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Disposition of Flavonoids via Recycling: Comparison of Intestinal versus Hepatic Disposition.

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ABSTRACT

The purpose of this study was to compare intestinal versus hepatic disposition of six flavonoids in order 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 K_m values (>2 folds). Likewise, the hepatic intrinsic clearance (IC, or a ratio of V_{max}/K_m) values of isoflavones were generally higher than their intestinal IC values (200%-2000% higher), except for prunetin, whose jejunal IC value was 50% higher than its hepatic IC. When comparing intestinal metabolism, the results showed that intestinal metabolism rates and V_{max} values of isoflavones were less when an additional A-ring electron-donating group was absent (i.e., daidzein and formononetin). In 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.

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INTRODUCTION

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 post-menopausal

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, a Chinese herb found to

be active against alcohol abuse (Lukas et al, 2005), is mainly found in Kudzu roots.

Currently, soy isoflavones are undergoing clinical trials for prostate cancer prevention

(Takimoto et al, 2003).

Even though there is substantial amount of information pointing to their anticancer and

other health beneficial effects, isoflavones have poor bioavailabilities (Setchell et al.,

2001; Setchell et al., 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 we can develop viable

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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 MRPs (Chen et al, 2004, Hu et al, 2003), 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.

MATERIALS AND METHODS

Materials. Isoflavones genistein, daidzein, glycitein, formononetin, and prunetin were purchased from Indofine Chemical Company (Wayne, NJ) or LC Laboratories (Woburn, MA). β-Glucuronidase with (Catalog#G1512) or without sulfatase (Catalog#G7396), sulfatase without glucuronidase (Catalog#S1629), uridine diphosphoglucuronic acid (UDPGA), alamethicin, D-saccharic-1, 4-lactone monohydrate, biochanin A, magnesium chloride, and Hank's balanced salt solution (HBSS, powder form) were purchased from Sigma Chem. Co. (St Louis, MO). HPLC grade acetonitrile and methylene chloride were purchased from Fisher Scientific (Pittsburgh, PA). Other chemicals were obtained from reputable commercial sources.

Microsomes Preparation from Rat Intestine or Liver. Adult male Sprague-Dawley rats (200-250 g) were employed 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 laboratories, CA) as described (Chen et al, 2003).

Isoflavones Glucuronidation Using the Microsomes from Rat Intestine or Liver.

Intestinal and hepatic glucuronidation of six selected isoflavones (i.e., genistein, daidzein, biochanin 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 170 µl 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 in "**HPLC Analysis of Isoflavones and Their Conjugates**".

Transport and Metabolism Experiments in Perfused Rat Intestinal Model. The procedures were approved by Washington State University's Institutional Animal Care and Uses Committee. The rat surgical procedure for this study was similar to our previous published paper (Chen et al., 2003; Hu et al, 1988; 1998) with one significant modification, in that the whole small intestine from the upper duodenum to the end of 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.

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Processing of Biological Samples from Rat. A 200 µl portion of the intestinal perfusate was mixed with 50 µl Stop Solution including the internal standard. The mixture was centrifuged at 13,000 rpm for 8 min, and supernatant was introduced into HPLC as described later in "**HPLC Analysis of Isoflavones and Its Conjugates"**.

Each blood or bile sample was divided into two portions for quantitative measurement of genistein 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 HPLC system for free genistein. The other portion of sample (e.g., 200 µl) was added β-glucuronidase to completely hydrolyze genistein glucuronide to aglycone. The hydrolyzed sample containing total genistein was then extracted and amounts measured as described above. The amounts of glucuronidated genistein were equal to the difference between total genistein and free genistein.

HPLC Analysis of Isoflavones and Their Conjugates. The conditions for HPLC analysis of biochanin A, prunetin and formononetin and their glucuronides were the same as a previously published method (Chen et al, 2003). We prolonged the elution time with a shallower gradient to analyze genistein, daidzein and glycitein and their glucuronides. The conditions were: HPLC system, Agilent 1090 with dioarray detector and Chemstation; column, Aqua (Phenomenex, Guilory, 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) triethylamine (pH2.8); mobile phase B, 100% acetonitrile. The

gradient used for elution was: 0-3 min, 2%B; 3-35 min, 2%-50%B; 35-37 min, 50%B.

There was a five-minute 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 were shown in Figure 1.

Data Analysis.

In the perfused rat intestinal model, the calculation methods for amounts of genistein

perfused or infused (M_{in}) through intestine, amounts of glucuronidated genistein excreted

into the intestinal lumen (M_{gut}), amounts of genistein absorbed (M_{ab}), and amounts of

glucuronidated genistein excreted via bile (M_{bile}) were the same as previously described

(Chen et al., 2003):

$$M_{in} = Q \, \tau \, C_{in} \tag{1}$$

$$M_{gut} = Q \, \tau \, C_{out} \tag{2}$$

$$M_{ab} = Q \, \tau \, (C_{in}\text{-}C_{out}) \tag{3}$$

$$M_{bile} = V \, C_{bile} \tag{4}$$

Q: infusion rate (ml/min); τ: sampling time interval (min); C_{out}: concentration of

isoflavone aglycone or its glucuronide in outlet perfusate (nmol/ml) which is corrected

for water flux using ^{14}C-PEG-4000; C_{in}: inlet concentration of isoflavone aglycone; V:

volume of bile excreted over 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

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Eadie-Hofstee plot is linear:

$$V = \frac{V_{max} \times C}{K_m + C} \qquad (5)$$

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 per mg protein or nmol/min/mg.

When Eadie-Hofstee plots suggest 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 the 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 (MAICE), 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 to the following equation:

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$$\text{Reaction rate} = \frac{[V_{\text{max-0}} + V_{\text{max}-d}(1 - e^{-CR})] \times C}{K_m + C} \qquad (6)$$

where

$V_{\text{max-0}}$ - maximum intrinsic enzyme reaction rate

$V_{\text{max-d}}$ - maximum inducible enzyme reaction rate

R -   rate of enzyme activity induction

C -   concentration of substrate

K_m -   concentration of substrate to achieve 50% of $(V_{\text{max-0}} + V_{\text{max-d}})$

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 to the following equation (7):

$$\text{Reaction rate} = \frac{V_{\text{max1}} \times C}{K_{m1} + C} + \frac{V_{\text{max2}} \times C}{K_{m2} + C} \qquad (7)$$

where

V_{max1} - maximum enzyme reaction rate of one UGT isoform

V_{max2} - maximum enzyme reaction rate of another UGT isoform

K_{m1} concentration of substrate to achieve 50% of V_{max1}

K_{m2}-   concentration of substrate to achieve 50% of V_{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 to the following equation:

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$$\text{Reaction rate} = \frac{V_{\max}}{(1 + (K_m / C) + (C / K_{si}))} \qquad (8)$$

where

$V_{\max}$ -   maximum enzyme reaction rate

C -   concentration of substrate

K_m -   concentration of substrate to achieve 50% of $V_{\max}$

K_{si} -   substrate inhibition constant

Statistical Analysis

One-way ANOVA 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 of 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 were different ($p<0.05$) in microsomes prepared from different segment 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 difference 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 µM) 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 in Jejunal Microsomes as a Function of Concentration. The glucuronidation rates of six isoflavones as a function of their concentrations in jejunal microsomes were determined since jejunum is 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

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kinetic equations using ADAPTII program.   The actual equation used was based on

shapes of 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 in the method section.   This approach

generated excellent fittings and kinetic parameters were shown in Table 1 and 2.   The

V_{max} value of genistein was the highest (3.17 nmol/min/mg protein), followed by

biochanin A (sum of two V_{max} values), glycitein, and prunetin (1.998, 1.92, and 1.62

nmol/min/mg protein, respectively). V_{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

µM (formononetin).   The K_m values of daidzein, glycitein, and prunetin were

approximately the same to each other (2.05-2.66 µM), and those of the other three

isoflavones (genistein, biochanin A and formononetin) were also approximately the same

to each other (6.628-7.25 µM).   The ratios of V_{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 IC of first component (0.276) and V_{max}

value of second component since K_m of second component is very small and intestinal

concentration of biochanin A is likely to significantly surpass that K_m value.   We have

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therefore, used the ratio of V_{max} over a concentration of 1 µM 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 µM is less than glycitein, prunetin and genistein, but higher than the other two isoflavones. The same approach will be used in liver metabolism when the K_m value (of second component) is less than 0.01 µM.

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 were also measured in liver microsomes (Fig. 4A). The corresponding kinetic parameters were determined using various kinetic models and listed in Table 1. All data were fit nicely with the chosen model except for glycitein, whose data were fit to substrate inhibition model and produced kinetic parameters that could describe the kinetic for concentrations up to 15 µM. We did not think this as a serious concern since in vivo portal vein concentration was expected to be much less than 1 µM. Based on the fitted parameters, V_{max} values of genistein and glycitein were the highest at ~3.7 nmol/min/mg protein followed by biochanin A, formononetin, and daidzein (2.89, 1.38, and 1.19 nmol/min/mg protein), respectively. The V_{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 value) in the liver microsomes than in intestinal microsomes, although the range was quite large 0.196 µM (biochanin A) to 23.6 µM (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 -folds (genistein) to ~20

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-folds (biochanin A).   The only exception was that the jejunal IC value of prunetin was

~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 other three regions of the intestine:

duodenum, ileum, and colon. The results were then combined with jejunal and hepatic

metabolism and plotted in Fig. 5.   Genistein glucuronidation was saturable and the

kinetics of glucuronidation (K_m, V_{max} and V_{max}/K_m values) were determined (Table 2).

Hepatic metabolism had the highest V_{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_{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, 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

μM) and excretion of genistein conjugates in male rats by perfusing the whole small intestine. In a steady-state perfusion, the amounts disappeared 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 30min to 120min 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_{in}) or 40.3% of absorbed amounts (M_{ab}) 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 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 µM and 35 µM using four-site perfusion model (Liu and Hu, 2002; Chen et al., 2003). The results of those studies clearly showed that intestinal excretion of metabolites were a major contributor to the overall metabolism and disposition of genistein. However, multi-site 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, 20002; 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 in order 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 µM through the whole small intestine, much similar to *in vivo* condition than four-site

perfusion.   A concentration of 10 µM was chosen because it was close to the K_m values

(2-7 µM) of microsomal glucuronidation found in jejunum.   The results demonstrated

that genistein is well absorbed and much more glucuronidated genistein conjugates was

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 glucuronidation of genistein in

vivo.   The bigger role played by intestinal glucuronidation was most likely the results of

much higher isoflavone concentration 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 suggest 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 if IC of glucuronidation can predict

relative contribution of intestine versus liver metabolism in flavonoid disposition.

We have previously used four-site perfusion model to determine if absorption and

metabolism of genistein and analogs are dependent on region of the intestine perfused.

This model, whereas 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 has a much higher V_{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_{max} value, 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 that of daidzein and formononetin, which lacks 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-hydroxyl group among the six, was

poorly metabolized and had the smallest V_{max} and IC values (Fig. 4A; Table 1).   Hence,

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7-hydroxyl position appeared to be a main site for glucuronidation in liver, which is quite different from the intestine. The observed importance of 7-hydroxyl group is consistent with our previous study in Caco-2 cells (Chen et al., 2005).

We can safely deduct from the comparison studies conducted in liver and intestinal microsomes that different isoforms of UGT are involved in the glucuronidation of same isoflavones when using different types of microsomes since they have drastically different kinetic parameters and same structural change do not elicit same response. Furthermore, kinetic studies suggest that different UGT isoforms are likely to contribute towards 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 were 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 isoform 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., Shelby et al, 2003; Grams et al, 2000). Their results, obtained using various PCR methods, shared similarities but also show some quantitative discrepancies.

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We will focus our discussion using Shelby et al's (2003) data since they used

Sprague-Dawley rats, whereas Grams et al used (2000) Wistar rats.

According to the paper by Shelby et al (2003), mRNA levels of UGT1A subfamily were

generally higher in intestine than liver except for UGT1A5 and UGT1A8.   However, the

mRNA levels of UGT1A5 and UGT1A8 were much (10-fold) lower than the main UGT

isoforms including UGT1A1, UGT1A2, UGT1A6, and UGT1A7 (Shelby et al, 2003).

On the other hand, mRNA level of UGT2B subfamily was always higher in the liver than

in the intestine except for UGT2B8.   Based on these data, we hypothesized that

UGT1As were responsible for the intestinal metabolism of isoflavones such as prunetin

since IC value was much higher in the gut than in the liver.   We further hypothesized

that prunetin was mainly metabolized by UGT1A6, which are highly expressed in both

gut and liver and therefore expected to rapidly metabolize the substrate.   In addition, gut

and liver microsomal metabolism of prunetin had similar K_m values and displayed one

component in Eadie-Hofstee plots.   Lastly, the difference in V_{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 isoflavone's structural differences, 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.

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Figure Legends:

Fig. 1. Chemical structures of six isoflavones and HPLC elution profiles of these isoflavones (i.e. genistein, biochanin A, glycitein, prunetin, daidzein, and formononetin) and their glucuronidated metabolites in rat duodenal microsomal incubation mixtures. A solution 12.5 μM of isoflavone was used in incubation solution. Glucuronidated isoflavone are labeled as M; biochanin A (Bio) and prunetin (Pru) formed two metabolites that are labeled as M1 (major metabolite) and M2 (minor metabolite). The formation of the major metabolites were measured in this study. Genistein, daidzein, glycitein and formononetin are labeled as Gen, Dai, Gly and For, respectively. Metabolic samples of genistein, daidzein and glycitein were eluted in a more shallow gradient method with a longer elution time (Fig. 1A) than that of biochanin A, prunetin, and formononetin (Fig. 1B). Testosterone was used as the internal standard (IS, 4 nmol), which had different retention times in two different elution gradients (see "HPLC Analysis of Isoflavones and Their Conjugates")

Fig. 2. Comparison of the six isoflavones glucuronidation in rat intestinal microsomes prepared from duodenum, jejunum, ileum and colon. An isoflavone (12.5 μM) was used in the reaction mixture. Each column represents the average of three determinations and the error bar is the S.D. There were significant statistical differences ($p < 0.05$) among glucuronidation rates of each isoflavone in microsomes prepared from different regions of the intestine, which are marked by the symbol "*" with underline covering the rates (of glucuronidation) being compared.

Fig. 3. Glucuronidation of the six isoflavones in rat jejunal microsomes as a function of concentration (Fig. 3A). The points are observed isoflavone glucuronide formation rates, and the curves are estimated based on fitted parameters generated by the Michaelis-Menten equation (1) and other equations that describe various enzyme kinetics. The apparent kinetics parameters are listed in Table 1. Eadie-Hofstee plots were generated to determine which equation to use for data fitting (Fig. 3B). Lower concentrations of prunetin and glycitein were used because their K_m values were low (<5 μM). Each data point is the average of three determinations and the error bar is the standard deviation of the mean.

Fig. 4. Glucuronidation of the six isoflavones in rat hepatic microsomes as a function of concentration (Fig. 4A). The points are observed values, and the curves are estimated based on fitted parameters generated by the Michaelis-Menten equation (1) and other equations that describe various enzyme kinetics. The kinetics parameters are also listed in Table 1. Eadie-Hofstee plots are also generated in Fig. 4B. Each data point is the average of three determinations and the error bar is standard deviation of the mean.

Fig. 5. Genistein glucuronidation as a function of concentration in rat hepatic and intestinal microsomes prepared from duodenum, jejunum, ileum and colon. The points are observed genistein glucuronidation rates and the curves are estimated based on fitted parameters generated by the Michaelis-Menten equation (1) and other equations that describe enzyme kinetics. The kinetics parameters are presented in Table 2. Eadie-Hofstee plots are also generated in Fig. 5B. Each data point is the average of

three determinations and the error bar is standard deviation of the mean.

Fig. 6. Amounts of genistein (Gen) absorbed (M_{ab}), and genistein glucuronide (Gen-glu) excreted into intestine lumen perfusate (M_{gut}) or bile (M_{bile}) as a function of time (Fig. 6A).   Rates of genistein infused through intestine, genistein absorbed in intestine, Gen-glu excreted back to intestine lumen perfusate and Gen-glu excreted via bile are shown in Fig. 6B.   A 10 µM solution of genistein is used as an inlet perfusate to infuse the whole small intestine at a flow rate of 0.382 ml/min using protocol described in the "Method" section.   Amounts of genistein infused (M_{in}) through intestine, M_{gut}, M_{ab}, and M_{bile} are calculated using equation (2) – (4), respectively.   Genistein infused rate is 3.82 nmol/min.   The rates of absorption, intestinal or excretion are presented in Fig. 6B. Each data point is the average of three determinations and the error bar is standard deviation of the mean.

Table 1. Kinetic parameters of six isoflavones glucuronidation in jejunal and hepatic microsomes

|	Parameters	Gen	Gly	Bio	Pru	Dai	For
Jejunum	$V_{max(total)}$ (nmol /min/mg)	3.171	1.92	1.841	1.62	0.646	0.495
	K_{m1} (µM)	6.628	2.66	6.652	2.05	2.439	7.250
	V_{max1}/K_{m1} (ml /min/mg)	0.478	0.72	0.276	0.79	0.265	0.068
	V_{maxO} (autoact) (ml/min/mg)	-	-	-	-	-	-
	V_{maxD} (autoact) (nmol /min/mg)	-	-	-	-	-	-
	V_{max2} (biphasic) (nmol/min/mg)	-	-	0.157	-	-	-
	K_{m2} (µM)	-	-	1.73E-4	-	-	-
	R^2	0.995	0.997	0.972	0.991	0.999	0.981
	R (autoact only)	-	-	-	-	-	-
	AIC	-19.13	-35.72	-15.83	-33.89	-52.96	-42.32
Liver	V_{max1} (nmol /min/mg)	3.713	3.720	1.250	0.343	1.190	1.384
	K_{m1} (µM)	23.6	1.034	0.196	0.834	1.119	2.225
	V_{max1}/K_{m1} (ml /min/mg)	0.157	3.598	6.351	0.411	1.063	0.622
	V_{max2} (biphasic) (nmol /min/mg)	0.826	-	1.647	-	-	-
	K_{m2} (µM)	0.71E-7	-	16.65	-	-	-
	Ksi (inhibition)	-	5920	-	-	-	-
	R^2	0.989	0.960	0.991	0.978	0.991	0.985
	R (autoact only)	-	-	-	-	-	-
	AIC	-17.9	-9.13	-27.01	-32.82	-42.93	-31.89

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Table 2. Kinetic parameters of genistein glucuronidation in different microsomes

Parameters	Duodenum	Jejunum	Ileum	Colon	Liver
V_{max1} (nmol /min/mg)	1.445	3.171	1.423	0.435	3.713
K_{m1} (μM)	4.04	6.628	8.239	3.186	23.6
V_{max1}/K_{m1} (ml /min/mg)	0.357	0.478	0.172	0.136	0.157
V_{max2} (Biphasic) (nmol /min/mg)	-	-	0.041	1.59E3	0.826
K_{m2} (μM)	-	-	7.994	2.74E5	0.71E-7
R^2	0.998	0.995	0.963	0.995	0.989
AIC	-55.67	-19.13	-21.85	-53.61	-17.9

Figure 1
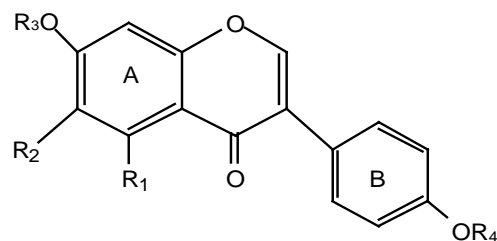
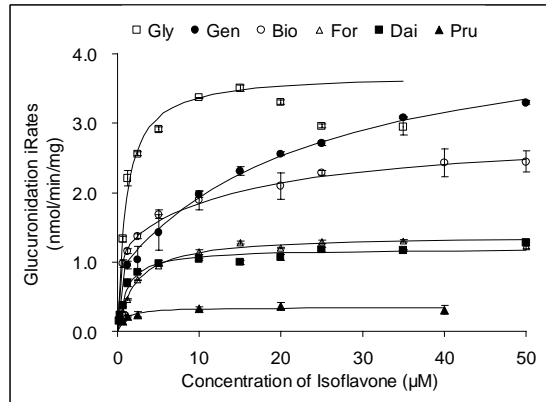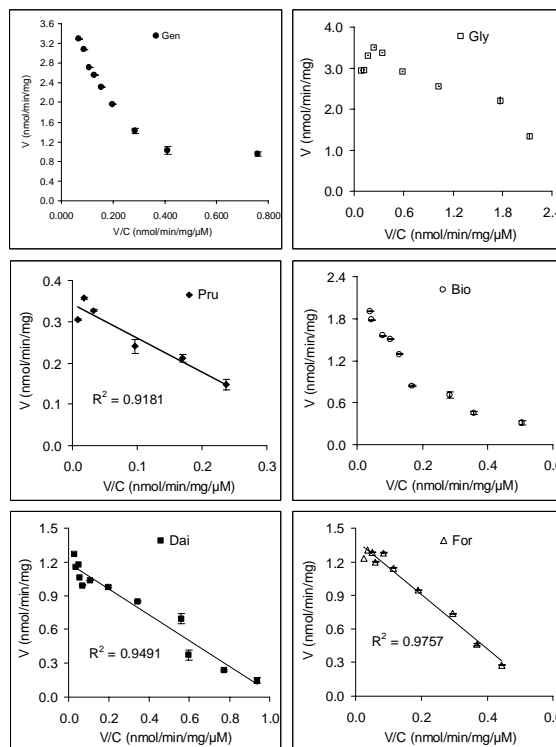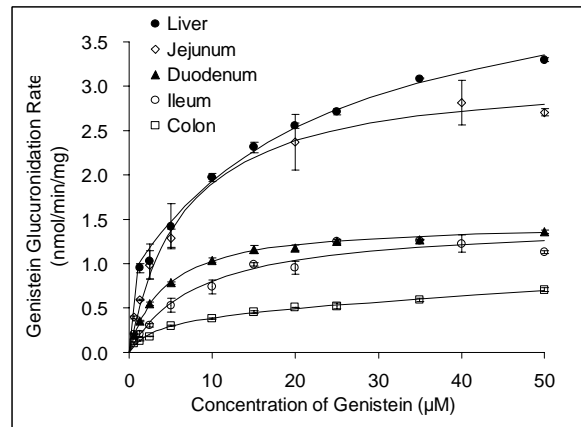| R_1	R_2	R_3	R_4	Compounds
H	H	H	H	Daidzein
OH	H	H	H	Genistein
H	OCH_3	H	H	Glycitein
H	H	H	CH_3	Formononetin
OH	H	H	CH_3	Biochanin A
OH	H	CH3	H	Prunetin
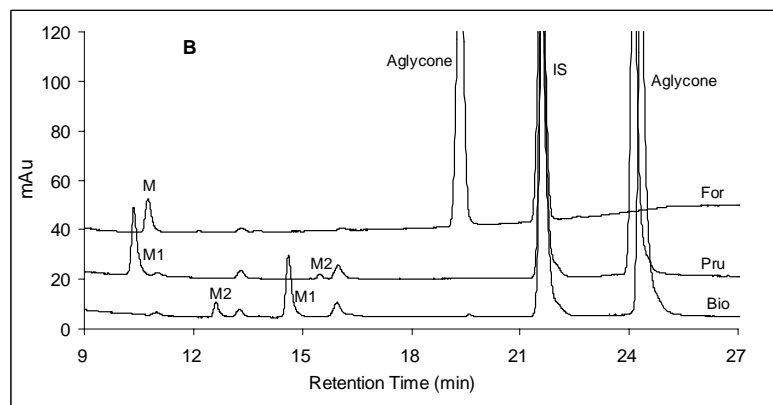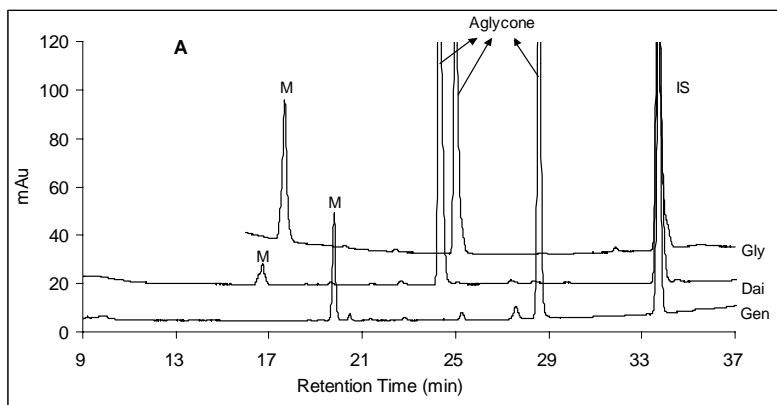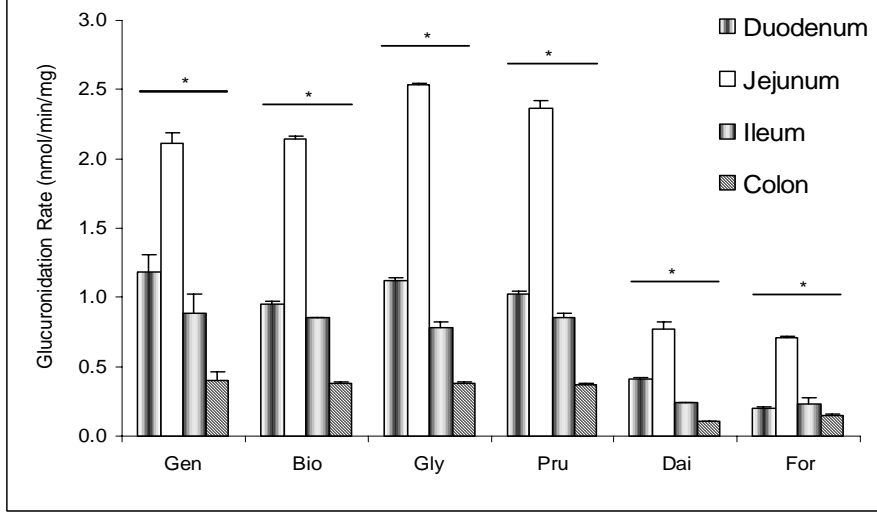
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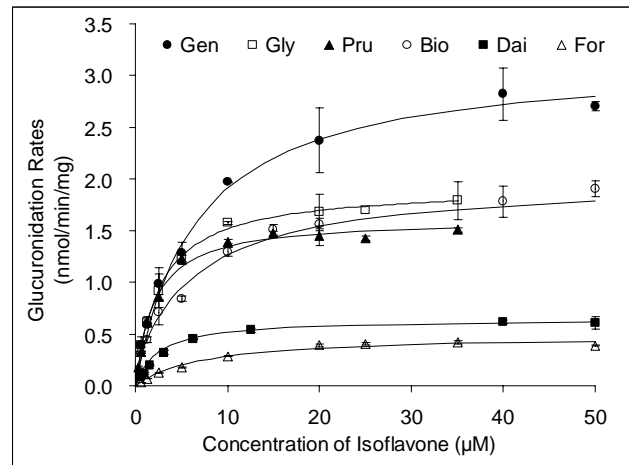
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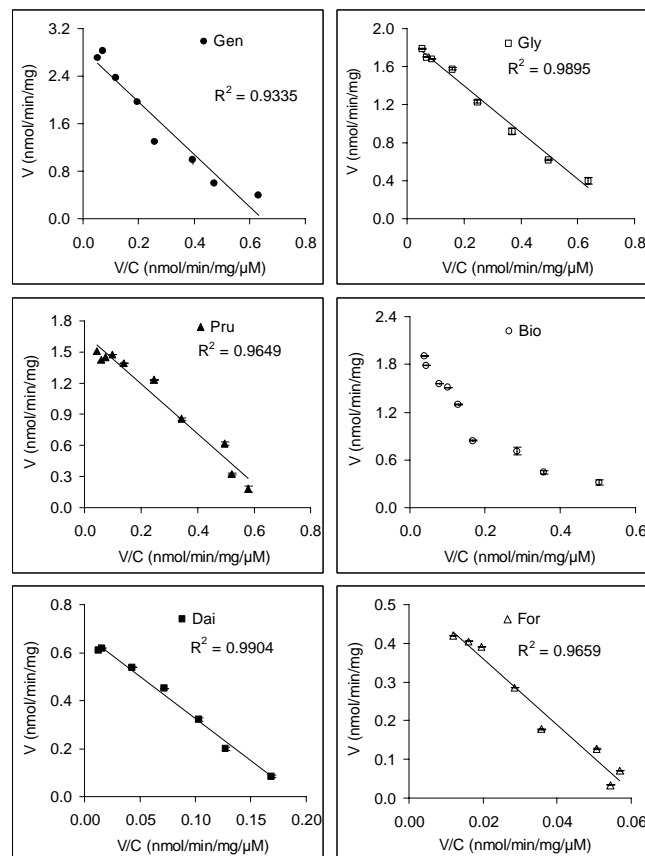
Figure 4A

Figure 4B

Fig.5A

Fig.5B

Figure 6A

Figure 6B