Pharmacokinetics of Equol, a Soy Isoflavone Metabolite, Changes with the Form of Equol (Dietary versus Intestinal Production) in Ovariectomized Rats

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ABSTRACT: Recent findings indicate that soy isoflavones and their metabolites may play a role in mitigating postmenopausal bone loss. Equol, a metabolite of the soy isoflavone daidzein produced by intestinal bacteria, has shown some potential, but only 30−50% of the U.S. population is capable of converting dietary daidzein to equol. There are limited data on the pharmacokinetics of dietary racemel equol and its metabolites. This study was conducted to assess the levels of equol and its conjugates in plasma for a 24 h period resulting from oral administration of dietary daidzein and racemic equol in ovariectomized Sprague−Dawley rats. Plasma samples were analyzed for conjugated and free forms of equol using LC-MS/MS. The maximum plasma concentration (Cmax) and time to reach it (tmax) for total equol (conjugated and unconjugated) were 8815 ± 2988 nmol/L and 2.17 ± 2.91 h and 3682 ± 2675 nmol/L and 20.67 ± 4.67 h, for dietary equol and daidzein, respectively. Although the majority of equol metabolites present were glucuronide conjugates (≥99%), there were low levels of equol monosulfate present. The changes in equol metabolism, specifically equol conjugates, due to the form of equol may play a role in the potential health benefits of equol.

KEYWORDS: pharmacokinetics, equol, soy isoflavones, ovariectomized rats, equol conjugates

INTRODUCTION

Soy isoflavones and their metabolites have been extensively studied for their various bone and cardiovascular health benefits. In particular, equol, a metabolite of intestinal bacterial conversion from daidzein (Figure 1), has emerged as a bioactive compound with potential for bone health benefits. Equol supplementation is associated with improved bone health in ovariectomized rats including increased trabecular microarchitecture at the proximal femur and lumbar spine as well as enhanced osteoporotic fracture healing. However, results from clinical studies on soy isoflavones have been mixed, which many have attributed to the varied equol-producing ability of the U.S. population. Equol production is primarily dependent on the intestinal bacterial composition of an individual and could play a role in achieving bone health benefits from soy. After classifying participants on equol-producing status, Setchell et al. observed a 2.4% increase in lumbar spine bone mineral density of equol producers (45% of participants) compared to a 0.6% increase in non-equol producers with a 2 year soy isoflavone intervention.

In addition to equol-producing ability, the form of circulating equol may also affect potential health benefits. The majority of circulating isoflavones are present primarily as glucuronidated conjugates with low amounts of sulfated conjugates, which could exert various health effects as well. Daidzein sulfate conjugates, but not daidzein, inhibited sterol sulfatase, which is involved in the development of breast cancer in vitro. Daidzein and genistein glucuronides have also been shown to augment activation of human natural killer cells in vitro and could aid in improved immunity defense against various cancers. To date, there is limited information on the conjugation profile of equol. Schwen and colleagues reported high glucuronidation of S-equol in male Sprague−Dawley rats as well as monkeys. Although there has been much research on the metabolism of soy isoflavones and their conjugates in rodents and humans, there are limited data on equol metabolism. Two studies explored the transport, absorption, and metabolism of equol in vitro. S-Equol is the only enantiomer produced by human intestinal bacteria from daidzein. Pharmacokinetic studies of dietary equol in young healthy adults and postmenopausal women showed rapid absorption of equol. Although there are a few studies of S-equol pharmacokinetics, there is only one in vivo study that evaluated enantiomer forms of equol on metabolism. Setchell et al. demonstrated the bioavailability of equol depends on the enantiomeric form of equol (R-equol, S-equol, racemic equol), which subsequently may affect health implications.

The aim of this pharmacokinetic study was to compare circulating levels of equol and its conjugates (Figure 2) from dietary racemic equol versus intestinal production from dietary daidzein in ovariectomized female Sprague−Dawley rats, a postmenopausal rodent model.

Received: July 10, 2013
Revised: January 14, 2014
Accepted: January 21, 2014
Published: January 21, 2014

dx.doi.org/10.1021/jf400097m
J. Agric. Food Chem. 2014, 62, 1294−1300
MATERIALS AND METHODS

Animal Procedures. Three-month-old female Sprague-Dawley rats were purchased from Harlan (Indianapolis, IN, USA) and underwent ovariectomy (OVX) surgery. Rats were maintained on an AIN-93 M diet with corn oil replacing soybean oil (to eliminate the potential presence of phytoestrogens in diet) for 1 week before undergoing pharmacokinetics. Animals were housed in individual cages in temperature- and humidity-controlled rooms with a 12 h−12 h on−off light cycle. After a 2 day acclimation period, animals were grouped into pairs and divided into two treatment groups: dietary daidzein (n = 6 pairs) and dietary equol (n = 6 pairs). Animals were matched on the basis of body weight, and pairs were selected to ensure similar body weight average across treatment groups.

Chemicals. Daidzein was obtained from a commercial source (Indofine Chemical Co., Hillsborough, NJ, USA). Racemic equol (50% R-equol, 50% S-equol) as well as equol sulfate conjugates (equol monosulfate and disulfate) were generously provided by the Helferich

Figure 1. Schematic of equol production from intestinal bacteria metabolism of daidzein.

Figure 2. Chemical structures of equol and its conjugates.
Table 1. Equol Metabolites Detected in Plasma of Ovarectomized Rats Fed a Single Oral Dose of Dietary Daidzein \((n = 4\) Pairs, 10 mg/mL) or Equol \((n = 5\) Pairs, 2 mg/mL)

| metabolite     | retention time (min) | \(m/z\) transition |
|----------------|----------------------|--------------------|
| equol          | 10.8                 | 241/119            |
| equol disulfate| 8.83                 | 401/321            |
| equol glucuronide| 8.72              | 417/241            |
| equol monosulfate| 9.76              | 321/121            |

(Materials. For formulation of oral gavage solutions, both daidzein and equol were suspended in 4.5% starch solution for single oral doses of 10 and 2 mg/mL, respectively. The oral equol dose represents a daily intake of 200 mg racemic equol/kg diet based on a physiologically relevant level for dietary racemic equol seen in earlier research in our laboratory. The oral daidzein dose (1000 mg daidzein/kg diet) was selected due to the fact it produced similar circulating equol levels as seen with the dietary dose of 200 mg racemic equol/kg diet.

Pharmacokinetics. Animals were implanted with jugular catheters for blood collection 2 days before pharmacokinetics. Animals received a single oral gavage solution (2 mL) of dietary daidzein (10 mg/mL) or dietary equol (2 mg/mL). Blood (0.2 mL) was drawn via jugular catheter from each rat at the following time points: 0, 0.5, 1, 2, 3, 4, 6, 8, 12, 16, 20, and 24 h. For each pair of animals, blood was pooled at each time point (total of 0.4 mL). Animals weighed between 200 and 250 g. To replenish animals from blood loss, fluids (50:50, saline/5% dextrose, 2 mL) were given after the 3 h time point. After the last blood collection, animals were euthanized by CO₂ inhalation. All procedures were approved by the Purdue University Animal Care and Use Committee (Protocol 08-112).

Sample Preparation, Extraction and Analysis. Plasma samples were processed through two separate sample preparations to determine total aglycone and equol conjugate concentrations. To determine total plasma aglycone equol, samples underwent enzymatic hydrolysis as described previously. In brief, two conjugates, phenolphthalein β-glucuronide (PHG) and 4-methylumbelliferone sulfate (4-MUS), were added to all samples as markers of effective hydrolysis along with an internal standard, chrysin (CHYS). Isoflavones were recovered by diethyl ether extraction. The ether extracts were evaporated under a stream of nitrogen and reconstituted with 100 μL of an 80% aqueous methanol solution for analysis.

To determine circulating levels of free equol and equol sulfate conjugates, plasma samples (200 μL) underwent protein precipitation. Methanol containing 1% acetic acid solution and a mixed set of internal standards (10 μM 4-MUS−PHG−CHYS) were added to each sample in a ratio of 4:1. Samples were centrifuged for 10 min at 3000 g and 4 °C. The supernatants were used for analysis.

Total unconjugated equol, free equol, equol monosulfate, and equol disulfate concentrations were identified and quantified using a highly sensitive and specific electrospray ionization liquid chromatography–multiple reaction ion monitoring (MRM) mass spectrometry method.

Figure 3. MS/MS chromatogram of control sample and product ion mass spectra of plasma samples from rats fed dietary equol (2 mg/mL). The presence of equol glucuronides was determined by monitoring the \(m/z\) 417/241 transition using a triple-quadrupole mass spectrometer (4000 QTRAP, ABSCIEX, Framingham, MA, USA) with chromatography details described previously.

The Journal of Agricultural and Food Chemistry
"Journal of Agricultural and Food Chemistry"
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as previously described\textsuperscript{35} with chromatography conditions detailed in earlier work\textsuperscript{35} and with the following modifications: equol monosulfate and disulfate were detected using their precursor to product ion transitions of $m/z$ 321/121 and $m/z$ 401/321, respectively. Identification and quantitation of equol and its sulfate conjugates were determined on the basis of comparison of MS/MS fragmentation pattern and retention time to reference material. LC-MS/MS method did not distinguish between R- and S-equol enantiomers. Due to limited availability of known standard for equol glucuronide, it was identified by its $m/z$ 417/241 transition, and concentrations were determined using the following equation, which assumes that only equol sulfate and glucuronide conjugates are present:

\[
\text{equol glucuronide conjugates (nM)} = \text{total aglycone equol (nM)} - \text{free equol (nM)} - \text{equol sulfate conjugates (nM)} \tag{1}
\]

The limit of detection for all analytes of interest was 5 nM. All parameters for metabolite identification including retention times and $m/z$ transitions are listed in Table 1. To validate calculation for equol glucuronide conjugates and ensure no other metabolites were present, a control plasma sample was analyzed along with a sample from the dietary equol group. All pertinent $m/z$ transitions were monitored. Representative chromatograms of equol glucuronide and equol sulfates are shown in Figures 3 and 4.

**Pharmacokinetic Parameters.** The following pharmacokinetic parameters were determined using noncompartmental methods (WinNonlin Pro version 4.01, Pharsight Corp., Mountain View, CA, USA): $C_{\text{max}}$ (maximum concentration), $t_{\text{max}}$ (time of maximum concentration), $t_{1/2}$ (elimination half-life), $AUC_{0-\infty}$ (area under the plasma concentration-time curve from 0 to infinity). The pharmacokinetic parameters for equol metabolites from dietary racemic equol are detailed in Tables 2 and 3. There were higher levels of equol glucuronides than equol monosulfate for both dietary daidzein and racemic equol groups. There were no detectable levels of equol disulfate.

### RESULTS AND DISCUSSION

Pharmacokinetic parameters for equol metabolites from dietary daidzein and racemic equol are detailed in Tables 2 and 3. There were higher levels of equol glucuronides than equol monosulfate, with greater animals needed to confirm if plasma increases are significant. Equol conjugates had lower clearance rates ($CL/F$) than unconjugated equol, which is unexpected due to polarity of conjugates, and warrants further investigation.

There were significantly higher maximum concentrations of all equol metabolites from dietary racemic equol compared to S-equol produced from dietary daidzein. Plasma concentrations of equol metabolites peaked around 2–3 h for dietary racemic equol and appeared in large levels at 8–13 h for dietary daidzein. The late appearance of equol metabolites with dietary daidzein was expected due to time required for bacterial conversion of daidzein to equol in the large intestine and therefore is a measure of intestinal transit.

Dietary equol led to rises in circulating equol and equol conjugates at 1, 4–5, and 8–9 h (Figure 5). The initial rise at 1 h is attributed to its rapid absorption in the small intestine, which is also seen in both in vitro\textsuperscript{27} and in vivo\textsuperscript{30} studies of equol metabolism. The second and third peaks at 4–5 and 8–9 h may be indicators of enterohepatic recycling and additional absorption occurring in the large intestine, respectively. Increases in equol metabolite levels during 16–24 h could also reflect enterohepatic recirculation, as previously reported with other isoflavones.\textsuperscript{18} Small rises in equol metabolite levels could also be due to high biological variation. Additional studies with greater animals are needed to confirm if plasma increases are significant. Equol conjugates had lower clearance rates ($CL/F$) than unconjugated equol, which is unexpected due to polarity of conjugates, and warrants further investigation.
The first appearance of equol metabolites (total equol and equol glucuronides) after administration of dietary daidzein occurred around 7–8 h (Figure 5A,D). This corresponds to findings from previous studies.\(^{15,17,38}\) In the dietary daidzein treatment group, total equol, unconjugated equol, and equol glucuronide levels from dietary daidzein rose between 8 and 13 h and all metabolites peaked between 20 and 24 h, consistent with earlier work assessing the bioavailability of daidzein and genistein conjugates in rats.\(^{15}\) None of the equol metabolites reached a plateau after 24 h (Figure 5). There were few data to accurately assess HL, CL/F, and V/F of equol metabolites produced from dietary daidzein. Both findings suggest that a longer time frame is needed to adequately assess the pharmacokinetic profile of equol metabolites from daidzein consumption.

Table 3. Pharmacokinetic Parameters of Equol Metabolites from a Single Oral Gavage of Dietary Equol Administered to Ovariectomized Rats (\(n = 5–6\) Pairs)\(^a\)

| metabolite                  | HL (h)   | \(t_{\text{max}}\) (h) | \(C_{\text{max}}\) (nmol/L) | AUC\(_{0–24}\) (nmol/L·h) | CL/F (L/h) | V/F (L)  |
|-----------------------------|----------|-------------------------|-----------------------------|--------------------------|------------|----------|
| total equol                 | 21.9 ± 8.02 | 2.17 ± 2.91            | 8815 ± 2988                | 104337 ± 20531           | 0.08 ± 0.03 | 2.32 ± 0.26 |
| unconjugated equol          | 9.41 ± 3.93 | 4.42 ± 4.50            | 9.46 ± 3.56                | 96.0 ± 41.9              | 179 ± 138   | 1,937 ± 692 |
| equol monosulfate           | 14.4 ± 9.26 | 3.20 ± 2.95            | 23.9 ± 8.54                | 234 ± 97                 | 43.7 ± 11.7 | 912 ± 703  |
| equol glucuronides          | 23.0 ± 2.91 | 2.17 ± 2.91            | 8775 ± 2976                | 10408 ± 20516            | 0.08 ± 0.03 | 2.38 ± 0.50 |

\(^a\)Values are means ± SD. Pharmacokinetic parameters: HL (elimination half-life), \(t_{\text{max}}\) (time of maximum concentration), \(C_{\text{max}}\) (maximum concentration), AUC\(_{0–24}\) (area under plasma concentration time curve from 0 to 24 h), CL/F (clearance rate) = D/AUC\(_{0–24}\), V/F (volume of distribution)

Figure 5. Pharmacokinetic curves over a 24 h period in ovariectomized rats after a single oral dose of dietary daidzein (\(n = 6\) pairs) (10 mg/mL) or equol (\(n = 6\) pairs) (2 mg/mL) for plasma (A) total aglycone equol, (B) unconjugated or free equol, (C) equol monosulfate, and (D) equol glucuronides. The equol glucuronide was calculated as the difference between the total plasma equol concentration and the sum of the plasma unconjugated and sulfated equol.
Our results indicate there were higher levels of circulating equol metabolites with dietary racemic equol compared to equol produced from dietary daidzein in ovariec-tomized rats. We also observed poor clearance of equol metabolites as well as high enterohepatic recirculation as seen previously with other soy isoflavones.\(^\text{12,29,30}\) Most of the circulating equol was conjugated equol, equally equol glucuronides, which agrees with findings from other in vivo studies. Pharmacokinetics studies of S-equol in various species (rat, monkey, human) show similar metabolism including rapid absorption.\(^\text{12,29,30}\) Future studies could utilize animal models to determine mechanisms of action for various forms of equol. More elucidation on the impact of the forms of equol (various enantiomers, dietary vs intestinal production) as well as the effect of equol conjugates on health benefits of equol is needed.

### ACKNOWLEDGMENTS

We thank Pamela Lachcik and Emily Hohman for their assistance with the study as well as William Helferich and the Helferich Laboratory for providing equol sulfate and the Hettling Laboratory for providing racemic equol and Nigel Botting and the Botting Laboratory for providing equol sulfate standards.

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