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Short communication

Bioavailability and urinary excretion of isoflavones in humans: Effects of soy-based supplements formulation and equol production☆

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Abstract

Soy isoflavones (IF) are of particular interest for their possible estrogenic effects on the symptoms of menopause. The bioavailability of IF is clearly a factor influencing their biological activity. The first aim of this study was to elucidate the impact of the matrix process and especially the formulation of soy-based capsules on IF bioavailability. Twelve healthy volunteers were recruited for a randomized, double-blind, two-way crossover trial and received a single dose of the two soy-based formulations, one containing a pure soy standardized extract of IF, and the other containing soy flour in addition to the standardized extract of IF. Using a new and validated ELISA method, we measured the plasma and urinary concentrations of genistein, daidzein and its metabolite equol. Based on European Medicine Evaluation Agency recommendations, the main pharmacokinetic parameters allowed us to demonstrate the bioequivalence of the two formulations, indicating that the presence or absence of soy flour did not alter either the absorption or the elimination of daidzein and genistein. As bioequivalence was demonstrated, we pooled data collected during the two study-periods to address another original issue: Did the ability to produce equol affect the bioavailability of daidzein? We demonstrated that daidzein excretion was significantly lower in equol producers compared with equol non producers over the entire elimination period of the soy IF. This difference disappeared when equol excretion was added to daidzein excretion in equol producers. Our results indicated that the production of equol could partly explain the difference in daidzein bioavailability after IF ingestion.

Keywords: Isoflavones; Equol; Bioavailability; Urinary excretion; ELISA

1. Introduction

Soy (*Glycine max*) isoflavones (IF) such as genistein and daidzein belong to the well known phytoestrogen family. These polyphenolic compounds from soy are of interest due to their estrogenic activity. They have been proposed as a replacement to estrogen deficiency consecutive to menopause. Indeed, the side effects of hormonal replacement therapy have caused a fair amount of apprehension amongst menopausal women [1–3]. For several years, a large volume of controversial scientific literature has been dealing with the efficacy of phytoestrogens in menopausal treatment. To our knowledge, few clear conclusive statements about the efficacy of soy IF on human health have been written [4,5]. In all cases, to assess the potential benefits of soy IF and their metabolites, and the mechanisms by which beneficial effects on health occur, it is essential to have a more complete understanding of the pharmacokinetics

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of IF after the consumption of soy IF, and particularly after the consumption of soy-based supplements. The differences in the processing methods for soy-based supplements and/or the inter-individual variabilities in IF metabolism could be responsible for these discrepancies [4,6,7]. Indeed, soy extracts are obtained through various industrial processes (alcohol or water extraction, resin separation, or combinations of these techniques), leading to numerous soy-based supplement formulations. Despite the great number of commercialized soy-based supplements, little data is available on soy IF bioavailability according to the raw materials used in the production of particular formulations.

Furthermore, the variability of IF bioavailability can also be due to inter-individual differences in IF metabolism [6]. As a matter of fact, only 20–35% of the adult population is able to convert daidzein into equol after ingesting soy or soy derivative products [8]. This conversion is carried out by the intestinal bacteria [9,10]. These inter-individual differences could, at least, partly explain the differences in the biological effects observed following soy consumption. In this case, the combination of the greater estrogenicity of equol [8] and the longer elimination half-life from systemic circulation could result in greater physiological effects in equol producers as suggested by Karr et al. [11]. Very little data exists on the impact of the ability to produce equol on the bioavailability of daidzein and its excretion in urine. Karr et al. and Lampe et al. both studied the impact of equol production on the urinary elimination of daidzein [11,12]. Both failed to find any significant difference in the daily urine IF excretion between equol excreters and non-excreters. However, this appears to be inconsistent due to the biotransformation of daidzein into equol. To investigate this issue further, soy-based capsules with high daidzein *versus* genistein ratio, were used. Moreover, this study was performed during a 48-h period, *i.e.* a longer period than that previously published [11,12].

The present paper gives new data on the bioavailability and the urinary excretion of IF in humans for ingested doses that reflect the IF concentrations of current soy-based supplements. The study was originally performed using an ELISA assay. The objective of this study was to determine the impact of (1) the formulation of soy-based supplements and (2) the inter-individual gut microflora metabolism of daidzein into equol, on plasma pharmacokinetics of IF and their urinary excretion.

2. Materials and methods

2.1. Phytosoya® capsules

Capsules containing soy extracts were provided by Arkopharma, Pharmaceutical Laboratories (Carros, France) and adjusted with soy extract or soy flour to 17.85 and 17.20 mg of IF glycosides, respectively for capsules A and B. Capsule A formulation was 53.0% soy extract, containing 10.0% total IF and 46.1% microcristallin cellulose as the excipient. Capsule B was 38.8% soy extract containing 10% total IF and 59.7% soy flour containing 2.5% total IF. According to a comparison test, there was no difference in appearance between capsules A and B. Before the clinical trial, each formulation was assessed for daidzein and genistein, measured in aglycone equivalents using the ELISA method developed in our laboratory [13,14]. Total IF ingestion per study period was 46.12 mg equivalent aglycone for form A and 40.27 mg equivalent aglycone for form B, *i.e.* five capsules.

2.2. Study subjects and design

Twelve healthy male volunteers, aged 21–35 years with a body mass index between 20 and 25 kg m^{-2}, gave informed consent to enter the study. Prior to the study, all subjects underwent a full clinical examination. None of the subjects had an allergy or intolerance to soy. The subjects had to abstain from consuming any drugs, especially antibiotics, for at least 30 days prior to the beginning of the study and, thereafter during the study. Soy foods and their derivatives were prohibited for 10 days prior to and during the study. The study was performed at the Clinical Investigation Center (Haut-Levêque Hospital, Pessac, France) and was approved by the local Medical Ethics Committee (Comité Consultatif pour la Protection des Personnes se prêtant à des Recherches Biomédicales, CCPPRB, Bordeaux, France).

The design was a randomized, double-blind, two-way crossover study. Volunteers were hospitalized at 12:00 a.m. for a 24-h period and randomly received a single dose of either Phytosoya® formulation. After intake of the soy-based capsules, volunteers had lunch at 12:00 a.m., dinner at 7:00 p.m. and breakfast at 7:00 a.m. the following morning. After two weeks wash-out period, the study was repeated in the same condition to complete the crossover design.

2.3. Sample collection and analytical methods

Ten milliliters blood samples were drawn into Vacutainer® glass tubes (Becton Dickinson, Le Pont-De-Claix, France) containing heparin and lithium as anticoagulants, through an indwelling cannula, before (0) and at 2, 4, 6, 8, 12, 18, 24 and 48 h after capsule intake. Plasma samples were prepared by centrifugation at 5000 × *g*, 5 min, 4 °C and stored frozen at −20 °C until further analysis. Urinary samples were collected before (0) and at 6, 12, 18 and 24 h after capsule ingestion. Volunteers were instructed to collect all their urine in plastic bottles containing ascorbic acid (1 g l^{-1}) during the second day of the experiment. A 10 ml aliquot of each urinary sample was removed and stored at −20 °C until analysis. Daidzein, equol and genistein concentrations in blood and urinary samples were measured by the ELISA method as previously described [13,14], based on homologous competition tests specific to each IF. Briefly, total compounds were assayed since samples were first hydrolysed with β-glucuronidase-aryl sulfatase (Roche, Mannheim, Germany) and extracted using ethyl acetate before assay. Standard solutions were prepared from synthetic phytoestrogens [15]. The techniques used polyclonal antibodies raised in rabbits. Each plate contained a standard curve run in parallel with unknown samples. The secondary antibody was swine anti-rabbit immunoglobulin linked to peroxidase. *o*-Phenylenediamine was used as substrate for peroxidase. Hydrolysis and extractions were checked on external standards. The inter- and intra-assay variations and the limit of detection of the

techniques are reported in previous works [13]. The technique was validated against HPLC method [7].

2.4. Determination of the serum and urinary IF pharmacokinetics

Non-compartimental pharmacokinetic (PK) analysis was used to analyse plasma drug concentration–time data. The parameters C_{max} (maximum observed concentration) and T_{max} (time to reach peak concentration) were obtained directly from experimental observations without interpolation. The terminal slope (K_e) of the concentration–time curve was determined by log-linear regression of at least the last three points. Elimination half-life ($T_{1/2}$) of the terminal log-linear phase was calculated following the equation $0.693/K_e$. Area under the plasma concentration–time curve extrapolated to infinity ($AUC_{0\to\infty}$) was determined by summing the areas from time (0) to the time of last quantifiable concentration by trapezoidal and log-trapezoidal methods ($AUC_{0\to t}$) and the extrapolated area. The extrapolated area was determined by dividing the last detectable concentration by the slope of terminal log-linear phase. The volume of distribution (Vd/F) was determined by dividing the administered dose (D) by the area under the plasma concentration–time curve extrapolated to infinity, and the terminal slope (K_e) of the concentration–time curve: $D/AUC_{0\to\infty} \times K_e$. The total body drug clearance (Cl/F) was determined by dividing the administered dose by the area under the plasma concentration–time curve extrapolated to infinity: $D/AUC_{0\to\infty}$.

2.5. Statistical analysis

All pharmacokinetic parameters, particularly C_{max} and $AUC_{0\to\infty}$ required in determining bioequivalence, and statistical evaluation of the crossover study design, were performed by the pharmacokinetic software PK-FIT version 1.2 (RDPP, Montpellier, France). Formulations A and B were considered bioequivalent if mean ratio (B/A) of C_{max}, $AUC_{0\to\infty}$, and their 90% confidence interval (CI) were within 70–143% for C_{max} and within 80–125% for $AUC_{0\to\infty}$ (recommendations of the European Medicine Evaluation Agency (EMEA) CMPM/EWP/QWP/1401/98). Pharmacokinetic parameters were evaluated using the two one-sided tests procedure for logarithmic transformed data.

The comparison of plasma pharmacokinetic parameters and urinary excretion between equol producer and non producer volunteers was analysed using Student's *t*-test. Differences were considered significant at $p < 0.05$.

3. Results and discussion

3.1. Pharmacokinetic parameters of IF

Fig. 1A represents the mean plasma concentration–time profiles of daidzein and genistein, respectively from 0 to 48 h after intake of the two formulations. The kinetics of daidzein and genistein appear very similar for both formulations. At baseline, patients had no detectable concentrations of genistein and daidzein. The absorption of IF is biphasic and takes place from $t = 0$ to 8 h, indicating an entero-hepatic recirculation as already suggested by several authors [6,16,17]. The elimination of IF is linear, which allowed us to determine the elimination $T_{1/2}$ of daidzein 9.7 ± 2.6 and 8.4 ± 2.4 h and the elimination $T_{1/2}$ of genistein 20.0 ± 6.7 and 14.9 ± 3.8 h for formulations A and B, respectively. Absorption and bioavailability parameters were in agreement with those classically described in literature [6] validating the assay technique, if still necessary. Nevertheless, elimination $T_{1/2}$ was found to be longer than $T_{1/2}$ previously described, especially for genistein. Only Richelle et al. reported a similar elimination half-life of 17.8 ± 2.7 h for ingested glycoside genistein. Ideally, the goal for pharmaceutical products and bioactive agents is to remain bioavailable for target tissues and cells for a long time in order to exert their health effects. Therefore, previous authors have microencapsulated IF in order to limit their absorption and increase their mean residence time [18]. With long elimination $T_{1/2}$, both genistein and daidzein are potentially able to accumulate in plasma, achieving a steady state level. Shorter $T_{1/2}$ do not allow such a kinetic pattern, except in the case of repeated ingestion throughout the day. Such a practice is common to Asian people consuming soy foods as a natural component of the traditional diet [18]. In western countries soy is not consumed as much, but soy capsules can be ingested at this rhythm. Therefore, such kinetic because they lead to a steady-state level [7] can lead to a reduction of daily IF capsule intake to achieve the same plasma level.

3.2. Assessment of bioequivalence

Table 1 reports the main pharmacokinetic parameters of IF. According to the statistical analysis and the EMEA criteria, the two formulations of soy-based supplements are bioequivalent. In previous studies, the bioavailability of IF was investigated using either pure compounds, whether glycosilated or not [19–24], or single soy foods [17,25–27]. To our knowledge, there is little data on the bioavailability of the different forms of soy capsules according to the extraction and preparation procedures of IF [20]. In the aim of capsules processing improvement, the present study brings some new relevant data.

3.3. Urinary excretion of IF

For formulations A and B respectively, 51.4% and 33.2% of the total ingested genistein was excreted in urine 48 h after soy-based capsule intake (Fig. 2A). From 0 to 24 h, the genistein excretion profile was a bell-curve with a maximal excretion peak at 12 h. The daidzein excretion profile is similar. For both formulations, 65% of ingested daidzein was eliminated during the study period (Fig. 2B). The mean Vd/F of daidzein was 61.8 and 53.8 l and the mean Cl/F was 4.85 and 4.54 $l\,h^{-1}$ for A and B formulations, respectively (Table 1). The lower urinary elimination recovery of genistein compared with daidzein on a 48-h study period is in accordance with literature [26] and with the longer elimination $T_{1/2}$ of genistein compared with daidzein.

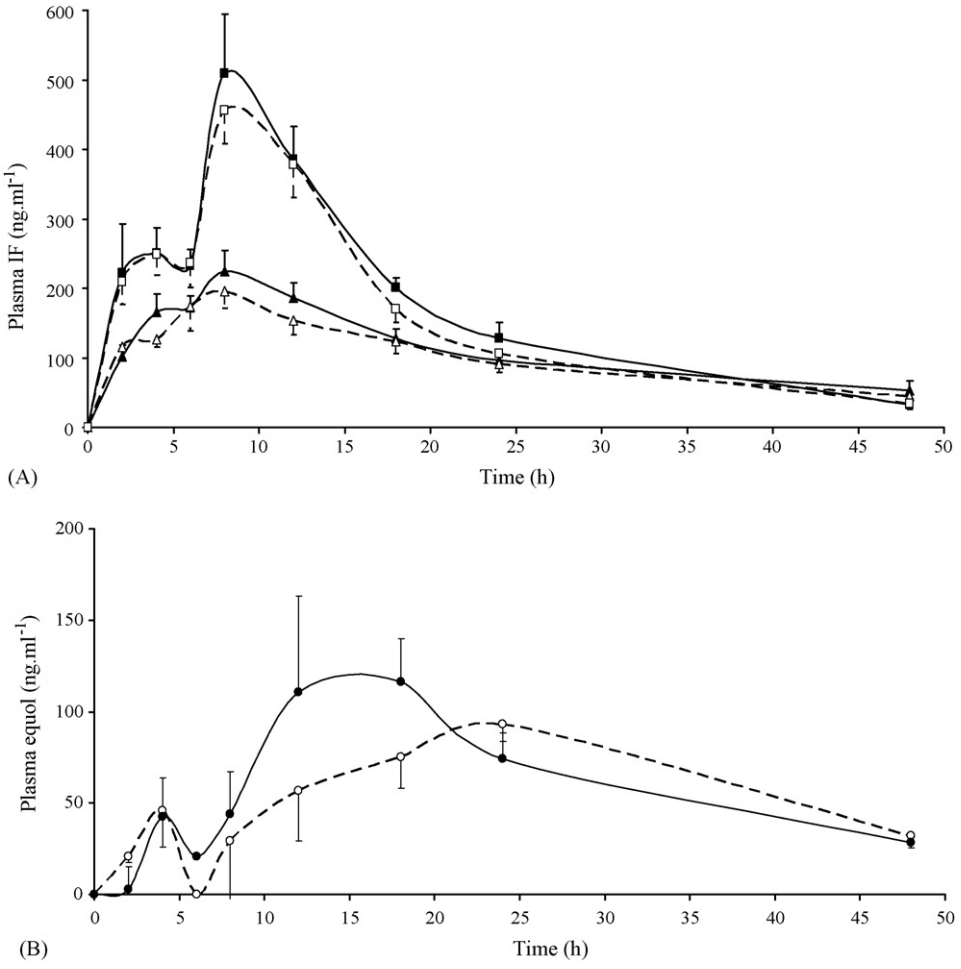

Fig. 1. (A) Time-course of plasma daidzein (square) and genistein (triangle) concentrations (mean ± S.E.M.) in 12 volunteers following formulation A (solid form) and B (open form) capsule intake. (B) Time-course of plasma equol concentrations (mean ± S.E.M.) in the four equol producer volunteers following formulation A (solid circle) and B (open circle) capsule intake.

3.4. Plasma kinetics and urinary excretion of equol

Four out of 12 volunteers were found to be equol producers. As shown in Fig. 1B, the pharmacokinetic profiles are very similar for both formulations. The mean C_{max} of equol were 133.6 ± 54.0 and 141.4 ± 23.10 ng ml^{-1} at 16.0 and 16.5 h for A and B formulations, respectively. Pharmacokinetic analysis of the plasma concentration–time curves determined that the mean $AUC_{0\rightarrow\infty}$ of equol was 3660 ± 872 and 3566 ± 904 ng ml^{-1}, respectively for formulation A and B. Volunteers with urine equol excretion greater than 2 μmol per day were defined as equol producers according to Lampe et al. [12]. The mean equol excretion of the 4 equol producers is represented in Fig. 2C. Equol appeared mainly at $t = 12$ h in urine. The maximal urinary equol excretion was observed at $t = 18$ h, although excretion continued from 18 to 48 h after capsule intake. Although equol concentration in plasma is lower than that of daidzein, some evidences indicate that it could be advantageous to convert daidzein

Table 1
Pharmacokinetic parameters for daidzein and genistein and relative bioavailability of the two formulations

	Daidzein			Genistein		
	Mean (95% CI)		Ratio (90% CI)	Mean (95% CI)		Ratio (90% CI)
	Formulation A	Formulation B	B/A	Formulation A	Formulation B	B/A
$AUC_{0\rightarrow\infty}$ (ng ml^{-1} h)	7978 (6220–9736)	8425 (6573–10276)	1.06 (0.93–1.18)	6150 (4483–7817)	5863 (4293–7432)	0.95 (0.80–1.10)
C_{max} (ng ml^{-1})	508.1 (414.6–601.7)	567.7 (379.6–756.0)	1.12 (0.84–1.39)	254.6 (182.3–327.0)	261.9 (197.3–326.4)	1.03 (0.75–1.30)
T_{max} (h)	8.67 (7.20–10.13)	8.17 (5.90–10.43)	0.94 (0.63–1.25)	7.67 (5.97–9.37)	7.67 (6.48–8.86)	1.00 (0.84–1.16)
$T_{1/2}$ (h)	9.71 (7.17–12.3)	8.43 (6.0–10.9)	0.87 (0.55–1.18)	20.0 (13.3–26.7)	14.9 (11.0–18.7)	0.74 (0.43–1.01)
Vd (l)	61.6 (49.5–73.8)	53.8 (37.5–70.0)	0.87 (0.79–1.08)	38.7 (29.2–48.2)	30.8 (24.0–37.5)	0.80 (0.75–1.16)
Clearance (l h^{-1})	4.85 (3.87–5.83)	4.54 (3.73–5.34)	1.09 (0.54–1.14)	1.59 (1.09–2.10)	1.57 (1.21–1.92)	0.98 (0.81–1.16)

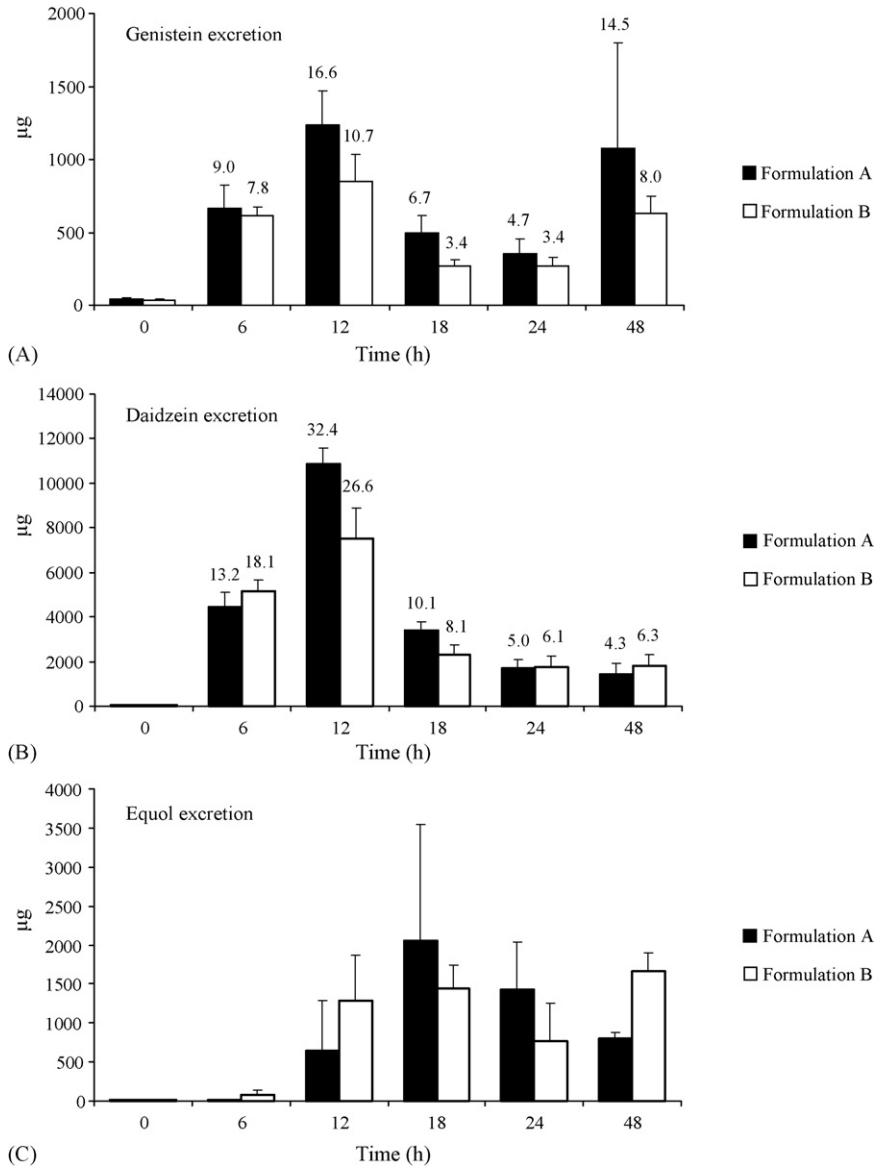

Fig. 2. Urinary genistein (A), daidzein (B) and equol (C) excretion profiles following the ingestion of formulation A capsules (black bars) and formulation B capsules (open bars). Results are expressed as mean ± S.E.M. Values indicated at the top of the bars represent the eliminated fraction as a percentage of intake.

into equol. First, 49.7% of equol circulates freely in plasma due to its greater solubility [28] compared to the 18.7% of free circulating daidzein [28]. Moreover, free equol is able to bind the two sub-types of estrogen receptor (ER) and shows a stronger affinity for ERβ than that of its precursor [29]. Only the unbound fraction is considered to be available for estrogen receptor activation. Consequently, it could be advantageous to convert daidzein into equol to enhance its estrogenic potency *in vivo* [30], especially in the case of estrogen deficiency occurring during menopause.

3.5. Comparison of pharmacokinetic parameters and urinary excretion of daidzein in equol producers and non producers

As bioequivalence between formulations A and B was demonstrated and no statistical period effect was found, data from the two crossover periods were pooled to address another question: Does equol production influence the bioavailability and excretion of daidzein? Interestingly, no statistical differences were found for the plasma PK parameters between equol and non equol producers (Table 2). This lack of significance could be due to (1) the low number of equol producer volunteers; (2) the large inter-individual variability for plasma PK parameter data; (3) the low daidzein/equol conversion rate in the volunteers; and (4) the low ingested dose of daidzein. Previous studies have also failed to find a significant difference in daily IF excretion between equol producers and non producers [11,12,31] even though, in all studies performed over a 24-h period, daidzein excretion tended to be lower for equol producers. On the contrary, we were able to show that equol producers excreted significantly lower amounts of daidzein than equol non producers after extending the analytical period to 48 h following IF ingestion (Fig. 3). Indeed, in our study the average daidzein concentration was still 117.26 ± 11.20 ng ml^{-1} at 24 h and there-

Table 2
Pharmacokinetic parameters for daidzein according to the ability of volunteers to produce equol and the relative influence of the biotransformation of daidzein into equol on the bioavailability of daidzein

	Daidzein		
	Equol producers Mean (S.E.M.)	Non equol producers Mean (S.E.M.)	*p*-Value of the equol production effect
$AUC_{0\rightarrow\infty}$ (ng ml^{-1} h)	8191 (6892–9490)	8207 (7188–9226)	0.99
C_{max} (ng ml^{-1})	585.1 (470.3–699.8)	514.4 (449.9–578.9)	0.57
T_{max} (h)	9.50 (8.54–10.5)	7.88 (7.53–8.23)	0.11
$T_{1/2}$ (h)	9.19 (7.28–11.1)	9.01 (8.29–9.73)	0.92
Vd (l)	56.5 (47.3–65.6)	58.3 (52.9–63.7)	0.86
Clearance (l h^{-1})	4.72 (3.94–5.50)	4.68 (4.28–5.08)	0.96

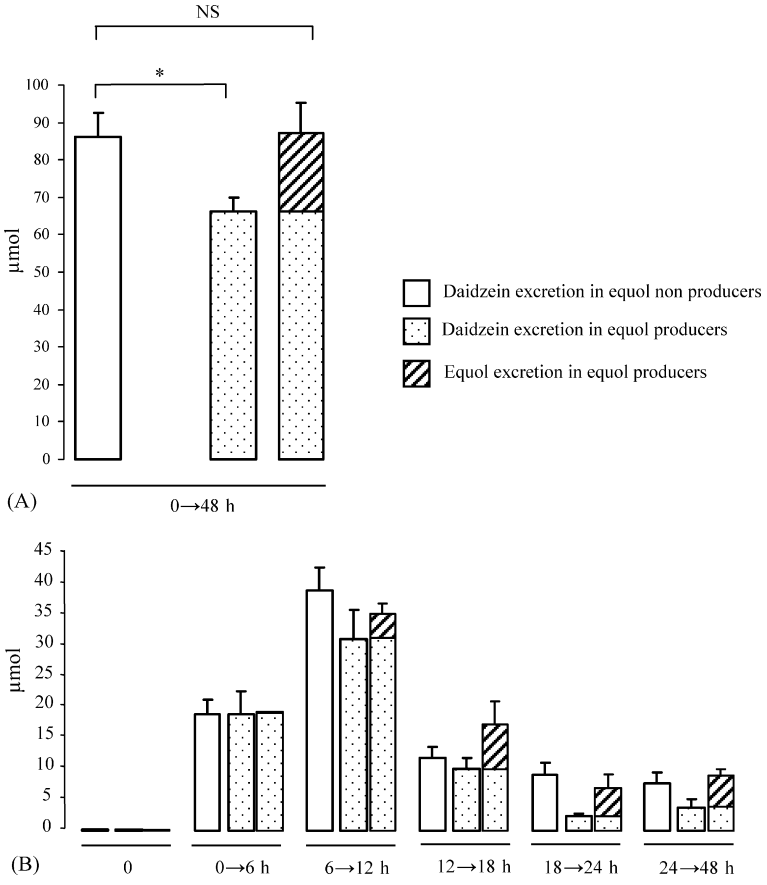

Fig. 3. Total (A) and detailed (B) urinary excretion of (□) daidzein in equol non producers; (⬚) daidzein in equol producers; and (▨) daidzein + equol in equol producers. Results are expressed as mean ± S.E.M.; (*) indicates significant difference using Student's *t*-test with $p < 0.05$, NS indicates a non significant difference.

fore, the elimination process was not finished by that time. When urinary excretion of daidzein is considered on its own, a significant difference is recorded between equol excretors and non excretors. However, when daidzein and its metabolite equol are considered the difference previously mentioned disappears. Several authors have concluded that the urine excretion parameter of IF is largely inter-variable [6,7,26]. Kelly et al. demonstrated that the inter-individual variations may reflect differences in gut microflora populations [32]. The present work adds some new evidence on the fundamental role of gut bacteria on the urinary excretion of IF and illustrates the importance of defining equol producers for further clinical studies aiming at studying soy IF.

4. Conclusions

This randomized double-blind, two-way crossover study has shown the bioequivalence between two formulations of a soy-based capsule, one containing a pure soy standardized extract of IF, and the other soy flour in addition to the standardized extract of IF. The matrix differences between the two formulations did not alter either the absorption or the elimination of IF. Elevated elimination $T_{1/2}$ sustained a long elimination process for both daidzein and genistein and therefore, a potential accumulation of daidzein and genistein during chronic ingestion, with the appearance of the steady state level in such ingestion conditions. Moreover, the data obtained in this study has

shown a significant difference in daidzein excretion between equol producers and non equol producers, while no difference in plasma pharmacokinetic parameters of IF was revealed between these population subtypes. This may be taken into account when IF effects are interpreted on the basis of urine excretion.

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