Kinetics of Genistein and Its Conjugated Metabolites in Pregnant Sprague-Dawley Rats Following Single and Repeated Genistein Administration

Nicole V. Soucy,1 Horace D. Parkinson, Mark A. Sochaski, and Susan J. Borghoff

CIIT Centers for Health Research, 6 Davis Drive, Research Triangle Park, North Carolina 27709

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Diets high in soy-based products are well known for their estrogenic activity. Genistein, the predominant phytoestrogen present in soy, is known to interact with estrogen receptors (ER) α and β and elicits reproductive effects in developing rodents. In the rat, genistein is metabolized predominantly to glucuronide and sulfate conjugates, neither of which is capable of activating ER. Therefore, it is critical to understand the delivery of free and conjugated genistein across the placenta to the fetus following maternal genistein exposure such that the potential fetal exposure to free genistein can be assessed. Genistein (4 or 40 mg/kg) was administered to pregnant Sprague-Dawley rats by oral gavage daily from gestation day (GD) 5 through 19 or on GD 19 alone. Maternal and GD 19 fetal tissues were collected 0.5, 1, 2, 4, 6, 8, 12, and 24 h following administration of the final dose on GD 19. Concentrations of genistein, genistein glucuronide, and genistein sulfate were quantitated by LC-MS/MS. In maternal plasma, genistein glucuronide was the predominant metabolite. In the fetal plasma, genistein glucuronide and genistein sulfate were the primary metabolites. Genistein levels in maternal and fetal plasma were much lower than its conjugates. The concentration of genistein in placental tissue was higher than either conjugate. Fetal concentrations of unconjugated genistein following administration of 40 mg/kg were above the EC50 for ERB activation. Repeated administration of 40 mg/kg genistein resulted in minor changes in genistein kinetics in the pregnant rat compared to single administration of the same dose. These data suggest that conjugated forms of genistein are not transported across the placenta. High placental concentrations of genistein indicate the placenta is a potential target organ for genistein action during gestation.

Key Words: genistein; genistein glucuronide; genistein sulfate; pharmacokinetics; placenta; gestation.

Dietary phytoestrogens are primarily present in legumes and grains. Soybean products contain very high concentrations of phytoestrogens, and these plant-derived constituents have been shown to display estrogen-like activities (Setchell, 1998; You, 2004). Like endogenous estrogens, the estrogenic effects of phytoestrogens are mediated through activation of estrogen receptors (ER). A diet rich in phytoestrogens has been associated with lower incidences of prostate and breast cancer, particularly for people in South East Asia, where large amounts of soy products are consumed (Dai et al., 2003; Strom et al., 1999).

Genistein, the predominant phytoestrogen found in soy-based products, is an isoflavone sharing similar chemical structural features with 17β-estradiol, particularly the phenolic ring and the distance between the 4’ and 7-hydroxy groups (Fig. 1) (Dixon and Ferriera, 2002). As such, genistein is an agonist for both ERα and ERβ. When compared with estradiol, genistein has a low affinity to ERα and comparable affinity to ERβ (Casanova et al., 1999; Kuiper et al., 1998; Mueller et al., 2004). Additional biological activity attributed to genistein occurs through inhibition of tyrosine kinases and DNA topoisomerase (Akiyama et al., 1987).

In Asian populations consuming diets high in soy-based products, the estimated genistein exposure is approximately 0.4–0.6 mg/kg/day (Barnes et al., 1995, 1996; Fukutake et al., 1996; Yamamoto et al., 2001). While the typical Western diet contains far lower levels of genistein, followers of strict vegetarian diets would be expected to have intake levels approaching that of the Asian diet. Infants fed soy-based formulas have particularly high phytoestrogen exposure levels approaching 4.5–8 mg/kg/day, of which ~65% is genistein, predominantly present in the glycosidic form (Setchell et al., 1998).

Proposed beneficial effects of genistein exposure include cancer prevention and decreased incidence of osteoporosis and cardiovascular disease (Albertazzi, 2002; Bhathena et al., 2002; Lamartiniere et al., 2002). However, at some critical life stages genistein exposure may be detrimental. There is specific concern that the differentiating reproductive organs of embryos, fetuses, and newborns may be particularly sensitive to hormonally active chemicals (Tuohy, 2003). This concern is partially based on the detrimental effects associated with other estrogenic compounds such as diethylstilbestrol (DES).

1 To whom correspondence should be addressed at CIIT Centers for Health Research, P.O. Box 12137, Research Triangle Park, NC 27709. E-mail: nsoucy@ciit.org.
In laboratory rodents, genistein has been reported to have multiple effects attributed to endocrine modulation. Genistein exposure during the period spanning gestation and weaning results in decreased anogenital distance (AGD) and other structural changes in male and female rodents (Fielden et al., 2003; Levy et al., 1995; Wisniewski et al., 2003), including increased masculinization of the female brain (Lewis et al., 2003) and, depending on study design, increased or decreased ovarian, prostate, and testes weight (Awoniyi et al., 2003; Levy et al., 2003; Wisniewski et al., 2003). Functional deficits such as persistent estrous, decreased fertility in females, and decreased copulatory function in males have also been associated with high-dose genistein exposure during the same gestation-weaning time frames (Lewis et al., 2003; Wisniewski et al., 2003). Along with demonstrating estrogen activity, genistein has also been shown to interact with a variety of molecular targets such as tyrosine kinases; however, in developmental studies, the estrogenic effects predominate and have been separated out from effects due to tyrosine kinase inhibition (Jefferson et al., 2002).

Genistein is metabolized in rats and humans to a number of metabolites, many of which have not been fully characterized for their specific estrogenic potential. Metabolites of genistein, identified in rat bile, include glucuronide and sulfate conjugates of the parent compound (Yasuda et al., 2002). Metabolites of genistein identified in rat urine include 4-hydrogenistein, 6'-hydroxy-O-desmethylgenistein, and 4-hydroxyphenyl-2-proprionic acid (HPA). Substantial gender-related differences in biotransformation of genistein have been reported (Coldham and Sauer, 2000). All of these metabolites, with the exception of HPA, have been found in human urine (Joannou et al., 1995; Fischer et al., 2004), suggesting that common pathways of genistein metabolism exist in rats and humans.

In contrast to the major metabolites identified in urine, genistein glucuronide (Gen-Gluc) and genistein sulfate (Gen-S) are reported to be the major metabolites present in plasma in both rats and humans (Barnes et al., 1996). In rats, the intestinal wall is most likely the primary site of glucuronidation of genistein (Barnes et al., 1996), and there is evidence that genistein undergoes extensive enterohepatic circulation in rats (Sfakianos et al., 1997; Yasuda et al., 1996). Enterohepatic circulation will impact pharmacokinetics of genistein, since it will delay excretion and prolong the length of time target tissues are potentially exposed to genistein.

Although there are a number of pharmacokinetics studies in nonpregnant rats, little is known about the kinetics of genistein in pregnant rats and the transfer of genistein and its metabolites to the developing fetus. Most kinetic studies report total genistein levels (after hydrolysis of all metabolic conjugates) in plasma with limited tissue data available (Coldham and Sauer, 2000) following a single dose of genistein. When pregnant Sprague-Dawley rats (gestation day (GD) 20) were administered a single oral dose of genistein, genistein and its conjugated metabolites were found to be transported to the fetus including delivery to fetal brain, an estrogen target organ (Doerge et al., 2001); however, this study did not differentiate between Gen-Gluc or Gen-S. It has been hypothesized that genistein conjugates do not cross the placenta (Lamartiniere et al., 2000), but this has not been rigorously tested. Previous studies investigating the placental transfer of thyroid hormone sulfoconjugates in rats have concluded that sulfated iodotironines cross the placenta (Wu et al., 2000); acetaminophen glucuronides do not (Wang et al., 1986). While genistein has been shown to activate ER in vitro, Gen-Gluc or Gen-S do not (Borghoff et al., 2004). Information on the dosimetry of the conjugated metabolites of genistein to the fetus will provide an improved understanding of the amount of genistein, unconjugated, that is available to interact with ER within the developing reproductive tract. Therefore, in this manuscript, genistein refers only to the unconjugated form. The relative fetal distributions of all three analytes will also provide an understanding of the fetal capacity to metabolize genistein to its inactive metabolites.

The objectives of this study were to examine the transport of genistein and its conjugated metabolites across the placenta to the fetus and determine the exposure of the fetus to unconjugated genistein following single and repeated administration of genistein. An additional objective was to evaluate the pharmacokinetics of genistein and conjugated metabolites in the pregnant rat, following both single and repeated oral administration during gestation. These studies are important, since reproductive and developmental toxicity studies are typically carried out over the entire period of gestation. In addition, repeated dosing more accurately reflects typical human exposures, where soy-based products serve as the significant source of phytoestrogens during daily dietary intake, possibly throughout gestation.
MATERIALS AND METHODS

Chemicals. Genistein was obtained from Indofine Chemical Company, Inc. (Somerville, NJ). If not otherwise indicated, all other chemicals were obtained from Sigma Chemical Company. The isolation and characterization of Gen-Gluc and Gen-S standards, from bile of rats administered genistein and chemical synthesis respectively, is described by (Borghoff et al., 2003).

Pharmacokinetic Studies

Animals. All animal studies were conducted in accordance with federal guidelines for the humane care and use of laboratory animals (National Institutes of Health, 1985). The study was approved by the Institutional Animal Care and Use Committee of the CIIT Centers for Human Health Research. Pregnant time-mated female Sprague-Dawley rats [Crl:CD(SD)] were obtained from Charles River Laboratories (Raleigh, NC) on GD 0 and housed in pairs. Room temperature was maintained at approximately 22 ± 2°C and relative humidity at approximately 30–70%, with a 12-h photo period (0700–1900 h for light phase). Rats were maintained on a soy- and alfalfa-free diet (SAFD) in which casein and corn oil were substituted for soy and alfalfa meal and soy oil, respectively. Chrysine was selected as the internal standard because it is quantification (LOQ) for these compounds were 2.90, 5.03, and 6.17 ng/ml plasma were 0.87, 1.51, and 1.85 ng/ml, respectively, and the limit of detection (LOD) for genistein from extracts prepared from SAFD was approximately 80 ng/ml, as well as Gen-S and Gen-Gluc (0–25 µg/ml). Each standard was spiked into control plasma, followed by internal standard (chrysine, 150 ng/ml). The samples were centrifuged, and supernatants were analyzed by LC-MS/MS. Tissue calibration standards were prepared by spiking the stock solution into tissue homogenate from untreated animals. The samples were mixed and evaporated to dryness under nitrogen gas. The residue was reconstituted with a 3:1 solution of acetonitrile/5 mM ammonium acetate and filtered prior to analysis.

Enzyme Activity Measurement

Microsome preparation. The S9 fraction and microsomes were prepared from maternal liver, fetal liver, and placenta from control animals maintained on the SAFD diet and killed on GD 19, as described by (Guenegger, 1989). Protein assays were conducted on microsomal fractions and the S9 fraction using the BCA assay with albumin as the standard.

Preparation of plasma and amniotic fluid for LC-MS/MS analysis. Rat plasma and amniotic fluid (50 µl) were diluted in methanol 1:1 with internal standard (IS) (150 ng/ml chrysine;acenontirile) added to the mixture. The sample was vortexed, followed by centrifugation at 8,000 × g for 5 min. The acetonitrile layer was removed and analyzed for IS, genistein, Gen-Gluc, and Gen-S by LC-MS/MS. The LODs for genistein, Gen-S, and Gen-Gluc in plasma were 0.87, 1.51, and 1.85 ng/ml, respectively, and the limit of quantification (LOQ) for these compounds were 2.90, 5.03, and 6.17 ng/ml respectively. Chrysine was selected as the internal standard because it is structurally similar to genistein, yet does not actually contain genistein as an impurity as does biochanin A, another commonly used internal standard for genistein analysis.

Preparation of tissues for analysis by LC-MS/MS. Tissues (liver, placenta, or brain) were minced and combined with a solution of acetonitrile/ chrysine and citrate buffer (pH 5.0, in a ratio of 4:1) 1:15 weight to volume. The samples were homogenized in a Mixer Mill MM301 (Retsch GmbH & Co. KG (Germany). The homogenate was centrifuged at 8,000 × g, and the supernatant was evaporated to dryness with N2 on the Turbo Vac system. The residue was reconstituted with a 3:1 solution of acetonitrile/5 mM ammonium acetate and filtered using a miniprep syringe (Whatman Inc., NJ).

LC-MS/MS analysis was performed using a PE Series 200 HPLC system interfaced with a PE Sciex API 3000 triple quadrupole mass spectrometer with an ESI interface. Chromatography was conducted on a Discovery HS C18 column (5 cm × 2.1 mm; 3 µm) eluted with (A) 5 mM ammonium acetate, pH 7.0, and (B) acetonitrile at a flow rate of 200 µl/min, with a gradient of 5–95% B over 11.0 min. The elution of genistein and its metabolites was monitored by Multiple Reaction Monitoring (MRM) in the negative ion mode for the following ions: genistein: m/z 269.3 to 133.1 (RT 5.2–5.8 min); chrysine (IS): m/z 253.3 to 209.2 (RT 6.2–6.6 min); Gen-Gluc: m/z 445.2 to 269.2 (RT 3.6–3.9); and Gen-S: m/z 349.2 to 269.2 (RT 4.2–4.5 min). The range in retention times reflected the different tissues analyzed. A representative chromatogram is presented in Figure 2. The ratio of peak areas for analyte to IS was plotted against the concentration of analyte in the solution. LOD values for genistein, Gen-S, and Gen-Gluc in the different tissues were: maternal liver, (1.06, 0.05 and 0.22 ng/g); fetal liver, (2.79, 0.92, and 1.54 ng/g); placenta, (1.45, 0.13, and 0.30 ng/g); and fetal brain, (2.785, 0.33, and 0.48 ng/g). LOQ for genistein, Gen-S and Gen-Gluc were: in maternal liver, (3.53, 0.15, 0.76 ng/g); fetal liver, (9.30, 3.06, and 5.14 ng/g); placenta, (4.85, 0.42, and 1.0 ng/g); and fetal brain (8.87, 1.11, and 1.61 ng/g). Values were excluded from analysis if they were below the LOD. If values were between the LOD and LOQ, they were set equal to the LOQ/2.

Calibration curve preparation. Gen-Gluc and Gen-S were diluted in 96% ethanol, and concentrations of stock solutions were determined using molar extinction coefficients (ε) (Franke et al., 1995; King and Bursill, 1998). The stock solutions were diluted to prepare calibration standards of genistein (0–0.5 µg/ml), as well as Gen-S and Gen-Gluc (0–25 µg/ml). Each standard was spiked into control plasma, followed by internal standard (chrysine, 150 ng/ml). The samples were centrifuged, and supernatants were analyzed by LC-MS/MS. Tissue calibration standards were prepared by spiking the stock solution into tissue homogenate from untreated animals. The samples were mixed and evaporated to dryness under nitrogen gas. The residue was reconstituted with a 3.1 solution of acetonitrile/5 mM ammonium acetate and filtered prior to analysis.

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FIG. 2. Representative LC-MS/MS chromatogram from a plasma sample of a pregnant rat administered genistein. Chromatography was similar in each tissue with slight shifts in retention times depending on the matrix.
β-Glucuronidase activity. β-Glucuronidase activity was measured in the S9 fraction from tissues (Lucier et al., 1977). The activity was determined by measuring the hydrolysis of p-nitrophenol β-glucuronide (PNP-β-glucuronide) at 37°C. The final incubation volume was 0.5 ml and contained 75 mM sodium citrate buffer (pH 4.5), 1.0 mM PNP β-glucuronide, and 2 mg of S9 protein. Blank values were obtained in the absence of substrate. The mixture was incubated for 10 min, and the reaction was stopped with 5.0 ml of 0.2 M of glycine buffer (pH 10.4). The product appearance (p-nitrophenol (PNP)) was measured spectrophotometrically at 405 nm. The amount hydrolyzed was determined using an external standard curve of PNP.

UDP-GT activity. UDP-GT activity was measured using microsomes prepared from untreated maternal and fetal liver and placenta (Goon and Klaassen, 1992). The final incubation volume was 0.5 ml and contained 0.1 mM genistein, Tris-HCl (200 mM, pH 7.7), magnesium chloride (10 mM), UDP-GA (4 mM), D-saccharic-1,4-lactone (1.25 mM), and microsomal protein (0.5–2 mg/ml). The assays were performed in duplicate at 37°C with mechanical agitation. The reactions were initiated by the addition of UDP-GA after 10 min of preincubation, and the reaction was stopped after 20 min by the addition of 0.5 ml ice-cold ethanol. The amount of Gen-Gluc was determined by HPLC chromatography using an external calibration curve of Gen-Gluc prepared in 200 mM Tris–HCl:ethanol. The glucuronide was eluted using a linear gradient from 30 to 50% B over 2 min, remaining at 50% B for 8 min, then to 100% B in 2 min, and remaining at 100% B for 3 min.

Statistical analysis. All values reported are the mean ± SD of samples from at least four rats. The pharmacokinetic parameters for the concentration–time curves of each metabolite in plasma and tissues measured by LC-MS/MS were determined using a noncompartmental model (WinNonlin, Version 4.5, Pharsight Corporation, Mountain View, CA). To test for statistical significance, Student’s t-test was performed, unless variances were unequal, in which case the Mann-Whitney test was used. To make multiple comparisons, a one-way ANOVA was used followed by Bonferroni’s. All statistical analysis was performed using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA, www.graphpad.com).

RESULTS
Repeated oral administration of genistein did not affect maternal body weight gain, terminal maternal liver weights, or fetal organ:fetal body weight ratios (data not shown). Gen-Gluc was the major metabolite in maternal plasma following repeated doses of 4 or 40 mg genistein/kg b.w. (Figs. 3A and 4A, respectively). The peak concentrations of genistein in maternal plasma occurred at 1 h and were 5.5 and 75 pmole/ml following 4 or 40 mg/kg b.w. genistein, respectively. In the placenta, genistein was the predominant analyte, peaking at 6 h (~100 pmoles/g tissue) following repeated dosing of 4 mg/kg genistein (Fig. 3B) and peaking at 4 h (~1500 pmoles/g tissue) following repeated dosing of 40 mg/kg genistein (Fig. 4B). The concentration of genistein in the placenta decreased to levels below the LOD between 12 and 24 h following administration. Although Gen-Gluc was the major metabolite present in fetal plasma (Figs. 3 and 4C), Gen-S also had a significant presence in the plasma following repeated administration of 4 or 40 mg/kg genistein to the dam. The concentration of both Gen-Gluc and Gen-S increased with time in fetal plasma. Peak concentrations were dependent on the dose and number of days that genistein was administered. Concentrations of genistein and conjugated metabolites were present in amniotic fluid.

FIG. 3. The concentration–time curves of genistein, Gen-S, and Gen-Gluc in maternal plasma (A), placenta (B), fetal plasma (C), and amniotic fluid (D) following oral administration of repeated doses (GD 5–19) of 4 mg genistein/kg b.w. Each symbol represents the mean ± SD (n = 4 rats) of the each analyte. Missing data points reflect the fact that the concentration of analyte fell below LOD and is therefore not reported.
The distributions of metabolites in amniotic fluid are shown in Figures 3D and 4D, where genistein was the major metabolite at early time points, and Gen-Gluc the major metabolite at later time points, with accumulation over time of all three analytes. Pharmacokinetic parameters were analyzed for maternal and fetal plasma, placenta, and amniotic fluid for both the 4-mg/kg and 40-mg/kg dose groups. Following a single dose of 4 mg genistein/kg b.w, there were no significant differences in the pharmacokinetic parameters when compared with repeated dosing; therefore, data are presented for the 40-mg/kg b.w dose group only. The concentration–time profile for genistein is presented in Figure 5 for maternal plasma (A), placenta (B), fetal plasma (C), and amniotic fluid (D). These data were analyzed using noncompartmental analysis of each individual animal, and the resulting pharmacokinetic parameters are listed as means in Table 1. Repeat administration of genistein (40 mg/kg) increased the area under the curve (AUC) from time 0 to the last sample for genistein in the placenta compared to a single dose. This corresponded to a significant decrease in clearance of Gen-S. There were no other significant differences in pharmacokinetic parameters for Gen-S in maternal or fetal plasma when comparing single verses repeated exposures.

The concentration–time profile for Gen-G is presented in Figure 7 for maternal plasma (A), placenta (B), fetal plasma (C), and amniotic fluid (D), with the pharmacokinetics parameters presented in Table 1. In placenta and maternal plasma, there was a significant increase in AUC for Gen-G following repeated dosing of 40 mg/kg b.w., as compared with a single dose. This corresponded to a decrease in the clearance of Gen-G, also from the placenta and maternal plasma. Genistein, Gen-S, and Gen-G were also measured in maternal liver; single versus repeated administration of genistein did not alter the liver concentration time courses for any of the analytes, and therefore these data are not reported. Gen-Gluc was the major metabolite in maternal liver, followed by genistein and Gen-S (data not shown). Concentrations of genistein and conjugated metabolites in the fetal liver and brain are reported in Figure 8. In fetal liver, Gen-G was the predominant metabolite, followed by genistein and Gen-S. In the fetal brain, Gen-G and Gen-S were above the LOD; however, these concentrations were very low compared to the
concentration of genistein. Attempts to measure genistein and conjugates in the pooled fetal reproductive organs were unsuccessful, since concentrations in these tissues were well below the LOD. It is interesting to note that the time course for the appearance of the metabolites in the fetal liver is very similar to the appearance of metabolites in the fetal plasma. As indicated in Figure 9, the ratio of the AUC for genistein to the AUC for Gen-S or the AUC for Gen-Gluc was higher in the

![Figure 5](http://toxsci.oxfordjournals.org/)

**FIG. 5.** The concentration–time curves of genistein in maternal plasma (A), placenta (B), fetal plasma (C), and amniotic fluid (D) following oral administration of a single dose (GD 19) or repeated doses (GD 5–19) of 40 mg genistein/kg b.w. Each symbol represents the mean ± SD (n = 4 rats) of the each analyte. Missing data points reflect the fact that the concentration of analyte fell below LOD and is therefore not reported.

<table>
<thead>
<tr>
<th></th>
<th>Maternal plasma</th>
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<th>Fetal plasma</th>
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<tr>
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<td>Single</td>
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<tr>
<td>Cmax (pmole/ml)</td>
<td>Genistein</td>
<td>95.6 ± 23.6</td>
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<td>551 ± 181</td>
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<td></td>
<td>Gen-S</td>
<td>1.5 ± 1.7</td>
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<td></td>
<td>Gen-Gluc</td>
<td>1.9 ± 1.9</td>
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<td>AUC&lt;sub&gt;a&lt;/sub&gt; (h × pmole/ml)</td>
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<td></td>
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<td>Gen-Gluc</td>
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<td>65521 ± 12501</td>
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<td>t&lt;sub&gt;1/2&lt;/sub&gt; (h)</td>
<td>Genistein</td>
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<td></td>
<td>Gen-S</td>
<td>4.3 ± 1.1</td>
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<td>Gen-Gluc</td>
<td>4.3 ± 0.8</td>
<td>4.7 ± 1.2&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>Cl&lt;sub&gt;obs&lt;/sub&gt; (l/h)</td>
<td>Genistein</td>
<td>77.3 ± 46.7</td>
<td>60.5 ± 31.7</td>
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<tr>
<td></td>
<td>Gen-S</td>
<td>13.2 ± 7.70</td>
<td>10.8 ± 3.91</td>
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<td></td>
<td>Gen-Gluc</td>
<td>0.61 ± 0.27</td>
<td>0.41 ± 0.06&lt;sup&gt;b&lt;/sup&gt;</td>
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**TABLE 1**

Concentration–Time Profiles Following Administration of 40 mg/kg Genistein to Pregnant Sprague-Dawley Rats

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**Note.** Noncompartmental analysis of concentration–time profiles following single (GD 19) or repeated (GD 5–19) administration of 40 mg/kg genistein to pregnant Sprague-Dawley rats.

<sup>a</sup>0–last time point.

<sup>b</sup>Compared with single administration (p < 0.05).
fetal brain than in the fetal liver. The ratios of genistein to Gen-S or Gen-Gluc are also significantly higher in the placenta than in maternal or fetal plasma.

Activities of UDP-glucuronyltransferase (UDP-GT) and β-glucuronidase, the key enzymes involved with glucuronidation and deglucuronidation of genistein, were measured in maternal and fetal liver and placenta from untreated pregnant rats (GD 19). The data are reported in Figure 10 and indicate that the ability to conjugate genistein to glucuronic acid is present in both the maternal and fetal liver. However, in the placenta,

FIG. 6. The concentration–time curves of Gen-S in maternal plasma (A), placenta (B), fetal plasma (C), and amniotic fluid (D) following oral administration of a single dose (GD 19) or repeated doses (GD 5–19) of 40 mg genistein/kg b.w. Each symbol represents the mean ± SD (n = 4 rats) of the each analyte. Missing data points reflect the fact that the concentration of analyte fell below LOD and is therefore not reported.

FIG. 7. The concentration–time curves of Gen-G in maternal plasma (A), placenta (B), fetal plasma (C), and amniotic fluid (D) following oral administration of a single dose (GD 19) or repeated doses (GD 5–19) of 40 mg genistein/kg b.w. Each symbol represents the mean ± SD (n = 4 rats) of the each analyte. Missing data points reflect the fact that the concentration of analyte fell below LOD and is therefore not reported.
UDP-GT activity was very low. Fetal liver UDP-GT and β-glucuronidase activities were 23% and 53% of maternal liver, respectively.

**DISCUSSION**

Genistein and two of its metabolites, Gen-Gluc, and Gen-S, were quantified in maternal and fetal tissues and amniotic fluid following single and repeated administration of either 4 or 40 mg/kg genistein. This study was conducted to determine the extent of transfer of genistein across the placenta and its distribution to the fetus. It is believed that genistein, but not its conjugated metabolites, acts via the ER to produce developmental effects in laboratory animals. It is important to determine the concentration of unconjugated genistein that reaches the fetus. In addition, the effects of single and repeated dosing of genistein on the pharmacokinetics of this compound in pregnant rats was carried out, as repeated dosing more accurately reflects the protocol used to assess developmental toxicity and is more relevant to human exposure.

In the current study, there was no change in the concentration time course for genistein or its metabolites in the maternal liver when comparing single versus repeated administration of genistein, again suggesting a lack of induction of metabolic enzymes. Data reported by Kishada *et al.* also support this conclusion, as dietary soy intake (~95 mg genistein/rat based on average feed intake) failed to result in induction of cytochrome P-450 mRNA in rat hepatic microsomes (Kishada *et al.*, 2004). In a previous study, Delclos *et al.* (2001) showed a significant reduction in body weight gain at the highest concentration of genistein (70–97 mg/kg/day) administered in the diet from GD 7 through 21. In this study, repeat administration of genistein had no effect on maternal body weight, maternal liver weight, or fetal organ to body weight ratios, as compared with a single dose on GD 19.

It has been reported that genistein has an EC50 of 34 pmoles/ml for ERα and 4.9 pmoles/ml for ERβ in a human hepatoma cell line (HEPG2) (Casanova *et al.*, 1999), with relative binding affinities (RBA) of 4 RBA (ERα) and 87 RBA (ERβ) based on an arbitrary binding affinity of 100 RBA for 17β estradiol, the endogenous ligand for these receptors (Kuiper *et al.*, 1998). The concentration of genistein seen in fetal plasma (50 pmoles/ml) following repeated administration of 40 mg/kg, although fairly low, would be sufficient to compete with estradiol for ERactivation. Even following genistein exposures that mimic human exposures (4 mg/kg b.w.), the fetal plasma concentration was approximately 5.5 pmoles/ml, slightly above the EC50 for ERβ, and sufficient to compete with estradiol for ERβ activation. This would suggest...
The first 6–8 h following genistein administration and the for Gen-Gluc accumulation in fetal plasma being linear over the placenta. This conclusion is supported by the time course suggestive that maternally formed conjugates do not cross genistein exposure; future work will examine this possibility. The placenta may be that transport proteins are being inhibited by for the altered kinetics of genistein and its metabolites in the well above the EC50 for both ERa and ERb. While not directly assessed in this study, our data are a potential for ER-mediated effects in the fetus following maternal administration of genistein.

In placenta, repeated administration of genistein increased the AUC and decreased clearance for all three analytes, and unlike other maternal tissues, genistein was the major metabolite. This was not expected, since Gen-Gluc is the major metabolite in maternal plasma and fetal plasma, and only a low concentration of genistein was measured in fetal plasma. Over the course of 24 h, the placental tissue contained high concentrations of genistein, peaking at 4–6 h and not decreasing until 12 h following administration to the maternal rat. The concentration of unconjugated genistein in the placenta between 4 and 12 h was approximately 1500 pmol/g, in the 40 mg/kg dose group, well above the EC50 for both ERa and ERb. One explanation for the altered kinetics of genistein and its metabolites in the placenta may be that transport proteins are being inhibited by genistein exposure; future work will examine this possibility.

While not directly assessed in this study, our data are suggestive that maternally formed conjugates do not cross the placenta. This conclusion is supported by the time course for Gen-Gluc accumulation in fetal plasma being linear over the first 6–8 h following genistein administration and the presence of low concentrations of Gen-G and Gen-S in the placenta, strongly suggesting that Gen-Gluc and Gen-S are not readily transported across the placenta to the fetus. Further support for this conclusion comes from the literature. Studies on the transplacental transport of DES-glucuronide in rhesus monkeys have shown negligible transfer across the placenta in either direction (Hill et al., 1980). In experiments using acetaminophen glucuronide and acetaminophen sulfate as test compounds, it was concluded that placental transfer of glucurononitated and sulfated acetaminophen is negligible (Wang et al., 1985, 1986). Due to the time-related increase in both Gen-S and Gen-Gluc in fetal plasma and liver, the presence of these metabolites is most likely due to conjugation of genistein to Gen-S and Gen-Gluc in the fetal liver.

The data from these studies suggest that the fetal liver has the ability to conjugate genistein to Gen-Gluc. UDP-GT activity in the fetal liver was 23% of the activity in the maternal liver. The fetal liver also maintains the ability to hydrolyze Gen-Gluc, with β-glucuronidase activity in the fetal liver 58% of the activity observed in the maternal liver on GD 19. Measurement of enzyme activities in the placenta demonstrated that UDP-GT activity was only 2% of the activity in the maternal liver and that β-glucuronidase activity was 28% of maternal liver activity. These data suggest that there is very little metabolism of genistein to Gen-G within the placenta and that, if Gen-G is transported into the placenta and is not being efficiently transported to the fetus, the placental β-glucuronidase activity is most likely hydrolyzing the conjugate and contributing to the higher genistein concentrations observed in this tissue. High placental β-glucuronidase activities are not limited to rats; data from human pregnancies indicate that term human placenta has high β-glucuronidase activity compared to placenta from first trimester pregnancies (Leung et al., 1978). Again, pharmacokinetic analysis suggests that there are altered kinetics in the placenta following repeat administration of genistein. The retention of genistein in the placenta at high concentrations may be due to a number of factors such as inhibition of transport across the placenta to the fetus, either due to the action of efflux proteins or genistein binding to ER. P-glycoprotein (PGP) is a major efflux protein in the placenta. In the rat, gene expression begins on GD 11, with PGP detected by Western blot from GD 13 onward (Novotna et al., 2004). Genistein has been shown to inhibit PGP activity without a reduction in protein expression of PGP (Limtrakul et al., 2005).

In addition to its role in transporting molecules between mother and fetus, the placenta is a major endocrine organ. The placenta is very rich in ERb, and genistein has been shown to have a higher affinity for ERβ than for ERa (Borghoff et al., 2004; Buchovsky et al., 2003). Diethylstilbestrol (DES), a chemical with high affinity to ER, has been shown to induce developmental changes in the placenta (Scott and Adejokun, 1980; Tremblay et al., 2001), and both ERα and ERβ have been shown to play a role in placentaion (Couse and Korach, 1999). The high concentrations of genistein in the placenta are...
consistent with data from dosimetry studies of other endocrine active compounds, but the implications of high genistein concentrations in the placenta are not apparent and have not been fully studied at this time. Schonfelder et al. reported a similar accumulation of parent bisphenol A (BPA) in human placenta collected from term pregnancies of women living in an urban area (Schonfelder et al., 2002). BPA, a compound present in flame retardants and resins, also has estrogenic activity and has been shown to have adverse effects in the developing reproductive system of laboratory animals (Kubo et al., 2001). Similar to genistein, the predominant metabolite, BPA-glucuronide, is biologically inactive (Matthews et al., 2001). This suggests a need to further investigate the potential of glucuronidation-deglucuronidation in the placenta and to determine what impact endocrine-active compounds may have in the organ.

In summary, repeated administration of genistein to the maternal rat resulted in fetal exposure to unconjugated genistein at concentrations that, although very low, were still above the reported EC50s for ER activation, indicating a potential mechanism for fetal effects observed in developmental toxicity studies in rodents. The fetal distribution of genistein, Gen-S, and Gen-Gluc, as well as the ratios of genistein, Gen-S, and Gen-Gluc, as well as the ratios of genistein to conjugates indicates sulfation may have a larger role in fetal metabolism as compared to maternal metabolism, with increased sulfation occurring in the fetus. The placenta appears to serve as a sink for unconjugated genistein. What effect genistein may have on the placenta is not known; however, trophoblast differentiation is dependent upon ERβ activation in the placenta, suggesting there may in fact be a placental target for genistein action (Buchovsky et al., 2003). Further evaluation of efflux proteins and their role in transporting genistein in the placenta will be necessary to fully understand the transport of genistein to the fetus. The kinetics of repeated genistein exposure are slightly altered, compared to a single dose of genistein, primarily in terms of increased AUC and decreased clearance; however, this only occurs at supra-physiological doses and is not observed in the fetal plasma. These data indicate that repeated administration does not increase the overall exposure of the fetus to unconjugated genistein. In addition, placental transport of genistein to the fetus appears to be inefficient, and the data are suggestive that the unconjugated forms do not cross the placenta. These results may explain the very low concentrations of unconjugated genistein observed in the fetus. These levels are sufficient for ERβ activation and may account for the relatively mild reproductive effects observed in rodents in laboratory studies.

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