Intestinal Uptake and Biliary Excretion of the Isoflavone Genistein in Rats\textsuperscript{1,2,3}

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ABSTRACT The intestinal absorption, biliary excretion and metabolism of genistein, a potent and specific protein tyrosine kinase inhibitor that occurs naturally in soy foods, was examined in anesthetized, adult female rats fitted with indwelling biliary canulas. \textsuperscript{4}\textsuperscript{14}C-Genistein, when infused into the duodenum, was rapidly absorbed from the intestine, taken up by the liver and excreted into the bile as its 7-O-\textbeta-glucuronide conjugate. Cumulative recovery of \textsuperscript{14}C-radioactivity in the bile over a 4-h period was 70\textendash75% of the dose. When genistein was infused into the portal vein, it was also taken up efficiently by the liver, conjugated with glucuronic acid and transported into bile. However, portal blood collected after duodenal infusions of genistein contained mostly genistein 7-O-\textbeta-glucuronide, suggesting that in vivo glucuronidation occurred in the intestinal wall rather than the liver. This was confirmed using everted intestinal sac preparations. Reinfusion of genistein 7-O-\textbeta-glucuronide into the duodenum or into the mid small intestine resulted in its reappearance in the bile, albeit more slowly than when genistein was infused. Over a 4-h collection period, the cumulative recovery of \textsuperscript{14}C-radioactivity in bile was 27 and 70\textendash75% of the administered dose for duodenal and ileal infusions, respectively. These data indicate that genistein is highly bioavailable in rats and because of its enterohepatic circulation may accumulate within the gastrointestinal tract.


KEY WORDS: • rats • biliary isoflavones • isoflavone uptake • portal blood • glucuronides

The soy isoflavone genistein (4',5,7-trihydroxyisoflavone) has recently emerged as an important dietary component associated with many health-related and clinical benefits (see March 1995 supplement to this journal). Much of this development occurred after the discovery that genistein is a potent and specific inhibitor of protein tyrosine kinases (Akiyama et al. 1987). As such, it is considered to be an important modulator of many mitogen-stimulated signal transduction events (Peterson and Barnes 1996).

Consumption of soy protein is associated with a reduction in the risk of several cancers (Messina et al. 1994) and causes a reduction in serum hypercholesterolemia in animals (Anthony et al. 1996, Carroll 1991) and in humans (Bakhit et al. 1994, Potter et al. 1993, Sitori et al. 1977). Evidence that the beneficial effects of soy are due to the isoflavones has been obtained from experiments in which the isoflavones were removed from the soy protein by alcohol extraction: the extracted protein no longer had its effect (Anthony et al. 1996, Barnes et al. 1994). As further evidence that this class of compounds has important biological effects, a synthetic isoflavone, ipriflavone (7-isopropyloxyisoflavone), has been successfully used in the treatment of postmenopausal (Agrusede et al. 1992) and senile osteoporosis (Passeri et al. 1992). Genistein administered in the diet also prevents bone loss in ovariec

Most soy products contain quite large amounts (1\textendash3 mg/g) of genistein and daidzein (4',7-dihydroxyisoflavone) (Coward et al. 1993). Extensive recent literature reports have demonstrated that genistein, and to a lesser extent daidzein, inhibits proliferative growth of many transformed and nontransformed cell lines in tissue culture experiments (Barnes and Peterson 1995, Peterson 1995, Peterson and Barnes 1996). Both soy (Barnes et al. 1990 and 1994, Hawrylewicz et al. 1991, Trott et al. 1980) and genistein (Helms and Gallaher 1995, Lamartiniere et al. 1995, Murrill et al. 1996, Pereira et al. 1994, Wei et al. 1995) have been shown to be effective in treating leukemia in a nude mouse in a model of pre-B cell human leukemia.

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\bibitem{Anthony et al. 1996} The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 USC section 1734 solely to indicate this fact.
\bibitem{Carroll 1991} To whom correspondence should be addressed.
\end{thebibliography}
et al. 1995) cause chemoprevention effects in vivo using animal models of cancer. These data have given support to the hypothesis that isoflavones (particularly genistein) are important contributors to the anticancer effect of soy (Adlercreutz et al. 1991, Barnes et al. 1990, Setchell et al. 1984).

 Crucial issues for determining how much genistein, either in soy foods or as a supplement, is required for its effects are those which govern the efficacy of other xenobiotics, i.e., absorption, distribution, metabolism and excretion (Barnes et al. 1996). Renal excretion of genistein after its ingestion has been used to evaluate its bioavailability (Xu et al. 1994). On this basis, because 3–10% of the dose appears in the urine, genistein was claimed to be poorly bioavailable. However, this approach does not take into account genistein absorbed from the intestine and then secreted in the bile in a conjugated form (sulfate ester or glucuronide), which might not be reabsorbed but excreted eventually in the feces either as genistein or its metabolites. Therefore, renal excretion can be regarded only as an apparent measure of genistein’s absorption from the gastrointestinal tract. The pharmacokinetics of genistein in mice when administered by oral, intramuscular and intravenous routes revealed that genistein is rapidly eliminated from the blood compartment, with an apparent systemic availability of 12% (Supko and Malspies 1995). Because of a prominent secondary peak in the plasma unconjugated genistein concentration 78 min after a bolus intravenous dose of genistein, these investigators suggested that genistein may undergo enterohepatic cycling.

 In a preliminary study conducted in this laboratory in a bile duct–cannulated rat model (Armstrong, H. and Barnes, S., unpublished observations), 40–50% of a dose of genistein administered in the stomach appeared in bile over a 4-h period, consistent with genistein undergoing an enterohepatic circulation. However, our technologies at that time did not allow us to be certain of the identity of the chemical forms of genistein in bile, or from which part of the intestine genistein is absorbed. In the present study using the bile duct–cannulated rat model, we have administered 4-14C-genistein to determine the proportion of the dose recovered in the bile, the role of the sites of absorption and their effect on the first pass metabolism of genistein.

 MATERIALS AND METHODS

 Materials. Genistin, the β-glucoside of genistein, was isolated from soy molasses, an aqueous alcohol extract of soy flour (Walter 1941). Genistin was prepared from genistin by hydrolysis in methanol/HCl (Walter 1941). These isoflavones, after recrystallization, were 98% pure (by reversed-phase HPLC), and had melting points, molecular weights (determined by atmospheric pressure chemical ionization mass spectrometry) and proton nuclear magnetic resonance (NMR)1 spectra consistent with genistin and genistein, as previously described (Coward et al. 1993 and 1996). Genistein 4-sulfate was synthesized chemically by a carbodiimide coupling procedure (Coward et al. 1996).

 4-14C-Genistin was custom synthesized (Moravek Biochemicals, Brea, CA) and had a radiochemical purity of ≥98% by HPLC and a specific radioactivity of 58.7 GBq/mmol. β-Glucuronidase and sulfatase were purchased from Sigma Chemical, St. Louis, MO. Acetonitrile and trifluoroacetic acid were sequencing grades and were obtained from Fisher Chemical, Norcross, GA, and Pierce Chemical, Milwaukee, WI, respectively. All other chemicals were of the highest grades obtainable. Sep-Pak C18 cartridges were purchased from Waters (Milford, MA).

 Animals. Female Sprague-Dawley rats (225–275 g) were bred in the Department of Nutrition Sciences Animal Care Facility, University of Alabama at Birmingham. Both fathers and dams (purchased from Harlan Sprague Dawley, Indianapolis, IN) were fed an isoflavone- and soy-free diet (AIN-76A, Harland Teklad, Madison, WI) before impregnation of the dams. The pregnant dams were maintained on this diet throughout pregnancy and weaning. The pups were also fed this diet after weaning and throughout the rest of the experiment. Another group of female adult rats, used in preliminary experiments, were fed a nonpurified Teklad 4% diet. The animal experimental procedures were approved by the Institutional Animal Care and Use Committee at the University of Alabama at Birmingham.

 Rats in these experiments were anesthetized with ketamine/ xylocaine (0.1 mL/100 g body weight); their body temperature was maintained at 38°C (monitored rectally) by placing them on a heating pad. After a midline incision, the bile duct was exposed and cannulated with PE-10 tubing, tied and secured with 5-0 silk (Ethicon, Somerville, NJ).

 In Experiment 1, a section of the duodenum (70 cm), proximal to the ligament of Trietz, was used to determine the rate of absorption from the small intestine. Each end of the intestinal segment was tied off around a piece of PE-50 polyethylene tubing. The perfusate consisted of 10 mmol/L sodium taurocholate, 10 mmol/L glucose, 154 mmol/L NaCl, and 10 mmol/L KCl. Variable amounts of genistein were added to this basic perfusate.

 After the initial surgery, the intestinal segment was infused with physiological saline for 20 min, followed by two 20-min periods with the basic perfusate, each at a flow rate of 70 μL/min. Test perfusates contained 4-14C-genistein (3.7 MBq/L) and 94, 188 or 376 μmol/L unlabeled genistein. The three genistein-containing perfusates were administered to each of four rats in random order from animal to animal. The infusates were warmed to 37°C and were administered for 1 h, followed by the basic perfusate for 20 min. Biles were collected over 20-min intervals throughout the experiment, as were perfusates that had passed through the intestinal segment. In a separate group of rats, portal blood (2 mL) was obtained after 1-h duodenal infusion of 14C-genistein.

 In Experiment 2, to more accurately assess the total intestinal recovery, 0.37 MBq (630 nmol) of 4-14C-genistein was infused (70 μL/min) over a 1-h period into the duodenum of a bile duct–cannulated rat; then, the basic perfusate was infused for the remainder of the experimental period (4 h). Unlike in the first experiment, the perfusates were allowed to proceed down the intestine without collection. Biles were collected over 20-min intervals throughout the experiment, as were perfusates that had passed through the intestinal segment. In a separate group of rats, portal blood (2 mL) was obtained after a 1-h duodenal infusion of 14C-genistein.

 In Experiment 3, to determine whether the biliary genistein metabolites also underwent an enterohepatic circulation, the biles collected over the first 60–100 min from Experiment 2 in which 14C-genistein was infused into the duodenum were pooled and diluted using the basic perfusate. This mixture was transferred into the duodenum or the proximal ileum for 60 min, followed by infusion of the basic perfusate for a further 4 h. Biles were collected through a biliary cannula at 20-min intervals throughout the experiment. The biles were removed and divided as described in Experiment 2.

 The hepatic uptake and biliary excretion of genistein were examined by infusion of 14C-genistein into the portal vein in the bile duct–cannulated rat. The dose of genistein was varied from 0.77 to 8.8 μmol/min; genistein was dissolved in freshly prepared rat serum which was infused at 4.5 μL/min for 1 h. Biles were collected through a biliary cannula at 5-min intervals throughout the experiment. Peripheral blood (2–3 mL) was collected by cardiac puncture at the end of the experiment.

 Intestinal uptake of genistein was also examined using everted intestinal sac preparations. Sections (~10 cm) representing the proximal, mid and distal small intestine were rapidly removed, flushed with physiological saline and everted on a glass rod. The everted intestinal segments were flushed with Tyrode solution (136 mmol/L NaCl, 2.7 mmol/L KCl, 1.4 mmol/L CaCl2, 1.0 mmol/L MgCl2, 12.0 mmol/L NaHCO3, 0.4 mmol/L NaH2PO4, and 16 mmol/L L-glutamic acid, pH 7.1), and the ends of the intestinal segments were tied off with

 Abbreviations used: FSAL, 2-fluoro-β-alanine; HPLC-MS, HPLC mass spectrometry; Km, infusion rate causing half maximal transport; NMR, nuclear magnetic resonance; Tmax, maximum predicted biliary excretion rate.

 1 NMR, nuclear magnetic resonance; KINF, infusion rate causing half maximal transport; NMR, nuclear magnetic resonance; Tm, maximum predicted biliary excretion rate.
5-O silk after filling the segments with Tyrode solution containing 27 μmol/L genistein. The everted intestinal segments were then placed in a 500-ml Erlenmeyer flask in Tyrode solution containing 27 μmol/L genistein and 3.7 MBq 14C-genistein/L. The incubation solution was perfused with 95% O2-5% CO2 throughout the experimental period. Incubations were conducted at 37°C for 3 h. Fluids inside the intestinal segments (the serosal side) were then removed and stored at -70°C until analyzed.

**Analytical methods.** 14C-labeled genistean was extracted from intestinal loops and biles by the method of SFAKIANOS ET AL. (1992) with some modifications. For the purest material, the partially purified material was treated with chorylglycine hydrolase to convert taurocholate to cholic acid and tauro, which were then removed by rechromatography on the Sephadex LH-20 column. The fractions containing genistein glucuronide were finally purified by preparative reversed-phase HPLC.

**HPLC-mass spectrometry.** Extracted samples of bile or urine were separated by reversed-phase HPLC on a 15 cm × 0.21 cm i.d. Brownlee Aquapore C8 column using a linear 0–50% gradient (5%/min) of acetonitrile in 10 mmol/L ammonium acetate at a flow rate of 0.2 mL/min. The column eluate was split 1:1, and one stream passed into the IonSpray interface of a PE-Sciex (Concord, ON, Canada) API III triple quadrupole mass spectrometer operating in the negative ion mode, with an orifice potential of -60 V. In the MS-MS mode, daughter ion spectra were obtained by selecting parent ions in the first quadrupole, which were then collided with argon/10% nitrogen gas in the second quadrupole and analyzed in the third quadrupole. The operation of the mass spectrometer and analysis of data were conducted using two MacIntosh Quadra 950 computers interfaced with an Ethernet link.

**RESULTS**

Preliminary experiments were conducted on rats that had been fed a nonpurified diet (Teklad 4% mouse/rat diet) until they were adults. Three days before the experiment, they were switched to isoflavone-freeAIN-76A diet. Nonetheless, bile obtained before the administration of genistein contained a large peak detected by reversed-phase HPLC at 262 nm. Treatment with β-glucuronidase/sulfatase resulted in its eluting much more slowly (and with the same retention time as genistein), strongly suggesting that it was a genistein conjugate. This dietary source of genistein made it difficult to determine the rate of excretion of the administered genistein. Therefore, all subsequent experiments were conducted with animals not previously exposed to genistein at any time during gestation, the neonatal period, or after weaning. This result was the first to suggest that genistein is not rapidly cleared from the body. When 4-14C-genistein was infused into the intestinal loop in the duodenum of an otherwise genistein-free, bile duct-cannulated rat, 14C-radioactivity appeared in the bile within 20 min, reaching equilibrium within 1 h (Fig. 1). The mass of genistein metabolites excreted into bile increased with the infusion rate of genistein into the intestinal loop. Compared with the rate of infusion of genistein into the intestinal loop, biliary output of genistein metabolites was 9.2 ± 1.1% for genistein infusion at 62 nmol/h, and declined to 7.7 ± 1.6% for infusion at 124 nmol/h and 6.7 ± 1.2% for infusion at 247 nmol/h. Genistein had no effect on the bile flow rate (data not shown).

When 14C-genistein was infused into the duodenum for 60 min and allowed to proceed down the small intestine (without collection as in the intestinal loop experiment), a concordant,
large peak of radioactivity was observed in bile (Fig. 2A). Radioactivity continued to be secreted into bile in decreasing amounts for the remaining 4 h of the experiment; a total of 70–75% of the infused dose was recovered via the biliary route (Fig. 2B). When these biles were pooled and analyzed by reversed-phase HPLC, a single peak of radioactivity eluting at 11.5 min was observed. Direct injection of unextracted bile onto the HPLC column led to a similar profile, indicating that the Sep-Pak C₁₈ cartridge recovered all of the hydrophilic genistein metabolites. In addition, there was full recovery of the radioactivity injected onto the HPLC column.

Treatment of the extracted bile with beta-glucuronidase led to a shift in the elution of the major peak of radioactivity to 21 min, coincident with genistein (data not shown). On the other hand, treatment with sulfatase did not alter the chromatographic mobility of the peak (data not shown).

When the biliary extract was analyzed by HPLC-MS in the negative ion mode, several peaks were detected for ions with a m/z value of 445 (the expected [M-H]⁻ ion for a glucuronide conjugate of genistein). One of these peaks had a mass spectrum which had an M+2 isotope excess due to the ¹⁴C label. Daughter ion spectra of each of the m/z 445 ions (during HPLC analysis) revealed that only this peak gave rise to the expected m/z 269 ion.

Proton NMR spectroscopy of genistein and the genistein glucuronide isolated from rat bile showed that the C₁₀ (9.63 δ) and C₇ (12.95 δ) hydroxyl proton resonances were observed for the metabolite as for genistein, but the C₆ hydroxyl proton resonance was absent. In addition, the chemical shifts for the C₇, C₈ proton resonances were unchanged (6.88 δ vs. 6.82 δ), whereas the chemical shifts for the C₁₀ (6.47 δ) and C₉ (6.73 δ) proton resonances were 0.25–0.3 δ downfield from those observed for genistein. As noted previously, these data are consistent with genistein 7-O-β-glucuronide as the biliary metabolite (Coward et al. 1996).

¹⁴C-Radioactivity analyzed by HPLC from portal blood collected after a 1-h duodenal infusion of genistein was predominantly genistein 7-O-β-glucuronide, rather than genistein (Fig. 3). This was independent of the dose rates of genistein administered in this study (data not shown).

**Uptake of ¹⁴C-genistein 7-O-β-glucuronide from the small intestine.** When the pooled bile from Experiment 2 was reinfused into the duodenum of rats for 1 h, ¹⁴C-radioactivity began to appear in bile immediately; however, when biliary radioactivity was plotted as a function of time, instead of there being a peak (as in Experiment 2), radioactivity continued to increase throughout the remainder of the 4-h collection period (data not shown). HPLC analysis of biliary radioactivity again showed that it gave rise to a single peak eluting at 11.5 min, i.e., genistein 7-O-β-glucuronide (data not shown).

Extraction of the luminal contents of the various parts of the intestines revealed that the radioactivity had reached two thirds of the way down the small intestine over the 4-h infusion period (Fig. 4A). HPLC analysis revealed that the small intestinal fractions contained a single peak of radioactivity eluting at 21 min, i.e., genistein (Fig. 4B). Radioactivity was also contained in the intestinal wall in the same fractions as it was found in the luminal contents (data not shown). When the pooled bile was infused more distally, into the mid small intestine, radioactivity appeared rapidly in bile, reaching a peak within 80 min and declining thereafter (Fig. 5A). Cumulative recovery of biliary radioactivity was 72% over the 4-h collection period (Fig. 5B). Again, the principal biliary metabolite

**FIGURE 1** Biliary excretion of ¹⁴C-radioactivity during administration of 4-¹⁴C-genistein over a 3-h period into a 10-cm section of the duodenum of anesthetized rats. The biliary excretion rate is expressed as the percentage of the duodenal infusion rate. Data are the means ± SEM for each 20-min collection period from bile samples obtained from three rats.

**FIGURE 2** A substantial proportion of duodenally administered 4-¹⁴C-genistein is excreted in the bile of anesthetized rats. The period of duodenal infusion of the 4-¹⁴C-genistein dose (0.37 MBq) is marked by the black horizontal bar. Genistein-free perfusate was then infused for the next 3 h. A) Biliary excretion of ¹⁴C-radioactivity in each 20-min period is shown. The data points are biliary radioactivity as the percentage of the duodenal infusion rate. B) Cumulative recovery of the dose in bile (as a percentage of the total dose) is given. These data are the means ± SEM of results obtained in 3 rats.
Hepatic uptake and biliary excretion of $^{14}$C-genistein. When $^{14}$C-genistein was infused into the portal vein, $^{14}$C-radioactivity rapidly appeared in bile (Fig. 6). At the lowest dose (0.77 nmol/min), the rate of biliary excretion of $^{14}$C-radioactivity approached that of the rate of infusion of $^{14}$C-radioactivity. However, at higher dose rates, evidence of saturation of hepatobiliary transport was observed (Fig. 7). The estimated $T_{max}$ was 10.2 nmol/min, and the half-maximal infusion rate was 11.7 nmol/min.

HPLC analysis of $^{14}$C-radioactivity in peripheral blood collected at the end of the 1-h infusion period revealed that, at the lower rates of portal vein infusion of $^{14}$C-genistein, only genistein 7-O-β-glucuronide was present in the peripheral circulation (Fig. 8A). At the highest infusion rate studied (8.8 nmol/min), ~half of the $^{14}$C-radioactivity in peripheral blood was genistein (Fig. 8B).

Uptake of $^{14}$C-genistein by everted intestinal sacs. Over a 3-h period, $^{14}$C-radioactivity accumulated inside the everted sac preparation. The concentration of $^{14}$C-radioactivity (per mL) was three to four times higher than that in the bathing medium. This concentrative effect was observed in each of the small intestinal segments so long as they were removed from the animal one at a time and the everted sac immediately prepared.

HPLC analysis of the $^{14}$C-radioactivity contained in the inside of the everted sac revealed that it was a mixture of genistein and genistein 7-O-β-glucuronide (Fig. 9). The proportion of genistein 7-O-β-glucuronide increased in relation to the size of the gradient across the everted sac. The concentration of genistein inside the everted sac was the same as in the perfusing buffer.

Urinary excretion of $^{14}$C-radioactivity. Radioactive metabolites were excreted in the urine in each of the experimental designs used for small intestinal infusions. However, the fraction of the dose appearing in the urine was significantly higher over the 4-h time period after infusion of genistein 7-O-β-glucuronide (7.4 ± 1.7%, mean ± SEM) than for genistein (2.4 ± 0.5%, mean ± SEM).

DISCUSSION

The data from the present study demonstrate directly for the first time that, at least in rats, the isoflavone genistein undergoes an efficient enterohepatic circulation. Genistein and its principal metabolite, genistein 7-O-β-glucuronide, are not only well absorbed from the intestines, but are efficiently extracted from the portal blood into the liver and excreted into bile. Biliary excretion and enterohepatic circulation have been previously described for many flavonoids (Hackett 1986), although the extent to which genistein and its biliary metabolite are absorbed is substantially greater than those previously reported. The possibility of enterohepatic cycling of dietary phytoestrogens was originally proposed by Setchell et al. (1982) and of genistein by Supko and Malspeis (1995).

Data obtained using perfusion of a short intestinal segment in the duodenum suggested that the initial absorption of genistein 7-O-β-glucuronide (data not shown).

FIGURE 3 The $^{14}$C-radioactivity in portal blood collected after infusion of $^{14}$C-genistein into the duodenum of an anesthetized rat for 1 h is genistein 7-O-β-glucuronide. Reversed-phase HPLC of portal blood extracts was conducted on a 25 cm × 0.46 cm i.d. C8 reversed-phase column. Radioactivity was eluted with a mobile phase consisting of a linear gradient of 0–50% acetonitrile in 10 mmol/L aqueous trifluoroacetic acid (see Materials and Methods). The peak eluting at 13 min was sensitive to treatment with β-glucuronidase and was shown by 1H NMR to be genistein 7-O-β-glucuronide.

FIGURE 4 $^{14}$C-Radioactivity in rat intestinal lumen after intraduodenal administration (into small intestinal segment #1) of the $^{14}$C-labeled genistein biliary metabolite is mostly unconjugated genistein. After intraduodenal administration of the $^{14}$C-labeled genistein biliary metabolite for 1 h, followed by infusate with genistein-free infusate for a further 3 h, the intestines were removed and divided into segments for determination of the residual radioactivity. A) Distribution of radioactivity in the intestines expressed as a percentage of the infused dose per segment. B) Reversed-phase HPLC analysis (see legend to Fig. 3) of $^{14}$C-radioactivity in fraction #3. The peak eluting at 13 min was genistein 7-O-β-glucuronide (GENGlca) and that eluting at 20 min was genistein (GEN).
UPTAKE AND EXCRETION OF GENISTEIN IN RATS

FIGURE 6 Increasing biliary excretion of 14C-radioactivity in rats with an indwelling biliary catheter after portal vein infusion of various doses of 14C-labeled genistein. Data are the mean ± SEM values from 3 rats at each infusion rate. Bile samples were collected over 5-min intervals.

Genistein was excreted in bile as a 7-O-β-glucuronide conjugate. This was demonstrated in two ways: first, by its sensitivity to hydrolysis to genistein by β-glucuronidase, but not to sulfatase or to solvolysis; and second, by HPLC-electrospray ionization mass spectrometry which revealed that the molecular weight of the biliary metabolite was 446, consistent with the addition of a single β-glucuronide group (by the 176 increase in the molecular weight compared with genistein). There was no evidence that genistein was converted to its sulfate or sulfate/glucuronide conjugates as was recently reported for daidzein in rats (Yasuda et al. 1994). It should be noted that even in a simple physiological fluid such as bile, the detection of the [M-H]− ion of genistein 7-O-β-glucuronide (m/z 445) by HPLC-MS was interfered with by other substances giving rise to 445 m/z ions. It was essential to conduct HPLC-MS-MS to identify the peak that was due to genistein.

FIGURE 5 The genistein biliary metabolite is reabsorbed more slowly, but just as completely as unconjugated genistein, from the small intestine in rats. Biliary excretion of 14C-radioactivity over a 4-h period from rats with an indwelling biliary catheter after reinfusion into the mid small intestine for 1 h of the 14C-labeled genistein biliary metabolite was investigated. Data are the mean ± SEM values for each 20-min collection period from 4 rats. A) Biliary excretion of 14C-radioactivity in each 20-min period is shown. The data points are biliary radioactivity as the percentage of the total dose infused. B) The cumulative recovery of the dose in bile (as a percentage of the total dose) is given.

FIGURE 7 Kinetic analysis of biliary excretion and portal blood infusion rate of genistein in rats. Replot of the reciprocals of the biliary 14C-radioactivity output rates vs. the reciprocals of the portal vein infusion rates in rats fitted with indwelling biliary catheters (see data in Fig. 6).
the β-glucuronide and sulfate esters of genistein accounted for 95% of the genistein in peripheral blood. However, in rats fed higher oral doses [148 μmol/(kg body wt·d)], peripheral blood and urine of rats contain a substantial proportion of unconjugated genistein (Sfakianos, J., Coward, L., Kirk, M. and Barnes, S., unpublished observations), a reflection of saturation of Phase II conjugation.

Intestinal conjugation of genistein was confirmed in experiments using everted intestinal sacs, establishing a lumenal-to-serosal concentration gradient of ¹⁴C-radioactivity as a result of the glucuronidation of genistein during transit through the intestinal wall. The observed concentration gradient of genistein 7-O-β-glucuronide to the serosal side is due to its much slower rate of back diffusion (serosal side to luminal side) than genistein. Genistein is therefore taken up by the liver mostly as its 7-O-β-glucuronide rather than as genistein.

When the genistein 7-O-β-glucuronide metabolite was reinfused into the duodenum of rats, radioactivity quickly appeared in bile, with 27% of the infused dose reexcreted over a 4-h study period. It was apparent, however, in this model, that intestinal absorption and biliary recovery were underestimated because, after duodenal infusion, the radioactivity had

![FIGURE 8](https://example.com/figure8.png)

The composition of genistein and its glucuronide metabolite in peripheral blood of rats depends on the infusion rate of genistein into portal blood. Composition of ¹⁴C-radioactivity in peripheral blood collected after infusion of ¹⁴C-genistein into the portal vein of anesthetized rats (3 rats at each infusion rate) for 1 h was determined by reversed-phase HPLC (as described in Fig. 3). A) A single peak was observed of genistein 7-O-glucuronide (GENGlcA) when the infusion rate of genistein was 0.77 nmol/min. B) Peaks corresponding to both genistein (GEN) and its glucuronide (GENGlcA) were observed when the infusion rate of genistein was 8.82 nmol/min.

![FIGURE 9](https://example.com/figure9.png)

Genistein is converted to its glucuronide during passage through the rat small intestinal wall. Composition of ¹⁴C-radioactivity from the serosal side of closed, everted, rat intestinal loops (from each of 2 rats) after incubation (on the luminal side) with ¹⁴C-labeled genistein was determined by reversed-phase HPLC (as described in Fig. 3). Each radiochromatogram [(A) duodenum, (B) jejunum] shows the presence of peaks corresponding to both genistein (GEN) and its glucuronide (GENGlcA).
reached only the mid small intestine. In subsequent experiments, to better assess intestinal reabsorption, the metabolite was reintroduced into the mid small intestine. In this mode, intestinal absorption and reexcretion of the genistein metabolite into bile was extensive and reached 72% over a 4-h period. The slowed intestinal recovery can be explained by the larger concentrations of intestinal bacteria in the more distal parts of the small intestine that cause hydrolysis of genistein 7-O-β-glucuronide to genistein (and hence allow passive absorption of genistein to occur). This was observed in these experiments. The slower rate of excretion in bile, compared with infusion of genistein, is therefore a function of the extent of hydrolysis of the glucuronide within the intestine. No evidence was obtained of a specific transport system for genistein 7-O-β-glucuronide in the distal small intestine as has been described for transport of another class of organic anions secreted in bile (the bile acids), which is localized in this region of the small intestine (Lack and Weiner 1961).

The consequences of an efficient enterohepatic circulation of genistein and its metabolites are as follows: 1) genistein may accumulate within the enterohepatic circuit, and 2) it may be excreted with a long half-life. This slow rate of excretion may have been missed by investigators who are overwhelmed by the rapid excretion observed in the first 24 h after administration of genistein. In this respect, genistein may behave like the anticancer drug 5-fluorouracil; after initial rapid urinary excretion of its metabolite, 2-fluoro-β-alanine (FBAL), this drug exhibits an extended half-life as a result of the conjugation of FBAL to bile acids (Sweeney et al. 1987), a consequence of the very efficient enterohepatic circulation of bile acid N-acetyl amidates (Zhang et al. 1991).

The observation that humans fed purified isoflavones have a lower urinary output of genistein relative to daidzein has been interpreted to mean that genistein has a lower bioavailability than daidzein (Xu et al. 1994). However, the present data indicate that genistein is absorbed from the intestines very well and is excreted into bile with only a small proportion appearing in urine. Because daidzein is converted to sulfate and sulfate/glucuronide conjugates in rats (Yasuda et al. 1994), it is likely to be eliminated more rapidly in the urine than genistein. The difference in the rates of elimination of genistein and daidzein would also explain why rats, which consumed the soy-containing nonpurified diets and were food-deprived overnight or consumed a soy-free diet for up to 3 d, had large amounts of genistein 7-O-β-glucuronide in their bile, but no measurable daidzein or its metabolites. Investigators should note that most animal diets contain soy, and hence genistein, but in unpredictable amounts.

An important factor that may alter the initial intestinal absorption and the enterohepatic recycling of genistein is bacterial metabolism. Xu et al. (1995) reported that two patients who had a renal excretion of 32–37% of the genistein dose (three times higher than five other subjects studied) also excreted large amounts of genistein in their feces.

Studies are required to examine the effect of glycoside conjugation of genistein on its rate of absorption from soy foods although it has been anticipated (Barnes et al. 1996) that the absorption will be efficient, if somewhat delayed, as was observed for the β-glucuronide of genistein in the present study. In a recently reported study, it was shown that after administration of genistein in rats, the plasma concentration of genistein reached a peak 2 h later; in contrast, when genistein glycoside conjugates (recovered from soy flour by ethanol extraction) were administered, the peak plasma concentration occurred after 8 h (King et al. 1996).

Although genistein, like many therapeutic drugs used in the treatment of cancer, could be used in the pill form for delivery as a chemopreventive agent, its delivery in soy foods would be far more economical. Such a food delivery mechanism is used by Southeast Asians who have the lowest breast and prostate cancer rates (Park et al. 1996) and lowest cardiovascular disease risk among nations of the world.

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LITERATURE CITED


