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Identification of Puerarin and Its Metabolites in Rats by Liquid Chromatography–Tandem Mass Spectrometry

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Puerarin (daidzein-8-*C*-glucoside) is the major bioactive isoflavone of kudzu root (the root of *Pueraria lobata*). Its metabolic fate, however, is not well-known. In this study, a sensitive and specific LC-ESI-MS/MS method for the determination of puerarin and its metabolites daidzein, dihydrodaidzein, and equol was developed for their analysis in biological samples. Two new metabolites of puerarin, mono- and dihydroxylated derivatives, were detected in the urine and feces of rats after oral administration. The persistence of puerarin in blood and urine as the principal metabolic form for the period of 4–72 h after oral administration suggested that puerarin is rapidly absorbed from the intestine without metabolism. Its presence in organs such as the brain suggests that this glucoside may enter tissues by specific transport pathways. Study of these metabolites may provide further understanding of the health beneficial effects of puerarin in kudzu dietary supplements.

KEYWORDS: Puerarin; metabolites; *C*-glucoside; kudzu; LC-MS/MS; rats

INTRODUCTION

Glucoside conjugates of isoflavonoids (*O*- and *C*-glucosides) are the major naturally occurring isoflavones in leguminous plants, including kudzu (*Pueraria lobata*). Flavonoid *O*-glycosides such as phlorizin are inhibitors of sodium-dependent glucose transporters (1, 2). This has led to the suggestion that bioflavonoid glycosides could be absorbed from the small intestine via these transporters (3). However, currently it is generally considered that the glycosidic forms of the isoflavones and other flavonoids must first be hydrolyzed in the intestine to release the respective aglycons by intestinal glucosidases/hydrolases (4, 5). In the case of *O*-glycosides, these are hydrolyzed by β -glucosidases in the small intestinal wall and appear in plasma mostly as metabolites within a short period of time (6, 7). Because the aglycons and their metabolites are more hydrophobic, they may be more efficiently transported across the wall of the gastrointestinal tract than their respective glucosides. However, there appears to be a complex interplay between the chemical structure of glycosides and their rate of transport (8, 9). The *C*-glycosides of isoflavones, which are resistant to hydrolysis by glucosidases, represent an important tool to evaluate whether bioflavonoid glycosides are capable of intestinal transport via the glucose transporter systems. This

prompted us to develop an LC-MS method for the analysis of the isoflavone-*C*-glycosides such as puerarin (daidzein-8-*C*-glucoside) and their in vivo metabolites.

Puerarin, the most abundant isoflavone-*C*-glucoside, isolated from *Pueraria Radix* (the root of kudzu, *P. lobata*), has been shown to have beneficial effects on cardiovascular, neurological, and hyperglycemic disorders (10–12). Because *Pueraria Radix* has been prepared in the form of commercially available dietary supplements that are consumed by the public, it is critical to understand the metabolic fate of its major isoflavones. However, little is known about the metabolism of puerarin. Understanding of its metabolism and bioavailability is very important in defining the pharmacological and toxicological profile of this compound and other similar *C*-glycosides. In the present work we have applied LC-MS and MS/MS methods to the measurement of puerarin in biofluids (serum and urine), feces, and tissues (heart and brain) of rats.

MATERIALS AND METHODS

Chemicals. Puerarin was purchased from Sigma Chemical Co. (St. Louis, MO). Apigenin and other standards, daidzein and equol, were purchased from Indofine (Somerville, NJ). Dihydrodaidzein was obtained from Dr. Kristiina Wähälä (University of Helsinki), Finland. All other HPLC solvents and reagents were purchased from Fisher (Norcross, GA) and were of HPLC grade.

Standard Solutions and Calibration Curve. Stock solutions of standards (100 μ M) and the internal standard, apigenin, were separately prepared by dissolving accurately weighed amounts in 80% aqueous methanol. These solutions were serially diluted with the same solvent to obtain calibration standards. The stock solutions were kept refriger-

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ated (4 °C). The calibration curves covered a wide range of concentrations (0.05, 0.1, 0.5, 1, 5, 10, 25, and 50 μ M) of the standards.

Animal Experiments and Sample Preparation. Sprague–Dawley male rats ($n = 9$) were obtained from Harlan Sprague–Dawley Inc., Indianapolis, IN. They were kept in a controlled environment at 23 °C and 55% relative humidity under a 12 h dark–light cycle, with free access to soy-free custom diet TD86369 (Harlan TekLAD, Madison, WI) and tap water for 2 weeks. The rats were then placed in metabolic cages with access to water and food. Puerarin (50 mg/kg of body weight) was orally administered in suspension by gavage to the rats four times at intervals of 72 h. Urine samples were collected at intervals of 0–4, 4–20, 20–27, 27–43, and 43–72 h. Blood was collected at 4 h after the first puerarin administration by inserting a cannula into the femoral artery, and serum was isolated by centrifugation of the blood at 3000g for 10 min at 4 °C. The rats were anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg). The heart, brain, and blood were collected 24 h after the final administration, and the organs were taken at sacrifice after perfusion with ice-cold normal saline. Feces were collected and combined at the end of the experiment. All samples were kept at –20 °C until analysis.

Heart and brain samples (wet weight = 1–2.2 g) were minced and added to 1.5 mL of double-distilled water. The tissue was homogenized with a Tissue Tearor homogenizer (Biospace Product Inc., Racine, WI), and 3.5 mL of MeOH and 10 μ L of 50 μ M apigenin (internal standard) were added for extraction of puerarin and its metabolites. The samples were vortex mixed and tumbled for 2 h at room temperature. The homogenate was centrifuged at 3000g for 10 min and the supernatant removed. The tissue was re-extracted by adding an additional 5 mL of 80% aqueous MeOH. The aqueous MeOH extracts were dried under air and reconstituted in the same solvent (100 μ L).

Thawed urine and serum samples (200 μ L) were placed in Eppendorf tubes, and 100 μ L of apigenin (50 μ M) was added. A further 200 μ L of acetonitrile was added to precipitate proteins. Samples were vortex mixed and then centrifuged (3000g for 10 min) in Eppendorf tubes. When needed, the extract of the urine samples was filtered through a syringe filter. The supernatant was transferred to an autosampler vial, and a 20 μ L aliquot was injected onto the LC-MS/MS system for analysis. Frozen fecal material (200 g) was extracted with 80% aqueous MeOH (250 mL \times 2) and centrifuged at 3000g for 10 min, and a 100 μ L aliquot was directly analyzed by ESI-MS.

LC-MS/MS Analysis of Puerarin and its Metabolites. LC-MS/MS analyses were performed using a system consisting of a model SIL-HT refrigerated Shimadzu autosampler (Shimadzu Scientific Instruments, Inc., Columbia, MD) and an API-III triple-quadrupole mass spectrometer (PE Sciex, Concord, Canada). Chromatography was carried out on a 100 \times 4.4 mm i.d. Aquapore RP-300 reversed-phase column (Perkin-Elmer, Shelton, CT) pre-equilibrated with 10 mM ammonium acetate (NH_4OAc). The mobile phase consisted of a gradient of 10–40% acetonitrile in 10 mM NH_4OAc with a flow rate of 1.0 mL/min. Multiple reaction monitoring (MRM) was used to perform mass spectrometric quantification of puerarin and its metabolites. The MRM delivers a unique product ion that can be monitored and quantified in the midst of a very complicated biological matrix. For MRM-MS, the gradient was over 15 min, whereas for LC-MS, it was over 30 min. The column effluent was introduced into the mass spectrometer using electrospray ionization in negative mode. The voltage on the ionspray interface was –4900 V, and the orifice potential was set at –50 V. Selected $[\text{M} - \text{H}]^-$ (deprotonated molecular ions) were analyzed by collision-induced dissociation with 90% argon–10% nitrogen gas, and product ion spectra were recorded. The MRM analysis was conducted by monitoring the precursor ion to product ion transitions from m/z 415/267 (puerarin), 415/295 (puerarin), 253/223 (daidzein), 255/149 (dihydrodaidzein), 241/119 (equol), and 269/149 (apigenin). MACQUAN software provided by PE SCIEX was used to process the quantification data.

Various procedures were performed to validate the assay. The lower limit of quantification (LLOQ) was defined as the lowest concentration in the standard curve for which the percentage coefficient of variation (%CV) was <15%. Specificity was established by the lack of interference peaks at the retention time for the internal standard and puerarin. Linearity was tested at eight levels of concentrations covering

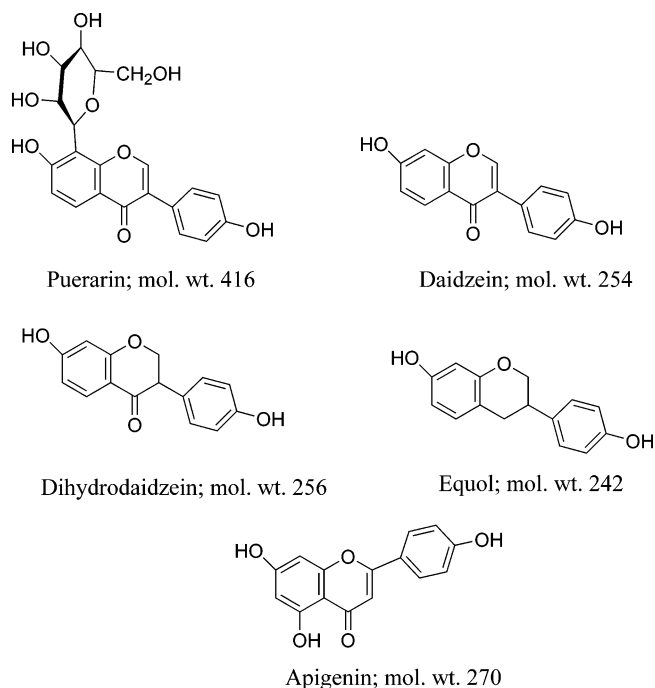


Figure 1. Structures of standards.

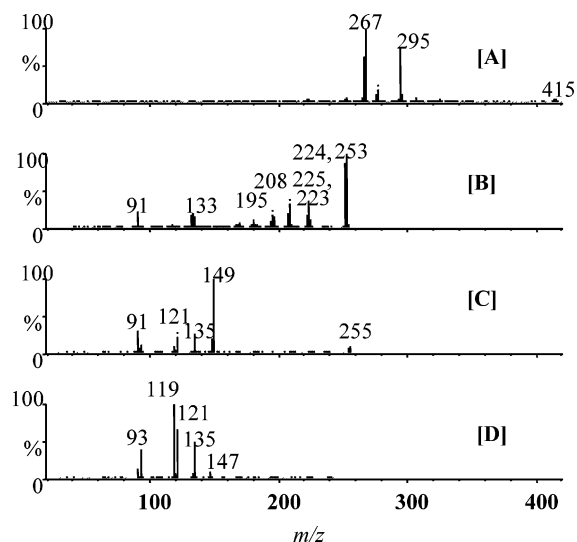


Figure 2. Product ion spectra of standards: puerarin [A], daidzein [B], dihydrodaidzein [C], and equol [D].

a range from 0.05 to 50 μ M. Puerarin, daidzein, dihydrodaidzein, and equol had linear response curves with correlation coefficients >0.99. The detection limit of injected puerarin was 50 fmol/ μ L.

RESULTS

LC-MS/MS Method Development. Puerarin and its metabolites in biological samples were investigated using LC-MS/MS methods. The first step of this work involved the characterization of the mass spectral properties of the parent compound and other standards.

The chemical structures and molecular weights of standards are shown in Figure 1. The product ion spectra of the $[\text{M} - \text{H}]^-$ ions of puerarin and the other standards, daidzein, dihydrodaidzein, and equol, are shown in Figure 2. The deprotonated molecular ion of puerarin showed prominent ions at m/z 295 and 267 due to the losses of 120 and 148 Da, respectively. We recently reported a detailed interpretation on the tandem mass spectrum of puerarin (13). An MS/MS experiment on the

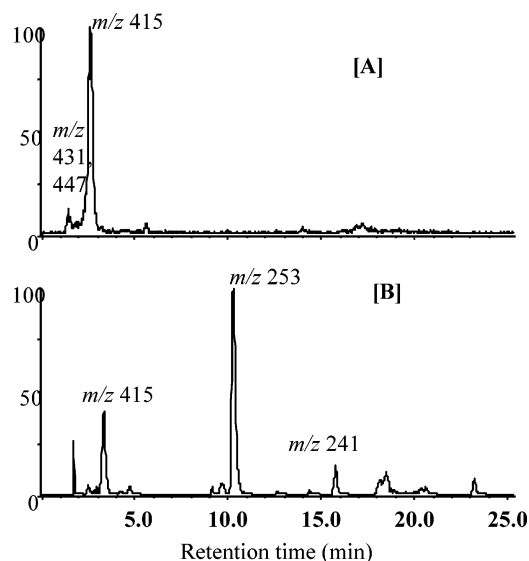


Figure 3. Representative LC-MS chromatogram of rat urine samples obtained during 4 h [A] and 43 h [B] after puerarin administration.

deprotonated molecular ion $[M - H]^-$ of daidzein (m/z 253) yielded a series of product ions due to the losses of 28, 29, 30, and 16 Da. A cluster of product ions appeared at m/z 223, 224, and 225 due to possible losses of CH_2O (30 Da), CHO (29 Da), and CO (28 Da), respectively. Likewise, dihydrodaidzein yielded product ions at m/z 91, 121, 135, and 149 as shown in **Figure 2**. In the case of equol, prominent ions were observed at m/z 93, 119, 121, 135, and 147. Most of these product ions are formed via a retro-Diels–Alder reaction in the MS/MS experiments. The product ions at m/z 295 and 267 of puerarin, at m/z 223 of daidzein, at m/z 149 of dihydrodaidzein, and at m/z 119 of equol were prominent and characteristic for each isoflavonoid. Apigenin, the flavonoid isomer of genistein, matched the chromatographic retention, recovery, and ionization properties with analyte isoflavonoids. Therefore, it was used as internal standard.

The gradient of 10–40% acetonitrile–10 mM NH_4OAc enabled efficient separation of all analytes with high specificity. Sample preparation for urine and serum is a simple one-step dilution with acetonitrile. No sample transfer and extraction steps are involved. The solvent serves to dissociate any non-covalent binding between analyte and matrix.

Metabolism of Puerarin. LC-MS analysis of rat urine samples obtained at 4 h (**Figure 3A**) and 43 h (**Figure 3B**) after puerarin administration revealed the presence of several isoflavonoids. A broad peak eluting at 2.9 min contained deprotonated molecular ions $[M - H]^-$ at m/z 415, 431, and 447. These peaks were not detected in the control urine samples. MS/MS experiments on the ions at m/z 415 and 431 showed product ions at m/z 295 and 311, respectively, due to the loss of 120 Da, a characteristic of isoflavonoid C-glucoside (13). By comparing these product ions with those of puerarin, the ions at m/z 415 and 431 were assigned to be puerarin and monohydroxylated puerarin, respectively (**Figure 4**). The species at m/z 447 is proposed to be dihydroxylated puerarin. The urine samples collected during 43 h after puerarin administration contained, in addition to puerarin, the deconjugated and reductive metabolites daidzein, dihydrodaidzein, and equol.

The time–excretion profile of puerarin and its metabolites daidzein, dihydrodaidzein, and equol in rat urine after administration is shown in **Table 1**. An average of 2 mL of urine was collected during 0–4 h, and the total concentration of

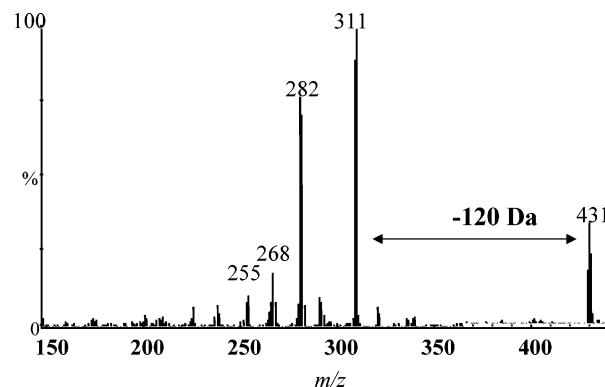


Figure 4. Product ion spectrum of the ion at m/z 431 in puerarin-treated rat urine sample.

Table 1. Excretion of Puerarin and Its Metabolites in Urine Samples over 72 h Following Oral Administration of Puerarin in Rats

time period (h)	average urine vol (mL)	output ($\mu\text{mol}/\text{period}$)			
		puerarin	daidzein	dihydrodaidzein	equol
0–4	2	2.30	nd	nd	nd
4–20	7	0.26	0.02	0.03	0.01
20–27	3.5	0.10	0.05	0.08	0.09
27–43	7	0.09	0.02	0.06	0.28
43–72	19	0.05	0.009	0.007	0.34

nd = not detected.

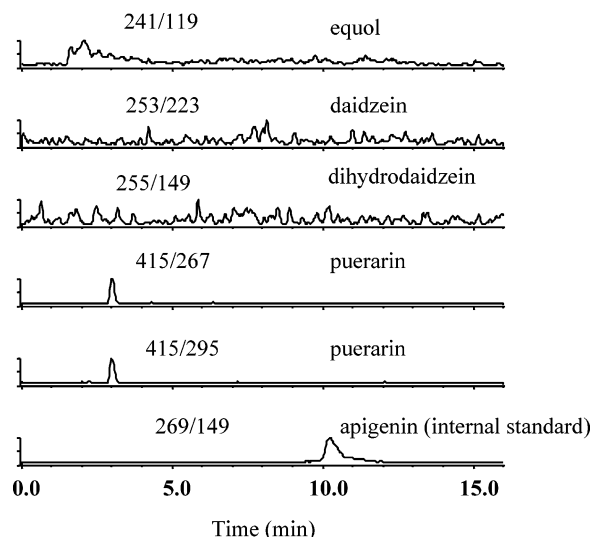


Figure 5. Representative LC-MS-MRM chromatogram of a rat serum sample collected during 4 h after puerarin administration.

puerarin excreted during this period was $2.30 \mu\text{mol}$. Although it decreased after this, it remained as a major component until 72 h. Hydrolyzed and reduced metabolites of puerarin were not present in the urine collected at 4 h. They first appeared in the 20 h urine collection. The maximum concentrations of daidzein and dihydrodaidzein were at 27 h, whereas for equol the maximum occurred during 43 h. Equol was the most abundant metabolite ($0.34 \mu\text{mol}$) in the 72 h urine sample.

Unmetabolized puerarin ($6.2 \mu\text{M}$) was detected in the serum samples collected at 4 h after oral administration of puerarin. No metabolites of puerarin were detected at this time (**Figure 5**). However, by 24 h the concentration of puerarin decreased drastically to 20 nM. In the 24 h sample, equol was also detected in very low quantity.

The aqueous methanolic extract of the feces of puerarin-treated rats contained the presence of unmetabolized puerarin,

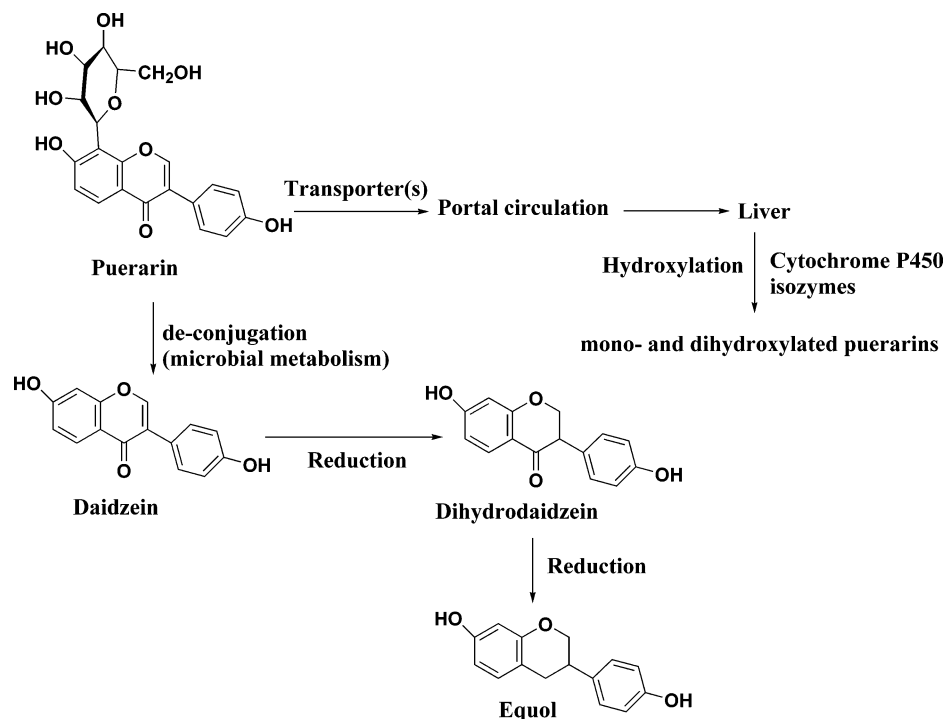


Figure 6. Proposed metabolic pathways of puerarin.

monohydroxylated puerarin, and small amounts of dihydrodaidzein and equol. Their structures were confirmed by MS/MS analysis.

Puerarin in Brain Tissues. The methanolic extracts of heart and brain samples were analyzed by the LC-MS/MS method. Interestingly, puerarin was detected in the brain sample perfused with ice-cold water prior to euthanasia using MRM experiments. The LC-MS/MS spectrum of the ion at m/z 415 further confirmed the presence of puerarin in the brain as it generated product ions at m/z 295, 267, and others similar to those of deprotonated puerarin (13). On the other hand, detection of puerarin in the heart tissue was not conclusive.

DISCUSSION

The chemical forms of isoflavones play a crucial role in their bioavailability and thus for their biological activity. We hypothesized that the carbon-carbon bond linking the isoflavone aglycon with glucose in puerarin would be resistant to hydrolysis by enzymes in the small intestine mucosa. Although the corresponding 7-*O*-glucoside (daidzin) is absorbed in the upper gastrointestinal tract, enterocytes play an important role in its uptake (14). It is proposed that this rapid uptake involves hydrolysis of daidzin by the lumenally exposed enzyme lactase phlorizin hydrolase (LPH) (15). The released aglycon (daidzein) is either absorbed by the intestine by passive mechanisms and extensively converted to β -glucuronides or further metabolized by intestinal microflora into several other products, including equol, dihydrodaidzein, and *O*-desmethylangolensin (16–18).

In this study, we analyzed the rat urine samples collected during 72 h after puerarin administration by LC-MS and MS/MS analyses. Detection of a large amount of unmetabolized puerarin and only small amounts of its oxidative metabolites, mono- and dihydroxylated puerarin, in the urine in the 0–4 h period indicated that puerarin is absorbed without hydrolysis and may undergo limited conversion to phase I oxidative reaction products. These metabolites could be of the catechol or pyrogallol types. It is of considerable interest now to investigate which cytochrome P450 isozymes are involved in

the formation of these metabolites and to study their biological properties. As catechol formation is considered to be a major pathway in the metabolism of endogenous estrogens E2 (19), puerarin may interfere with E2 metabolism if they share the same P450 forms.

In agreement with the high content of puerarin in the urine collected within the first 4 h after oral administration, the concentration of puerarin in blood serum at 4 h reached 6.2 μ M. This observation clearly demonstrated that deconjugation of isoflavone-*C*-glycosides is not a prerequisite for its absorption in rats.

The concentration of puerarin decreased substantially with the increase in the concentrations of daidzein, dihydrodaidzein, and equol in the urine samples collected 4 h after puerarin administration. By 72 h equol was the most abundant metabolite in the urine. Equol is produced after reductive metabolism of daidzein by intestinal bacteria. Therefore, these results suggest that puerarin is hydrolyzed to daidzein by bacterial enzymes in the large intestine and subsequently reduced to dihydrodaidzein and equol. Because puerarin is absorbed from the gastrointestinal tract without being hydrolyzed, it is possible that puerarin is transferred to the portal circulation by the sodium-dependent glucose transporter 1. Because it may also be taken up by tissues such as the liver, it can undergo oxidative metabolism involving certain cytochrome P450 isozymes. On the basis of these assumptions, we propose the metabolic pathways for puerarin as shown in Figure 6. One of the routes of excretion of puerarin could be through the bile into the intestine and hence to the colon, where it is likely to be metabolized in part by colonic microflora and then reabsorbed. Detection of small amounts of equol in blood samples collected at 24 h supports this proposition. Further experiments are underway to more firmly establish the metabolic pathways. It is probable that metabolites of puerarin are re-absorbed into the systemic circulation, and significant amounts appear in the urine.

As mentioned earlier, puerarin has been reported to have a beneficial effect against memory impairment in the model of aging-mice induced by D-galactose (20). It has also been

reported that puerarin can protect apoptotic endothelial cells induced by chemical hypoxia—ischemia markedly, and the effect was performed partly by decreasing caspase-3 expression (21). Emerging evidence has suggested that physiological barriers such as the blood—brain barrier could be crossed selectively by using glucose or glucose derivative/drug conjugates (22). We hypothesized that puerarin, being a C-glucoside, might be able to cross the blood brain barrier. We therefore determined whether puerarin is in the heart and brain tissues. Consistent with this hypothesis, puerarin was detected in the extract of brain tissues of puerarin-treated rats. It is assumed that blood—brain barrier-specific influx transporters may be involved for the brain delivery of puerarin because drug penetration into the brain is restricted under normal conditions. It remains to be seen which transporter(s) is (are) involved in transporting puerarin into the brain.

In summary, we identified and characterized puerarin and its metabolites in rats after administration. The oxidative metabolites of puerarin are of particular interest because of their biological activities. To our knowledge, this is the first study on the metabolism and in vivo uptake of puerarin in rats using mass spectrometric methods. Although concentrations of phytoestrogens in specific brain regions have been reported (23, 24), it is also the first time that an isoflavone-C-glucoside such as puerarin has been detected in rat brain tissues.

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