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Isoflavone Urine Kinetics after Giving Soymilk to Healthy Adults in Taiwan

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Abstract

This study was aimed to investigate the urine kinetics of isoflavones after oral intake of soymilk in healthy adults in Taiwan. Nine volunteers received 800 mL of soymilk equivalent to 200 g of soybeans and the urine were collected from 0 to 48 h. Daidzein, equol and genistein in urine were assayed by HPLC before and after hydrolysis with β-glucuronidase and sulfatase. The results revealed that the parent forms of daidzein and genistein were not detected in the urine with major forms being the glucuronides (G) and sulfates (S), mainly G. Besides, equol G and S, the metabolites of daidzein, were detected in the urine of all subjects. In conclusion, G and S of daidzein and genistein, mainly G, were excreted in the urine of healthy adults in Taiwan after ingestion of soymilk. In addition, G and S of equol were found in urine of all nine subjects.

Introduction

Dietary flavonoids, a class of semi-essential food components[1], have long been thought to exert protective effects against many diseases, in particular cardiovascular disease and even cancer[2]. Soybean, an essential part of Asian diet, is a rich source of isoflavones, a subfamily of flavonoids. There are many epidemiological reports describing the health effects of soy-rich food on the prevention of estrogen-related cancers, cardiovascular diseases and osteoporosis[3]. Due to the adverse effect of hormone replacement therapy (HRT), isoflavone supplements are becoming popular alternatives for HRT[4].

Soymilk at breakfast has long been a habit for many Chinese people. The popularity of soymilk has also spread to the West as a substitute for cow milk. Soymilk contains several isoflavones including daidzin, genistin, daidzein and genistein, all of which are possibly beneficial to health[3,4]. The *in vitro* activities of soy isoflavones have been shown to include chemoprevention[5-7], antioxidation[8-10]and estrogenic effect[11,12]. In addition, isoflavone metabolites were found to be weakly estrogenic and capable of activating human natural killer cells[13,14]. Furthermore, recent *in vivo* studies have found that soy isoflavones increase the bone mass in osteoporotic mice[15], and suppress the growth of colon tumors in rats[16], as well as improve aging and Alzheimer's disease in mice[17].

The absorption of daidazin and genistin through intestine is limited because of its poor lipophilicity, while cleavage of the sugar moiety by enteral microflora converted them into more lipophilic aglycones which are absorbable by the intestine. Moreover, the aglycones daidzein and gensitein were then subject to further metabolism[18,19]. Despite many studies reporting the metabolism and bioavailability of soy isoflavones in adults after intake of soy products[12,20-22], there is limited information concerning isoflavone pharmacokinetics of soymilk in Chinese subjects[23,24]. This study investigated the urine kinetics of isoflavones in healthy adults in Taiwan given soymilk.

Materials and Methods

Subjects

Nine healthy volunteers (6 males and 3 females), 22 - 57 years old and weighing 52 - 72 kg, provided their informed consents. Routine biochemical tests indicated that their hepatic and renal functions were in good condition. In this study, all of the subjects took regular diets including soy products once a while. Only one of them was habitual drinker of soymilk. They did not smoke or drink alcoholic beverages and have not taken soy products for at least 2 weeks before and throughout the experiment. The present study was conducted according to the guidelines in the Declaration of Helsinki, and all protocols involving human subjects were approved by the Medical Ethics Committee of China Medical University.

Chemicals

Daidzin (purity 98%), daidzein (purity 98%), equol (purity 99%), genistin (purity 98%), genistein (purity 98%), ethylparaben (purity 99%), 5,7-dimethoxycoumarin (purity 98%), sulfatase (Type H-1, 14,400 units/g, from *Helix pomatia*, containing 574,000 unit/g of β-glucuronidase) and β-glucuronidase (type B-1, 666,400 units/g, from bovine liver) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). L(+)-Ascorbic acid was obtained from RdH Laborchemikalien GmbH & Co. KG (Seelze, Germany). Milli-Q plus water (Millipore, Bedford, MA, USA) was used for

all preparations. Acetonitrile, methanol and ethyl acetate were of LC grade and purchased from Mallinckrodt Baker, Inc. (Phillipsburg, NJ, U.S.A.). Other reagents were of HPLC grade or analytical reagent grade.

Instrumentation

An HPLC apparatus (Shimadzu LC-10AT, Japan) was equipped with an autoinjector (SIL-10A) and a photodiode array detector (SPD-M 10AVP). The column used is a Cosmosil C18 (5 μm, 250×4.6 mm) with a guard column (MetaGuard 4.6 mm Polaris 5 μm C18-A, MetaChem, Torrance, USA).

Preparation of soymilk and quantitation of isoflavones

Soybeans (3 kg) were macerated in water for 12 h and then homogenized with 12 L water by using a food processor. The homogenate was filtered with cloth, and the filtrate was heated with stirring for 30 min and kept simmering for 10 min; then sufficient hot water was added to make 12 L. Soymilk (3.0 mL) was mixed with 7 mL of 60% acetonitrile and centrifuged, the residue was extracted again with 10 mL of 60% acetonitrile with ultrasonic shaking for 30 min[25]. The extracts were combined and 60% acetonitrile was added to make 20 mL and then frozen at -30°C pending analysis. The mobile phase of HPLC comprised 0.1 % phosphoric acid (A) and acetonitrile (B) with gradient program as follows: A/B: 89/11 (0 min), 82/18 (10 min), 50/50 (20 min), 89/11 (35 min). The UV detector was set at 250 nm and the flow rate

was 1.0 mL/min.

Daidzin, daidzein, genistin and genistein were accurately weighed and dissolved in methanol to afford a series of standards in the concentration range of 7.8 - 100.0 μg/mL. An equal volume of internal standard solution (5,7-dimethoxycoumarin in methanol, 100.0 μg/mL) was added to each standard to afford a final concentration of 50.0 μg/mL. Calibration curves were plotted by linear regression of the peak - area ratios (daidzin, daidzein, genistin and genistein to internal standard) against concentrations of correspondent isoflavone.

The precision and accuracy of the assay method were evaluated by intra-day and inter-day analysis of triplicate standards in methanol at 7.8 - 100.0 μg/mL within one day and over a period of three consecutive days. A recovery study was carried out by spiking appropriate amounts of daidzin, daidzein, genistin and genistein into soymilk, which has been quantitatively analyzed in triplicates to afford additional concentrations of 50, 25 and 6.25 μg/mL, respectively.

Soymilk ingestion and urine collection

After overnight fasting, each volunteer ingested 800 mL of warm soymilk (equivalent to 200 g of soybeans) within 30 min. Food was withheld for another 3 h. Urine samples were collected before and over 0 - 2, 2 - 4, 4 - 6, 6 - 8, 8 - 10, 10 - 12, 12 - 24, 24 - 36 and 36 - 48 h period after intake of soymilk. In each collection time

interval, the volume of pooled urine was recorded and 20 mL of homogeneous aliquots were stored at – 30 °C before analysis.

Quantitation of the parent forms and isoflavone metabolites

The parent forms and conjugated metabolites of isoflavones in urine were determined before and after treatments with β-glucuronidase and sulfatase,. For the quantitation of glucuronides (G), 400 µL urine was added with 100 µL β-glucuronidase (1,000 units/mL) in acetate buffer, pH 5, and 100 µL ascorbic acid (200 mg/mL), and incubated at 37℃ for 4 h. For the quantitation of sulfates/ glucuronides (S/G), 100 µL sulfatase (1,000 units/mL containing 39,861 unit/mL β-glucuronidase) and 100 µL ascorbic acid (200 mg/mL) were added to 400 µL urine and incubated at 37℃for 2 h. After hydrolysis, 600 µL of the mixture was partitioned with 600 µL of ethyl acetate (containing 5.0 µg/mL of ethyl paraben as internal standard). The ethyl acetate layer was evaporated under nitrogen to dryness and resolubilized with 50 µL of mobile phase which comprised 0.1% phosphoric acid (A), acetonitrile (B) and methanol (C) with A:B:C= 64:26:10.

For calibrator preparations, blank urine was spiked with various standard solutions of daidzein, equol and genistein to afford urine standards in concentration range of 0.6 - 20.0 µg/mL. The pretreatment of urine standards and HPLC condition were identical with those described above for urine samples except the addition of

enzyme, which was substituted with equal volume of pH 5.5 buffer. The calibration curves for urine were plotted by linear regressions of the peak area ratios of daidzein, equol and genistein to internal standard against concentrations of each isoflavone.

Validation of assay method for urine

The precision and accuracy of the analytical method was evaluated by intra-day and inter-day analysis of triplicate urine standards within one day and over a period of three days. By spiking daidzein, equol and genistein into blank urine and water in triplicates to afford concentrations of 2.5, 5.0 and 10.0 μg/mL, the recoveries of each isoflavone were evaluated by comparing their concentrations obtained from spiked urine to the corresponding ones from spiked water. LOQ (Limit of Quantitation) represents the lowest concentration of analyte that can be determined with acceptable precision and accuracy with coefficients of variation and relative errors below 15.0% and 20%, respectively. LOD (Limit of Detection) represents the lowest concentration of analyte that can be detected with $S/N > 3$.

Data analysis

Owing to the presence of considerable amount of β-glucuronidase in the sulfatase used in this study, treatment of sulfatase hydrolyzed both G and S. Accordingly, the concentrations of S were obtained from the difference of released aglycones between treatments with sulfatase and glucuronidase. The amount of G and

S of daidzein, equol and genistein excreted in the specific sampling time was calculated by multiplying their concentrations by respective urine volume collected in each time interval. The renal excretion rates were calculated by dividing the urinary recovery over collection time. The apparent elimination rate constants were estimated by Sigma–Minus method in which the slope of the regression line was obtained when the natural logarithm of the amount not yet excreted was plotted versus time and then transformed into half-lives.

Results

Daidzin, genistin, daidzein and genistein in soybean milk as well as the internal standard were well resolved within 35 min as shown in Fig. 1. Good linearity were shown in the range of 7.8 to 100.0 μg/mL for daidzin, genistin, daidzein and genistein. Validation of this assay method indicated that all coefficients of variation for intraday and interday analysis were below 5.0%, and the relative errors were below 8.6%. The recoveries of daidzin, genistin, daidzein and genistein from soymilk were 89.1 - 97.2%, 89.1 - 90.4%, 87.0 - 94.2% and 81.9 - 87.1 %, respectively. Quantitation results indicated that 800 mL of soymilk contained daidzin 96.9 μmol (40.3 mg), daidzein 65.1 μmol (16.6 mg), genistin 254.0 μmol (109.7 mg) and genistein 58.4 μmol (15.8 mg).

The chromatograms of urine extract before and after enzymolysis were shown in Fig. 2~~,~~. Good linearities of daidzein, equol and genistein in the range of 0.6 - 20.0 μg/mL. All coefficients of variation for intraday and interday analysis were less than 9.3%, and the relative errors were below 9.6%. The recoveries of daidzein, equol and genistein from urine were 94.3 - 100.5%, 94.7 - 95.4% and 94.3 - 96.5%, respectively. The limits of quantitation (LOQ) were 0.1 μg/mL, 0.2 μg/mL, 0.2 μg/mL, and the limits of detection (LOD) were 0.02, 0.03, 0.03 μg/mL for daidzein, equol and genistein, respectively.

Using the present protocol of urine analysis, no free forms of daidzein, equol and genistein were detected. The concentrations of the G and S of daidzein, equol and genistein were determined by HPLC through enzymolysis. Our study found the optimal time needed for the hydrolysis with G and S were 4 h and 2 h, respectively, in the presence of ascorbic acid. The G and S of daidzein, equol and genistein were detected in the urine of all volunteers. The cumulative urinary recoveries of G and S of each isoflavone over 48 h were shown in Fig. 4. Most G and S of daidzein and genistein were excreted during the first 12 hours, whereas the majority of equol G and S were eliminated during 6-36 h.

The urinary excretion amount and the elimination half-lives of the G and S of daidzein, equol and genistein were shown in Table 1. The amounts of isoflavone conjugates ranked in the order of daidzein G＞daidzein S＞genistein G＞equol G＞ equol S, genistein S. The G of daidzein and genistein were about 2 folds of their correspondent S. The total excretion of daidzein G/S were significantly higher than that of genistein G/S by 217.9%. The excretion amount of equol G/S was 26.1% of daidzein G/S, and comparable with genistein G/S. The excretion ratios of daidzein G and daidzein S, equol G and equol S accounted for 78.3% and 38.9%, 17.8% and 12.7% of the total intake of daidzin/daidzein, whereas genistein G and genistein S were 12.8% and 6.3% of the total intake of genistin/genistein, respectively. The mean elimination

half-lives of daidzein G, daidzein S, genistein G and genistein S were 4.9 h, 6.5 h, 5.7 h and 7.6 h, respectively, while those of equol G and equol S were 9.9 h and 13.3 h, respectively.

Discussions

The present study is the first work to illustrate the urine kinetic profiles of G and S of isoflavones after ingestion of soymilk by adults in Taiwan. The result indicated that the parent forms of daidzein and genistein were not present in human urine, whereas the G and S of daidzein, equol and genistein in urine were predominant, which echoes previous reports[19,27].

The cumulated urinary excretion of daidzein G/S was 3-fold of genistein G/S, although the content of genistin/genistein was 2-fold of daidzin/daidzein in the soymilk, which was consistent with related studies[20,27,28]. Contrary to the findings in urine, previous studies have shown that the concentrations of genistein G/S in serum were consistently higher than those of daidzein G/S[29,30]. The higher excretion of daidzein G/S than those of genistein G/S in urine can be accounted for by a greater fractional excretion of the latter via bile. The difference in excretion pathway may explain the lower recovery of genistein G/S from urine. The total urinary excretion of daidzein G/S and equol G/S was found higher than the intake amount of daidzin/daidzein, which can be explained by the presence of other ester derivatives of daidzein in soymilk in addition to daidzin, such as malonyl daidzin and acetyl daidzin[25].

Following ingestion of soymilk, G of daidzein and genistein were found significantly higher than corresponding S in urine, especially during 0-8 h as shown in Fig. 3. This result was in good agreement with a previous women study, which reported that the ratios of G were 73% and 71% of the total daidzein and genistein excreted in urine[31]. Furthermore, previous studies have pointed out that G of daidzein and genistein were the major forms in the circulation, confirming that glucuronidation and sulfation demonstrated marked selective kinetics of conjugation for daidzein and genistein[32,33].

Equol was not a constituent in soymilk and detected after incubation of daidzein with fecal bacteria under anoxic condition, bringing to light that equol was a metabolite of daidzein in gut lumen[34]. Equol underwent extensive conjugation metabolism and the excretion of equol G/S lagged behind daidzein G/S and genistein G/S. The half lives of equol G/S were longer than those of daidzein G/S and genistein G/S. The amount of equol S excreted in urine was comparable with that of equol G. An *in vitro* cell line study has reported that 7-hydroxy group of daidzein and genistein was the main site for glucuronidation, whereas the 4'-hydroxy group was the only site for sulfation[35]. In regard to the physical properties, the pKa values of 7-hydroxy group of daidzein, equol and genistein were 7.5, 9.8 and 7.8, respectively[36]. The weaker acidity of equol makes the relative lower glucuronidation of equol than daidzein and genistein explicable.

In this study, equol G/S were detected in the urine of all nine subjects. In contrast, previous studies have reported that the prevalences of equol-producer in Japanese, Korean and Chinese were 46%, 59% and 60%, respectively[24], whereas only 14-34% of equol producers was reported in Caucasian[24,37]. In addition, the proportion of equol producers in postmenopausal Japanese women was 55% (six out of 11 subjects)[18]. Because this study only included nine volunteers, whether the adults in Taiwan are 100% equol-producers needs to be confirmed by future study. Asian populations have higher proportion of equol-producer than Western populations was suspected to be associated with habitual diet. For instance, the equol-producers had a higher intake of carbohydrates, phytoproteins and fiber than those of non equol-producers[21,38,39]. In addition, vegetarians had much higher percentage of equol-producers than nonvegetarians in Japan[40]. We thus speculate that adults in Taiwan often having high intake of soy products in their daily diets might result in more equol-producers.

For equol-producers, besides prevention of bone loss and fat accumulation in early postmenopausal women[41], a reduced risk of breast cancer has been reported in premenopausal women[42]. Moreover, epidemiology studies of Asian and also some Mediterranean countries discovered that equol-producers had lower incidence of

prostate cancer[37,43]. We therefore speculate that equol G/S might contribute beneficial effects on hormone related diseases.

In conclusion, daidzein G/S and genistein G/S were the major isoflavones excreted in the urine of Taiwan adults after ingestion of soymilk. Equol G/S, the metabolites of daidzein, were produced in all nine subjects.

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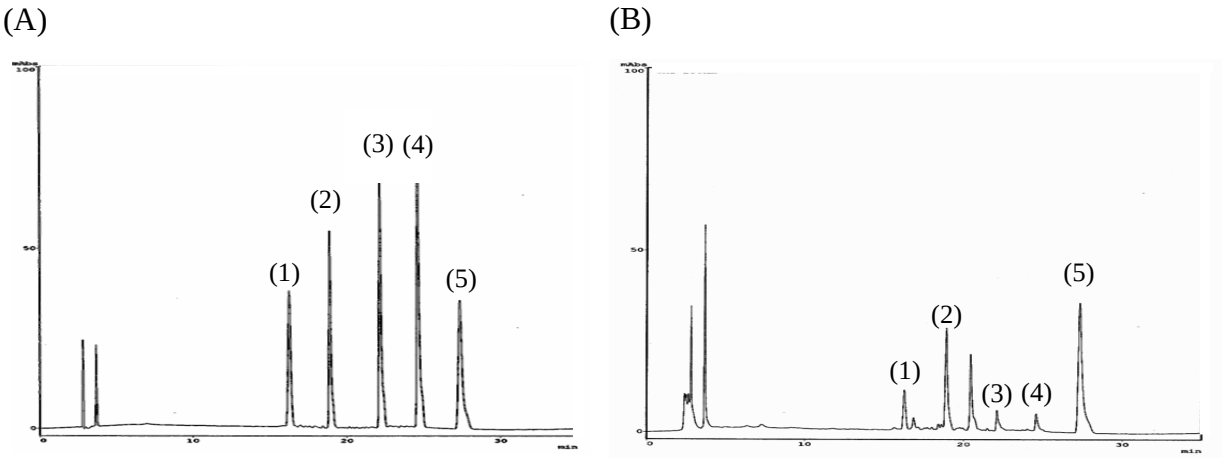

Fig. 1 Chromatograms of standards (12.5 μg/mL) in methanol (A) and soymilk sample (B).

1: daidzin (16.5 min), 2: genistin (19.5 min), 3: daidzein (22.0 min), 4: genistein (24.9 min), 5: 5,7-dimethoxycoumarin (internal standard, 27.5 min).

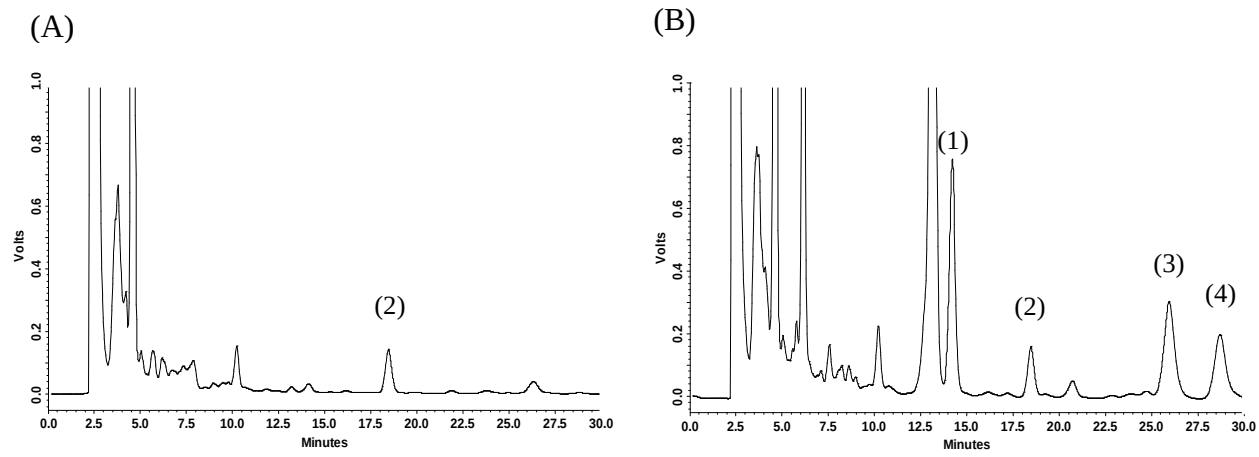

Fig. 2 Chromatograms of an urine sample before hydrolysis (A) and after hydrolyzed with sulfatase/glucuronidase (B).

1: daidzein (18.6 min), 2.: ethylparaben (internal stardard, 14.4 min), 3: equol (26.1 min), 4: genistein (28.8 min).

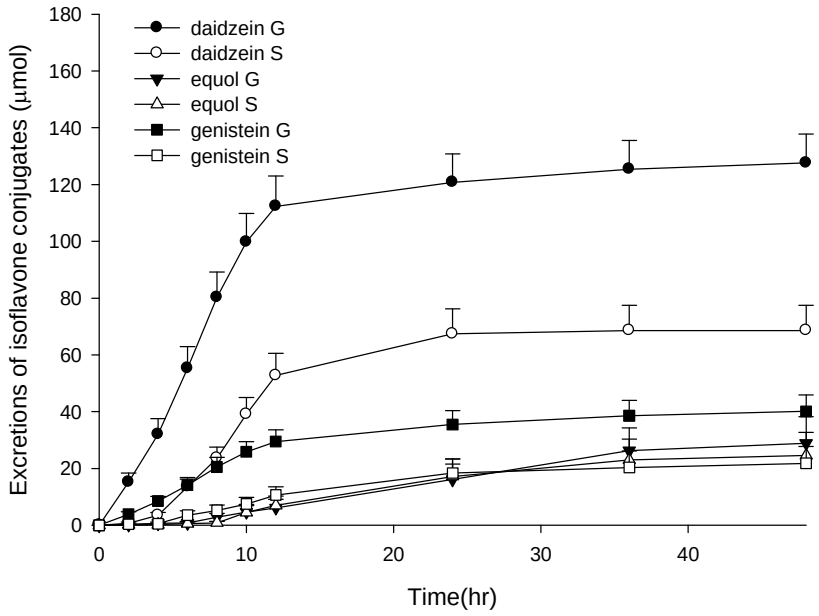

Fig. 3 Mean (±SE) cumulative urinary recoveries of glucuronides (G) and sulfates (S) of daidzein, equol and genistein after intake of 800 mL soymilk in nine adults.

Table 1. Urinary excretion of isoflavones after intake of 800 mL soymilk in nine volunteers.

	Amount excreted (μmol)	% in intake dose[a]	$T_{1/2}$ (h)
Daidzein G	126.9 ±10.2*	78.3	4.9 ±1.1
Daidzein S	63.0 ±10.8	38.9	5.7 ±1.1
Equol G	28.9 ±9.3	17.8	9.9 ±1.8
Equol S	20.6 ±8.5	12.7	13.3 ±4.5
Genistein G	40.1 ±5.7*	12.8	6.5 ±1.2
Genistein S	19.5 ±6.3	6.3	7.6 ±1.7

Data expressed as Mean ± SE

G: glucuronides

S: sulfates

* $P<0.05$, compared to corresponding S

[a] 800 mL of soymilk contained daidzin 96.9 μmol, daidzein 65.1 μmol, genistin 254.0 μmol and genistein 58.4 μmol. The intake dose was based on the total amount of daidzin/daidzein or genistin/genistein.