

# Disposition of Flavonoids via Enteric Recycling: Enzyme-Transporter Coupling Affects Metabolism of Biochanin A and Formononetin and Excretion of Their Phase II Conjugates

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**ABBREVIATIONS:** HBSS, Hank's balanced salt solution; UGT, UDP-glucuronosyltransferases;  $P_{BA}$ , secretory permeability; NADP, nicotinamide-adenine-dinucleotide phosphate (NADP);  $M_{ab}$ , amounts of isoflavones absorbed;  $M_{gut}$ , amounts of conjugated isoflavones excreted into the intestinal lumen;  $M_{bile}$ , amounts of conjugated isoflavones excreted via the bile;  $M_{total}$ , total amounts of isoflavones absorbed from all four perfused segment;  $P_{eff}$ , dimensionless effective permeabilities

## ABSTRACT

The purpose of this study was to continue our effort to determine how enzyme-transporter coupling affect disposition of flavonoids. The rat intestinal perfusion and Caco-2 cell models were used together with relevant microsomes. In perfusion model, isoflavone (i.e., formononetin and biochanin A) absorption and subsequent excretion of its metabolites were always site-dependent. Maximal amounts of intestinal and biliary conjugates excreted per 30 min were 31 and 51 nmol for formononetin, more than that for pure biochanin A (12 and 20 nmol). When a standardized red clover extract (biochanin A :formononetin =10:7) was used, the results indicated that more metabolites of biochanin A than formononetin were found in the perfusate (36.9 vs. 22.8 nmol) and bile (78 vs. 51 nmol). In metabolism studies, rat intestinal and liver microsomes always glucuronidated biochanin A faster ( $p<0.05$ ) than formononetin, whereas intestinal microsomes glucuronidated both isoflavones faster ( $p<0.05$ ) than liver microsomes. However, rapid metabolism in the microsomes did not translate into more efficient excretion in either the rat perfusion model as shown previously, or in the Caco-2 model. In the Caco-2 model, both isoflavones were rapidly absorbed, efficiently conjugated, and the conjugates excreted apically and basolaterally. More formononetin conjugates were excreted than biochanin A when used alone but much more biochanin A conjugates were found when using the isoflavone mixture. In conclusion, efficiency of enzyme-transporter coupling controls the amounts of metabolites excreted by the intestine and liver, and determines the relative contribution of enteric and enterohepatic recycling to the in vivo disposition of isoflavones.

Red clover (*Trifolium pratense* L.) extracts are sold as dietary supplements in supermarkets and health food stores in both industrialized and developing countries. Several defined extracts of Red clover are sold in the United States to treat women who suffer from menopausal related symptoms (Rijke et al., 2001; Oleszek and Stochmal, 2002). The extracts are available as tablets (e.g. Promensil<sup>TM</sup>), capsules, tea, liquid preparations and several other forms. Limited clinical studies have demonstrated the effectiveness of Promensil<sup>TM</sup> in the management of hot flashes (Peter et al, 2002), though the efficacy of such products is not always consistent (Baber et al., 1999; St. Germain et al., 2001).

Red clover extract contains many of the same isoflavones as soy (Lin et al., 2000). However, a majority of isoflavones in red clover are present in aglycone forms whereas they are present as glucosides in soy and soy extracts. Furthermore, the principal isoflavones are biochanin A and formononetin in red clover but are genistein and daidzein in soy. Nevertheless, these isoflavones all have similar biological activities and biochanin A and formononetin can be converted to genistein and daidzein by cytochrome P450 (Tolleson et al, 2002; Hu et al, 2003a). Moreover, daidzein can be metabolized to highly active equol (Xu et al., 1995; Moyad, 2000; Sathyamoorthy and Wang, 1997).

Isoflavones are the main class of phytoestrogens that has been gathering increasing interest in clinical nutrition and disease prevention (Tham et al., 1998; Setchell and Cassidy, 1999). Isoflavones exert purported health benefits in aging-related and hormone-dependent disorders, including but are not limited to cancer, menopausal symptoms, cardiovascular disease and osteoporosis (Tham et al., 1998; Setchell and Cassidy, 1999; for reviews, see Kurzer and Xu, 1997; Birt et al., 2001; Yang et al., 2001).

Despite their reported biological activities *in vitro*, in several animal models and in limited human studies, isoflavones have poor bioavailability (generally in the range of less than 5%) and absorption varies considerably among individuals (Kelly et al., 1995; Setchell, 1998; Setchell et al, 2001; Busby et al, 2002; Birt et al, 2001). This was attributed in part to differences in the intestinal microflora because it was thought that the naturally occurring isoflavone glycosides must be

cleaved first for more rapid absorption. However, recent investigation from several investigators including us has demonstrated the presence of intestinal lactase phlorizin hydrolase that are capable of rapidly hydrolyzing the glucosides (Liu and Hu, 2002; Day et al, 2003; Sesink et al, 2003; Wilkinson et al, 2003). The presence of this enzyme in the upper intestinal tract makes it more important to study the absorption and metabolism of isoflavone aglycones since we showed equally efficient absorption of glucosides in rats when comparing to that of aglycones (Liu and Hu, 2002; Liu et al, 2003).

Previously, single-dose pharmacokinetic study suggested that the bioavailability of genistein and daidzein differs in different mixes of the two isoflavones (Busby et al., 2002). In the present study, we investigated the intestinal absorption and metabolism of biochanin A and formononetin, two of the predominant isoflavones in red clover, which are also prodrugs of genistein and daidzein, respectively (Knight et al, 1996, Hu et al, 2003a). We also studied how these two compounds are absorbed and metabolized in the intestine when it given as a standard red clover extract. This investigation represents our continued effort in testing the hypothesis that efflux transporters play a more important role in determining the extent and rate of cellular (e.g., intestinal) excretion of hydrophilic phase II conjugates than phase II enzymes themselves. The studies will be conducted using the Caco-2 and intestinal perfusion models, which has been used previously for the study of flavonoid disposition in the intestine via the enteric recycling mechanisms. Enteric recycling is a concept we proposed to explain the in vivo disposition of flavonoids (Liu and Hu, 2002). It is similar to enterohepatic recycling except the conjugates are excreted by the enterocytes.

## MATERIALS AND METHODS

**Materials.** Cloned Caco-2 cells, TC7, were a kind gift from Dr. Monique Rousset (Institut National de la Sante et de la Recherche Medicale U178, Villejuif, France). Biochanin A and formononetin were purchased from Indofine Chemicals (Somerville, NJ). A popular red clover product, Promensil™ tablets (Lot#2L0449R3106) made by Novogen Ltd (Australia, lot #) were purchased from Drugstore.com.  $\beta$ -Glucuronidase with (catalog no. G1512) or without sulfatase (catalog no. G7396), sulfatase without glucuronidase (catalog no. S1629), uridine diphosphoglucuronic acid, alamethicin, D-saccharic-1,4-lactone monohydrate, magnesium chloride, Tris, and Hanks' balanced salt solution (HBSS, powder form) were purchased from Sigma-Aldrich (St Louis, MO). All other materials (typically analytical grade or better) were used as received.

**Cell Culture.** The culture conditions for growing Caco-2 cells have been described previously (Hu et al., 1994a,b; Liu and Hu, 2002). The seeding density ( $100,000 \text{ cells/cm}^2$ ), growth media (Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum), and quality control criteria were all implemented in the present study as they were described previously (Hu et al., 1994a,b). Caco-2 TC7 cells were fed every other day, and the monolayers ( $4.2 \text{ cm}^2$  total areas) were ready for experiments from 19 to 21 days post seeding.

**Transport Experiments in the Caco-2 Cell Culture Model.** Experiments in triplicate were performed in pH 7.4 HBSS (Hu et al., 1994a,b). The protocol for performing cell culture experiments is the same as that described previously (Liu and Hu, 2002; Chen et al, 2003; Hu et al, 2003b).

**Animals.** Male Sprague-Dawley rats (150–170 days old) weighing between 400 and 450 g were from Simonsen Laboratory (Gilroy, CA). The rats were fed with Teklad F6 rodent diet (W) from Harlan Laboratories (Madison, WI). The rats were fasted overnight before the day of the experiment. No flavonoids were found in pH 7.4 HBSS buffer that had been perfused through a segment of jejunum, indicating minimal presence of dietary flavonoids in the intestine.

**Animal Surgery.** The procedures were approved by Washington State University's Institutional

Animal Care and Uses Committee. The intestinal surgical procedures were modified from our previous publications (Hu et al., 1988, 1998), in that we perfused four segments of the intestine simultaneously (a “four-site model”) and added a bile duct cannulation. The circulation to the liver and intestine was not disrupted in this model. We have described the surgical procedures (Chen et al., 2003). To keep the temperature of the perfusate constant, the inlet cannulate was insulated and kept warm by a 37°C circulating water bath.

**Transport and Metabolism Experiments in Perfused Rat Intestinal Model.** A single-pass perfusion method that was described previously was used here by simultaneously perfusing four segments of the intestine (duodenum, upper jejunum, terminal ileum, and colon) (Chen et al, 2003). A flow rate of 0.191 ml/min was used, and four perfusate and bile samples were collected every 30 min as described (Chen et al, 2003). The outlet concentrations of test compounds in the perfusate were determined by HPLC. Bile samples were diluted (1:10) with buffer, added glucuronidase + sulfatase, and reacted for 6 h to release the aglycones for HPLC measurement.

**Rat Intestinal Microsomes and Liver Microsomes Preparation.** Rat intestinal microsomes and male rat liver microsomes were prepared from adult Sprague-Dawley rats using a procedure adopted from the literature with minor modification (Okita et al., 1993; Chen et al., 2003). The resulting microsomes were suspended in 250 mM sucrose solution, and separated into microcentrifuge tubes, and stored at -80°C until use.

**Measurement of Protein Concentration.** Protein concentrations of microsomes and cell lysates were determined using a protein assay (Bio-Rad, Hercules, CA), using bovine serum albumin as the standard.

**Measurement of UGT Activities Using Microsomes.** The incubation procedures for measuring UGT activities using microsomes were used as described previously (Chen et al, 2003).

**Conversion of Biochanin A to Genistein Using Rat Liver Microsomes.** The incubation procedures were as follows: 1) mix microsomes (0.4 mg protein/ml), magnesium chloride (0.88 mM), NADP, glucose-6-phosphate dehydrogenase, glucose-6-phosphate; different concentrations



of substrates in a 100 mM potassium phosphate buffer (pH 7.4); 2) incubate the mixture (final volume=200  $\mu$ l) at 37°C for 30 min; and 3) stop the reaction by the addition of 50  $\mu$ l of 94% acetonitrile/6% glacial acetic acid containing 100  $\mu$ M testosterone as an internal standard.

**Sample Extraction.** To confirm the formation of a particular conjugate, we selectively extracted samples with methylene chloride to remove >90% of aglycones. The resulting sample was then divided into two parts, one of which was analyzed directly, whereas the other part was analyzed after glucuronidase or sulfatase hydrolysis. The difference in amount of aglycones found in these two samples was the amount of metabolites formed. The relationship between the peak areas of the metabolites before hydrolysis and the peak areas of aglycones after the hydrolysis is used to establish the conversion factor used to quantify the amounts of biochanin A and formononetin conjugates as described previously (Liu and Hu, 2002).

**Red Clover Extraction.** Promensil™ tablets were scraped to remove the coating and grinded into powder. A 900mg portion of the powder was extracted with 8 ml ethanol, sonicated for 30min, and centrifuged at 1000rpm for 5 min. The concentration of formononetin and biochanin A in the supernatant ethanol extract were 6 mM and 8.5 mM, respectively.

**HPLC Analysis of Isoflavones and Their Conjugates.** The conditions for analyzing biochanin A, formononetin, and their conjugates were as follows: system, Agilent 1090 with diode array detector and ChemStation; column, Aqua (Phenomenex, Gilroy, CA), 5  $\mu$ m, 150×0.45 cm; mobile phase A, water (0.04% phosphoric acid and 0.09% triethylamine, pH 6.0); mobile phase B, 100% acetonitrile; gradient, 0 to 3 min, 15% B, 3 to 15, 15 to 19% B, 15 to 52, 19 to 41% B; wavelength, 254 nm (for formononetin, biochanin A and the internal standard); and injection volume, 200  $\mu$ l. There was a 4-min interval between the end of the run and the next injection to allow the column to be reequilibrated with 15% mobile phase B (Fig. 1). For biochanin A, two major metabolite peaks eluted earlier than the parent compound (designated as biochanin A-M1 and biochanin A-M2) (Fig.1). On the other hand, there was only one glucuronidated metabolite for formononetin (designated as formononetin-M) (Fig. 1).

HPLC condition for separating various phase I metabolites of biochanin A and formononetin from their parent compounds were published previously (Hu et al, 2003a).

**Data Analysis.** Permeability of the parent isoflavones were represented by  $P^*_{\text{eff}}$ , which was obtained as described previously (Hu et al., 1988, 1995; Liu et al., 2002). Amounts of isoflavones absorbed ( $M_{\text{ab}}$ ), amounts of conjugated isoflavones excreted into the intestinal lumen ( $M_{\text{gut}}$ ), amounts of conjugated isoflavones excreted via the bile ( $M_{\text{bile}}$ ) and the percent absorbed and percent metabolized values were calculated as described (Chen et al., 2003). Rates of metabolism in intestinal or liver microsomes were expressed as amounts of metabolites formed per min per mg protein or nmol/min/mg.

One-way ANOVA or an unpaired Student's *t* test (Microsoft Excel) was used to analyze the data. The prior level of significance was set at 5%, or  $p < 0.05$ .

## RESULTS

**Regional Absorption and Metabolism of Pure Formononetin and Biochanin A.** Absorption of formononetin and biochanin A (10  $\mu$ M each) was rapid (Table 1) with minimal effects on water flux (i.e., water flux < 0.5%/cm of perfused segment). However, there were differences between amounts absorbed for these two compounds in different regions of the intestine (Fig. 2). For formononetin, amounts absorbed was the highest in the colon (39.1 nmol/30min or 67%, normalized to 10 cm of intestine for all perfusion results) and the lowest in the terminal ileum (13.5 nmol/30min or 23%) ( $p < 0.05$ , Fig. 2A). For biochanin A, amounts absorbed in duodenum (47.7 nmol/30min or 81% of perfused amounts) and colon (50.8 nmol/30min or 89%) were higher ( $p < 0.05$ ) than that in jejunum (31.5 nmol/30min or 49%) and terminal ileum (30.5 nmol/30min or 54%).

In the rat intestine and liver, biochanin A was metabolized into two glucuronidated metabolites, designated as biochanin A-M1 and biochanin A-M2. Both are glucuronides based on hydrolysis studies using sulfatase-free glucuronidases. On the other hand, formononetin was metabolized into a single glucuronic acid metabolite. Based on the structures of these two isoflavones and the fact that 7-OH group is most common site of glucuronidation, biochanin A-M1 and biochanin A-M2 are likely to be 7-OH glucuronic acid and 5-OH glucuronic acid, respectively. Alternatively, biochanin A-M2 would be biochanin A-5,7-diglucuronic acid, but this is unlikely since a much longer reaction time did not result in higher formation of biochanin A-M2, as would be expected from a secondary metabolite (see results presented under microsome studies). The formononetin metabolite is 7-OH-glucuronic acid since that is the only position available for glucuronidation and the peak is hydrolyzed by sulfatase-free glucuronidases.

There were also significant differences in conjugates excretion. For example, larger amounts of formononetin conjugates were excreted ( $p < 0.05$ ) than biochanin A conjugates in all regions of the intestine except for colon (Fig. 2B). In addition, for both compounds, conjugates excretion was much higher in duodenum than that in jejunum, terminal ileum and colon (Fig. 2B). The maximal differences in excretion were about 26-fold for biochanin A (32% of amounts absorbed or  $M_{ab}$  excreted as conjugates in duodenum vs. 1.2% in colon). The differences may be even higher for

formononetin (80% of  $M_{ab}$  excreted as conjugates in ileum, but none in colon). We also determined the amounts of formononetin and biochanin A absorbed during perfusion (10  $\mu$ M each,  $M_{total}=229$ nmol for all four segments every 30 min or 57nmol for each 10 cm segment every 30 min), and found that  $M_{ab}$  was more rapid for biochanin A (total absorption from 4 segments combined: 89nmol for formononetin, and 160nmol for biochanin A;  $p<0.05$ ).

**Regional Absorption and Metabolism of Red Clover Isoflavones.** In the red clover extract, the main isoflavones were biochanin A and formononetin. In Promensil<sup>TM</sup> tablet extract, the ratio of formononetin : biochanin A was approximately 7:10. When the extract was used, absorption of the two isoflavones was also rapid and region-dependent (Table 1, Fig. 3). For example, for formononetin,  $M_{ab}$  was the highest in the colon (28.6 nmol/30min or 77%) and the lowest in the terminal ileum (15.8 nmol/30min or 42%) ( $p<0.05$ ; Fig. 3A). For biochanin A,  $M_{ab}$  was highest in the colon (46.3 nmol/30min or 85% of perfused amounts,  $p<0.05$ ), which were much higher than that in duodenum (36.2 nmol/30min or 66%), jejunum (27.6 nmol/30min or 50%) and terminal ileum (31.8 nmol/30min or 58%). As to the conjugates excreted by the intestine, major metabolites of biochanin A and formononetin were similarly excreted at all regions of the intestine (Fig.3B). For both compounds, conjugates excretion was much higher in duodenum than that in jejunum, terminal ileum and colon (Fig. 3B). We also determined the amounts of formononetin and biochanin A absorbed during perfusion (9.7  $\mu$ M biochanin A,  $M_{total}=222$ nmol for all four 10 cm segments every 30 min; 6.7 $\mu$ M formononetin,  $M_{total}=153$ nmol for all four 10 cm segments every 30 min), and found that  $M_{ab}$  (81nmol for formononetin, and 142nmol for biochanin A;  $p<0.05$ ) was approximately equal to what we saw using pure isoflavones.

**Intestinal versus Biliary Excretion of Phase II Conjugates of Pure Formononetin and Biochanin A.** Intact aglycones were not found in the bile or plasma collected during the perfusion experiments, even though large percentages of aglycones were absorbed (Fig. 2). Therefore, we analyzed amounts of conjugates excreted in bile after their hydrolysis with  $\beta$ -glucuronidase + sulfatase (Fig. 4). The results indicated that for biochanin A amounts of conjugates excreted into the intestinal lumen or  $M_{gut}$  (10 nmol or 7% of  $M_{ab}$  on average and 29 nmol maximum per 30 min) were similar to that excreted into the bile or  $M_{bile}$  (20 nmol or 12% of  $M_{ab}$  on average and 27 nmol maximum) ( $p>0.05$ ) (Fig. 4B). In contrast, for formononetin,  $M_{bile}$  (48 nmol or 46% of  $M_{ab}$  on

average and 51 nmol maximum) were much larger than  $M_{\text{gut}}$  (30 nmol or 29% of  $M_{\text{ab}}$  on average and 31 nmol maximum) ( $p < 0.05$ ) (Fig. 4A). The highest observed conjugated formononetin concentration in bile was about 246  $\mu\text{M}$  with an average steady-state concentration at approximately 190  $\mu\text{M}$ , whereas the highest observed conjugated biochanin A concentration in bile was slightly more than 104  $\mu\text{M}$  with an average of approximately 76  $\mu\text{M}$ . The steady-state concentrations of formononetin and biochanin A conjugates in bile were significantly higher than the starting perfusate concentration of 10  $\mu\text{M}$ .

**Intestinal versus Biliary Excretion of Phase II Conjugates of Red Clover Isoflavones.** For formononetin,  $M_{\text{bile}}$  (49 nmol or 50% of  $M_{\text{ab}}$  on average and 51 nmol maximum) was found to be much larger than  $M_{\text{gut}}$  (16 nmol or 20% of  $M_{\text{ab}}$  on average and 23 nmol maximum per 30 min) ( $p < 0.05$ ) (Fig. 5A). For biochanin A,  $M_{\text{bile}}$  (50 nmol or 35% of  $M_{\text{ab}}$  on average and 78 nmol maximum) were also much larger than  $M_{\text{gut}}$  (23 nmol or 16% of  $M_{\text{ab}}$  on average and 37 nmol maximum) ( $p < 0.05$ ) (Fig. 5B). The highest observed conjugated formononetin concentration in bile was about 119  $\mu\text{M}$  with an average steady-state concentration at approximately 91  $\mu\text{M}$ , whereas the highest observed conjugated biochanin A concentration in bile was slightly more than 184  $\mu\text{M}$  with an average at approximately 110  $\mu\text{M}$ . The steady-state concentrations of formononetin and biochanin A conjugates in bile were significantly higher than the starting perfusate concentration of 7 and 10  $\mu\text{M}$ .

**Glucuronidation of Pure Formononetin and Biochanin A in Intestinal Microsomes Prepared From Different Region of the Intestine.** The main conjugation reaction of formononetin and biochanin A was glucuronidation. Metabolism of formononetin and biochanin A (10  $\mu\text{M}$  each) was determined using intestinal microsomes prepared from different regions of the rat intestine (i.e., duodenum, jejunum, ileum, and colon) (Fig. 6). The results indicated that there were significant differences in the metabolism of these two compounds using microsomes prepared from different regions of the rat intestine. Similar to what was observed in the perfusion studies, biochanin A was metabolized faster than formononetin. However, metabolism of these two compounds was always the highest in duodenum microsomes, and occurred readily in the colon, even though very little or no metabolites were found in the colon perfusate. The difference in metabolism rate between duodenum and colon was 2.5-fold for biochanin A and 1.7-fold for formononetin. In contrast, the

differences in amounts of metabolites excreted in duodenum versus colon were 37-fold for biochanin A, whereas no metabolite of formononetin was found in colon perfusate.

### **Kinetics of Glucuronidation of Pure Formononetin and Biochanin A by Microsomes.**

Metabolism of formononetin and biochanin A was determined using rat liver and intestinal microsomes at concentrations ranging from 0.625 to 50 $\mu$ M. The results indicated that metabolism of both compounds was saturated in the intestinal microsomes and liver microsomes at the highest concentration tested (50 $\mu$ M) (Fig. 7). We also determine the apparent kinetic parameters of the conjugation of formononetin and biochanin A using different microsomes. The results indicated that  $K_m$  values were similar (5-8.4  $\mu$ M) when using pure formononetin. They were more different ( $K_m$  range: 2.7 to 7  $\mu$ M) when using pure biochanin A. The differences in  $V_{max}$  values were also bigger for biochanin A than for formononetin. When we calculated the intrinsic clearance ( $V_{max}/K_m$ ) of pure formononetin, we found that duodenum had the highest value (0.14  $\mu$ l/min/mg) whereas the liver has the lowest value (0.041  $\mu$ l/min/mg), a difference of 3 times. When we calculated the intrinsic clearance ( $V_{max}/K_m$ ) of pure biochanin A using biochanin A-M1 formation, we found that duodenum had the highest value (0.40  $\mu$ l/min/mg) whereas the liver has the lowest value (0.12  $\mu$ l/min/mg), a difference of 3.3 times. Biochanin A-M2 formation had similar intrinsic clearance values for duodenum and ileum but smaller intrinsic clearance values for jejunum, colon and liver. However, the  $V_{max}$  values for biochanin A-M2 formation were very much smaller than that for biochanin-M1 formation (4-13 times smaller).

### **Glucuronidation of Red Clover Isoflavones in Liver and Jejunum Microsomes.**

Metabolism of red clover extract was determined using rat liver and jejunum microsomes at biochanin A concentrations ranging from 0.625 to 50 $\mu$ M. The results indicated that metabolism of biochanin A was saturated in the jejunum microsomes at the highest concentration tested (50 $\mu$ M) (Fig. 8). Metabolism of biochanin A in the liver microsomes was also saturated at 50 $\mu$ M (Fig. 8) but metabolite of formononetin was not found. We also determined the metabolism of red clover isoflavones (containing 10 $\mu$ M biochanin A) using rat liver microsomes from 0.5h to 12h. The results indicated that formation of biochanin A-M1 increased rapidly between 0.5h to 4h and became steady afterwards. On the other hand, biochanin A-M2 increased slowly before 4 hr, continue to increase until 6 hours but became steady after than, indicating that it is not a secondary

metabolite of biochanin A-M1, which is expected to be biochanin A-5,7-diglucuronic acid and formed more rapidly after the formation of biochanin A-M1. Very small amounts of formononetin glucuronic acid were found at the beginning and metabolism of formononetin increased slowly from 1h to 8h (Fig. 9). We also determine the apparent kinetic parameters of the conjugation of biochanin A when it is present in the red clover extract (Table 2). Compared to using pure biochanin A, the intrinsic clearance values for both intestinal and liver microsomes were decreased.

**Conversion of Biochanin A to Genistein.** Daidzein and genistein were found in bile after their hydrolysis with  $\beta$ -glucuronidase + sulfatase (Fig. 1). The bile concentration was about 19 $\mu$ M for daidzein or 13 $\mu$ M for genistein when pure formononetin or biochanin A was used alone in the perfusion experiments. When red clover isoflavones were perfused, about 7 $\mu$ M daidzein and 11 $\mu$ M genistein were collected in the bile. To confirm the conversion of formononetin and biochanin A to daidzein and genistein as observed previously in human liver microsomes (Hu et al, 2003a), we incubated these two compounds individually with rat intestinal microsomes and found measurable conversion. Kinetic studies of conversion of biochanin A to genistein using rat liver microsomes generated a saturable kinetic profile with  $V_{max}$  of 490 $\pm$ 26 pmol/min/mg protein and  $K_m$  of 64.5 $\pm$ 9.04 $\mu$ M (Fig. 10). The Eadie-Hofstee plot generated a curvilinear relationship.

#### **Absorption and Metabolism of Pure Biochanin A and Formononetin in the Caco-2 Model.**

Significant amounts of formononetin and biochanin A (10  $\mu$ M each at the donor side) were metabolized into glucuronidated metabolites during their transport across the Caco-2 cell monolayers. More formononetin was conjugated and excreted to the basolateral side than biochanin A, though similar amounts were excreted apically (Table 3). Amounts of formononetin glucuronic acid found in the basolateral and apical media were 1.6 times of biochanin A glucuronic acid, whereas the rate of excretion was 70% faster. A small amount of sulfated metabolites were also found in both apical and basolateral sides, but are not reported here due to their very low concentrations.

#### **Absorption and Metabolism of Red Clover Isoflavones in the Caco-2 Model.**

Significant amounts of biochanin A (10  $\mu$ M at the donor side) were metabolized into glucuronidated metabolites during their transport across the Caco-2 cell monolayers, but no

glucuronidated metabolite of formononetin was found (Table 3). Conjugated metabolites of biochanin A were excreted into both apical and basolateral sides (Table 3). Interestingly, the rate of apical excretion of biochanin A-M1 increased 77% ( $P < 0.05$ ), but the rate of its basolateral excretion did not change, when given as a red clover isoflavone mixture. Additionally, we could not find the presence of sulfates in red clover incubation samples.



## DISCUSSION

One of the main reasons why flavonoids have poor bioavailabilities is because they are extensively metabolized and participate in metabolic recycling processes, which are enabled by the coupling of efflux transporters and conjugating enzymes (or “enzyme-transporter coupling”) in gut and liver. The best known recycling process (i.e., enterohepatic recycling), which has been shown to be important for the disposition of many drugs (Roberts et al., 2002), is proposed as one of the main reasons why these compounds have poor bioavailabilities. Recently, we proposed that enteric recycling involving microbial hydrolysis of flavonoid conjugates excreted by the enterocytes is also very important in the disposition of flavonoids (Liu and Hu, 2002; Chen et al, 2003). It is generally believed that these recycling processes may provide a prolonged period of exposure for dietary flavonoids. Therefore, the present studies represent our continued effort in studying the components of these recycling processes, namely intestinal metabolism and excretion of flavonoid conjugates. We also conducted studies to determine if and how a mixture of isoflavone will be handled by the enterocytes using a red clover extract obtained from Promensil™ tablets.

We first determined the absorption of formononetin and biochanin A when given alone or in a red clover extract using the four-site intestinal perfusion model, which perfuses  $\approx 50\%$  of total intestinal length. The results showed that absorption was rapid (Table 1, Fig.2A, Fig.3A) and site-dependent (Fig.2A, Fig.3A) regardless whether these isoflavones were given alone or in a red clover extract (formononetin: biochanin A $\approx 7:10$ ). Nearly identical results were obtained in Caco-2 model (Table 3). Our results support the notion that change in isoflavone matrix had minimal effect on the absorption. This is expected since these compounds are highly permeable with  $P_{\text{eff}}$  greater than 1 in the perfusion model (Table 1) and permeability greater than  $10 \times 10^{-6}$  cm/sec in the Caco-2 model (Table 3).

We then determined the excretion of phase II conjugates of formononetin and biochanin A, via bile or from four perfused segments of the intestine, when given alone or in a red clover extract (Fig.2-Fig.5). We found that excretion of metabolites was rapid and site-dependent ( $p < 0.05$ ), regardless if it is given as pure compound or as a red clover mixture. For pure formononetin (10  $\mu\text{M}$ ), the excretion of conjugates was fastest in duodenum, followed by ileum and jejunum, but

none was found in the colonic perfusate. In addition, more conjugates were excreted via the bile than from the perfused segments. For pure biochanin A (10  $\mu$ M), site-dependent excretion is similar to formononetin but less extensive. Taken together, our results indicated that intestinal metabolite excretion is site-dependent, and more formononetin conjugates were excreted than biochanin A conjugates in both intestine and liver.

The excretion of isoflavones was found to change greatly and unexpectedly when given as a red clover mixture. We expected decreases in the excretion of both conjugates because of the expected competition for the same enzymes and efflux transporters. Interestingly, however, total amounts of biochanin A-M1 excreted (into the lumen) increased (~30%) in the small intestine, whereas that of the formononetin-M1 decreased about 30-50% (Fig.2B, Fig.3B). As a result, slightly more ( $p>0.05$ ) biochanin A conjugates were found in the perfusate than formononetin conjugates (Fig.3B). Similarly, when formononetin and biochanin A were used alone in the Caco-2 cells, more formononetin-M was excreted than biochanin A-M1 (Table 3). When they were used together in the red clover mixture, however, only biochanin A-M1 were excreted. Moreover, apical biochanin A-M1 was increased when an isoflavone mixture was used in the Caco-2 study, similar to what we seen in rat luminal excretion (Fig.5, Table 3).

To account for this unexpected change when isoflavone mixture was used, we examined two factors that will affect the intestinal excretion of conjugated isoflavones: conjugate formation and subsequent excretion of the conjugates. We performed microsomal metabolism studies to determine if enzyme action could explain the difference. We found that microsomes, prepared from rat intestine and liver, always glucuronidated biochanin A faster than formononetin ( $p<0.05$ ), as evidenced by much larger intrinsic clearance values (2-4 times) for biochanin A (Table 2). When a red clover extract was used, glucuronidation of biochanin A by intestinal and liver microsomes decreased significantly (~33% decrease in intrinsic clearance), whereas glucuronidation of formononetin was severely limited (not measurable within 30 min) and much less than biochanin A-M1 formation in a long-term incubation study (Fig.9). Therefore, these results suggest that the inhibition of enzyme action is most likely responsible for the difference seen when using a red clover isoflavone mixture.

Is the action of the conjugating enzyme always predictive of intestinal conjugate excretion, as shown above? The answer is no. This is based on our novel finding that a faster glucuronidation in the intestinal or liver microsomes (Table 2 and Fig.7), did not translate into a more extensive metabolite excretion in the intestine or the liver (Fig.2, Fig.3). On the contrary, formononetin conjugates were excreted more rapidly than biochanin A conjugates when given alone, even though it has a much lower intrinsic clearance value in all regions of the intestine and the liver (Table 2). The same statement can be made when using  $V_{\max}$  as the indicator of conjugate formation. This novel finding again demonstrates the importance of studying the enzyme-transporter coupling process as a whole using the intact cell system, where one can measure the dynamic interplay between conjugating enzymes and efflux transporters.

This enzyme-transporter coupling has been proposed to account for the intestinal disposition of apigenin in Caco-2 cells (Hu et al, 2003b). Here, we have showed again that the overall efficiency of enzyme-transporter coupling could affect *in vivo/in situ* excretion. For example, a more rapid excretion of formononetin conjugates into lumen or bile was caused by more rapid excretion of these conjugates since formononetin was glucuronidated much slower than biochanin A. Additionally, the percent decrease in the excretion of metabolites (about 3 times) was far less than percent decrease in metabolism of formononetin (>10 times) when using the red clover extract. Therefore, we found formononetin conjugates in the bile and luminal perfusate even though the same metabolites could not be found within 30 min using the microsomes. Taken together, these results demonstrate again that cellular excretion of conjugated isoflavones is under the control of enzyme-transporter coupling. As such, the use of an isoflavone mixture can significantly impact the *in vivo* disposition of its individual component in a way not readily expected from performing only one set of studies (either microsomal metabolism or perfusion).

The enzyme-transport coupling may also be used to compare how efficient the coupling process functions in the intestine versus liver. Although intestinal microsomes metabolized biochanin A and formononetin much more efficiently (higher intrinsic clearance values) than liver microsomes (Fig.7, Table 2), there were more metabolites in bile (40-50% more) when a pure isoflavone was perfused (Fig.4). Assuming biliary metabolites are produced in liver, the hepatic coupling process must be more efficient than intestinal coupling process, even if we have perfused 100% of the

intestine (i.e., double the length). This is because the concentration of these isoflavones in the hepatocytes (probably less than 1  $\mu\text{M}$ ) is expected to be much lower than that in the enterocytes, which is expected to be closer to the perfusate concentration of 10  $\mu\text{M}$ . An alternative explanation for the higher biliary excretion is that the intestinal conjugates are transported into the hepatocytes and then excreted into the bile. This possibility is supported by a recent study by O'Leary et al (2003), which showed a slow but measurable uptake of phase II conjugates by human liver HepG2 cells. Lastly, the large number of hepatocytes that can contribute to the conjugate excretion via MRP2 may also be used to explain the difference. Future studies are needed to sort if any or all of these factors are involved.

Besides phase II metabolism, there was also significant phase I metabolism. Daidzein and genistein were found in bile after treating the bile with  $\beta$ -glucuronidase + sulfatase (Fig. 1). In addition, conversion of biochanin A to genistein in rat liver microsomes was found to be rapid and saturable (Fig. 10). Multiple cytochrome P450 isoforms probably participate in this demethylation reaction since the Eadie-Hofstee plot is curvilinear, which was shown previously using human liver microsomes (Tolleson et al, 2002, Hu et al, 2003a). Nevertheless, demethylation in rats appeared to be faster than that in human liver microsomes (Tolleson et al, 2002).

In conclusion, our studies clearly showed that formononetin and biochanin A are metabolized by multiple isoforms of UGT. In addition, intestinal and hepatic excretion of isoflavone conjugates is affected by the coupling efficiency of the conjugating enzymes and efflux transporters. As a consequence, the disruption of the major component will significantly decrease the excretion of formononetin conjugates. Taken together, our studies indicate that the efficiency of organ specific coupling will determine the relative magnitudes of intestinal versus hepatic excretion of isoflavone conjugates, which in turn will affect the relative contribution of enteric recycling versus enterohepatic recycling.

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## Figure Legends

**Fig. 1.** HPLC chromatographs of Red Clover (formononetin and biochanin A) and their Phase II metabolites. Compounds were labeled as internal standard (IS, testosterone, 46.1 min), formononetin-M or formononetin-7-O-glucuronic acid (13.0 min), biochanin A-M2 or biochanin A-5-O-glucuronic acid (14.3 min), biochanin A-M1 or biochanin A-7-O-glucuronic acid (22.1 min), daidzein (23.8 min), genistein (32.2 min), formononetin (40.5 min), and biochanin A (51.5 min). Panel A shows the HPLC chromatograms of a perfusate solution before it enters the perfused segment (solid) or after it exits the perfused segment (dashed line). Panel B shows the HPLC chromatograms of a diluted bile before (dashed line) or after (solid line) treatment of glucuronidases+sulfatases. Panel C shows chromatograms of a microsomal reaction mix before (solid line) or after (dashed line) glucuronidation reaction. The HPLC condition is specified in the “Materials and Methods.”

**Fig. 2.** Amounts of pure aglycones absorbed (Fig.2A), and amounts of conjugated excreted (Fig.2B) in a four-site rat intestinal perfusion model (number of replicates, or  $n=4$ ). Perfusate containing 10  $\mu$ M formononetin (solid columns) or biochanin A (slashed columns) was used, and four segments of the intestine (i.e., duodenum, upper jejunum, terminal ileum, and colon) were perfused simultaneously at a flow rate of 0.191 ml/min. Amounts absorbed and conjugates excreted, normalized to 10-cm intestinal length, were calculated using Equations 1 to 3. Each column represents the average of four determinations and the error bar is the S.E.M. There were statistically significant differences ( $p<0.05$ ) between amount absorbed and conjugated excreted at different regions of the intestine for biochanin A and formononetin, according to a one-way ANOVA analysis (“a” and “b”). In the same region of the intestine, amounts of absorption of biochanin A were higher than amounts of absorption of formononetin ( $p<0.05$ , arrows) except in jejunum (Fig.2A). In the same region of the intestine, amounts of formononetin conjugates excreted were significantly higher ( $p<0.05$ , arrows) than amounts of biochanin A conjugates excreted except for colon (Fig.2B).

**Fig. 3.** Amounts of aglycones of Red Clover (Promensil™) absorbed (Fig.3A), and amounts of conjugated excreted (Fig.3B) in a four-site rat intestinal perfusion model (number of replicates, or

$n=4$ ). Perfusate containing 6.7  $\mu\text{M}$  formononetin (solid columns) or 9.7  $\mu\text{M}$  biochanin A (slashed columns) was used, and four segments of the intestine (i.e., duodenum, upper jejunum, terminal ileum, and colon) were perfused simultaneously at a flow rate of 0.191 ml/min. Amounts absorbed and conjugates excreted, normalized to 10-cm intestinal length, were calculated using Equations 1 to 3. Each column represents the average of four determinations and the error bar is the S.E.M. There were statistically significant differences ( $p<0.05$ ) between amount absorbed and conjugated excreted at different regions of the intestine for biochanin A and formononetin, according to a one-way ANOVA analysis (“a” and “b”). In the same region of the intestine, amounts of absorption of biochanin A were higher than amounts of absorption of formononetin ( $p<0.05$ , arrows) (Fig.3A). In the same region of the intestine, amounts of formononetin conjugates excreted were similar to amounts of biochanin A conjugates excreted ( $p>0.05$ ) except for colon (Fig.3B).

**Fig. 4.** Relative contribution of intestinal (solid columns) versus biliary (slashed columns) excretion of pure formononetin (Fig.4A) and biochanin A (Fig.4B) conjugates ( $n=4$ ). Amounts of metabolites excreted in the perfusate ( $M_{\text{gut}}$ ) and bile ( $M_{\text{bile}}$ ) over a 30-min sample interval were calculated as described under *Data Analysis* using Equations 2 and 3. In the figures, the time (e.g., 60 min) indicates the time when the sampling ends. Each column represents the average of four determinations and the error bar is the S.E.M. In Fig.4A, amounts of formononetin conjugates excreted into the lumen were lower ( $P<0.05$ , arrows) than amounts of formononetin conjugates excreted into the bile. In Fig.4B, the amounts of biochanin A conjugates excreted into the lumen were similar to amounts of biochanin A conjugates excreted into the bile ( $p>0.05$ ).

**Fig. 5.** Relative contribution of intestinal (solid columns) versus biliary (slashed columns) excretion of conjugates of two red clover isoflavones formononetin (Fig.5A) and biochanin A (Fig.5B) ( $n=4$ ). Amounts of metabolites excreted in the perfusate ( $M_{\text{gut}}$ ) and bile ( $M_{\text{bile}}$ ) over a 30-min sample interval were calculated as described under *Data Analysis* using equations 2 and 3. In the figures, the time (e.g., 60 min) indicates the time when the sampling ends. Each column represents the average of four determinations and the error bar is the S.E.M. In Fig.5A, amounts of formononetin conjugates excreted into the lumen were lower ( $P<0.05$ , arrows) than amounts of formononetin conjugates excreted into the bile. In Fig.5B, the amounts of biochanin A conjugates excreted into the lumen were significantly lower ( $p<0.05$ , arrows) than amounts of biochanin A conjugates

excreted into the bile .

**Fig. 6.** Formation of formononetin-M (solid columns), biochanin A-M1 (slashed columns) and biochanin A-M2 (open columns) in microsomes prepared from different regions of the rat intestine ( $n=3$ ). The concentration of both aglycones was  $10\mu\text{M}$  and the reaction time was 30 min. Each column represents the average of four determinations and the error bar is the S.D. There were statistically significant differences ( $p<0.05$ ) between rate of metabolism using microsomes prepared from different regions of the intestine for biochanin A and formononetin, according to a one-way ANOVA analysis (“a”, “b” and “c”). In microsomes prepared from the same region of the intestine, formation rates of formononetin-M was significant lower ( $p<0.05$ , arrows) than that of biochanin A-M1.

**Fig. 7.** Metabolism of pure formononetin (Fig.7A) and biochanin A (Fig.7B, Fig.7C ) in rat liver duodenal, jejunal, ileal, and colonic microsomes ( $n=3$ ). Rates of metabolism were determined from 0.625 to  $50\mu\text{M}$ , and reaction time was for 30 min. Each data point represents the average of three determinations and the error bar represents the standard deviation of the mean.

**Fig. 8.** Metabolism of red clover isoflavones in rat liver and jejunal microsomes ( $n=3$ ). Rates of metabolism were determined from containing 0.625 to  $50\mu\text{M}$  of biochanin A, and reaction time was for 60 min. Each data point represents the average of three determinations and the error bar represents the standard deviation of the mean.

**Fig. 9.** Metabolism of Red Clover isoflavones in rat liver microsomes ( $n=3$ ). Rates of metabolism were determined from 0.5h to 12 h at containing  $10\mu\text{M}$  biochanin A. Each data point represents the average of three determinations and the error bar represents the standard deviation of the mean.

**Fig. 10.** Demethylated metabolism of biochanin A as a function of concentrations by rat liver microsomes. Simple Michaelis-Menten equation was used to fit the data. The points are experimental values (Investigated) and curve (Estimated) is calculated number based on the fitted parameters (Fig.10A). Genistein formation was saturated with a  $K_m$  value of  $64.5\pm 9.04\mu\text{M}$  and a  $V_{\text{max}}$  value of  $490\pm 26\text{ pmol/min/mg}$ . The ratio of  $V_{\text{max}}/K_m$  (or intrinsic clearance) is  $7.59\mu\text{l/min/mg}$ .

Eadie-Hofstee plot was used to test if multiple isoforms are involved in the demethylation (Fig.10B). In Fig.10B, “V” stands for genistein formation rate, and “C” for biochanin A concentration.

**TABLE 1**

Effective Permeabilities or  $P^*_{\text{eff}}$  of Pure Formononetin, Biochanin A and Two Main Red Clover Isoflavones (i.e., biochanin A and formononetin) in Four-site Perfused Rat Intestinal Model

Sites	Duodenum	Jejunum	Ileum	Colon
Pure Formononetin	2.38±0.50	2.17±0.34	1.06±0.07	3.46±0.30
Pure Biochanin A	4.13±0.30	2.49±0.34	2.73±0.46	4.52±0.21
Formononetin in Red Clover	2.51±0.09	2.19±0.31	2.15±0.30	3.93±0.28
Biochanin A in Red Clover	3.36±0.76	2.56±0.23	2.94±0.27	4.30±0.23

**TABLE 2**

Kinetic Parameters of Glucuronidation of Formononetin and Biochanin A in Pure Form or in Red Clover Extract by Rat Liver and Intestinal Microsomes

Site	Duodenum		Jejunum		Ileum		Colon		Liver		
	M1	M2	M1	M2	M1	M2	M1	M2	M1	M2	
Formononetin	$V_{max}$ (nmol/min/mg)	0.791		0.471		0.413		0.485		0.209	
	$K_m$ ( $\mu$ M)	5.653		6.794		4.972		8.419		5.138	
	$V_{max}/K_m$ ( $\mu$ l/min/mg)	0.140		0.069		0.083		0.058		0.041	
	$R^2$	0.999		0.995		0.963		0.994		0.999	
Biochanin A	$V_{max}$ (nmol/min/mg)	2.349	0.666	1.291	0.256	0.970	0.254	0.790	0.140	0.323	0.024
	$K_m$ ( $\mu$ M)	6.994	2.116	5.358	2.101	5.653	2.066	3.636	1.213	2.694	0.628
	$V_{max}/K_m$ ( $\mu$ l/min/mg)	0.397	0.416	0.241	0.163	0.172	0.148	0.217	0.115	0.120	0.038
	$R^2$	0.998	0.998	0.998	0.996	0.999	0.998	0.997	0.990	0.973	0.995
Biochanin A In Red Clover	$V_{max}$ (nmol/min/mg)			0.336	0.337					0.613	0.275
	$K_m$ ( $\mu$ M)			2.089	20.693					8.349	18.876
	$V_{max}/K_m$ ( $\mu$ l/min/mg)			0.161	0.016					0.073	0.015
	$R^2$			0.986	0.996					0.994	0.999



**TABLE 3**

Permeabilities of formononetin and biochanin A as well as rates and amounts of their metabolites excreted in the Caco-2 model ( $n=3$ ). Amounts of metabolites in the apical (AP) and basolateral (BL) media at the end of 4-h experiments were determined. Permeability values were calculated using first three data in the amount transported versus time plot.

Isoflavone	Isoflavone Aglycone	Isoflavone Glucuronic Acid (Average $\pm$ SD)			
		AP Side		BL Side	
Pure or Mixture	Permeability ( $10^{-6}$ cm/s)	Rate of Excretion (nmol/hr/monolayer)	Amount Excreted (4 hr) (nmol)	Rate of Excretion (nmol/hr/monolayer)	Amount Excreted (4 hr) (nmol)
Pure Formononetin	16.7 $\pm$ 0.22	0.150 $\pm$ 0.024	0.60 $\pm$ 0.09	0.375 $\pm$ 0.004	1.50 $\pm$ 0.01
Pure Biochanin A	16.3 $\pm$ 0.10	0.122 $\pm$ 0.016	0.49 $\pm$ 0.06	0.161 $\pm$ 0.036	0.64 $\pm$ 0.14
Red Formononetin	13.5 $\pm$ 0.07	-	-	-	-
Clover Biochanin A	17.3 $\pm$ 0.07	0.216 $\pm$ 0.031	0.86 $\pm$ 0.13	0.161 $\pm$ 0.004	0.65 $\pm$ 0.01

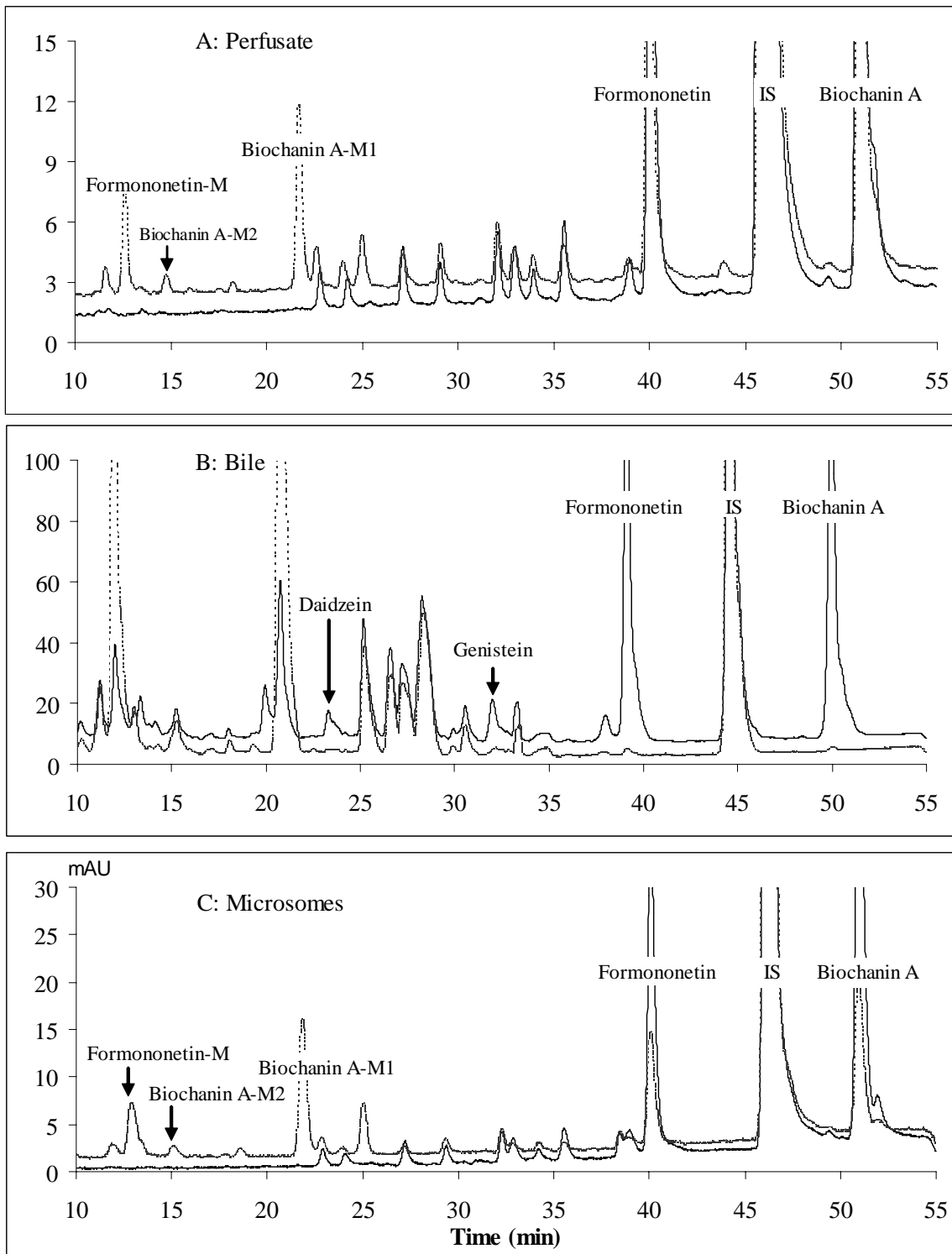


Fig. 1

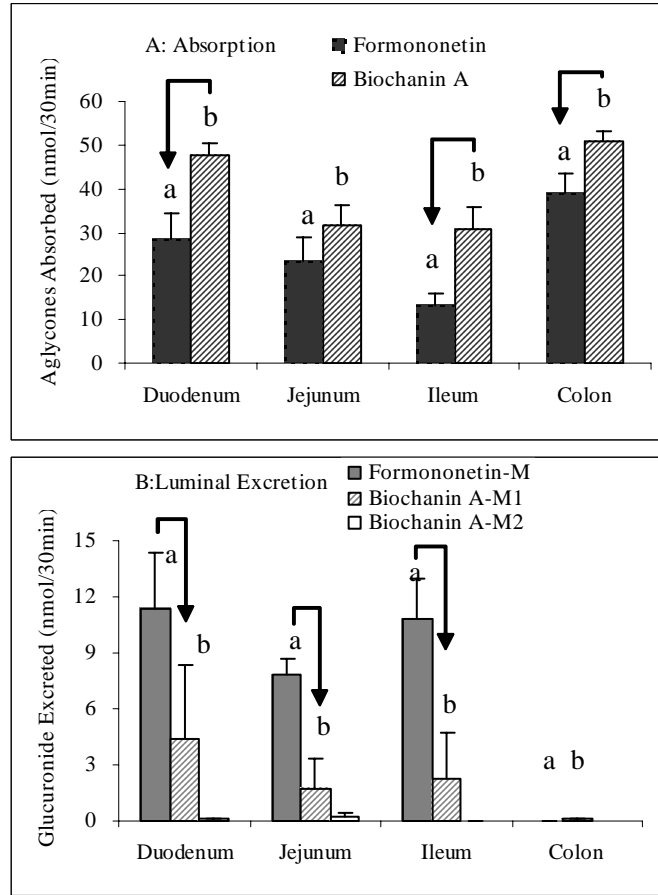


Fig 2

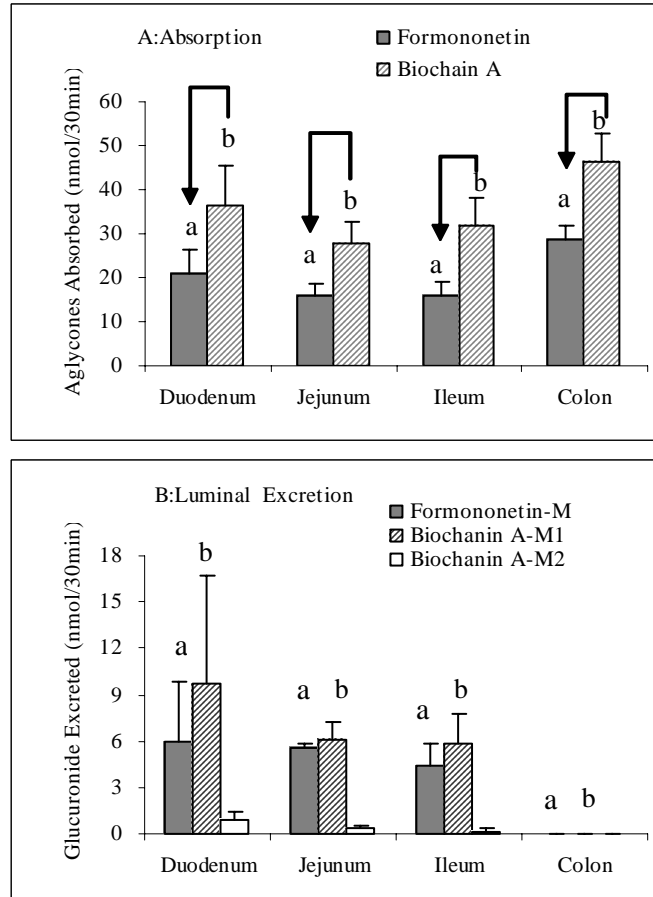


Fig 3

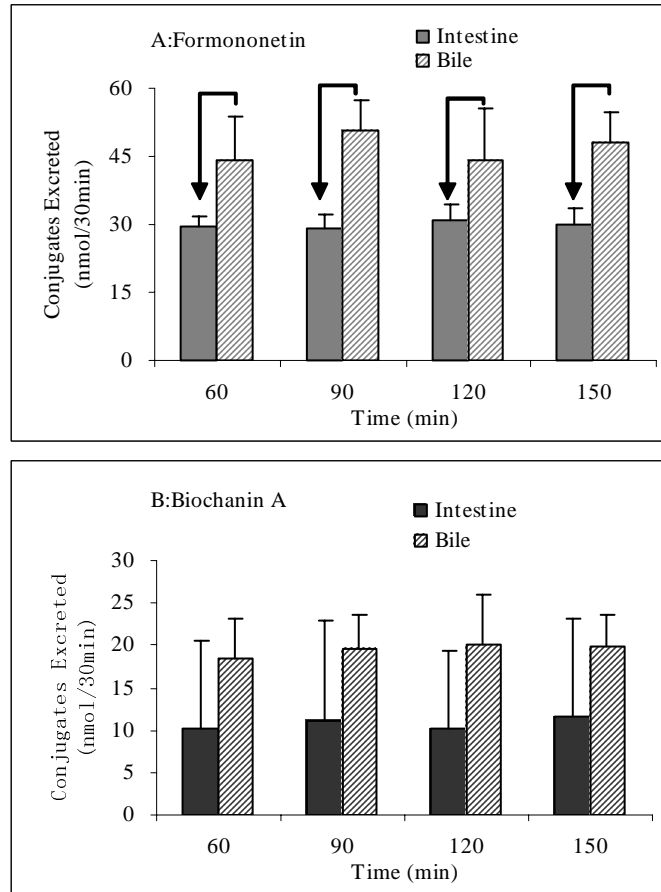


Fig 4

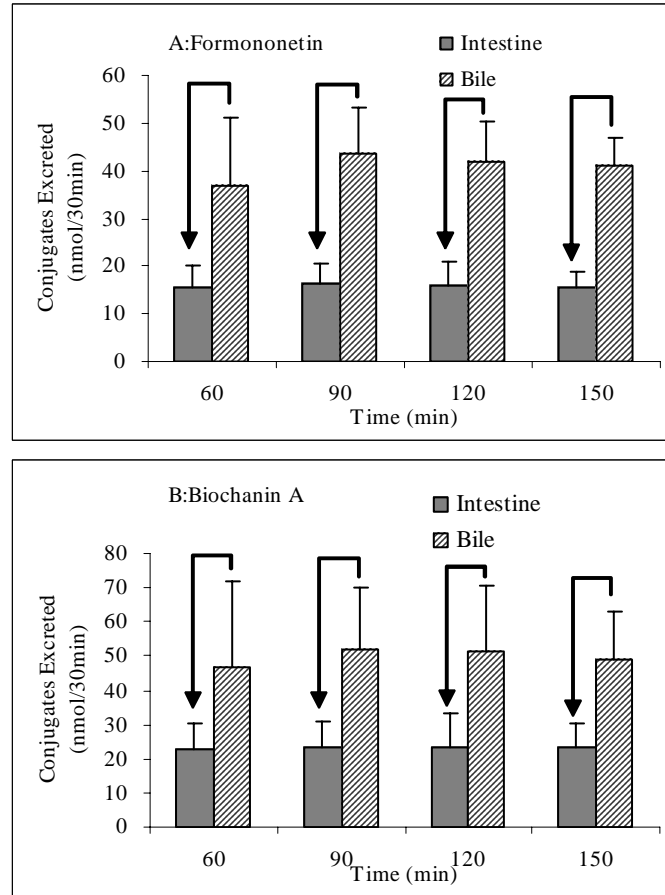


Fig 5

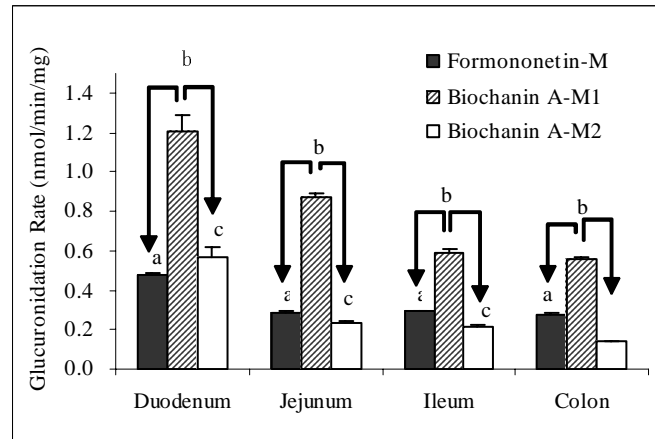


Fig 6

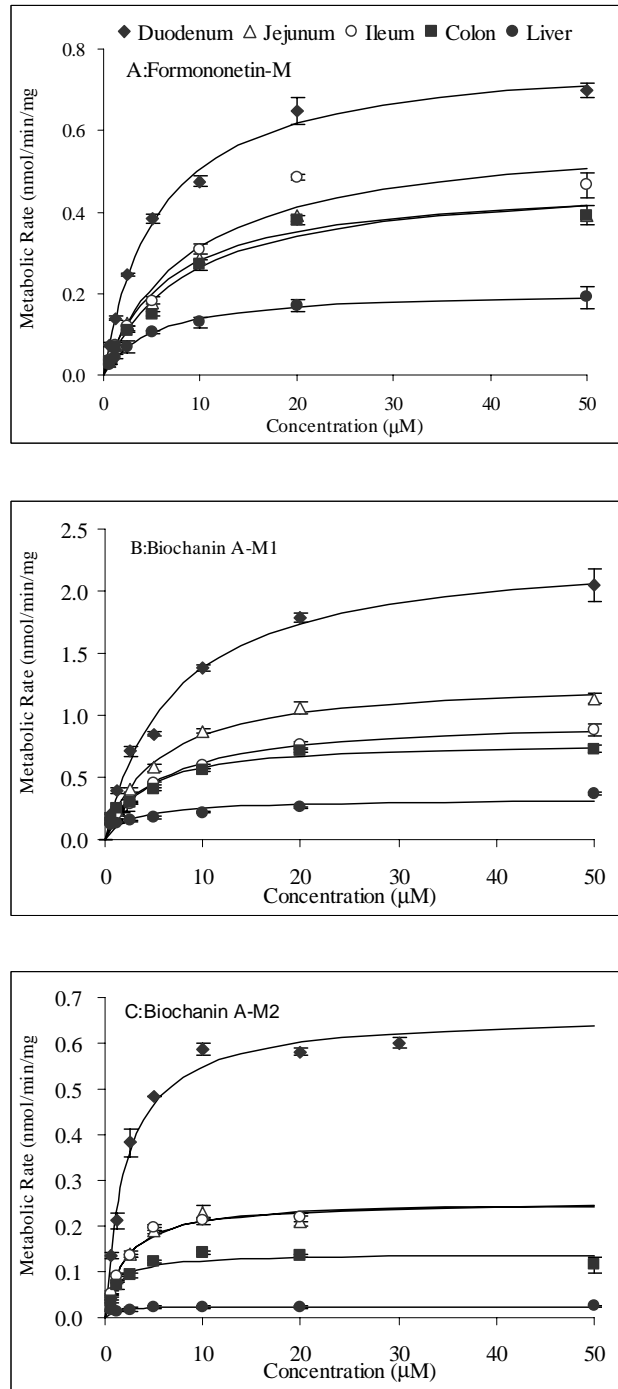


Fig 7



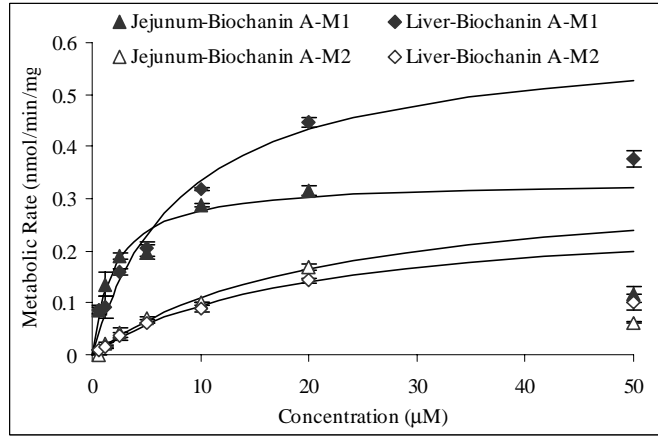


Fig 8

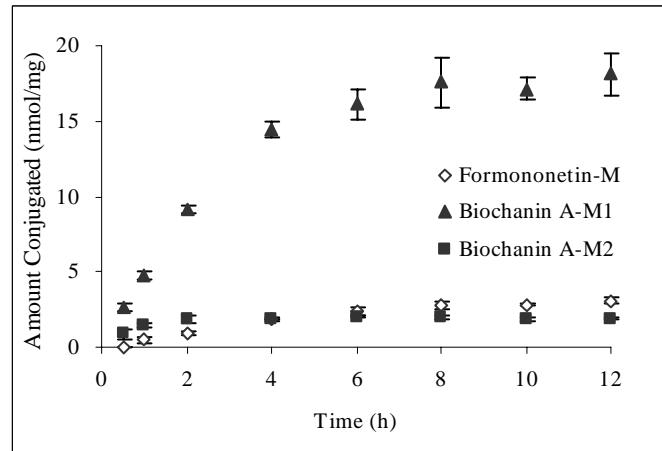


Fig. 9

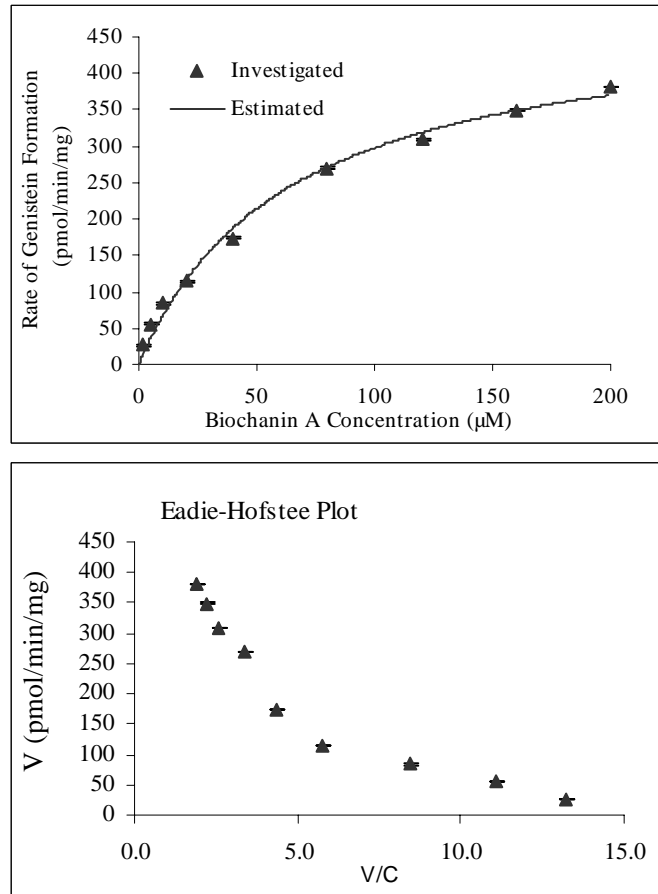


Fig. 10