. 1996). It is not known whether the preventive effect is from the soy protein or the isoflavones therein. However, another study conducted by Anderson et al. (1995) demonstrated genistein can act similarly to an estrogen in preventing trabecular bone loss.

It is known that isoflavones are absorbed, conjugated and then excreted in the urine as glucuronidated and sulfated compounds (Adlercreutz et al. 1995) and possibly to the bile. Excretion rates of isoflavones have begun to be evaluated. In two separate studies, a majority of daidzein and genistein from a single dose of mixed isoflavones (24.7 mg daidzien and 19.3 mg genistein) were excreted within twenty-four hours with minimal levels detected the second

day (Xu et al. 1994; Kelly et al. 1994). The relationship between amounts of isoflavones consumed and isoflavone excretion patterns has not been reported. Therefore, in the following study graded amounts of daidzein and genistein were fed to human subjects and the dose dependent excretions of isoflavones were monitored over time.

MATERIALS AND METHODS

Study Design

Eight subjects (4 females and 4 males) consumed one of four doses of soy protein at seven day intervals. The treatments were randomly assigned to individuals chosen for the study and the dosing schedule is shown in Table 1.

Table 1. Soy protein dose assignment*

			# Packets Consumed for Week			
Sex	**Subject**		**1**	**2**	**3**	**4**
F	A	-->	2	3	1	4
M	B	-->	3	2	4	1
F	C	-->	3	2	4	1
M	D	-->	1	3	2	4
F	E	-->	1	4	3	2
F	F	-->	2	1	3	4
M	G	-->	4	3	2	1
M	H	-->	2	1	4	3

*The indicated number of packets, containing equal amounts of soy protein, were consumed as a dose at the specified time on the same day of each week, for four weeks.

Subjects & Recruiting

Subjects were recruited from among the graduate students and faculty of the Food Science and Human Nutrition Department at M.S.U. All persons interested in participating in the study were given a complete verbal description of the project by Dr. Maurice Bennink, Professor of Human Nutrition in the Department of Food Science and Human

Nutrition. All subjects (22 to 50 yrs of age) typically consumed an omnivorous diet and abstained from any oral antibiotics one month prior to the study to avoid intestinal micro floral changes during the feeding trial. Informed written consent was obtained from the subjects prior to participation in the study and subjects were moderately compensated for their assistance (Appendix A). The study protocol and the informed consent form (Appendix A) were approved by the University Committee on Research Involving Human Subjects (UCRIHS IRB#94-521). Subjects were required to omit foods containing soy ingredients during the feeding trials.

Dietary Supplement Composition

The soy protein supplement is manufactured by Nutritious Foods, Inc., St. Louis, Missouri and is commercially available. The primary ingredient in the soy protein supplement is Supro Brand Isolated Soy Protein manufactured by Protein Technologies International, St. Louis, Missouri. Table 2 outlines the manufacturer's label claims for proximate, vitamin and mineral composition.

Supplement Consumption

The powdered soy protein supplement was mixed in juice, water or soft drink prior to consumption. Subjects took the

Table 2. Supplement composition*

1 packet - 28 grams.
100 Kcal per packet.
10 Kcal from fat.

Proximate	% RDA	Typical
Moisture	NA	0.63 g
Fat	2%	1.1 g
Total CHO (by diff.)	1%	4.28 g
Protein	40%	18.76 g
Kcal		102
Vitamins		
Vitamin A	10%	1060 IU
Vitamin D	25%	132 IU
Riboflavin (B_2)	25%	450 mg
Folacin	15%	74 ug
Pantothenic Acid	8%	0.8 mg
Vitamin C	4%	2.7 mg
Thiamine (B_1)	6%	130 ug
Vitamin (B_6)	6%	220 ug
Vitamin (B_{12})	15%	2.34 ug
Niacin		0.4 mg
Minerals		
Sodium	8%	200 mg
Calcium	70%	600 mg
Magnesium	10%	55 mg
Iron	20%	3.6 mg
Phosphorous	50%	500 mg
Potassium	NA	200 mg
Zinc	6%	2.41 mg

* Daily % values are approximate only. Actual values vary slightly day to day due to manufacturing variation. The values shown are for the soy protein supplement utilized in this study.

assigned treatments at approximately 8:00 a.m. (±1 hr) as a meal or with other foods. The subjects consumed the designated number soy protein packets in less than 10 min. intervals between packets and the time of consumption was noted for all treatments taken (Appendix B).

Urine Sample Collection

All urine voids for 24 hours were collected separately with the times and volumes of each recorded (Appendix C). The investigator was blinded as to subject, date and time of urine sample until all samples were analyzed. Samples were frozen at -20°C until analysis. Samples were collected according to the following approximate schedule in Table 3. Subjects were instructed to have urine voids with no more than 2 hours between voids. The number of samples collected varied according to fluid intake and time spent sleeping. The collection times shown in Table 3 were for the minimal number of samples.

Urinary Isoflavone Analysis

A modified method (Lundh et al. 1988) utilized for the analysis of daidzein, formononetin, coumesterol and equol in bovine urine was utilized in this study. Urine samples were thawed and brought to room temperature before sub sampling and analysis. Figure 1 outlines the procedure for isoflavone

Table 3. Urine sample collection schedule

Packet Consumption Time	Maximum Time Between Voids (Hrs.)						
7 - 9 a.m.	+2	+4	+6	+8	+10	+12	+14 -> 24

(Baseline voids were morning voids taken upon rising collected prior to all assigned soy protein supplement treatment assignments).

extraction from urine samples. Isoflavones were separated and quantified by HPLC. One hundred ul of prepared urine sample was injected into a manual Rheodyne injector (20 ul loop), pumped by a Waters M-45 pump (Milford, Massachusetts) through a Microsorb MV, 5u, 100 A, C_{18} column (Rainin Instrument Co., Woburn, MA). Isoflavones were eluted with an isocratic (50:50 MeOH/H_20 + 1% HAc) solvent system over 20 minutes. The isoflavones were detected at 260 nm with a LDC Analytical Spectro Monitor 4100 Dual Wavelength Detector. Sample data were collected via a personal computer utilizing Peak 2 Integration software (SRI Instrument, Torrance, CA). Peak areas and retention times were compared with standards and totals were quantified by the external standard method.

Evaluation of Standards

References suggest the maximal absorbance for daidzein is 250 nm and for genistein 262.5 nm (The Merck Index,

Figure 1. Protocol for total urinary isoflavone recovery (as aglycone)

Combine 2.5ml urine + 7.5ml 0.2M sodium-acetate (pH 5.5) + 50ul *B*-glucuronidase/sulfatase and vortex.

↓

Incubate sample preparation for 16 hours @ 37°C.

↓

Add 10ml, 10mM sodium phoshate buffer (pH 7.0) to sample preparation and mix.

↓

Pour entire sample preparation onto a QE20 Extralut™ Column and allow to saturate packing until solvent front is visibly stable (2-3 minutes). Rinse sample tubes with 10ml aliquot of ethyl acetate and apply to column.

↓

Elute isoflavones from column with additional 60mls of ethyl acetate into a 100 ml round bottom flask.

↓

Roto-Vap™ sample to dryness, dissolve residue in 2ml 100% EtOH then dilute to 10ml with 8ml, H_2O.

↓

Acidify solution with 200ul 1M HCL and mix.

↓

Prepare 3cc, C_{18} Sep-Paks™ by passing 5ml 100% MeOH and 5ml HPLC H_2O through the column.

↓

Apply sample to prepared C_{18}, Sep-Pak™ column. Rinse sample tube with 2ml H_2O and apply to Sep-Pak™ (discard polar compounds that passed through column).

↓

Elute isoflavones from column with 3ml 80:20 methanol/water (80% methanol) into screw capped tubes and freeze until analysis by HPLC.

10th ed.). A mixed daidzein (10.13 ng/ml) and genistein (11.45 ng/ml) solution of 80% methanol and water was scanned over a UV range of 220-300 nm with maximum absorbances at approximately 260-265 nm. 260 nm was the wavelength used for detecting mixed isoflavonoids in methanol and water solution based on my findings and as reported by Seo and Morr (1984). Isoflavone peaks were identified by comparing retention times of individual and mixed isoflavone standards. Urine samples containing isoflavones and isoflavone blank urine samples were spiked with single and mixed standards. Peaks in the spiked blank urine samples corresponded with standard peak retention times. Urine samples containing daidzein and genistein plus genistein and daidzein standards resulted in peaks which were cumulative proportionally to the standards added.

Statistical Analysis

Data on cumulative urinary excretions of daidzein and genistein were statistically analyzed as a split plot design using the general linear models procedure of SAS 6.20(SAS® Institute, Cary, NC). Main plot variables were time and amount of soy isoflavones consumed. Subplot variables were individual subjects and sex. There was no significant differences between males and females so the data for all subjects were combined for statistical evaluation. Treatment

sums of squares (SS) was further divided into single degrees of freedom orthogonal comparisons for linear, quadratic and cubic effects. Linear regression analysis was used to determine the degree of correlation between isoflavones excreted in urine and amounts of isoflavones consumed. Quantities of daidzein and genistein found in pre-treatment void samples were not included in total outputs.

RESULTS AND DISCUSSION

Isoflavone Detection

Individual and mixed standards were utilized initially to determine appropriate HPLC conditions for quantifying isoflavones. A linear gradient of two solutions - A = (90:10:2) H_2O/MeOH/Acetic Acid (HAc) and B = (98:2) MeOH/HAc - from 0% B -> 100% B over 60 minutes was utilized to determine the approximate MeOH concentration necessary to elute the isoflavones. Subsequently it was determined that an isocratic (49:49:2) methanol/water/HAc mobile phase provided good separation of the urinary isoflavones within a 20 min. period. A typical chromatogram of sample and standard is shown in Figure 2. Injections of 0.1, 0.2, 0.4, 1.0 and 2.0 ug of daidzein and genistein standards were utilized to generate a seven point standard curve. The resulting regression equation for daidzein was: y = 6226(x) + 12; r = 0.9997 and for genistein: y = 9767(x) + 11;

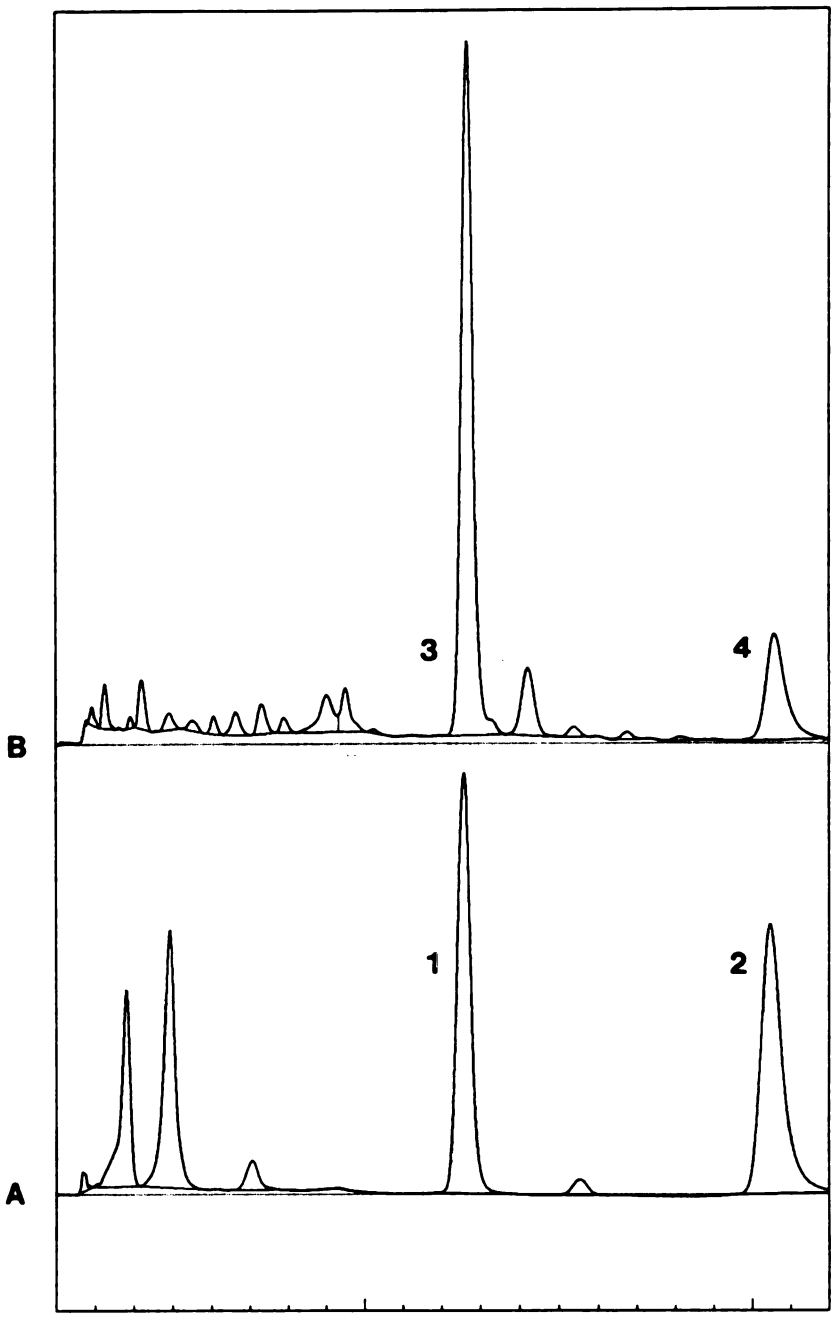


Figure 2. HPLC chromatograms. Standards are shown on (A) with daidzein (1) and genistein (2). Urine sample (B) with identification of daidzein (3) and genistein (4).

r = 0.9997. Isoflavone concentrations in urine were calculated by substituting the areas determined from HPLC analysis for y and solving for x.

Reproducibility

Reproducibility over the duration of a clinical trial is critical when metabolic samples are collected over extended time periods. Injection reproducibility over time establishes the consistency of the HPLC system being utilized. Method reproducibility establishes the effectiveness of the preparation method in reproducing similar results on a given sample. A method which can be consistently reproduced on a given sample can be relied upon to produce accurate results. The following results summarize injection and sample preparation reproducibility.

Injection Reproducibility Over Time

Two mixed standards (A = daidzein @ 10.4 ng/20ul and genistein @ 8.8 ng/20ul; B = daidzein @ 31.2 ng/20ul and genistein @ 26.4 ng/20ul) were injected daily and monitored over a 6 month period. The resulting coefficients of variance (CV) were: A - daidzein = 3.96%, genistein = 5.83%; B - daidzein = 2.37%; genistein = 2.71%.

Sample Preparation Reproducibility

A single sample was run in quadruplicate through the sample preparation in Figure 1. Each preparation was injected 3 times with results as follows: CV for 4 preparations was 1.34% for daidzein and 0.88% for genistein.

Isoflavone Recoveries

A urine sample was spiked with one of four concentrations of daidzein and genistein (D = 0.699, 6.99, 27.95 and 69.87 ng; G = 0.669, 6.69, 26.75 and 66.87 ng). These quantities of daidzein and genistein approximated the concentrations of daidzein and genistein typically found in urine samples obtained from subjects consuming soy protein in a clinical study. Samples were analyzed in duplicate. The average recovery was 92.30% for daidzein and 90.01% for genistein.

Minimum Detectable Levels

Minimum detectable levels were defined as 3 times the background noise level (0.501 ng/injection for daidzein and 0.686 ng/injection for genistein).

Isoflavone Analysis of Treatment

The distribution of isoflavones in the soy supplement (Table 4) is similar to that found in soybeans with the exception that approximately two times higher levels of aglycones were found in the supplement than is typically found in soybeans. Malonyl-glucoside conjugates of daidzin and genistin made up 65 to 90% of all isoflavones found in seven varieties of soybeans (Tsukamoto et al. 1995). The glucosides of daidzin and genistin made up the remaining isoflavones with other forms such as the aglycones, acetyl-glucosides or glycitin conjugates comprising <1% of the total soybean isoflavones. It should be noted that there is a significant amount of variation in isoflavone content between varieties of soybeans. The soy protein in the supplement would be reflective of the variety or mix of varieties used in its manufacture. The supplement contains a higher percentage of aglycone and a lower percentage of malonyl-glycosides than raw soybean. Table 5 shows the amount of daidzein and genistein consumed for each treatment and Table 6 shows the amount of the isoflavones recovered in urine.

The urinary excretion of daidzein and genistein as a function of isoflavone concentration is shown in Figure 3. Dose response was significant ($P < 0.05$) and urinary excretions increased linearly for both daidzein and genistein.

Table 4. Isoflavone content of the soy protein supplement*

Conjugate	Aglycone	
	DAIDZEIN	GENISTEIN
Glucoside - (Daidzin and Genistin)	5.99 mg	11.3 mg
% of total isoflavones	24.4	38.44
Malonyl Glucoside - (Daidzin and Genistin)	12.8 mg	16.7 mg
% of total isoflavones	52.04	56.61
Aglycone -	5.76 mg	1.46 mg
% of total isoflavones	23.52	4.94
Total Isoflavone**	24.50 mg	29.48 mg
% of total isoflavones	43.8	52.7

*Results are reported per packet or 18.76 g protein.
**Total Isoflavones (All daidzein and genistein forms) - 1.928 mg/g supplement.

Table 5. Isoflavone consumption per treatment*

Dose	(mg of aglycone)		(umol)	
	DAIDZEIN	GENISTEIN	DAIDZEIN	GENISTEIN
1	24.50	29.48	96.38	109.1
2	49.0	58.96	192.8	218.2
3	73.5	88.44	289.1	327.3
4	98.0	117.9	385.5	436.4

*Total aglycone from all forms. Presented in mg and umol for ease of interpretation only.

Table 6. Daily urinary excretion of isoflavones*

# of packets consumed	AGLYCONES (mg)		OUTPUT AS AGLYCONES (umol)	
	DAIDZEIN	GENISTEIN	DAIDZEIN	GENISTEIN
1	4.54	1.74	17.86 ± 5.24	6.42 ± 6.25
2	7.78	2.13	30.61 ± 11.8	7.87 ± 6.08
3	13.68	3.75	53.79 ± 22.8	13.88 ± 15.1
4	16.04	4.24	63.10 ± 10.3	15.68 ± 11.6

*Values are averages from eight subjects ± 1SD

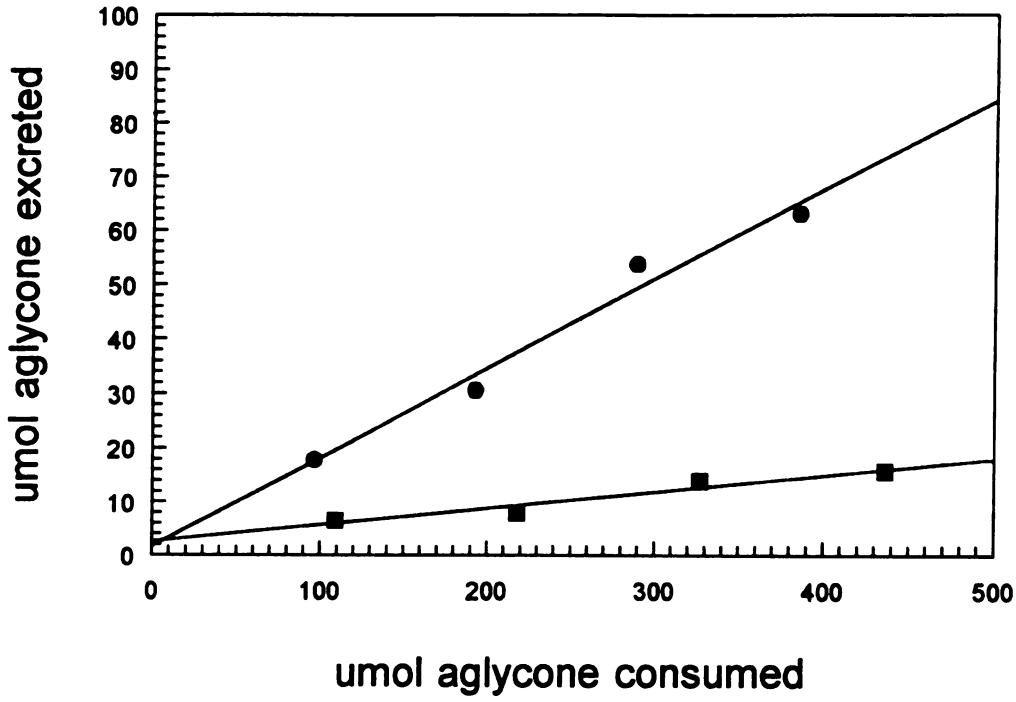


Figure 3. Twenty-four hour excretion of daidzein and genistein as a function of dose. Average daidzein (●) and genistein (■) excretion for all subjects with linear regression for each isoflavone(r = 0.9873 for daidzein and r = 0.9677 for genistein). SEM's (pooled) were 3.81 for daidzein and 2.53 for genistein.

Amounts recovered for each individual treatment are summarized in Appendices G and H. The excretion of daidzein and genistein (as a percentage of the amount excreted in 24 hr.) were plotted for each subject and for each dose. These graphs were used to estimate daidzein and genistein excretion at two hour intervals and the average excretion for all subjects are shown in Figures 4 and 5. Fifty-percent of daidzein is excreted in 7.5 hours and ninety-percent in 16.5 hours. Fifty-percent of the genistein is excreted in 6.5 hours and ninety-percent is excreted in 16 hours. The extra hydroxyl group on genistein may make it more readily available for Phase II conjugation reactions and slightly quicker clearance thus the faster times for excretion. In this study samples were collected for twenty-four hours only. Kelly et al. (1993) found that isoflavones from a 40 g dose of soy protein (98 mg genistein and 80 mg daidzein) can be excreted and detected by GC/MS for up to 72 hrs. However, quantities of daidzein and genistein returned to baseline levels after 2-3 days. Detectable levels of daidzein were found in the last voids of all subjects for all treatments in this study. Only one subject (G) did not have any genistein in the final sample of the 24 hr collection period. The lowest dose treatment for subject G was cleared by 11.4 hrs and the seven subsequent samples also contained no genistein. It is possible that further collection of

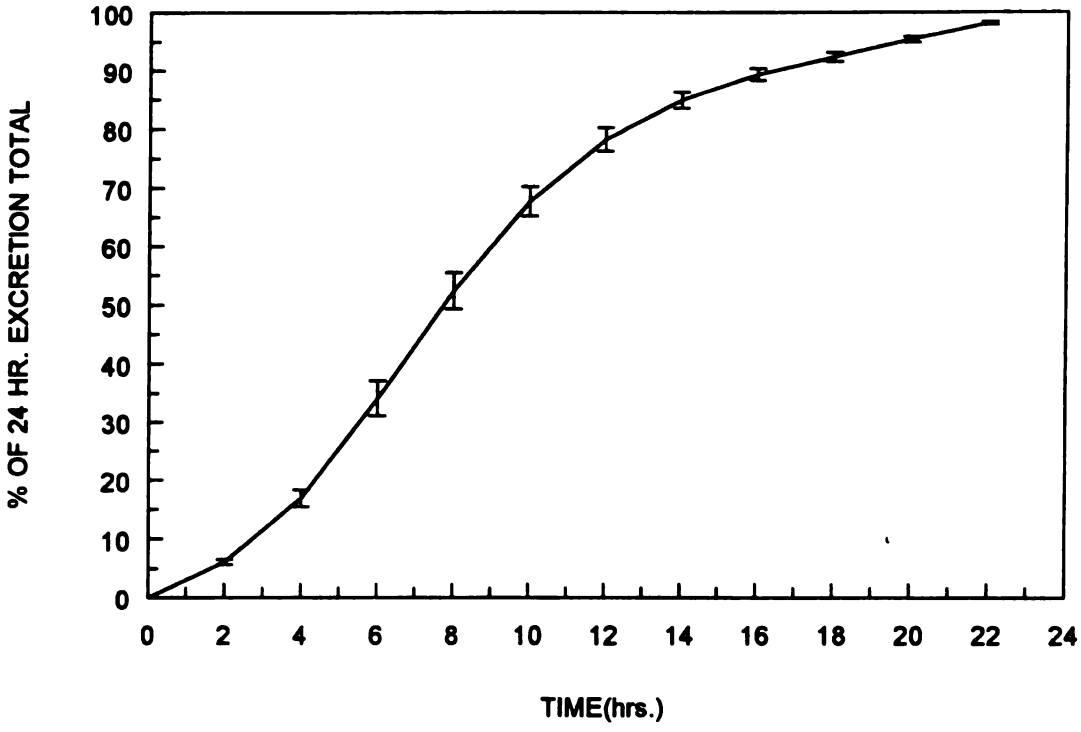


Figure 4. Percentage of twenty-four hour excretion total for daidzein*

*The isoflavone quantities for each void (across all subjects and for all treatments) were calculated as a percentage of the 24 hr total. Percentages were then plotted cumulatively. Percentages were taken at 2 hr intervals and plotted. SEM is shown for each interval.

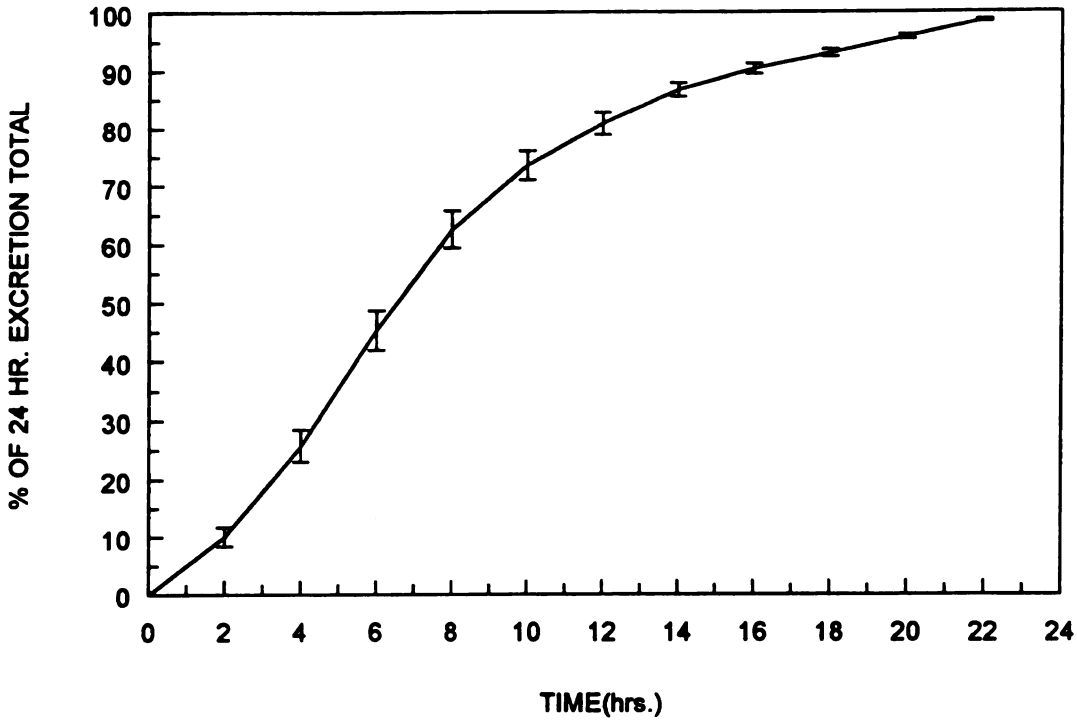


Figure 5. Percentage of twenty-four hour excretion total for genistein*

*The isoflavone quantities for each void (across all subjects and for all treatments) were calculated as a percentage of the 24 hr total. Percentages were then plotted cumulatively. Percentages were taken at 2 hr intervals and plotted. SEM is shown for each interval.

urine samples, past twenty-four hours, would have increased isoflavone totals in some of the subjects.

Some clinical trials may require subjects to fast for 14 hours prior to obtaining blood and urine specimens. Therefore, the data was evaluated to determine if daidzein and genistein were being excreted at 14 hours after consuming a dose of soy protein. Table 7 shows 14 hour detect ability when utilizing genistein excretion as an indicator of soy consumption.

Table 7. Genistein detection at 14 hours*

SUBJECT	Dose 1	2	3	4
A	+	+	+	+
B	+	+	+	+
C	- [1]	+	- [2]	+
D	+	+	+	+
E	+	+	+	+
F	+	+	+	+
G	- [3]	-[4]	+	+
H	+	+	+	+

*First urine sample collected during the 24 hour period that contained no evidence of genistein

[1]Genistein detected at 9.0 hour sample only

[2]" " 11.3 hour sample only

[3]" " 11.4 " "

[4]" " 10.9 " "

When one packet of supplement was consumed, genistein in urine samples could still be detected at 14 hours for seventy-five percent of the subjects. The consumption of two or three packets resulted in eighty-eight percent of the subjects having detectable levels of genistein in the urine and when four packets were consumed all subjects had detectable levels of genistein in their urine at 14 hours.

The first urine void with no detectable genistein did not result in all subsequent samples being devoid of genistein. Subjects frequently would have detectable levels evident after one or more previous samples without genistein. The reoccurrence of genistein in urine may be indicative of enterohepatic recirculation of genistein following initial absorption. Genistein conjugated in the liver might be partitioned to the bile, secreted and then hydrolyzed by intestinal bacteria and then reabsorbed as aglycone.

This study suggests that genistein can be cleared from the body in less than 12 hours if no more than 40 mg of genistein is consumed. As more genistein is consumed, more time is required to clear genistein from the body. The results also demonstrate that measurement of urinary genistein and to a lesser degree daidzein can be used to assess soy isoflavone consumption. Utilization of genistein to monitor soy isoflavone consumption is best applied to

non-fasting subjects; however, acceptable results are achievable even if subjects are required to fast prior to sample collection providing genistein dose and length of fast are considered.

This study follows the hour to hour excretion of isoflavones thus giving an indication of clearance and residence times in the body. The type of isomer conjugate absorbed and the extent of digestion still need to be evaluated to find out what species is the active component in soy foods. Recent findings suggest that a compound similar in structure to isoflavones, the flavanone quercitin, may have its absorbance enhanced by the presence of a glucoside moiety (Hollman et al. 1995). This suggested that a glucose receptor mediated transport might be possible. The absorbance of intact glucosides of daidzein and genistein has not been confirmed. Further study will help to evaluate the absorbance and metabolism of different conjugates.

CHAPTER 3.

Evaluation of the Isoflavones Daidzein and Genistein As Compliance Monitors for Soy Consumption

ABSTRACT

Urinary excretion of the isoflavones daidzein and genistein was evaluated as an approach to monitor subject compliance in a double-blind clinical study that required subjects to consume soy isolate or casein supplements. Urine samples collected from 4 male and 4 female subjects instructed to abstain from foods containing soy, were analyzed by HPLC at a wavelength of 260 nm to define criteria for monitoring compliance in subjects not consuming soy. In urine samples devoid of soy, a peak eluted which overlapped the daidzein retention time. When evaluated at an additional wavelength of 212 nm and compared to a daidzein standard, the conflicting peaks were confirmed not to be daidzein. In the same urine samples there were no peaks which conflicted with genistein detection at either 260 or 212 nm. Specificity was increased and false positive detections were decreased by using dual wavelength detection (212 and 260 nm) and a low level detection limit equivalent to 13.7 ng of genistein per ml of urine. Daidzein was found to be effective as a secondary compliance marker to genistein only. 128 samples from 24 subjects were analyzed for genistein and daidzein content. Genistein was identified in 96% (94:98) of the samples from subjects consuming soy and 7% (2:28) of the samples from subjects consuming casein.

INTRODUCTION

Recent evidence suggests that dietary intervention might decrease the occurrence of atherosclerotic heart disease and cancer, the two major causes of non-accidental death in the U.S. The potential of dietary soy protein to reduce the risk of cardiovascular disease and cancer is being evaluated in a clinical trial being conducted by Dr. Bennink and Dr. Mayle (1996) at Michigan State University . Such clinical trials require compliance monitoring to confirm the absence or presence of soy in the diet. Evaluation of compliance in human studies is often difficult, but necessary. Compounds such as para-amino benzoic acid (PABA) have been utilized in human clinical trials but excretion is often not proportional to intake and renal function may also affect PABA excretion (Bingham et al. 1992). The isoflavones daidzein and genistein are excreted in urine after soy products are consumed. A previous study (Chapter 2) demonstrated that isoflavone excretion is proportional to intake. The objectives of this study were: 1) to determine if subjects that do not consume soy excrete daidzein or genistein in urine and 2) to evaluate patient compliance in a double-blind clinical study.

MATERIALS AND METHODS

Study Design

Experiment 1 evaluated daidzein and genistein output in the urine of subjects who had eliminated soy from the diet. Four males and four females were recruited from the graduate student body of the Food Science Department at Michigan State University. Subjects were instructed to abstain from soy foods at least five days prior to urine sample collection.

Experiment 2 evaluated the effectiveness of daidzein and genistein detection in urine as a way to evaluate compliance in a double-blind clinical trial evaluating the effect of dietary soy protein on cardiovascular disease and colon cancer that was ongoing. Subjects were recruited from two gastrointestinal clinics from a list of patients who previously had colon polyps removed.

Dietary Supplement Composition

The soy and casein supplements are manufactured by Nutritious Foods, Inc., St. Louis, Missouri and are commercially available. The primary ingredient in the soy protein supplement is Supro Brand Isolated Soy Protein manufactured by Protein Technologies International, St. Louis, Missouri. The primary ingredient in the casein supplement is calcium-caseinate. Table 1 outlines

Table 1. Composition of the Nutritional Beverage Powder*

1 packet - 28 grams
100 KCal per packet.
10 KCal from fat.

Supplement	(Casein) A	B	C
Moisture (g)	0.63	0.72	0.80
Protein (g)	18.76	20.49	18.95
Fat (g)	1.10	0.25	1.10
Ash (g)	2.78	1.83	1.10
CHO (g, by difference)	4.28	4.21	4.00
Kcal	102	101	102
Vitamin A (IU)	1060	952	880
Vitamin D (IU)	132	139	130
Vitamin C (mg)	2.70	3.16	3.15
Vitamin B_1 (mg)	0.13	0.16	0.22
Vitamin B_2 (mg)	0.45	0.36	0.54
Niacin (mg)	0.40	0.29	0.44
Vitamin B_6 (mg)	0.22	0.21	0.29
Folacin (ug)	74	19	45
Vitamin B_{12} (ug)	2.34	1.24	1.29
Calcium (g)	0.60	1.01	0.05
Magnesium (mg)	55	53	53
Zinc (mg)	2.41	3.46	2.04

* Manufacturers analysis

the manufacturers label claims for proximate, vitamin and mineral composition of each of the supplements.

Supplement Consumption

Subjects in Experiment 1 were instructed to abstain from eating soy foods at least five days prior to urine sample collection.

Subjects in Experiment 2 consumed the soy protein or casein supplement mixed in juice, water or soft drinks. Subjects took the assigned treatments any time during the day. The subjects consumed the two packets each day for one year. Table 2 shows the daily isoflavone consumption of subjects assigned the soy supplement. Casein supplements contained no isoflavones.

Table 2. Isoflavone consumption per day*

dose	(mg of aglycone)		(umol)	
	DAIDZEIN	GENISTEIN	DAIDZEIN	GENISTEIN
(2 packets)	49.0	58.96	192.8	218.2

*Total aglycone from all forms

Sample Collection

Urine samples collected in Experiment 1 were collected in the morning upon rising. Four samples from each subject

were collected at weekly intervals (32 samples total).

Urine samples were collected from subjects in the double blind clinical study (Experiment 2) for isoflavone content analysis. Subjects consumed two packets of protein supplement per day (casein (control) or soy (treatment)). Subjects may have fasted 14 hours prior to urine sample collection as fasting blood samples were also taken midway through the clinical trial. Urine samples were collected at the physician's office bi-monthly and frozen until analyzed.

Urinary Isoflavone Analysis

Isoflavones in urine were analyzed according to the protocol in Figure 2 (p. 34) and were quantified as aglycones of the glucuronidated and sulfated parent compounds. All urine samples were coded so that the analyst was unaware of subject and treatment. Minimal detectable levels were defined as 3 times the background noise level which is: 0.501 ng of daidzein per injection and 0.686 ng of genistein per injection.

RESULTS AND DISCUSSION

Urine samples from 4 male and 4 female subjects (32 samples total) were collected at weekly intervals for 4 weeks. Subjects were instructed to abstain from consuming soy products. Table 3 shows the results when the 32 samples were analyzed according to the method for quantifying isoflavones described in Chapter 2. Ten samples had peaks with retention times similar to daidzein and/or genistein. Since these samples should not have contained daidzein or genistein, the method for isoflavone detection was examined. Dual wavelength detection can improve specificity, therefore the samples were also monitored at 212 nm. Genistein and daidzein standards monitored at 212 nm consistently resulted in peak areas that were ninety-two and seventy-three percent of the areas detected at 260 nm. The same ratio was found for daidzein and genistein in urine samples.

6 of 10 samples had peaks overlapping the daidzein retention time zone but had no indication of genistein when monitored at 260 nm. The compounds that were suspected as being daidzein absorbed light more strongly at 212 nm and the peak areas were much greater at 212 than at 260 nm. In addition, there were slight shifts in retention times at 212 nm when compared to a standard. The difference in absorbance and slight changes in retention time suggest that these

Table 3. Putative genistein and daidzein detection at 260 nm

SUBJECT	Dose 1	2	3	4
A	+	-	-	-
B	-	-	+	-
C	-	+	-	+
D	-	-	+	-
E	-	+	-	+
F	-	-	-	-
G	-	-	-	+
H	+	-	-	+

compounds were not daidzein.

When samples A1, G4 and H1 were analyzed at 212 and 260 nM, profiles characteristic of daidzein and genistein were found. Subjects A and G consumed food with small amounts of isoflavones within twenty-four hours prior to collection of the urine sample. However, closer examination of the diets showed that for sample H1 a dietary source of soy could not be identified. It is possible that some other food contains these isoflavones and that the presence of daidzein and genistien has not been reported yet.

Sample D3 contained peaks that with casual examination would be identified as daidzein and genistein at 260 nm. However, closer scrutiny at 212 nm showed slight shifts in retention time compared to retention times of standards. Detection at 212 nm produced peaks with large areas that had similar but not identical retention times to daidzein and genistein. Since the peak areas were not proportional when detected at 212 nm and then compared to areas at 260 nm, it was concluded that sample D3 did not contain daidzein or genistein.

Daidzein has a greater potential for incorrect identification than genistein. Therefore, the presence or absence of genistein is a better indicator of soy consumption. The presence of both daidzein and genistein peaks; however, is the best overall indication of soy consumption when

monitoring at single or dual wavelengths. Single wavelength detection is inadequate for making decisions regarding compliance when trace amounts of daidzein or genistein may be present in the urine.

Experiment 1 showed that detection of daidzein and genistein at 260 nm is less specific than dual wavelength detection at 212 nm and 260 nm. Therefore, compliance monitoring in subjects that were instructed to consume soy or instructed to abstain from soy containing foods was done with dual wavelength detection of daidzein and genistein. Table 4 shows that 96% of the samples from subjects consuming the soy protein supplements had daidzein and genistein present in urine. In some instances urine was collected from subjects that had been fasted for 12 - 14 hours because they also had blood drawn for lipid analysis during the same visit to the clinic. Subjects who consume one packet of soy protein in the morning and one in the evening would most likely have detectable levels of genistein in the urine even when fasted for 12 - 14 hours. However, urinary genistein may be difficult to detect if an individual has unusually fast clearance of isoflavones from the body. At this time we cannot determine if samples from subjects without genistein in the urine were non-compliant or if the subjects had a faster rate of genistein clearance and we were simply unable to detect genistein in the urine.

There was no detectable genistein in 23 of 28 samples collected from subjects consuming the casein supplement. Two samples (from separate subjects) had peaks that eluted at the same time as genistein. The samples also contained peaks which were at the retention times for daidzein. Both samples had characteristic profiles of soy consumption when evaluated at both wavelengths (212 and 260 nm). At this time we cannot determine if the subjects consumed a soy product

Table 4. Urinary genistein from subjects consuming soy isolate or casein supplement

Treatment	soy isolate	casein
# of samples from subjects consuming	98	28
(+) for Genistein	94	2
(-) for Genistein	4	26

such as tofu, miso, or soy milk or if a compound with similar retention time to genistein is excreted by some individuals. Three subjects were eliminated as being positive for soy consumption as evaluated by dual wavelength detection. One subject had two samples with peaks that matched daidzein and genistein at 260 nm. Evaluation at 212 nm revealed large areas of absorbance at the retention times for daidzein and genistein and were not at all character-

istic of soy consumption (non-proportional). The final sample contained a peak for genistein only and was characteristic at both wavelengths. The sample did not contain any indication of daidzein at either wavelength.

Even though genistein makes up the largest quantity of isoflavone present in the supplement it is not the predominant isoflavone excreted in urine (Kelly et al. 1993 ;Xu et al. 1994). Daidzein is excreted at levels 2-3 times that of genistein even when daidzein is present at lower levels than genistein. Differences in the excretion pattern might be due to the glucoside profiles of each compound. Daidzein is present in the supplement as 23.5% aglycone and genistein present as 4.94% aglycone. Preferential absorption of the aglycone may account for some of the difference in excretion rates. However, another possibility might due to differences in partitioning to the bile. Molecular weight differences have been associated with different rates of partitioning between urinary or biliary secretion (Sipes & Gandolfi 1991). It is possible that genistein is within a molecular weight range that allows it to be partitioned to the bile. Genisteins partitioning to the bile due to a higher molecular weight would make it less evident in the urine when compared to daidzein.

Urine samples from subjects in the clinical study are still being received. Supplement packets returned are

counted. Evaluating subject compliance based on actual packet consumption will help in eliminating questions of compliance. Supplement consumption history may also help to evaluate compliance test results by establishing when supplements were consumed. Interviews with subjects assigned casein supplements might also help to confirm if patients consumed soy containing foods.

Utilization of genistein as a compliance monitor for soy consumption would be best utilized for non-fasting subjects. Regular consumption of soy could be readily detected in subjects consuming soy foods as a regular part of the diet. Daidzein is best utilized as a secondary indicator of soy consumption when multiple peaks on a chromatogram make genistein identification less distinquishable.

Recommendations For Future Research.

Daidzein is metabolized to equol in some human subjects (Adlercreutz et al. 1995; Kelly et al. 1995). Equol possess estrogenic activity (Molteni et al. 1995) and may influence estrogenic processes, such as the menstrual cycle and the maturation process in children, as well as the formation of estrogenic dependant breast cancer. The induction of estrogenic effects has been documented in animals (Shutt & Braden 1968) but effects on humans have not been thoroughly investigated. Individuals that have the ability to metabolize daidzein to equol may be of interest in understanding the estrogenic effects or lack thereof in humans.

The metabolism of genistein in humans has not been clarified. The formation of a compound similar to that formed from daidzein via microbial metabolism (equol) is possible. A compound such as 5-hydroxy equol (4',5,7 isoflavandiol) formed from genistein is likely, but has yet to be identified. It is possible that this compound has shown up as peaks on HPLC chromatograms and need only be confirmed. This compound might also have estrogenic activity that may exceed that of genistein, daidzein or equol. Identification of all possible metabolites of the isoflavones daidzein and genistein would further our understanding of some very bioactive compounds.

APPENDICES

APPENDIX A

INFORMED CONSENT

TITLE: Absorption and Excretion of Genistein

Investigators: R. Grabiel and M. R. Bennink

Source of Support: Department of Food Science & Human Nutrition

Description: If I agree to participate in this study, I will consume 1-4 packets of a soy protein beverage supplement. The reconstituted beverage is essentially soy milk. The supplement has been received directly from Soy Protein Technologies and are available commercially as "Altima". Maximum intake of 4 packets of the supplement is equal to 1.5 chicken breasts in protein content.

Risks: Dr. Bennink has explained the risks and possible complications associated with consuming 19.0 - 76.0 g of soy isolate protein in one meal. I understand that the most likely side effects of consuming soy protein are possible allergenic reaction and abdominal bloating due to drinking 8 to 32 oz of liquid. I also am aware that drawing blood by venipuncture could cause discomfort, bruising, infection or clotting at the blood drawing site.

I understand that breast feeding or pregnant individuals will not be able to take part in this study. If I become pregnant, my participation in the study will be ended.

Testing: I understand that I will be required to collect all urine for the 24 hour period following consumption of the soy supplement. In addition, I will be required to consume 2 packets of the supplement (12 hours apart) for one week and on the eighth day, four blood samples will be drawn, one every three hours (40 ml of blood in total). All urine will be collected during that same 12 hour period. Urine and blood samples will be analyzed for genistein and its metabolites. All results and findings are to be confidential with all subject identities remaining anonymous in any and all reports. Upon request, results of the study will be made available to me.

Costs: I understand that I will not incur any costs as a subject in this study, except possibly as outlined in the **"compensation for illness or injury"** section.

APPENDIX A (continued)

Payments: I understand that I will be paid $10.00 for each day that I am assigned to collect urine specimens and that payment will be received upon delivery of the specimens. In addition, I will receive $5.00 per blood sample with payment at the end of the 12 hour period when blood samples are taken.

Right to withdraw: I understand that I am free to refuse to participate in this study or withdraw at any time and that my decision will not adversely affect evaluation of my academic performance.

Compensation for Illness or Injury: I understand that if I am injured as a result of my participation in this research project, Michigan State University (or my health care provider) will provide emergency medical care if necessary, but these and any other medical expenses must be paid from my own health insurance program. I may contact Dr. Bennink at Michigan State University (353-9512) regarding questions about the research and what to do in the event of a research related injury. After 5:00 pm and on weekends, I can call Michigan State University answering service to reach the "on-call" physician at 355-4757. Any questions concerning my rights as a research subject will be answered by the University Committee on Research Involving Human Subjects (UCRIHS) at 355-2180.

Voluntary Consent: I have read this consent form and understand it. I have been given the opportunity to ask questions, and have received answers concerning any information which I said I did not understand. I willingly give my consent to participate in this study. I understand that I am free to contact the principal investigators of this study regarding any additional questions. I understand that after this consent is signed, I will be given a copy for my own information.

Signature of subject_____ Date___/___/___

Signature of witness_____ Date___/___/___

Signature of Investigator_____ Date___/___/___

APPENDIX B

SI Packet Consumption Questionnaire

Name_____ **Date** _____

Have you consumed any products containing soy or soy based derivatives within the last (4) days? check one: **Yes**____**No**____

(If yes, please note products and time consumed on back of this sheet).

Please Note: One urine sample is required prior to SI Packet consumption.

Before Sample: Date _____Time_____

Volume_____Sample #_____

All specimens are to be collected in individual containers with a minimum void time of 2 hours between samples.

SI Packet consumption should occur between the hours of 7 - 9 am with the collection of one urine sample before (note above).

(All packets should be consumed within 40 minutes).

SI Packets Consumed: Date _____ Time _____ # packets ____

Were all packets assigned consumed within 40 minutes (please limit time between packets to 10 mins)? check one: **Yes**____**No**____

(Please note the times the individual packets were consumed -

#1 _____ #2 _____ #3 _____ #4 _____)

Please Note: All urine samples must be collected immediately after the consumption of the SI Packets and continuously for 24 hours!

Sample collection times, volumes and randomly assigned container numbers are to be recorded on the following page (sample containers have been prelabelled for your convenience).

APPENDIX C

Metabolite Collection Record

Name_____ **Date**_____

Specimen Collection Table:

Time _____ Volume _____ Sample # _____

Time _____ Volume _____ Sample # _____

Time _____ Volume _____ Sample # _____

Time _____ Volume _____ Sample # _____

Time _____ Volume _____ Sample # _____

Time _____ Volume _____ Sample # _____

Time _____ Volume _____ Sample # _____

Time _____ Volume _____ Sample # _____

Time _____ Volume _____ Sample # _____

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Time _____ Volume _____ Sample # _____

Time _____ Volume _____ Sample # _____

Time _____ Volume _____ Sample # _____

Time _____ Volume _____ Sample # _____

APPENDIX D

DAILY URINARY EXCRETION OF ISOFLAVONES(AS AGLYCONES).

Subject/Dose	SEX	AGLYCONE EXCRETED (24 hour total) DAIDZEIN (mg)	DAIDZEIN (umol)	GENISTEIN (mg)	GENISTEIN (umol)	% OF AGLYCONE CONSUMED* DAIDZEIN	GENISTEIN
SUBJECT A	(F)						
1		4.76	18.71	2.68	9.93	82.59	184.2
2		7.80	30.67	4.85	17.95	67.71	166.5
3		24.06	94.62	13.54	50.08	139.2	309.6
4		17.07	67.13	11.03	40.80	74.07	189.2
SUBJECT B	(M)						
1		4.68	18.41	0.93	3.42	81.24	63.46
2		5.31	20.89	0.55	2.04	46.12	18.94
3		13.11	51.56	3.43	12.67	75.84	78.36
4		15.99	62.87	6.45	23.85	69.37	110.6
SUBJECT C	(F)						
1		2.45	9.63	0.16	0.58	42.50	10.78
2		3.60	14.15	0.93	3.43	31.25	31.85
3		6.21	24.43	0.87	3.21	35.93	19.84
4		12.44	48.91	2.16	7.98	53.96	37.00

APPENDIX D (continued)

SUBJECT D (M)						
1	6.88	27.03	5.39	19.93	119.3	369.7
2	13.34	52.46	7.69	28.43	115.8	263.7
3	18.86	74.15	10.94	40.47	109.1	250.2
4	18.52	72.81	10.88	40.25	80.33	186.6
SUBJECT E (F)						
1	3.80	14.94	0.74	2.74	65.93	50.82
2	6.14	24.14	0.93	3.44	53.30	31.86
3	11.79	46.37	2.06	7.61	68.21	47.02
4	17.19	67.60	1.95	7.21	74.59	33.42
SUBJECT F (F)						
1	4.43	17.40	0.91	3.38	76.81	62.69
2	7.22	28.39	1.61	5.97	62.68	55.37
3	15.48	60.88	3.94	14.89	89.55	90.21
4	17.51	68.86	3.89	14.79	75.97	66.67
SUBJECT G (M)						
1	5.66	22.27	2.24	8.29	98.28	153.8
2	9.17	36.05	1.94	7.18	79.57	66.56
3	12.19	47.95	2.35	8.69	70.53	53.75
4	18.11	71.22	3.52	13.02	78.58	60.38

APPENDIX D (continued)

SUBJECT H (M)						
1	3.69	14.50	0.86	3.19	64.01	59.12
2	9.70	38.15	4.50	16.64	84.22	154.4
3	7.73	30.38	1.79	6.62	44.69	40.92
4	11.55	45.41	2.97	11.00	50.10	51.00

*% = (excreted aglycone mg/ # packets(5.762 mg daidzein/packet or 1.457 mg genistein/packet)*100. Assumes 100% absorption of aglycones present.

Isoflavone Totals (as aglycones).

	Daidzein	Genistein	Total D & G
1	5.7624 mg/pkt	1.4574 mg/pkt	7.220 mg/pkt
2	11.52	2.9148	14.44
3	17.29	4.3722	21.66
4	23.05	5.8296	28.88

APPENDIX E

Total urine samples collected per treatment.*

SUBJECT	Dose 1	2	3	4	TOTAL
A	10	9	11	10	40
B	12	12	14	13	51
C	8	8	9	6	33
D	10	10	10	12	42
E	10	10	10	12	42
F	12	14	12	13	52
G	19	16	14	13	62
H	11	11	10	12	44

*Number of samples collected includes one pre-treatment void per treatment.

APPENDIX F

Total urine volumes collected per treatment.*

SUBJECT	Dose 1	2	3	4
A	2093	1177	1576	1632
B	1440	2069	1389	1719
C	3203	2408	3590	2425
D	2278	3176	2625	2590
E	951	1118	1373	1584
F	1117	2116	1975	2550
G	5705	4348	3350	2361
H	1377	1638	1485	1387

* Volumes are for all collections after supplement consumption and do not include pre-treatment voids.

APPENDIX G

Total output of daidzein for 24 hour recovery (umole of aglycones).

SUBJECT	Dose 1	2	3	4
(umol)	(96.38)	(192.8)	(289.1)	(385.5)
A	18.7	30.7	94.6	67.1
B	18.4	34.9	51.6	62.9
C	9.63	14.2	24.4	48.9
D	27.0	52.5	74.2	72.8
E	14.9	24.1	46.4	67.6
F	17.4	28.4	60.9	68.9
G	22.3	36.1	47.9	71.2
H	14.5	38.2	30.4	45.4
ave =	17.9	30.6	53.8	63.1
SE =	1.85	4.17	8.06	3.65
SD =	5.24	11.8	22.8	10.3

APPENDIX I

24 hour urinary daidzein recovery (%).

SUBJECT	Dose 1	2	3	4
(umol)	(96.38)	(192.8)	(289.1)	(385.5)
A	19.4	15.9	32.7	17.4
B	19.1	18.1	17.8	16.3
C	9.99	7.34	8.45	12.7
D	28.1	27.2	25.7	18.9
E	15.5	12.5	16.0	17.5
F	18.1	14.7	21.1	17.9
G	23.1	18.7	16.6	18.5
H	15.0	19.8	10.5	11.8
ave =	18.5	16.8	18.6	16.4
SE =	1.93	2.05	2.79	0.94
SD =	5.46	5.80	7.89	2.67

APPENDIX J

24 hour urinary genistein recovery (%).

SUBJECT	Dose 1	2	3	4
(umol)	(109.1)	(218.2)	(327.3)	(436.4)
A	9.07	8.25	15.3	9.35
B	3.13	2.10	3.88	5.48
C	0.53	1.57	0.98	1.64
D	18.2	2.91	2.30	1.64
E	2.51	1.58	2.32	1.65
F	3.10	2.74	4.46	3.30
G	7.60	3.29	2.66	2.98
H	2.92	7.61	2.02	2.52
ave =	5.88	3.76	4.24	3.57
SE =	2.02	0.94	1.63	0.94
SD =	5.72	2.65	4.60	2.67

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