Research Article

Bioavailability of soy isoflavones in rats Part I: Application of accurate methodology for studying the effects of gender and source of isoflavones

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There are limited and controversial reports about the effects of gender and source of isoflavones on their bioavailability. Moreover, several previous studies have not used appropriate methodology to determine the bioavailability of soy isoflavones, which requires comparing the area under the plasma concentration-time curve after both oral and intravenous injection (IV) administration. Therefore, the present study was conducted to determine the bioavailability of isoflavones from different sources following both oral and IV administration in male and female rats. Three sources of isoflavones; Novasoy™ (a commercial supplement), a mixture of synthetic aglycones (daidzein, genistein and glycitein) and a mixture of synthetic glucosides (daidzin, genistin and glycitin) were tested. Following administration, blood samples were collected at several time points (0, 10, 30 min and 1, 2, 8, 24, 48 h post oral gavage and 0, 10, 30, 45 min and 1, 2, 3, 4, 8 h post-IV dosing) and plasma isoflavones were measured by LC/MS. Bioavailability values for daidzein, genistein and glycitein were significantly (p <0.05) higher (up to sevenfold) in Novasoy™ and the glucoside forms of isoflavones compared with those of the aglycone forms. Moreover, significant (p <0.05) gender differences in the bioavailability of 7-hydroxyl-3-(4′-hydroxyphenyl)-chroman (a metabolite of daidzein), glycitein and daidzein were observed for Novasoy™, with higher values in male rats. In summary, the source of isoflavones and the sex of rats had significant effects on isoflavone bioavailability.

Keywords: Isoflavones / Liquid chromatography / Mass spectrometry / Pharmacokinetics / Photo diode array detector

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1 Introduction

Phytoestrogens have gained increasing interest in recent years because of their potential health benefits in cases of certain cancers, cardiovascular diseases, and alleviation of symptoms of menopause and bone loss in postmenopausal women [1 – 3]. These naturally occurring plant compounds with estrogenic or anti-estrogenic activities comprise three major classes: isoflavones, lignans and coumestans [1 – 3]. Soybeans, soy protein products and soy-based infant formula are the most significant sources of isoflavones [4]. Moreover, a number of isoflavone-rich supplements have become available for over the counter sale.

The major isoflavones present in soy are predominantly in their glycoside forms (daidzin, genistin, and glycitin), with daidzin and genistin being the most abundant [1, 5, 6]. However, in fermented soy products such as miso or tem-

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Abbreviations: AUC, area under the concentration-time curve; $\mathbf{C_{max}}$, maximum observed peak plasma; **equol**, 7-hydroxyl-3-(4′-hydroxyphenyl)-chroman; **IV**, intravenous; $\mathbf{Cl_{or}}$, plasma clearance; $\mathbf{t_{1/2}}$, elimination half-life; $\mathbf{Vd_{or}}$, volume of distribution

peh, the unconjugated aglycones (daidzein, genistein, glycitein) are the predominant forms, because the fermentation process causes cleavage of the glucoside bond [6].

Isoflavones, when ingested predominantly as the β-glucoside form, are hydrolyzed in the gut by both intestinal mucosal and bacterial β-glucosidases releasing the aglycones. Aglycones 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) [7, 8].

Controversial evidence exists regarding the bioavailability of the aglycone and glucoside forms of isoflavones in animal and human studies [9–11]. Some studies have reported that aglycones were absorbed more efficiently than glucosides [9], while other data suggest that the resulting bioavailability of daidzein and genistein was greater when soy isoflavones were ingested as glucosides rather than aglycones [10]. However, in one study, there was no difference in bioavailability following consumption of aglycone or glucoside tablets [11].

There is also limited information regarding gender influences on the bioavailability of isoflavones in humans or animal models. Cassidy *et al*. [8] reported significant effects of gender and food matrix on the area under the concentration-time curve (AUC) of isoflavones in humans, and Chang *et al*. [12] reported significant gender differences in the elimination half-life ($t_{1/2}$) and AUC in adult rats.

In most of these earlier studies, the determination of bioavailability was based on the oral administration of isoflavones, which is an acceptable method for the determination of bioavailability in food sources. Some isoflavones such as genistein, are also considered to be pharmaceutical agents. Therefore, the bioavailability of these compounds (as drugs) should be determined by comparing the AUC of the plasma concentration-time curve after intravenous (IV) administration with the AUC after oral administration [13–15].

The objectives of the present study were to develop accurate methodology for measurement of bioavailability based on oral and IV administration of purified synthetic isoflavones (daidzein, genistein, glycitein and their respective β-glucosides) and Novasoy™, a commercial dietary isoflavone supplement, and to investigate the effects of the three isoflavone sources and of gender on the bioavailability and pharmacokinetics of soy isoflavones in rats.

2 Materials and methods

2.1 Chemicals

The following chemical with the indicated specifications were used.

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

(ii) Hydrolytic enzyme. *Helix pomatia* type H-5 (S3009), containing 29 units/mg solid sulfatase activity was purchased from Sigma- Aldrich (St. Louis, MO, USA). A solution containing 23 units of sulfatase activity was prepared by dissolving an appropriate amount of enzyme in 1.0 mL of the sodium citrate buffer [16].

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

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

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 reversed-phase column (4.6 × 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 #152–400) soy isoflavone (Archer Daniels Midland Company, Decatur, IL, USA). One gram of Novasoy™ contains 248 mg isoflavones (the remainder was moisture, carbohydrate, protein, fat and ash). The total content of genistein, daidzein and glycitein in the Novasoy™ was determined by Waters HPLC linear gradient with UV detection monitored at 254 nm [6].

To prepare the 20 mg/kg oral dose of Novasoy™, 80.645 mg of Novasoy™ powder was used according to the following calculation: (1 mg × 20 mg/kg)/0.248 mg = 80.645 mg/kg rat body weight.

(ii) Daidzin, genistin, glycitin, daidzein, genistein, glycitein. – LC Labs. (Woburn, MA, USA).

The ratio of genistein, daidzein and glycitein in Novasoy™ was 1.0 : 0.5 : 0.2, respectively. Synthetic glucosides and respective aglycones 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 aglycone 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 aglycones are reported in Table 1.

Table 1. Molecular weight for isoflavones glucoside and aglycone

Isoflavone glucoside	MWa	MWg	MWa/MWg
Genistin	270.23	438.37	0.616
Daidzin	254.23	416.36	0.611
Glycitin	284.27	446.21	0.637

Table 2. Composition of the casein based isoflavone-free diet

Ingredient	g/kg
Vitamin free casein[a)]	222.20
Corn starch	477.30
Sucrose	100.00
Fiber (Sulfa-Floc)	50.00
Soybean oil	100.00
AIN-93-G Mineral Mix[b)]	35.00
AIN-93-V Vitamin Mix[b)]	10.00
Choline Bitartrate	2.50
DL-methionine	3.00
Tert-Butylhydroquinone	0.01
Actual isoflavone content[c)], mg/kg diet	0.00

a) Casein from ICN Biomedicals contains 90% crude protein.
b) AIN-93-G Mineral Mix [18] and AIN-93-V Vitamin Mix [33] were from ICN Biomedicals.
c) The actual content of isoflavones was determined by Waters HPLC linear gradient with UV detection monitored at 254 nm [6].

(iii) Casein protein (90% purity, ICN Biomedicals, Costa Mesa, CA, USA). The composition of the casein based isoflavone-free diet is reported in Table 2.

2.4 Preparation of diets

2.4.1 Oral gavage

A mixture of synthetic glucosides (genistin, daidzin, glycitin); or a mixture of synthetic aglycones (genistein, daidzein, 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 one hour prior to the oral administration.

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 aglycones (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. This amount of DMSO has been shown to be well tolerated by mice when administered by rapid IV or extra vascular injection [17].

2.5 Animals, isoflavones administration and samples collection

Forty female and male 90-day-old (250–400 g) Sprague-Dawley rats (Charles River, St-Constant, QC, Canada), were individually caged in rooms where the temperature was maintained at 23 ± 2°C and lights were on a 12-h light/dark cycle. 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-93G) recommendations [18]. After the adjustment period, rats of each sex were randomly assigned to groups of four animals; 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 aglycones 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 aglycones 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

Blood samples (0.4 mL) were collected from the saphenous vein in lithium heparin microtainers according to the method of Hem *et al*. [19] at 0, 10, 30 min, and 1, 2, 8, 24, 48 h (post oral) for aglycone and Novasoy™ source and at 0, 2, 8, 24, 48 h (post oral) for glucoside source.

To investigate the possible presence of the multiple peaks observed in the previous studies reported by Janning *et al*. [13] and Supko and Malspeis [17] before 2 h, three time points (10, 30, 60 min) were added to the original (0, 2, 8, 24, 48 h) blood collection for the two groups of 90-day-old male and female Sprague-Dawley rats as the minor amendment to the original protocol. A total of 16 (8 male and 8 female) rats were gavaged with Novasoy™ and synthetic aglycone at 20 mg/kg dose.

Blood samples were also collected at 0, 10, 30, 45 min, 1, 2, 3, 4, 8 h (post IV) for aglycone and glucoside groups. Accumulative multiple sampling was maintained at about 15% of the total rat blood circulatory volume [20].

Collected blood was centrifuged at 4°C, 8000 rpm in an IEC Centra MP4R refrigerated centrifuge (Needham Heights, MA, USA) for 3 min and 250-μL aliquots were stored frozen 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 [16]. Briefly, following

enzymatic hydrolysis of isoflavone conjugates with mixed glucuronidase/sulfatase enzyme, the resultant aglycones 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 into a Zorbax SB-CN reversed-phase column (4.6 × 75 mm, 3.5-μm particle size). The chromatographic run time was 16.0 min, with a delay of 10 min/injection.

2.8 Determination of plasma isoflavones pharmacokinetics

Pharmacokinetic analysis of plasma isoflavone profiles for each rat was conducted using non-compartmental analysis software (PK Solutions™ version 2.0.2 package, Summit Research Services, Ashland, Ohio, USA). The pharmacokinetic parameters were calculated using the residuals method of analysis, assuming first order disposition kinetics. The depletion kinetics was modeled for the elimination phase after oral dosing. The pharmacokinetic parameters determined were: the terminal half-life, $t_{1/2}$ (the time taken for the plasma concentration to decrease by half and thereby reflecting the rate of elimination); C_{max} (the maximum observed peak plasma isoflavone concentration); t_{max} (time point at C_{max}); $AUC_{(0\text{-}t)}$, 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); $AUC_{(0\text{-}t)}$ was estimated using the linear trapezoidal rule and calculated using data to the last quantifiable time point. All pharmacokinetic analyses of the rats dosed with glucoside, aglycone and Novasoy™ were calculated using five time points (0, 2, 8, 24, 48 h). However, only minor differences in bioavailability parameters were observed when all eight time points were used for the analysis.

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 and female rats.

$$\%\ \text{Bioavailability} = [(AUC_{oral}) \times Dose_{iv}/(AUC_{iv}) \times Dose_{oral}] \times 100$$

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

2.9 Statistical analyses

Statistical analyses were carried out using Sigma stat version 3.1 (2004 Systat Software, Richmond CA, USA). All data were expressed as mean ± SD, and were analyzed using two-way ANOVA involving two main effects (source of isoflavones, and gender). The interaction between these two main effects (source of isoflavones × gender) was also analyzed. When warranted, post hoc analysis was performed using Holm-Sidak test. Differences were considered significant at $p < 0.05$.

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 aglycone source and Novasoy™ to male and female 90-day-old SD rats are presented in Figs. 1 – 3.

Plasma concentration-time curves from rats dosed with the isoflavone glucosides revealed a significant effect of time (daidzein, $p < 0.001$; glycitein, $p < 0.001$; equol, $p = 0.018$; and genistein, $p < 0.001$) and gender × time interactions (geniestin, $p = 0.025$) but no significant effect of gender (Fig. 1). Rats dosed with the aglycone form showed a significant effect of time (daidzein, glycitein, genistein, $p < 0.001$); gender (daidzein, $p = 0.020$); and gender × time interactions (daidzein, $p = 0.001$; genistein, $p = 0.036$) on the plasma concentration-time curves (Fig. 2). Rats dosed with Novasoy™ showed a significant effect of time (daidzein, glycitein, equol and genistein, $p < 0.001$); gender (daidzein, $p = 0.001$, and genistein, $p = 0.023$) and gender × time interactions (daidzein, $p < 0.001$; equol, $p = 0.037$; and geniestin, $p < 0.001$) on the plasma concentration-time curves (Fig. 3).

Pharmacokinetic data analysis from the plasma curves of the synthetic isoflavone aglycone, glucosides and Novasoy™ were examined post single-bolus oral exposure to 90-day-old SD rats (Tables 3 – 6).

The maximum observed peak plasma (C_{max}) daidzein concentration was significantly higher ($p < 0.05$) in glucoside- (~3-fold) and Novasoy™- (~7-fold) dosed rats compared with aglycone-dosed rats. In addition, gender differences in daidzein C_{max} were observed in rats dosed with glucoside ($p = 0.002$) and Novasoy™ ($p = 0.000$) (Table 3). Genistein C_{max} in rats dosed with Novasoy™ was significantly higher (~ 2 fold; $p < 0.05$) than in rats dosed with glucoside and aglycone, and both daidzein and genistein concentrations were ~60% higher in male rats compared with female rats (Table 3). Glycitein concentration reached their maximum levels in rats dosed with Novasoy™ significantly earlier, compared with glucoside- ($p < 0.048$) and aglycone- ($p = 0.000$) dosed rats (Table 3).

A significant effect of gender ($p = 0.004$) on $t_{1/2}$ for glycitein was observed within the glucoside treatment with longer $t_{1/2}$ (~ 2.4-fold) in female rats (Table 6).

The AUC_{or} values post oral administration of daidzein and glycitein were found to be significantly greater ($p < 0.05$) for the glucoside-dosed rats than the aglycone-

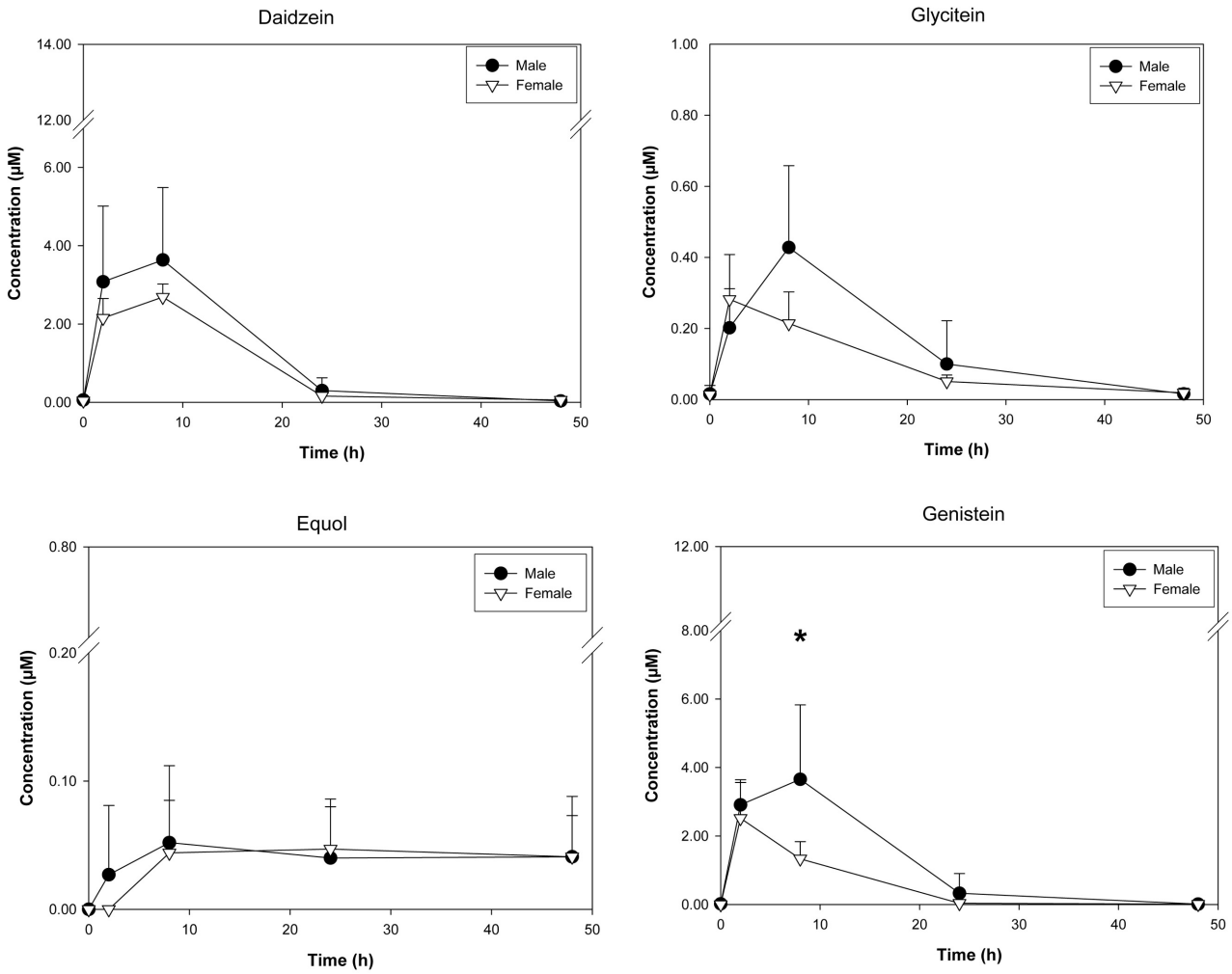

Figure 1. Plasma profiles of isoflavones in male and female (3 months) SD rats post oral gavage of a mixture of synthetic isoflavone glucosides at 20 mg/kg rat body weight. Results are expressed as mean ± SD on a sample size of four rats/group. An effect of gender at specified time points is denoted by an asterisk (*), $p < 0.05$.

dosed rats. Following Novasoy™ treatment, the AUC_{or} for daidzein, genistein and glycitein were significantly higher ($p < 0.05$) compared with both the aglycone and glucoside treated rats. In addition, a significant effect of gender ($p < 0.05$) was obtained on AUC_{or} for daidzein and genistein where greater values (~ 2-fold), were obtained for male rats (Tables 4–6).

Volume of distribution (Vd_{or}) was significantly larger ($p < 0.05$) for daidzein, genistein and glycitein in rats dosed with aglycones compared with rats dosed with glucosides or Novasoy™. Glycitein and daidzein exhibited larger Vd_{or} values (>10-fold and 0.5-fold, respectively) compared with genistein in rats dosed with the aglycones (Tables 4–6).

Plasma clearance (Cl_{or}) rates for daidzein, genistein and glycitein were significantly faster ($p < 0.05$) in rats dosed with aglycones compared with rats dosed with glucosides or Novasoy™. Also, a significant effect of gender on the Cl_{or} rates for both daidzein ($p = 0.000$) and genistein ($p = 0.012$) were obtained in rats dosed with aglycones. Cl_{or} rates of daidzein and genistein were faster (>1.5-fold and >0.5-fold, respectively) in females than males dosed with aglycone source (Tables 4–6).

3.2 Phase 2 (IV injection)

Following IV administration of two sources of isoflavones, the plasma profile of isoflavones demonstrated a rapid increase in plasma concentration of isoflavones followed by an elimination phase (Figs. 4, 5). The profiles of plasma isoflavones in 90-day-old SD rats post-IV injection of mixtures of isoflavone glucosides or aglycones reached C_{max} for daidzein, genistein and glycitein within 2 h post-administration of the doses (Figs. 4, 5).

Plasma concentration-time curves for rats dosed with the mixture of glucosides revealed a significant effect of time (daidzein, $p < 0.001$; glycitein, $p < 0.001$; equol, $p = 0.013$;

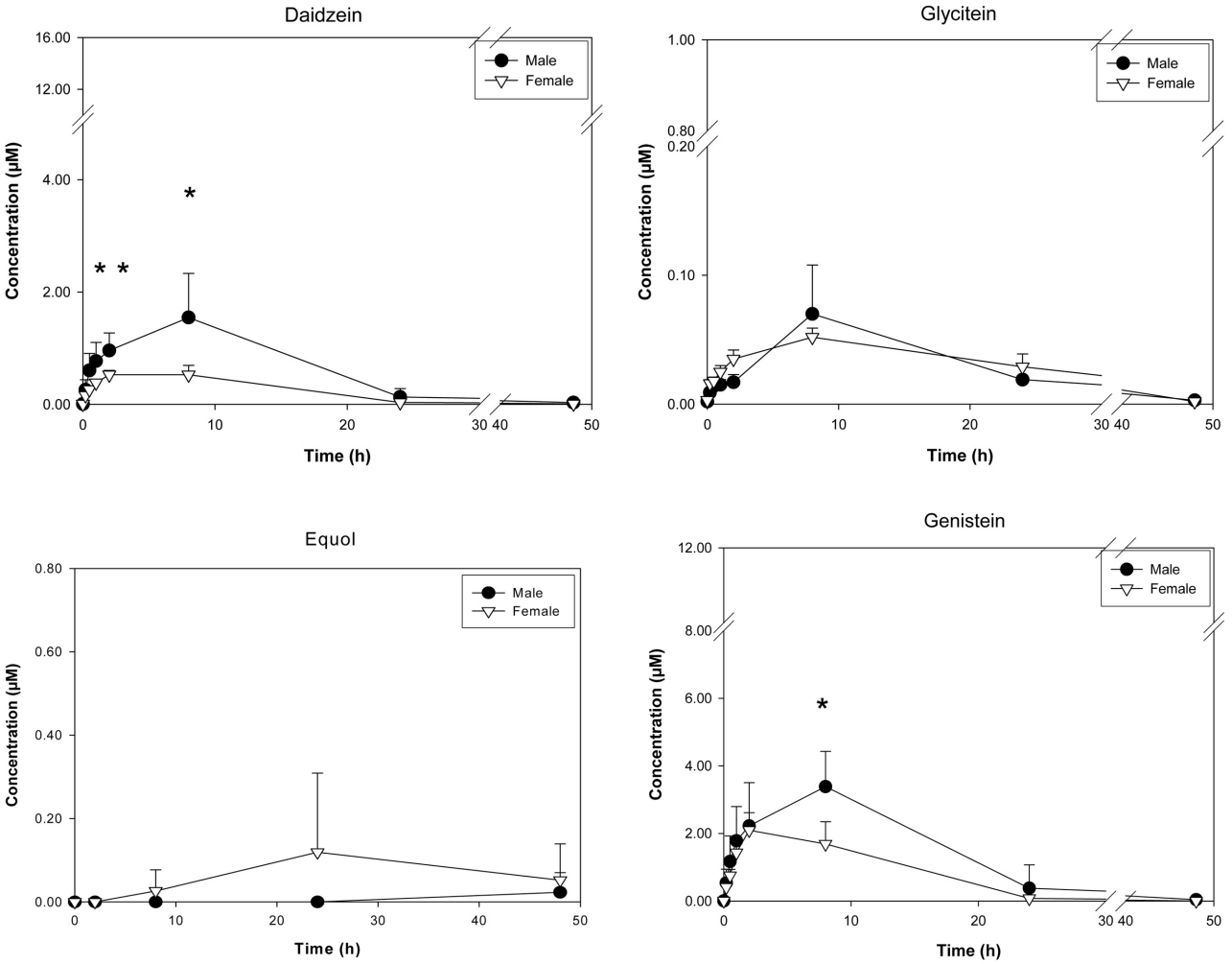

Figure 2. Plasma profiles of isoflavones in male and female (3 months) SD rats post oral gavage of a mixture of synthetic isoflavone aglycones at 20 mg/kg rat body weight. Results are expressed as mean ± SD on a sample size of four rats/group. An effect of gender at specified time points is denoted by an asterisk (*), $p < 0.05$.

and genistein, $p < 0.001$); a significant effect of gender (daidzein, $p = 0.038$; genistein, $p = 0.041$); and gender × time interactions (daidzein, $p < 0.001$; glycitein, $p = 0.005$; equol, $p = 0.013$; and genistein, $p < 0.001$) (Fig. 4). Rats dosed with a mixture of isoflavone aglycones showed a significant effect of time (daidzein, glycitein, genistein, $p < 0.001$); a significant effect of gender (daidzein, $p = 0.002$); and significant gender × time interactions (daidzein, $p < 0.001$; genistein, $p = 0.009$) on the plasma concentration-time curves (Fig. 5).

The AUC_{iv} values post-IV administration for daidzein, genistein and glycitein were not significantly different for the glucoside and aglycone dosed rats but significant effects of gender ($p < 0.05$) within both glucoside- and aglycone-dosed rats were found for daidzein ($p < 0.05$) and genistein ($p < 0.05$) where higher values (~ one to threefold) were obtained for males (Tables 4–6).

3.3 Formation of equol

Plasma profiles (Figs. 1–5) 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. Equol production also differed with gender, with significantly ($p = 0.035$) higher concentrations produced by female rats post oral administration of 20 mg/kg of three different mixtures of soy isoflavones. However, no significant effects of source or gender × source interactions were obtained (Fig. 6).

The AUC for plasma equol in male and female rats after IV injection of 10 mg/kg of the two different mixtures of soy isoflavones are shown in Fig. 7. The plasma concentration-time curve revealed no significant effects of gender ($p = 0.356$), time ($p = 0.448$) or gender × time interactions

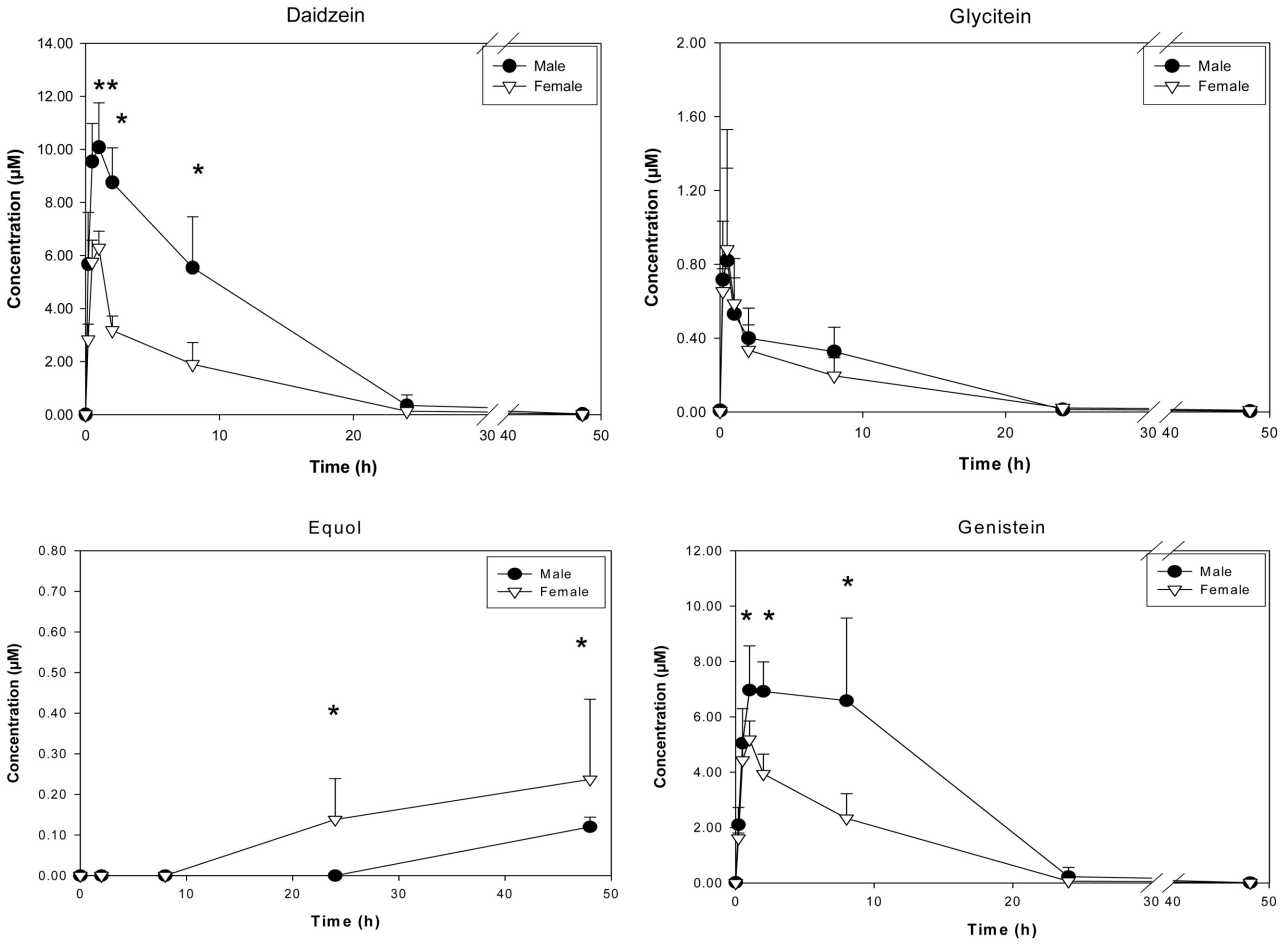

Figure 3. Plasma profiles of isoflavones in male and female (3 months) SD rats post oral gavage of a mixture of isoflavone Novasoy™ at 20 mg/kg rat body weight. Results are expressed as mean ± SD on a sample size of four rats/group. An effect of gender at specified time points is denoted by an asterisk (*), $p < 0.05$.

($p = 0.448$) for equol post aglycone injection. However, significant effects of time ($p = 0.013$) and gender × time interactions ($p = 0.013$) were observed for equol, post glucoside injection (Fig. 7).

4 Discussion

The importance of isoflavones in the prevention of a range of chronic diseases, and data from human and animal studies attest to the biological activity of these compounds and their potential role in human health [2, 7, 21]. If soy isoflavones are to be effective in preventing diseases, they must be bioavailable, and thus understanding the factors, which may alter their bioavailability, such as gender and food sources, need to be elucidated. Most published studies on the pharmacokinetics of soy isoflavones have focused on systemic studies of the absorption, metabolism and excretion of isoflavones (mainly daidzein and genistein) using the areas under the concentration-time curves following oral administration of isoflavones in humans or animal models [8–11, 22]. Several reports have measured only the concentration of isoflavones excreted in urine, which may only be deemed appropriate if the compound is 100% bioavailable and recovered completely in the urine. As is evident from many studies, this is not the case for soy isoflavones; [23–25]. Therefore, conflicting results are present in the literature regarding the bioavailability of the aglycone and glucoside forms of isoflavones, mainly daidzein and genistein [9–11]. Accurate measurements of bioavailability would ideally compare the AUC after both oral and IV administration of the pure compounds [13, 17, 26]. The accuracy of this determination is dependent upon obtaining multiple blood samples during the elimination phase and sampling times should ideally be extended to at least five half-lives beyond the time that steady-state levels are reached in the blood [21, 23]. Only a few studies of isoflavone bioavailability have considered the above points [10, 27–29], and several studies have only used two time points to compute the pharmacokinetics [9, 22]. These inconsis-

tencies in study design may account for discrepancies in the available literature.

The present study is the first part of isoflavone bioavailability study in our laboratory that measures bioavailability based on AUC of both oral and IV administration of two sources of isoflavones; a mixture of synthetic aglycones (daidzein, genistein and glycitein) and a mixture of synthetic glucosides (daidzin, genistin and glycitin). Glycitein

Table 3. Pharmacokinetic parameters of plasma isoflavones in male and female (3 months) SD rats following gavage of a single dose (20 mg/kg) of three different sources of soy isoflavones[a)]

Sources of isoflavones	Daidzein		Genistein		Glycitein	
	C_{max} μmol/L	t_{max} h	C_{max} μmol/L	t_{max} h	C_{max} μmol/L	t_{max} h
Aglycone						
Male	1.63 ± 0.69	6.50 ± 3.00	3.53 ± 1.03	6.50 ± 3.00	0.05 ± 0.06	8.00 ± 0.00
Female	0.60 ± 0.08	5.00 ± 3.46	2.38 ± 0.31	5.00 ± 3.50	0.05 ± 0.06	8.00 ± 0.00
Glucoside						
Male	4.28 ± 1.13	5.00 ± 3.46	3.93 ± 1.87	5.00 ± 3.46	0.45 ± 0.24	8.00 ± 0.00
Female	2.20 ± 0.41	3.50 ± 3.00	2.58 ± 0.93	3.50 ± 3.00	0.30 ± 0.14	3.50 ± 3.00
Novasoy™						
Male	8.78 ± 1.31	2.00 ± 0.00	7.68 ± 1.67	4.75 ± 3.77	0.45 ± 0.13	3.50 ± 3.00
Female	3.15 ± 0.54	2.00 ± 0.00	3.93 ± 0.71	2.00 ± 0.00	0.28 ± 0.15	3.50 ± 3.00
Statistics (ANOVA)				*P*-value		
Source effect	<0.001	0.035	<0.001	0.315	<0.001	0.002
Gender effect	<0.001	0.367	<0.001	0.143	0.080	0.100
Source x gender	<0.001	0.809	0.085	0.895	0.433	0.075
Holm-Sidak						
Novasoy™ *vs.* aglycone	0.000	0.011	0.000		0.000	0.000
Novasoy™ *vs.* glucoside	0.000	0.106	0.000		0.000	0.048
Glucoside *vs.* aglycone	0.000	0.272	0.627		0.000	0.025
Gender within aglycone	0.091	0.433	0.197		1.000	1.000
Gender within glucoside	0.002	0.433	0.133		0.155	0.008
Gender within Novasoy™	0.000	1.000	0.000		1.000	1.000

a) Values are mean ± SD, $n = 4$ rats in each gender were tested in each dietary treatment group.

Table 4. Pharmacokinetic parameters of plasma daidzein in male and female (3 months) SD rats after oral and IV exposure to three different sources of soy isoflavones[a)]

	$t_{1/2\,or}$ h	AUC_{or} μmol · h/L	Vd_{or}/kg L/Kg bw	Cl_{or}/Kg L/h/kg bw	AUC_{iv} μmol · h/L	Bioavailability %
Daidzein						
Aglycone						
Male	9.34 ± 1.48	8.46 ± 3.15	14.80 ± 7.29	1.08 ± 0.45	18.85 ± 0.42	23.00 ± 4.48
Female	8.29 ± 2.25	3.68 ± 0.38	14.13 ± 3.39	2.77 ± 0.58	7.48 ± 1.38	25.07 ± 3.57
Glucoside						
Male	7.74 ± 2.39	23.26 ± 10.17	3.33 ± 1.94	0.28 ± 0.11	17.03 ± 4.15	56.93 ± 8.04
Female	7.21 ± 0.49	13.89 ± 1.77	5.15 ± 0.60	0.50 ± 0.06	10.45± 1.60	54.49 ± 7.76
Novasoy™						
Male	8.26 ± 2.66	40.28 ± 11.11	1.65 ± 0.52	0.14 ± 0.03	–	97.66 ± 2.63
Female	6.39 ± 2.39	18.13 ± 3.23	3.70 ± 1.64	0.40 ± 0.08	–	88.46 ± 13.09
Statistics (ANOVA)						
Source effect	0.314	<0.001	<0.001	<0.001	0.979	<0.001
Gender effect	0.194	<0.001	0.460	<0.001	<0.001	0.157
Source x gender	0.811	0.002	0.688	<0.001	0.046	0.027
Holm-Sidak						
Novasoy™ *vs.* aglycone		0.000	0.000	0.000	–	0.000
Novasoy™ *vs.* glucoside		0.000	0.378	0.456	–	0.000
Glucoside *vs.* aglycone		0.001	0.000	0.000	0.147	0.000
Gender within aglycone		0.309	0.786	0.000	0.000	0.858
Gender within glucoside		0.036	0.465	0.326	0.046	0.599
Gender within Novasoy™		0.000	0.413	0.257	–	0.004

a) Values are mean ± SD, $n = 4$ rats in each gender were tested in each dietary treatment group.

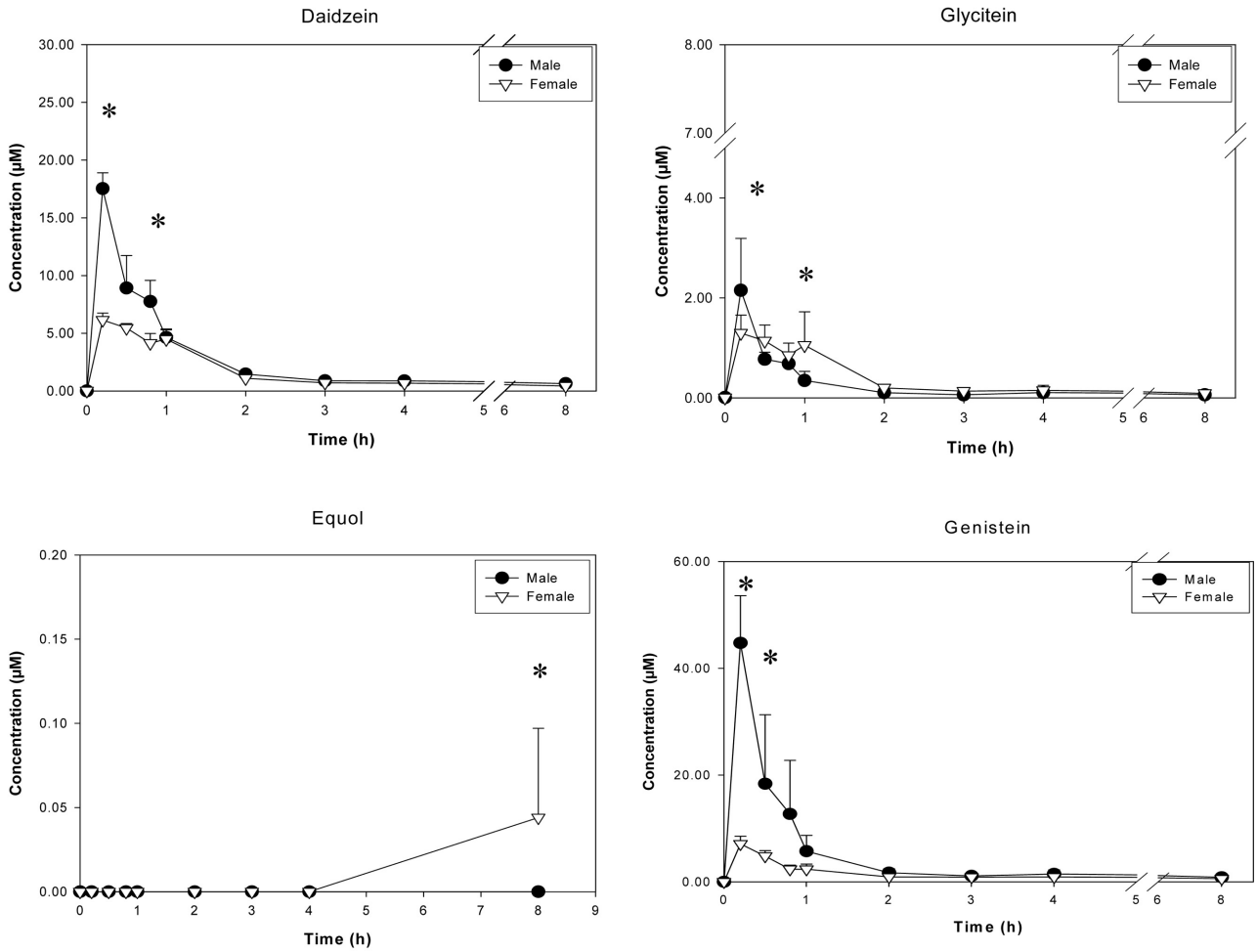

Figure 4. Plasma profiles of isoflavones in male and female (3 months) SD rats post IV injection of a mixture of synthetic isoflavone glucosides at 10 mg/kg rat body weight. Results are expressed as mean ± SD on a sample size of four rats/group. An effect of gender at specified time points is denoted by an asterisk (*), $p < 0.05$.

was included in our study because it may also contribute to soy's health effects. Knowing the bioavailability of glycitein is important because many commercially available supplements contain high levels of glycitein and limited information exists about its biological properties [10].

Our data clearly show that the bioavailability of daidzein and glycitein in rats dosed with these glucosides is significantly ($p < 0.05$) higher compared with the bioavailability of daidzein and glycitein (> twofold) in aglycone-dosed rats. The bioavailability of isoflavones was also significantly ($p < 0.05$) higher in rats dosed with Novasoy™ than in rats dosed with pure sources of isoflavone glucosides and aglycones. The glucoside moiety in isoflavones glucosides and in Novasoy™, in conjunction with other compounds in Novasoy™ (phytic acid, saponins, oligosaccharides, proteases inhibitors and phytosterols), may be protecting against biodegradation of the isoflavone structure [10]. Our results are in agreement with the previously published report [10]. This increase of absorption from Novasoy™ compared with pure chemicals may be an important consideration for regulators.

In our study, the lower bioavailability in rats dosed with aglycone sources is most likely explained by the reduced absorption of isoflavones with increasing level of intake (20 mg/kg body weight of rats). Earlier studies have demonstrated a linear dose–response relationship for isoflavone absorption at low doses [10]; however, at higher doses (0.4–1.8 mg/kg body weight) a curvilinear relationship was evident [23]. Usually, when a compound exhibits nonlinear pharmacokinetics, the AUC_{or} increases in a manner that is disproportionate to the applied dose. When the AUC_{or} is lower than would be expected from a linear relationship, this is indicative of either increased elimination, or reduced absorption [10, 23].

Cl_{or} rates for daidzein, genistein and glycitein in rats dosed with Naovasoy™ and glucosides, were significantly

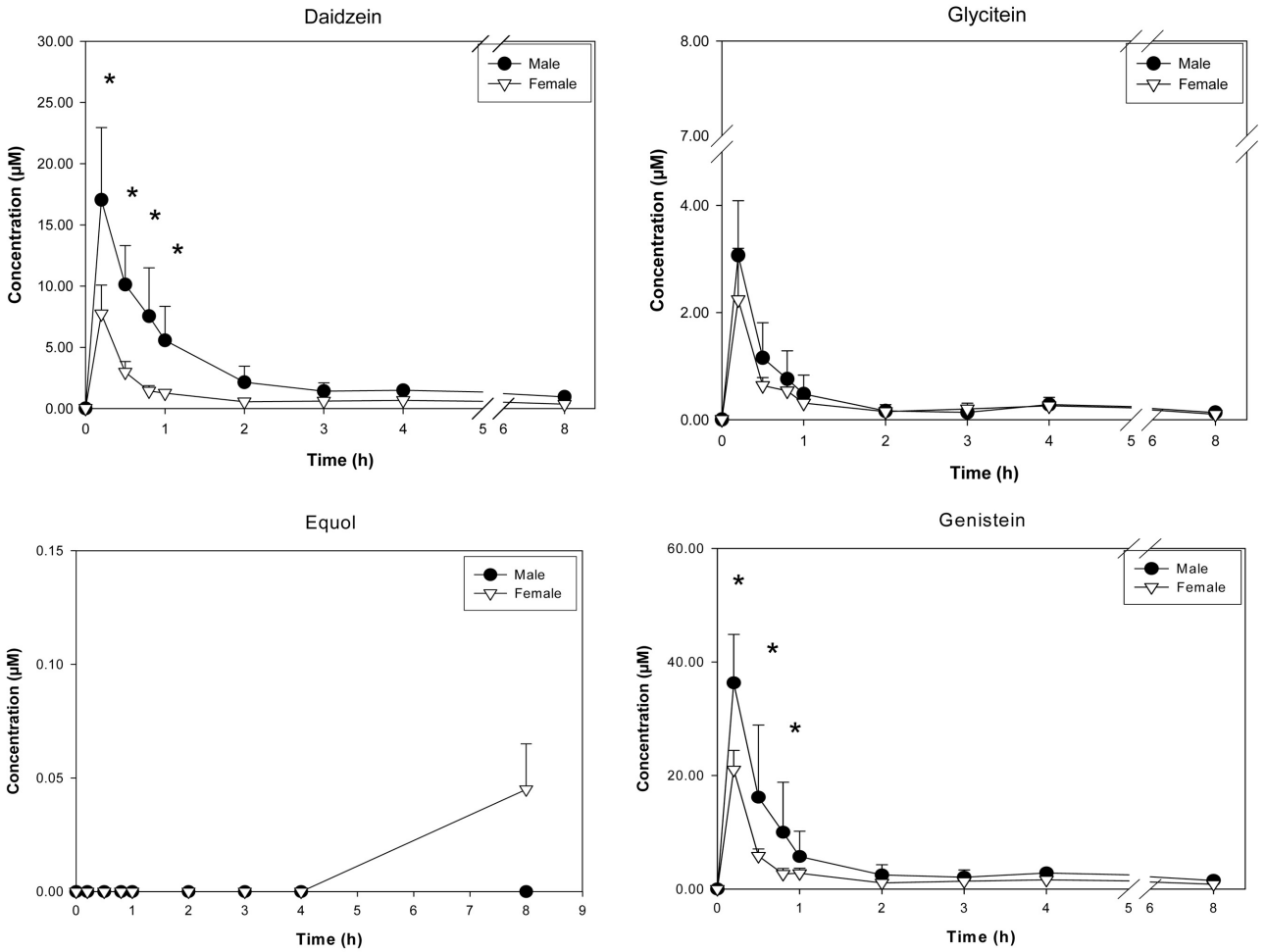

Figure 5. Plasma profiles of isoflavones in male and female (3 months) SD rats post IV injection of a mixture of synthetic isoflavone aglycone 10 mg/kg rat body weight. Results are expressed as mean ± SD on a sample size of four rats/group. An effect of gender at specified time points is denoted by an asterisk (*), $p < 0.05$.

($p < 0.05$) slower compared with the aglycone dosed rats, consistent with the high bioavailability of these isoflavones in Novasoy™- and glucoside-dosed rats compared with the aglycone-dosed rats. The pharmacokinetic data from the analysis of the plasma curves showed that Cl_{or} of daidzein (~1-fold) and genistein (>1.5-fold) were significantly higher in females than males.

In this study, the Vd_{or} was significantly ($p < 0.05$) larger for daidzein, genistein and glycitein in rats dosed with aglycones compared with rats dosed with glucosides or Novasoy™, indicating extensive tissue distribution of isoflavone aglycones. Furthermore, within the aglycone-dosed rats, genistein was more bioavailable (~1 – 6-fold) than daidzein and glycitein. The Vd for both glycitein (~108 L/kg bw) and daidzein (~14 L/kg bw) exhibited much higher values than genistein (~9 L/kg bw), indicating extensive tissue distribution of glycitein and daidzein in rats dosed with aglycone source. The lower Vd_{or} of genistein compared with daidzein and glycitein in the aglycone-dosed rats explains why the AUC_{or} for genistein in plasma is exceeded by daidzein and glycitein. When isoflavones reach the circulation, it is possible that they are bound extensively to proteins such as albumin (the most abundant protein in plasma), lipoproteins, sex hormone-binding globulin and α_1-acid glycoprotein. It has been reported that at 0.5 h, and 5 h post oral dosing of genistein, in male rats 80.6 ± 2.0 and 77.3 ± 4.7% and in female rats 97.7 ± 6.2 and 78.1 ± 4.7% were bound to plasma protein, respectively [14]. This will limit the distribution of isoflavones into tissues, resulting in a lower Vd, which may be the case for rats dosed with Novasoy™ and the glucosides. It is also possible that in aglycone-dosed rats, isoflavones bind preferentially to tissues at the expense of plasma and therefore, the plasma concentration of isoflavones would be significantly lower compared with the glucoside and Novasoy™-dosed rats. This may be the reason for the very large Vd_{or} values, which were even larger than the actual volumes of the rats themselves (>1 L/kg).

Table 5. Pharmacokinetic parameters of plasma genistein in male and female (3 months) SD rats after oral and IV exposure to three different sources of soy isoflavones[a)]

	$t_{1/2\,or}$ h	AUC_{or} μmol · h/L	Vd_{or}/kg L/Kg bw	Cl_{or}/Kg L/h/kg bw	AUC_{iv} μmol · h/L	Bioavailability %
Genistein						
Aglycone						
Male	7.02 ± 1.77	19.05 ± 6.69	9.43 ± 4.69	0.92 ± 0.43	34.55 ± 13.29	29.14 ± 12.35
Female	8.29 ± 2.25	13.41 ± 1.16	10.30 ± 5.30	1.60 ± 0.38	17.60 ± 3.62	38.93 ± 5.78
Glucoside						
Male	3.59 ± 2.65	30.38 ± 21.08	2.58 ± 1.08	0.64 ± 0.41	31.33 ± 16.23	57.78 ± 17.40
Female	4.65 ± 2.11	14.10 ± 4.90	7.28 ± 2.89	1.16 ± 0.41	10.40 ± 2.91	62.34 ± 8.07
Novasoy™						
Male	5.75 ± 2.14	49.54 ± 10.35	2.20 ± 0.76	0.27 ± 0.07	–	62.34 ± 8.07
Female	5.79 ± 2.09	20.38 ± 2.08	5.93 ± 2.90	0.67 ± 0.17	–	90.46 ± 5.00
Statistics (ANOVA)						
Source effect	0.236	<0.001	0.006	<0.001	0.351	0.013
Gender effect	0.568	<0.001	0.038	0.001	0.004	0.094
Source x gender	0.229	0.005	0.515	0.736	0.718	0.522
Holm-Sidak						
Novasoy™ *vs.* aglycone		0.000	0.003	0.000	–	0.006
Novasoy™ *vs* glucoside		0.000	0.617	0.022	–	0.136
Glucoside *vs.* aglycone		0.534	0.009	0.048	0.351	0.143
Gender within aglycone		0.250	0.719	0.012	0.046	0.643
Gender within glucoside		0.090	0.065	0.048	0.017	0.541
Gender within Novasoy™		0.000	0.137	0.110	–	0.065

a) Values are mean ± SD, $n = 4$ rats in each gender were tested in each dietary treatment group.

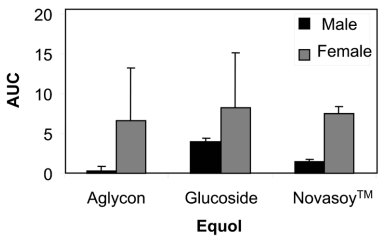

Figure 6. Area under the curve of plasma equol in male and female (3 months) SD rats after consumption of single oral dose (20 mg/kg) of three different mixtures of soy isoflavones. Results are expressed as mean ± SD on a sample size of four rats/group.

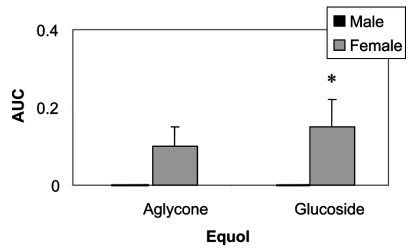

Figure 7. Area under the curve of plasma equol in 3-month-old male and female SD rats after IV injection of (10 mg/kg) of two different mixtures of soy isoflavones. Results are expressed as mean ± SD on a sample size of four rats/group. An effect of gender at specified time points is denoted by an asterisk (*), $p < 0.05$.

We observed significant ($p < 0.05$) gender differences in the bioavailability of daidzein and glycitein for the isoflavone Novasoy™ ($p < 0.05$). Gender related differences in bioavailability might be due to differences in absorption of isoflavones and differences in the biotransformation, excretion and enterohepatic circulation [14]. Previously published studies have reported peak plasma concentrations at time points within 2 h of the oral administration of isoflavones [13, 17, 26]. They also reported fluctuations in the plasma concentration at time points within the first 2 h of the oral administration of the doses and attributed them to entro-hepatic circulation. In other studies [10, 27, 28], peak concentrations of daidzein or genistein were achieved within 2 – 8 h post oral administration of daidzein and/or genistein and a sharp decrease was observed 8 h after the administration. In order to investigate the possible presence of the multiple peaks observed in the previous studies reported by Janning *et al*. [13] and Supko and Malspeis [17] before 2 h, three time points (10, 30, 60 min) were added to the original (0, 2, 8, 24, 48 h) blood collection for the two groups of 90-day-old male and female Sprague-Dawley rats as the minor amendment to the original protocol. A total of 16 (8 male and 8 female) rats were gavaged with Novasoy™ and synthetic aglycone at 20 mg/kg dose. In our study, we could not detect an early peak before C_{max} that would represent entero-hepatic circulation. In spite of the addition of extra time points, however, the study was designed to obtain bioavailability data. Future studies may address entro-hep-

Table 6. Pharmacokinetic parameters of plasma glycitein in male and female (3 months) SD rats after oral and IV exposure to three different sources of soy isoflavones[a)]

	$t_{1/2\,or}$ h	AUC_{or} μmol · h/L	Vd_{or}/kg L/Kg bw	Cl_{or}/Kg L/h/kg bw	AUC_{iv} μmol · h/L	Bioavailability %
Glycitein						
Aglycone	9.21 ± 2.84	0.28 ± 0.13	108.03 ± 71.68	7.68 ± 3.35	2.85 ± 0.66	4.85 ± 2.10
Male	8.72 ± 2.32	0.29 ± 0.01	76.05 ± 23.28	6.00 ± 0.85	2.28 ± 0.46	6.59 ± 1.11
Female						
Glucoside						
Male	5.59 ± 0.25	1.54 ± 0.36	7.43 ± 4.59	0.90 ± 0.53	1.60 ± 0.17	57.81 ± 15.75
Female	12.78 ± 5.97	2.28 ± 0.76	19.55 ± 10.35	1.03 ± 0.13	2.35 ± 0.97	38.32 ± 4.82
Novasoy™						
Male	6.03 ± 2.26	2.59 ± 0.96	8.83 ± 3.37	1.07 ± 0.45	–	68.14 ± 19.92
Female	7.82 ± 1.53	1.76 ± 0.67	7.98 ± 2.59	1.40 ± 0.37	–	40.18 ± 16.52
Statistics (ANOVA)						
Source effect	0.291	<0.001	<0.001	<0.001	0.132	<0.001
Gender effect	0.037	0.165	0.594	0.524	0.969	0.013
Source x gender	0.060	0.432	0.368	0.364	0.089	0.108
Holm-Sidak						
Novasoy™ *vs.* aglycone	0.200	0.000	0.000	0.000		0.000
Novasoy™ *vs.* glucoside	0.157	0.489	0.950	0.729		0.382
Glucoside *vs.* aglycone	0.886	0.000	0.000	0.000		0.000
Gender within aglycone	0.825	0.974	0.164	0.135		0.858
Gender within glucoside	0.004	0.488	0.589	0.905		0.058
Gender within Novasoy™	0.420	0.084	0.970	0.755		0.009

a) Values are mean ± SD, $n = 4$ rats in each gender were tested in each dietary treatment group.

atic circulation by increasing the number of rats in each group and bleeding each rat only at three or four time points [14, 30].

IV injection of isoflavones bypasses first-pass metabolism [14]. The metabolic transformation on first-pass absorption is important in both human and rats, because it is the unconjugated form of isoflavone that is available for receptor occupancy. IV administration of isoflavones will enable a more substantial role for renal excretion since first pass metabolism and enterohepatic circulation of isoflavones will be reduced [14].

By contrast, oral administration of isoflavones leads to significant phase II metabolism and isoflavone glucuronides become the major circulating forms in plasma [31]. This conjugation happens during transport across the enterocytes and in the liver [30]. An additional factor to consider is the extent of protein binding which differs considerably for daidzein, genistein and equol. The intestine provides a key barrier to limiting the biological activity of isoflavones administered orally. Not only do the enterocytes conjugate isoflavones on first pass, but there are also specific intracellular efflux pumps, such as the multidrug resistance protein (MRP2) that shunt isoflavones back into the intestinal lumen. Therefore, events with the lumen and across the enterocytes serve to account for the limited absorption of isoflavones when given orally [31].

In this study, daidzein C_{max} was significantly higher (p <0.05) in glucoside and Novasoy™-dosed rats (>2 and >6 times, respectively) compared with aglycone-dosed rats (Table 3). Genistein C_{max} in rats dosed with Novasoy™ was significantly higher (~ 2-fold; p <0.05) compared with glucoside- and aglycone-dosed rats, and both daidzein and genistein concentrations were 60% higher in male rats compared with female rats (Table 3). Based on the chemical structure of isoflavones, unconjugated genistein and daidzein are very lipophilic, which favors retention and accumulation of those compounds in other tissues such as liver [30]. Higher disposition of isoflavones in the liver would allow the reduction of the systemic plasma concentration of genistein and daidzein as reflected by lower plasma pharmacokinetic values for C_{max}. Gender-related differences in tissue concentrations of isoflavones have already been reported in rat liver due to a number of factors, including the presence of binding proteins, biotransformation, and clearance at the site [30]. C_{max} of ingested isoflavone aglycone and glucoside were attained within 5.0–8.0 h and 3.5–8.0 h, respectively. This is in agreement with other human and rodent studies [8, 10, 27, 28, 32]. Values of $t_{1/2}$ for aglycone and glucoside dosed rats in the present study, are similar to $t_{1/2}$ for genistein in mice [17], and for genistein and daidzein in humans [8, 10, 27, 28]. Moreover, significant (p <0.05) gender differences on $t_{1/2}$ for glycitein were observed within the glucoside treatment where the $t_{/2}$ in female rats was longer compared with males. These values are similar to the previously reported $t_{1/2}$ for the pure compounds [10, 23] and are similar to values reported by others allowing for some limitations in the methods used [8, 27, 28, 32]. It is clear that the t_{max} and $t_{1/2}$ values for the pure compounds are longer than for Novasoy™, which is also consistent with previously reported data [27, 28, 32].

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 colon region of the large intestine [8, 10]. Rats dosed with glucoside and Novasoy™ produced more equol compared with aglycone-dosed rats (Fig. 6). Earlier studies using an *in vitro* model of human colonic fermentation, found that in a high carbohydrate environment, colonic fermentation was stimulated and this increased the rate of conversion of daidzein to equol [1, 2, 33]. Equol production also differed with gender, with significantly higher concentrations produced by female rats post oral and IV administration of the glucoside source, possibly due to the enhanced ability of female rats to convert more daidzein to equol compared with male rats. It has been reported that variation in equol production is due to the absence of certain bacterial species in the intestine [34, 35]. For example, only about 25% of women possess the gut microorganisms to produce equol, whereas the gut flora of rats produce very large quantities of equol [36]. The appearance of equol in blood following IV administration of isoflavones indicates the transfer of daidzein into the intestine since the conversion to equol is thought to be mediated only by the gut microflora. It is possible that once absorbed by the liver, some daidzein will be excreted in the bile, which would allow for the transfer of daidzein into the intestine. Recently, transfer to the bile has been indicated for genistein metabolites [37].

Establishing the validity of a model species for investigating the bioavailability of isoflavones is important in order to extrapolate their effects in humans. The pharmacokinetic parameters (C_{max}, t_{max}, and $t_{1/2}$) for genistein, daidzein and glycitein in the present study were very similar to those reported for humans [8, 10, 27, 28, 32] and support the use of rats as a model species for human isoflavone pharmacokinetics.

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