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SCIENCE DIRECT
Journal of Pharmaceutical and Biomedical Analysis 41 (2006) 957–965
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Concentrations of isoflavones in plasma and urine of post-menopausal women chronically ingesting high quantities of soy isoflavones

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Received 24 October 2005; received in revised form 23 January 2006; accepted 25 January 2006
Available online 2 March 2006

Abstract

Soy food or food supplements based on soy containing isoflavones (Isos) are increasingly available in Western countries. However, the variability of Isos levels in urine and plasma in humans during chronic ingestion is poorly documented. Nevertheless, this is the way these compounds will most probably be used in the future, especially if the soy-based supplements market goes on increasing. Here, glycosilated Isos in an enriched extract of Prevastein equal to 100 mg of equivalent Isos aglycone was given daily to 27 post-menopausal women for 30 days and to 12 post-menopausal women for 60 days. Volunteers were given Prevastein in a cereal bar (25 mg Isos) and in a yoghurt (25 mg Isos) both at breakfast and dinner. Plasma samples were collected after overnight fasting. Urine samples were aliquots of a 24 h collection checked on volume and creatinin excretion levels. Genistein, daidzein and equol were measured at day 0 and every 15 days afterwards, using original specific ELISAs. Constant levels were reached from the 15th day. About 59.2% of the volunteers were significant equol producers in the first experiment and 58.3% in the second. A large variability in plasma and urine levels was observed among post-menopausal women consuming 100 mg Isos per day, although remaining relatively stable in each individual subject. This could partly account for the controversial effects of Isos recorded so far in clinical studies. So Isos plasma levels would have to be assayed during chronic exposures, and could help to better understand the large variability of the effects classically observed in clinical studies. ELISA techniques could be easily exported to analytical laboratories to help physicians and nutritionists with their prescriptions.

Keywords: Isoflavones; Post-menopausal women; ELISA; Plasma; Urine; Chronic exposure

1. Introduction

Soy isoflavones (Isos), and especially genistein and daidzein from soy extracts, are now increasingly used in Western countries as diet supplements for post-menopausal women. Indeed, more than 100 supplements based on soy Isos were recently inventoried in France in April 2005 (Phytohealth CP4, WP1). In this specific case, supplement providers sometimes indicate a two times daily intake: one in the morning and one in the evening. However, very little data is so far available on the plasma bioavailable fraction of soy Isos with such a dosage. Soya is also advertised as a source of these Isos for post-menopausal women. These soy-based foodstuffs contain Iso amounts similar to food supplements based on soy [1]. The main claims of these foodstuffs or diet supplements are the reduction of climacteric symptoms [2], cardiovascular prevention and bone sparing effects, although these commercialised preparations are still controversial [3]. Indeed, for climacteric symptoms no clear dose – response effect can be detected from the data published in the literature [2,4,5]. The preservation of bone density is a promising issue, since, up to now, studies conducted on animals, in vitro on human and animal bone cells and in clinical investigation all showed a bone sparing effect either with high soy intake or with treatment with high concentrations of Isos [6–13]. However, the mechanisms of the action of Isos remain to be clearly determined before Soy or Isos could be used to prevent osteoporosis. In any case, if the bone sparing effect is conclusively proven, this effect could be of great interest nowadays as elderly women represent an ever-increasing percentage of the Western population. Even though Isos are sometimes considered as a good alternative to HRT [2], controversy still remains as to their effect on a developing breast cancer [14–17]. As long as patients and physicians

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doi:10.1016/j.jpba.2006.01.051

are aware of such restrictions in usage, Isos can probably be used safely in most cases.

In this context, and because soy-based supplements are currently taken for several weeks once or twice a day, and because soy food as healthy food can be consumed regularly, it seemed interesting to measure the plasma fraction of genistein and daidzein (i.e. the main soy Isos) in such conditions of usage. Daidzein's main metabolite, equol, that appears now to be a very active compound [18–20], was also investigated. Several studies performed on a unique intake [21–23] have already reported the plasma and urinary pharmacokinetic parameters of Isos. More recently, other studies [24–27] contributed to confirm the pharmacokinetic parameters first obtained, although minor discrepancies sometimes appeared in T1/2 and T_{max} values.

Few studies so far have provided information about Isos plasma levels during chronic ingestions [1,21,28–30]. In this context the present work, which is a double blind clinical study, was carried out on post-menopausal women chronically supplemented (for 30 or 60 days) with soy-enriched food providing 100 mg Isos/day, expressed in aglycone equivalents to assess Isos bioavailability.

2. Experimental

2.1. Volunteers

Thirty-nine post-menopausal women aged 47–73 years old (median age: 59) and with a Body mass index (BMI) between 17.5 and 34.6 (median: 26.0) were enrolled in two clinical studies. All were recent post-menopausal women and had their last menstruations one year before. Their LH levels were in accordance with a post-menopausal status. In the first study, 27 volunteers accepted to take Isos in yoghurt and cereal bars for 30 days. In the second study, 12 other volunteers accepted to take Isos in yoghurt and cereal bars for 60 days. Compliance was encouraged with regular phoning and checked weekly on remaining food items. No problem of any kind was noticed. None of them had HRT or digestive troubles or a diet that could possibly interfere with the study (vegetarian, high fiber or regular soy consumption). Dietary records were collected and analysed which showed that the dietary Isos intake, excepting that of the treatment, ranged from 0.026 to 0.1 mg/day according to the French Isos data-base established by AFSSA [31]. This is within the range of standard French consumption. Each volunteer was informed about the study. They were not involved in other studies and they all agreed to consume yoghurts or cereal bars. The (comité Consultatif de Protection des Personnes se prêtant à des Recherches Biomédicales) CCPPRB of Clermont - Ferrand granted ethical approval. Volunteers affected with food allergies, or having taken antibiotic medications during the study or up to three months before, were excluded.

2.2. Isoflavone intake

At recruitment, women were asked not to eat soy or soy products except soy lecithin (devoid of Isos) for 3 weeks before the study started and for the whole duration of the study. Except for this restriction, all women maintained their usual feeding habits. In the first experiment, all 27 women received 100 mg of Isos (expressed in aglycone equivalents) per day, 50 mg in the morning at breakfast and 50 mg in the evening at dinner. Isos were concealed in a cereal bar (25 mg) and in a yoghurt (25 mg). This supplementation was carried out for 30 days. In parallel, 12 post-menopausal women accepted to have the same yoghurt and cereal bars at the same rhythm for 60 days. Although they were present in food as glycosylated forms, Isos were always expressed in aglycone equivalents. According to the volunteers bodyweight, the intake ranged from 1.6 to 1.4 mg/kg bw. This is higher than the dose recently recommended by the French Food Agency for consumers, but in this study a medical survey was organized and the test only lasted for 60 days at the very maximum. In addition, in several studies scientists previously used even higher doses of isoflavones without any trouble [32,33].

2.3. Compliance control

The Isos preparation Prevastein (Prevastein®HC, Eridania Beghin-Say, Thumeries, France) was assayed before starting the experiment and was determined to contain 46.19 g of total Isos expressed in the aglycone form per 100 g of preparation. The proportions were as follows: genistin 55–75%, daidzin 20–40%, glycitin 1–5%. Blood sampling was performed in the morning after over-night fasting at day 0 and every 15 days thereafter. Twenty-four hours urine samples were obtained from the volunteers at the same time during the two studies. In addition, a 24 h urine collection was organized at day 5 and urine samples were analyzed for equol production. Checked on both volume collected and the levels of creatinin in the urine, the compliance for the 24 h collection was found to be 87.8% in the first 30 days experiment and 84.5% in the second 60 days experiment. Blood was taken using heparinised vacutainers and spun for 10 min at 10,000 g in order to separate the plasma and blood cells. Plasma samples were collected and stored at −20 °C until analysis. The twenty-four hours urine samples were collected onto ascorbic acid (1 g/L) and 5 mL aliquots were collected after homogenisation and before being stored at −20 °C. Creatinin in urine was assayed according to Jaffé's reaction [34].

2.4. Chemicals used

All chemicals came from SIGMA, (L'isle d'Abeau Chesne, France) unless otherwise mentioned. Secondary antibody was supplied from Dako (Trappes, France). β-glucuronidase arylsulfatase was supplied from Roche Diagnostique (Meylan, France). Ethyl acetate and ethanol came from ICS (Belin-Beliet, France).

2.5. The ELISA technique

All assays were performed using specific ELISA techniques for genistein, daidzein and equol. These techniques were performed as described elsewhere [1,35,36]. Briefly, samples were hydrolysed with β glucuronidase-aryl sulfatase for 48 h at 37 °C, and extracted using ethyl acetate before assay. Enzyme activ-

ity was checked on a genistin solution run in parallel in the same conditions. The extraction procedure was checked on a genistein solution extracted in parallel. Standard solutions were prepared using phytoestrogens synthesized according to previously described methods [37] and stored at 4 °C when not being used. Microtitration plates were coated with thyroglobulin-hapten conjugates. Antibodies were raised in rabbits against the same hapten but linked to bovine serum albumin. Serial dilutions of the analytes were prepared as standard curves and run on each microtitration plate in parallel with unknown samples. The secondary antibody was swine anti-rabbit immunoglobuline linked to peroxidase. *o*-phenylenediamine was used as substrate for peroxidase. The reaction was stopped with H_2SO_4. ODs were read at 490 nm. The inter- and intra-assay variations as well as the sensitivity of the technique, are reported in previous works [1]. As validation of the ELISA technique, day 5 urine samples were analysed using an HPLC method according to [38]. The results obtained on urine samples treated with both techniques fitted correctly.

2.6. Statistical analysis

Statistical analysis was performed using Jump Software fifth version for Windows. Variance comparison was performed using the Log-Anova test and mean comparisons were performed using Student *t*-test.

3. Results

3.1. Measurements at day 0 and day 5

Measurements were performed on plasma and urine samples at day 0 before the experiment started. None of the Isos assayed were found to be detectable. Therefore, dispersion and correlation values will not be presented at day 0 hereafter. Although no data on plasma are available at day 5, 24 h urine collection allowed Isos measurements in urine samples. Values recorded for the first experiment were compared to those measured later during the experiment and were not found to be significantly different. Indeed, in the first experiment genistein, daidzein, equol and total Isos mean concentration ± S.E.M. were respectively: 52.57 ± 7.74, 73.53 ± 7.35, 15.93 ± 2.71 and 134.37 ± 13.24 μMole excreted per 24 h. In the second experiment, the values for genistein, daidzein, equol and total Isos were 40.55 ± 6.84, 66.97 ± 10.71, 15.72 ± 5.73, 115.38 ± 17.10 μMole excreted per 24 h, respectively.

3.2. The evolution of mean concentrations with time

The evolution of mean plasma concentrations ± S.E.M. are presented in Fig. 1a for the 30 days experiment and in Fig. 1b for the 60 days experiment. Fig. 1a and b show that the concentrations measured at day 15 (D15) were maintained thereon.

Indeed, the mean plasma concentrations were not significantly different from D15 to D60. The same pattern is noticed in urine profiles (Fig. 2a and b). It can be noted that, although the concentration of genistein is higher than that of daidzein in plasma, the situation is inverted in urine. Indeed, the daidzein excretion is consistently higher than that of genistein for both experiments, according to the T and Anova tests.

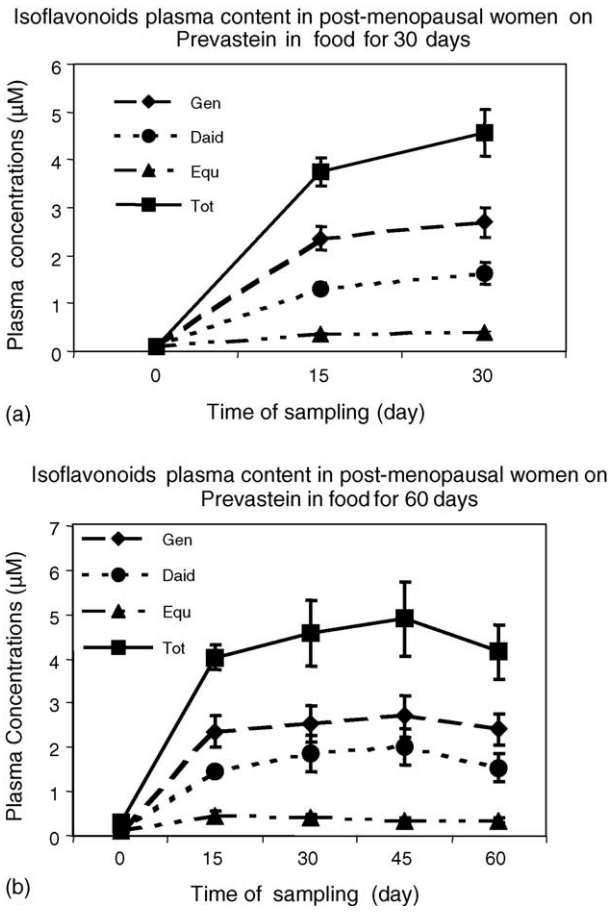

Fig. 1. Evolution of isoflavone plasma levels in menopausal women on French habitual diet supplemented with 100 mg prevastein. Plasma samples were collected 11 h post-dosing. Prevastein was delivered in yoghurt (25 mg) and cereal bars (25 mg) taken together both on breakfast and dinner. 1a give data obtained on a 30 days intake ($n = 27$) and 1b data obtained on a 60 days intake ($n = 12$).

3.3. Equol production

Sixteen out of the 27 women (59.2%) involved in the 30 days study produced equol, as measured in their plasma and urine samples. In the second experiment lasting 60 days, 7 out of 12 women (58.3%) were found to be equol producers. Equol production does not seem to be markedly modified when Isos consumption is maintained. Indeed, in some cases, the production was always elevated with, plasma concentrations ranging from 0.56 to 0.72 μM from D15 to D60 while in some other cases it always remained low with values ranging from 0.23 to 0.29 μM from D15 to D60. All the equol producers detected after 15 days of treatment remained producers during the whole duration of the study. On the other hand, when a volunteer was detected as a non equol producer at day 15, she remained a non producer during the whole study, i.e., her equol production remained non detectable during the study. There was only one exception.

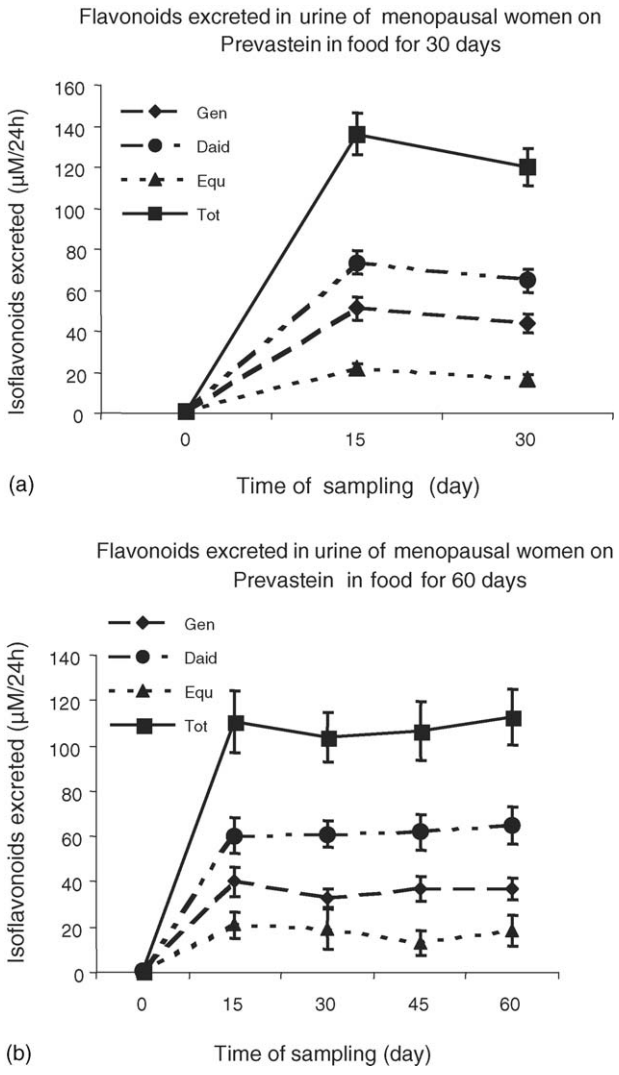

Fig. 2. Evolution of isoflavone urine excretion in menopausal women on French habitual diet supplemented with 100 mg prevastein. Plasma samples were collected 11 h post-dosing. Prevastein was delivered in yoghurt (25 mg) and cereal bars (25 mg) taken together both on breakfast and dinner. 1a give data obtained on a 30 days intake ($n = 27$) and 1b data obtained on a 60 days intake ($n = 12$).

3.4. Dispersion of values

Tables 1 and 2 give the dispersion criteria, including mean, min and max and the variation coefficients (VC) for the two experiments. These data show that Min–Max values are sometimes very different, sustaining the idea of a great variability in individual bioavailability of Isos. In addition, the VCs were relatively high (from 49 to 66% for the 30 days exposure and from 42 to 76% for the 60 days exposure). In plasma, the highest variability was recorded for daidzein (Table 1). The pattern is identical for urine data (data not shown).

For the second experiment, mean concentrations and dispersion criteria are given in Table 2. Data are in accordance with that of Table 1 and the variability is identical at all sampling times. The pattern is identical for data obtained on urine samples (data not shown).

Table 1
Dispersion criteria on plasma data collected during 30 days ($n = 27$)

	Day 15	Day 30
Genistein		
Mean	2.35	2.44
Min	0.72	0.92
Max	5.00	6.08
VC	53.11	55.42
Daidzein		
Mean	1.31	1.53
Min	0.58	0.43
Max	3.82	4.27
VC	56.21	65.77
Equol		
Mean	0.36	0.38
Min	0.04	0.03
Max	0.73	0.87
VC	51.46	60.70
Total		
Mean	3.74	4.56
Min	1.36	1.61
Max	7.02	9.96
VC	49.31	54.85

Plasma samples were collected 10 to 11 h post-dosing.

3.5. Correlation between plasma and urine levels

Table 3a presents correlation coefficients (R) calculated from the data collected at day 15 and 30 for the first experiment and plotted separately. Coefficients were calculated on 22 samples for genistein, daidzein and total Isos and 14 samples for equol. Table 3b presents correlation coefficients (R) calculated on data from the 12 volunteers participating to the second experiment. All data collected from day 15 to day 60 were plotted on the same

Table 2
Dispersion criteria on plasma data collected during 60 days ($n = 12$)

	Day 15	Day 30	Day 45	Day 60
Genistein				
Mean	2.35	2.53	2.70	2.42
Min	1.21	0.71	0.82	0.84
Max	5.26	4.69	5.55	4.15
VC	51.61	57.36	58.72	50.44
Daidzein				
Mean	1.46	1.85	2.00	1.54
Min	0.67	0.45	0.56	0.39
Max	2.77	4.54	4.56	3.54
VC	49.28	76.72	72.24	71.14
Equol				
Mean	0.44	0.40	0.34	0.34
Min	0.22	0.21	0.13	0.17
Max	0.72	0.62	0.65	0.64
VC	52.74	46.32	57.26	44.90
Total				
Mean	4.03	4.58	4.90	4.16
Min	2.42	1.34	1.41	1.87
Max	8.03	9.23	10.11	7.55
VC	42.90	57.47	58.87	51.03

Plasma samples were collected 10 to 11 h post-dosing.

Table 3a
Correlation coefficient (*R*) between plasma concentration (μM) measured 10–11 h post-dosing and urinary excretion (μM/24 h) at 15 and 30 days of treatment

(*R*) Plasma vs. Urine	D15	D30
Genistein	0.56	0.81
Daidzein	0.15	0.23
Equol	0.60	0.35
Total	0.16	0.33

Table 3b
Correlation coefficient (*R*) between plasma concentration (μM) measured 10 to 11 h post-dosing and corresponding urinary excretion (μM/24 h) of volunteers from 15 to 60 days of treatment

(*R*) Plasma vs. Urine	D15–D60
Genistein	0.59
Daidzein	0.36
Equol	0.76
Total	0.31

graph in order to produce statistically significant coefficients. These correlation coefficients were calculated on 39 samples for genistein, daidzein and total Isos, and 31 samples for equol. It can be observed that the correlation coefficients (*R*) between Isos plasma levels and Isos excreted in urine were globally low, ranging from 0.15 to 0.81.

Table 4 presents data obtained on 4 volunteers from the second experiment Tho 11, Gro 4, Cub 29 and Sal 34. It can be seen that Tho 11 presented high plasma Iso concentrations (mean: 7.03 μM; S.D.: 3.51) and high urinary excretion (mean: 153.11 μM/24 h; S.D.: 76.55) for each sampling time. In the meantime Gro 4 and Sal 34 respectively presented significantly lower plasma levels (mean: 2.70 μM; S.D.: 1.35) and (mean: 3.17 μM; S.D.: 1.59). However, their urinary excretions were not significantly different from that of Tho 11. Under the same conditions, Cub 29 constantly presented relatively low plasma concentrations (mean: 3.57 μM; S.D.: 1.79) not significantly different however from Tho 11 and high urinary excretion (mean 149.37 μM/24 h; S.D. 74.68) nearly equivalent to that of Tho 11 ($p = 0.8615$). Finally when Cub 29 and Sal 34 excretion values were compared, they appeared to be significantly different ($p < 0.0001$) while their plasma levels were nearly equivalent ($p = 0.5006$).

4. Discussion

The specific ELISA techniques used in this study were previously validated in an international ring test organised by Acatris International [1]. It was shown that for low Iso concentrations in food, the assay is as good as other physicochemical techniques, including HPLC-MS techniques. In the present study, the assays were validated through comparison with HPLC-UV and HPLC-CAD [39] (data not shown). All urinary samples collected at day 5 were doubly analysed in order to identify equol producers and validate the ELISA technique. These assays were shown to be reliable and sensitive enough to assess low doses in plasma samples and in urine samples. They offer the opportunity to assay large numbers of samples in one run with a simple extraction procedure before assaying, thus reducing costs [1].

At day 0 no Isos were detected in the volunteers' urine and plasma, which is explained by the fact that the volunteers were asked not to eat soy-food, except soy lecithin, for at least 3 weeks before the experiment began nor during the rest of the experiment. Day 0 represents the controlled Isos intake. The volunteers filled in the food questionnaires, and the only soy product which appeared to be consumed during the experiment, was soy lecithin. Indeed, it is known that lecithin contains only very low levels of Isos [1]. In addition, the AFSSA-AFSSAPS database [31] gives Isos consumption from food other than soy in the typical French diet. It reports that a traditional French diet globally delivers to the consumers, quantities of daidzein plus genistein from 0.026 and 0.100 μg per day. This is below the estimation calculated for other European countries [40–45]. It was then found that the global Isos intake in traditional French foodstuffs is low (below 1 mg/day) and was considered to be negligible compared with the 100 mg of Isos delivered everyday during the testing periods. Therefore, the Isos delivered by the usual diet of each volunteer should have no effect on the large individual variations in plasma and urine levels registered in the study.

Table 4
Plasma and Urine Isoflavonoids concentrations in 4 volunteers from the second experiment

	Plasma concentrations in four volunteers (μM)				Urine excretion in four volunteers (μM/24 h)			
	Tho11	Gro 4	Cub 29	Sal 34	Tho 11	Gro 4	Cub 29	Sal 34
D15	8.03	3.32	2.42	3.17	200.20	87.31	139.60	35.82
D30	6.57	2.59	3.23	3.61	146.59	90.03	163.22	50.69
D45	6.89	2.60	4.83	3.36	161.01	75.71	154.35	37.84
D60	6.62	2.30	3.80	2.55	104.63	121.02	140.29	18.06
Mean	7.03	2.70	3.57	3.17	153.11	93.52	149.37	35.60
SD	3.51	1.35	1.79	1.59	76.55	46.76	74.68	17.80
Stat group	a	b	ab	b	a	ab	a	ac
Significance		.0001	.0013	.0001		.035	.86	.0013

Group of values shearing the same letter are not significantly different. Significance is given from levels measured in Tho 11.

Blood samples were always collected between 7:00 and 9:00 am after an overnight fasting, i.e., 10–12 h after the last Isos intake. According to previous studies [1,21,22,26], at this time Isos concentrations in plasma are known to rapidly decline. Although it can sound questionable to tend to correlate Isos plasma levels from spot samples collected several hours post dosing, many other authors have already done this [23,32,45,46]. Commonly, the other authors performed their measurements 6–8 h post ingestion of Isos since it is known that the T_{max} values for isoflavones range from 6 to 12 h [21,22,47–49]. However, in chronic administration, a steady-state level is expected and therefore Isos plasma levels are thought not to fluctuate a great deal around the plateau value. Therefore, the sampling time post ingestion does not necessarily have to be at T_{max} as long as it is constant for the whole study. For urine, 24 h excretions were measured and corrected by creatinin excretion. Because the study was performed in a double blind pattern all samples were initially assayed but when urine volumes and creatinin did not match the classical physiological data, the concentrations were not used in the analysis.

Indeed, Ritchie and collaborators have shown that samples of 24 h urine collections are reliable for Isos excretion measurements as long as they are checked with *p*-aminobutyric acid (PABA) [45,50]. In this experiment, PABA could not be used for technical reasons.

As previously mentioned, 27 volunteers participated in the first experiment (lasting 30 days) and 12 volunteers in to the second (lasting 60 days). Plasma levels recorded after overnight fasting reached a plateau as early as at day 15 in both experiments and did not evolve thereon. The plateau may have been reached earlier but no plasma samples were collected to verify this. As mentioned earlier, a 24 h urine collection was organized at day 5 initially to check equol production among the volunteers and validate the ELISA technique on this matrix. All classical Isos were measured on these samples and the levels were not found to be significantly different from those collected later. Although these are urine samples and may not exactly reflect the plasma compartment, this may indicate that the pharmacokinetic plateau had already been reached at day 5. However, this information should ideally have been verified on plasma samples and this still remains to be validated. These findings may be in discrepancy with some previous findings obtained on animals and humans, which reported a modification of the pharmacokinetic properties of Isos in consumers after chronic Isos ingestions. Namely, this modification was characterized by a decrease in Isos concentrations in plasma and urine after several days of chronic ingestion [47,51–53]. However, the present data (Fig. 1a and b) shows that plasma levels remain constant overall during a 60-day-period in post-menopausal women chronically exposed to 100 mg of Isos per day. This suggests that the kinetic remained unchanged. Several authors [1,21,22,26] already suspected this.

It was shown in this chronic exposure that, although daidzein concentrations in plasma were lower than those of genistein and reflected the ingested proportions, the reverse was the case in the urine samples. This is in accordance with previous studies undertaken on humans and rats [26] showing that daidzein excretion is higher than that of genistein. This is also in accordance with the half-life of the compounds as measured previously in human subjects by Setchell and collaborators [21,54,55] and as reported by Rowland [56]. This, with the possible conversion of daidzein into equol, may account for the poor correlation globally observed between the daidzein plasma levels and those recorded in urine (see Table 3a and b). Because urine levels do not reflect the ingested proportions of genistein and daidzein in this study, they may not be the best markers of chronic Isos intake.

Equol producers in this study appeared to represent between 58% and 60% of the volunteers. This proportion is higher than that reported in other studies (40% generally) [18,20,54,57] but it could be due to the small size of the tested population or to the sensitivity of the ELISA technique. Indeed, some of the equol producers exhibited constantly low plasma and urine levels during the experiment. If the ELISA technique had been less sensitive these volunteers would have been considered as non-equol producers. It must be noticed that one of the volunteers who participated in the second study had undetectable equol levels at the beginning of the experiment and low detectable levels at day 30 and after. It is known that equol is produced by the colon microflora through the metabolic transformation of daidzein [23,54,55,58]. Recently, Decroos et al. found a mixture of colon bacteria able to perform this transformation [59] but this mixture is not always present in human gut. This explains why human subjects can be either equol producers or non-equol producers. According to this study, it appears that the bacterial flora responsible for equol production cannot be simply induced by the introduction of its precursor i.e. by a daidzein supplementation. However, this ability to produce equol appears to be very important from a health point of view, and according to several authors, this compound seems to have particularly interesting health effects [20,23,60]. Equol production via the management of human intestinal flora may be a crucial issue in the years to come.

The levels of genistein, daidzein and equol in volunteers' plasma and urine were globally highly variable from one subject to another (Tables 1 and 2), although the Isos intake was always the same. This is confirmed by Table 4 in which statistical analysis was performed on plasma or urine levels collected from the same volunteer at each sampling time. In this table, the problems of urine collection cannot explain the lack of coherence between plasma and urine levels. All data obtained on urine samples were checked for both volume and creatinin excretion. Furthermore, if urine collection had been incomplete, urine levels would have presented much larger variations from one sample time to another for the same subject. All our results are in accordance with previous studies first reporting high variation in women's urine excretion after 6–10 days of consumption of moderate to high Isos quantities [23,25,32,61]. These results are in accordance with those given in Table 3a and b that show poor correlations between plasma and urine Isos levels. However, there is a discrepancy between our results and those of Ritchie and collaborators [45,50]. These authors found correlations above 0.80 between plasma and urine levels when they tested various Isos intakes. In their studies, they worked with

21 volunteers, having an Isos intake via Scottish food ranging from 1.2 to 3.7 mg/day plus a supplementation of 28 mg of Isos. They followed urine excretion with PABA and managed to distinguish the higher from the lower Isos intake, based on the urinary excretion of Isos. Another study by Grace et al. [46], showed an excellent correlation between plasma and urine levels with Isos intake lower than in this study (3.9 and 14 mg/day). The discrepancy between the results cited above and ours may then be linked to the amount of Isos used. Indeed, Slavin et al., who checked the urinary excretion of Isos after 6–9 days of soy protein ingestion, also found good dose–response urinary recovery. However, in their study Isos intake was moderate, from 9 to 36 mg/day. The authors concluded only on low to moderate doses suggesting that recovery may be different at high doses [61]. Furthermore, Tsangalis et al. [62] tested Isos doses from 20 to 80 mg, in chronic ingestion. They showed a higher inter-individual variation in urinary recovery when Isos were consumed in glycoside forms (which is the case here) rather than in fermented soymilk containing Isos predominantly in aglycone forms. They also mentioned that the correlation between plasma and urine Isos levels was worse with glycosylated forms, and that urine recovery decreased when the ingested quantity increased.

All this suggests that several enzymes involved in Isos bioavailability and excretion may be saturated by high doses of Isos. Indeed, in these studies Isos intakes were repeated for one or two months with a very regular rhythm. This rhythm resembles that of a medical prescription. In this particular case, it is well known from pharmacological studies that repeated ingestions of the same drug can lead to a steady-state level. The range of this steady-state level is directly influenced by the intestinal resorption and total clearance of the administrated drug. For a constant resorption, a subject who exhibits a high clearance (i.e., in our case a rapid renal excretion) will exhibit a lower plasma steady-state level. Both resorption and renal elimination of xenobiotics are influenced by enzyme activities like gut β glycosidase, hepatic or kidney phase I and II enzymes [63,64] as well as by phase III transporters [65]. Indeed, enzymatic activities are always influenced by genetic background [66] and environmental factors (other nutrients or pollutants) [67]. In this respect large inter-individual variations in resorption and renal elimination were expected. In addition, Table 4 shows that low, medium and high renal excretion could be observed among the volunteers (for Sal 34, Gro 4, Tho 11 and Cub 29, respectively). These results explain those presented in Tables 1 and 2. The present results would suggest that urinary excretion might not be the best way to evaluate plasma levels consecutive to chronic ingestion of large amounts of Isos.

Anyway, most of the health effects demonstrated with Isos were shown to follow dose-response patterns. Indeed, in this kind of situation it is much more important to consider the dose reaching the target tissues rather than the dose excreted. Therefore, although urine excretion could help determining Isos intake, plasma measurements appear to be much more reliable in helping understand the effectiveness of a treatment. Providing these measurements are always performed at the same point of the kinetic curve they are a good way to evaluate the range of a steady-state level in chronic intakes. Therefore, plasma levels would be of great help in interpreting the variability of a clinical effect.

Although the rhythm of Isos ingestion applied in this study is comparable to that possibly advertised for food supplements, it was performed under foodstuff supplementation. It must then be considered that the food matrix may have influenced the bioavailability of the Isos [68]. Therefore studies are still required to check if the same patterns are obtained with food supplements. Nevertheless, in this study all subjects received the same preparations in the same conditions and therefore conclusions on inter-individual variations are relevant.

5. Conclusion

When French post-menopausal women were exposed to 100 mg of Isos per day for 30 or 60 days, plasma levels measured after over night fasting, remained stable from D15 until the end of the experiment and mean urine levels were globally constant from day 5 till day 60. In this study there were between 58 and 60% of equol producers. In each case, the level of production maintained was nearly constant (either high, or low) for the whole of the experimentation period regardless of the daidzein ingested. Only one person became a low producer from D30 after being considered as a non-producer at first. As shown in this study, Isos concentrations were highly variable from one subject to another in both plasma and urine after chronic oral administration, even though all women received the same amount of compounds. This is in accordance with previous data [47]. Considering the complex pathways involved in Isos bioavailability and leading to resorption and clearance phenomena, such variability was not surprising. Urine and plasma levels were not very well correlated when data from different persons were plotted together. Therefore, urine levels were considered not to be the best biomarkers of Isos plasma levels in these conditions of intake. Therefore, if clinical studies are further undertaken to check for the activity of Isos in humans, it would be of great interest to systematically monitor plasma levels. Indeed, because Isos have already been found to act following a dose-response pattern, the large variability in plasma concentrations could then account for at least a part of the variability in the clinical responses. This hypothesis has already been formulated in the past [26] but needs to be validated in future clinical approaches. If it is found that plasma levels explain clinical activity, a more rational approach to the effects of Isos treatment could be undertaken based on the measurement of Isos concentrations in plasma. The ELISA techniques could help greatly in that respect.

Acknowledgements

This work was supported in part by The French Ministry of Research (AQS program A01347), the Region Aquitaine and by Danone, Nutrition Santé and Beghin-Meiji. We are grateful to Beghin-Meiji for kindly supplying the powdered soybean Isos concentrate (Prevastein®HC), Danone for yoghurts and Nutrition Santé for cereal bars. Special thanks are addressed to

Claudine Manach (U3M Clermont-Ferrand) for the validation of ELISAs using HPLC-UV and HPLC-CAD. We are grateful to Russell Wallace (ENITA Bordeaux) for his help with the English.

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