Urinary and Biliary Metabolites of Puerarin in Rats

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Examination was made of the urinary and biliary excretion of the metabolites of puerarin, the major component of the roots of Pueraria lobata Ohwi (Leguminosae) in rats. The urine of rats administered puerarin orally contained puerarin and four major metabolites, daidzein 4’,7-di-O-sulfate (M-I), daidzein 7-O-β-D-glucuronide (M-II), daidzein 4’-O-sulfate (M-III), daidzein (M-IV), as determined from spectroscopic and chemical data. Total cumulative amounts of the puerarin and four metabolites excreted in the urine at 48 h following the oral administration of puerarin were approximately 3.6% of the doses administered. The bile of rats administered puerarin orally contained puerarin and two major metabolites, which were identified as puerarin 4’-O-sulfate (PB1) and puerarin 7-O-β-D-glucuronide (PB2) on the basis of chemical and spectroscopic data. These experimental data suggest that C-glycoside puerarin is partially hydrolyzed to aglycone in the body, but mainly excreted in the urine as unchanged puerarin.

Key words: puerarin; Pueraria Radix; metabolite; urinary excretion; biliary excretion

Puerarin is the major ingredient of the roots of Pueraria lobata Ohwi (Leguminosae), used as herbal medicine in Japan and China. Puerarin has hypoglycemic activity and increases coronary artery blood flow. Pharmacokinetics of puerarin such as absorption, distribution and elimination have been examined by thin layer chromatography (TLC) and ultraviolet (UV) spectrophotometry. The structures of its metabolites have yet to be determined.

Thus, in this study, the urinary and biliary metabolites of orally administered puerarin were isolated from rats and their structures were elucidated. The total cumulative amounts of the puerarin and four metabolites excreted in the urine during 48 h after oral administration of puerarin were estimated. The metabolism of puerarin is discussed based on the results of a comparison with that of O-glycoside daidzin in the previous paper.

MATERIALS AND METHODS

Apparatus The apparatus was essentially the same as in the previous study. 1H- and 13C-NMR spectra for PB3 were measured at 400 and 100 MHz, respectively, on a JEOL JMN-GSX400 spectrometer with tetramethylsilane as internal standard.

Reagents Puerarin was isolated from Puerariae Radix according to Hayakawa et al. Other chemicals were of special grade.

Animals Male SD (Japan SLC, Inc.) rats weighing 150—200 g were used. They were deprived of food but had free access to water for 18 h prior to the experiments.

Animal Experiments Bile Sample: Under light anesthesia with ether, bile duct cannulation using polyethylene tubing was carried out on all the animals. To identify and determine the metabolites, 100 mg/kg of daidzin suspended in 0.5% CMC-Na solution was administered to each rat and bile samples were collected in CH3OH with cooling for 3—6 h. To isolate biliary metabolites, twelve rats were orally administered 300 mg/kg of finely ground puerarin suspended in 0.5% CMC-Na solution and bile was collected in CH3OH with cooling for 30 h. Urine samples:

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For metabolite isolation, the rats were orally administered 300 mg/kg of finely ground puerarin suspended in 0.5% CMC-Na solution and urine was obtained over 24 h by using a metabolic cage. To identify and determine the metabolites, five rats were orally administered 100 mg/kg of puerarin suspended in 0.5% CMC-Na solution and urine samples were obtained at 4, 8, 12, 24, and 48 h. All samples were stored below −20 °C until use.

HPLC Conditions For the identification and quantitation of metabolites, a 5 μm octadecyl silica (ODS) column, TSKgel ODS-120T (Tosoh, Tokyo, Japan, 250 mm × 4.6 i.d.) was used. The mobile phase contained the following: Solvent A, 0.05% trifluoroacetic acid (TFA). Solvent B, 30% CH3CN with 0.05% TFA. Conditions: linear gradient (A/B = 100/0 → 0/100, 45 min, 40 °C) and a flow rate 1 ml/min. For preparative HPLC, a TSKgel ODS-120T (Tosoh, Tokyo, Japan, 300 mm × 7.8 i.d.) was used. The mobile phase contained Solvent A, 5% CH3CN. Solvent B, 15% CH3CN. Conditions: linear gradient (A/B = 100/0 → 0/100, 60 min, room temperature) and a flow rate 2 ml/min.

General and Enzymatic Procedures The procedures for acid hydrolysis, carbonization and product identification were essentially the same as in the previous study.

Isolation of Metabolites Urine samples (about 0.9 l) from the rats were combined and subjected to Sephadex LH-20 column chromatography (0.05 N HCl → H2O → CH3OH) to obtain the metabolites (puerarin, M-III and M-I, M-II and M-IV) fractions. Further fractionation and purification by Sephadex LH-20 column chromatography gave puerarin (19 mg), M-I (8 mg), M-II (5 mg), M-III (16 mg) and M-IV (15 mg) (Chart 1).

Bile samples from the rats were combined and subjected to Sephadex LH-20 column chromatography (0.05 N HCl → H2O → CH3OH) to obtain the metabolites (PB1, PB2 and puerarin) fraction. Further fractionation and purification by preparative HPLC gave PB1 (8 mg), PB2 (7 mg) and puerarin (4 mg).

Quantitation of Urinary Metabolites Urine samples were diluted with CH3OH to a final volume of 10 ml. An aliquot of the sample was filtered through a 0.45 μm
membrane filter. 20 μl of the solution were subjected to HPLC. Quantification of the metabolites was done by measuring peak areas. Calibration plots of the peak area of each metabolite against metabolite concentration were linear from 1.7—170 μg/ml for puerarin, 2.2—220 μg/ml for M-I, 1.0—100 μg/ml for M-II, 0.8—80 μg/ml for M-III and 0.6—60 μg/ml for M-IV. The recovery of each metabolite in urine ranged from 78—108%, based on determination of standard samples added to drug-free urine.

Puerarin: white powder. mp 186—189°C. EI-MS m/z: 416 (M+), 3410 (OH), 1630 (C=O). 1H-NMR: as given in Table I.

M-I: white powder. mp 166—167°C. FAB-MS m/z: 435 (M+Na-H), 333 (M-H-SO3)−, 253 (M-H-SO3-SO3)−. IR (KBr)cm⁻¹: 1626 (conjugated C=O), 1510 (arom. C=C, 1055 (−O−SO3−).

M-II: white powder. mp 255—258°C. FAB-MS m/z: 429 (M-H), 253 (M-H-C₆H₅O₆)−. IR (KBr)cm⁻¹: 3401 (OH), 1625 (conjugated C=O), 1518 (arom. C=C).

M-III: white powder. mp 243—245°C. FAB-MS m/z: 355 (M+Na-H), 333 (M-H), 253 (M-H-SO3)−. IR (KBr)cm⁻¹: 3471 (OH), 1630 (conjugated C=O), 1510 (arom. C=C), 1060 (−O−SO3−).

M-IV: white powder. mp 318—320°C. EI-MS m/z: 254 (M+), 3215 (OH), 1614 (conjugated C=O), 1518 (arom. C=C).

PBI: white powder. mp 214—218°C. FAB-MS m/z: 495 (M-H), 415 (M-H-SO3)−. IR (KBr)cm⁻¹: 3423 (OH), 1637 (conjugated C=O), 1509 (arom. C=C). 1H-, 13C-NMR: as given in Table I.

PB2: white powder. mp 229—232°C. FAB-MS m/z: 591 (M-H), 415 (M-H-C₆H₅O₆)−. IR (KBr)cm⁻¹: 3423 (OH), 1638 (conjugated C=O), 1510 (arom. C=C). 1H-, 13C-NMR: as given in Table I.

RESULTS

Separation of Urinary Metabolites and Determination of Chemical Structures of the Metabolites: Puerarin was orally administered to rats deprived of food and urine samples were taken. Reversed-phase HPLC of urine indicated puerarin and four distinct peaks due to the metabolites of puerarin: M-I, M-II, M-III and M-IV in order of retention time (tR) (Fig. 1).

Puerarin and M-I—M-IV were isolated from urine by repeated chromatographic separation on a Sephadex LH-20 column as shown in Chart 1.

M-I—M-IV exhibited absorption due to conjugated carbonyl (1614—1626 cm⁻¹) and aromatic functions (1510—1518 cm⁻¹) in the IR spectra and maximal absorption at 249—251 nm in the ultraviolet (UV) spectra. M-I—M-IV was thus suggested to have an isoflavone skeleton.

Puerarin was identified by a direct comparison with the reference sample.

M-I—M-IV were identified as daidzein 4',7-di-O-sulfate (M-I), daidzein 7-O-β-D-glucuronide (M-II), daidzein 4'-O-sulfate (M-III) and daidzein (M-IV) by a direct spectroscopic and chemical data comparison for each compound in the previous paper.⁴

Based on the above, the chemical structures of the puerarin and four metabolites are shown in Chart 2.

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Fig. 1. HPLC Chromatogram of Urine Excreted during 24h after Oral Administration of Puerarin (100 mg/kg) to Rats

—, metabolic urine; ——, control urine.
Cumulative Excretion of the Following Oral Administrations of Pueraerin The urinary excretion profile of metabolites after administering 100 mg/kg of pueraerin is shown in Fig. 2. Total amount excreted during 48 h was 3.63% ± 0.05% (mean ± S.E., n = 5) of the pueraerin administered.

Separation of Biliary Metabolites and Determination of Chemical Structures of the Metabolites Pueraerin was orally administered to bile duct cannulated rats and by which bile samples were obtained. Reversed-phase HPLC of bile indicated pueraerin and two distinct peaks due to the metabolites of pueraerin: PB1 and PB2 in the order of tR (Fig. 3).

PB1, PB2 and pueraerin were isolated from bile by repeated chromatographic separation on a Sephadex LH-20 column and preparative HPLC. PB1 and PB2 exhibited absorption due to conjugated carbonyl (1637–1638 cm⁻¹) and aromatic functions (1509–1510 cm⁻¹) in the IR spectra and maximal absorption at 245–251 and 303–304 nm in UV spectra. PB1 and PB2 were thus suggested to each have a isoflavone skeleton.

The enzymatic hydrolysis of PB1 with arylsulfatase gave pueraerin, as indicated by agreement with tR on HPLC. The intense absorption at 1050 cm⁻¹ in the IR spectrum and $\text{SO}_4^2^-$ formation on carbonization suggested a sulfate-conjugated structure for PB1. Negative FAB-MS of PB1 showed a molecular ion peak corresponding to (M−H)$^-$ at $m/z$ 495 and ion peaks corresponding to (M−H+Na)$^-$ and (M−H−SO$_3^-$) at $m/z$ 517 and 415. A sulfate group is thus shown to be present in PB1. A comparison of the $^{13}$C-NMR spectrum of PB1 with that of pueraerin indicated the C-4' signal of PB1 to have shifted 4.0 ppm upfield, accompanied by downfield shifts of C-3' (5.0 ppm) and C-5' (5.0 ppm). These shifts suggest a sulfate group at C-4'. PB1 was thus concluded to be pueraerin 4'-O-sulfate.

The enzymatic hydrolysis of PB2 with β-glucuronidase gave pueraerin, as indicated by agreement with tR on HPLC. The $^1$H-NMR spectrum showed a β-anomeric proton at δ 5.03 (d) and in negative FAB-MS, a molecular ion peak at $m/z$ 591 (M−H)$^-$ corresponding to the mono-

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Fig. 2. Cumulative Urinary Excretion Profiles of the Metabolites in Rats after Oral Administration of 100 mg/kg of Pueraerin (n = 5) 
○ – ○, pueraerin; ● – ●, M-I; ○○○○, M-II; ○●○●, M-III; □–□, M-IV. 
■ – ■, total.

Fig. 3. HPLC Chromatogram of Bile Excreted during 3–6 h after Oral Administration of Pueraerin (100 mg/kg) to Rats
– – –, metabolic bile; -----, control bile.
DISCUSSION

Many flavonoid glycosides undergo microbial hydrolysis in the gastrointestinal tract.5,7 The results of our previous study5 indicated that the urine of rats given O-glycoside daidzin orally to contain metabolites possessing free hydroxyl, sulfated or glucuronidated hydroxys at the C-7 position and that the unchanged compound, daidzin, could not be detected.

The present study shows the urine of rats given C-glycoside puerarin orally to mainly contain the unchanged compound puerarin, in addition to the same urinary metabolites as obtained with daidzin. C-Glycoside puerarin could thus appear not to undergo appreciable hydrolysis by intestinal microflora in the gastrointestinal tract. Most flavonoids are reported to be metabolized to various phenolic acid derivatives through ring fission,7 but in this study we could not be detected those compounds on the above HPLC conditions.

The total cumulative amounts of the puerarin and four metabolites excreted during the period of 48 h constituted to 3.63 ± 0.05% (mean ± S.E., n = 5) of the puerarin administered. Puerarin excretion was slightly less than that of daidzin or daidzein. The puerarin and its metabolites may possibly have been continuously excreted in the urine after 48 h (Fig. 2).

As biliary metabolites of puerarin, puerarin 4'-O-sulfate (PB1) and puerarin 7-O-β-d-glucuronide (PB2), so far not detected in urine, were observed in the bile, in along with the unchanged compound, puerarin. The biliary metabolites of puerarin were composed primarily of PB1 and PB2 with high polarity and large molecular weight, both these parameters facilitate the excretion of flavonoids into the bile.7 Blood constituents of puerarin are present by being studied.

REFERENCES


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TABLE 1. 1H- and 13C-NMR Spectral Data for the Biliary Metabolites (δ in DMSO-d6)

<table>
<thead>
<tr>
<th>Position</th>
<th>PB1</th>
<th>PB2</th>
<th>Puerarin a</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>δH</td>
<td>δC</td>
<td>δH</td>
</tr>
<tr>
<td>2</td>
<td>8.40 s</td>
<td>153.0</td>
<td>8.43 s</td>
</tr>
<tr>
<td>3</td>
<td>—</td>
<td>126.1</td>
<td>—</td>
</tr>
<tr>
<td>4</td>
<td>—</td>
<td>174.6</td>
<td>—</td>
</tr>
<tr>
<td>5</td>
<td>7.95 d (9)</td>
<td>126.5</td>
<td>8.07 d (9)</td>
</tr>
<tr>
<td>6</td>
<td>6.98 d (9)</td>
<td>115.1</td>
<td>7.29 d (9)</td>
</tr>
<tr>
<td>7</td>
<td>—</td>
<td>161.3</td>
<td>—</td>
</tr>
<tr>
<td>8</td>
<td>—</td>
<td>112.5</td>
<td>—</td>
</tr>
<tr>
<td>9</td>
<td>—</td>
<td>157.1</td>
<td>—</td>
</tr>
<tr>
<td>10</td>
<td>—</td>
<td>116.6</td>
<td>—</td>
</tr>
<tr>
<td>1'</td>
<td>122.7</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>2'</td>
<td>7.48 d (9)</td>
<td>129.2</td>
<td>7.40 d (9)</td>
</tr>
<tr>
<td>3'</td>
<td>7.20 d (9)</td>
<td>119.9</td>
<td>6.81 d (9)</td>
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<tr>
<td>4'</td>
<td>—</td>
<td>153.1</td>
<td>—</td>
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<tr>
<td>5'</td>
<td>7.20 d (9)</td>
<td>119.9</td>
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<td>6'</td>
<td>7.48 d (9)</td>
<td>129.2</td>
<td>7.40 d (9)</td>
</tr>
</tbody>
</table>

7-O-GlcUA

1'       | 5.03 d (7) | 101.3 |
2'       | —   | 73.0 |
3'       | —   | 75.7 |
4'       | —   | 71.8 |
5'       | —   | 73.2 |
6'       | —   | 171.5 |

8-C-Glc

1'       | 4.82 d (10) | 73.4 | 4.88 d (10) | 73.5 | 4.82 d (10) |
2'       | 70.7 | —   | 69.5 | —   |
3'       | 78.7 | —   | 78.8 | —   |
4'       | 70.4 | —   | 69.5 | —   |
5'       | 81.7 | —   | 81.1 | —   |
6'       | 61.3 | —   | 60.4 | —   |

δ values in ppm. Values in parentheses are coupling constants (Hz). a) 1H-NMR spectrum was measured at 400 MHz. 13C-NMR spectrum was not obtained because of small amount of the PB3 sample.

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glucuronide, thus indicating one glucuronide group, PB2 is thus present. A comparison of the 13C-NMR spectrum of PB2 with that of puerarin showed the C-7 signal of PB2 to have shifted 0.7 ppm upfield, accompanied by downfield shifts of C-6 (3.4 ppm) and C-8 (1.5 ppm). These shifts suggest a glucuronide group to be situated at C-7. PB2 was thus identified as puerarin 7-O-β-d-glucuronide.

Puerarin was identified on the basis of agreement with tR on HPLC and a direct comparison with the reference sample.

Based on the above, the chemical structures of the two metabolites are presented in Chart 2. 1H-, 13C-NMR data are given in Table I.