

## Disposition of Flavonoids via Enteric Recycling: Enzyme Stability Affects Characterization of Prunetin Glucuronidation across Species, Organs, and UGT Isoforms

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**Abstract:** We characterized the in vitro glucuronidation of prunetin, a prodrug of genistein that is a highly active cancer prevention agent. Metabolism studies were conducted using expressed human UGT isoforms and microsomes/S9 fractions prepared from intestine and liver of rodents and humans. The results indicated that human intestinal microsomes were more efficient than liver microsomes in glucuronidating prunetin, but rates of metabolism were dependent on time of incubation at 37 °C. Human liver and intestinal microsomes mainly produced metabolite 1 (prunetin-5-*O*-glucuronide) and metabolite 2 (prunetin-4'-*O*-glucuronide), respectively. Using 12 human UGT isoforms, we showed that UGT1A7, UGT1A8, and UGT1A9 were mainly responsible for the formation of metabolite 1, whereas UGT1A1, UGT1A8, and UGT1A10 were mainly responsible for the formation of metabolite 2. This isoform-specific metabolism was consistent with earlier results obtained using human liver and intestinal microsomes, as the former (liver) is UGT1A9-rich whereas the latter is UGT1A10-rich. Surprisingly, we found that the thermostability of the microsomes was isoform- and organ-dependent. For example, human liver microsomal UGT activities were much more heat-stable (37 °C) than intestinal microsomal UGT activities, consistent with the finding that human UGT1A9 is much more thermostable than human UGT1A10 and UGT1A8. The organ-specific thermostability profiles were also evident in rat microsomes and mouse S9 fractions, even though human intestinal glucuronidation of prunetin differs significantly from rodent intestinal glucuronidation. In conclusion, prunetin glucuronidation is species-, organ-, and UGT-isoform-dependent, all of which may be impacted by the thermostability of specific UGT isoforms involved in the metabolism.

**Keywords:** Glucuronidation; prunetin; UGT; bioavailability; stability; flavonoids; isoflavone

### Introduction

Glucuronidation is the major metabolic pathway for the disposition of flavonoids in humans and in experimental animals such as rodents. Catalyzed by the UDP-glucuronosyltransferases (UGT), an important superfamily of enzymes, the glucuronidation pathway is responsible for metabolic

clearance of drugs (e.g., SN-38),<sup>1</sup> phytochemicals (e.g., genistein),<sup>2</sup> carcinogens (e.g., PhIP),<sup>3</sup> and endogenous substances (e.g., serotonin).<sup>4</sup> Therefore, many investigators have determined if one or several expressed UGT isoforms are responsible for the metabolism of a particular drug because

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of the need to predict metabolic interactions and pharmacogenetics that can impact a drug's safety or efficacy or both.

In general, the characterization of a compound's glucuronidation pathway involves the use of multiple UGT isoforms, one to three concentrations of substrates, one incubation time, and microsomes prepared from different organs. This is based on a survey of published literature, where characterization is usually done using one incubation time and mostly one concentration, but sometimes two and seldom three concentrations.<sup>5–12</sup> Therefore, one of the purposes of the present study is to test the validity of this approach and to establish a viable approach useful for the characterization of other isoflavones and flavones.

We have chosen prunetin as the model isoflavone for several reasons. First, prunetin, an isoflavone that is also a naturally occurring methylated prodrug of genistein, is

biologically active<sup>13</sup> and present in various plants, including Kudzu roots.<sup>14</sup> Second, in rats, liver metabolism was the slowest for prunetin among the six isoflavones (genistein, daidzein, glycitein, biochanin A, formononetin, and prunetin) studied,<sup>15</sup> but the intestinal metabolism of prunetin was not.<sup>15</sup> Third, there appears to be species-specific effects as the metabolism of prunetin in human intestinal Caco-2 cell lysates also appeared to be the slowest among the same six isoflavones.<sup>16</sup> Therefore, the use of prunetin as a model compound affords us the opportunity to fully characterize all facets of a flavonoid's glucuronidation: incubation time, concentration, species, organ, and UGT isoforms.

In addition, because prunetin is metabolized into two glucuronidated compounds in human microsomes in our preliminary studies, additional characterization studies are conducted to determine if formation of a particular metabolite is (expressed UGT) isoform-specific and whether isoform-specific metabolism could predict formation of these two metabolites in human liver and intestinal microsomes. This is especially important for the characterization of flavonoid metabolism, as the vast majority of flavonoids have more than one phenolic group.

## Materials and Methods

**Materials.** Expressed human UGT isoforms (Supersomes), pooled female human liver, jejunum, and ileum microsomes were purchased from BD Biosciences (Woburn, MA). Prunetin was purchased from Indofine Chemical (Summerville, NJ).  $\beta$ -D-Glucuronidase without sulfatase (catalog no. G7396), uridine diphosphoglucuronic acid (UDPGA), alameithicin, D-saccharic-1,4-lactone monohydrate, magnesium chloride, and Hanks' balanced salt solution (powder form) were purchased from Sigma-Aldrich (St Louis, MO). All other materials (typically analytical grade or better) were used as received.

**Enzymatic Activity of Expressed UGTs.** The incubation procedures for measuring UGTs' activities using microsomes were as follows: (1) mix microsomes (final concentration  $\approx$  0.05 mg of protein/mL), magnesium chloride (0.88 mM), saccharolactone (4.4 mM), alamethicin (0.022 mg/mL), different concentrations of substrates in a 50 mM potassium phosphate buffer (pH 7.4), and UDPGA (3.5 mM, add last); (2) incubate the mixture (final volume = 200  $\mu$ L) at 37 °C

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for a predetermined period of time (e.g., 1 h); and (3) stop the reaction by the addition of 50  $\mu\text{L}$  of 94% acetonitrile/6% glacial acetic acid containing 100  $\mu\text{M}$  testosterone as an internal standard. This is essentially the same as our published procedures.<sup>15,17</sup>

**Hydrolysis of Prunetin Glucuronides.** To confirm the presence of prunetin glucuronides, metabolite samples were divided into two parts, one of which was added to water and then analyzed directly via ultraperformance liquid chromatography (UPLC, see below), whereas the other was analyzed after  $\beta$ -D-glucuronidase was added to hydrolyze O-glucuronides. The difference between the amounts of aglycones found in these two samples was usually regarded as the amount of metabolites formed. Matching the decreased peak areas of the metabolites and the increased peak areas of aglycone after the hydrolysis is used to confirm the presence of prunetin O-glucuronides. Moreover, development of the relationship between a decrease in metabolite peak area and an increase in aglycone peak area is used to quantitate metabolite concentration in the reaction mixture, as was done previously.<sup>18</sup>

**UPLC Analysis of Prunetin and its Glucuronides.** We analyzed prunetin and prunetin glucuronides by using the following: system, Waters Acquity UPLC with photodiode array detector and Empower software; column, BEH C18, 1.7  $\mu\text{m}$ , 2.1  $\times$  50 mm; mobile phase B, 90% acetonitrile in water, mobile phase A, 10% acetonitrile in aqueous buffer (0.06%  $\text{C}_6\text{H}_{15}\text{N}$  and 0.045%  $\text{HCOOH}$ ); gradient, 0 to 0.3 min, 0% B, flow rate = 1 mL/min, 0.3 to 1.80 min, 0–50% B, flow rate = 0.925 mL/min, 1.80 to 2.10 min, 50–100% B, flow rate = 0.925 mL/min, 2.10 to 2.40 min, 100% B, flow rate = 0.925, 2.40 to 2.50 min, 100%–0% B, flow rate = 1 mL/min; wavelength, 254 nm; and injection volume, 10  $\mu\text{L}$ . The retention times were 1.01 min for metabolite 1, 1.23 min for metabolite 2, 1.16 min for metabolite 3, 1.62 min for testosterone (internal standard, IS), and 1.70 min for prunetin. Representative analysis of the metabolic samples is shown in Figure 1A.

**LC-MS/MS Analysis of Prunetin and Its Glucuronides.** Prunetin and its glucuronides were separated by the same UPLC system but using slightly different chromatographic conditions because of mass spectrometer requirements. Here, mobile phase A was an ammonium acetate buffer (pH 7.5) and mobile phase B was 100% acetonitrile with the gradient as follows: 0 to 2.0 min, 10–35% B, 2.0 to 3.0 min, 35–70% B, 3.2 to 3.5 min, 70–10% B, 3.5 to 3.7 min, 10% B. The flow rate was 0.5 mL/min. The effluent was introduced into an API 3200 Qtrap triple-quadrupole mass spectrometer (Applied Biosystem/MDS SCIEX, Foster City, CA) equipped with a TurboIonSpray source. The mass spectrometer was operated in the negative ion mode to perform the analysis

of prunetin and prunetin glucuronides. The main working parameters for the mass spectrometers were set as follows: ion-spray voltage,  $-4.5\text{ kV}$ ; ion source temperature,  $400\text{ }^\circ\text{C}$ ; nebulizer gas (gas1), nitrogen, 40 psi; turbo gas (gas2), nitrogen, 40 psi; curtain gas, nitrogen, 20 psi. Prunetin and prunetin glucuronides were identified by MS and MS2 full scan modes (Figure 1B).

**Purification of Metabolite 2 and Its Structural Identification via NMR.** A large amount of metabolite 2 was produced using human intestinal microsomes. The prepared metabolite 2 solution was concentrated using a C-18 solid-phase extraction procedure, where the coenzyme and other water-soluble components were washed off first. The compound was eluted by using 40% acetonitrile in water, whereas the microsomal proteins were retained on the column. The metabolite 2 solution was then lyophilized to remove water and organic solvent. The resulting residue was dissolved in methanol-*d* and used for NMR spectrum acquisition. The  $^1\text{H}$  NMR spectrum was recorded on a Bruker Avance 800 MHz NMR spectrometer. Coupling constants are given in hertz, and chemical shifts are represented in  $\delta$  (ppm).

**Thermal Stability of Human UGTs.** We first incubate the expressed UGTs at  $37\text{ }^\circ\text{C}$  for various time periods (1, 4, 8, or 24 h) with everything needed for the glucuronidation reaction to proceed except the substrate. The substrate was then added to the mixture after 1, 4, 8, or 24 h to allow the reaction to proceed for 1 h as would normally be done for any studies that measure glucuronidation rates. The rates were then determined by measuring the amount of metabolites formed. We also added dithiothreitol (DTT, final concentration in reaction mixture = 5 mM), a strong antioxidant, to the reaction mixture to determine if DTT could mitigate the thermal instability of the UGTs.

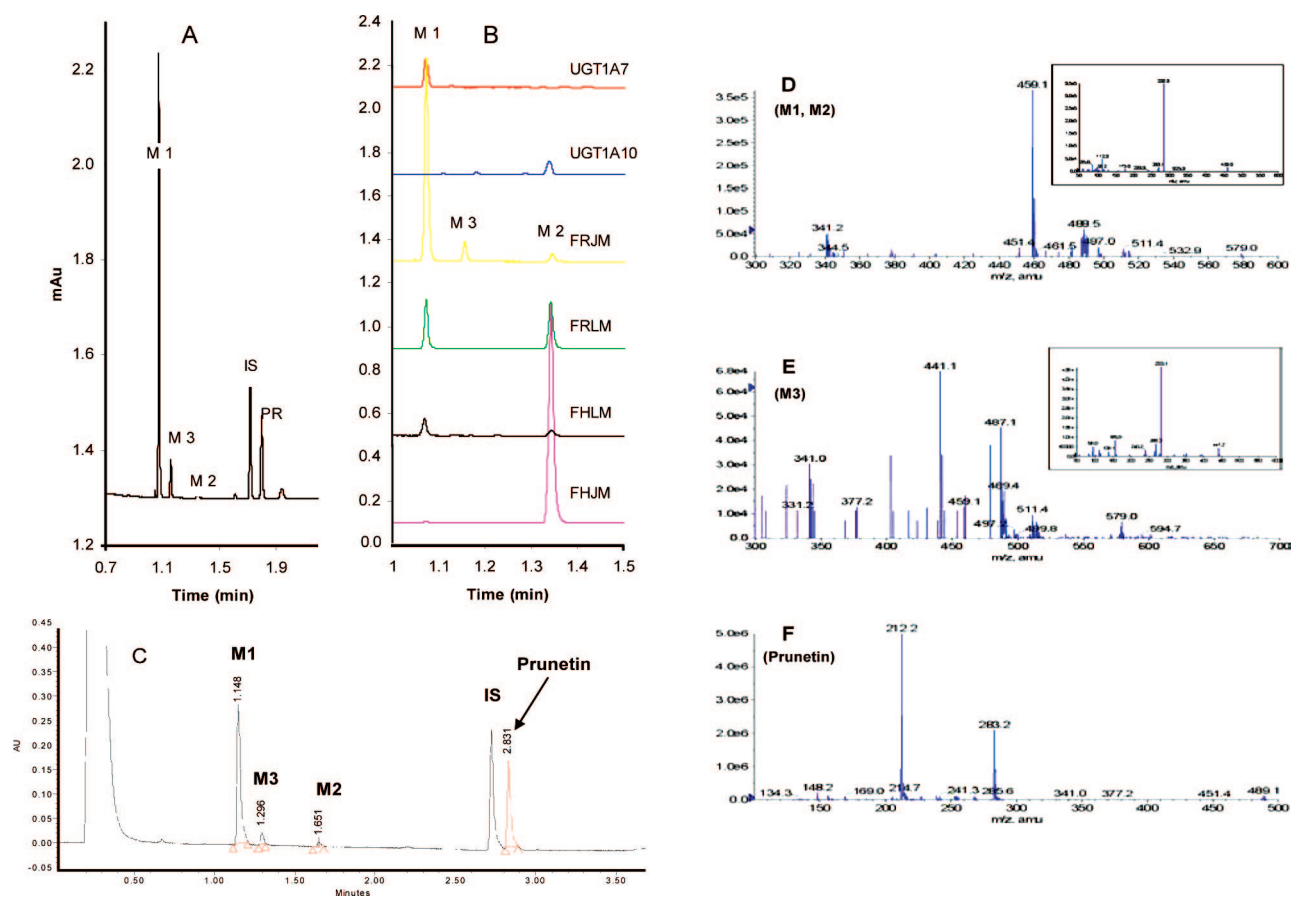
**Statistical Analysis.** One-way ANOVA or Student's *t* tests were used to analyze the data. The prior level of significance was set at 5%, or  $p < 0.05$ .

## Results

**Prunetin Glucuronidation in Human Liver, Jejunum, and Ileum Microsomes.** Microsomal glucuronidation of prunetin was site-/organ-dependent. At 40  $\mu\text{M}$ , glucuronidation was faster in intestinal microsomes than liver microsomes, in that 90% was metabolized by intestinal microsomes whereas 12% of prunetin was metabolized by liver microsomes in 8 h. Intestinal glucuronidation of prunetin mainly formed metabolite 2, whereas the liver glucuronidation mainly formed metabolite 1. Specifically, the rate of formation of metabolite 2 was 72 times higher in the jejunal microsomes ( $2.20 \pm 0.10\text{ nmol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$  of protein), and 65 times higher in the ileal microsomes ( $1.99 \pm 0.10\text{ nmol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$  of protein) than that in the liver microsomes ( $0.030 \pm 0.001\text{ nmol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$  of protein) (Figure 2, top). On the other hand, the formation rate of metabolite 1 using liver microsomes ( $0.073 \pm 0.001\text{ nmol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$  of protein) was about 7 times higher than that using intestinal microsomes ( $0.010 \pm 0.001\text{ nmol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$  of protein). Taken together, the rate of

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**Figure 1.** UPLC chromatograms of prunetin and prunetin metabolites following UV absorbance (A–C) or MS/MS spectral analysis (D–F). The UPLC methods are described in the Materials and Methods section. Panel A shows the UPLC trace of 40  $\mu$ M prunetin metabolism by female rat jejunal microsomes (FRJM), with the peaks of metabolite 1 (M1), metabolite 2 (M2), metabolite 3 (M3), internal standard (IS), and prunetin (PR) labeled. Panel B shows metabolites 1–3 (if present) of 40  $\mu$ M prunetin in female human jejunal microsomes (FHJM), female human liver microsomes (FHLM), female rat liver microsomes (FRLM), female rat jejunal microsomes (FRJM), expressed human UGT1A10 (UGT1A10), and expressed human UGT1A7 (UGT1A7). Notice the distinct pattern of metabolite formation. Panel C shows an alternative UPLC tracing using UV absorbance, which shows prunetin and its three glucuronidated metabolites. Panels D–F show the MS3 spectra of prunetin *O*-glucuronide, prunetin *C*-glucuronide, and prunetin, respectively. The insets in panels D and E show the MS2 full scan for the corresponding metabolite.

metabolite 2 formation in jejunal microsomes was 30 times higher than the rate of metabolite 1 formation in liver microsomes.

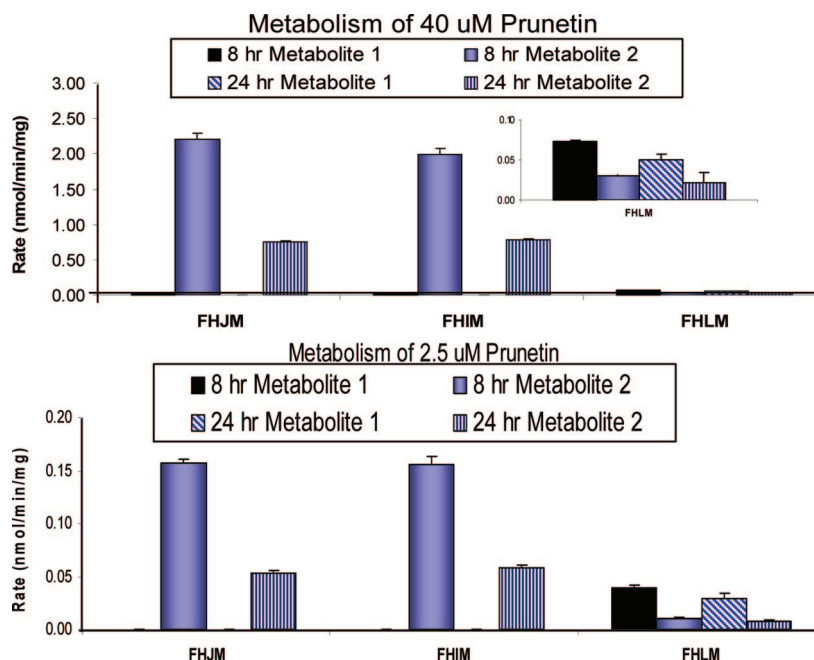
We also determined the effect of concentration change on prunetin metabolism. At 2.5  $\mu$ M, 34% of prunetin was metabolized in 8 h by liver microsomes and 99% by intestinal microsomes. The formation of metabolite 1 was only detectable in liver microsomes at a rate of  $0.040 \pm 0.002$  nmol $\cdot$ min $^{-1}\cdot$ mg $^{-1}$  of protein, whereas the formation of metabolite 2 was at least 15 times higher in jejunal and ileal microsomes ( $0.156 \pm 0.01$  nmol $\cdot$ min $^{-1}\cdot$ mg $^{-1}$  of protein) than in liver microsomes ( $0.010 \pm 0.001$  nmol $\cdot$ min $^{-1}\cdot$ mg $^{-1}$  of protein) (Figure 2, bottom). The formation rate by intestinal microsomes was underestimated at 2.5  $\mu$ M since more than 98% of the substrate has been exhausted at 8 h.

We also determined how prolongation of incubation time changed metabolism and the results indicated that after 24 h incubation, 30% prunetin (concentration = 40  $\mu$ M) was metabolized by liver microsomes whereas 100% was me-

tabolized by the intestinal microsomes. For 2.5  $\mu$ M, the rates of intestinal microsomal metabolism were not quite meaningful since substrate was nearly exhausted by 8 h, but liver microsomal metabolic rates were still meaningful as the results showed a 70% substrate exhaustion at 24 h. In addition, the retention time of two metabolite peaks did not change, and no additional metabolite peak was visible at longer incubation time, suggesting the absence of diglucuronide formation as aglycone was exhausted.

**Identification of Prunetin Metabolites.** There are only two *O*-glucuronides identified in metabolism studies conducted using human microsomes. These two glucuronides have the same molecular weight ( $m/z = 459$  in negative ion mode, Figure 1B), corresponding to two phenolic groups available for *O*-glucuronidation in prunetin. Therefore, we need to purify only one of the metabolites to identify the structures of both. M2 was selected for purification because glucuronidation of prunetin in human intestinal microsomes produced this metabolite almost exclusively (>98% of the





**Figure 2.** Metabolism of prunetin by female human jejunal microsomes (FHJM), female human ileal microsomes (FHIM), and female human liver microsomes (FHLM) at two concentrations (40 and 2.5  $\mu\text{M}$ ) and two incubation times (8 and 24 h). The experiments were conducted at 37  $^{\circ}\text{C}$  for two incubation times and substrate concentrations, and the amounts of metabolite 1 and metabolite 2 were measured using UPLC. Each bar is the average of three determinations, and the error bars are the standard deviations of the mean.

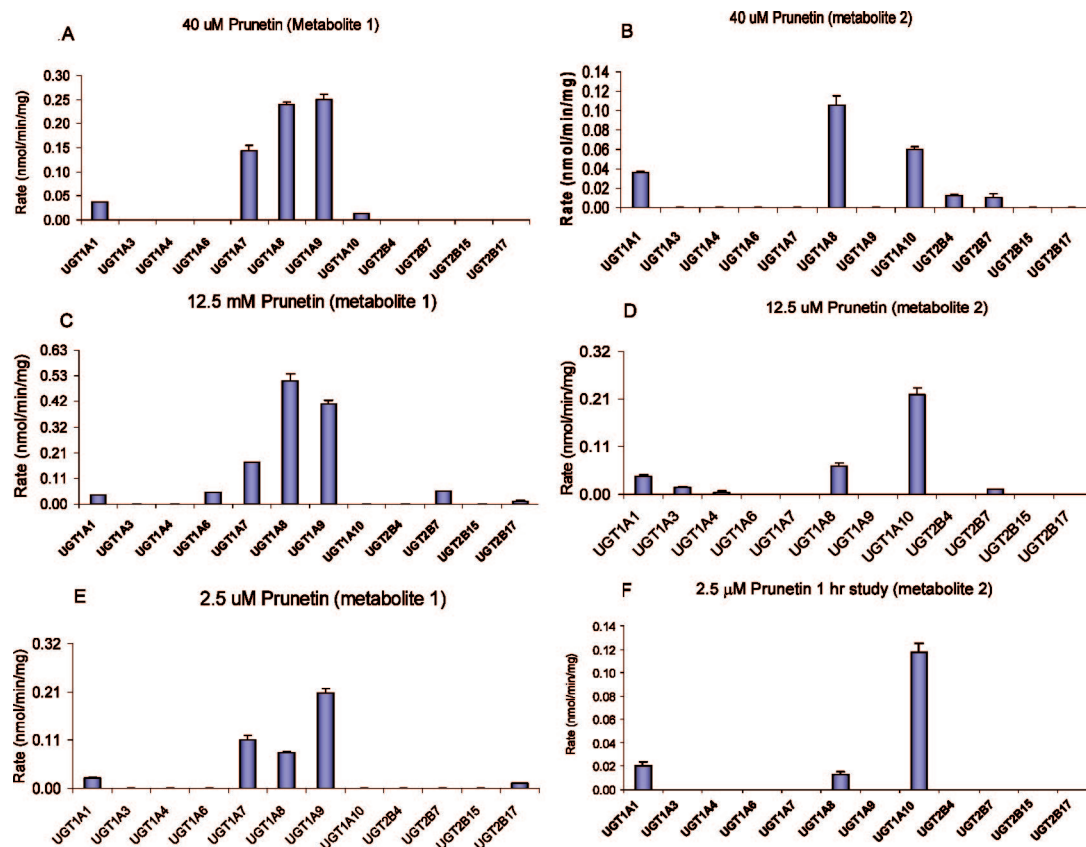
glucuronides). The structure of the main metabolite (M2) was identified by MS and NMR spectra. The positive ESI/MS spectrum showed that M2 and M1 have the same molecular weight as the monoglucuronide derivative of prunetin ( $m/z = 459$ , Figure 1B). Its  $^1\text{H}$  NMR spectrum suggested the presence of a  $\beta$ -D-glucuronopyranosyl moiety in M2, as evidenced by the anomeric proton at  $\delta$  4.86 (1H, d,  $J = 7.2$  Hz). By comparing the  $^1\text{H}$  NMR spectrum of M2 with that of the parent compound, downfield shifts of H-2',6' ( $\Delta\delta_{\text{H}} = +0.27$  ppm) and H-3',5' ( $\Delta\delta_{\text{H}} = +0.12$  ppm) were observed, indicating that the glucuronyl group is located at C-4'. This conclusion is further evidenced by the disappearance of the signal of 4'-OH ( $\text{D}_2\text{O}$  exchanged) at  $\delta_{\text{H}}$  9.62 ( $\text{D}_2\text{O}$  exchanged) in the  $^1\text{H}$  NMR spectrum of M2. On the basis of the above spectroscopic evidence, M2 was identified as prunetin 4'-O- $\beta$ -D-glucuronide.

**Metabolism of Prunetin by Expressed Human UGT Isoforms.** Prunetin glucuronidation rates were measured using 12 expressed human UGT isoforms since drastically different patterns of prunetin metabolism in the human intestinal and liver microsomes (as shown earlier) were likely the results of organ-specific expression of UGT isoforms.

To investigate which UGT isoforms may be responsible, we first used one incubation time and a high substrate concentration to determine the contribution of UGT isoforms, as has been done previously.<sup>5-9</sup> At a high concentration of prunetin (40  $\mu\text{M}$ ) and short incubation period (1 h), UGTs 1A7, 1A8, and 1A9 produced significant amounts of metabolite 1, and the averaged rates of glucuronidation were  $0.14 \pm 0.0123$ ,  $0.24 \pm 0.0058$ , and  $0.25 \pm 0.0109$

$\text{nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$  of protein, respectively (Figure 3A). Among other tested UGT isoforms, only UGT1A3 and UGT1A10 made minor amounts of metabolite 1 (5-O-glucuronide), whereas other UGT1A and UGT2B subfamily members (UGT2B4, 2B7, 2B14, and 2B17) did not (Figure 3A). On the other hand, UGTs 1A1, 1A8, and 1A10 produced significant amounts of metabolite 2 (4'-O-glucuronide), and the glucuronidation rates were  $0.04 \pm 0.001$ ,  $0.11 \pm 0.01$ , and  $0.06 \pm 0.002$   $\text{nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$  of protein, respectively (Figure 3B). Finally, UGT2B4 and UGT2B7 produced minor but measurable amounts of prunetin metabolite 2 (4'-O-glucuronide), whereas other isoforms did not (Figure 3B).

**Effect of Concentration Change on Prunetin Metabolism by UGT Isoforms.** Some investigators have argued for the use of more than one (mostly two) substrate concentrations to probe isoform-specific metabolism,<sup>10-12</sup> probably because different UGT isoforms will likely have different  $K_{\text{m}}$  and  $V_{\text{max}}$  values, resulting in different rates at different concentrations. Therefore, we also examined the UGT isoform-specific metabolism profiles using more physiological relevant concentration of 12.5 and 2.5  $\mu\text{M}$ , again for the same short incubation time of 1 h. At 12.5  $\mu\text{M}$ , metabolite 1 (5-O-glucuronide) was formed by UGTs 1A7, 1A8, and 1A9 at rates of 0.17,  $0.50 \pm 0.03$ , and  $0.41 \pm 0.02$   $\text{nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$  of protein, respectively (Figure 3C), whereas metabolite 2 (4'-O-glucuronide) was formed by UGTs 1A1, 1A8, and 1A10 at rates of  $0.04 \pm 0.003$ ,  $0.06 \pm 0.01$ , and  $0.22 \pm 0.02$   $\text{nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$  of protein,



**Figure 3.** Effects of concentration on the isoform-specific metabolism of prunetin. The experiments were conducted at 37 °C for 1 h, and the amounts of metabolite 1 and metabolite 2 were measured using UPLC. Each bar is the average of three determinations, and the error bars are the standard deviations of the mean.

respectively (Figure 3D). At this concentration, several isoforms produced minor amounts of metabolites 1 and 2 (Figure 3C,D). At 2.5 μM, UGTs 1A7, 1A8, and 1A9 were still the main isoforms that made significant amounts of metabolite 1 (5-*O*-glucuronide) at rates of  $0.11 \pm 0.01$ ,  $0.08 \pm 0.003$ , and  $0.21 \pm 0.01$  nmol·min<sup>-1</sup>·mg<sup>-1</sup> of protein, respectively, whereas metabolite 2 (4'-*O*-glucuronide) was formed in significant amounts by 1A1, 1A8, and 1A10 at rates of  $0.02 \pm 0.004$ ,  $0.01 \pm 0.003$ , and  $0.12 \pm 0.01$  nmol·min<sup>-1</sup>·mg<sup>-1</sup> of protein, respectively. Two isoforms (UGT1A1 and 2B4) made minor contributions to the formation of metabolite 1, whereas other isoforms did not make a detectable contribution to the formation of either metabolite. Looking at the isoform-specific metabolism profiles of metabolite 1 (Figure 3A,C,E), dominant isoforms changed with substrate concentration. This change in dominant isoforms was more obvious for metabolite 2 (see Figure 3B,D,F).

#### Effect of Incubation Time on Metabolite Formation.

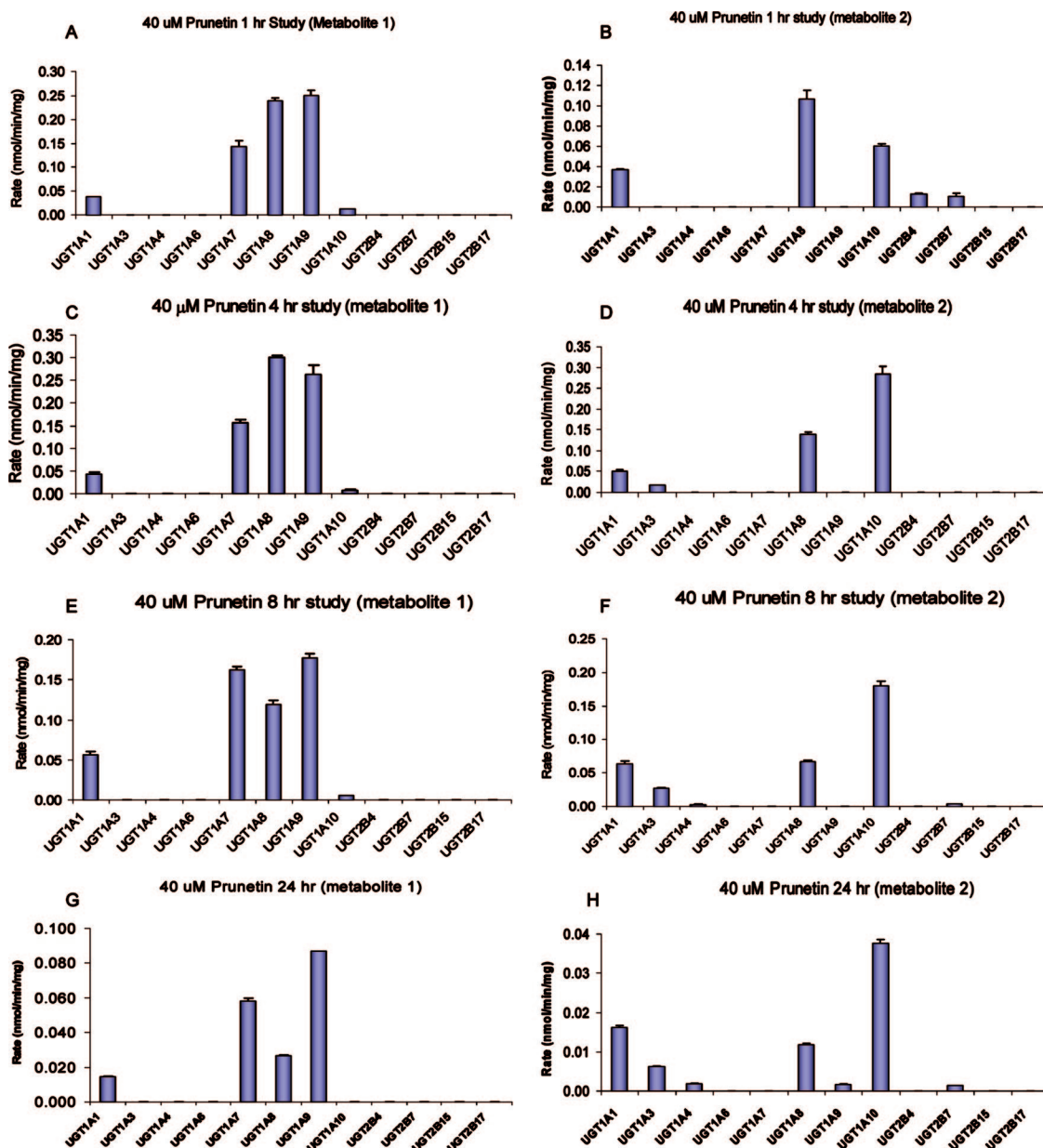
We were interested in determining the effect of time on metabolite formation for four reasons. First, metabolism of some isoflavones followed autoactivation.<sup>15,19</sup> Second, the

metabolite could be a diglucuronide, and if it is, its formation may continue to increase even if the substrate is depleted after a long incubation time, as diglucuronide can be formed from monoglucuronide. Third, as a glucuronide is accumulated in the reaction mixture, it may inhibit the function of a particular isoform, resulting in a change in the isoform-specific metabolite profile. The latter was observed in intact Caco-2 cell metabolism of raloxifene, where the pattern of metabolism was different in cell lysate versus intact cells as a result of the longer incubation time.<sup>20</sup> Last, certain UGT isoforms may be heat sensitive.

At 40 μM, prunetin (the substrate of reaction) was not exhausted, with a maximum of 70% being metabolized by UGT1A8 at 24 h. The rates of metabolite 1 (5-*O*-glucuronide) formation measured at 1, 4, 8, and 24 h following the incubation were fairly constant, with a statistically significant ( $p < 0.01$ ) but small change of the most active form from UGT1A8 and UGT1A9 to UGT1A9 only (Figure 4A,C,E,G). On the other hand, the formation rates of metabolite 2 (4'-*O*-glucuronide) at 1, 4, 8 and 24 h following the incubation fluctuated substantially, and the main isoform changed from UGT1A8 (at 1 h) to UGT1A10 (for 4 h or longer) (Figure

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**Figure 4.** Effects of incubation time on the isoform-specific metabolism of prunetin at 40  $\mu$ M. The experiments were conducted at 37  $^{\circ}$ C for various times (1, 4, 8, or 24 h), and the amounts of metabolite 1 and metabolite 2 were measured using UPLC. Each bar is the average of three determinations, and the error bars are the standard deviations of the mean.

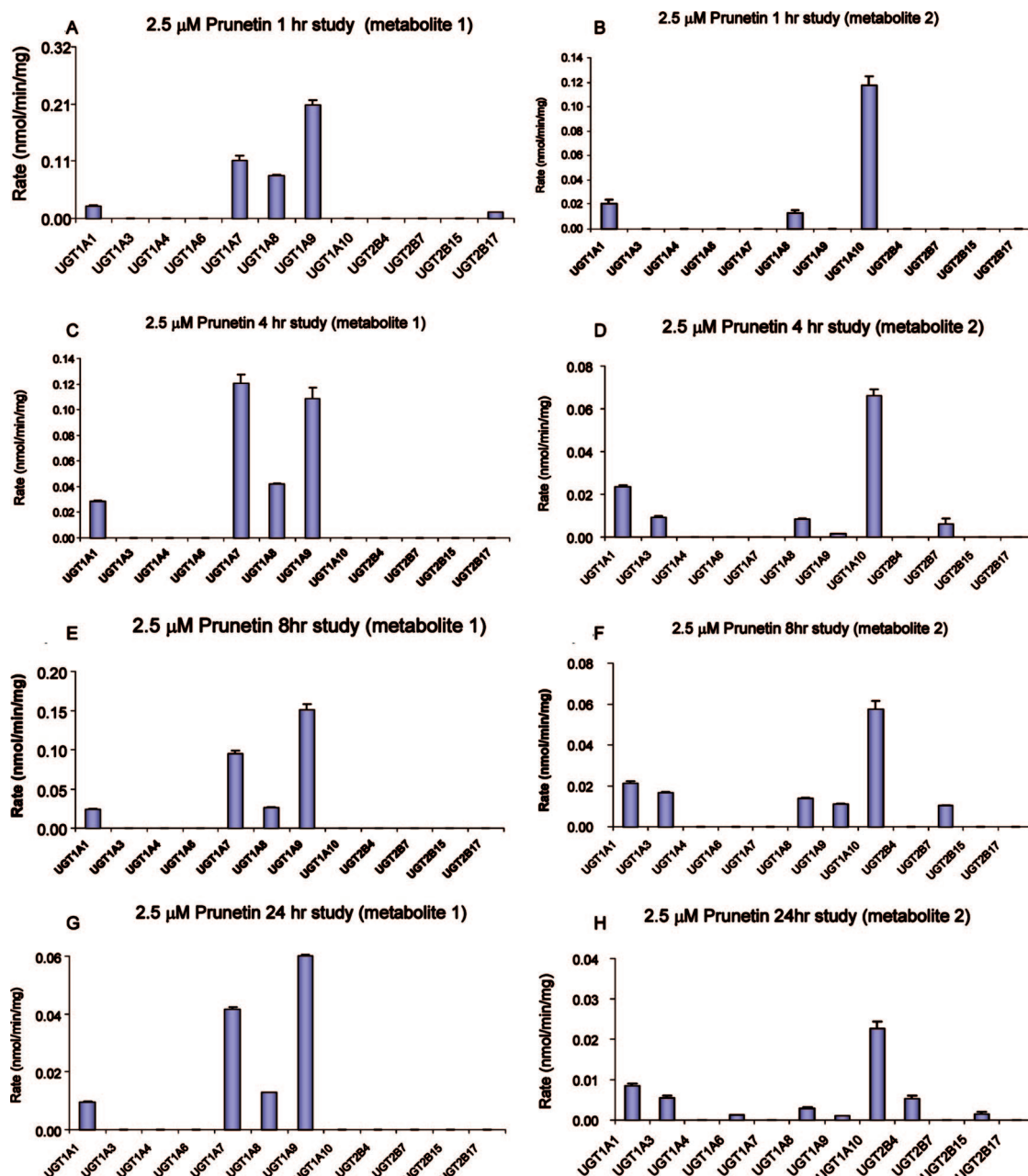
4B,D,F,H). This is not due to substrate exhaustion, since only 15% of the substrate was metabolized at 4 h using the present experimental conditions.

At 2.5  $\mu$ M, which is a more physiological concentration, the substrate prunetin was 100% metabolized by UGT1A9 in less than 24 h since more than 40% of prunetin was metabolized by UGT1A7 (49%) and UGT1A9 (45%) at 4 h, with even higher percentages by 8 h. The isoform-specific metabolic profile for metabolite 1 (5-*O*-glucuronide) formation changed from UGT1A9 dominant to UGT1A7 and UGT1A9 dominant, and then back to UGT1A9 dominant with a significant contribution from UGT1A8 (Figure 5A,C,E,G). The isoform-specific metabolic profile for metabolite 2 was consistently dominated by UGT1A10 (Figure

5B,D,F,H). In addition, many minor contributing isoforms were found at a longer reaction time, including UGT1A3 and UGT2B7.

**Thermal Stability of Expressed UGTs.** We were somewhat surprised by the fact that there was a strong dependence of the UGT “isoform-specific metabolic profiling” on the length of incubation. Since the incubation was conducted at 37  $^{\circ}$ C, a series of experiments were performed to determine the stability of expressed isoforms. The results indicated that human intestinal UGT isoforms such as UGT1A8 and UGT1A10 were not stable, whereas liver UGT isoform UGT1A9 and ubiquitous UGT1A1 were relatively stable (Figure 6A,B). As a result, the liver UGTs were more stable





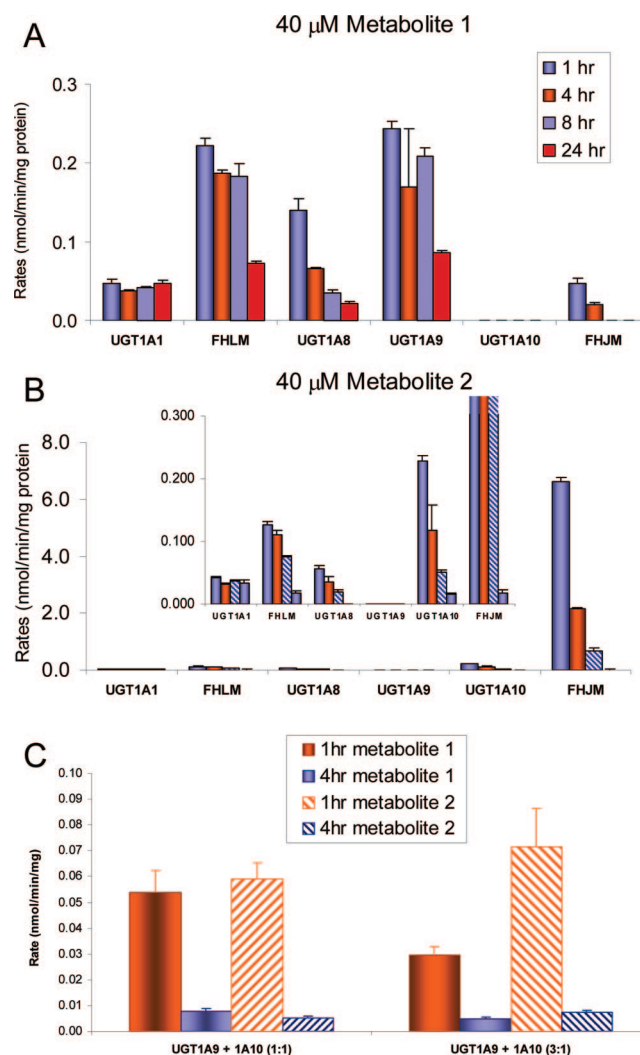
**Figure 5.** Effects of incubation time on the isoform-specific metabolism of prunetin at 2.5  $\mu$ M. The experiments were conducted at 37  $^{\circ}$ C for various times, and the amounts of metabolite 1 and metabolite 2 were measured using UPLC. Each bar is the average of three determinations, and the error bars are the standard deviations of the mean.

than intestinal UGTs (Figure 6A,B). Addition of DTT to the incubation media did not stabilize the UGTs, and in fact it inhibits the activities of UGT1A8 and UGT1A10, two thermolabile isoforms. Mixing of thermally unstable UGT isoforms with thermostable UGT isoforms also did not stabilize the thermally labile UGTs (Figure 6C).

**Comparison of Human and Rat Metabolism of Prunetin.** We also determined glucuronidation of prunetin in rat liver and jejunal microsomes using the same concentration(s) of microsomal protein (0.05 mg/mL in final reaction mixture) and prunetin (40 and 2.5  $\mu$ M) and the same incubation time (8 and 24 h) for the purpose of comparing these results with those obtained using human intestinal and

liver microsomes. The results indicated that rat glucuronidation shares only *limited similarities* in that metabolite 1 (5-*O*-glucuronide) was the main metabolite in the rat liver microsome as well, but only at the low concentration of 2.5  $\mu$ M (Figure 7A). In addition, intestinal microsomes were more sensitive to length of incubation time than liver microsomes, although formation rates of metabolite 3 in the rat intestinal microsomes were not heat sensitive. Other characteristics appeared to be quite different. Among them, metabolite 1 is no longer the main metabolite in the rat liver microsomes at the higher concentration (40  $\mu$ M), whereas it was in human liver microsomes. Metabolite 1 is the main metabolite in rat intestine (Figure 7) even though human





**Figure 6.** Testing of the thermal stability of UGT activities using human microsomes and expressed UGT microsomes. The abilities of UGTs to form metabolite 1 or metabolite 2 were followed as a function of preincubation time (or the length of time when microsomes were incubated with everything but substrate). The concentration used was 40  $\mu$ M, and the actual reaction time was 1 h. Each bar is the average of three determinations, and the error bars are the standard deviations of the mean.

intestine almost did not produce it (Figure 2). We then performed  $\beta$ -O-D-glucuronidase hydrolysis experiment to determine which peaks will decrease or disappear following O-glucuronide hydrolysis experiments (Figure 8). Because both M1 and M2 peaks disappeared following  $\beta$ -O-D-glucuronidase treatment and had the same retention time and molecular weight (in MS/MS) as the human microsomes-generated O-glucuronides, M1 was determined to be 5-O- $\beta$ -D-glucuronide and M2 4'-O- $\beta$ -D-glucuronide. In addition to these two O-glucuronides, a third glucuronidated metabolite (metabolite 3) appeared in the rat only. Since M3 was not hydrolyzable using the  $\beta$ -O-D-glucuronidase, M3 is not a  $\beta$ -O-D-glucuronide but perhaps a C-glucuronide, which is consistent with its molecular weight of 441 (*m/z*) (Figure

1B). The position of this C-glucuronide is likely to be C-8, since prunetin has a naturally occurring C-8 glucoside: puerarin, derived from the plant Kudzu. This C-glucuronide is rather rare and has not been previously reported for UGT glucuronidation of flavonoids in mammals, based on a PubMed search (conducted on September 4, 2007) using as keywords "c-glucuronide flavonoid" or "c-glucuronide flavone" or "c-glucuronide isoflavone".

#### Mouse Intestinal and Liver Metabolism of Prunetin.

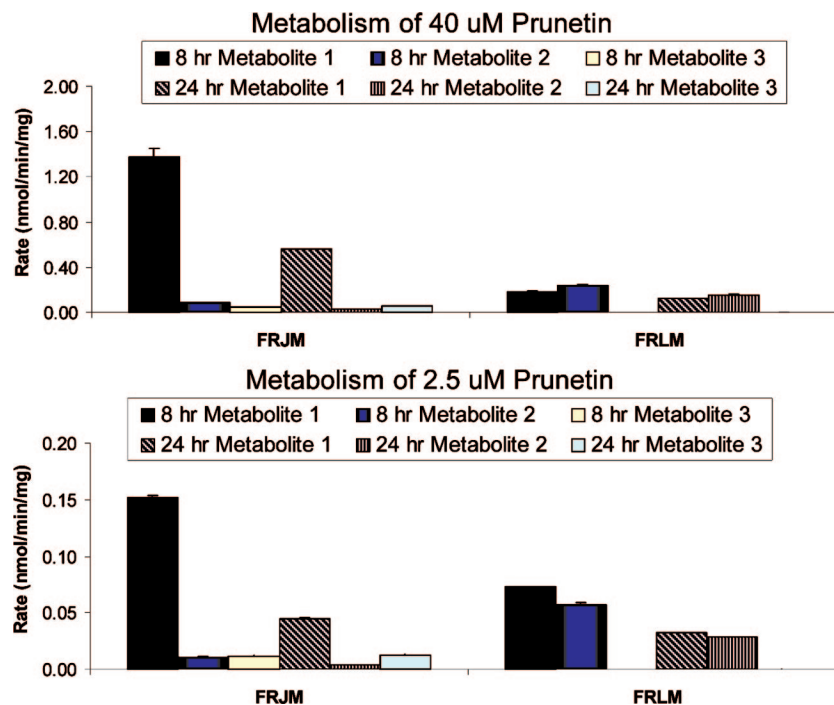
We also determined glucuronidation of prunetin in mouse liver and jejunal S9 fractions, using the same prunetin concentrations (40 and 2.5  $\mu$ M) and the same incubation times (8 and 24 h) for the purpose of comparing these results with those obtained previously using rats and humans. The results indicated that mouse S9 fractions formed only two metabolites (Figure 9A), similar to humans (Figure 2). The mouse intestinal S9 fraction metabolizes prunetin faster than liver microsomes, into two metabolites. Metabolite 1 is the major metabolite in mouse intestinal S9 fraction, but metabolite 2 is the major metabolite in mouse liver S9 fraction. In addition, apparent rates of metabolism decreased as the incubation time increased at high prunetin concentration, where only a small percentage (<30%) of prunetin is metabolized.

## Discussion

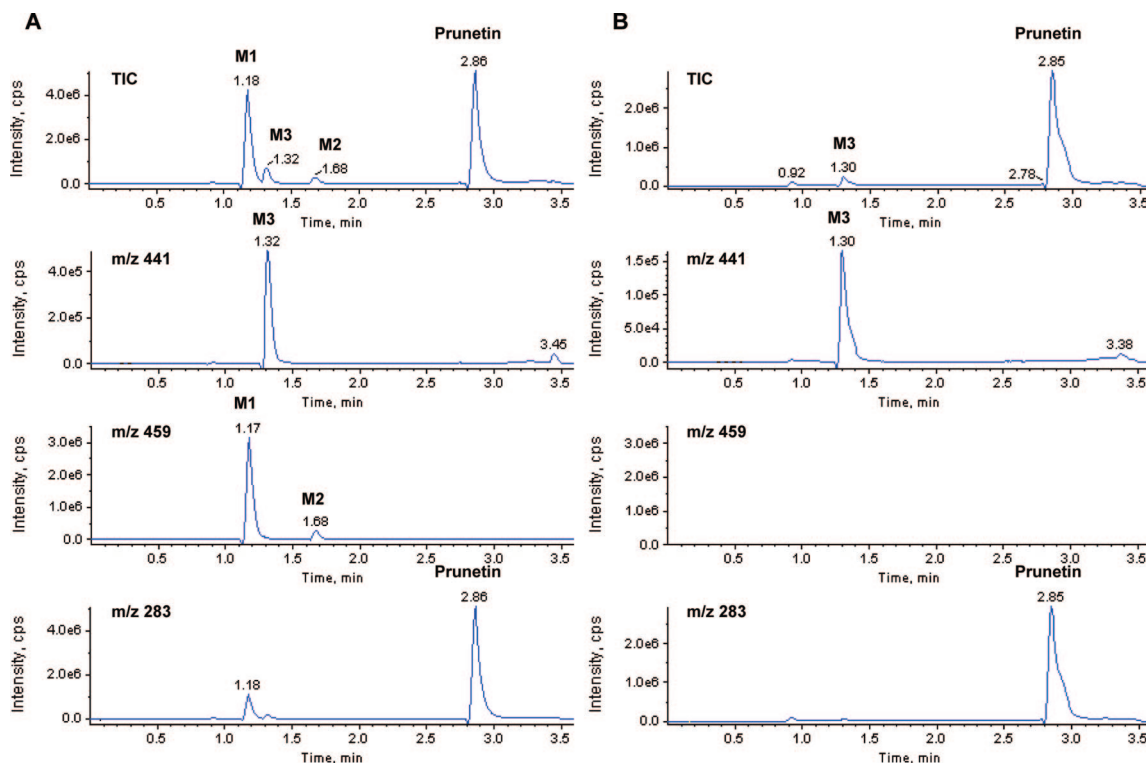
In our continued effort to characterize the intestinal disposition of flavonoids, we have begun in this paper to determine how human subcellular fractions such as microsomes and expressed human UGTs metabolize isoflavones. Earlier, we conducted comprehensive studies of six isoflavones using rat intestinal and liver microsomes.<sup>15</sup> Therefore, the present work represents first systematic and comprehensive characterization of prunetin glucuronidation in human liver and intestinal microsomes and in expressed human UGT isoforms. We also contrasted the metabolism of prunetin in human liver and intestinal microsomes to that of rat or mouse liver and intestinal microsomes/S9 fractions. In addition to these two sets of conventional studies, we also performed pioneering study of how the thermostability of selected UGT isoforms and human intestinal and liver UGTs affected prunetin metabolism.

One of the results of our conventional studies was that intestinal microsomes produced metabolite 2 (4'-O-glucuronide) at rates substantially faster than metabolite 1 (5-O-glucuronide) by intestinal or liver microsomes. On the basis of the distribution pattern of human UGT1As, we expected that prunetin would be mainly metabolized into metabolite 2 by UGT1A8 and 1A10 since they are mainly expressed in the intestine.<sup>21</sup> We expected that prunetin would be metabolized into metabolite 1 by UGT1A9 and UGT1A1 since they are abundantly expressed in the liver.<sup>22</sup> The results of our

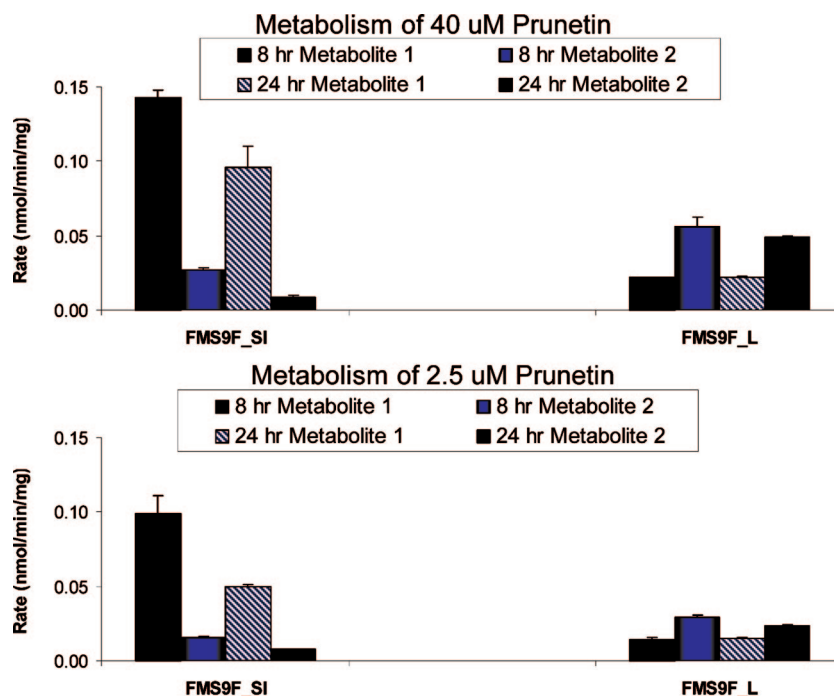
(21) Radominska-Pandya, A.; Little, J. M.; Pandya, J. T.; Tephly, T. R.; King, C. D.; Barone, G. W.; Raufman, J. P. UDP-glucuronosyl-transferases in human intestinal mucosa. *Biochim. Biophys. Acta* **1998**, *1394* (2–3), 199–208.



**Figure 7.** Metabolism of prunetin by female rat jejunal microsomes (FRJM) and female rat liver microsomes (FRLM) at two concentrations (40 and 2.5  $\mu$ M) and two incubation time (8 and 24 h). The experiments were conducted at 37  $^{\circ}$ C, and the amounts of metabolite 1, metabolite 2, and metabolite 3 were measured using UPLC. Each bar is the average of three determinations, and the error bars are the standard deviations of the mean.



**Figure 8.** Total anion scan (TIC) or selective anion scan ( $m/z$  441, 459, and 283) of rat microsomal metabolism samples containing prunetin and three of its glucuronides before (A) and after (B) hydrolysis with  $\beta$ -D-glucuronidases (24 h). Clearly shown in this figure is that *C*-glucuronide ( $m/z$  441) is not hydrolyzable by  $\beta$ -D-glucuronidases. There was some cross-talk between prunetin ( $m/z$  283) and its glucuronides, as these glucuronides are not very stable during the ionization process.



**Figure 9.** Metabolism of prunetin by female mouse jejunal S9 fraction (FMS9F\_SI) and female mouse liver S9 fraction (FMS9F\_L) at two concentrations (40 and 2.5  $\mu$ M) and two incubation times (8 and 24 h). The experiments were conducted at 37 °C, and the amounts of metabolite 1 and metabolite 2 were measured using UPLC. Each bar is the average of three determinations, and the error bars are the standard deviations of the mean.

study confirmed our expectations and were consistent with the results of an earlier study showing more rapid glucuronidation of genistein (but not daidzein) in human colon microsomes than in human liver microsomes.<sup>2</sup> We then performed additional studies to characterize the isoform-specific metabolism of prunetin using the so-called “isoform-specific metabolism profiling” technique, which is the determination of UGT isoform(s) responsible for the glucuronidation of a specific substrate to form a specific metabolite. This is often performed because of the need to identify possible drug interaction potentials and to search out single nucleotide polymorphism of a UGT isoform that may impact the metabolism of a therapeutically important drug. It is also done to find out whether we can predict organ-specific metabolism on the basis of the isoform distribution patterns.

We found that UGT “isoform-specific metabolism profiles” were metabolite- and substrate concentration-dependent (Figure 3), which was quite consistent with what other investigators have found using other UGT substrates. However, we were surprised that incubation time impacted the UGT “isoform-specific metabolism profiling” (Figure 4), since this was not expected as long as the concentration of prunetin (40  $\mu$ M) was sufficient to sustain the reaction rate. This is a chance discovery, since our initial aim was to use a long incubation time to determine if there is a diglucuronide during phase II reactions. In other words, we expected the diglucuronide peak to increase as the aglycone was ex-

hausted, but this did not happen. What did happen was that the rates of metabolism decreased when incubation time increased, even if substrate concentrations were high enough to sustain constant reaction rates.

This led us to determine why reaction rates decreased as a function of incubation time, in that the longer the incubation time, the slower the rates of metabolism, which is especially evident in the intestinal microsomes. We hypothesized that this could be the result of isoform-specific thermal instability. Previously, SULT1A1 was found to be more heat stable than many other SULT isoform.<sup>23,24</sup>

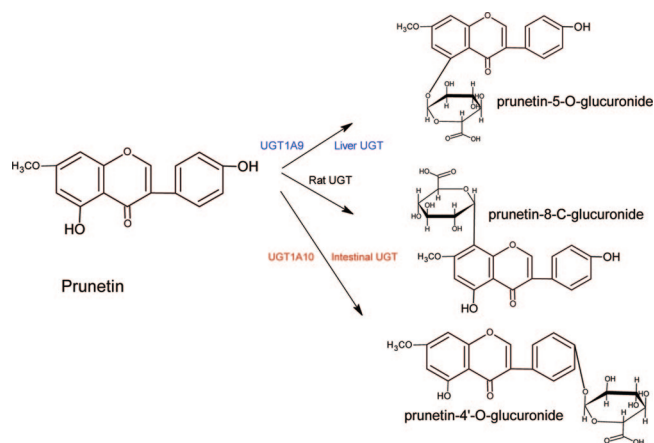
The results of our studies showed that certain isoforms, such as UGT1A9 and UGT1A1, were substantially more thermostable than UGT1A10 and UGT1A8 (Figure 6A). Interesting, the thermolabile isoforms appear to be mostly intestinal isoforms such as UGT1A10. In fact, UGT enzyme activities are more thermostable in liver than in intestinal microsomes of humans. Using the same criteria, it appears that UGT activities in liver microsomes of rodents are also more thermostable than those in the intestinal microsomes (Figures 7 and 9).

At present, we do not know why certain UGT isoforms are heat labile whereas the other isoforms are heat resistant.

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**Figure 10.** Metabolic pathways of prunetin in intestine and liver of rodents and humans. The position of C-glucuronide remains to be confirmed using NMR.

Oxidation did not appear to be the reason, since the use of a strong antioxidant (5 mM DTT) failed to stabilize these isoforms. Mixing of heat-stable and heat-labile UGT isoforms also did not stabilize the heat-labile isoforms. Further studies are also needed to identify the mechanism by which heat deactivates these intestinal UGT isoforms but not the liver UGT isoforms.

Finally, we also compared the similarities and differences in UGT metabolism of prunetin between humans and rodents (Figure 10). Our studies indicated that the intestinal microsomes were much more active than liver microsomes in all three species, a rare similarity. However, metabolite 1 was the predominant metabolite formed in both rat liver and intestinal microsomes, where metabolite 2 was the dominant human intestinal metabolite. On the basis of human and rat isoform expression pattern, we could assign this difference to the lack of UGT1A10 in rats.<sup>25</sup> In contrast, high expression

levels of UGT1A1 and UGT1A7 in the rat liver and intestine<sup>26</sup> but not in the human liver or intestine (e.g., expression of UGT1A7 limited to the stomach in humans) probably explain why rat liver microsomes appeared to be more active than those of humans. It also explains why metabolite 1 was always the major metabolite in both organs. There was little difference in the pattern of metabolism and type of metabolites formed between rat and mouse metabolism of prunetin. A major difference is the presence of C-glucuronide in rat intestinal microsomes but not in the intestinal or liver subcellular fractions of mice (Figure 8). C-Glucuronide of prunetin was also not found in studies conducted using human microsomes.

In conclusion, UGT-mediated metabolism of prunetin was organ-, concentration-, and UGT isoform-specific, all of which may be impacted by the heat stability of the UGTs involved in the glucuronidation. Because UGT isoforms have differential heat stability, more studies are needed to carefully characterize the heat stability of various isoforms so that we can better measure UGT activities under various conditions. Finally, our results suggest that we should conduct the “isoform-specific metabolism profiling” study at two or more concentrations and for at least two time periods. If those conditions provide consistent results, then no additional studies are required to complete the profiling study. Care must be taken in interpreting the results when only one concentration and one time are used for the characterization.

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