Urinary Metabolites of Daidzin Orally Administered in Rats
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In a study on the metabolism of flavonoids, the isoflavone glycoside daidzin was orally administered to rats. Urine samples were collected and treated with β-glucuronidase and arylsulfatase. Aglycone daidzein (M3) and other three metabolites, 3',4',7-trihydroxyisoflavone (M1), 4',7-dihydroxyisoflavone (M2) and 4',7-dihydroxyisoflavan (M4) were isolated from the urine following treatment with enzymes. The structures of M1, M2 and M4 were determined on the basis of chemical and spectral data.

Key words daidzin; Pueraria lobata Oswi; urinary metabolite; 3',4',7-trihydroxyisoflavone; 4',7-dihydroxyisoflavone; equol

Puerariae Radix, the root of Puerariae lobata Oswi (Leguminosae), is a very important Chinese traditional medicine and is used as a spasmylic and antipyretic agent. The chemical constituents of this plant have been studied extensively and various isoflavonoids have been found.1) Isoflavone glycoside daidzin is the major ingredient of Puerariae Radix, and shown to inhibit cyclic AMP phosphodiesterase3) and to induce differentiation in murine erythroleukemia cells.2) The spasmylic activity of daidzein, the aglycone of daidzin, was confirmed by a Magnus experiment using excised small intestine of mice.4) The hydrolysis of daidzin was previously demonstrated following its oral administration to rat and urinary and biliary excretion of glucuronide and/or sulfate conjugates of the aglycone daidzin was noted.5) Examination has also been made of the urinary metabolites of orally administered daidzin in rats.

The present paper reports the structures of the urinary metabolites of orally administered daidzin rats following treatment with β-glucuronidase and arylsulfatase.

MATERIALS AND METHODS

Apparatus Melting points were determined on Yanagimoto micro melting point apparatus and are not corrected. Optical rotations were measured with a Jasco DIP-360 automatic polarimeter. IR spectra were measured with a Perkin Elmer FT-IR 1725X spectrometer. UV spectra were taken on a Beckman DU-64 spectrometer. NMR spectra were recorded on a JEOL JNM-EX 270 with tetramethylsilane as the internal standard and chemical shifts are given as δ values. Mass spectra (MS) were measured with a JEOL DX-303 mass spectrometer. The HPLC system consisted of a CCPM pump, CO-8010 column oven (Tosoh, Tokyo, Japan) and model M9911 photodiode array detector (Waters Millipore, Milford, MA).

Reagents Daidzin was isolated from Puerariae Radix by the method of Hayakawa et al.6) 4',7-Dihydroxyisoflavane (equol) was prepared according to Lamberton et al.7) All other reagents were of special grade.

Animals Male SD rats (Japan SLC Inc.), 6 weeks old, were used. They were fasted but had free access to water for 18 h prior to the experiments.

Preparation of Plasma and Urine Samples a) Plasma Sample: Under light anesthesia with ether, daidzin (100 mg/kg) was administered orally and blood specimens were drawn from the portal vein at 8 h after administration with a heparin treated syringe. The collected blood samples were centrifuged at 3000 rpm for 10 min to obtain plasma. 4.0 ml of plasma were transferred to a test tube to which was added 6.0 ml of 0.2 M sodium acetate buffer (pH 5.5) and 50 μl of β-glucuronidase/aryl sulfatase solution (type H-1, Sigma, U.S.A.) followed by incubation at 37°C for 24 h. The incubation mixture was transferred to a separating funnel and extracted with ethyl acetate (30 ml) three times. The organic layer was washed with 1 ml of water and evaporated to dryness at 40°C. The residue was dissolved in 1 ml of methanol and a 20 μl sample was injected onto an HPLC column.

b) Urine Sample: Daidzin (100 mg/kg) was administered orally and urine specimens were obtained over a 24 h period using a metabolic cage. 3.0 ml of urine were transferred to a test tube followed by the addition of 7.0 ml of 0.2 M sodium acetate buffer (pH 5.5) and 50 μl of β-glucuronidase/aryl sulfatase solution (type H-1, Sigma, U.S.A.). Following the procedure above, a 10 μl sample was injected onto an HPLC column.

HPLC Conditions A stainless steel column (250× 4.6 mm i.d.), packed with reversed phase TSKgel ODS-120T (5 μm, Tosoh Company Ltd., Tokyo, Japan) was used. The mobile phase was a linear gradient system comprised of 10 mM sodium phosphate buffer (pH 6.5) (solvent A) and 100% methanol (solvent B), A/B=95/5 (0 min)→60/40 (45 min)→60/40 (70 min). The flow rate was 1.0 ml/min at 40°C.

Isolation of Urinary Metabolites For isolation of urinary metabolites, 1.3 g of daidzin were orally administered in portions to each of 12 rats at 100 mg/kg/d over a period of 30 d and urine samples were collected using metabolic cages. The combined urine samples (440 ml) were dissolved in 100 ml of 0.2 M sodium acetate buffer (pH 5.5) to which 5 ml of β-glucuronidase/aryl sulfatase solution were added and the solution was incubated at 37°C for 24 h. The incubated solution was extracted with ethyl acetate (1000 ml) three times. The organic layer was evaporated to dryness at 40°C. The residue (500 mg) was dissolved in a small amount of methanol and chromatographed on Sephadex LH-20 with methanol as the eluant. The fractions containing metabolites (M1, M2 and M4) were subjected to preparative HPLC under the following conditions: column, TSKgel ODS-120T (10 μm, 300×7.8 mm i.d., Tosoh Company Ltd., Tokyo, Japan); mobile phase, 20% methanol (solvent A) and 100% methanol (solvent B), linear gradient system, A/B=100/0

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(0 min)→0/100 (200 min). Flow rate was 2.0 ml/min at room temperature. Each metabolite fraction was evaporated to dryness at 40°C in vacuo to give M1 (4 mg), M2 (5 mg) and M4 (7 mg), respectively.

M1 (3',4',7'-Trihydroxyisoflavone): Pale brown powder, mp 271—272°C. [α]D^20 +16.8° (c=0.6, methanol). UV (methanol) λ max (log ε): 247 (3.99), 259 (3.96), 290 (3.82) nm. IR (KBr) ν max 3268, 1626 cm^-1. 1H-NMR (CD3OD, 270 MHz) δ: 6.85 (1H, d, J=8.2 Hz, H-5'), 6.90 (1H, d, J=2.0 Hz, H-8), 6.95 (1H, dd, J=8.2, 2.1 Hz, H-6'), 6.99 (1H, dd, J=8.2, 2.0 Hz, H-6), 7.18 (1H, d, J=2.1 Hz, H-2'), 8.05 (1H, d, J=8.7 Hz, H-5), 8.11 (1H, s, H-2). 13C-NMR (CD3OD, 60 MHz) δ: 103.2 (C-8), 115.7 (C-6), 115.9 (C-5'), 117.3 (C-2'), 118.6 (C-10), 121.4 (C-6'), 125.1 (C-1'), 125.2 (C-3), 128.5 (C-5), 145.5 (C-3'), 146.0 (C-4'), 153.2 (C-2), 158.8 (C-9), 175.6 (C-4). El-MS m/z: 270 [M]+ (100), 213 (11), 137 (48), 134 (20), 32 (45). HREI-MS m/z: 270.0493 (calcd for C12H12O3, 270.0528).

M2 (4',7-Dihydroxyisoflavonoid): White powder, mp 227—230°C. [α]D^20 ±0° (c=2.0, methanol). UV (methanol) λ max (log ε): 226 (3.60), 276 (3.58), 310 (3.39), 318 (sh) (3.34) nm. IR (KBr) ν max 3423, 1610 cm^-1. 1H-NMR (CD3OD, 270 MHz) δ: 3.85 (1H, t, J=6.6 Hz, H-3). 6.42 (1H, d, J=2.3 Hz, H-8), 6.59 (1H, dd, J=8.6, 2.3 Hz, H-6), 6.79 (2H, d, J=8.7 Hz, H-3',5'). 7.14 (2H, d, J=8.7 Hz, H-2',6'), 7.74 (1H, d, J=8.6 Hz, H-5). 13C-NMR (CD3OD, 60 MHz) δ: 51.8 (C-3), 72.7 (C-2), 103.4 (C-8), 111.4 (C-6), 115.3 (C-10), 116.2 (C-3',5'), 128.0 (C-1'), 130.1 (C-5), 130.6 (C-2',6'), 157.6 (C-4'), 164.4 (C-9), 165.2 (C-7), 191.0 (C-4). El-MS m/z: 256 [M]+ (15), 137 (100), 120 (49), 108 (11), 91 (21), 32 (15).

HREI-MS m/z: 256.0721 (calcd for C12H12O3, 256.0736).

M4 (4',7-Dihydroxyisoflavone): White powder, mp 156—159°C. [α]D^20 -10.9° (c=0.6, methanol). UV (methanol) λ max (log ε): 223 (3.48), 281 (3.21), 285 (sh) (3.17) nm. IR (KBr) ν max 3436, 1510 cm^-1. CD (methanol) [θ] (nm): +4198 (237), -3277 (287). 1H-NMR (CDCl3—CD3OD, δ: 2.90 (1H, d, J=8.4 Hz, H-4), 3.07—3.18 (1H, m, H-3), 3.95 (1H, t, J=10.6 Hz, H-2α), 4.26 (1H, dd, J=10.6, 2.6 Hz, H-2β), 6.37 (d, J=2.5 Hz, H-8), 6.41 (1H, dd, J=8.2, 2.5 Hz, H-6), 6.84 (2H, d, J=8.7 Hz, H-3',5'), 6.89 (1H, d, J=8.2 Hz, H-5), 7.09 (2H, d, J=8.7 Hz, H-2',6'). 13C-NMR (CDCl3—CD3OD, δ=5:1, 60 MHz) δ: 32.1 (C-4), 38.2 (C-3), 71.3 (C-2), 103.3 (C-8), 108.4 (C-6), 113.7 (C-10), 115.8 (C-3',5'), 128.4 (C-2',6'), 130.3 (C-5), 132.8 (C-1'), 156.0 (C-4'), 155.2 (C-9), 156.4 (C-7). El-MS m/z: 242 [M]+ (57), 147 (5), 135 (27), 123 (67), 120 (100), 107 (30), 91 (20), 32 (58). HREI-MS m/z: 242.0947 (calcd for C12H12O3, 242.0943).

RESULTS AND DISCUSSION

The three dimensional (3D)-HPLC profile of urine samples from rats after oral administration of daidzin showed 4 distinct peaks tentatively designated as M1, M2, M3 and M4 in decreasing order of polarity (Fig. 1). Furthermore, M2, M3 and M4 were also detected in blood samples after oral daidzin administration (Fig. 2). M3 was identified as daidzin by direct comparison of UV spectrum and retention time with those of an authentic sample in 3D-HPLC. M1, M2 and M4 from urine treated with enzymes were isolated by chromatographic separation on a Sephadex LH-20 column.

![Fig. 1. 3D-HPLC Profiles of (A) Ethyl Acetate Extract of Control Urine and (B) Ethyl Acetate Extract of Urine Samples Excreted during 24 h Following the Oral Administration of Daidzin (100 mg/kg) to Rats](image-url)
Fig. 2. 3D-HPLC Profiles of (A) Ethyl Acetate Extract of Control Plasma and (B) Ethyl Acetate Extract of Plasma Samples at 8 h after the Oral Administration of Daidzein (100 mg/kg) to Rats

and repeated preparative HPLC as described in the experimental section followed by determination of these structures. 

**M1** was obtained as a pale brown powder, mp 271—272 °C, [α]_D^20 +16.8° (methanol). Its molecular formula was C_{12}H_{10}O_4 based on high-resolution mass spectrum (HR-MS). The UV spectrum of **M1** in methanol exhibited major absorbance at 247 nm, typical of the isoflavone skeleton. The IR spectrum showed absorption bands at 3268 and 1626 cm^{-1}, characteristic of hydroxyl and conjugated carbonyl groups. The isoflavone skeleton was further confirmed from the ¹H-NMR spectrum containing the characteristic singlet at δ: 8.11 due to the C-2 proton. MS of **M1** showed typical retro Diels–Alder fragmentation that gave rise to m/z 270 [M^+], 137 and 134. The ring A of the isoflavone skeleton was thus shown to have one hydroxyl group and ring B, two hydroxyl groups. In the ¹H-NMR of **M1**, the three signals coupled to each other at δ: 6.90 (1H, d, J=2.0 Hz), 6.99 (1H, dd, J=8.7 and 2.0 Hz) and 8.05 (1H, d, J=8.7 Hz) were assigned to H-8, H-6 and H-5, respectively and another set of three signals [δ: 6.85 (1H, d, J=8.2 Hz), 6.95 (1H, dd, J=8.2 and 2.1 Hz) and 7.18 (1H, d, J=2.1 Hz)] were assigned to ring B of the isoflavone skeleton. Comparison of the ¹H-NMR chemical shifts with those of daidzein indicated a signal attributable to the C-3' of daidzein to be shifted downfield from δ: 115.8 to 145.5 in **M1** and the signals of C-2', C-4' and C-6', located ortho and para to C-3', to be shifted to a higher field by 13.8, 12.2 and 9.7 ppm. The positions of the hydroxyl groups were the same as those in 3',4'-dihydroxyisoflavone, based on a comparison of chemical shifts of ring B proton signals. **M1** was thus concluded to be 3',4',7-trihydroxyisoflavone.

**M2** was obtained as a white powder, mp 227—230 °C, [α]_D^20 ±0° (methanol) with molecular formula determined as C_{12}H_{14}O_4 from its HR-MS. The UV spectrum in methanol exhibited major absorbance at 276 and 310 nm typical of the isoflavonone skeleton. Structural features were also demonstrated by ¹C signals at 72.7 (C-2) and 51.8 (C-3) differing from those of flavanone derivatives. The IR spectrum showed absorption bands at 3423 and 1610 cm^{-1}, characteristic of hydroxyl and carbonyl groups. The ¹H-NMR spectrum suggested an isoflavonone skeleton with a signal at δ: 4.62 (2H, d, J=6.6 Hz, H-2) and 3.85 (1H, t, J=6.6 Hz, H-3), and three signals coupled to each other at δ: 6.43 (1H, d, J=2.3 Hz), 6.59 (1H, dd, J=8.6 and 2.3 Hz) and 7.74 (1H, d, J=8.6 Hz) which were assigned to H-8, H-6 and H-5, respectively and another set of A_2B_2 type signals at δ: 6.79 (2H, d, J=8.7 Hz)
and 7.14 (2H, d, J=8.7 Hz) which were assigned to B-ring protons (H-3, ‘-5’ and H-2, ‘-6’). The structure of M2 was thus concluded to be 4’,7-dihydroxyisoflavananone. M2 seems to be racemic, as can be judged from its optical rotation of about 0°.

M4 was obtained as a white powder, mp 156—159°C, [α]20 D +10.9° (methanol) with a molecular formula determined as C13H14O3 from HR-MS. The UV spectrum in methanol exhibited major absorbance at 281 nm, typical of the isoflavon skeleton. The IR spectrum showed absorption bands at 3436 and 1510 cm⁻¹, characteristic of the hydroxyl group and aromatic ring. In the 1H-NMR spectrum, signals assignable to H-2α, H-2β, H-3 and H-4 of the isoflavon skeleton appeared at δ: 3.93 (1H, t, J=10.6 Hz), 4.26 (1H, dd, J=10.6 and 2.8 Hz), 3.07—3.18 (1H, m) and 2.90 (2H, d, J=8.4 Hz), respectively. Observation in the 1H-NMR spectrum of an ABX type aromatic proton signals at δ: 6.37 (1H, d, J=2.3 Hz), 6.41 (1H, dd, J=8.2 and 2.3 Hz) and 6.89 (1H, d, J=8.2 Hz) and an A,B type aromatic proton signals at δ: 6.84 (2H, d, J=8.7 Hz) and 7.09 (2H, d, J=8.7 Hz) indicated M4 to possibly have a hydroxyl group at C-7 and C-4‘. These data indicate the structure to be the known isoflavon, 4’,7-dihydroxyisoflavon (equol). Direct comparison of M4 by IR, MS and NMR spectra with an authentic sample synthesized from daidzein confirmed M4 to be identical with equol. The absolute configuration at C-7 was determined to be S from the circular dichroism spectrum, which showed a negative Cotton effect at 287 nm.50 M4 is thus shown to be (3S)(-)-equol. Carbon signals were assigned based on the 13C-1H COSY and Heteronuclear multiple-bond correlation spectroscopy (HMBC) spectra.

Little attention has been directed to the metabolism of isoflavonoids. Studies of the metabolism of formononetin and daidzein in sheep and rat have been reported by Braden et al.60 and Griffiths et al.,61 respectively. Shutt et al.62 reported that formononetin and daidzein are converted into equol in sheep, and the estrogenic effect of subterranean clover has been attributed to equol produced in this manner. Earlier work reported that many flavonoid glycosides undergo microbial hydrolysis in the gastrointestinal tract and are excreted in the urine as various phenolic acid derivatives through ring-fission.63 It is noteworthy that, although phenolic ring-fission products were not detected in the urine and plasma of rats, isoflavonane derivative (M2) in addition to hydroxylated metabolite (M1) were newly isolated from the urine after oral administration of daidzin in vivo.

In previous papers64,65 we reported that daidzin orally administered to rat was hydrolyzed to the aglycone daidzin, which then was metabolized to conjugated forms (sulfates or glucuronides) and finally excreted into the urine and bile via the blood stream. Daidzin was shown in this study to be metabolized to M1, M2, M3 and M4. M1 and M2 were previously isolated from the fermentation broth of Streptomyces sp. OH-104966 and heartwood of Periplocapsa mooniana,67 respectively, but their isolation as urinary metabolites of rats receiving orally administered daidzin is reported here for the first time. In Fig. 3, we propose a metabolic pathway in which daidzin is hydrolyzed to aglycone M3. Next, M3 is partially hydroxylated to give M1. M3 is hydrogenated to M2 and/or reduced to give M4.

Daidzein has papaverine-like antispasmodic action68 and inhibits cyclic AMP phosphodiesterase.21 M1 has potent antioxidant activity.15 The known isoflavon, equol (M4) was found to be weakly estrogenic, with 10⁻³—10⁻⁵ times the activity of estradiol-17β, with which it has some structural similarity17 and is antagonistic to estradiol-17β through its competing for cytoplasmic estrogen receptors.58 The presented metabolites would thus appear to be the active forms of orally administered daidzin in rat. Traditional medicines are taken as decoctions and some natural compounds become bioactive after being metabolized.19—21 Further studies are now being made of the biological activity of these metabolites.

REFERENCES
