Urinary and Biliary Metabolites of Daidzin and Daidzein in Rats

Takaaki YASUDA,^a Yoshihiro KANO,^b Ken-ichi SAITO,^b and Keisuke OHSAWA^{*,a}

Tohoku College of Pharmacy,^a 4-1, Komatsushima 4 chome, Aoba-ku, Sendai, Miyagi 981, Japan and Hokkaido Institute of Pharmaceutical Science,^b 7-1, Katsuraoka-cho, Otaru 047-02, Japan.

Received March 28, 1994; accepted June 17, 1994

Examination was made of the urinary and biliary excretion of metabolites of daidzin and daidzein, the major components of roots of *Pueraria lobata* OHWI (Leguminosae) in rats. The urine of rats administered daidzin orally contained four major metabolites, daidzein 7,4'-di-*O*-sulfate (M-1), daidzein 7-*O*- β -D-glucuronide (M-2), daidzein 4'-*O*-sulfate (M-3), daidzein (M-4), as determined from spectroscopic and chemical data. The urine of rats treated with daidzein contained M-2—M-4 in the above metabolites.

Total cumulative amounts of the four metabolites excreted in the urine at 48 h following the oral administration of daidzin and daidzein were approximately 4.8% and 4.6% of the doses administered, respectively. The bile of rats administered daidzin orally contained M-1—M-4. Daidzein 7-*O*- β -D-glucuronide 4'-*O*-sulfate (M-5), a major biliary metabolite, was identified by the high-performance liquid chromatography (HPLC), liquid chromatography-mass spectrometry (LC-MS) and nuclear magnetic resonance (NMR) spectra. At least daidzin appeared to be hydrolyzed to aglycone after absorption in the body, and as a part of metabolites, M-1—M-4 having free hydroxyl, glucuronidated or sulfated hydroxyls at the C-7 position, may then be excreted in the urine and bile.

Keywords daidzin; daidzein; *Puerariae Radix*; metabolite; urinary excretion; biliary excretion

Daidzin and the aglycone daidzein are major ingredients of the roots of *Pueraria lobata* OHWI (Leguminosae), used as herbal medicine in Japan and China. Both inhibit cyclic AMP phosphodiesterase¹⁾ and induce differentiation in murine erythroleukemia cells.²⁾ Daidzein has 1/3 of the antispasmodic activity of papaverine.³⁾

The pharmacokinetics of daidzin have so far not been studied. Those of daidzein such as absorption, distribution and elimination have been examined using ¹⁴C-labelled daidzein.⁴⁾ The structures of its metabolites, however, have yet to be determined. The metabolism of flavonoids has been studied by Abe *et al.*, for clarification of the structures of the biliary metabolites of orally administered baicalin, baicalein⁵⁾ and hesperetin⁶⁾ in rats. The metabolism of isoflavone derivatives have yet to be reported.

Thus, in this study, the urinary metabolites of orally administered daidzin and the aglycone daidzein were isolated from rats and their structures were determined. Total amounts of metabolites were estimated. The biliary metabolites of orally administered daidzin were analyzed by HPLC, LC-MS and NMR spectra.

MATERIALS AND METHODS

Apparatus Melting points were determined on Yanagimoto micro melting point apparatus and not corrected. Infrared (IR) spectra were measured with a Perkin elmer FT-IR 1725X spectrometer. NMR spectra were recorded on a JEOL JNM-EX 270 (¹H-, ¹³C-NMR: 270 MHz) spectrometer. Chemical shifts are given in δ values (ppm) downfield from tetramethylsilane (s, singlet; d, doublet; dd, double doublet). Fast atom bombardment mass spectra (FAB-MS) were measured with a JEOL JMS-DX 303 mass spectrometer and LC-MS (Electrospray ionization (ESI) method), with a Finnigan MATTSQ-700. The HPLC system consisted of a CCPM pump, CO-8010 column oven (Tosoh, Tokyo, Japan) and model M991J photodiode array detector (Waters Millipore, Milford, MA, U.S.A.).

GLC was carried out on a Shimadzu GC-4CMPF unit.

Reagents Daidzin and daidzein were isolated from *Puerariae Radix* according to Hayakawa *et al.*⁷⁾ Authentic samples were purchased from Funakoshi (Tokyo, Japan). For column chromatography, Sephadex LH-20 (Pharmacia, Uppsala, Sweden) or Kieselgel 60 (Merck, Darmstadt, Germany) was used. Arylsulfatase and β -glucuronidase were from Sigma (St. Louis, MO, U.S.A.). Ether, methanol, acetonitrile, trifluoroacetic acid (TFA) (Wako Pure Chemical Industries, Osaka, Japan) and citrate buffer (Kanto Chemical, Tokyo, Japan) of special grade were used. Carboxymethylcellulose (CMC)-Na was purchased from Kanto Chemical (Tokyo, Japan).

Animals The rats for this study were male SD (Japan SLC, Inc.) weighing 120—260 g. They were deprived of food but had a free access to water for 18 h prior to the experiments.

Animal Experiments **Bile Samples:** Under light anesthesia with ether, bile duct cannulation using polyethylene tubing was carried out on all the animals. For identification and determination of metabolites, 100 mg/kg of daidzin suspended in 0.5% CMC-Na solution were administered to each rat and the bile samples were collected in CH₃OH with cooling for 2—6 h. To isolate M-5, fourteen rats were orally administered 300 mg/kg of finely ground daidzin suspended in 0.5% CMC-Na solution and bile was collected in CH₃OH with cooling for 30 h.

Urine Samples: For isolation of metabolites, the rats were orally administered 300 mg/kg of finely ground daidzin 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 daidzin or 60 mg/kg of daidzein suspended in 0.5% CMC-Na solution and urine samples were taken at 4, 8, 12, 24, 36 and 48 h. All samples were stored below -20 °C until use.

HPLC Conditions For identification and quantitation of metabolites, a 5 μ m octadecyl silica (ODS) column,

TSKgel ODS-120T (Tosoh, Tokyo, Japan 250 mm $\times$ 4.6 mm 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 $\rightarrow$ 0/100, 45 min, 40°C) and a flow rate 1 ml/min. For LC-MS, a 4 μ m ODS column, Supersphere 100 (Merck, Darmstadt, Germany, 250 mm $\times$ 4.6 mm i.d.) was used. The mobile phase consisted of: Solvent A, 25 mM CH₃COONH₄ with 10% CH₃OH. Solvent B, CH₃OH.

Conditions: linear gradient (A/B=100/0 $\rightarrow$ 0/100, 100 min) and a flow rate of 1 ml/min.

GLC Conditions A glass capillary column (20 m $\times$ 0.3 mm i.d. Scot type) was used. Column oven temperature was maintained at 170°C. The nitrogen carrier gas flow rate was 50 ml/min.

Isolation of Metabolites Urine samples (about 21) from rats were combined and subjected to Sephadex LH-20 column chromatography (0.05 N HCl $\rightarrow$ H₂O $\rightarrow$ CH₃OH) to obtain the metabolite (M-1—M-4) fraction. Further fractionation and purification (M-1, M-2—M-4) by Sephadex LH-20 column chromatography gave M-1 (90 mg), M-2 (28 mg) and M-3 (50 mg). The M-4 fraction was recrystallized from 50% C₂H₅OH and to give M-4 (15 mg) (Chart 1).

M-5 fraction was repeated chromatographic separation on a Sephadex LH-20 column (0.05 N HCl $\rightarrow$ H₂O $\rightarrow$ CH₃OH) to obtain M-5 (14 mg).

Acidic Hydrolysis of Metabolites M-2 (1 mg) was refluxed in 2 ml of 2 N HCl–50% aqueous dioxane (1:1) for 3 h. The reaction mixture was filtered and the filtrate evaporated to dryness *in vacuo*. The residue was trimethylsilylated by the usual method and subjected to GLC analysis to obtain glucuronolactone (main) and glucuronic acid (trace).

Enzymatic Hydrolysis of Metabolites M-1 and M-3 (each 1 mg) were incubated with crude arylsulfatase (0.1 ml, type H-1) in 0.1 M citrate buffer (pH 5.2) for 2 h at 37°C. M-2 (1 mg) was treated with β -glucuronidase (0.1 ml, type H-2) in the same way as above. Each reaction mixture was extracted with ether and the organic layer was washed with 1 N HCl and H₂O, and evaporated *in vacuo* to give the aglycone.

Detection of Sulfate Function in M-1, M-3 and M-4 M-1, M-3 and M-4 (each 1 mg) were carbonized in a Pt crucible and extracted with 0.3 ml of H₂O. After centrifugation, the supernatant was treated with 0.2% BaCl₂ to afford a white precipitate (BaSO₄).

Quantification of Urinary Metabolites Urine samples were diluted with CH₃OH to a final volume of 10 ml. An aliquot of the sample was filtrated through a 0.45 μ m membrane filter. 20 μ l 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 2.2—220 μ g/ml for M-1, 1.0—100 μ g/ml for M-2, 0.8—80 μ g/ml for M-3 and 0.6—6.0 μ g/ml for M-4. The recovery of each metabolite in urine ranged from 93—106% based on determination of standard samples added to drug-free urine.

M-1: White powder. mp 148—150°C. FAB-MS *m/z*:

435 (M+Na-H)⁻, 413 (M-H)⁻, 333 (M-SO₃H)⁻, 253 (M-2SO₃H)⁻. IR (KBr) cm⁻¹: 1056 (-O-SO₃H). ¹H-, ¹³C-NMR: as given in Table I.

M-2: White powder. mp 264—265°C. FAB-MS *m/z*: 429 (M-H)⁻, 253 (M-H-C₆H₈O₆)⁻. IR (KBr) cm⁻¹: 3431 (OH). ¹H-, ¹³C-NMR: as given in Table I.

M-3: White powder. mp 238—240°C. FAB-MS *m/z*: 355 (M+Na-H)⁻, 333 (M-H)⁻, 253 (M-SO₃H)⁻. IR (KBr) cm⁻¹: 1060 (-O-SO₃H). ¹H-, ¹³C-NMR: as given in Table I.

M-4: White powder. mp 316—318°C. EI-MS *m/z*: 254 (M⁺). IR (KBr) cm⁻¹: 3235 (OH). ¹H-, ¹³C-NMR: as given in Table I.

M-5: White powder. mp 198—200°C. ESI-MS *m/z*: 509 (M-H)⁻, 333 (M-H-C₆H₈O₆)⁻, 253 (M-C₆H₈O₆-SO₃H)⁻. IR (KBr) cm⁻¹: 1054 (-O-SO₃H). ¹H-, ¹³C-NMR: as given in Table I.

RESULTS

Separation of Urinary Metabolites and Determination of Chemical Structures of Metabolites Daidzin was orally administered to rats deprived of food and urine samples were taken. Reversed-phase HPLC of urine indicated four distinct peaks due to the metabolites of daidzin: M-1, M-2, M-3 and M-4 in order of retention time (*t_R*) (Fig. 1). M-1—M-4 was isolated from urine by repeated chromatographic separation on a Sephadex LH-20 column as shown in Chart 1.

M-1—M-4 exhibited absorption due to conjugated carbonyl (1621—1630 cm⁻¹) and aromatic functions (1508—1518 cm⁻¹) in the IR spectra and maximal absorption at 248—250 nm in the ultraviolet (UV) spectra. M-1—M-4 was thus concluded have a isoflavone skeleton.

The enzymatic hydrolysis of M-1 with arylsulfatase gave the aglycone daidzein by agreement with *t_R* on HPLC. The intense absorption at 1056 cm⁻¹ in the IR spectrum and SO₄²⁻ formation on carbonization suggested a sulfate-conjugated structure for M-1. The negative FAB-MS of M-1 showed a base ion peak corresponding to (M-SO₃H)⁻ at *m/z* 333, and ion peaks corresponding to (M-H+Na)⁻, (M-H)⁻ and (M-2SO₃H)⁻ at *m/z* 435, 413 and 253, respectively, thus indicating two sulfate groups in M-1. A comparison of the ¹³C-NMR spectrum of M-1 with that of daidzein showed the C-7 signal of M-1 to have shifted 4.5 ppm upfield, accompanied by downfield shifts of C-6 (2.7 ppm) and C-8 (4.6 ppm). The C-4' signal of M-1 also shifted 3.9 ppm upfield, accompanied by downfield shifts of C-3' (5.1 ppm) and C-5' (5.1 ppm). These shifts indicate possibly two sulfate groups at C-7 and C-4'.^{5,6,8)} M-1 could thus be identified as daidzein 7, 4'-di-*O*-sulfate.

The enzymatic hydrolysis of M-2 with β -glucuronidase gave the aglycone daidzein by agreement with *t_R* on HPLC. The ¹H-NMR spectrum showed one β -anomeric proton at δ 5.08 (d) and in negative FAB-MS, a molecular ion peak at *m/z* 429 (M-H)⁻ corresponding to monoglucuronide. One glucuronide group is thus present M-2. A comparison of the ¹³C-NMR spectrum of M-2 with that of daidzein indicated the C-7 signal of M-2 to have shifted 1.1 ppm upfield, accompanied by downfield shifts

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

—, metabolic urine; ----, control urine.

Chart 1. Isolation of the Metabolites Excreted in Urine

of C-6 (0.4 ppm) and C-8 (1.1 ppm). These shifts suggest glucuronide groups at C-7. Thus, M-2 was identified as daidzein 7-*O*- β -D-glucuronide.

The enzymatic hydrolysis of M-3 with arylsulfatase gave the aglycone daidzein by agreement with t_R on HPLC. The intense absorption at 1060 cm^{-1} in the IR spectrum and SO_4^{2-} formation on carbonization suggested a sulfate-conjugated structure for M-3. The negative FAB-MS of M-3 showed a base ion peak corresponding to $(M-H+Na)^-$ at m/z 355, and ion peaks corresponding to $(M-H)^-$ and $(M-\text{SO}_3\text{H})^-$ at m/z 333 and 253, respectively. A sulfate group is thus present in M-3. A comparison of the ^{13}C -NMR spectrum of M-3 with that of daidzein showed the C-4' signal of M-3 to have shifted 4.0 ppm upfield, accompanied by downfield shifts of C-3' (5.1 ppm) and C-5' (5.1 ppm). These shifts suggest a sulfate group at C-4'. Thus, M-3 was identified as daidzein 4'-*O*-sulfate.

M-4 was identified as daidzein by a direct comparison with the reference sample.

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

	R ₁	R ₂
daidzin	Gl c	H
M-1	SO ₃ ⁻	SO ₃ ⁻
M-2	Gl c UA	H
M-3	H	SO ₃ ⁻
M-4	H	H
M-5	Gl c UA	SO ₃ ⁻

Gl c UA : β -glucuronic acid

Chart 2. Structures of Daidzin and Its Metabolites

Other ^1H - and ^{13}C -NMR data are given in Table I.

The authors were thus performed to investigate the urinary excretion of metabolites following daidzein administration. HPLC analysis indicated the urine of rats given daidzein to contain the same metabolites (M-2—M-4) except M-1 as those obtained with daidzin, as apparent from the chromatogram in Fig. 2.

Urinary Excretion of Metabolites Following Oral Daidzin and Daidzein Administration Urinary excretion profiles of metabolites after administering 100 mg/kg of daidzin and 60 mg/kg of daidzein are shown in Fig. 3.

Total amounts excreted during 48 h were $4.80 \pm 0.08\%$ (mean $\pm$ S.E., $n=5$) of administered daidzin and $4.57 \pm 0.24\%$ (mean $\pm$ S.E., $n=5$) of daidzein administered. The two groups were essentially the same with respect to percentages of metabolites excreted during the 48 h.

Separation and Determination of Biliary Metabolites and Chemical Structure of M-5 Daidzin was orally administered to rats by bile-duct cannulation and by which bile samples were obtained. HPLC analysis indicated bile to contain the same urinary metabolites (M-1—M-4) as those obtained with daidzin, as evident from the chromatogram in Fig. 4. Peak M-5, suggestive of a major biliary

TABLE I. ¹H- and ¹³C-NMR Spectral Data for Daidzein and the Metabolites (δ in DMSO-*d*₆)

Position	M-1		M-2		M-3		M-4		M-5		Daidzein ^{a)}	
	δ _H	δ _C	δ _H	δ _C	δ _H	δ _C	δ _H	δ _C	δ _H	δ _C	δ _H	δ _C
2	8.45 s	153.8	8.37 s	153.1	8.36 s	153.2	8.28 s	152.7	8.45 s	153.2	8.28 s	152.7
3	—	126.4	—	123.5	—	126.5	—	123.3	—	126.3	—	123.4
4	—	174.6	—	174.6	—	174.4	—	174.6	—	174.4	—	174.6
5	8.05 d (9)	126.3	8.03 d (9)	126.7	7.98 d (9)	127.1	7.96 d (9)	127.1	8.06 d (9)	126.7	7.96 d (9)	127.2
6	7.27 dd (9, 2)	117.8	7.13 dd (9, 2)	115.5	6.94 dd (9, 2)	115.0	6.93 dd (9, 2)	115.0	7.15 dd (9, 2)	115.6	6.92 dd (9, 2)	115.1
7	—	158.0	—	161.4	—	162.4	—	162.4	—	161.5	—	162.5
8	7.45 d (2)	106.8	7.23 d (2)	103.3	6.88 d (2)	102.0	6.86 d (2)	101.9	7.25 d (2)	103.4	6.86 d (2)	102.2
9	—	156.3	—	156.8	—	157.3	—	157.0	—	156.9	—	157.1
10	—	118.8	—	118.2	—	116.5	—	116.5	—	118.2	—	116.5
1'	—	123.3	—	122.1	—	123.1	—	122.4	—	123.3	—	122.4
2'	7.49 d (9)	129.3	7.39 d (9)	129.8	7.46 d (9)	129.2	7.38 d (9)	129.9	7.49 d (9)	129.2	7.38 d (9)	130.0
3'	7.22 d (9)	119.9	6.81 d (9)	114.8	7.21 d (9)	119.9	6.80 d (9)	114.8	7.21 d (9)	119.9	6.80 d (9)	114.8
4'	—	153.2	—	157.2	—	153.1	—	157.3	—	153.7	—	157.1
5'	7.22 d (9)	119.9	6.81 d (9)	114.8	7.21 d (9)	119.9	6.80 d (9)	114.8	7.21 d (9)	119.9	6.80 d (9)	114.8
6'	7.49 d (9)	129.3	7.39 d (9)	129.8	7.46 d (9)	129.2	7.38 d (9)	129.9	7.49 d (9)	129.2	7.38 d (9)	130.0
7-O-GlcUA												
1''			5.08 d (7)	99.7					5.08 d (7)	99.8		
2''			—	72.9					—	72.9		
3''			—	76.4					—	76.5		
4''			—	71.9					—	71.9		
5''			—	73.4					—	73.3		
6''			—	171.7					—	171.6		

δ values in ppm. Values in parentheses are coupling constants (Hz). a) These data were obtained from authentic sample.

Fig. 2. HPLC Chromatogram of Urine Excreted during 24 h after Oral Administration of Daidzein (60 mg/kg) to Rats
—, metabolic urine; ----, control urine.

Fig. 3. Cumulative Excretion of Metabolites as a Function of Time Following Oral Administration of Daidzin (100 mg/kg) and Daidzein (60 mg/kg)
(□), M-1; (⊕), M-2; (⊙), M-3; (●), M-4; (○), total. Each point represents the mean ± S.E. of five rats.

Fig. 4. HPLC Chromatogram of Bile Excreted during 2–6h after Oral Administration of Daidzin (100mg/kg) to Rats
—, metabolic bile; ----, control bile.

Fig. 5. LC-MS Chromatogram (Parent Ion Scan) of Bile sample

Fig. 6. ESI Mass Spectral (Daughter Ion Scan) of M-5

metabolite, was observed at t_R 24 min. The structures of these metabolites (M-1—M-5) were determined by LC-MS. Peaks on the chromatogram, obtained through parent ion scanning as the mother nucleus for daidzein, were identified as M-1, M-2, M-3 and M-4 by agreement with t_R on HPLC and daughter ion scanning of mass spectra corresponding to the compounds (M-1—M-4) (Fig. 5).

Intense absorption at 1054 cm^{-1} in the IR spectrum, SO_4^{2-} formation following carbonization and a β -anomeric proton at $\delta 5.08$ (d) in $^1\text{H-NMR}$ were observed. The mass spectrum (Daughter ion scan) indicated the base ion peak at m/z 333 ($\text{M-H-C}_6\text{H}_8\text{O}_6$) $^-$ and ion peaks corresponding to (M-H) $^-$ and ($\text{M-C}_6\text{H}_8\text{O}_6\text{-SO}_3\text{H}$) $^-$ at m/z 509 and 253 (Fig. 6). M-5 would thus appear to contain one glucuronide- and one sulfate-

conjugated of daidzein. A comparison of the ^{13}C -NMR spectrum of M-5 with that of daidzein showed that the C-7 signal of M-5 was shifted 1.0 ppm upfield, accompanied with downfield shifts of C-6 (0.5 ppm) and C-8 (1.2 ppm). These shifts were closely correlated with those of M-2 and suggested a glucuronide group to be present at C-7. The C-4' signal of M-5 shifted 3.4 ppm upfield, accompanied by downfield shifts of C-3' (5.1 ppm) and C-5' (5.1 ppm). These shifts were closely correlated with those of M-3, and suggested a sulfate group to be at C-4'. M-5 was thus concluded to be daidzein 7-*O*- β -D-glucuronide 4'-*O*-sulfate.

DISCUSSION

Many flavonoid glycosides undergo microbial hydrolysis in the gastrointestinal tract.^{9,10)}

In our previous study, the urine of rats given daidzin orally was shown to contain metabolites possessing free hydroxyl, sulfated or glucuronided hydroxyls at the C-7 position. At least daidzin would thus appear to be hydrolyzed to aglycone in the body.

The excretion of daidzein, the aglycone of daidzin, was similar to that of daidzin and daidzein showed basically the same percentages of metabolites excreted during the 48 h period. M-1 was detected by HPLC analysis as a slight broad peak (Fig. 2), however, it could not be identified by UV spectrum.

M-1—M-4 was excreted in the bile of rats orally

administered daidzin. As a major biliary metabolite of daidzin, daidzein 7-*O*- β -D-glucuronide 4'-*O*-sulfate (M-5), high in molecular weight and so far not detected in urine, was observed in the bile.

M-1—M-3, M-5 metabolites showed high polarity and large molecular weights. These properties promote the excretion of flavonoids in the bile and urine.

Blood constituents and C-glycoside, puerarin will be our next topics of research.

REFERENCES

- 1) T. Nikaido, T. Ohmoto, U. Sankawa, T. Hamanaka, K. Totsuka, *Planta Med.*, **46**, 162 (1982).
- 2) T. Kinoshita, U. Sankawa, T. Takuma, K. Asahi, N. Takahashi, *Chem. Pharm. Bull.*, **33**, 4109 (1985).
- 3) H. Nakamoto, Y. Iwasaki, H. Kizu, *Yakugaku Zasshi*, **97**, 103 (1977).
- 4) S. Ghengye, Z. Xiuyuan, *Acta Pharm. Sin.*, **14**, 129 (1979).
- 5) K. Abe, O. Inoue, E. Yumioka, *Chem. Pharm. Bull.*, **38**, 208 (1990).
- 6) K. Abe, Y. Nakada, A. Suzuki, E. Yumioka, *Shoyakugaku Zasshi*, **47**, 43 (1993).
- 7) J. Hayakawa, N. Noda, S. Yamada, K. Uno, *Yakugaku Zasshi*, **104**, 50 (1984).
- 8) K. Abe, H. Katayama, A. Suzuki, E. Yumioka, *Shoyakugaku Zasshi*, **47**, 402 (1993).
- 9) L. A. Griffiths, G. E. Smith, *Biochem. J.*, **130**, 141 (1972).
- 10) L. A. Griffiths, "The Flavonoids: Advances in Research," ed. by J. B. Harbone, T. J. Mabry, Chapman and Hall, London, 1982, pp. 692—694.