Disposition and Biotransformation of the Estrogenic Isoflavone Daidzein in Rats

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Daidzein is an estrogenic isoflavone present in many plants and therefore consumed in relatively high doses by humans. Daidzein has a low affinity for the estrogen receptor (3 orders of magnitude lower than estradiol) and has been demonstrated to have estrogenic effects in rodents after administration of high doses. We have studied the disposition and biotransformation of daidzein in rats fed a diet low in isoflavone content. Four male and four female Fischer 344 rats were orally administered 100 mg/kg daidzein; excreted urine and feces were collected for 96 h and unchanged daidzein as well as formed metabolites were quantified by HPLC.

In urine of male rats, daidzein, daidzein-glucuronide, and daidzein-sulfate were excreted; in females, only unchanged daidzein and daidzein-glucuronide were present. Total urinary excretion of daidzein accounted for < 10% of dose in both males and females. The major pathway of daidzein elimination was excretion of unchanged daidzein with feces. Reductive daidzein-metabolites likely formed by intestinal microflora (equol, O-desmethylandoligenin) were excreted with feces in small amounts (< 5% of dose). Excretion of daidzein and metabolites with urine and feces was rapid with elimination half-lives of less than 12 h; daidzein concentrations in urine and feces were below the limit of detection 36 h after daidzein administration. The results suggest that daidzein is only poorly absorbed from the gastrointestinal tract in rodents. Absorbed daidzein is rapidly eliminated both unchanged and as conjugates with urine. The inefficient absorption of daidzein from the gastrointestinal tract and the rapid excretion may explain the weak estrogenicity of daidzein seen in vivo in rodents when compared to other estrogenic chemicals with comparatively low affinity to the estrogen receptor.

Key Words: daidzein; estrogen; isoflavone; disposition.

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Daidzein is an isoflavone present as a glucoside in many plants used in human diets. Daidzein is especially concentrated in soy and soy-based products used for human consumption, resulting in relative large daily doses of daidzein in humans (Reinli and Block, 1996; Setchell et al., 1997).

Daidzein has been found to act as an estrogen receptor agonist and induces receptor-mediated estrogenic responses both in in vivo and in vitro assays for estrogenicity. For example, daidzein induces receptor-mediated increases in cell proliferation in human breast cancer cells (E-screen) and a dose-dependent weight increase of the uterus of rats (disruption of ovarian cyclicity; Kaldas and Hughes, 1989). In humans, high consumption of soy and soy-based food has also been connected with estrogenic responses in humans (Baird et al., 1995; Cassidy et al., 1994; Hargreaves et al., 1999; Setchell et al., 1987), along with other effects such as antioxidative and antiproliferative responses (Setchell, 1998) and a lower incidence of cardiovascular diseases in Asia (Murkies et al., 1998). In some animal species, consumption of diets rich in phytoestrogens has been associated with toxic effects on reproduction (Kaldas and Hughes, 1989).

Humans are also exposed to a variety of industrial compounds that are weak estrogens in different assays used to determine estrogenicity. These chemicals are sometimes called "environmental estrogens" or "endocrine disrupters" (Guillette et al., 1995; Sharpe and Skakkebaek, 1993; Toppari et al., 1996). In general, these compounds are much weaker estrogens than daidzein and humans are exposed to much lower quantities of these compounds. For example, the EC₅₀ values for the transactivation of the rat estrogen receptors are in the range of 10⁻⁷ to 10⁻⁸ M for daidzein and related isoflavones, whereas EC₅₀ values for bisphenol A, one of the most intensively studied "environmental estrogens," are in the range of 10⁻⁴ to 10⁻³ M (Casanova et al., 1999; Kuiper et al., 1998; Zava et al., 1997). Environmental estrogens have been implicated in a variety of effects in humans such as increased incidences of breast cancer, decreased sperm counts, and developmental effects (Colborn et al., 1993; Davis et al., 1993). However, since human exposure to phytoestrogens is much larger as compared to industrial chemicals with estrogenic effects and phytoestrogens are more potent estrogens than most environmental estrogens, health risks of human exposure to environmental estrogens may be much lower as compared to risks of phytoestrogen exposure (Safe, 1995) or phytoestrogens may also act as "endocrine disrupters" (Santti et al., 1998; Setchell et al., 1997).

In rodents, daidzein in vivo is only a relatively weak estrogen despite relatively high affinity to the estrogen receptor suggesting that toxicokinetics may be a major determinant of...
estrogenic potency in vivo. Therefore, the low potency of daidzein in vivo may be due to rapid biotransformation and excretion or inefficient absorption, therefore lowering the amount of daidzein available for receptor binding. The experimental toxicokinetics of daidzein and other endocrine modulators should be integrated into the process of extrapolation of toxicological data, from in vitro to in vivo, and from animals to humans, and into the risk assessment process of endocrine chemicals.

In this work we have studied the disposition, biotransformation, and kinetics of excretion of daidzein in rodents in order to elucidate the apparent discrepancies between estrogenic activities of daidzein in vivo and in vitro.

MATERIALS AND METHODS

Chemicals. All chemicals used were of the highest purity commercially available. Daidzein (98%), 4-hydroxybenzoic acid (99%), and (4-hydroxy-phenyl)-2-propionic acid (98%) were obtained from Sigma-Aldrich (Steinheim, Germany). Equol and O-desmethylangolensin were synthesized in a purity of 98% (HPLC) as described (Franke et al., 1995) and characterized by UV-spectroscopy and GC/MS after trimethylsilylation. Bis-trimethylsilyl-acetamide and bis-trimethylsilyl-trifluoroacetamide were obtained from Fluka (Buchs, Switzerland).

Instrumental analysis. HPLC was performed with a Millipore Waters pump system (pumps 501 and 510 and gradient controller) and a HP 1050 UV-detector coupled to a Shimadzu-sil 9-A autosampler. Peak integration was performed by a Waters-Millipore data module 740 integrator. Separations were performed using a 4.6 x 250 mm steel column filled with Partisol ODS-3, 5 µm. Samples were analyzed by gradient elution using a linear gradient from 100% H₂O (adjusted to pH 2 with trifluoroacetic acid) to 100% acetonitrile over 60 min at a flow rate of 1 ml/min. A different gradient (20% to 80% acetonitrile in water in 40 min) was used to identify unchanged daidzein. To record online UV-spectra, some urine samples were also analyzed by HPLC by a Hewlett-Packard 1040 series II diode array detector coupled to a Hewlett-Packard 1050 HPLC pump. Spectra were recorded from 220–460 nm and HPLC separation conditions were identical as described above.

GC-MS in the electron impact mode (70 eV) was performed with a HP 5973 MSD using a DB-1 fused silica capillary column (30 m x 0.18 mm I.D., 0.5 µm film thickness; J & W Scientific, Folsom, CA) with a linear temperature gradient of 20°C/min from 100°C to 320°C. Split injection with a split ratio of 20:1 and helium at a flow rate of 1 ml/min was used as carrier gas.

HPLC-MS/MS was performed with an Applied Biosystems 140B HPLC-pump coupled to a TQ 7000 tandem mass spectrometer system equipped with an electrospray ionization interface (Finnigan MAT, Bremen, Germany). Data acquisition and evaluation were conducted on a DEC 5000/33 using the ICSI 8.1 software (Finnigan MAT). Samples were separated using a 100 x 2 mm ID steel column filled with Eurospher 100-C18 (Merck and Nagel, Düren, Germany) and a linear gradient from 100% H₂O (adjusted to pH 2 with trifluoroacetic acid) to 100% acetonitrile over 60 min at a flow rate of 0.2 ml/min. Capillary temperature was 250°C and electrospray voltage was 3.5 kV. Spectra were collected in the positive ion mode.

Half-lives were calculated using exponential regression in Microsoft Excel® spreadsheets using the curve fitting function of the program. Excretion data correlated with first-order kinetics with r² values > 0.8.

Animals and treatment. Daidzein (100 mg/kg, dissolved in corn oil) was administered by gavage to 4 male (280–300 g) and 4 female (160–190 g) Fischer F344 rats (Charles-River-Wiga, Sulzfeld, Germany). After administration, the animals were individually kept in metabolic cages and urine and feces were collected at 4°C at predetermined intervals for 96 h. For acclimatization, animals were kept in metabolic cages for 72 h before daidzein administration and control urine and feces were collected during this time. Animals had free access to food and tap water during the sample collection period and were given a diet low in soy proteins and in isoflavones (No 1324, Altromin, Langen, Germany) for 1 week before daidzein administration and during the sampling period. Daidzein and genistein, 2 major isoflavones usually present in soy, could not be detected in this diet by HPLC or by GC/MS after methanol extraction (Casanova et al., 1999). Only very low levels (less than 2 nmol/ml) of daidzein and daidzein-metabolites were detected in urine and feces collected from the animals for 24 h before daidzein administration.

Separation and quantification of daidzein-metabolites. A solution (10 µl) of the internal standard flavone (2-phenyl-4H-1-benzopyran-4-one, 40 µmol/ml in methanol) was added to rat urine samples (20 µl) and the obtained solution was diluted with 770 µl of methanol. After centrifugation, 50 µl of this solution were separated by HPLC. For quantification of daidzein and metabolites, the eluate from the column was monitored at 302 nm. Quantitation was performed relative to the content of flavone and referenced to calibration curves with fortified aliquots of urine samples from controls containing 0–80 nmol/ml daidzein. The method was linear in the range of concentrations used and calibration standards were analyzed with every sample series (usually 20–30 samples). The method permitted the quantitation of 2 nmoles of daidzein/ml of urine with a signal to noise ratio of 5:1. When identical samples were repeatedly analyzed, deviations of the obtained quantitative results were lower than 10%. Daidzein-glucuronide and daidzein-sulfate were quantified based on calibration curves obtained with daidzein due to identical UV-absorption.

Quantitation of daidzein excretion with feces. Feces collected at different time points were lyophilized. To extract daidzein and daidzein-metabolites, 250 mg of freeze-dried feces samples were suspended in 20 ml of ethyl acetate and agitated at 60°C for 48 h in closed vials. After filtration, the ethyl acetate extracts were adjusted to a volume of 25 ml, 10 µl of the internal standard flavone were added to aliquots of the extract (0.79 ml) and 50 µl of the obtained solution were separated by HPLC as described above. Recovery of daidzein from spiked samples of control feces collected from the rats before daidzein administration was >90%.

Identification of minor daidzein-metabolites in feces. Minor daidzein-metabolites in feces were identified by GC/MS in the ethyl acetate extracts prepared as described above. The solvent from aliquots (5 ml) of the extracts was removed under reduced pressure and the obtained residues were treated with 0.5 ml of bis-trimethylsilyl trifluoroacetamide at 100°C. After 1 h, 2 µl of the solutions were analyzed by GC/MS. Standards of O-desmethylangolensin, equol, 4-hydroxybenzoic acid, and 2-(4-hydroxyphenyl)propanoic acid were added to feces obtained from control rats to demonstrate recovery.

RESULTS

Identification of Daidzein-Metabolites Excreted in Urine

To study biotransformation and kinetics of excretion of daidzein, daidzein was orally administered to rats and urine and feces were collected for 96 h. To detect metabolites, urine samples from treated animals were separated by HPLC and the obtained chromatograms were compared to chromatograms of urine samples from the same animals before daidzein exposure (Fig. 1). HPLC separation of a 12-h urine sample from daidzein-treated male rats showed the presence of 3 new peaks in the chromatogram; 2 new peaks were seen in urine samples from daidzein treated female rats. The recorded UV-spectra of all new peaks were identical and peak 3 (Fig. 1) had an identical retention time as unchanged daidzein using different HPLC-separation conditions, conclusively identifying this metabolite as unchanged daidzein. The compound in this peak
was also confirmed as daidzein by GC/MS of the bis-trimethylsilyl ether. The UV-spectra suggested that the metabolites represent daidzein-conjugates and both metabolites were converted to daidzein upon treatment of urine samples with glucuronidase/sulfatase or by concentrated acid. To conclusively identify these conjugates, urine samples were subjected to LC-MS/MS. The electrospray mass spectrum of peak 2 (Fig. 1) is shown in Figure 2A and exhibited a single peak with m/z 335 (M + H)^+. When applying a collision energy of 15 eV to obtain structural information, a fragment with m/z 255 representing daidzein was formed. The mass difference of 80 Da suggests the presence of a sulfate of daidzein. The mass spectrum of peak 1 (Fig. 1) is shown in Figure 2B and exhibited a single ion at m/z 431 (M + H)^+ that was also fragmented to give m/z 255 (daidzein). The loss of 176 Da suggests the loss of glucuronic acid indicating that this conjugate represents a glucuronide. The mass spectrometry data conclusively confirm daidzein, daidzein-glucuronide, and daidzein-sulfate as urinary excretion products of orally administered daidzein.

**Identification of Daidzein-Metabolites in Feces**

In ethyl acetate extracts from the feces of daidzein-treated rats, HPLC-analysis showed the presence of large amounts of unchanged daidzein; daidzein-conjugates were not detected in the samples collected (data not shown). GC/MS-analysis of the extracts confirmed the presence of large amounts of daidzein in the feces (Fig. 3); in addition, the separations indicated the presence of 2 small peaks with identical mass spectra and retention times as the bis-trimethylsilyl-ether of equol and the tris-trimethylsilylether of O-desmethylangolensin identifying...
these compounds, which are known daidzein-metabolites in humans, as metabolites formed from daidzein in rodents in small amounts. The feces-extracts did not contain detectable amounts of 4-hydroxybenzoic acid and 2-(4-hydroxyphenyl)propanoic acid, which potentially could be formed by reductive ring cleavage of daidzein in rats analogous to the biotransformation of the related isoflavone genistein (Coldham and Sauer, 2000).

**Kinetics of Excretion of Daidzein and Its Metabolites**

The major pathway of excretion for orally administered daidzein was with feces as unchanged parent compound with a recovery of more than 85% of the administered dose (Table 1) in both male and female rats. Only a minor part of the administered daidzein-dose was recovered in rat urine, in part as parent compound (due to some water solubility) and as conjugates (Table 1). In urine of male rats, daidzein-sulfate was the major excretory product, whereas in the urine of daidzein-administered female rats, daidzein-sulfate was only formed in small amounts and daidzein-glucuronide was the major metabolite excreted. Excretion of daidzein and its conjugates with urine occurred with first-order kinetics and was rapid in both male and female rats and followed first-order kinetics with elimination half-lives between 3 and 5 h (Fig. 4, Table 1). The concentrations of daidzein-metabolites and parent compound were below the limit of detection 36 h after oral administration and most of the administered dose was recovered in urine within 24 h after administration. In both male and female rats, elimination of unchanged daidzein with feces was also rapid and was essentially complete 36 h after administration (Fig. 5).

**DISCUSSION**

The objectives of this study were to evaluate the toxicokinetics of daidzein in rodents to contribute to the database for improved risk assessment of this widely ingested food constituent. Moreover, the studies were aimed to elucidate a possible role of toxicokinetics in the differences of estrogenic potencies of daidzein observed in intact animals and in *in vitro* assays to determine estrogenicity.

Of the applied daidzein dose, only a minor part was recovered in urine both as unchanged parent compound, and, as expected, in the form of conjugates. No indication for phase I oxidation reactions in daidzein biotransformation is indicated by the structure of metabolites found in urine; due to the presence of hydroxyl groups, daidzein is efficiently conjugated to glucuronic acid and to sulfate and the highly water soluble conjugates are rapidly excreted. The exact regiochemistry of the conjugates was determined previously, glucuronic acid is

### TABLE 1

<table>
<thead>
<tr>
<th></th>
<th>Males</th>
<th>Females</th>
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<tr>
<td>nmol</td>
<td>% of Dose</td>
<td>nmol</td>
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<tr>
<td><strong>Urine</strong></td>
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<tr>
<td>Daidzein</td>
<td>113,475</td>
<td>67,358</td>
</tr>
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<td>Daidzein</td>
<td>949 ± 375</td>
<td>826 ± 395</td>
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<tr>
<td>t\textsubscript{1/2} = 3.6 h</td>
<td>t\textsubscript{1/2} = 4.5 h</td>
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<tr>
<td>Daidzein-glucuronide</td>
<td>2852 ± 854</td>
<td>4485 ± 1785</td>
</tr>
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<td>t\textsubscript{1/2} = 2.9 h</td>
<td>t\textsubscript{1/2} = 3.1 h</td>
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<tr>
<td>Daidzein-sulfate</td>
<td>6189 ± 3082</td>
<td>144 ± 23\textsuperscript{a}</td>
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<tr>
<td>Σ urine</td>
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<td>5455</td>
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<tr>
<td>8.8 ± 3.1</td>
<td>8.2 ± 2.8</td>
<td></td>
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<tr>
<td><strong>Feces</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Σ feces</td>
<td>98,140 ± 26,807</td>
<td>57,537 ± 5,767</td>
</tr>
<tr>
<td>86.2 ± 24.2</td>
<td>86.3 ± 8.6</td>
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<tr>
<td>Total</td>
<td>95.0</td>
<td>94.6</td>
</tr>
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*Note.* Male and female rats were orally administered a dose of 100 mg/kg b.w. Data are mean ± SD from 4 male and 4 female rats.

*\textsuperscript{a}Due to low amounts seen only in the 6-h urine sample, half-life could not be determined.*
coupled to the oxygen atom in position 7 to give daidzein-7-O-β-D-glucuronide and sulfate is coupled to the oxygen atom in 4'-position to daidzein-4'-O-sulfate (Yasuda et al., 1994) (Fig. 6).

The gender differences in the excretion of daidzein-sulfate, which is the predominant daidzein-conjugate excreted in males and only a minor product excreted in female rats, are likely due to gender differences in the expression of specific sulfotransferases in the liver of male rats. Phenol sulfotransferase is present in much higher activity in male as compared to female rats (Falany and Wilborn, 1994). Based on substrate requirements and structure activity relationships between sulfotransferase substrates, it may be concluded that the male rat specific phenol sulfotransferase has a major contribution to daidzein-sulfate formation in male rats.

The high recovery of administered daidzein in feces may indicate an inefficient absorption of daidzein from the gastrointestinal tract of rats or an intensive conjugation of daidzein in rat liver, enterohepatic circulation of the formed conjugates and cleavage of the conjugates by glucuronidase, and/or sulfatase in intestinal bacteria resulting in daidzein excretion with feces. Likely, an inefficient absorption of daidzein is responsible for the high percentage of administered dose excreted with feces. This assumption is supported by the expected elimination of the highly water-soluble daidzein-sulfate from the liver into the blood stream to be excreted in urine and therefore not subjected to enterohepatic circulation. In case of...
enterohepatic circulation, the differences in the extent of daidzein-sulfate formation between male and female rats should therefore result in differences in the extent of daidzein excretion with feces and a lower recovery of the administered dose in feces of male animals. The lack of gender difference in fecal excretion suggest that enterohepatic circulation of daidzein-conjugates is unlikely to have a major impact on daidzein disposition. Moreover, enterohepatic circulation of daidzein-conjugates is also not supported by the rapid excretion of unchanged daidzein with feces and of daidzein and daidzein-conjugates with urine. Structurally similar compounds to daidzein such as bisphenol A, which undergo enterohepatic circulation in rats, are only slowly excreted with urine and feces over a prolonged period of time (Pottenger et al., 2000).

A minor part of the applied daidzein in rats was reduced to O-desmethylangolensin and to equol, likely by intestinal bacteria (Adlercreutz et al., 1986). Reductive metabolism of daidzein by intestinal bacteria has also been observed in humans. Species differences in the content and type of intestinal bacteria may explain species differences in the extent of daidzein reduction. A reductive cleavage of daidzein resulting in ring fission as observed with genistein (Coldham and Sauer, 2000) was not seen in our studies with daidzein. Genistein is reduced to give 4-hydroxyphenyl-2-propionic acid and dihydrogenistein, the expected products from daidzein to be formed by an identical mechanism, 4-hydroxybenzoic acid and 4-hydroxyphenyl-2-propionic acid were not detected in the feces of rats despite application of sensitive and specific methods.

In humans, disposition of daidzein from dietary sources was reported to be different from the disposition data observed in this study in rats. Daidzein in human diet may be slowly but more efficiently absorbed from the gastrointestinal tract in humans (Lu et al., 1995, 1996b; Xu et al., 1995, 1994). Therefore, higher blood levels of daidzein in humans as compared to rats may be the result. Indeed, daidzein and other isoflavones seem to be relatively potent estrogens in humans since estrogenic effects were seen in people consuming diets rich in isoflavones for a prolonged time (Lu et al., 1996a).

In summary, our results show that daidzein absorption from the gastrointestinal tract in rodents is not efficiently absorbed and absorbed daidzein is rapidly conjugated and excreted. Thus, only low concentrations of daidzein in rat blood and in organs are to be expected resulting in only weak estrogenic effects despite administration of relatively high doses and a relatively high affinity of daidzein to the estrogen receptor. The inefficient absorption of daidzein is therefore likely correlated to the low potency of this compound as an estrogen in vivo; a low estrogenic potency (Hopert et al., 1998) and low rate of absorption of daidzein in comparison to other estrogenic isoflavonoids was also observed (Breinholt et al., 2000).

Disposition and biotransformation data as described here may be important contributors to the risk assessment process of “environmental estrogens” and will have to be included in comparisons of relative contributions of estrogenic chemicals from different sources to the total “estrogen load” of an organism. Species differences in extent of absorption, disposition, and biotransformation may further complicate the risk assessment process for these compounds. As a conclusion, in vitro data on estrogenicity such as the E-screen (Andersen et al., 1999; Arnold et al., 1996; Soto et al., 1995) and other systems should only be used as a prescreen to obtain information on potential estrogenicity of a compound. An assessment of estrogenic effects in vivo in rodents and comparative toxicokinetics in rodents and humans need to be included in the assessment of potentially adverse effects of chemicals with possible endocrine modulating activity (Kavlock et al., 1996; Safe et al., 1997).

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