

Orally Administered Isoflavones Are Present as Glucuronides in the Human Prostate

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Better knowledge of the bioavailability and metabolism of isoflavones in prostate tissue is needed to further investigate their mechanisms of action in the context of prostate cancer prevention. A total of 12 men with benign prostatic hyperplasia received soy extract supplementation (3 Evestrel[®] capsules, providing a total of 112.5 mg isoflavones aglycone eq/day) for 3 days before prostate surgery. Blood and prostate tissues were sampled and metabolites were identified using electrospray ionization liquid chromatography tandem mass spectrometry (LC-ESI-MS/MS) and chemically synthesized standards of glucuronidated isoflavones. The main metabolites were the same in prostate tissue and in plasma, namely, 2 monoglucuronides of daidzein and 2 monoglucuronides of genistein. Concentrations of total isoflavones measured in prostate reached 1.05 ± 0.62 nmol/g tissue (range 0.30–2.23) at the time of sampling, 12 h after the last isoflavone supplementation. At that time point, prostate concentrations were lower than plasma concentrations in all volunteers: 0.47 nmol/g vs. 0.66 μ M for daidzein and 0.58 nmol/g vs. 0.78 μ M for genistein. Isoflavone mechanisms

of action should thus be investigated in *in vitro* cell studies using physiological conditions, intracellular concentrations below 5 nmol/g and no intracellular deconjugation of the monoglucuronide metabolites.

INTRODUCTION

Prostate cancer is the most frequent cancer and one of the leading causes of death for men in Western countries (1,2). In fact, it is so frequent in aged men that it might be regarded as a normal aging-related phenomenon. The development of the disease spreads over decades, making it particularly open to modulation by environmental factors such as nutrition. The traditional Asian diet is believed to slow down the progression of latent prostate intraepithelial neoplasia toward clinically apparent tumors, at least in Asian populations (3). Soy and its exclusive micronutrients, isoflavones, have been recognized by worldwide experts as potential chemopreventive agents and are actively studied. However, existing data supporting the protective effects of isoflavones are still limited, although studies have shown that mortality from prostate cancer is lowest in areas of high intake of soy and isoflavones, especially in Asia (4).

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Increased risk of prostate cancer for Asian men who migrated to Western countries or adopted a Westernized lifestyle, highlighted the key role of environmental factors beyond genetic factors (5–7). A recent meta-analysis compiling 2 cohort studies and 6 case-control studies on Western and Asian populations estimated that soy food consumption can be related to a 30% reduction in prostate cancer risk (8). Studies on animal models have further supported the protective role of isoflavones. Isoflavones have been reported to reduce both incidence and tumor sizes in more than 40 studies carried out with a variety of rodent models (3,9). In addition, isoflavones have inhibited proliferation of all cultured cell lines classically used to study prostate cancer (10,11).

These epidemiological and experimental data suggest a protective role for soy isoflavones in the prevention of prostate cancer; nevertheless, they do not provide direct evidence for their preventive effects. Some clinical trials have investigated the effect of isoflavone intake on some prostate cancer related endpoints such as prostate-specific antigen (PSA) level (12,13). However, these studies were only designed to evaluate the pharmacological effects of isoflavones, using short-term exposures to high doses of isoflavones, on men with already diagnosed prostate cancer. Demonstrating the preventive effects of long-life exposure to isoflavone-rich food on healthy men is much more challenging and cannot be realized with such trials. The complexity of the human diet, possible interactions with other nutrients, variability in interindividual responses to the diet due to genetic polymorphisms and diverse microbiota, multiplicity of possible biological targets, and the low amplitude of the chronic effects expected from nutrition are some of the factors that make the demonstration of isoflavone-preventing effects difficult. In addition, validated biomarkers for early stages of prostate cancer development are not available to date. One possible way to progress in the understanding of the role of isoflavones in prostate cancer prevention is to clarify their molecular mechanisms of action in animal and cellular models. A number of studies have provided clear evidence that isoflavones are active compounds. They have been shown to bind estrogen receptors $ER\alpha$ and $ER\beta$ and to induce agonist or antagonist responses depending on tissues and conditions (14). Many other molecular mechanisms have been proposed such as modulation of steroid biosynthesis, transport, and metabolism; modulation of growth factors signaling pathways (epidermal growth factor, insulin-like growth factor-1, nuclear factor- κ B, Akt) and inhibition of tyrosine kinase activities; modulation of xenobiotic metabolism; regulation of genes involved in the cell cycle; induction of apoptosis; reduction of oxidative stress; and inhibition of angiogenesis (10,15). However, the issue of bioavailability has not been taken into account in the design and interpretation of mechanistic studies. Cell cultures have been generally exposed to concentrations far exceeding the concentrations that may be achieved in the body.

The aim of our study was to look for the presence of isoflavone metabolites in the human prostate after ingestion

of an isoflavone-rich supplement to compare the concentrations achieved in the prostate tissue to the plasma concentrations and to determine the nature of the metabolites present in plasma and prostate tissue. The underlying objective is to provide the information needed to design more relevant *in vitro* studies to investigate the mechanisms of action of dietary isoflavones involved in the prevention of prostate cancer.

MATERIALS AND METHODS

Materials

Standards of daidzein, genistein, daidzin, genistin, and equol were purchased from Extrasynthese (Genay, France). A standard of glycitein was purchased from Interchim (Montluçon, France). Standards of daidzein 7-glucuronide and genistein 7-glucuronide were chemically synthesized according to the method previously published (16). β -glucuronidase/sulfatase from *Helix pomatia* was obtained from Sigma (L'Isle d'Abeau Chesnes, France).

Isoflavone Content of the Evestrel[®] Supplement

Evestrel capsules were provided by Théraxem (Monte Carlo, Monaco). The isoflavone composition of the Evestrel extract was analyzed as follows. A total of 20 mg extract powder were homogenized in 10 ml methanol/water (70/30, vol/vol) using a Polytron mixer. Concentrations of isoflavone aglycones and glycosides were determined by high-performance liquid chromatography (HPLC)-CoulArray as described following before and after acid hydrolysis. Acid hydrolysis was carried out by addition of 0.25 volume 6M HCl followed by incubation for 2 h at 90°C. All glycosylated forms of isoflavones are hydrolyzed into aglycones by this procedure.

HPLC analysis was performed using a system consisting of 2 pumps (Model 580, ESA, Chelmsford, MA) for high-pressure gradient, a temperature-controlled autosampler (Gilson, Villiers-le-Bel, France), a 150 × 2.1 mm SymmetryShield RP18-5 μ column (Waters, Milford, MA) and an 8-channel CoulArray detector (model 5600, Eurosep, Cergy, France). Mobile phases consisted of a 30 mmol/l NaH_2PO_4 buffer (pH 3) containing 5% acetonitrile (A) and 40% acetonitrile (B). Separation was achieved using a gradient elution (flow = 0.4 ml/min): 0–25 min, linear gradient from 100% A to 100% B; 25–29 min, 100% B. Potentials were set at 200, 320, 490, 550, 600, 680, 750, and 800 mV (Pd as reference).

Each capsule contained a total of 37.5 mg isoflavones, including 65.7% genistein, 31.7% daidzein, and 2.5% glycitein in aglycone equivalents. Daidzein and daidzin represented 24.9% and 75.1% (in aglycone eq.) of the total daidzein forms. Genistein and genistin represented 22.2% and 54.9% (in aglycone eq.) of the total genistein forms, the rest corresponding probably to malonylated and acetylated glycosides also hydrolyzed into aglycones by our procedure. Glycitein represented 29% of the total glycitein forms.

Subjects

Sixteen men with benign prostatic hyperplasia (BPH) scheduled for surgery were included consecutively in the study. All the patients presented severe symptoms related to BPH. For all of them, conservative medical treatment had failed to improve their urinary symptoms. Preoperative evaluation included physical examination with digital rectal examination, free uroflowmetry, measurement of post void residual volume, blood analysis, coagulation parameters, PSA determination, and measurement of prostate volume using ultrasound. Due to the volume of the prostate, surgical procedure was a transurethral resection in 15 cases and an open prostatectomy for 1 patient.

Characteristics of the patients (mean \pm SD) were weight 80 ± 5 kg (range 69–130), body mass index 27 ± 2 kg/m² (range 22.4–44.9), and 67 ± 2 years old (range 54–77). Patients had normal PSA level (<4 ng/ml). Exclusion criteria included prostate cancer, diabetes, kidney or liver failure, antibiotic treatment within 3 months as well as a strict vegetarian or vegan diet or a high isoflavone consumption through soy-based food or supplement intake evaluated by a diet history questionnaire.

Study Design

Volunteers were randomly allocated to the group of 12 men who received soy extract supplementation for 3 days before prostate resection or to the control group of 4 men who did not receive any supplementation before the surgical treatment.

During the whole experimental period and the 3 days before, the volunteers were asked to maintain their normal food intake and to avoid isoflavone consumption. They were given a list of prohibited food items. All soy-based foods (tofu, tempeh, miso, natto, soymilk, soybeans, soy cheese, soy-based yogurts, and desserts) as well as cereal bars, breakfast cereals, foods for vegetarians, nuggets, burgers, and meal substitutes were prohibited.

The isoflavone supplementation consisted of 3 Evestrel capsules per day, providing a total of 112.5 mg total isoflavones (aglycone eq.). Volunteers were asked to take the 3 capsules at dinner the 3 days before the procedure.

Blood was sampled at the beginning of the surgical operation; 5 to 10 g of the inner prostatic tissue were collected in the transitional zone and immediately clamped in liquid nitrogen. The whole operation lasted about 60 min.

For each volunteer, a pool of prostate chips (about 1 g) was ground in liquid nitrogen to obtain homogenized powder that was stored at -80°C until analyses. The remaining chips were also stored at -80°C .

The protocol was approved by the regional Biomedical Research Ethics Committee (CCPPRB, Clermont-Ferrand, France), and all participants gave their written informed consent.

Sample Treatment

For quantitative analysis, plasma samples were acidified to pH 4.9 with 10 mmol/l acetic acid and incubated for 18 h in the presence of 1,000 units β -glucuronidase and 45 units sulfatase

(from *Helix pomatia*, Sigma G0876). Samples were then treated with 4 vol methanol/HCl 200 mM and centrifuged 5 min at 12,500 g. For qualitative analysis, plasma samples were directly extracted with 4 vol methanol/HCl 200 mM and centrifuged 5 min at 12,500 g. Supernatants were analyzed by LC-MS/MS as described following.

For quantitative analysis in prostate tissue, 150 mg prostate tissue powder were homogenized in 9 vol of cooled methanol/water (70/30, vol/vol) using a Polytron mixer (Kinematica, Lucerne, Switzerland). After centrifugation (3,500 rpm, 10 min, 4°C), and keeping the supernatant, the pellet was extracted again with 4 vol of cooled methanol/water (70/30, vol/vol). Pooled supernatants were evaporated to dryness and then reconstituted in 1 vol sodium acetate buffer 0.1 M pH 4.9 before incubation with β -glucuronidase/sulfatase (*Helix pomatia*) at 37°C for 18 h (2,000 units β -glucuronidase and 90 units sulfatase). After incubation, isoflavones were extracted with 4 vol methanol/HCl 200 mM. We performed 5 replicates of extraction and analysis for each sample.

For determination of the nature of tissue metabolites, prostate chips rather than prostate tissue powder were used because of possible metabolite hydrolysis during grinding. Prostate chips (150 mg) were extracted with 4 vol methanol/H₂O (70/30) containing 200 mM HCl using the Polytron mixer. After centrifugation at 3,500 rpm, 4°C for 10 min, supernatants were analyzed by LC-MS/MS.

As aglycones and conjugated derivatives, such as glucuronides, have quite different polarity and stability, giving different behavior during extraction and analysis, it was considered not relevant to use an aglycone as internal standard because a high proportion of conjugated forms of isoflavones could be present in our plasma and tissues samples. The extraction recovery and the efficiency of hydrolysis were checked using control plasma and prostate tissue supplemented with known concentrations (2 and 5 $\mu\text{mol/l}$ in duplicate) of aglycones and chemically synthesized standards of isoflavones glucuronides and were treated the same way as the samples. The recovery rate ranged from 85% to 103% for aglycones and glucuronides in plasma and from 88% to 106% in prostate tissue. Glucuronides were not detectable after hydrolysis with β -glucuronidase/sulfatase in the conditions described previously.

LC-MS/MS Analysis of Isoflavone Metabolites

Liquid chromatography (LC) analyses were performed using a Hewlett-Packard 1100 HPLC system (Agilent Technologies, Waldbronn, Germany) equipped with a quaternary pump and an autosampler. An Applied Biosystems API 2000 triple quadrupole mass spectrometer (PE Sciex, Concord, Ontario, Canada), equipped with a Turbo IonSpray source ionizing in the positive mode at 500°C , was used to obtain the mass spectrometry (MS) and MS/MS data. SymmetryShieldTM RP18 column (Waters, Milford, MA), 2.1×150 mm id, 5 μm , was used for chromatographic separation (injection volume = 20 μl).

Gradient elution was carried out with 0.5% acetic acid in 20% acetonitrile as mobile Phase A and 0.5% acetic acid in 80% acetonitrile as mobile Phase B: 0–20 min from 100% A to 100% B at a constant flow rate of 400 $\mu\text{l}/\text{min}$. The column was reequilibrated for 10 min.

Parameters for the detection of aglycones and metabolites were the following: capillary voltage 5,500 V, collision gas 5 (arbitrary units), and curtain gas 20 (arbitrary units). Declustering potential, focusing potential, entrance potential, and collision energy were optimized with infusion experiments of daidzein (51, 400, 15, and 55, respectively), genistein (50, 400, 12, and 60, respectively), daidzein-7-glucuronide (30, 375, 5, and 25, respectively), genistein-7-glucuronide (20, 200, 10, and 30, respectively), equol (19, 370, 9, and 17, respectively), and glycitein (30, 375, 10, and 50, respectively).

To determine the nature of isoflavone metabolites present in nonhydrolyzed prostate and plasma, MS data were collected in multiple reaction monitoring (MRM) mode by monitoring specific transitions of parent and product ions for each metabolite (Table 1). Dihydrodaidzein, dihydrogenistein, dihydroglycitein, and O-desmethylangolensin were checked using selected-ion monitoring (Table 1). For keeping the sensitivity in LC-MS/MS, we monitored 5 transitions for each analysis in the MRM mode, with a dwell time of 400 ms.

For quantitative analyses in hydrolyzed prostate and plasma samples, the MRM mode was used with a dwell time of 500 ms, monitoring 4 transitions for each analysis: daidzein (255/91), genistein (271/91), equol (243/123), and glycitein (285/270). Calibration curves were prepared in human plasma by spiking control pools with known concentrations of genistein, daidzein, glycitein, and equol (0; 0,08; 0,16; 0,32; 0,64; 1,28; and 2,56 $\mu\text{mol}/\text{l}$). The limit of detection was estimated as 12 nmol/l for daidzein and genistein, 19 nmol/l for glycitein, and 2.1 $\mu\text{mol}/\text{l}$ for equol. The limit of quantification was estimated as 41 nmol/l for daidzein and genistein, 65 nmol/l for glycitein, and 7.1 $\mu\text{mol}/\text{l}$ for equol.

HPLC-CoulArray Analysis of Equol

Because the limit of detection was high for equol in our LC-MS/MS conditions, equol was analyzed using CoulArray detection. Mobile phases consisted of a 30 mmol/l NaH_2PO_4 buffer (pH 3) containing 20% acetonitrile (A) and 40% acetonitrile (B). Separation on a 150 \times 2.1 mm SymmetryShield RP18-5 μ column (Waters) using a gradient elution (flow = 0.4 ml/min): 0–15 min, linear gradient from 100% A to 100% B; 15–19 min, 100% B; 19.01–25 min, 100% A. Potentials were set at 200, 320, 490, 550, 600, 680, 750, and 800 mV (Pd as reference) on the HPLC-CoulArray system described previously. The detection limit was 8 nmol/l.

Statistics

Values are given as mean \pm SD. The significance of differences was determined using the paired *t*-test (INSTAT, GraphPad

Software, San Diego, CA). A *P* value < 0.05 was considered significant.

RESULTS

After daily supplementation with 112.5 mg isoflavones (aglycone eq, composed of 31.7% daidzein eq, 65.7% genistein eq, and 2.5% glycitein eq) for 3 days, plasma concentrations of total isoflavones measured by LC-ESI-MS/MS ranged from 0.40 to 2.54 μM , with a mean value of $1.45 \pm 0.77 \mu\text{M}$, whereas concentrations measured in prostate tissues ranged from 0.30 to 2.23 nmol/g tissue, with a mean value of $1.05 \pm 0.62 \text{ nmol/g}$ tissue (Fig. 1). Significant interindividual variability was observed regarding total concentrations of isoflavones, but prostate concentrations were lower than plasma concentrations for all volunteers.

The main aglycones recovered after β -glucuronidase/sulfatase hydrolysis were daidzein and genistein, with low concentrations of glycitein. The genistein/daidzein ratio was 1.3 in prostate (0.58/0.47 nmol/g) as well as in plasma samples (0.78/0.66 $\mu\text{mol}/\text{l}$).

As measurements were only made at 1 time point, it is not possible to compare the absorption/elimination efficiency for genistein and daidzein.

Equol, the well-known microbial metabolite of daidzein produced by only 30% of the Western population, was detected in 2 volunteers only. Equol concentrations in prostate samples from equol producers were 0.36 and 0.12 nmol/g vs. 0.95 and 0.33 $\mu\text{mol}/\text{l}$ in the corresponding plasma samples.

The nature of prostate and plasma metabolites was determined using LC-ESI-MS/MS and chemically synthesized standards of glucuronidated conjugates of isoflavones. The major metabolites were the same in prostate tissue and in plasma, namely, 2 monoglucuronides of daidzein and 2 monoglucuronides of genistein (Fig. 2). Daidzein 7-*O*-glucuronide and genistein 7-*O*-glucuronide were identified by comparison with the chemically synthesized standards. We assume that the other major metabolites detected with the MS transitions 431/255 and 447/271 are 4'-*O*-glucuronides of daidzein and genistein, respectively, which are the other isoflavone glucuronides that have already been reported in human plasma. The relative proportion of 7-*O*-glucuronides and 4'-*O*-glucuronides in plasma and prostate samples varied between the volunteers.

Table 1 summarizes the MRM transitions that have been searched in nonhydrolyzed plasma and prostate samples and the metabolites that were detected in addition to the monoglucuronides of daidzein and genistein. Daidzein and genistein aglycones were detected in prostate tissue of only 5 volunteers among the 12. Their concentration represented less than 10% of the total aglycone concentrations measured after β -glucuronidase/sulfatase hydrolysis of the prostate samples.

Very low concentrations of diglucuronides and sulfates of daidzein and genistein were also detected for a few volunteers but in trace amounts not quantifiable in our conditions.

TABLE 1
Isoflavone Metabolites Detected in Nonhydrolyzed Plasma and Prostate of 12 Volunteers Who Received 112.5 mg Isoflavones/Day for 3 Days^a

Metabolite	MRM Transitions	Nonhydrolyzed Plasma	Nonhydrolyzed Prostate
Daidzein-glucuronide	431/255	++	++
Genistein-glucuronide	447/271	++	++
Daidzein	255/91	+/-	+/-
Genistein	271/91	+/-	+/-
Daidzein-diglucuronide	607/255	tr	tr
Genistein-diglucuronide	623/271	tr	tr
Daidzein-sulfate	335/255	tr	tr
Genistein-sulfate	351/271	tr	tr
Glycitein-glucuronide	461/285	tr	tr
Glycitein	285/270	Nd	Nd
Equol	243/123	Nd	Nd
Equol-glucuronide	419/243	Nd	Nd
Dihydrodaidzein-glucuronide	433/257	Nd	Nd
Dihydrogenistein-glucuronide	449/273	Nd	Nd
Dihydroglycitein-glucuronide	463/287	Nd	Nd
Glycitein-sulfate	365/285	Nd	Nd
Equol-sulfate	323/243	Nd	Nd
Daidzein-sulfoglucuronide	511/255	Nd	Nd
Genistein-sulfoglucuronide	527/271	Nd	Nd
Glycitein-sulfoglucuronide	540/285	Nd	Nd
Equol-sulfoglucuronide	499/243	Nd	Nd
Glycitein-diglucuronide	637/285	Nd	Nd
Equol-diglucuronide	595/243	Nd	Nd
Daidzein-disulfate	415/255	Nd	Nd
Genistein-disulfate	431/271	Nd	Nd
Glycitein-disulfate	445/285	Nd	Nd
Equol-disulfate	403/243	Nd	Nd
Daidzin	417/255	Nd	Nd
Genistin	433/271	Nd	Nd
Glycitin	447/285	Nd	Nd
Daidzein-glycine	312/255	Nd	Nd
Genistein-glycine	328/271	Nd	Nd
Glycitein-glycine	342/285	Nd	Nd
Equol-glycine	300/243	Nd	Nd
Dihydrodaidzein	257	Nd	Nd
Dihydrogenistein	273	Nd	Nd
Dihydroglycitein	287	Nd	Nd
O-desmethylangolensin	259	Nd	Nd
Daidzein-glutathione	560/255	Nd	Nd
Genistein-glutathione	576/271	Nd	Nd
Glycitein-glutathione	590/285	Nd	Nd
Equol-glutathione	548/243	Nd	Nd

^aAbbreviations are as follows: MRM, multiple reaction monitoring; Nd, not detected; tr, trace; +/-, present in some but not all samples.

Sulfoglucuronides, glycosides, disulfates, glycine or glutathione conjugates, dihydrodaidzein, dihydrogenistein, dihydroglycitein, and O-desmethylangolensin were not detected in any plasma or prostate sample.

DISCUSSION

The chosen daily dose (112.5 mg total isoflavones, aglycone eq) was quite high but achievable with natural food sources. It is equivalent to the highest nutritional intake reported for

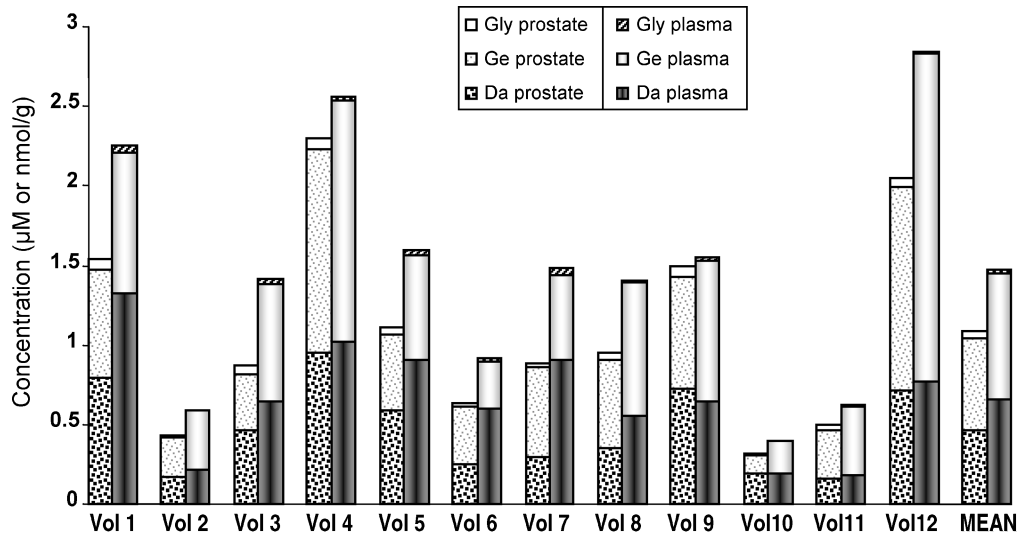


FIG. 1. Isoflavone concentrations measured by liquid chromatography electrospray ionization tandem mass spectrometry after β -glucuronidase/sulfatase hydrolysis in plasma and prostate samples from 12 volunteers supplemented for 3 days with 112.5 mg/d total isoflavones (aglycone eq). Gly, glycitein; Ge, genistein; Da, daidzein.

Asian rural populations and can be achieved occasionally with 2–3 servings of soy-based foods (17). This dose was chosen to ensure sufficient levels in prostate, allowing quantitative and qualitative analysis of the metabolites.

Using this dose, isoflavone concentrations reached 1.05 ± 0.62 nmol/g in the prostate tissue and 1.45 ± 0.77 μ mol/l in plasma when measured 12 h after the last intake of isoflavones. The time of sampling was imposed by anesthesia constraints. Considering that $t_{\max} = 4\text{--}7$ h and $t_{1/2} = 5\text{--}8$ h for isoflavones in plasma, concentrations measured at this stage should still represent around 50% of peak plasma concentrations (18). The kinetics of penetration and elimination of isoflavones in tissues is not known. Assuming that it follows the plasma pharmacokinetics, twice these concentrations may have been reached in the prostate tissue of the volunteers at earlier time points.

Two previous studies have measured isoflavone concentrations in human prostate or prostatic fluid without prior supplementation. First, Morton et al. (19) determined the daidzein concentration in the prostatic fluid of men living in Hong-Kong, in Portugal, or in the United Kingdom. The basal level of daidzein was 6-fold to 15-fold higher for men living in Hong Kong (0.275 μ mol/l) than for men living in Europe. Prostatic fluid was more concentrated than plasma (0.275 μ mol/l vs. 0.123 μ mol/l, respectively). Hong et al. (20) determined the concentration of isoflavones in plasma and prostate tissues of 15 Korean men who had benign prostatic hyperplasia. Hong et al.'s results were consistent with ours, with isoflavone concentrations in prostate being about half the plasma concentrations: 0.167 nmol/g vs. 0.382 μ mol/l for daidzein and 0.242 nmol/g vs. 0.695 μ mol/l for genistein, respectively. Similar concentrations were measured in volunteers with normal prostate. Two additional studies have focused on Western men receiving isoflavone

supplementation. In one, 45 healthy American men were classified into low or high isoflavones consumers and supplemented with a soy beverage for 1 wk (42–60 mg isoflavones/day) (21). Daidzein was 4 times more concentrated in the prostatic fluid than in plasma: 1.88 vs. 0.44 μ mol/l for low consumers and 2.78 vs. 0.54 μ mol/l for high consumers. In contrast, genistein did not concentrate in the prostatic fluid and was present at lower concentration ($0.4\text{--}0.48$ μ mol/l). In the last study, Rannikko et al. (22) supplemented men with prostate cancer with a high dose of red clover phytoestrogens. After 2 wk, genistein as well as daidzein concentrations were twice as high in prostate tissue than in plasma: 1.38 and 1.07 vs. 0.66 and 0.49 μ mol/l, respectively.

Compilation of these limited data indicates that concentrations as high as 1–2.5 nmol/g of each isoflavone may be achieved in human prostate or prostatic fluid after short-term supplementation with high nutritional doses of isoflavones and that in Asian men, basal levels of total isoflavones are about 0.4 nmol/g.

Hedlund et al. (21) as well as Morton et al. (19) have reported significantly higher daidzein and equol concentrations in prostatic fluid from high isoflavone consumers compared to low consumers. This raises the question of a possible accumulation of isoflavones in prostate or prostatic fluid with regular soy intake. For daidzein, concentrations achieved remain modest, suggesting that elevated concentrations may reflect the last intakes of isoflavones in preceding days rather than a real storage in tissue. The situation may be different for equol for which striking prostatic fluid/plasma concentration ratios have been observed in high isoflavone consumers but not in low isoflavone consumers. The equol concentration was 40-fold higher in the prostatic fluid of men from Hong Kong than in their plasma (19) and 22-fold higher in the prostatic fluid of high isoflavone

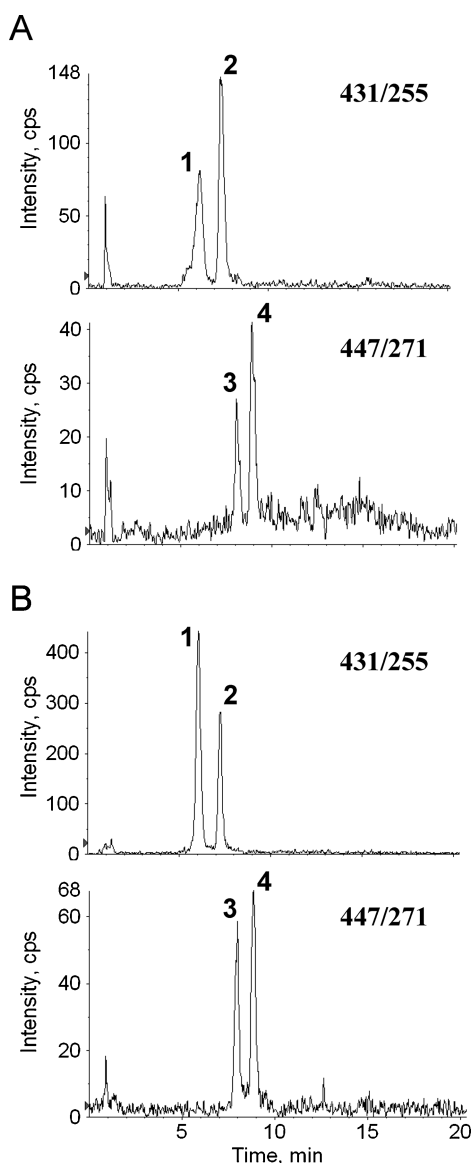


FIG. 2. Nature of the major isoflavone metabolites detected by liquid chromatography electrospray ionization tandem mass spectrometry in plasma (A) and prostate (B) samples from 12 volunteers supplemented for 3 days with 112.5 mg/day total isoflavones (aglycone eq). Compounds 1 and 3 were identified as daidzein 7-*O*-glucuronide and genistein 7-*O*-glucuronide by comparison with chemically synthesized standards. Compounds 2 and 4 were tentatively identified as daidzein 4'-*O*-glucuronide and genistein 4'-*O*-glucuronide on the basis of ion transitions and retention times. cps, counts per second.

consumers than in their plasma in the study of Hedlund et al. (21). However, this observation relies on a very small number of volunteers. We did not observe higher concentrations of equal in prostate compared to plasma in this study. More data are needed on a larger number of volunteers from various populations.

Due to extensive intestinal and hepatic metabolism, isoflavone glycosides and aglycones present in foods are recovered in human plasma primarily as monoglucuronidated metabolites along with low amounts of aglycones and sulfate

esters (23–25). Specific uptake of some metabolites by the cells as well as intracellular metabolism, especially possible deglucuronidation, may lead to differences in the nature and concentration of isoflavone metabolites present in plasma and in inner tissues such as prostate.

Our study shows for the first time that daidzein and genistein are mainly recovered as glucuronides in prostate tissue. We only detected very low concentrations of aglycones in some prostate samples and observed that artifactual hydrolysis of glucuronides into aglycones easily occurred during sample preparation. Extraction had to be realized rapidly in ice. We looked for the presence of various other metabolites that may be present due to classical conjugation reactions known for xenobiotics (26). Trace amounts of diglucuronides and sulfates were detected for a few volunteers. Other metabolites were not detected. However, their presence in low amount can not be ruled out because we were not able to optimize extraction and detection conditions for these compounds due to the lack of pure standards. It is worth noting that tumoral cells may contain more aglycones than normal cells because glucuronidase activity has been reported to be markedly higher in tumors (27).

Data on the nature of tissue metabolites is crucial because the biological properties of conjugated metabolites may differ markedly from those of aglycones. For instance, the affinity of isoflavone glucuronides for estrogen receptors has been reported to be 10- to 40-fold lower than that of the aglycones (28). Sulfation of isoflavones was also shown to decrease their antioxidant activity and their effect on platelet aggregation, inflammation, cell adhesion, and chemotaxis (29,30).

Most *in vitro* studies that have investigated the mechanisms of action of isoflavones have used concentrations as high as 50 to 100 $\mu\text{mol/l}$ isoflavones provided as aglycones or glycosides. Such concentrations have never been achieved in the human body, even with pharmacological doses. Peak plasma concentrations of isoflavone metabolites generally range between 0.4 and 4 $\mu\text{mol/l}$, with a mean value of 2 $\mu\text{mol/l}$, after consumption of 50 mg aglycone equivalent dose of isoflavone, which is about the mean intake in Asian countries (18). The highest isoflavone concentrations reported so far were detected in the plasma of volunteers challenged with a pharmaceutical dose of genistein to test its potential genotoxic effects (300 mg/day for 28 days, then 600 mg/day for 56 days) (31). A high interindividual variability was observed in the study, and maximum plasma concentrations ranged between 4 and 27 $\mu\text{mol/l}$ genistein among the 20 volunteers. The results suggest that plasma concentrations higher than 10 $\mu\text{mol/l}$ can only be achieved in a low proportion of individuals and after intake of pharmacological doses at least sixfold higher than nutritional doses.

Our study shows that the isoflavone concentration in prostate remains below 5 nmol/g after a high-dose supplementation.

In vitro studies using concentrations exceeding these physiological concentrations may provide erroneous information on

isoflavone mechanisms of action and contribute to delays in our understanding of their complex health effects. Future studies on isoflavone activities in prostate cancer prevention will thus have to use conditions of isoflavone exposure that lead to intracellular isoflavone concentrations below 5 nmol/g. Furthermore, glucuronide metabolites will have to be tested in addition to the classical aglycones under conditions in which they are not hydrolyzed into aglycones inside the cultured cells.

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REFERENCES

- Jemal A, Siegel R, Ward E, Murray T, Xu JQ, and Thun MJ: Cancer statistics, 2007. *CA Cancer J Clin* **57**, 43-66, 2007.
- Hill C and Doyon F: The frequency of cancer in France in year 2002, and trends since 1968. *Bull Cancer* **93**, 7-11, 2006.
- Messina MJ: Emerging evidence on the role of soy in reducing prostate cancer risk. *Nutr Rev* **61**, 117-131, 2003.
- Hsing AW, Tsao L, and Devesa SS: International trends and patterns of prostate cancer incidence and mortality. *Int J Cancer* **85**, 60-67, 2000.
- Shimizu H, Ross RK, Bernstein L, Yatani R, Henderson BE, et al.: Cancers of the prostate and breast among Japanese and white immigrants in Los Angeles County. *Br J Cancer* **63**, 963-966, 1991.
- Cook LS, Goldoft M, Schwartz SM, and Weiss NS: Incidence of adenocarcinoma of the prostate in Asian immigrants to the United States and their descendants. *J Urol* **161**, 152-155, 1999.
- Sim HG and Cheng CWS: Changing demography of prostate cancer in Asia. *Eur J Cancer* **41**, 834-845, 2005.
- Yan L and Spitznagel EL: Meta-analysis of soy food and risk of prostate cancer in men. *Int J Cancer* **117**, 667-669, 2005.
- Pollard M and Suckow MA: Dietary prevention of hormone refractory prostate cancer in Lobund-Wistar rats: a review of studies in a relevant animal model. *Comp Med* **56**, 461-467, 2006.
- Magee PJ and Rowland IR: Phyto-oestrogens, their mechanism of action: current evidence for a role in breast and prostate cancer. *Br J Nutr* **91**, 513-531, 2004.
- Hempstock J, Kavanagh JP, and George NJR: Growth inhibition of prostate cell lines in vitro by phyto-oestrogens. *Br J Urol* **82**, 560-563, 1998.
- Messina M, Kucuk O, and Lampe JW: An overview of the health effects of isoflavones with an emphasis on prostate cancer risk and prostate-specific antigen levels. *J AOAC Int* **89**, 1121-1134, 2006.
- Bemis DL, Katz AE, and Buttyan R: Clinical trials of natural products as chemopreventive agents for prostate cancer. *Expert Opin Investig Drugs* **15**, 1191-1200, 2006.
- Morrissey C and Watson RW: Phytoestrogens and prostate cancer. *Curr Drug Targets* **4**, 231-241, 2003.
- Sarkar FH and Li Y: The role of isoflavones in cancer chemoprevention. *Front Biosci* **9**, 2714-2724, 2004.
- Al-Maharik N and Botting NP: A facile synthesis of isoflavone 7-O-glucuronides. *Tetrahedron Lett* **47**, 8703-8706, 2006.
- Messina M, Nagata C, and Wu AH: Estimated Asian adult soy protein and isoflavone intakes. *Nutr Cancer* **55**, 1-12, 2006.
- Manach C, Williamson G, Morand C, Scalbert A, and Remesy C: Bioavailability and bioefficacy of polyphenols in humans: I. review of 97 bioavailability studies. *Am J Clin Nutr* **81**, 230S-242S, 2005.
- Morton MS, Chan PS, Cheng C, Blacklock N, Matos-Ferreira A, et al.: Lignans and isoflavonoids in plasma and prostatic fluid in men: samples from Portugal, Hong Kong, and the United Kingdom. *Prostate* **32**, 122-128, 1997.
- Hong SJ, Kim SI, Kwon SM, Lee JR, and Chung BC: Comparative study of concentration of isoflavones and lignans in plasma and prostatic tissues of normal control and benign prostatic hyperplasia. *Yonsei Med J* **43**, 236-241, 2002.
- Hedlund TE, Maroni PD, Ferucci PG, Dayton R, Barnes S, et al.: Long-term dietary habits affect soy isoflavone metabolism and accumulation in prostatic fluid in Caucasian men. *J Nutr* **135**, 1400-1406, 2005.
- Rannikko A, Petas A, Rannikko S, and Adlercreutz H: Plasma and prostate phytoestrogen concentrations in prostate cancer patients after oral phytoestrogen supplementation. *Prostate* **66**, 82-87, 2006.
- Setchell KD, Brown NM, Desai P, Zimmer-Nechemias L, Wolfe BE, et al.: Bioavailability of pure isoflavones in healthy humans and analysis of commercial soy isoflavone supplements. *J Nutr* **131**, 1362S-1375S, 2001.
- Busby MG, Jeffcoat AR, Bloedon LT, Koch MA, Black T, et al.: Clinical characteristics and pharmacokinetics of purified soy isoflavones: single-dose administration to healthy men. *Am J Clin Nutr* **75**, 126-136, 2002.
- Zhang Y, Hendrich S, and Murphy PA: Glucuronides are the main isoflavone metabolites in women. *J Nutr* **133**, 399-404, 2003.
- Levsen K, Schiebel HM, Behnke B, Dotzer R, Dreher W, et al.: Structure elucidation of phase II metabolites by tandem mass spectrometry: an overview. *J Chromatogr A* **1067**, 55-72, 2005.
- de Graaf M, Boven E, Scheeren HW, Haisma HJ, and Pinedo HM: Beta-glucuronidase-mediated drug release. *Curr Pharm Des* **8**, 1391-1403, 2002.
- Zhang Y, Song TT, Cunnick JE, Murphy PA, and Hendrich S: Daidzein and genistein glucuronides in vitro are weakly estrogenic and activate human natural killer cells at nutritionally relevant concentrations. *J Nutr* **129**, 399-405, 1999.
- Turner R, Baron T, Wolfram S, Minihane AM, Cassidy A, et al.: Effect of circulating forms of soy isoflavones on the oxidation of low density lipoprotein. *Free Radic Res* **38**, 209-216, 2004.
- Rimbach G, Weinberg PD, de Pascual-Teresa S, Alonso MG, Ewins BA, et al.: Sulfation of genistein alters its antioxidant properties and its effect on platelet aggregation and monocyte and endothelial function. *Biochim Biophys Acta* **1670**, 229-237, 2004.
- Miltyk W, Craciunescu CN, Fischer L, Jeffcoat RA, Koch MA, et al.: Lack of significant genotoxicity of purified soy isoflavones (genistein, daidzein, and glycitein) in 20 patients with prostate cancer. *Am J Clin Nutr* **77**, 875-882, 2003.

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