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Research Article

Absorption, Distribution and Excretion of [14C]-S-equol in the Rat and Cynomolgus Monkey

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ABSTRACT

Introduction: S-equol is a potent, selective estrogen receptor β agonist that is currently in development for various nutritional and therapeutic uses. S-equol is produced in the gut by the microbial reduction of daidzein, a chemical found in soy protein. Previously, we reported the similarities in drug metabolites in the rat, monkey and man. The present study investigated the absorption, tissue distribution and routes of excretion of orally-dosed [14C]-S-equol in the rat and cynomolgus monkey to support their use in toxicology studies.

Materials and Methods: Radiolabeled [14C]-S-equol was administered as a single oral dose to male Sprague-Dawley rats (2 mg/kg) and male cynomologus monkeys (1 mg/kg). Total collections of urine, feces, cage rinse, and expired air were obtained in continuous 12 and 24 h intervals through 96 h after dosing. Plasma and tissue samples were collected at various timepoints to determine the pharmacokinetics of radiolabel in plasma and clearance of all drug-related materials from tissues.

Results: [14C]-S-equol was rapidly absorbed with peak plasma concentrations occurring between 0.5 and 1 h in both animal species. S-equol (ng-eq./g) was rapidly cleared from the circulation with radioactivity below the limits of quantitation by 48 h. Terminal half-lives of [14C]-S-equol in the rat and monkey were 10.7 h and 4.3 h, respectively. For the rat, urine and feces were the major routes of excretion (total 73.5%) and for the monkey urine accounted for most of the administered dose (62.2%). Tissue levels of radioactivity in both species were negligible at 96 h.

Discussion: The present studies demonstrate that [14C]-S-equol is rapidly absorbed and excreted in urine and feces after a single oral dose in both the rat and monkey. Excretion of radioactivity from tissues in both species was rapid and essentially complete by 96 h. These data, along with the similarity in metabolite profiles in rat, monkey and man, support the use of the rat and monkey as appropriate species for toxicology studies for the development of S-equol for various uses in man.

Key words: Absorption, Distribution, Excretion, [14C]-S-equol.

INTRODUCTION

Historically, hormone therapy (HT) has been the major treatment option for vasomotor symptoms (VMS) in menopausal women. However, the Women's Health Initiative (WHI) showed that after 5 years of treatment with conjugated equine estrogens plus the progestin, medroxyprogesterone acetate, there was increased risk of invasive breast cancer, cardiovascular disease, stroke and venous thromboembolic events [1]. As a result, women throughout the world are reluctant to take estrogens for their menopausal symptoms, and they have resorted to other therapies, including non-hormonal approaches [2].

One potential non-hormonal alternative to HT is S-equol, an isoflavone produced by the gut biotransformation of daidzein, a component of soy [3]. This diet-related exposure to S-equol has been shown to be associated with health benefits in not only women, but in men. The potential health benefits of soy and their relationship to an individual's ability to convert daidzein to S-equol have been reviewed [4-6]. Direct evidence that S-equol is effective in the treatment of VMS has been provided by Ishiwata et al. [7]; women receiving a dietary supplement with the equivalent of 10 mg S-equol three-times a day showed a reduction of menopausal symptoms.

Preclinical and clinical studies have been conducted as part of a development program of S-equol for nutritional and therapeutic uses in man [8-11]. These studies included the use of the rat as the rodent species, and the cynomolgus monkey as the non-rodent species in support of the required safety evaluation for small molecules [12]. In addition, we have characterized the metabolic profiles of

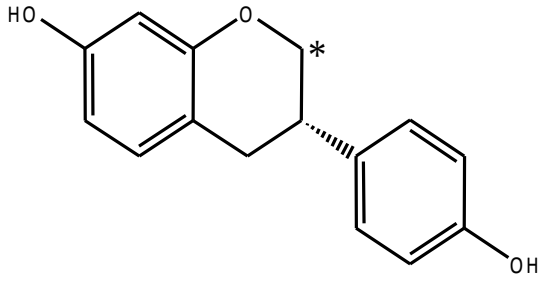

Fig 1: Chemical structure of S-equol. The asterisk shows the position of the ^{14}C radiolabel, located at the 2 position in the molecule.

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S-equol in the rat and cynomolgus monkey, and demonstrated their similarity to that in human hepatocytes [10]. These studies showed that the 4'-glucuronide conjugate, the 7-sulfate conjugate, and the 7-sulfate-4'-glucuronide diconjugate were the major metabolites in plasma and excretia of the rat and monkey, and in hepatocyte extracts of rat, monkey and man [10]. The present studies were undertaken to further characterize the pharmacokinetics of absorption, tissue distribution and excretion of S-equol and metabolites in the rat and cynomolgus monkey using [14C]-S-equol to facilitate quantitative tracking of the compound and all related metabolites. The results provide the toxicological rationale for the use of the rat and monkey as appropriate animal species for the risk assessment of S-equol exposure in man.

METHODS

Materials: [2-14C]-S-equol [S-3, 4–dihydro-3-(4-hydroxy-phenyl)–2H-1 benzo- pyran–7-ol] with radiochemical, chemical and chiral purity of 99.8%, 98.8% and 98.3%, respectively, and S-equol (purity 99.1%) were synthesized by Girindus Solvay America, Inc. (Cincinnati,OH, USA). The radiolabeled compound was synthesized with the 14C at the 2-position to allow tracking of the parent compound and metabolites (Figure 1). All other reagents were purchased from commercial sources.

Studies in rats and monkeys: All animal studies were reviewed and approved by the Institutional Animal Care and Use Committee of Charles Rivers Laboratories, Inc., and included review and approval of radiochemical use. Male Sprague–Dawley rats (225-300 g) were placed on an alfalfa- and soy- free diet (2016-C Tekland global 16% protein rodent diet; Harlan, Fredrick MA, USA) for two weeks prior to the study to minimize diet-related exposure to phytoestrogens. Animals were housed individually in polycarbonate cages (animals for plasma sampling), or glass metabolism cages (animals for collection of urine, feces and expired air). The temperature was 18–26°C, relative humidity was 30–70%, and the light cycle was 12-hour light/12-hour dark. Fresh filtered tap water and food were provided ad libitum, and all other husbandry conditions were maintained as described in the Guide for the Care and Use of Laboratory Animals (National Research Council, Washington D.C., National Academy Press, 1996). Groups of three rats per time point received a single oral dose of 2 mg/kg [14C]-S-equol (150 µCi/kg, dose volume 5 mL/kg) as a suspension in 0.5% methylcellulose/0.1% Tween 80. Dose solutions for both rats and monkeys were prepared on the day of use, with concentrations of S-equol and radiolabel confirmed by subsequent analyses [10]. The dose was selected based upon an FDA guideline and use of allometric scaling to provide the human equivalent dose of 20 mg of S-equol in a 60 kg person [13, 14]. Total collections of urine, feces, cage rinse, and expired air were obtained in continuous 12 and 24 h intervals through 96 h after dosing. After each collection of urine and feces, the remaining cage residue ("cage rinse") was collected by rinsing with 50:50 (v/v) alcohol:water, with the last rinse including gauze pads for wiping the cage. Expired air was collected using glass metabolism cages and 6 M potassium hydroxide to trap the expired carbon dioxide. Blood samples were collected at the indicated timepoints in animals by intracardiac puncture following anesthesia; plasma was obtained by centrifugation at 2000 x g for 10 min at a temperature between 2-9°C. Following euthanasia by carbon dioxide asphyxiation and terminal blood collection, tissues from rats were collected, rinsed, blotted dry, and weighed. Gastrointestinal contents for each animal were placed into separate containers and weighed.

The cynomolgus monkey was selected as the non-rodent species for this program since the primate's reproductive system is more similar to that in man [15, 16]. Male non-naïve cynomolgus monkeys (3-4 kg) were kept on a special diet (PMI Nutrition International, Certified Primate Diet 5C8V; Quality Lab Product Elkridge, MA, USA) which did not include soy or alfalfa products for the 2 weeks prior to dosing and throughout study procedures. The diet was supplemented daily with small bits of bananas, carrots or yams. Tap water was available ad libitum to each animal from drinking bottles. Animals were housed individually in stainless steel metabolism cages. Animals were maintained with ventilation greater than 10x 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 D.C., National Academy Press, 1996). Groups of three monkeys received a single oral dose of 1 mg/kg [14C]-S-equol (50 µCi/kg, dose volume 5 mL/kg) based upon the allometric scaling equivalent of a 20 mg/60 kg human dose [13, 14]. A lower specific activity was used compared to the rat studies due to the larger size of the cynomolgus monkey. 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 as noted above for rats; expired air was not collected for the monkey since the experiments in rats showed this was not a route of excretion of the radiolabel. Blood samples were collected at the indicated timepoints by direct venipuncture of a saphenous or femoral vein, and plasma was obtained by centrifugation at 2000 x g for 10 min at a temperature between 2-9°C. At the designated time points for euthanasia, the animals were initially immobilized with ketamine HCl (10 mg/kg, IM). Nembutal® (10-30 mg/kg, IV) was administered before the euthanizing dose of sodium pentobarbital. Euthanasia was performed by deep anesthesia (IV sodium pentobarbital) followed by exsanguination, consistent with accepted guidelines.

Analyses of samples for [14C]-S-equol derived radioactivity: 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

Table 1: Pharmacokinetic parameters after a single oral dose of [^{14}C]-S-equol.

Parameter (Units)	Rat (2 mg/kg)	Monkey (1 mg/kg)
C_{max} (ng-eq./g)	1050	2550 ± 401
T_{max} (h)	1.0	0.5 ± 0
$AUC_{0-\infty}$ (h·ng-eq./g)	5950	6060 ± 1537
$T_{1/2}$ (h)	10.7	4.3 ± 0.8

The values represent the mean ±SD (n=3 animals/group). Values for the rat are based upon pharmacokinetic analysis of a single curve (n=3 rats per time point, see Methods section for details).

Table 2: Levels of [^{14}C]-S-equol in tissues of the rat. Mean concentration ± SD of S-equol and metabolites (ng-eq./g) in male rat tissues after a single oral dose (n=3).

Tissue	1 h	12 h	96 h
Bone (Femur)	48.4 ± 30.9	0	0
Bone Marrow	55.9 ± 29.3	5.60 ± 9.70	0
Brain	14.6 ± 3.02	1.94 ± 0.321	0.736 ± 0.670
Epididymis	122 ± 48.4	15.7 ± 3.31	0
Esophagus	2270 ± 676	30.3 ± 10.4	0
Eyes	62.5 ± 21.8	10.9 ± 1.68	0
Fat	58.6 ± 17.5	16.6 ± 16.0	0
Heart	217 ± 85.9	17.4 ± 4.51	0
Cecum	208 ± 48.0	7600 ± 751	22.0 ± 22.6
Colon	582 ± 292	2130 ± 700	9.18 ± 8.48
Duodenum	9780 ± 4590	1560 ± 419	0.79 ± 1.37
Illeum	965 ± 619	4150 ± 2460	25.8 ± 28.4
Jejunum	12000 ± 8380	2620 ± 577	12.7 ± 8.53
Rectum	620 ± 554	477 ± 315	1.66 ± 1.98
Kidneys	1320 ± 607	177 ± 60.4	19.5 ± 14.1
Liver	2600 ± 1180	299 ± 23.4	5.35 ± 3.15
Lungs	364 ± 85.0	26.4 ± 8.02	0
Pancreas	309 ± 76.9	90.3 ± 42.6	0
Pituitary	315 ± 136	90.9 ± 104	0
Plasma	1050 ± 394	102 ± 23.0	0
Prostate	234 ± 173	65.5 ± 46.3	0
Salivary Gland	142 ± 40.0	26.5 ± 16.2	0.615 ± 0.538
Seminal Vesicles	72.1 ± 21.7	23.6 ± 12.8	0.313 ± 0.542
Skeletal Muscle	103 ± 47.8	7.83 ± 6.79	0
Skin	220 ± 84.3	26.5 ± 7.8	0
Spleen	78 ± 23.3	28.1 ± 4.94	0
Stomach	20100 ± 12000	44.1 ± 6.99	0.690 ± 1.20
Testes (Tunic)	78.6 ± 39.4	14.1 ± 5.22	0
Thymus	88.8 ± 41.2	8.64 ± 2.15	0
Urinary Bladder	1150 ± 227	227 ± 109	0

Table 3: Percent recovery of S-equol and metabolites in tissues of rats.

	Time after dosing					
	1 h	4 h	12 h	24 h	48 h	96 h
Tissues	77.67 ± 7.60	86.19 ± 6.19	33.11 ± 5.74	21.54 ± 2.06	2.08 ± 0.91	0.17 ± 0.14
Carcass	5.24 ± 1.55	2.06 ± 0.22	0.73 ± 0.08	0.42 ± 0.13	0.09 ± 0.05	0.32 ± 0.37
Total	82.91	88.25	33.84	21.96	2.17	0.49

Male rats received a single oral dose of [^{14}C]-S-equol (2 mg/kg). The values represent mean % ±SD of the administered dose of radioactivity in 3 animals. Values for intestinal contents are included as part of tissues value.

analyzed for total radioactivity. All samples were stored at −20°C until analyzed. Pharmacokinetic parameters were determined as described previously [8]. In rats, eyes, pituitary, ileum, esophagus, and urinary bladder were analyzed in toto, whereas duplicate representative samples of fat, bone, skeletal muscle, and skin were taken for analysis. A single sample of bone marrow was taken from the femur. Samples were combusted and then analyzed by liquid scintillation counting (LSC). The remaining carcass was solubilized in potassium hydroxide (2 M) in methanol and analyzed directly by LSC. Samples were combusted using a Model 307 Sample Oxidizer (PerkinElmer, Inc., Boston, MA, USA), with the 14CO2 trapped in Carbo-Sorb E (PerkinElmer, Inc.) and mixed with Permafluor E+

Table 4: Levels of [^{14}C]-S-equol in tissues of the monkey. Mean concentration ± SD of S-equol and metabolites (ng-eq./g) in male monkey tissues after a single oral dose (n=3).

Tissue	6 h	12 h	96 h
Bone	6.30 ± 10.9	0	0
Bone Marrow	51.4 ± 59.1	0	0
Brain	2.23 ± 3.86	0	0
Epididymis	203 ± 53.7	0	0
Esophagus	153 ±7.0	123 ± 83.0	0
Eyes	48.8 ± 29.6	0	0
Fat	196 ± 214	0	0
Heart	90.6 ± 53.3	12.6 ± 15.6	0
Cecum	5910 ± 3010	768 ± 117	7.57 ± 13.1
Colon	740 ± 749	452 ± 40.3	4.95 ± 4.33
Duodenum	485 ± 109	261 ± 271	0
Illeum	3740 ± 3850	323 ± 103	0
Jejunum	606 ± 252	206 ± 93.6	3.43 ± 5.95
Rectum	137 ± 14.6	22.4 ± 38.9	0
Kidneys	1670 ± 1300	237 ± 111	5.17 ± 8.95
Liver	328 ± 120	72.0 ± 23.3	0
Lungs	163 ± 74.0	32.7 ± 14.3	0
Pancreas	104 ± 17.2	64.1 ± 34.8	0
Pituitary	90.0 ± 156	0	0
Plasma	419 ± 255	0	0
Prostate	455 ± 340	152 ± 131	0
Salivary Gland	119 ± 47.8	0	0
Seminal Vesicles	1100 ± 1060	228 ± 353	0
Skeletal Muscle	17.7 ± 30.7	0	0
Skin	53.8 ± 3.74	0	0
Spleen	55.8 ± 39.5	0	0
Stomach	229 ± 183	39.8 ± 30.4	0
Testes (Tunic)	108 ± 77.9	0	0
Thymus	58.5 ± 13.1	0	0
Urinary Bladder	3500 ± 2840	239 ± 73.9	0

(PerkinElmer, Inc.) scintillation fluid prior to analysis by LSC using a Model LS 6500 liquid scintillation counter (Beckman Instruments, Inc., Fullerton, CA, USA), as described previously [10]. Tissues with radioactivity less than twice background were assigned values of zero. For monkeys, duplicate representative samples of fat, bone, bone marrow, skeletal muscle, and skin were taken, homogenized, combusted, and then analyzed by LSC, as was done for the rat samples. Intestinal contents from both species were collected at euthanasia, analyzed by combustion, with radiolabel values included as part of total recovery calculations. Duplicates of all other tissues were solubilized in potassium hydroxide (2 M) in methanol prior to direct analysis by LSC. Values for total recovery in tissues were determined based upon the measured concentration in tissue and the organ weight, or estimates of tissue as a percent of total body weight.

RESULTS

Absorption and pharmacokinetics of [14C]-S-equol in rats and monkeys: Rats receiving a single oral dose of 2 mg/kg of [14C]-S-equol had a plasma concentration of 1050 ng-eq./g (Cmax) at 1 h indicating rapid absorption (Figure 2A). Plasma concentrations of radiolabel then declined and were below the detection limit of 5.1 ng-eq./g. by 48 h. Pharmacokinetic parameter estimates for S-equol equivalents in plasma are shown in Table 1. The estimated terminal half-life (T1/2) was 10.7 h and the estimated area under the curve (AUCo.∞) postdose was 5950 h•ng-eq./g. Male monkeys given a single oral dose of [14C]-S-equol (1 mg/kg) had a mean maximum concentration of S-equol equivalents of 2550 ng-eq./g (Cmax) at 0.5 h (tmax) postdose, also indicating rapid absorption (Figure 2B). Plasma levels of radioactivity then declined in an apparent multi-phasic manner, and were below the detection limit of 7.4 ng-eq./g by 48 h. The estimated terminal half-life in monkeys was 4.3 h and the estimated area under the curve (AUCo.∞) postdose was 6060 h•ng-eq./g. (Table 1).

Excretion of [14C]-S-equol and metabolites: The mean cumulative excretion of [14C]-S-equol derived radioactivity in urine, feces, expired air traps, and cage residue for rats is shown in Figure 3A. In the rat, greater than 70% of the radioactivity was excreted in urine and feces, with most excreted in the first 24 h. The negligible excretion of radiolabel in expired air suggested that the ring structure of S-equol was not degraded, consistent with our previous report [10]. The total recovery of the oral dose of radioactivity through 96 h was 31.3% in urine,

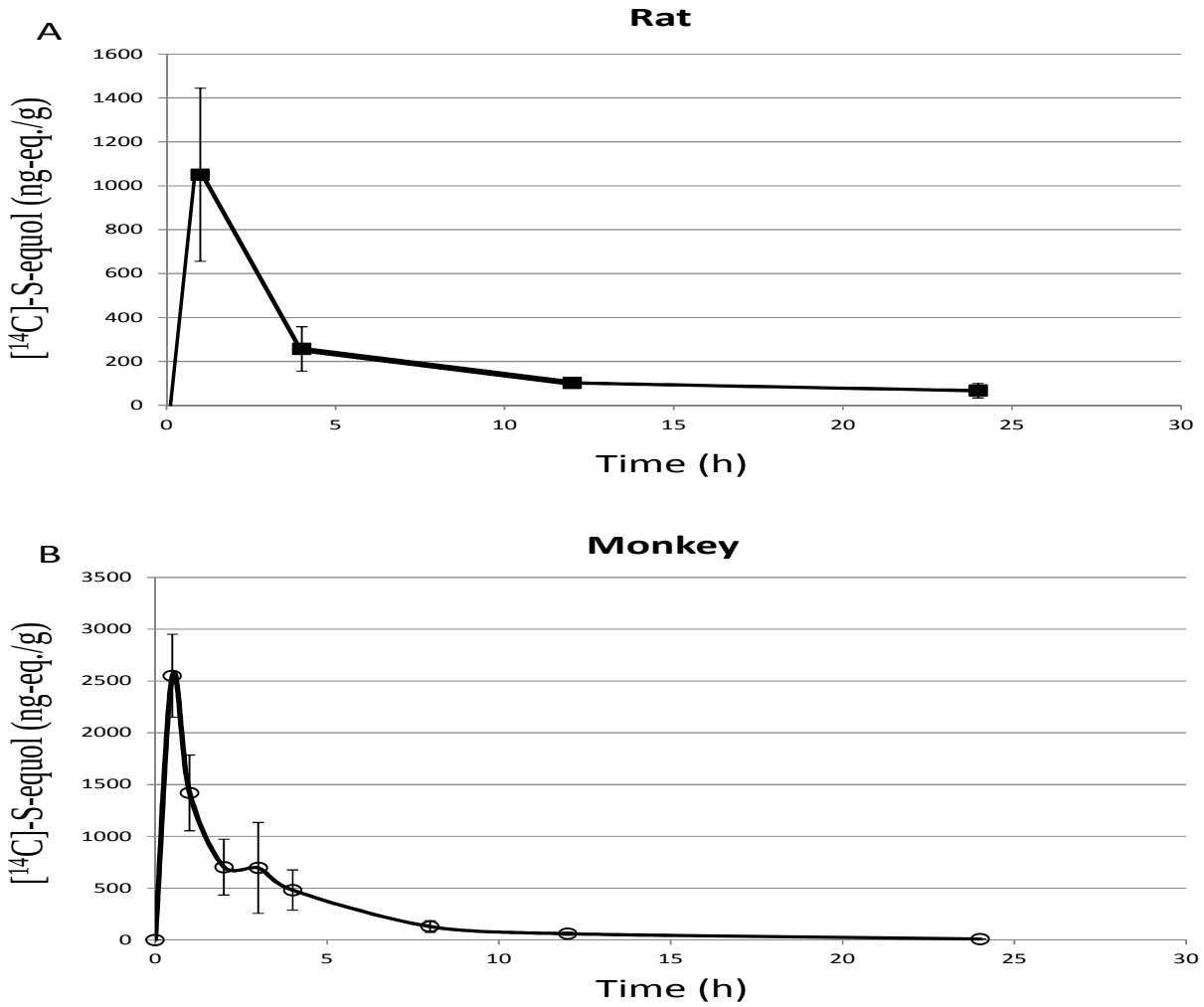

Fig 2: Pharmacokinetics of plasma [^{14}C]-S-equol. Values represent levels of S-equol (plus metabolites) after a single oral dose of [^{14}C]-S-equol in the rat (2 mg/kg) (A) and monkey (1 mg/kg) (B). The values represent the mean ± SD of 3 animals each. The limit or quantitation was 5.1 ng-eq./g for the rat study, and 7.4 ng-eq./g for the monkey study. Values for both studies were below quantitation limits by 48h.

42.2% in feces, 0.04% in expired air traps, 8.2% in cage rinse, and 0.49% in tissues. Elimination of radioactivity appeared to be rapid, and the mass balance at 96 h postdose showed that 81.8% of the oral dose was excreted by this timepoint. It is possible that incomplete collection of cage rinse, which contains a high percent of dose, may have contributed to the lower than expected total recovery.

In the monkey, [14C]-S-equol-derived radioactivity was excreted rapidly, primarily in urine (Figure 3B). A combined total of 63.5% of the administered dose of radioactivity was recovered in urine, feces, and cage rinse at 12 h. Elimination in urine appeared to be rapid, as a majority of the administered radioactivity (54.7%) was recovered in urine within the first 12 h of dosing. Elimination of S-equol-related radioactivity in feces was lower, with only 3.71% recovered in feces within 24 h and 5.74% within 48 h after dosing. Consequently, it is likely that most of the radioactivity recovered in cage debris samples from 0-12 h postdosing (8.83%) was related to urinary excretion, which would bring the total recovery in urine to >70%. Mean total recoveries of radioactivity in monkeys through 96 h were 62.2% in urine, 6.1% in feces, 16.2% in cage residues, and 0.10% in tissues. The mass balance at 96 h in the monkey was 84.6% of the orally administered dose. As with the rat study, incomplete collection of cage rinse may have contributed to theobserved total recovery.

Tissue Distribution and clearance of [14C]-S-equol in rats and monkeys: The distribution of [14C]-S-equol derived radioactivity in tissues of the rat is shown in Table 2. The tissues having the highest maximal mean concentrations at 1 h were those of the gastrointestinal tract (ileum, stomach, jejunum, and duodenum), likely related to the route of administration, with highest levels in the stomach (20,100 ng-eq./g). The lowest amount of radioactivity was found in the brain (14.6 ng-eq./g). For the three non-intestinal tract tissues, liver, kidneys, and urinary bladder, the amount of radioactivity was greater than the plasma Cmax of 1050 ng-eq./g. The mean concentration of radioactivity for non- intestinal tissues declined with time after 4 h postdosing

(data not shown) and by 12 h the values were < 30% of the 1 h time point. By 96 h postdose, the only tissues with remaining radioactivity were those associated with the GI

Table 5: Percent recovery of S-equol and metabolites in tissues of monkeys.

	Time after dosing		
	6 h	12 h	96 h
Tissues	22.01 ± 7.11	8.17 ± 4.82	0.10 ± 0.06

Male monkeys received a single oral dose of [^{14}C]-S-equol (1 mg/kg). The values represent mean % ± SD of the administered dose of radioactivity in 3 animals and include values for intestinal contents.

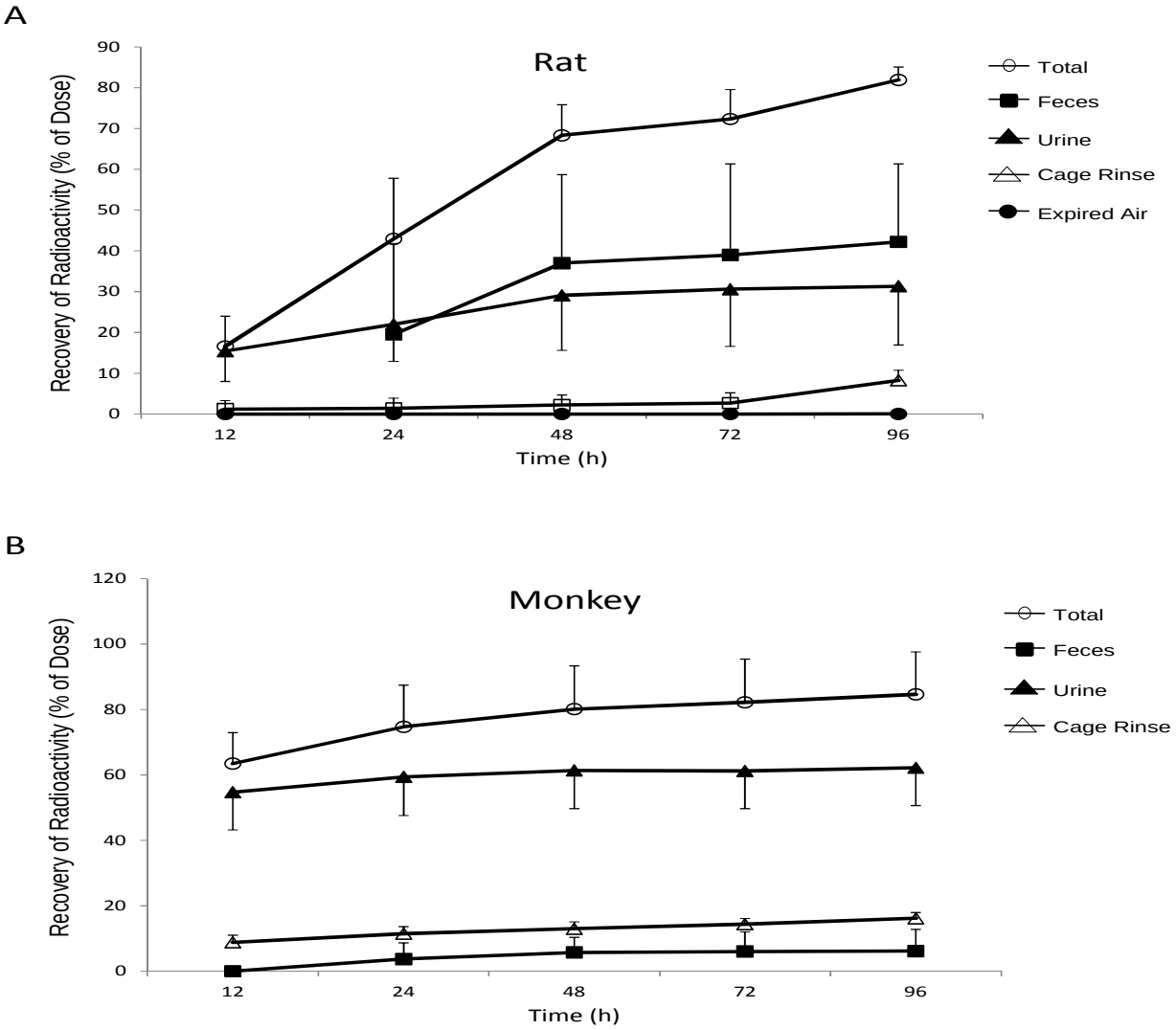

Fig 3: Excretion of [^{14}C]-S-equol. Values represent levels of [^{14}C]-S-equol (plus metabolites) after a single oral dose of [^{14}C]-S-equol in the rat (2 mg/kg) (A) and monkey (1 mg/kg) (B). The values represent the percent of the administered dose and are the mean ±SD of 3 animals.

tract, plus the brain, liver, kidney, salivary gland and seminal vesicle. The total recovery of radioactivity in tissues and carcass also declined with time after 4 h and was < 1% (0.49%) of the oral dose at 96 h (Table 3), indicating a rapid rate of clearance and almost complete elimination of radioactivity from tissues.

The distribution of radioactivity in tissues of the monkey is shown in Table 4. The tissues with the highest mean concentrations at 6 h postdosing were those involved with excretion (cecum, ileum, and urinary bladder; 5910, 3740, and 3500 ng-eq./g, respectively), followed by kidneys and seminal vesicles (1670 and 1100 ng-eq./g, respectively). At 6 h, the concentrations in colon, jejunum, duodenum and prostate were greater than the plasma concentration of 419 ng-eq./g. The lowest concentrations were observed in brain and bone (2.23 and 6.30 ng-eq./g, respectively). Mean concentrations of radioactivity at 12 h postdosing were lower than at 6 h in all tissues. The highest concentrations at 12 h were for cecum, colon, and ileum (768, 452, and 323 ng-eq./g, respectively). Radioactivity was below the quantitation limit (twice background) in many tissues at 12 h postdose. By 96 h, concentrations were quantifiable only in cecum, colon, kidneys, and jejunum. The total percent of the oral dose of radioactivity recovered in tissues declined with time after dosing and in the monkey was only 0.10% at 96 h (Table 5).

DISCUSSION

The present studies were conducted to characterize the absorption, distribution and excretion of S-equol plus

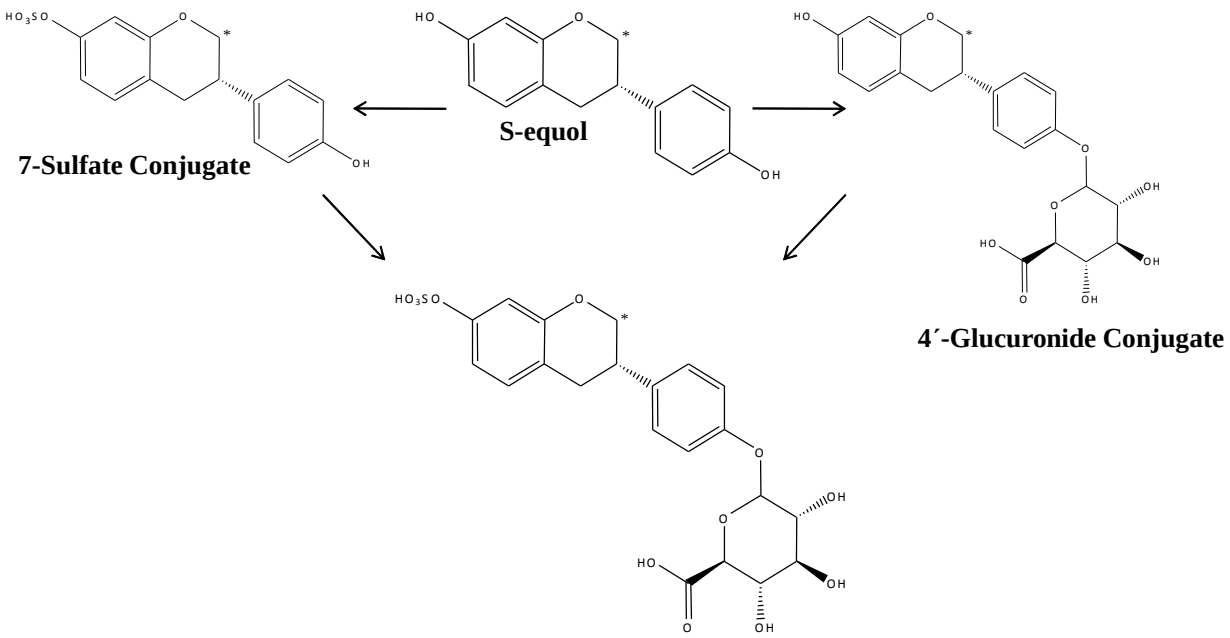

Fig 4: Metabolic pathway of S-equol, based upon metabolite identification studies in rat, monkey and man [10]. The asterisk indicates the position of the ^{14}C radiolabel.

metabolites in the rat and cynomolgus monkey in order to justify their use in a safety program for S-equol. The use of a custom synthesized [14C]-S-equol, dosed at pharmacologic levels, allowed for a comprehensive assessment of exposure and clearance of drug-related material from tissues and the whole body. The findings show that S-equol and related metabolites are rapidly absorbed and cleared from the body and tissues of both species.

We previously reported that in rats and monkeys, the vast majority of the radiolabel in plasma, urine and feces consisted of the 4'-glucuronide conjugate, the 7-sulfate conjugate, and the 7-sulfate-4'-glucuronide diconjugate [10]. These studies allowed for the metabolic pathway in rats and monkeys to be defined [Figure 4]. We also showed that similar S-equol metabolite profiles were produced in hepatocytes from the rat, monkey and man, which further validated the use of the rat and monkey as appropriate species for use in toxicology studies of S-equol. Separate repeat-dose studies in rats and monkeys (once daily doses, for 28 days) using higher oral doses of S-equol also confirmed that conjugates were the major drug-related material in plasma, with unconjugated S-equol accounting for less than 1% of the total drug-related material [9].

In the present studies, the pharmacokinetics of [14C]-S-equol-related radioactivity in plasma showed a mean T1/2 of 10.7 h in the rat and 4.3 h in the monkey; in man the mean value for total equol (S-equol plus conjugates) for a single dose of 20 mg was 9.6 h in the fasted state [8]. The observed half-life of plasma radioactivity in the present study indicates that S-equol and conjugated metabolites are not expected to show any signs of accumulation beyond steady state, with the anticipated twice-daily dosing regimen for use in man. The total exposure to [14C]-S-equol-related radioactivity in the rat and the monkey were very similar (5950 and 6060 h•ng-eq./ml). In man the AUCo.∞ value was 3442 h•ng/ml after a single oral dose of 20 mg [8]. These results confirm the suitability of the use of allometric scaling to select appropriate doses for the rat and monkey studies.

With regard to tissue distribution of 14C-related material, both the rat and monkey showed highest tissue levels in the GI and the organs related to excretion (kidneys, liver, bladder). Radioactivity decreased relatively quickly with time from all tissues, and there appeared to be no tissues that showed a prolonged retention of radioactivity. Prolonged retention or high accumulation in tissues after repeat dose would be a signal for potential toxicity in that tissue. While repeat-dose tissue distribution and/or quantitative whole body autoradiography studies would be required to confirm this, the observed half-life of radioactivity in plasma and the rapid wash-out from tissues in the present studies suggests that twice-daily dose would not lead to high accumulation or toxicity in tissues. Repeat dose toxicity studies in the rat and monkey showed plasma pharmacokinetics similar to the present study [9] and did not show any toxicity in the uterus, which is a potential target organ for the class of estrogens. Repeat twice-daily oral doses of S-equol in man demonstrated achievement of steady state in plasma after 1-2 days of dosing, with no signs of toxicity after 14 days of dosing [8].

Excretion of [14C]-S-equol-related radioactivity was relatively rapid in both the rat and monkey with > 70% of the administered dose eliminated after 48 h. In the monkey the route of excretion was mainly urine whereas in the rat it was equally excreted in the feces and urine. Our prior demonstration that the primary drug-related material in the feces were conjugates of S-equol suggests the potential for biliary excretion and enterohepatic circulation of S-equol

conjugates, which is consistent with the demonstrated capacity of hepatocytes from rat, monkey and man to produce these conjugates [10].

The present studies in the rat also collected radioactivity in expired air, in the event that the S-equol molecule (14C in the 2 position of the ring) was oxidatively degraded to the point of generation of [14C] O2. The lack of radiolabel in these samples suggests that the S-equol is not extensively degraded in the rat *in vivo*. These results are consistent with our prior metabolite identification studies, which did not identify any ring oxidation metabolites of S-equol in extracts of plasma or excretia of rats and monkeys, or in incubations of hepatocytes from rats, monkeys and man [10]. Lack of an oxidation pathway in the *in vivo* disposition of S-equol is also consistent with our previous demonstration that S-equol is not a substrate for the oxidative P450 cytochromes 1A2, 2C9, 2C19, 2D6, or 3A4/5 [9].

Our previous report described the pharmacokinetics of S-equol in a single dose Phase 1 study and a 14-day repeat dose Phase 1 study using twice-daily dosing in normal volunteers [8]. S-equol was rapidly absorbed with peak plasma concentrations occurring between 1.5 to 3 h after a single dose. Cmax and area under the curve (AUCo.∞) increased proportionally with doses from 10 to 320 mg/day. Metabolism was extensive, with S-equol conjugates accounting for over 99% of the drug-related material in plasma after an oral dose. The half-life of total S-equol (S-equol plus conjugates) was 9.6 h, and is similar to that observed for total radioactivity in the present rat and monkey study. This similarity in metabolism and clearance of S-equol related material in rat, monkey and man further supports use of the rat and monkey in future toxicology studies of S-equol.

The therapeutic dose of S-equol for the treatment of vasomotor symptoms in postmenopausal women is predicted to be 10-40 mg per day. This projection is based, in part on a study in women given EffisoyTM containing 43 mg of daidzein [17]. The women in this study who were equol producers and demonstrated a reduction of vasomotor symptoms (hot flashes) had fasting plasma levels of S-equol of 13.1 ng/ml; a dose of 10 mg given twice a day would achieve this plasma level of S-equol [8]. In another study [7] in which S-equol was given as a food supplement, a positive outcome for menopausal symptoms in Japanese women was observed when 10 mg of S-equol was given three times a day, consistent with the above predictions. Based on this information, allometric scaling determined that for a 60 kg human subject and a daily dose of 20 mg, the pharmacologically-equivalent dose in the rat and monkey used in the present studies would be 2 mg/kg and 1 mg/kg, respectively.

S-equol is a potent estrogen receptor β (ERβ) agonist with a Ki of 0.73 nM [3] or 0.18 ng/ml. Thus, even after 12 h of dosing, the concentration of S-equol in most tissues of the rat and monkey would be sufficient to saturate ERβ binding. One proposed use of S-equol is for treatment of vasomotor symptoms (hot flashes) in post-menopausal women [8]. The hypothalamus controls body temperature with ERα and ERβ being the targets of interest. In preclinical studies, orally dosed S-equol has been shown to decrease the tail temperature in an ovariectomized rat model [18] and in an opiate-dependent rat model [19]. At 1 h after dosing in the rat, the concentration of [14C]-S-equol equivalents in the brain was 14.9 ng-eq/g; in the monkey at 6 h the levels were 2.33 ng-eq/g, both sufficient concentrations for binding to ERβ. Although it is not known whether the [14C]-S-equol in the brain is unconjugated S-equol or a metabolite, it is more likely unconjugated S-equol since it is less polar and thus more able to cross membranes.

CONCLUSIONS

The present studies show that rats and monkeys are similar in regard to the rapid excretion of [14C]-S-equol in urine and feces, and rapid elimination from tissues. We have previously published data demonstrating the similarity in metabolites of S-equol in plasma, urine and feces for the rat and monkey, and in hepatocytes from the rat, monkey and man. The general similarities in the plasma pharmacokinetics and metabolism of S-equol in the rat, cynomolgus monkey and man suggest that the rat and monkey are appropriate species for pharmacology and toxicology studies related to the development of S-equol for various nutritional and therapeutic uses in man. The relatively rapid excretion and clearance of [14C]-S-equol related material from tissues of the rat and monkey also support further development of orally-dosed S-equol for various uses in man.

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