Elucidation of the Metabolic Pathway of S-equol in Rat, Monkey and Man

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Abbreviations: HT, Hormone Therapy; WHI, Women’s Health Initiative; LC-RAM-MS, Liquid Chromatography-Radioactivity Monitor-Mass Spectrometry; MS/MS, Tandem Mass Spectrometry; TIC, Total Ion Content; XIC, Extracted Ion Chromatograms; HPLC, High Pressure Liquid Chromatograph

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ABSTRACT

S-equol is a selective estrogen receptor β (ERβ) agonist which is produced in certain individuals after ingestion of its precursor daidzein, an isoflavone present in soy. S-equol is thought to provide certain health benefits, including reduced menopausal symptoms. The metabolic profile of S-equol was determined in vivo in Sprague-Dawley rats and cynomolgus monkeys, and in vitro using hepatocytes from rat, monkey, and human. High resolution MS fragmentation patterns indicated that the major metabolite of S-equol in rat plasma and urine was the 4'-glucuronide conjugate, with lesser amounts of unconjugated S-equol, the 7-sulfate conjugate, and the 4'-glucuronide-7-sulfate diconjugate. Monkeys also showed extensive metabolism, with the major species in plasma being the 4'-glucuronide and the 7-sulfate-4'-glucuronide diconjugate; urine contained primarily the 4'-glucuronide, as seen in the rat. In vitro metabolism by hepatocytes was extensive and similar in all species, with fragmentation patterns also indicating that the 4'-glucuronide was the major metabolite. No oxidative metabolites of [C\(^{14}\)] S-equol were detected in either in vivo or in vitro studies. These findings show that glucuronidation is the primary pathway for the metabolism of S-equol in rat, monkey and man, and that all metabolic routes of S-equol observed in vitro were also observed in vivo.

KEYWORDS:

S-equol
Metabolism
Hepatocytes
Metabolite Identification
1. Introduction

S-equol (the S-enantiomer of 7-hydroxy-3-(4'-hydroxyphenyl)-chroman) is a natural product that is produced by the biotransformation of the soy isoflavone daidzein (Figure 1). Individuals, particularly Asians, who consume large amounts of soy and have the appropriate gut bacteria, carry out the biotransformation of daidzein to S-equol (Adlercreutz et al., 1991; Setchell et al., 2002; Atkinson et al., 2005; Setchell et al., 2005; Setchell and Clerici, 2010). In both man and animals, only the S-enantiomer of equol is produced from daidzein (Setchell et al., 2005). S-equol is a selective estrogen receptor β (ERβ) agonist with weaker activity for ERα (Setchell et al., 2005, Muthyala et al., 2004). In contrast, 17-β estradiol has equal binding activity to both estrogen receptors. The Women’s Health Initiative (WHI) showed that postmenopausal women on hormone therapy (HT), receiving conjugated estrogens plus a progesterone had a greater risk of developing cardiovascular diseases, breast cancer, stroke and venous thrombosis (Rossouw et al., 2002). For this reason, there is much interest in developing S-equol as an alternative to HT for menopausal symptoms and osteoporosis in postmenopausal women (Ishiwata et al., 2009; Jackson et al., 2011; Tousen et al., 2011).

It is known that S-equol undergoes rapid first-pass metabolism after oral administration in man (Jackson et al., 2011; Setchell et al., 2009a; Setchell et al., 2009b; Plomley et al., 2011). The purpose of the present study was to characterize the metabolic pathways for S-equol after the oral administration of radiolabeled S-equol. The strategy of using [14C]-S-equol allowed us to determine the relative ratios of the major metabolites of S-equol and to identify any minor components that might be present. Plasma, urine, and feces were analyzed for S-equol metabolites in the Sprague-Dawley rat and cynomolgus monkey. In vitro metabolic studies were also carried out with cryopreserved hepatocytes from rat, monkey and man. In all species, high resolution fragmentation patterns indicated that the 4’-glucuronide conjugate was the major metabolic product.
2. Materials and Methods

2.1. Materials.

[2-14C]-S-equol [(S)-3, 4-dihydro-3-(4-hydroxy-phenyl)-2H-1 benzopyran-7-ol] with radiochemical, chemical and chiral purity of 99.8%, 98.8% and 98.3%, respectively, and S-equol (purity 98.8%) were obtained from Girindus Solvay America, Inc. (Cincinnati, OH, USA). The specific activity was 53.8 mCi/mmol. Other chemicals used in the assays were purchased from commercial suppliers and for chromatography were typically LC-MS grade.

2.2. Studies in rats and monkeys.

Male Sprague–Dawley rats (225-300 g) were obtained from Charles River Laboratories (Shrewsbury, MA, USA). The animals were placed on an alfalfa- and soy-free diet (2016-C Teklad global 16% protein rodent diet; Harlan, Frederick, MA, USA) for two weeks prior to and during the study. Male non-naïve cynomolgus monkeys (3-4 kg) were obtained from Charles River Laboratories (Shrewsbury, MA, USA). For the 2 weeks prior to dosing and throughout study procedures, monkeys were kept on a special diet which did not include soy or alfalfa ingredients (PMI Certified Lab Diet 1812242; Quality Lab Products, Elkridge, MA, USA). This diet was supplemented with fruit or vegetables 2-3 times weekly, and small bits of fruit, vegetables, and other treats were occasionally given to the animals. Tap water was available ad libitum to each animal from drinking bottles. During sampling of urine and feces, rats were housed individually in stainless steel metabolism cages under standard conditions (12 h light/12 h dark photoperiod, temperature range 18–26°C; relative humidity range 30–70%). Monkeys were maintained with ventilation greater than 10 air exchanges per h with 100% fresh air, a 12-h light/12-h dark photoperiod, and room temperature between 18°-29°C. Other husbandry conditions were maintained as described in the Guide for the Care and Use of Laboratory Animals (National Research Council, Washington DC, National Academy Press, 1996). Monkeys were housed individually in stainless steel metabolism cages under the same environmental conditions during collection of samples. At the designated time points for euthanasia, rats were euthanized by carbon dioxide asphyxiation. Monkeys were euthanized by initial immobilization with ketamine HCl (10 mg/kg, IM), followed by Nembutal® (10-30 mg/kg, IV, for deep anesthesia) and a euthanizing dose of sodium pentobarbital; exsanguination was performed in accordance with accepted guidelines.

2.3. Animal dosing and sampling procedures.

In these studies, three rats received a single oral dose of 2 mg/kg [14C]-S-equol (150 μCi/kg) and three monkeys received a single oral dose of 1 mg/kg [14C]-S-equol (50 μCi/kg); these doses were chosen based on allometric scaling of an equivalent human dose of 20 mg for a 60 kg individual (Boxenbaum and DiLea, 1995). S-equol was dosed by oral gavage as an aqueous suspension in 0.5% w/v carboxymethyl cellulose and 0.1% Tween 80. Total collections of urine, feces and cage wash were obtained from rats and monkeys in continuous 12-h and 24-h intervals for 96 h after dosing. For rats, expired air was collected using glass metabolism cages and 6 M potassium hydroxide to trap the expired carbon dioxide. Since excretion of radioactivity in expired air of rats was negligible, expired air was not trapped for monkeys.
The amount of radioactivity in feces was determined in triplicate following homogenization with a 50:50 mixture of ethanol:water (~20% w/w homogenate) followed by combustion in a Packard oxidizer. Urine samples were collected in chilled containers. The total volume of each voided urine sample was recorded and analyzed for total radioactivity content. In rats, terminal blood samples were collected at 1, 4, 12, 24 and 48 h (3 rats/group) after administration of the radioactive dose by intracardiac puncture following anesthesia. In monkeys, 25 mL whole blood samples were collected for metabolite analysis by direct venipuncture of a saphenous or femoral vein at 6 h, 12 h and 96 h. Plasma was obtained by centrifugation at 3000 rpm for 10 min at a temperature between 2-9°C. All samples were stored at −20°C until analyzed. For the rat, the collection times for plasma, urine and feces with the highest radioactivity were 1 h, the 0-12 h collection, and the 0-24 h collection, respectively. For the monkey, the times for plasma, urine and feces were 6 h, the 0-12 h collection, and the 12-24 h collection, respectively.

2.4. Studies with Cryopreserved Hepatocytes.

The in vitro metabolism of [14C]-S-equol was determined using pooled cryopreserved hepatocytes from male Sprague-Dawley rats, cynomolgus monkeys, and humans (In Vitro Technologies, Chicago, IL, USA). The metabolic functionality of hepatocyte incubations was confirmed by assaying for ethoxycoumarin O-demethylation (phase I enzymes) and 7-hydroxycoumarin glucuronide conjugation (phase II enzymes) in duplicate, in the presence and absence of hepatocytes at two time points (0 and 120 min); metabolic products were monitored by HPLC with UV detection. Cryopreserved hepatocytes (~0.25 x 10^6 cells/incubation in William’s E medium) from the rat, monkey and human were incubated with 10 μM [14C]-S-equol (53.8 mCi/mmol) at 37°C for 0, 60 and 180 min. Incubations were carried out in 24-well plates aerated with a 5% CO2/95% O2 mixture. Control incubations without hepatocytes were performed for 0 and 180 min. Cell viability was determined throughout the study duration using trypan blue exclusion. Reaction mixtures were quenched with an equal volume of ice-cold acetonitrile containing 0.1% formic acid, centrifuged at 12,000 rpm for 10 min, and the supernatant fractions were analyzed for metabolites as described below. All incubations were carried out in duplicate.

2.5. Extraction of [14C]-S-equol metabolites.

The extraction methods and efficiency were optimized for consistency, precision and reproducibility using control urine, feces and plasma samples spiked with radiolabeled S-equol. Urine samples were extracted by the addition of 4 volumes of ice-cold methanol containing 0.1% formic acid. Plasma samples were extracted by addition of 4 volumes of ice-cold acetonitrile acidified with 0.1% formic acid. For feces, homogenates were extracted by addition of 10 volumes of ice-cold methanol containing 0.1% formic acid. Samples were vortexed, sonicated, and then centrifuged at 12000 rpm (approx. 24,000 x g) at 4°C for 15 min. The supernatant fractions were quantitatively transferred to a new test tube, and pellets were re-extracted. Samples were then vortexed, sonicated and centrifuged at 12000 rpm at 4°C for 15 min. Supernatant fractions were combined and then evaporated to dryness with nitrogen; residues were then reconstituted with the initial mobile phase for subsequent injection into the HPLC system.

Extracts from plasma, urine, feces and hepatocytes were analyzed by HPLC with radiochemical detection using a Gemini C$_{18}$, 5 µm, 4.6 x 250 mm column (Phenomenex, Torrance, CA, USA) and a gradient of 0.1% formic acid (solvent A) in water and 0.1% formic acid in acetonitrile (solvent B). Samples were eluted using a linear gradient of 10–90% (v/v) solvent B over 20 min, followed by isocratic elution of 90% solvent B for 2 min, and then a linear gradient of 90-100% solvent B for an additional 3 min at 35°C; re-equilibration time was 10 min. The HPLC system consisted of a Waters 2790 system (Waters, Milford, MA, USA) and a Perkin-Elmer (Woodbridge, Ontario, CAN) Radiomatic Flow Scintillation Analyzer. The flow rate of mobile phase was 1 mL/min. Prior to on line radioactivity analysis, the eluent from the HPLC column was mixed with Ultima Flo scintillation cocktail (Packard Instruments Co.) at a ratio of 1:3 (v/v).

2.7. Identification of S-equol metabolites.

Metabolites were analyzed using an Agilent 1100 HPLC system coupled to a QSTAR XL mass spectrometer (Applied Biosystems/MDS Sciex, Framingham, MA, USA) and a Perkin-Elmer (Woodbridge, Ontario, CAN) Radiomatic Flow Scintillation Analyzer. Extracts were separated on a XTerra MS C$_{18}$ (5 µm, 2.1 x 150 mm) column (Waters, Milford, MA, USA) with a gradient of 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B). A linear gradient elution of 10-60% (v/v) solvent B for 12 min was followed by an isocratic elution of 60% solvent B for 2 min, and then a linear gradient of 60-10% (v/v) solvent B for 6 min. The column was equilibrated for 10 min prior to each injection. The HPLC effluent (0.5 ml/min) was split so that 40% of the flow was introduced to the mass spectrometer via a TurboIonSpray$^{\text{TM}}$. A separate software package (Flo One, v3.61, PerkinElmer) collected the analog output from the radioactivity detector. An in-line check valve was used to prevent any scintillation cocktail from entering the mass spectrometer. Samples were analyzed under the following MS experimental conditions in the negative ion mode: capillary voltage of -4.0 V; declustering potentials DP and DP 2 of -150 V and -20 V, respectively; nebulizer gas (GS 1) and heater gas (GS 2) with a value of 40; curtain gas (CUR) of 35, and collision energy of -15 eV, -20 eV and -30 eV. Total ion count (TIC) chromatograms were converted into extracted ion chromatograms (XIC) in order to eliminate a large offset due to background noise. The mass accuracy for the MS experiments was greater than 0.01 amu.
3. Results


A representative HPLC chromatogram with radiochemical detection of extracts from rat plasma, urine and feces after oral dosing of $[^{14}C]$-S-equol is shown in (Figure 2A-C) and for monkey plasma and urine in (Figure 3A, B). The column conditions provided excellent separation of the parent compound $[^{14}C]$-S-equol (retention time 13.6 min). Three major conjugates of S-equol were observed (M1, M2, M3) with distinct retention times of 12.0, 10.0 and 8.9 min, respectively. A summary of the distribution of $[^{14}C]$-S-equol and its metabolites in the rat and monkey is shown in Table 1. In rats receiving an oral dose of $[^{14}C]$-S-equol, absorption was rapid and conjugation was efficient, with the glucuronide conjugate averaging 67% of the radioactivity at 1 h; the other analytes detected in this sample included unconjugated S-equol (4%), sulfate conjugate (5%) and a sulfate-glucuronide diconjugate (11%). The conjugates were readily excreted in urine, with the majority of the radiolabelled material in the 0-12 h sample being the glucuronide (65%), with lesser amounts of the unconjugated S-equol (12%), sulfate conjugate (10%), and diconjugate (4%). The 0-24 h feces sample from the rat contained primarily unconjugated $[^{14}C]$-S-equol with only trace amounts of the other three conjugates.

Monkeys showed an even greater conjugation efficiency, with the primary species in the 6 h plasma sample being the glucuronide conjugate and diconjugate. Unconjugated S-equol was not detectable at the 6 h time point suggesting more extensive conjugation as the parent compound continues to undergo enterohpatic circulation. The metabolic profile in monkey urine (0-12 h sample) was similar to that seen for the rat. However, the excretion of $[^{14}C]$-S-equol and metabolites in the feces of monkeys was too low to allow for metabolite profiling.

3.2. In Vivo Metabolism of $[^{14}C]$-S-equol.

The structures of the metabolites of S-equol were proposed based on the accurate masses of the deprotonated molecules and their diagnostic product ions. The major fragment ions for the unchanged drug and its metabolites are summarized in Table 2. Since the structures of the major metabolites of S-equol were the same in both the rat and monkey, only the monkey data are presented below.

3.3. $[^{14}C]$-S-equol.

MS/MS spectrum of $[^{14}C]$-S-equol is shown in (Figure 4A). The diagnostic fragmentation of the deprotonated parent molecular ion [M-H]$^-$ with a mass of 243.0972 amu (C$_{15}$H$_{13}$O$_3^-$, radiolabeled S-equol) was the cleavage of the C-C bond between the chroman-7-ol and the phenol moiety resulting in the mass fragment ions at $m/z$ 146.9873 (C$_8$H$_7$O$_2^-$) and $m/z$ 93.0406 (C$_6$H$_5$O$^-$); the latter was considered a key fragment ion as its presence was indicative of an unmodified phenol structure. Further fragmentation of the $m/z$ 147 ion resulted in the two product ions detected at $m/z$ 135.0524 (C$_8$H$_7$O$_2^-$) and $m/z$ 121.0296 (C$_7$H$_5$O$_2^-$). The proposed fragmentation pathways of $[^{14}C]$-S-equol are shown in (Figure 4B).
3.4. Monosulfate Conjugate (M1).

The representative MS/MS spectrum for the M1 metabolite in monkey urine is shown in (Figure 5A). This metabolite, characterized by its [M-H] ion of m/z 321.1031 (C\textsubscript{15}H\textsubscript{13}O\textsubscript{6}S\textsuperscript{-}, non-radiolabeled S-equol), which was 80 amu more than the unchanged drug, was determined to be an S-equol monosulfate conjugate. The diagnostic fragmentation pattern (Figure 5B) was the loss of 80 amu (a sulfate moiety) to m/z 241.1248 (C\textsubscript{15}H\textsubscript{13}O\textsubscript{3}, S-equol molecular ion mass). Other fragment ions with m/z 121, 135 and 93 in the spectrum correspond to those observed in the product ion spectrum of the S-equol parent compound and are consistent with previous findings (Plomley et al., 2011). The presence of the intact phenolic product ion with m/z 93, which was not observed in the product ion spectra of the other conjugates (see below), indicated that sulfation occurred on the chroman 7-ol moiety.

3.5. Monoglucuronide Conjugate (M2).

MS/MS analysis of the M2 radioactive peak in monkey urine revealed the presence of the S-equol monoglucuronide conjugate (Figure 6A). This was also the major metabolite in rat urine and plasma, and was characterized by its [M-H] ion of m/z 417.1725 (C\textsubscript{21}H\textsubscript{21}O\textsubscript{9}, which was 176 amu more than the unchanged drug. The diagnostic fragmentation was the loss of 176 amu, i.e., an unsaturated glucuronic acid, to m/z 241.1317 (C\textsubscript{15}H\textsubscript{13}O\textsubscript{3}, which corresponded to the mass of the [M-H] ion of S-equol (Figure 6B). The absence of an intact product ion with m/z 93 in the spectrum (i.e., a free phenolic ion, as seen for S-equol) indicated that the glucuronide acid group was attached to the phenol moiety.

3.6. Sulfate-Glucuronide Diconjugate (M3).

The representative product ion spectrum for the M3 metabolite in monkey urine, with the exact mass of 497.1727 amu (C\textsubscript{21}H\textsubscript{21}O\textsubscript{12}S\textsuperscript{-}) is shown in (Figure 7A). The diagnostic fragmentation pattern was the loss of the sulfate moiety (-80 amu) to m/z 417.2057 (C\textsubscript{21}H\textsubscript{21}O\textsubscript{9}, S-equol -monoglucuronide ion mass) and the loss of an unsaturated glucuronic acid (-176 amu) to m/z 321.1039 (C\textsubscript{15}H\textsubscript{13}O\textsubscript{6}S\textsuperscript{-}, S-equol -monosulfate ion mass). The m/z 417 and 321 ions further fragmented to lose 176 and 80 amu, respectively, to m/z 241.1183 (C\textsubscript{15}H\textsubscript{13}O\textsubscript{3}, S-equol molecular ion mass). These results support the assignment of the M3 metabolite as a sulfate-glucuronide diconjugate, with the site of glucuronidation at the phenol moiety, and the site of sulfation at the chroman 7-ol moiety.

3.7. In Vitro Metabolism of [\textsuperscript{14}C]-S-equol.

The in vitro metabolites of [\textsuperscript{14}C]-S-equol produced by cryopreserved hepatocytes from the rat, monkey and man were determined by HPLC with radiometric detection. The profiles in rat, monkey and human were qualitatively similar, with all species producing the same three metabolites M1, M2 and M3 at retention times similar to those described above (12.1 min, 9.9 min and 9.0 min, respectively). A representative chromatogram for human hepatocytes is shown in (Figure 8A). The parent compound [\textsuperscript{14}C]-S-equol was nearly completely metabolized over the 180 min incubation period. The distribution of S-equol and the three metabolites in hepatocytes extracts of the rat, monkey and human are shown in Table 3. The M2 metabolite
(glucuronide) was the major metabolite in each species, with lesser amounts of the M1 (sulfate) and M3 (diconjugate) metabolites.

To confirm metabolite structures in hepatocyte incubations, individual samples from hepatocyte incubations from each species (0 and 180 min) were analyzed using a full-scan LC-MS acquisition combined with simultaneous radioactivity monitoring. Since all species showed identical metabolic profiles (data not shown), equal aliquots from each supernatant fraction were pooled, evaporated to dryness under a nitrogen flow and reconstituted in the initial mobile phase for analysis by LC-RAM-MS. A representative LC-RAM-MS chromatogram of a pooled sample is shown in (Figure 8B). The radioactivity response for the M1 metabolite was detected at 8.6 min and the corresponding extracted ion chromatogram response at 8.3 min with an experimental mass of 323 amu (+80 amu mass shift) of the $[^{14}\text{C}]$-S-equol indicating that the M1 metabolite was the monosulfate conjugate. Metabolite M2 was associated with its molecular ion of $m/z$ 419 (+176 amu mass shift of the $[^{14}\text{C}]$-S-equol) corresponding to a monoglucuronide. Similarly, analysis of the M3 metabolite revealed the presence of a sulfate-glucuronide diconjugate, characterized by its [M-H]-1 molecular ions of $m/z$ 499 (+256 amu mass shift of the $[^{14}\text{C}]$-S-equol). The fragmentation patterns of hepatocytes-derived M1, M2 and M3 were nearly identical to those found in plasma and urine from monkeys and rats.
4. Discussion

The present study provides the first full characterization of the in vivo metabolism of S-equol dosed orally in the rat and monkey, as well as the in vitro metabolism in hepatocytes from rat, monkey and man. S-equol was rapidly metabolized after oral dosing in both the rat and monkey. Conjugation was the only metabolic process in each species. MS/MS fragmentation patterns indicated that the major conjugates consisted of the 4'-glucuronide conjugate, the 7-sulfate conjugate, and the 7-sulfate-4'-glucuronide diconjugate. While confirmation of these structures would require isolation of the metabolites or synthesis of authentic standards, followed by NMR analysis, the use of a radiolabeled S-equol and the observed differences in fragmentation patterns allowed structures to be determined by deductive analysis. The in vitro incubations in hepatocytes from rat, monkey and man each showed a high metabolic capacity with similar metabolite profiles. Radiolabeled metabolite profiling combined with MS/MS fragmentation analysis of the radiolabeled peaks indicated that the 4'-glucuronide conjugate was the most abundant metabolite in all three species, with lesser amounts of the monosulfate conjugate and sulfate-glucuronide diconjugate. Furthermore, the metabolite profiles in hepatocytes were qualitatively similar to those observed in the rat and monkey after oral dosing, suggesting that the in vitro hepatocyte model was able to predict the in vivo hepatic biotransformation of S-equol. Based on these studies, the overall metabolic pathway for S-equol is shown in (Figure 9).

Glucuronide and sulfate conjugates of equol have been previously reported in man and animals after oral exposure to its precursor isoflavone daidzein (Axelson et al., 1984; Rüfer et al., 2006). Although these studies report the presence of equol conjugates, the specific structures and sites of conjugation were not identified. In the study by Gu et al. (2006), the metabolic profiles in female rats, cynomolgus monkeys, pigs and women were determined after oral exposure to a soy protein isolate containing daidzein. Pigs and the women in the study were not equol producers. Thus, no metabolites of equol were identified. Consistent with the present study, the major conjugate in rat serum and urine was glucuronide-equol with lower levels of the sulfate and unconjugated equol.

In the monkey, our results differ from those previously reported by Gu et al. (2006). They reported that unconjugated S-equol was the major metabolite in urine and in plasma it was sulfated equol in contrast to glucuronide and diconjugate in the present study. Differences in the design of the two studies should be noted. In the Gu et al. (2006) study, monkeys were fed a diet formulated with soy protein isolate that provided 3.7 mg daidzein per kg body weight; the animals were fed the diet for 5 weeks, and only occasional urine samples were analyzed. In contrast, the monkeys in the present study were given a single oral dose of [14C]-S-equol, with 0-12h urine collection. It is possible that long-term feeding of soy itself affects the metabolite profile for S-equol (via phase II enzyme regulated expression) and may explain the differences in the results, or that the metabolic pathway for S-equol produced from the biotransformation of daidzein may be different from that after dosing with pure S-equol.

In the present study, there were no phase I oxidative metabolites of S-equol identified in any in vivo or in vitro samples. In contrast, Rüfer et al. (2006) identified eleven mono- and di-hydroxy metabolites of racemic equol produced by hepatic microsomes from rats induced with Arochlor 1254, and six oxidative metabolites produced from human liver microsomes; the major
metabolite in the rat was 3'-hydroxy equol and in man 6-hydroxy equol. In these studies, phase I enzymes were activated with exogenous cofactors (NADPH-regenerating system) to produce oxidation. The present studies were designed to determine the metabolic profile of $^{14}$C-S-equol in more normal systems representative of the in vivo metabolic environment. The normal hepatocyte cultures that were used contained the full complement of hepatic drug-metabolizing enzymes (phase I and phase II) maintained within the intact cells. In these studies, high resolution mass spectroscopy did not detect any molecular ions indicative of monohydroxy ($m/z$ 259), dihydroxy ($m/z$ 275), or trihydroxy ($m/z$ 291) metabolites, or the presence of the oxidation product dehydroequol ($m/z$ 241). Also, these oxidative metabolites were not observed in in vivo studies. This consistency in results indicates that oxidation is not a significant metabolic pathway for S-equol in animals or in in vitro systems which retain full capacity for both phase I and phase II metabolic reactions. The data also suggest that cytochromes P450 and flavin-containing monooxygenase systems have little, if any, involvement in the metabolism of S-equol.

In these studies, it is important to note that samples chosen for metabolic profiling were those with highest levels of radioactivity, in order to maximize analytical sensitivity for detection of even minor metabolites. Thus, it is possible that the ratios of the three major conjugates may be different at other time points. Also, the present study did not address the sequence of conjugation related to the formation of the 7-sulfate-4'-glucuronide diconjugate (Figure 9). Whether the sulfate or glucuronide conjugate is formed first remains to be determined. It is also unknown what level of enterohepatic circulation of S-equol and conjugates is occurring, as conjugates are likely re-hydrolyzed and re-absorbed. Also of interest regarding the conjugates is what biological activity they may possess, and what relative binding affinities they may have for ERβ. Since hydroxyl groups on estrogen receptors are required for ligand binding (Minutolo et al., 2011; Hsieh et al., 2006), it is unlikely that conjugates of S-equol would have high binding affinity or biological activity. This is supported by Pugazhendhi et al. (2008) who showed that 7-sulfate-equol (as a racemic mixture) had only 50% receptor binding to ERα even at a 100,000 molar excess of equol. Thus, it is likely that the conjugated metabolites of S-equol have less biological activity compared to unconjugated compound.

The present studies have demonstrated the similarity in metabolic pathways for S-equol in the rat, monkey and man. On the bases of MS spectrum data, glucuronidation occurs at the 4' position, while sulfation occurs at the 7 position of the S-equol molecule, in each species. This similarity supports the use of the rat and cynomolgus monkey as suitable species for comparative pharmacology and toxicity testing of S-equol. The identification of the importance of several routes of conjugation, and apparent lack of a role of cytochrome P450, supports the expectation of a low potential for drug interaction for S-equol. The linear pharmacokinetics of S-equol over a wide dose range, observed in previous clinical studies (Jackson et al., 2011), is also evidence that this conjugation pathway is a high capacity process in man, and thus less subject to drug interaction in regard to metabolism. These results together support the use of the rat and monkey as suitable species for pharmacology and toxicology testing of S-equol, and further support the development of S-equol as a nutritional or oral drug product.
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References


Figure Legends

Figure 1: Biotransformation of daidzein to S-equol. Various bacteria have been shown to carry out this process.

Figure 2: Representative HPLC radiochromatograms of [14C]-S-equol and metabolites from A) rat plasma (at 1 h), B) rat urine (0-12 h collection) and C) rat feces (0-24 h collection).

Figure 3: Representative HPLC radiochromatograms of [14C]-S-equol and metabolites from A) monkey plasma (6 h sample) and B) monkey urine (0-12 h collection).

Figure 4: LC-MS/MS spectrum of the ion [M-H]− at m/z 241 (A), m/z 243 (B), and the proposed fragmentation scheme for S-equol.

Figure 5: LC-MS/MS spectrum of the ion [M-H]− at m/z 321 and the proposed fragmentation scheme for M1.

Figure 6: LC-MS/MS spectrum of the ion [M-H]− at m/z 417 and the proposed fragmentation scheme for M2.

Figure 7: LC-MS/MS spectrum of the ion [M-H]− at m/z 497 and the proposed fragmentation scheme for M3.

Figure 8: A) Representative radiochromatogram for an extract from human hepatocytes incubated with [14C]-S-equol, and B) Representative overlapped extracted ion chromatogram of a pooled sample for m/z 343, 419 and 499.

Figure 9: *In vitro and in vivo* metabolic pathways of [14C]-S-equol in rat, monkey and human. Asterisks indicate the position of the [14C]-label.
TABLE 1

Distribution of $[^{14}C]$-S-equol and metabolites in plasma, urine and feces after oral dosing$^1$

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Rat</th>
<th>Monkey</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Plasma (1 h)</td>
<td>Urine (0-12 h)</td>
</tr>
<tr>
<td>$[^{14}C]$-S-equol</td>
<td>4.2 (6.7)</td>
<td>12.0 (9.2)</td>
</tr>
<tr>
<td>Glucuronide conjugate (M2)</td>
<td>67.1 (17.8)</td>
<td>64.6 (8.5)</td>
</tr>
<tr>
<td>Sulfate conjugate (M1)</td>
<td>5.9 (6.5)</td>
<td>10.2 (2.9)</td>
</tr>
<tr>
<td>Sulfate-glucuronide diconjugate (M3)</td>
<td>10.9 (12.7)</td>
<td>3.5 (2.5)</td>
</tr>
</tbody>
</table>

$^1$The values represent the mean % distribution of total radioactivity in the selected, most-radioactive plasma, urine and feces samples from 3 animals; no other peaks or compounds were quantifiable. Total recovery was less than 100% per sample due to background noise. Values in parentheses are the standard deviation. Rat urine and feces samples contained on average 15.3% and 19.5% of the oral dose of radioactivity, respectively; for the monkey these values were 54.7% and 3.7%, respectively. ND=not detectable.
TABLE 2

Summary of $^{14}$C-S-equol and metabolites in rat and monkey$^1$

<table>
<thead>
<tr>
<th>Molecule</th>
<th>$m/z$ of [M-H]-</th>
<th>$m/z$ of characteristic product ions</th>
<th>RT (min) mass spectrometer</th>
<th>RT (min) Radiodetector</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{14}$C-S-equol</td>
<td>243</td>
<td>147, 135, 121, 93</td>
<td>10.3</td>
<td>10.0</td>
</tr>
<tr>
<td>Glucuronide conjugate (M2)</td>
<td>417</td>
<td>241, 175, 113</td>
<td>7.1</td>
<td>7.2</td>
</tr>
<tr>
<td>Sulfate conjugate (M1)</td>
<td>321</td>
<td>241, 135, 121, 93</td>
<td>9.8</td>
<td>9.7</td>
</tr>
<tr>
<td>Sulfate-glucuronide diconjugate (M3)</td>
<td>497</td>
<td>417, 321, 241</td>
<td>6.8</td>
<td>6.7</td>
</tr>
</tbody>
</table>

$^1m/z$ mass/charge ratio; RT retention time.
### TABLE 3

Distribution of major metabolites after *in vitro* incubation of hepatocytes with $[^{14}\text{C}]-\text{S-equol}$

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Rat</th>
<th>Monkey</th>
<th>Human</th>
</tr>
</thead>
<tbody>
<tr>
<td>$[^{14}\text{C}]-\text{S-equol}$</td>
<td>0.8</td>
<td>Trace</td>
<td>Trace</td>
</tr>
<tr>
<td>Glucuronide conjugate (M2)</td>
<td>82.0</td>
<td>95.2</td>
<td>73.3</td>
</tr>
<tr>
<td>Sulfate conjugate (M1)</td>
<td>2.7</td>
<td>1.1</td>
<td>21.8</td>
</tr>
<tr>
<td>Sulfate-glucuronide diconjugate (M3)</td>
<td>13.4</td>
<td>2.0</td>
<td>6.3</td>
</tr>
</tbody>
</table>

1 Values represent the average percent of total radioactivity in duplicate samples after incubation for 180 min.
Figure 1

Daidzein (a soy isoflavone) is converted by gut bacteria into S-equol.

Gut Bacteria

- Eggerthella
- Lactobacillus
- Lactococcus
- Slackia

(Others)
Figure 2

A

Retention time (min)

DPM

M2

B

Retention time (min)

DPM

M2

[14C]-S-equol

M1

C

Retention time (min)

DPM

[14C]-S-equol

M1
Figure 3
* Localization of the $^{14}$C-label
A

![Intensity Counts Graph]

**Figure 5**

B

*Localization of the $^{13}$C-label*
**Figure 6**

A

![Graph showing mass spectrometry data with m/z values and intensity counts.]

B

![Diagram showing molecular structures with labeled mass values.]

C_{15}H_{15}O_3
Exact Mass: 241.087

C_{11}H_{11}O_2
Exact Mass: 175.076

C_{7}H_{13}O
Exact Mass: 113.097

C_{21}H_{21}O_9
Exact Mass: 417.119

*C Localization of the $^{14}$C-label*
Figure 7

A

B

* Localization of the $^{14}$C-label
Figure 8

A

Retention time (min)

B

Retention time (min)
Figure 9

Sulfate Conjugate (M1) → S-equol

Glucuronide Conjugate (M2)

Sulfate-Glucuronide Diconjugate (M3)

Sulfate Conjugate (M1) S-equol

Glucuronide Conjugate (M2)

Sulfate-Glucuronide Diconjugate (M3)
Research Highlights

$^{14}$C-S-equol metabolites were studied in plasma, urine and feces of rats and monkey.

The 4’-gluconronide, the 7-sulfate, and the diconjugate were major metabolites.

Similar metabolites were seen in hepatocytes from the rat, monkey and man.