DISPOSITION OF FLAVONOIDS VIA RECYCLING: COMPARISON OF INTESTINAL VERSUS HEPATIC DISPOSITION

Jun Chen,1 Stephen Wang,1 Xiaobin Jia,1 Susan Bajimaya, Huimin Lin, Vincent H. Tam,1 and Ming Hu1

Department of Pharmaceutical Sciences, College of Pharmacy, Washington State University, Pullman, Washington

Received January 11, 2005; accepted August 23, 2005

ABSTRACT:
The purpose of this study was to compare intestinal versus hepatic disposition of six flavonoids to fully characterize their first-pass metabolism. The perfused rat intestinal model and microsomes prepared from rat liver, duodenum, jejunum, ileum, and colon were used. The results indicated that isoflavone (12.5 μM) glucuronidation was highly variable among different microsomes prepared from liver or intestine. Comparing to liver metabolism, the intestinal metabolism had higher Km values (>2-fold). Likewise, the hepatic intrinsic clearance (IC, or a ratio of Vmax/Km) values of isoflavones were generally higher than their intestinal IC values (200–2000% higher), except for prunetin, for which the jejunal IC value was 50% higher than its hepatic IC. When comparing intestinal metabolism, the results showed that intestinal metabolism rates and Vmax values of isoflavones were less when an additional A-ring electron-donating group was absent (i.e., daidzein and formononetin). In the rat perfusion model using the whole small intestine, genistein (10 μM) was well absorbed (77% or 352 nmol/120 min). The first-pass metabolism of genistein was extensive, with 40% of absorbed genistein excreted as conjugated metabolites into the intestinal lumen. In contrast, the bile excretion of genistein conjugates was much less (6.4% of absorbed genistein). In conclusion, intestinal glucuronidation is slower in isoflavones without an additional A-ring substitution. Perfusion studies suggest that intestine is the main organ for genistein glucuronide formation and excretion in rats and may serve as its main first-pass metabolism organ.

Genistein, daidzein, glycitein, biochanin A, prunetin, and formononetin are six isoflavones that are commonly consumed and widely available as dietary supplements in pharmacies and health food stores. They belong to a class of phytochemicals called phytoestrogens, with significant estrogenic activities. Claimed health benefits of isoflavones include reduction of cancer risk, relief of peri- and postmenopausal symptoms, and maintenance of cardiovascular health (Tham et al., 1998; Setchell and Cassidy, 1999.). Additionally, isoflavones are thought to have protective effects against hormone-related malignancies such as breast and prostate cancer (Kurzer and Xu, 1997; Birt et al., 2001; Yang et al., 2001 and references therein). Genistein, daidzein, glycitein, and their glycosides are abundant in soybeans and represent the major active components in soy products. On the other hand, formononetin, biochanin A, genistein, and daidzein are found as a mixture in red clover (Lin et al., 2000). Prunetin is mainly found in Kudzu roots, a Chinese herb found to be active against alcohol abuse (Luksas et al., 2005). Currently, soy isoflavones are undergoing clinical trials for prostate cancer prevention (Takimoto et al., 2003).

Even though there is a substantial amount of information pointing to their anticancer and other health-beneficial effects, isoflavones have poor bioavailabilities (Setchell et al., 2001, 2003; Busby et al., 2002), which may prevent their development into viable chemopreventive agents. Much work is still needed to understand the absorption mechanism and metabolic pathways of isoflavones so that we can develop viable strategies to overcome their poor bioavailability.

Our previous studies have shown that intestinal conjugation and subsequent excretion of phase II metabolites via intestine is a main component of first-pass metabolism of genistein (Chen et al., 2003). Additional studies of isoflavones in Caco-2 cells showed that metabolism of isoflavones and excretion of their conjugates were strongly influenced by their structure (Chen et al., 2005). Since excretion of phase II conjugates is dependent on metabolite formation by conjugating enzymes such as UGT and subsequent efflux of phase II metabolites by transporters such as multidrug resistance-associated proteins (Hu et al., 2003; Chen et al., 2005), the present study will focus on metabolite formation through the main conjugation pathway, glucuronidation. Therefore, the purposes of this study are to characterize the intestinal and hepatic glucuronidation of six common isoflavones (i.e., genistein, daidzein, glycitein, formononetin, biochanin A, and prunetin), to study the effects of structural changes on glucuronidation, and to further define the main organ responsible for the first-pass metabolism of genistein, the main active soy isoflavone.

ABBREVIATIONS: UGT, UDP-glucuronosyltransferase; HPLC, high-performance liquid chromatography; AIC, Akaike’s information criterion; IC, intrinsic clearance.
Materials and Methods

Materials. The isoflavones genistein, daidzein, glycitein, formononetin, and prunetin were purchased from INDOFINE Chemical Co. (Hillsborough, NJ) or LC Laboratories (Woburn, MA). B-Glucuronidase with [catalog #G1512] or without sulfatase [catalog #G7396], sulfatase without glucuronidase [catalog #S1629], uridine diphosphoglucuronic acid, alamethicin, t-saccharic-1,4-lactone monohydrate, biochalcon A, magnesium chloride, and Hank’s’ balanced salt solution (powder form) were purchased from Sigma-Aldrich (St. Louis, MO). HPLC-grade acetonitrile and methanol were purchased from Fisher Scientific Co. (Pittsburgh, PA). Other chemicals were obtained from reputable commercial sources.

Microsome Preparation from Rat Intestine or Liver. Adult male Sprague-Dawley rats (200–250 g) were used for the isolation of liver microsomes or intestinal microsomes in four segments: duodenum, jejunum, ileum, and colon. The detailed procedures have been described previously (Chen et al., 2003). The microsomal pellets were suspended in 250 mM sucrose and stored at −80°C until use. The concentration of microsomal protein was determined by the Bio-Rad protein assay (Bio-Rad, Hercules, CA) as described (Chen et al., 2003).

Isoflavone Glucuronidation Using the Microsomes from Rat Intestine or Liver. Intestinal and hepatic glucuronidation of six selected isoflavones (i.e., genistein, daidzein, biochalcon A, glycitein, formononetin, and prunetin) were measured using the method described in our previous study (Chen et al., 2003). The incubation procedures for measuring UGT activities using microsomes were as follows: 1) mix microsomes (final concentration ~0.05 mg protein/ml), magnesium chloride (0.88 mM), saccharolactone (4.4 mM), and alamethicin (0.022 mg/ml), different concentrations of substrates in a 50 mM potassium phosphate buffer (pH 7.4), and uridine diphosphoglucuronic acid (3.5 mM, add last); 2) incubate the mixture at 37°C for 10 or 30 min; and 3) stop the reaction by the addition of a solution of 94% acetonitrile/6% glacial acetic acid containing 100 μM testosterone as an internal standard. Formation of isoflavone conjugates was confirmed using conjugate hydrolysis by glucuronidase or sulfatase as described (Liu and Hu, 2002). All samples generated from microsomal studies were centrifuged at 13,000 rpm for 8 min, and supernatants were used for HPLC assay as described later (“HPLC Analysis of Isoflavones and Its Conjugates”).

Transport and Metabolism Experiments in Perfused Rat Intestinal Model. The procedures were approved by Washington State University’s Institutional Animal Care and Use Committee. The rat surgical procedure for this study was similar to that in our previous published papers (Chen et al., 2003) with one significant modification: the whole small intestine from the upper duodenum to the end of the ileum was perfused at a flow rate of 0.384 ml/min. Bile and portal vein were also cannulated for the collection of bile and blood samples.

Processing of Biological Samples from Rat. A 200-μl portion of the intestinal perfusate was mixed with 50 μl of stop solution including the internal standard. The mixture was centrifuged at 13,000 rpm for 8 min, and supernatant was introduced into the HPLC system as described later (“HPLC Analysis of Isoflavones and Its Conjugates”).

Each blood or bile sample was divided into two portions for quantitative measurement of genistin aglycone and glucuronide conjugates, as described previously (Chen et al., 2003). One portion of the sample (e.g., 200 μl) was extracted with methylene chloride (6 ml), and the organic phase was separated and then evaporated. The dried sample was reconstituted in 200 μl of 50% methanol in water and analyzed by the HPLC system for free genistin. The other portion of the sample (e.g., 200 μl) was added to β-glucuronidase to completely hydrolyze genistin glucuronide to aglycone. The hydrolyzed sample containing total genistin was then extracted and amounts measured as described above. The amounts of glucuronidated genistin were equal to the difference between total genistin and free genistin.

HPLC Analysis of Isoflavones and Their Conjugates. The conditions for HPLC analysis of biochalcon A, prunetin, and formononetin and their glucuronides were the same as in a previously published method (Chen et al., 2003). We prolonged the elution time with a shallower gradient to analyze genistin, daidzein, and glycitein and their glucuronides. The conditions were: HPLC system, Agilent 1090 with diode array detector and Chemstation; column, Aqua (Phenomenex, Torrance, CA), 5 μm, 150 × 0.45 cm; detection wavelength, 254 nm; injection volume, 200 μl; mobile phase A, 0.04% (w/v) phosphoric acid plus 0.06% (v/v) triethyamine (pH 2.8); mobile phase B, 100% acetonitrile. The gradient used for elution was 0 to 3 min, 2% B; 3 to 35 min, 2% to 50% B; 35 to 37 min, 50% B. There was a 5-min interval between the end of the run and the next injection to allow the column to be re-equilibrated. The HPLC profiles of six isoflavones and their conjugates, along with chemical structures of the isoflavones, are shown in Fig. 1.

Data Analysis. In the perfused rat intestinal model, the calculation methods for amounts of genistin perfused or infused (M_in) through intestine, amounts of glucuronidated genistin excreted into the intestinal lumen (M_out), amounts of genistin absorbed (M_ab), and amounts of glucuronidated genistin excreted via bile (M_bile) were the same as those previously described (Chen et al., 2003):

\[ M_{in} = Q \tau C_{in} \]  
\[ M_{mi} = Q \tau C_{mi} \]  
\[ M_{mi} = Q \tau (C_{in} - C_{out}) \]  
\[ M_{bile} = V \times C_{in} \]

where \( Q \) is infusion rate (ml/min); \( \tau \) is sampling time interval (minutes); \( C_{out} \) is concentration of isoflavone aglycone or its glucuronide in outlet perfuse (nmol/ml), which is corrected for water flux using 14C-PEG 4000; \( C_{in} \) is inlet concentration of isoflavone aglycone; and \( V \) is volume of bile excreted over the sampling time period.

In microsomal studies, formation rates (V) of isoflavone glucuronides at various substrate concentrations (C) were fitted to the standard Michaelis-Menten equation if Eadie-Hofstee plot is linear:

\[ V = \frac{V_{max} \times C}{K_m + C} \]

where \( K_m \) is the Michaelis constant and \( V_{max} \) is the maximum formation rate. Rates of metabolism in microsomes were expressed as amounts of metabolite formed per min/mg protein or nmol/min/mg.

When Eadie-Hofstee plots suggested atypical kinetics (autoactivation, biphasic kinetics, and substrate inhibition) (Houston and Kenworthy, 2000; Hutzler and Tracy, 2002), the data from these atypical profiles were fitted to alternative Michaelis-Menten equations (see below) using the ADAPT II program (D’Argenio and Schumitzky, 1997). To determine the best-fit model among various available models, the model candidates were discriminated using Akaike’s information criterion (AIC) (Yamaoka et al., 1978). The rule of parsimony was also applied, which states that fitted estimations with minimum AIC are considered the best fit to the experimental data. Therefore, using this method of minimum AIC estimation, a fit with a smaller negative AIC value (i.e., −54.2) was considered a better fit to the data than those with a positive AIC value (i.e., 0.8) or a larger negative value (−9).

When reaction kinetic data showed autoactivation kinetics based on Eadie-Hofstee plot (Houston and Kenworthy, 2000; Hutzler and Tracy, 2002), formation rates (V) of isoflavone glucuronides at various substrate concentrations (C) were fit using the following equation:

\[ \text{Reaction rate} = \frac{[V_{max1} + V_{max2}(1 - e^{-K_m C})] \times C}{K_m + C} \]

where \( V_{max0} \) is maximum intrinsic enzyme reaction rate, \( V_{max1} \) is maximum inducible enzyme reaction rate, \( R \) is the rate of enzyme activity induction, \( C \) is the concentration of substrate, and \( K_m \) is the concentration of substrate needed to achieve 50% of \( (V_{max0} + V_{max2}) \).

When reaction kinetic data showed biphasic kinetics (in which two isoforms with different kinetic behaviors are responsible for the glucuronidation), formation rates (V) of isoflavone glucuronides at various substrate concentrations (C) were fit using the following equation (eq. 7):

\[ \text{Reaction rate} = \frac{V_{max1} \times C}{K_{m1} + C} + \frac{V_{max2} \times C}{K_{m2} + C} \]

where \( V_{max1} \) is the maximum enzyme reaction rate of one UGT isoform, \( V_{max2} \) is the maximum enzyme reaction rate of another UGT isoform, \( K_{m1} \) is the...
concentration of substrate needed to achieve 50% of $V_{\text{max1}}$, and $K_{m2}$ is the concentration of substrate needed to achieve 50% of $V_{\text{max2}}$.

When reaction kinetic data showed substrate inhibition kinetics (in which the substrate compound inhibits the glucuronidation especially at higher concentrations), formation rates ($V$) of isoflavone glucuronides at various substrate concentrations ($C$) were fit using the following equation:

$$\text{Reaction rate} = \frac{V_{\text{max}}}{1 + \left(\frac{K_{m}}{C} + \left(\frac{C}{K_{si}}\right)\right)} \quad (8)$$

where $V_{\text{max}}$ is the maximum enzyme reaction rate, $C$ is the concentration of substrate, $K_{m}$ is the concentration of substrate needed to achieve 50% of $V_{\text{max}}$, and $K_{si}$ is the substrate inhibition constant.

Statistical Analysis. One-way analysis of variance or Student’s $t$ test was used to analyze the data. The prior level of significance was set at 5% or $p < 0.05$.

Results

Glucuronidation of Genistein and Its Isoflavone Analogs in Duodenal, Jejunal, Ileal, and Colonic Microsomes. Glucuronidation rates of genistein, daidzein, biochanin A, glycitein, formononetin, and prunetin (12.5 μM each) were determined using intestinal microsomes prepared from rat duodenum, jejunum, ileum, and colon (Fig. 2). The results indicated that glucuronidation rates of the six isoflavones varied significantly among different regions of the intestine. Genistein, daidzein, and glycitein were glucuronidated at lower rates compared to biochanin A, prunetin, and formononetin.
vones were different ($p < 0.05$) in microsomes prepared from different segments of the intestine. Reaction rates were always the highest ($p < 0.05$) in jejunal microsomes followed by duodenal, ileal, and, lastly, colonic microsomes. The maximal differences in metabolism rates between jejunal and colonic microsomes were 4.3-fold for genistein and biochanin A, 5.7-fold for glycitein, 5.4-fold for prunetin, 6.1-fold for daidzein, and 3.9-fold for formononetin, respectively.

Among these six isoflavones, the glucuronidation rates (at 12.5 $\mu$M) of genistein, biochanin A, glycitein, and prunetin were fairly similar (2.11–2.53 nmol/min/mg) in each type of intestinal microsomes, but they were much faster than that of daidzein and formononetin (~0.7–0.8 nmol/min/mg).

**Glucuronidation of Genistein and Its Isoflavone Analogs as a Function of Concentration.** The glucuronidation rates of six isoflavones as a function of their concentrations in jejunal microsomes were determined since jejunal microsomes are the most active in metabolizing isoflavones. The results indicated that glucuronidation was saturable (Fig. 3A). The kinetic parameters were then obtained by fitting data to various kinetic equations using the ADAPT II program. The actual equation used was based on shapes of the Eadie-Hofstee plot as described previously (Table 1) (Houston and Kenworthy, 2000; Hutzler and Tracy, 2002). A standard Michaelis-Menten equation was used when Eadie-Hofstee plots were linear. A more complex Michaelis-Menten equation was used when the plots were nonlinear as described under Materials and Methods. This approach generated excellent fittings, and kinetic parameters are shown in Tables 1 and 2. The $V_{\text{max}}$ value of genistein was the highest (3.17 nmol/min/mg protein), followed by biochanin A (sum of two $V_{\text{max}}$ values), glycitein, and prunetin (1.998, 1.92, and 1.62 nmol/min/mg protein, respectively). $V_{\text{max}}$ values for glucuronidation of daidzein and formononetin were much smaller (0.646 and 0.495 nmol/min/mg protein, respectively). The corresponding Michaelis constant ($K_m$) values ranged from 2.05 (prunetin) to 7.25 $\mu$M (formononetin). The $K_m$ values of daidzein, glycitein, and prunetin were approximately the same to each other (2.05–2.66 $\mu$M), and those of the other three isoflavones (genistein, biochanin A, and formononetin) were also approximately the same to each other (6.628–7.25 $\mu$M). The ratios of $V_{\text{max}}/K_m$, representing in vitro intrinsic clearance (IC) value, were also calculated. IC value was the highest for prunetin at 0.79 ml/min/mg protein, followed closely by glycitein (0.72 ml/min/mg protein), and then by genistein and biochanin A (0.478 and 0.433 ml/min/mg protein). The IC values of daidzein and formononetin were much smaller (0.27 and 0.068 ml/min/mg protein, respectively). We used an IC value of 0.433 ml/min/mg protein for biochanin A since it follows biphasic kinetic profiles, and we summed the IC of the first component (0.276) and the $V_{\text{max}}$ value of the second component since the $K_m$ of the second component is very small and the intestinal concentration of biochanin A is likely to significantly surpass that $K_m$ value. We have therefore used the ratio of $V_{\text{max}}$ over a concentration of 1 $\mu$M as an approximation to the likely IC for the second component. This approximation is fairly reasonable since the rate of biochanin A glucuronidation at 0.625 $\mu$M is less than that of glycitein, prunetin, and genistein, but higher than those of the other two isoflavones. The same approach will be used in liver metabolism when the $K_m$ value (of the second component) is less than 0.01 $\mu$M.

**Glucuronidation of Genistein and Its Isoflavone Analogs in Liver Microsomes as a Function of Concentration.** The glucuronidation rates of six isoflavones as a function of concentration in jejunal microsomes were determined since jejunal microsomes are the most active in metabolizing isoflavones. The results indicated that glucuronidation was saturable (Fig. 3A). The kinetic parameters were then obtained by fitting data to various kinetic equations using the ADAPT II program. The actual equation used was based on shapes of the Eadie-Hofstee plot as described previously (Table 1) (Houston and Kenworthy, 2000; Hutzler and Tracy, 2002). A standard Michaelis-Menten equation was used when Eadie-Hofstee plots were linear. A more complex Michaelis-Menten equation was used when the plots were nonlinear as described under Materials and Methods. This approach generated excellent fittings, and kinetic parameters are shown in Tables 1 and 2. The $V_{\text{max}}$ value of genistein was the highest (3.17 nmol/min/mg protein), followed by biochanin A (sum of two $V_{\text{max}}$ values), glycitein, and prunetin (1.998, 1.92, and 1.62 nmol/min/mg protein, respectively). $V_{\text{max}}$ values for glucuronidation of daidzein and formononetin were much smaller (0.646 and 0.495 nmol/min/mg protein, respectively). The corresponding Michaelis constant ($K_m$) values ranged from 2.05 (prunetin) to 7.25 $\mu$M (formononetin). The $K_m$ values of daidzein, glycitein, and prunetin were approximately the same to each other (2.05–2.66 $\mu$M), and those of the other three isoflavones (genistein, biochanin A, and formononetin) were also approximately the same to each other (6.628–7.25 $\mu$M). The ratios of $V_{\text{max}}/K_m$, representing in vitro intrinsic clearance (IC) value, were also calculated. IC value was the highest for prunetin at 0.79 ml/min/mg protein, followed closely by glycitein (0.72 ml/min/mg protein), and then by genistein and biochanin A (0.478 and 0.433 ml/min/mg protein). The IC values of daidzein and formononetin were much smaller (0.27 and 0.068 ml/min/mg protein, respectively). We used an IC value of 0.433 ml/min/mg protein for biochanin A since it follows biphasic kinetic profiles, and we summed the IC of the first component (0.276) and the $V_{\text{max}}$ value of the second component since the $K_m$ of the second component is very small and the intestinal concentration of biochanin A is likely to significantly surpass that $K_m$ value. We have therefore used the ratio of $V_{\text{max}}$ over a concentration of 1 $\mu$M as an approximation to the likely IC for the second component. This approximation is fairly reasonable since the rate of biochanin A glucuronidation at 0.625 $\mu$M is less than those of glycitein, prunetin, and genistein, but higher than those of the other two isoflavones. The same approach will be used in liver metabolism when the $K_m$ value (of the second component) is less than 0.01 $\mu$M.

**Materials and Methods**. This study was approved by the Institutional Animal Care and Use Committee of the University of Connecticut. Rats were housed under standard laboratory conditions (22 ± 2°C, 12-h light-dark cycle) and given access to standard rat chow and water ad libitum. Glucuronidation was assayed by incubating microsomes (20 μg protein) in 100 mM sodium phosphate buffer (pH 6.2), 1 mM magnesium chloride, 2 mM dithiothreitol, and various concentrations of isoflavones in a final volume of 0.1 ml. Incubations were initiated by the addition of isoflavones and were stopped after 15 min by the addition of ice-cold acetonitrile and centrifugation (2300 g for 5 min). Glucuronides were precipitated by centrifugation (10,000 g for 20 min) and were extracted twice with ethyl acetate. The organic solvent was evaporated under a stream of nitrogen, and the dry residue was reconstituted in 100 μl of PBS (pH 7.4) and assayed by reverse-phase HPLC. The apparent kinetics parameters are listed in Table 1. B, Eadie-Hofstee plots were generated to determine which equation to use for data fitting. Lower concentrations of prunetin and glycitein were used because their $K_m$ values were low (<5 $\mu$M). Each data point is the average of three determinations and the error bar is the standard deviation of the mean.

![FIG. 3. A. glucuronidation of the six isoflavones in rat jejunal microsomes as a function of concentration. The points are observed isoflavon glucuronide formation rates, and the curves are estimated based on fitted parameters generated by the Michaelis-Menten equation (eq. 1) and other equations that describe various enzyme kinetics. The apparent kinetics parameters are listed in Table 1. B, Eadie-Hofstee plots were generated to determine which equation to use for data fitting. Lower concentrations of prunetin and glycitein were used because their $K_m$ values were low (<5 $\mu$M). Each data point is the average of three determinations and the error bar is the standard deviation of the mean.](image-url)
(2.89, 1.38, and 1.19 nmol/min/mg protein), respectively. The \( V_{\text{max}} \) value for prunetin (0.343 nmol/min/mg protein) was particularly small in comparison to other isoflavones. The Michaelis constants (\( K_m \)) were generally smaller (with at least one hepatic \( K_m \) less than intestinal \( K_m \)) in the liver microsomes than in intestinal microsomes, although the range was quite large, 0.196 \( \mu \text{M} \) (biochanin A) to 23.6 \( \mu \text{M} \) (genistein). The hepatic metabolic IC values of glycitein, biochanin A, formononetin, daidzein, and genistein were much higher than their jejunal IC values, and the difference ranged from 2-fold (genistein) to 20-fold (biochanin A). The only exception was that the jejunal IC value of prunetin was \( \sim 50\% \) higher than its hepatic IC value.

**Glucuronidation of Genistein in Intestinal and Liver Microsomes as a Function of Concentration.** Genistein is the most potent among six isoflavones and is the main active ingredient of soy isoflavones. Therefore, we further determined its glucuronidation rates in microsomes prepared from the other three regions of the intestine: duodenum, ileum, and colon. The results were then compared with jejunal and hepatic metabolism and plotted in Fig. 5.

Genistein glucuronidation was saturable and the kinetics of glucuronidation (\( K_m \), \( V_{\text{max}} \), and \( V_{\text{max}}/K_m \) values) were determined (Table 2). Hepatic metabolism had the highest \( V_{\text{max}} \) value (3.71 nmol/min/mg protein). On the other hand, the IC values were in the order of liver, jejunum, duodenum, ileum, and colon, and ranged from 0.136–0.983 ml/min/mg protein. Again, we used the sum of IC and \( V_{\text{max}} \) for total hepatic IC since the second \( K_m \) value was very small. In contrast, \( K_m \) values for ileum and colon were quite large and, therefore, the second IC component contributed very little to the total IC and was neglected.

**Intestinal Absorption, Metabolism, and Excretion of Genistein in Rat.** To verify the relative metabolic contribution of intestine versus liver, we measured intestinal and biliary excretion of phase II conjugates in a rat perfusion model. This was important since gut isoflavone concentrations are expected to be quite different from liver concentrations, and, therefore, a direct comparison of the IC values would not reflect the relative contribution of each organ to isoflavone metabolism. Here, we measured absorption of genistein (10 \( \mu \text{M} \)) and excretion of genistein conjugates in male rats by perfusing the whole small intestine. In a steady-state perfusion, the amounts that disappear from the perfusate are equal to the amounts absorbed (Amidon et al., 1988; Chen et al., 2003). This is because genistein is chemically stable in the intestinal perfusate and adsorption is minimal in a steady-state perfusion model (Liu and Hu, 2002). Furthermore, the conjugating enzymes are located inside the enterocytes. Therefore, in the rat perfusion model, genistein was found to be well absorbed (76.6 ± 3.6\% of the perfused amounts were absorbed) (Fig. 6). The accumulated amounts of genistein absorbed were linear from 30 min to 120 min with an average rate of 2.9 ± 0.09 nmol/min or 0.048 ± 0.0034 nmol/cm/min (\( n = 3 \), assuming a total length of 60 cm) (Fig. 6A).

Glucuronides of genistein were found in perfusate, bile, or plasma collected during the perfusion experiments (not shown). Glucuronidation was the major pathway for its metabolism in the rat perfusion, and glucuronides were mainly excreted into the lumen. Approximately 30.9 ± 5.2\% of perfused amounts (\( M_a \)) or 40.3\% of absorbed amounts (\( M_{a,b} \)) were excreted at a rate of 1.2 nmol/min. In contrast, 4.9 ± 0.7\% of perfused genistein was excreted via bile at a rate of 0.2 nmol/min (Fig. 6), and aglycone accounted for less than 5\% of the biliary excretion (below detection). Therefore, the results indicated

---

**TABLE 1**

Kinetic parameters of the glucuronidation of six isoflavones in jejunal and hepatic microsomes

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Gen</th>
<th>Gly</th>
<th>Bio</th>
<th>Pru</th>
<th>Dai</th>
<th>For</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jejunum</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( V_{\text{max}} ) (nmol/min/mg)</td>
<td>3.171</td>
<td>1.92</td>
<td>1.841</td>
<td>1.62</td>
<td>0.646</td>
<td>0.495</td>
</tr>
<tr>
<td>( K_m ) (( \mu \text{M} ))</td>
<td>6.628</td>
<td>2.66</td>
<td>6.652</td>
<td>2.05</td>
<td>2.439</td>
<td>7.250</td>
</tr>
<tr>
<td>( V_{\text{max}}/K_m ) (ml/min/mg)</td>
<td>0.478</td>
<td>0.72</td>
<td>0.276</td>
<td>0.79</td>
<td>0.265</td>
<td>0.068</td>
</tr>
<tr>
<td>Liver</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( V_{\text{max}} ) (nmol/min/mg)</td>
<td>3.713</td>
<td>3.720</td>
<td>1.250</td>
<td>0.343</td>
<td>1.190</td>
<td>1.384</td>
</tr>
<tr>
<td>( K_m ) (( \mu \text{M} ))</td>
<td>23.6</td>
<td>1.034</td>
<td>0.196</td>
<td>0.834</td>
<td>1.119</td>
<td>2.225</td>
</tr>
<tr>
<td>( V_{\text{max}}/K_m ) (ml/min/mg)</td>
<td>0.157</td>
<td>3.598</td>
<td>6.351</td>
<td>0.411</td>
<td>1.063</td>
<td>0.622</td>
</tr>
<tr>
<td>( K_{m2} ) (( \mu \text{M} ))</td>
<td>6.628</td>
<td>0.478</td>
<td>0.256</td>
<td>0.79</td>
<td>0.265</td>
<td>0.068</td>
</tr>
</tbody>
</table>

**TABLE 2**

Kinetic parameters of genistein glucuronidation in different microsomes

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Duodenum</th>
<th>Jejunum</th>
<th>Ileum</th>
<th>Colon</th>
<th>Liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>( V_{\text{max}} ) (nmol/min/mg)</td>
<td>1.445</td>
<td>1.371</td>
<td>1.423</td>
<td>0.435</td>
<td>3.713</td>
</tr>
<tr>
<td>( K_m ) (( \mu \text{M} ))</td>
<td>4.04</td>
<td>6.628</td>
<td>8.239</td>
<td>3.186</td>
<td>23.6</td>
</tr>
<tr>
<td>( V_{\text{max}}/K_m ) (ml/min/mg)</td>
<td>0.357</td>
<td>0.478</td>
<td>0.172</td>
<td>0.136</td>
<td>0.157</td>
</tr>
<tr>
<td>( K_{m2} ) (( \mu \text{M} ))</td>
<td>0.157</td>
<td>3.598</td>
<td>6.351</td>
<td>0.411</td>
<td>1.063</td>
</tr>
<tr>
<td>( R^2 )</td>
<td>0.998</td>
<td>0.995</td>
<td>0.995</td>
<td>0.999</td>
<td>0.985</td>
</tr>
<tr>
<td>AIC</td>
<td>−55.67</td>
<td>−19.13</td>
<td>−21.85</td>
<td>−53.61</td>
<td>−17.9</td>
</tr>
</tbody>
</table>
that genistein glucuronide was excreted to intestinal lumen at rates that were 460% faster than that to bile \((p < 0.05)\).

**Discussion**

Enzyme-transporter coupling, proposed in our previous studies, has been used to describe the intestinal disposition of polyphenolic compounds including isoflavones and raloxifene (Jeong et al., 2004; Jia et al., 2004). Participation in the processes of dual enteric and enterohepatic recycling may explain why flavonoids have poor bioavailabilities (Liu and Hu, 2002; Chen et al., 2003; Jia et al., 2004). Previously, genistein was perfused at 100 \(\mu M\) and 35 \(\mu M\) using a four-site perfusion model (Liu and Hu, 2002; Chen et al., 2003). The results of those studies clearly showed that intestinal excretion of metabolites was a major contributor to the overall metabolism and disposition of genistein. However, a multisite perfusion model was not ideal to mimic the in vivo disposition process, since perfusate containing genistein was introduced to four segments of the intestine simultaneously. In the present studies, we modified the perfusion model by perfusing the whole small intestine as one segment, which better mimicked the in vivo condition. We also determined the glucuronidation of genistein using microsomes prepared from different segments of the intestine, since previous studies always showed that amounts of glucuronides excreted were dependent on the segment perfused, not on amounts of isoflavones absorbed from that segment (Liu and Hu, 2002; Chen et al., 2003). These studies represent our continuing effort to characterize the enteric and enterohepatic recycling processes of genistein and its isoflavone analogs. The characterization is essential to understand the intestinal and hepatic metabolism and disposition of flavonoids, and how these disposition processes cause poor bioavailability.

In the present model of perfused rat intestine, genistein was perfused at a concentration of 10 \(\mu M\) through the whole small intestine, more similar to the in vivo condition than four-site perfusion. A concentration of 10 \(\mu M\) was chosen because it was close to the \(K_m\) values (2–7 \(\mu M\)) of microsomal glucuronidation found in jejunum. The results demonstrated that genistein is well absorbed, and many more glucuronidated genistein conjugates were excreted from intestine (40.3% of \(M_{ab}\)) than from the bile (6.4% of \(M_{ab}\)). Therefore, we believe that intestine is the major organ responsible for glucuronida-
tion of genistein in vivo. The bigger role played by intestinal glucuronidation was most likely the result of much higher isoflavone concentrations in intestinal lumen than in portal vein. Hence, the first-pass intestinal glucuronidation appeared to be the most important factor for genistein disposition and excretion, which suggests that the enteric recycling is likely to be more important than the enterohepatic recycling in determining the disposition of genistein. Additional perfusion studies that investigate other isoflavones which have higher hepatic IC than intestinal IC appear to be warranted to prove whether IC of glucuronidation can predict the relative contribution of intestine versus liver metabolism in flavonoid disposition.

We have previously used a four-site perfusion model to determine whether absorption and metabolism of genistein and analogs are dependent on the region of the intestine perfused. This model, although less similar to in vivo conditions, is more useful to determine the contribution of different regions of the intestine to the disposition of flavonoids. We found that amounts of glucuronides excreted were always region-dependent (Liu and Hu, 2002; Chen et al., 2003; Jia et al., 2004). Furthermore, the fold differences in rates of metabolism between different intestinal microsomes were often much less dramatic than fold differences in rates of excretion between different intestinal segments (Chen et al., 2003). The latter may be due to the actions of various efflux transporters that are responsible for excretion of phase II flavonoid conjugates. In the present studies, we also measured genistein glucuronidation using microsomes prepared from liver and four regions of the intestine. The results indicated that the glucuronidation of genistein was dependent on the type of microsomes used. In intestine, jejunal and duodenal glucuronidation of genistein have a much higher \( V_{\text{max}} \) and IC values than microsomes prepared from other regions of the intestine. However, for genistein, the hepatic microsomes have higher total IC and larger \( V_{\text{max}} \) values, suggesting that the liver remains a formidable barrier to improving genistein bioavailability.

In the present studies, we also noticed that there are substantial differences in the intestinal metabolism of genistein and its five isoflavone analogs. We examined which functional group and its position could affect the metabolism, and found that isoflavones with two electron-donating groups (–OH or –OCH\(_3\)) on the A-ring of isoflavones could significantly increase their glucuronidation rates (Fig. 1). For example, glucuronidation rates of genistein, biochanin A, glycitein, and prunetin were similar but were much faster than those of daidzein and formononetin, which lack an additional electron-donating group in the A-ring (Fig. 1). The functional group effect observed in the intestinal microsomes did not translate directly into similar effects in the liver microsomes, which appeared to have different and more complex structural effects. For example, prunetin, the only isoflavone with a blocked (methylated) 7-hydroxy group among the six, was poorly metabolized and had the smallest \( V_{\text{max}} \) and IC values (Fig. 4A; Table 1). Hence, the 7-hydroxy position appeared to be a main site for glucuronidation in liver, which is quite different from the intestine. The observed importance of the 7-hydroxy group is consistent with our previous study in Caco-2 cells (Chen et al., 2005).

We can safely deduce from the comparison studies conducted in liver and intestinal microsomes that different isoforms of UGT are involved in the glucuronidation of the same isoflavones when using different types of microsomes since they have drastically different kinetic parameters and the same structural changes do not elicit the same response. Furthermore, kinetic studies suggest that different UGT isoforms are likely to contribute toward the metabolism of different isoflavones in the same type of microsomes since Eadie-Hofstee plots are not linear for some isoflavones. For example, more than one UGT isoform was involved in biochanin A glucuronidation in both jejunal and hepatic metabolism. However, one isoform played a dominant role in the glucuronidation of formononetin and daidzein in jejunum and liver.

We recognized the difficulty in identifying which particular isoform is responsible for the metabolism of a particular isoflavone because expressed rat UGT isoforms are not commercially available. We took the approach of matching published expression profiles with our kinetic data because isofrom-specific monoclonal antibodies against UGT are not commercially available. Several groups have published the distribution of UGT isoforms (mRNA) in the tissues/ organs, including the gastrointestinal tract, in rodents (e.g., Grams et al., 2000; Shelby et al., 2003). Their results, obtained using various polymerase chain reaction methods, shared similarities but also showed some quantitative discrepancies. We will focus our discussion using the data of Shelby et al. (2003) since they used Sprague-Dawley rats, whereas Grams et al. (2000) used Wistar rats.

According to the paper by Shelby et al. (2003), mRNA levels of the UGT1A1 subfamily were generally higher in intestine than in liver except for UGT1A5 and UGT1A8. However, the mRNA levels of UGT1A5 and UGT1A8 were much (10-fold) lower than those of the main UGT isoforms including UGT1A1, UGT1A2, UGT1A6, and UGT1A7 (Shelby et al., 2003). On the other hand, the mRNA level of the UGT2B subfamily was always higher in the liver than in the intestine except for UGT2B8. Based on these data, we hypothesized...
that UGT1A6s were responsible for the intestinal metabolism of isoflavones such as prunetin, since the IC value was much higher in the gut than in the liver. We further hypothesized that prunetin was mainly metabolized by UGT1A6, which is highly expressed in both gut and liver and therefore is expected to rapidly metabolize the substrate. In addition, gut and liver microsomal metabolism of prunetin had similar \( K_{\text{m}} \) values and displayed one component in Eadie-Hofstee plots. Lastly, the difference in \( V_{\text{max}} \) values (of prunetin) between intestine and liver (4.4:1) approximates the difference in mRNA level (3.3:1). UGT2Bs were probably involved in the metabolism of isoflavones other than prunetin since they were metabolized more rapidly (with higher IC) in the liver. We did not attempt to assign a particular UGT2B isoform since we did not have data to support their assignment.

In conclusion, a modified whole intestinal perfusion model was more appropriate to determine the relative contribution of intestine versus liver to the disposition of flavonoids such as genistein. We found that intestinal and hepatic conjugation of isoflavones was significantly impacted by the structural differences of isoflavone, and that microsomal metabolism was often more rapid in liver microsomes than in intestinal microsomes. However, intestinal metabolism of genistein and subsequent excretion of its conjugates demonstrate that intestine is still the major first-pass metabolic organ for genistein. In other words, even though genistein is metabolized faster in the liver microsomes than in intestinal microsomes, concentration differences in intestinal lumen and portal vein can change the dynamics of genistein metabolism and disposition in vivo.

References


Address correspondence to: Dr. Ming Hu, Department of Pharmacological and Pharmaceutical Sciences, College of Pharmacy, University of Houston, Houston, TX 77030. E-mail: mhu@uh.edu