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Research Article

Effect of glycosidation of isoflavones on their bioavailability and pharmacokinetics in aged male rats

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There are limited reports on the bioavailability and pharmacokinetics of isoflavones in elderly humans and aged animals. The present study was conducted to assess the effect of glycosidation of isoflavones on their bioavailability and pharmacokinetics in aged (20 month old) male Fischer-344 (F-344) rats. The F-344 rat, developed by the National Institute on Aging, is an inbred rat model that is commonly used for aging studies and resembles many features of aging humans. Three sources of isoflavones; Novasoy™ (a commercial supplement), a mixture of synthetic aglycons (daidzein, genistein and glycitein), and a mixture of synthetic glucosides (daidzin, genistin, and glycitin) were tested. Following administration, blood samples were collected at different times (0–48 h post-oral gavage and 0–8 h post-IV dosing). Plasma isoflavones and 7-hydroxy-3-(4′-hydroxyphenyl)-chroman (a metabolite of daidzein) were measured by LC/MS. The extent of absorption was determined by comparing the area under the curve (AUC) of the plasma-concentration time curve after intravenous (IV) administration with that following oral administration. The extent of bioavailability was then calculated as: $\%\text{bioabailability} = (AUC_{or}/AUC_{IV}) \times (Dose_{IV}/Dose_{or}) \times 100$. Bioavailabilities for genistein were significantly ($p = 0.013$) higher for the aglycon (35 ± 9%) compared with the glucoside forms (11 ± 3%). In contrast, the bioavailabilities for glycitein were significantly ($p = 0.011$) higher in Novasoy (27 ± 13%) and the glucoside form (21 ± 10%) compared with the aglycon (8 ± 3%). No significant differences in the bioavailability of daidzein were observed in aged rats dosed with aglycon, glucoside or Novasoy. However, aged rats were able to produce equol as early as 8 h post-dosing. In summary, the source of isoflavones had significant effects on genistein and glycitein bioavailability in aged male rats.

Keywords: Bioavailability / Isoflavones / LC-MS / Plasma Pharmacokinetics

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1 Introduction

Phytoestrogens are a diverse group of polyphenolic plant compounds with hormonal activity and structural similarity to estrogen [1]. Three main classes of phytoestrogens are isoflavones, lignans, and coumestans [1, 2]. Isoflavones exist primarily in soybeans, in most soy foods and soy infant formula as a complex mixture of conjugated and unconjugated isoflavones. The β-glucosides, genistin, daidzin, glycitin and the 6′-*O*-malonyl glucosides and 6′-*O*-acetyl glucoside of genistein, daidzein and glycitein [3, 4]. The absorption, distribution, metabolism, and excretion of isoflavones from glucoside and aglycon forms have been investigated in animals and humans [5–12]. After inges-

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tion, the glucoside forms of isoflavones are hydrolyzed to the aglycon form in the jejunum [11]. The released aglycon forms of isoflavones are either absorbed into the enterocytes where extensive glucuronidation occurs or further metabolized by intestinal microflora into several products, including equol (7-hydroxyl-3-(4′-hydroxyphenyl)-chroman) a metabolite of daidzein, *p*-ethyl phenol, a metabolite of genistein and di-hydroglycitein, a metabolite of glycitein before absorption [11].

It is believed that dietary intake of soy isoflavones provides a number of health benefits [1]. Epidemiological studies suggest that Southeast Asians, who consume a soy-based diet, experience lower incidences of prostate cancer [13], cardiovascular disease [14], osteoporosis [15], age related disease, and inflammation than do Eastern and Western populations [1, 16, 17]. Within a few generations migrants to the West experience an increase in the incidence of cancer, approaching the rates seen in Western populations. Epidemiological studies, therefore, support the role of dietary and/or life style factors against the development of cancers and cardiovascular diseases [14].

The potential use of soybean protein products in the USA increased significantly following the Food and Drug Administration (FDA) decision [18] that allowed food manufacturers to make cardiovascular health claims for soy protein products; and the United States Department of Agriculture's (USDA) rule change allowing suitable alternative protein products (such as soybean products) to replace 100% of meat products in the National School Lunch Program, the Summer Food Service Program, and the Child and Adult Care Food Program [18, 19]. In North America, isoflavone supplements are available for sale in pharmacies and health food stores with wide ranging health claims but there is little regulation regarding their manufacture or efficacy which has raised concerns about potentially dangerous effects from self-administered mega doses of these compounds [20]. Soy isoflavones, as isolated pure compounds, are now marketed as concentrated powders, and tablets may soon become available for sale over-the-counter [20].

In 2004, it was reported that 465 million people (7.2% of the world's population) were over the age of 65 [21]. About 52% of the world total elderly were in Asia, 25% in Europe, 8.6% in North America, and 6.4% in Africa [21].

Aging has been defined as the progressive loss of function accompanied by decreasing fertility and increasing mortality and disability [22]. Age has a significant influence on food metabolism which is particularly important for the elderly [23]. With advancing age, liver blood flow, gastric, and pancreatic secretions decrease. Alterations in biliary secretion, increases in intestinal mucosa permeability, immunological changes, and impairment of intestinal motility may all alter the rate of food metabolism [24, 25]. Alterations in the pharmacokinetics of drugs and nutrients are now recognized as one of the consequences of increased longevity [26].

A recent Health Canada study on protein digestibility in young (5 wk old) and old (20 month old) Fischer-344 (F-344) rats indicated that old rats were more susceptible to nutritional insults, such as the presence of protein-associated bioactive components, compared with young rats [27]. Rats are commonly used as animal models of human diseases and biological studies because they are easily available, relevant, and appropriate [27, 28]. The motivation for using the rat model for study of age-related pharmacokinetics and bioavailability in men in this project is, in part, because the F-344 rat developed by the National Institute on Aging, is an inbred rat model that is commonly used for aging studies. It has a longer lifespan, smaller body size, lower spontaneous tumor rate than non-inbred rats, conveniently available and resembles many features of aging humans [29].

The effect of gender on isoflavone bioavailability has been previously reported [4, 30, 31]. To date there are limited data on the effect of aging on isoflavone bioavailability [4, 32, 33]. Therefore, the objective of the present study was to investigate the effects of the three isoflavone sources (purified synthetic isoflavones (daidzein, genistein, glycitein, and their respective β-glucosides) and Novasoy™ (a commercial dietary isoflavone supplement)) on the bioavailability and pharmacokinetics of soy isoflavones in aged male rats.

2 Materials and methods

2.1 Chemicals

The following chemicals with the indicated specifications were used.

(i) Sodium citrate buffer (25 mM, pH 5.0).

(ii) Hydrolytic enzyme – a mixture of sulfatase and glucuronidase from *Helix pomatia* type H-5 (S3009), containing 400–600 U/mg of glucuronidase activity and 15–40 U/mg of sulfatase activity was purchased from Sigma–Aldrich (St. Louis, MO, USA). A solution containing 23 U of sulfatase activity was prepared by dissolving an appropriate amount of enzyme in 1.0 mL of the sodium citrate buffer [34].

(iii) DMSO –99.9% HPLC grade (Sigma–Aldrich)

(iv) Water – deionized, NANO-pure (Diamond UV ultrapure water purification system; Barnstead International, Essex, UK).

All other chemicals including HPLC grade ACN, and ethyl acetate were purchased from EMD Chemicals Inc. (Gibbstown, NJ, USA).

2.2 Apparatus

(i) HPLC – the LC separations were performed using a Waters (Milford, MA, USA) Alliance 2695 liquid chromatograph equipped with a Zorbax SB-CN RP column

Table 1. Composition of the Novasoy diet

Ingredient	%
Total isoflavones	40
Other natural soy phytocomponents	42
Protein	9
Sugar	1
Fat	1
Ash	3
Moisture	4

Adapted from the technical data sheet for Novasoy (concentrate no. 152–400) soy isoflavone (Archer Daniels Midland Company).

(4.6 mm × 75 mm, 3.5 μm particle size; Agilent Technologies, Wilmington, DE, USA).

(ii) MS system, a Waters Micromass ZQ single quadrupole mass spectrometer was operated in the positive ion SIM mode. The entire system from sample injection to data acquisition was computer-controlled with Empower software (Waters).

2.3 Experimental diets

(i) Novasoy (concentrate no. 152–400) soy isoflavone (Archer Daniels Midland Company, Decatur, IL, USA). One gram of Novasoy contains 248 mg isoflavones as determined based on the method of Wang and Murphy, 1994 [35]. About 98% of isoflavones in Novasoy are present in their natural glycosidic form and the remainder are protein, sugar, fat, ash, and moisture (Table 1). The total content of genistin, daidzin, and glycitin in the Novasoy was determined by Waters HPLC linear gradient with UV detection monitored at 254 nm [35].

To prepare the 20 mg/kg oral dose of Novasoy-derived isoflavones 80.645 mg of Novasoy powder was used according to the following calculation:

(20 mg isoflavones/kg)/0.248 mg isoflavones/mg Novasoy = 80.645 mg Novasoy /kg rat body weight.

(ii) Daidzin, genistin, glycitin, daidzein, genistein, glycitein – LC Laboratories (Woburn, MA, USA). The ratio of genistin, daidzin, and glycitin in Novasoy was 1.0:0.5:0.2, respectively. Synthetic glucosides and the respective aglycons were prepared with the same ratio of 1.0:0.5:0.2 for genistein, daidzein, and glycitein, respectively. Thus, the aglycone diet (20 mg/kg body weight) composed of 11.76 mg genistein, 5.88 mg daidzein, and 2.36 mg glycitein.

The glucoside dose (20 mg/kg body weight) was prepared taking into account the differences in molecular weight between the aglycon and glucoside form. Therefore, the glucoside dose was composed of 19.09 mg genistin, 9.62 mg daidzin, and 3.70 mg glycitin. The molecular weights for isoflavone glucosides and aglycons are reported in Table 2. Thus, both the aglycon and glucoside doses were equimolar with respect to the isoflavone moieties.

Table 2. Molecular weights for isoflavone glucosides (MW_g) and aglycons (MW_a)

Isoflavone glucoside	Isoflavone aglycon	MW_g	MW_a	MW_a/MW_g
Genistin	Genistein	438.37	270.23	0.616
Daidzin	Daidzein	416.36	254.23	0.611
Glycitin	Glycitein	446.21	284.27	0.637

Table 3. Composition of the casein based isoflavone-free diet (g/kg diet)

Ingredient	Casein
Vitamin free casein[a)]	222.2
Sucrose	100.0
Cornstarch	477.3
Fiber (sulka-floc)	50.0
Soybean oil	100.0
Mineral mix[b)]	35.0
Vitamin mix[b)]	10.0
Choline bitartrate	2.5
dl-Methionine	3.0
tert-Butylhydroquinone	0.014
Total isoflavones (mg/kg diet)[c)]	0.0
Genistein (mg/kg diet)[c)]	ND
Daidzein (mg/kg diet)[c)]	ND
Glycitein (mg/kg diet)[c)]	ND

ND, not detectable.

a) Casein from ICN Biomedicals contains 90% crude protein.

b) AIN-93G Mineral mix and AIN-93G vitamin mix were from ICN biomedicals.

c) The actual content of isoflavones (genistein, daidzein, and glycitein) was determined by Waters HPLC linear gradient with UV detection monitored at 254 nm.

(iii) Casein protein (90% purity, ICN Biomedicals, Costa Mesa, CA, USA). The composition of the casein-based isoflavone-free diet is reported in Table 3.

2.4 Preparation of diets

2.4.1 Oral gavage

A mixture of synthetic glucosides (genistin, daidzin, and glycitin); or a mixture of synthetic aglycons (genistein, daidzein, and glycitein) or Novasoy were suspended in ultra-pure water and the administered volume was adjusted to 2.5 mL to provide a dose of 20 mg/kg body weight of rats. Solutions were sonicated for 1 h prior to the oral administration. The isoflavones oral dose of 20 mg/kg body weight of rats is representative of a high dietary level experience by infants fed on soy-based formulae [36, 37].

2.4.2 Intravenous (tail vein) injection

A mixture of synthetic glucosides (genistin, daidzin, glycitin in the same proportion as for oral gavage); or a mixture of synthetic aglycons (genistein, daidzein, glycitein, also in the same proportion as for oral gavage) was suspended in 50% v/

v aqueous DMSO and the volume adjusted to provide 10 mg/kg body weight of rats such that the intended doses were delivered in a volume of 1.0 μL/g rat body weight.

Novasoy source was not injected IV, however bioavailability parameters were estimated by comparing AUC_{oral} post-Novasoy administration by AUC_{IV} post-glucoside IV injection.

2.5 Animals, isoflavones administration, and samples collection

Twenty male retired breeder 20 month old (400 g) F-344 rats (National Institute on Aging; Bethesda, MD, USA), were individually caged in rooms where the temperature was maintained at 23 ± 2°C and lights were on a 12 h light/dark cycle. Animal handling and care followed the guidelines of the Canadian Council for Animal Care and all aspects of the experimental protocol were reviewed and approved by the Health Canada, Ottawa Animal Care Committee.

During an adjustment period of 10 days, rats were fed an isoflavone-free casein-based control diet formulated according to the American Institute of Nutrition (AIN-93M) recommendations [38]. After the adjustment period, rats were randomly assigned to five groups of four; those rats destined for oral administration were gavaged with a single oral dose of one of the three sources of isoflavones (Novasoy, a mixture of synthetic aglycons or a mixture of synthetic glucosides). Rats assigned for IV injection, were injected (tail vein) with one of the two sources of isoflavones (a mixture of synthetic aglycons or a mixture of synthetic glucosides) by using a 26-gauge needle (Becton Dickinson, Rutherford, NJ, USA) without anesthesia. Post-oral or IV administration, rats were assigned to metabolic cages with free access to isoflavone-free casein-based diet and tap water for the total period of the study.

2.6 Rat plasma sample collection

Individual blood samples of approximately 400 μL were collected from the saphenous vein of the same rat in lithium heparin microtainers at 0, 2, 8, 24, and 48 h (post-oral) for aglycon, glucoside, and Novasoy sources and at 0, 10, 30, and 45 min, 1, 2, 3, 4, and 8 h (post-IV) for aglycon and glucoside groups as described previously [30]. Plasma was separated by centrifugation for 3 min at 4°C, 8000 rpm in an IEC Centra MP4R refrigerated centrifuge (Needham Heights, MA, USA) and stored at −80°C until the day of analysis.

2.7 Plasma isoflavone analysis

Plasma concentrations of isoflavones were determined by LC/MS as previously described [34]. Briefly, following enzymatic hydrolysis of isoflavone conjugates with mixed glucuronidase/sulfatase enzyme, the resultant aglycons were extracted with ethyl acetate, centrifuged and the supernatant was diluted with mobile phase (0.1% formic acid in 85:15 water/ACN) and injected onto a Zorbax SB-CN RP column (4.6 mm × 75 mm, 3.5 μm particle size). The chromatographic run time was 16.0 min, with a delay of 10 min/injection.

2.8 Pharmacokinetic analysis

Pharmacokinetic parameters were first derived using non-compartmental methods (PK Solutions™ version 2.0.2 package, Summit Research Services, Ashland, OH, USA). The pharmacokinetic parameters were calculated using the residuals method of analysis, assuming first order disposition kinetics. The depletion kinetics were modeled for the elimination phase after oral dosing. The pharmacokinetic parameters determined were: the terminal half-life, $t_{½}$ (the time taken for the maximum plasma concentration to decrease by half); C_{max} (the maximum observed peak plasma isoflavone concentration); t_{max} (time point at C_{max}); $AUC_{(0-t)}$, the area under the concentration-time curve (reflecting the exposure of plasma to isoflavone from time zero to time t when the plasma concentration of isoflavones returned to baseline); $AUC_{(0-t)}$ was estimated using the linear trapezoidal rule and calculated using data to the last quantifiable time point.

The absolute oral bioavailability was calculated from the percentage ratio of the AUCs derived from plasma isoflavone concentrations after oral and IV dosage of different sources of isoflavones to male rats.

$$\%\,\text{Bioavailability} = \frac{AUC_{or}}{AUC_{IV}} \times \frac{Dose_{IV}}{Dose_{or}} \times 100$$

2.9 Statistical analyses

Statistical analyses were done using SigmaStat version 3.1 (2004 Systat Software, Richmond CA, USA). All pharmacokinetic data were expressed as mean ± SD, and were analyzed using one-way ANOVA for its source effect of isoflavones. Statistically significant differences between sources were identified using the Holm – Sidak test. A probability value of $p < 0.05$ was considered statistically significant.

3 Results

3.1 Phase 1 (oral gavage)

The profiles of plasma isoflavones (daidzein, genistein, glycitein, and equol) following oral administration of the synthetic glucoside source, synthetic aglycon source and Novasoy (20 mg/kg body weight) to aged (20 month old) male F-344 rats are presented in Fig. 1.

The results of the non-compartmental pharmacokinetic analysis from the plasma curves of the synthetic isoflavone

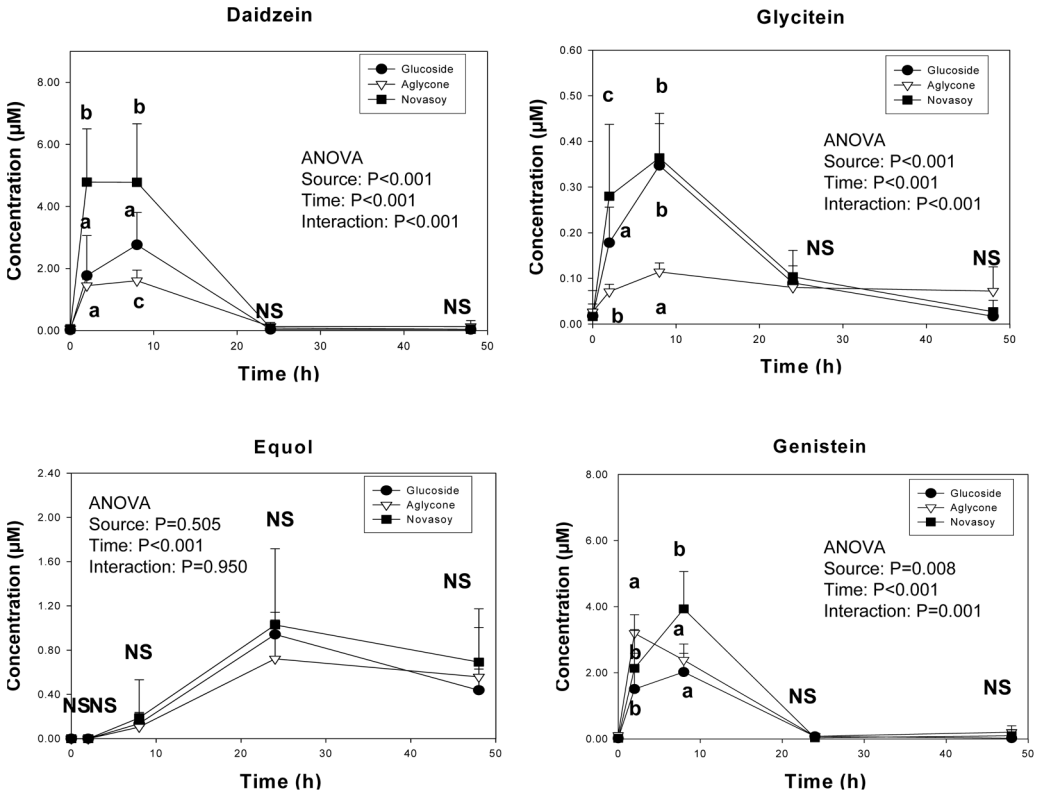

Figure 1. Plasma profiles of isoflavones in aged (20 month old) male F-344 rats following oral gavage of a mixture of synthetic isoflavone glucosides, aglycons or Novasoy at 20 mg/kg body weight. Results are expressed as means ± SD on a sample size of four rats/group. Significant effects of source at specific time points are indicated by different superscripts ($p < 0.05$). NS indicates no significant differences.

Table 4. Pharmacokinetic parameters of plasma isoflavones in aged (20 month old) male F-344 rats following gavage of a single dose (20 mg/kg/body weight) of three different sources (mixtures) of soy isoflavones

Sources of isoflavones	Daidzein		Genistein		Glycitein	
	C_{max} [a)] (μmol/L)	t_{max} (h)	C_{max} [a)] (μmol/L)	t_{max} (h)	C_{max} [a)] (μmol/L)	t_{max} (h)
Aglycon	1.63 ± 0.35^{A}	6.50 ± 3.00	3.23 ± 0.52^{AB}	3.50 ± 3.00	0.10 ± 0.00^{A}	8.00 ± 0.00
Glucoside	2.80 ± 1.05^{A}	6.50 ± 3.00	2.08 ± 0.82^{B}	6.50 ± 3.00	0.35 ± 0.13^{AB}	8.00 ± 0.00
Novasoy	5.15 ± 1.68^{B}	6.50 ± 3.00	3.95 ± 1.13^{A}	8.00 ± 0.00	0.40 ± 0.08^{B}	6.50 ± 3.00

Means in a column with different superscript alphabets differ significantly ($p < 0.05$).
a) Values are means ± SD, $n = 4$.

aglycon, glucosides and Novasoy post-single-bolus oral exposure to aged male rats are summarized in Tables 4 and 5.

The maximum observed peak plasma (C_{max}) daidzein and glycitein concentrations were significantly higher (up to four-fold; $p < 0.05$) in Novasoy dosed rats compared with aglycon-dosed rats (Table 4). Additionally, the C_{max} daidzein and genistein concentrations were significantly higher ($p < 0.05$) in Novasoy (up to two-fold) dosed rats compared with glucosides-dosed rats (Table 4).

The AUC_{or} values post-oral administration of daidzein and glycitein were found to be significantly greater (up to three-fold) for the Novasoy-dosed rats than the aglycon-dosed rats (Table 5). Following glucoside treatment, the AUC_{or} values for daidzein and glycitein were significantly higher (up to two-fold) compared with the aglycon treated rats (Table 5).

Volume of distribution (Vd_{or}) was significantly larger ($p < 0.05$) for glycitein in rats dosed with aglycons compared with rats dosed with glucosides (up to six-fold) and Novasoy (up to seven-fold). Additionally, Vd_{or} was significantly larger ($p < 0.05$) for daidzein in rats dosed with aglycons compared with rats dosed with Novasoy (up to three-fold) (Table 5).

Table 5. Pharmacokinetic parameters of plasma daidzein, genistein, and glycitein in aged (20 month old) male F-344 rats after oral or IV exposure to three different sources (mixtures) of soy isoflavones[a)]

	$t_{½\ or}$ h	AUC_{or}[a)] (μmol/h)/L)	Vd_{or}/kg[a)] L/kg bw	Cl_{or}/kg[a)] L/h/kg bw	AUC_{IV} (μmol/h)/L)	% Bioavailability
Daidzein						
Aglycon	6.23 ± 2.94	10.66 ± 1.72^{B}	7.00 ± 2.50^{B}	0.82 ± 0.17^{B}	16.30 ± 4.61	34 ± 6
Glucoside	6.88 ± 1.58	15.39 ± 7.73^{C}	4.28 ± 2.40^{AB}	0.41 ± 0.16^{A}	32.35 ± 18.46	26 ± 5
Novasoy	5.75 ± 1.11	32.37 ± 11.79^{A}	1.68 ± 0.57^{A}	0.22 ± 0.10^{A}	–	45 ± 18
Genistein						
Aglycon	5.92 ± 2.98	20.01 ± 1.74	8.75 ± 5.06	1.00 ± 0.12^{B}	30.68 ± 11.58	35 ± 9^{A}
Glucoside	6.25 ± 0.57	12.11 ± 6.65	9.03 ± 3.12	1.00 ± 0.31^{B}	43.30 ± 27.13	11 ± 3^{B}
Novasoy	5.63 ± 4.43	21.31 ± 7.67	4.60 ± 1.21	0.37 ± 0.20^{A}	–	21 ± 7^{AB}
Glycitein						
Aglycon	7.34 ± 5.45	0.65 ± 0.13^{B}	67.65 ± 8.87^{B}	1.39 ± 1.59	4.15 ± 0.85	8 ± 3^{B}
Glucoside	9.28 ± 1.69	1.77 ± 0.66^{A}	11.00 ± 3.52^{A}	0.83 ± 0.25	3.45 ± 0.24	21 ± 10^{A}
Novasoy	8.15 ± 0.62	2.23 ± 0.79^{A}	9.23 ± 5.14^{A}	0.77 ± 0.39	–	27 ± 13^{A}

Means in a column for individual isoflavones with different superscript alphabets differ significantly ($p < 0.05$).
a) Values are means ± SD, $n = 4$.

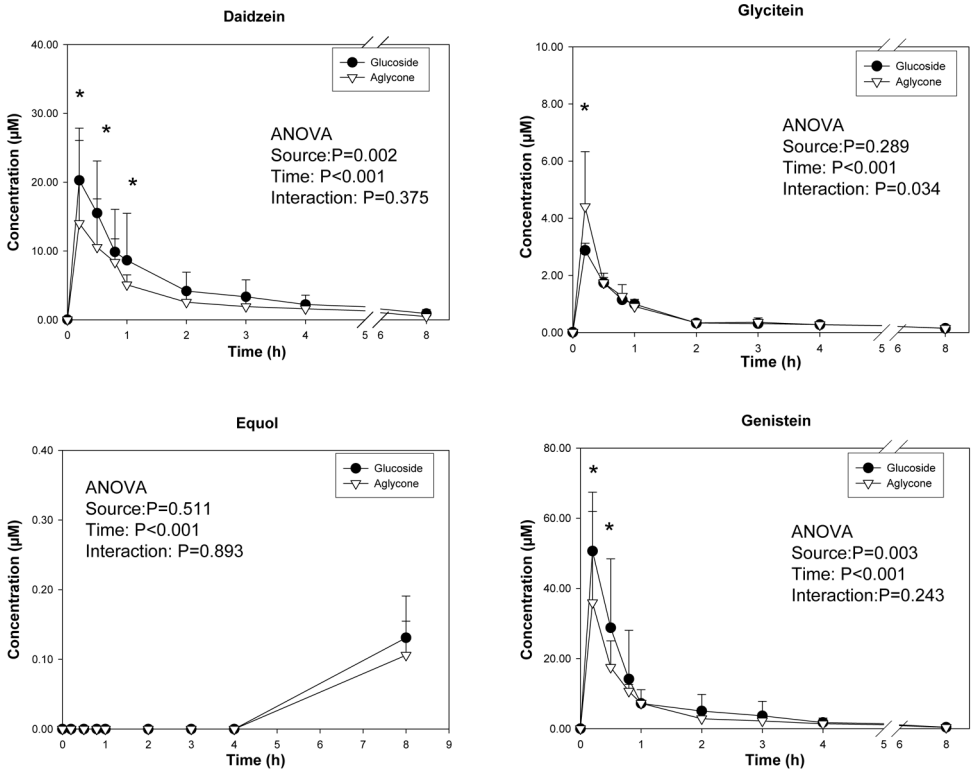

Figure 2. Plasma profiles of isoflavones in aged (20 month old) male F-344 rats following IV injection of a mixture of synthetic isoflavone glucosides or aglycons at 10 mg/kg body weight. Results are expressed as means ± SD on a sample size of four rats/group. Significant effects of source at specified time points are denoted by an asterisk (*), $p < 0.05$.

3.2 Phase 2 (IV injection)

Following IV administration of two sources of isoflavones (10 mg/kg body weight), the plasma profile of isoflavones demonstrated a rapid increase in plasma concentration of isoflavones followed by an elimination phase (Fig. 2). The profiles of plasma isoflavones in old male (20 month old) F-344 rats post-IV injection of mixtures of isoflavone glucosides or aglycons reached C_{max} for daidzein, genistein and glycitein within the first 10 min post-administration of the doses (Fig. 2).

3.3 Bioavailability of isoflavones

Bioavailability of daidzein did not significantly differ when administered as the aglycon, glucoside, or Novasoy. The bioavailability value for genistein was significantly ($p < 0.05$) higher (up to three-fold) in the aglycon form compared with those in the glucoside forms of isoflavones (Table 5). Bioavailability values for glycitein were significantly ($p < 0.05$) higher (up to four-fold) in Novasoy and the glucoside forms of isoflavones (up to three-fold) compared with those in aglycon forms (Table 5).

3.4 Formation of equol

Plasma profiles of equol exhibited a time lag in its appearance after a single-bolus of the isoflavones, and it took ≥8 h before equol appeared in substantial amounts after either oral or IV administration of the doses (Figs. 1 and 2).

4 Discussion

The bioavailability and pharmacokinetics of the isoflavones from glucoside and aglycon forms have been extensively investigated in animals and humans [4, 6, 10, 12, 18, 30, 39–45]. However, relatively few studies have characterized the bioavailability and pharmacokinetics of these compounds in elderly humans and aged animals [4, 32, 33, 46].

The present study is the second part of an isoflavone bioavailability study in our laboratory that determined bioavailability based on the AUC of both oral and IV administration of different sources of isoflavones, in aged (20 month old) rats. In our earlier study, the effects of the different isoflavone sources and of gender on bioavailability and pharmacokinetics of soy isoflavones in rats were investigated in detail [30].

The dietary dose selected for use in this study was relevant to infants consuming soy formulas. A recent National Centre for Toxicological Research/National Toxicology program study of life time exposure to dietary genistein demonstrates this connection [31]. In that study, rats consuming 5 μg/g and soy-free control diets had serum levels of total genistein (0.01–0.06 μM), similar to those in humans consuming a typical Western diet containing little or no soy; rats consuming a 100 μg/g genistein diet produced serum levels (0.6–0.9 μM) similar to those measured in adults consuming typical Asian diets [47]; and rats consuming a 500 μg/g genistein diet had serum total genistein levels (6.0–7.9 μM) similar to infants consuming soy formulas [48].

In the context of bioavailability, the main difference between the IV and oral doses is the fact that in oral administration, chemicals are absorbed by the digestive system and enter the liver *via* the hepatic portal system before reaching the rest of the body [49]. The liver metabolizes the chemicals sometimes to such an extent that only a small amount of active chemical emerges from the liver to reach the rest of the circulatory system [49]. Alternative routes of administration such as IV, avoid the first-pass effect and chemicals can be absorbed directly into the systemic circulation and therefore their bioavailability is 100% [49].

Our data clearly show that the bioavailability of genistein was significantly ($p < 0.05$) higher (up to three-fold) in aglycon treated rats compared with glucoside forms of isoflavones (Table 5). It is presumed that in aged rats genistein in the glucoside form is poorly absorbed from the gut compared with the aglycon, which might be due to the higher hydrophilicity and greater molecular weight of isoflavone glucosides [10, 50]. Factors such as intestinal bacterial fermentation, intestinal transit time, and age are expected to have an influence on the metabolism and bioavailability of isoflavones [6, 11, 51, 52]. Additionally, the observed short t_{max} (3.5 h) for genistein in plasma in rats treated with the aglycon mixture is attributed to the absorption of the aglycon form from the stomach, as was found in the study by Piskula *et al*. [6].

Glycitein comprises less than 10% of the total isoflavone amount in soybeans and soybean foods, which may be the reason that its absorption and metabolism has not been well-studied [53, 54]. However, the bioavailability of glycitein is important as many commercially available supplements contain high levels of glycitein and limited information exists about its biological properties [18]. Our data show that bioavailabilities for glycitein were significantly ($p < 0.05$) higher (up to four-fold) from Novasoy and the glucoside mixture (up to three-fold) compared with those from the aglycon mixture (Table 5).

The relatively low bioavailability of glycitein in aglycon dosed rats might be due to extensive first pass metabolism (*i.e*., enterohepatic recycling) [55]. Oral administration of glycitein in rats dosed with the aglycon mixture resulted in a significantly more rapid clearance rate (1.39 L/h/kg body weight) than from Novasoy (0.77 L/h/kg body weight) and the glucosides mixture (0.83 L/h/kg body weight) and furthermore, a larger volume of distribution (~67.65 L/kg body weight) compared with the glucosides (11 L/kg body weight) and Novasoy (9.23 L/kg body weight) sources. This suggests that the aglycon form of glycitein in aged rats is more likely to enter, and perhaps be sequestered by tissues, faster than the glucoside and Novasoy forms of glycitein. The rapid clearance rate is likely due to rapid metabolism of glycitein. The major pathways of glycitein metabolism by human intestinal microflora are reduction to dihydroglycitein (7,4′dihydroxy-6methoxy-isoflavanone) followed by demethylation to produce dihydro-6,7,4′-trihydroxyisoflavone (6,7,4′-trihydroxyisoflavanone) or, alternatively, C ring cleavage of dihydroglycitein to produce 5′-methoxy-*O*-desmethylangolensin [54]. Additionally, the chemical structure of glycitein is similar to daidzein with the exception of the methoxy group at the six-position, and in fact, Setchell *et al*. [18] and Simons *et al*. [54] have reported that

humans fed glycitein showed minute concentrations of daidzein in plasma due to direct demethoxylation of glycitein at the six-position. Therefore, minor pathways include direct demethoxylation of glycitein to daidzein (7,4′-dihydroxyisoflavone) and reduction of dihydroglycitein to 6-OMe-equol (7,4′-dihydroxy-6-methoxy-isoflavan) [54]. Since demethoxylation of glycitein to daidzein may not be a major pathway of glycitein metabolism in humans and rats, the exact identification of glycitein metabolites warrants further investigation.

There was no difference in the bioavailability of daidzein from aglycon or glucoside forms in aged rats. Additionally, there was no difference in t_{max} and C_{max} values for daidzein between the aglycon and glucoside forms. Our finding agrees with some reports indicating that the bioavailability of daidzein is not influenced by the presence of free or conjugated forms in the diet of humans [11, 56].

We observed age differences in the bioavailability of daidzein (57 *vs*. 26%), genistein (58 *vs*. 11%), and glycitein (58 *vs*. 21%) for the isoflavone glucosides and daidzein (98 *vs*. 45%), genistein (62 *vs*. 21%), and glycitein (68 *vs*. 27%) for the Novasoy sources, where lower bioavailability values (∼40%) were observed in aged (20 month old) male rats compared with young adult (3 month old) male Sprague Dawley rats reported earlier [30]. The age differences are likely due to the changes in physiology associated with advancing age, particularly decreases in total body water and lean body mass and, most importantly, a decline in renal function as well as differences in absorption of isoflavones and differences in the biotransformation, excretion, and enterohepatic circulation [34, 39, 57]. However, such a comparison between different strains of rats has drawbacks that future research may address.

When isoflavones are ingested, the conjugated isoflavones are hydrolyzed in the gut by both intestinal mucosal and bacterial β-glucosidases releasing the aglycons [4]. Aglycons are in turn either absorbed intact or further metabolized by intestinal microflora in the large intestine into other metabolites such as 7-hydroxyl-3-(4′-hydroxyphenyl)-chroman (equol) or *O*-desmethylangolensin (*O*-DMA) from daidzein, *p*-ethyl phenol (from genistein) and di-hydroglycitein (from glycitein) [4]. The next metabolic step in the metabolism of aglycons involves the re-conjugation of the aglycon with glucuronide or sulfate within the intestinal adsorbing cells and in the liver [58]. As a result, isoflavones are found primarily as conjugates in serum, with only a small portion present as free aglycons [59]. There are two conjugation sites on genistein and daidzein, and each of these sites can be sulfated and/or glucuronidated. Thus, there are monoglucuronides, monosulfates, diglucuronides, disulfates, and mixed conjugates of aglycons with one site glucuronidated and one site sulfated [58]. Structural determination based on LC/MS and ^{1}H NMR showed that both 7-and 4′-glucoronides of genistein were present in rat and human blood [60]. Circulating concentrations of genistein aglycon, measured after oral administration, are quite low in adult Sprague Dawley rats (about 2% of total genistein [61]) and in humans (about 1%; 18). This is in agreement with our previous report [34] where LC/MS chromatograms of isoflavones in rat plasma samples, following oral administration of isoflavones without enzyme hydrolysis, revealed very low concentrations of free isoflavone aglycons and also that the isoflavone glucoside concentrations were below the LOD of the reported method. An article by Janning *et al*. [41], reported daidzein conjugates (glucuronides/sulfates) are also the main circulating metabolites in female Da/Han rats post-oral and IV administration of daidzein.

Further metabolism of isoflavones and their metabolites occurs through their incorporation into bile acids followed by enterohepatic circulation including reabsorption into the bloodstream [59]. Bile excreted into the intestine is met by bacterial β-glucuronidase and sulfatase-enzymes which deconjugate the isoflavones and isoflavone metabolites and allow them to be reabsorbed into the circulation [59].

In the present study, the late appearance (∼8 h) of equol in plasma samples after both oral and IV administration is consistent with its production being in the large intestine [4, 18]. It is probable that enterohepatic circulation is the probable mechanism by which IV injected daidzein may reach the colon, where it can be converted to equol. Interestingly, we found aged (20 month old) male rats are capable of producing equol at 8 h post-IV administration of aglycon (0.106 ± 0.04 μM) and glucoside (0.131 ± 0.06 μM) sources of isoflavones compared with undetectable equol production in young adult (3 month old) male rats [30]. Additionally, a higher concentration of equol was produced in aged male rats following oral administration of aglycon (0.72 ± 0.4 μM), glucoside (0.94 ± 0.1 μM), and Novasoy (1.02 ± 0.6 μM) than in young adult male rats (aglycon and Novasoy (0.0 ± 0.0 μM) and glucoside (0.04 ± 0.02 μM)) [30]. This might be due to the enhanced ability of aged (20 month old) male rats to convert more daidzein to equol compared with young adult male rats. It has been reported that variation in equol production is due to the absence or presence of certain bacterial species in the intestine [10, 62–64] as evidenced from the finding that infants fed soy formula up to the age of 4 months [48, 65] and germ-free rats fed soy-containing diets do not produce equol [66]. Rats and mice are equol producers [67]. In contrast, humans are unique among animals as only 20–35% of the adult population produce equol after ingesting soy foods or pure isoflavones [67]. It is unknown why the colonization of equol-producing bacteria varies between individuals. Diet has been suggested as a contributing factor for equol production, especially when it contains prebiotics and/or probiotics [68]. A few bacterial groups make up 50–70% of the dominant bacteria in the human intestinal tract, comprising mainly the *Clostridium coccoides-Eubacterium rectale* cluster, the genus *Bacteroides*, the *Clostridium leptum* sub-

group (including the genus *Faecalibacterium*), and the genus *Bifidobacterium* [69]. Three strains of bacteria which are able to convert daidzein to equol *in vitro* are *Streptococcus intermedius*, *Ruminococcus productus*, and *Bacteroides ovatus* [70]. Although several studies have investigated bacterial populations in the adult large bowel [71 – 74], relatively little information is available concerning the effect of age on gut microflora. With advancing age, species such as bifidobacteria are thought to decline in numbers, whereas clostridia and enterobacterial populations increase [71, 75]. Determination of gut microflora types prior to and after soy isoflavones intake would be useful in identifying those bacteria that are involved in equol production.

In conclusion, our data clearly show that the glycosidation of isoflavones had significant effects on isoflavone genistein and glycitein bioavailability and pharmacokinetics in aged male (20 month old) rats. These findings will provide valuable information to policy and regulatory agencies in assessing safety and efficacy of isoflavones with regards to elderly men.

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