Plasma Profiling of Intact Isoflavone Metabolites by High-Performance Liquid Chromatography and Mass Spectrometric Identification of Flavone Glycosides Daidzin and Genistin in Human Plasma after Administration of Kinako

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ABSTRACT:

The roles of isoflavones in the prevention of several hormone-dependent cancers and osteoporosis are of great interest. Despite many pharmacokinetics studies of the isoflavones, the actual types of conjugates circulating in the body and the position(s) of conjugation sites on the flavone skeleton are still uncertain because, in general, conjugated compounds in biological fluids have been evaluated by measuring the free aglycones obtained after selective enzymatic hydrolysis. Using an high-performance (HPLC)-UV-diode-array detector (DAD) method combined with solid-phase extraction, we have obtained HPLC profiles of isoflavone glycosides [daidzin (Din) and genistin (Gin)] and of intact isoflavone metabolites in human plasma: daidzein, genistein, daizein-7-glucuronide, daidzein-4'-glucuronide, genistein-7-glucuronide, genistein-4'-glucuronide, daidzein-7-sulfate, daidzein-4'-sulfate, genistein-7-sulfate, and genistein-4'-sulfate. We investigated the plasma profile of intact isoflavone metabolites in plasma obtained 1 to 7 h after orally administration of 50 g of kinako (baked soybean powder) to two healthy volunteers. The results of DAD analysis indicated that the main isoflavone metabolite peaks were identified on the HPLC chromatogram. Furthermore, the intact glycosides Din and Gin were detected in 1-h plasma samples by their positive electrospray ionization mass spectra, demonstrating that the glycosides Din and Gin can be absorbed from the gut.

Isoflavonoid phytoestrogens occur naturally in the plant kingdom, and they generally are present as glycosides in the usual human diet (Setchell and Cole, 2003). A number of epidemiological studies and animal experiments have indicated that phytoestrogens could play a role in the prevention of several hormone-dependent diseases such as breast cancer, prostate cancer, and osteoporosis (Adlercreutz, 2002; Setchell and Lydeking-Olsen, 2003; McCue and Shetty, 2004; Bektic et al., 2005). Japanese individuals consume 63.2 to 70.2 g of soybeans per capita per day according to the 1975 to 2002 National Dietary Survey Data. The traditional custom of consuming soy products, such as miso (fermented soybean paste) soup, tofu (soybean curd), natto (fermented soybeans) and so on, may be important in cancer prevention for the Japanese (Adlercreutz et al., 1993; Yamamoto et al., 2003; Hirose et al., 2005).

It is generally considered that after digestion, the biologically active forms of the naturally occurring isoflavone glycosides daidzin (Din) and genistin (Gin) are either aglycone daidzein (Dein) or genistein (Gein) or their unconjugated metabolites (Yuan et al., 2003). However, several investigators have reported that the conjugates may have biological activity themselves, or they may be precursors of biologically active compounds at or within target cells. For example, a sulfoconjugate of Dein, Dein-7,4'-disulfate, inhibits sterol sulfatase in hamster liver microsomes (Wong and Keung, 1997). Genin glucuronides have weak estrogenic activity and can activate human natural killer cells within the concentration range of 0.1 to 10 μM in vitro (Zhang et al., 1999).

Flavonoid glycosides are known to be biotransformed to their corresponding aglycones and sugar moieties by enteral microorganisms (Németh et al., 2003). Therefore, it is commonly accepted that the glycosides are not absorbed intact from the human gut (Setchell et al., 2002). However, it is still ambiguous whether or not isoflavones are present as glycosides in plasma. Isoflavones are metabolized and then excreted as glucuronide, sulfate, and sulfoglucuronide conjugates in human urine (Doerge et al., 2000; Shelnutt et al., 2002).
Conjugated compounds are generally converted to free aglycones by selective enzymatic hydrolysis and then analyzed by HPLC (Morand et al., 2001) or LC-MS methods (Maskarinec et al., 2007) that require less sample preparation than gas chromatography or gas chromatography-MS methods (Adlercreutz et al., 1995; Heinonen et al., 2003). These methods, however, provide data only on total glucuronides, sulfates, and free aglycones. Furthermore, any glycosides that might have been absorbed as intact glycosides would be completely cleaved by the enzymatic hydrolysis step. The resulting estimates based on the enzymatically released aglycones do not provide detailed information on the type of conjugates or on the conjugation position(s) on the flavone skeleton for conjugated compounds circulating in plasma. Intact phytoestrogen sulfates and glucuronides in human urine have been measured using an isotope dilution LC-MS method (Clarke et al., 2002), but there have been no similar reports for human plasma.

We reported previously on the use of solid-phase extraction methods for extraction and clean-up of intact flavonoid glycosides possessing high polarity in biological fluids. This led to the successful development of HPLC-UV methods (Ishii et al., 2000, 2001). The aim of the present study was to directly measure the isoflavone metabolites simultaneously, without any sample treatment process affecting the ratios of glycosides, their aglycones and conjugates, by using HPLC-UV-DAD combined with solid-phase extraction. In this study, Dwing and Gin, and their aglycones (Dein and Gein), and conjugated metabolites of Dein and Gein (7-glucuronides, 4'-glucuronides, 7-sulfates, and 4'-sulfates) were measured by HPLC after oral administration of kinako (baked soybean powder) to two healthy volunteers. The plasma profiling of isoflavone glycosides, aglycones, and their conjugated metabolites should provide useful information for investigating the pharmacokinetics and pharmacodynamics of isoflavones in humans.

### Materials and Methods

**Chemicals.** Daidzein (7-hydroxy-3-(4-hydroxyphenyl)-4H-1-benzyopyran-4-one), daidzin (daidzein-7-glucoside), and genistin (genistin-7-glucoside) were purchased from LC Laboratories (Woburn, MA). Genistein (5,7-dihydroxy-3-(4-hydroxyphenyl)-4H-1-benzyopyran-4-one, 4', 5,

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**Fig. 1.** Structures of isoflavones and the conjugated metabolites considered in this study.

**Fig. 2.** HPLC chromatograms of D-7-G and D-4'-G (A) and G-7-G and G-4'-G (B) synthesized by UGT1A1.
7-trihydroxyisoflavone) was purchased from Extrasynthese (Genay, France). Daidzein-7-sulfate (D-7-S), daidzein-4'-sulfate (D-4'-S), genistein-7-sulfate (G-7-S), and genistein-4'-sulfate (G-4'-S) were synthesized in our laboratory (Nakano et al., 2004). Phosphoric acid (99.999%) was purchased from Aldrich Chemical Co. (Milwaukee, WI). Kinako (baked soybean powder) was purchased from a retail store. All other chemicals and solvents were of analytical grade and were used without further purification.

Preparation of Reference Substances. Synthesis of D-7-G. D-7-G was synthesized from 4',7-di-O-acetyldaidzein according to the method of Needs and Williamson (2001). 4',7-Di-O-acetyldaidzein was prepared by treatment of Dein with acetic anhydride in the presence of concentrated sulfuric acid (Walz, 1931). To a solution of 4',7-di-O-acetyldaidzein (3.0 g, 8.9 mmol) dissolved in tetrahydrofuran (375 ml) at 30°C, 375 ml of 1 mol/l acetic anhydride (6.8 mmol) was added. The mixture was stirred for 15 min at 30°C and then adjusted to pH 4.5 with acetic acid. One-half (750 ml) of the reaction mixture was extracted three times with 100 ml of dichloromethane. The combined organic extracts were washed sequentially with water, saturated aqueous Na2CO3, and water and then dried with MgSO4. After evaporation under reduced pressure, the residue (1.5 g) was recrystallized from ethanol. The yield of 4',7-di-O-acetyldaidzein was 1.8 g (70%). A suspension of 4',7-di-O-acetyldaidzein (1.6 g, 5.4 mmol) and methyl 2,3,4-tri-O-acetyl-β-D-α-D-glucopyranosyluronate bromide (2.7 g, 6.8 mmol) in 72 ml of dichloromethane was stirred in an 81% yield (Walz, 1931). To a solution of 4',7-di-O-acetyldaidzein-7-yl β-D-α-D-glucopyranosyluronate was suspended in 30 ml of methanol and cooled to 1°C. To this suspension was added 30 ml of 0.5 mol/l LiOH precooled to 1°C. The mixture was then stirred at 1°C for 16 h. A Dowex 50WX2–100 ion-exchange resin (H+ form, Aldrich Chemical Co.) of 20-ml volume was added to this solution. The reaction mixture was eluted through a 50 × 22 mm i.d. column of Dowex, and the latter was eluted with methanol. The combined eluate was evaporated to dryness (yield, 148 mg). The crude D-7-G was dissolved in 1 ml of methanol and purified by preparative HPLC.

Synthesis of G-7-G. G-7-G was synthesized from 4',5-di-O-acetylgensiestein according to the preparation procedure for D-7-G. 1H-NMR δ (600 MHz, DMSO-d6): 8.38 (s, H-2), 8.05 (d, J = 8.72 Hz, H-5), 7.15 (d, J = 8.35 Hz, H-6), 7.27 (s, H-8), 7.40 (d, J = 8.04, H-2', 6'), 6.82 (d, J = 8.05, H-3', 5'), 9.57 (s, H-4'), 5.56 (s, H-1'), 4.01 (s, H-5'). ESI-MS: m/z 431 [M + H]+, 255 [M – glucuronic acid + H]+.

Enzymatic synthesis of glucuronide conjugates. As reference compounds for isolation of 4'-glucuronides in urine extracts, a reference mixture containing 7- and 4'-glucuronide conjugates of Dein and Gein was prepared on a micro scale using microsomal recombinant human UDP-glucuronosyltransferase (UGT) 1A1 (EC 2.4.1.17) (Wako Pure Chemicals, Tokyo, Japan) according to a method published previously (Doerge et al., 2000). A mixture of 200 mM Dein or Gein and 1 mM UDP-glucuronic acid in 0.1 mol/l potassium phosphate buffer at pH 8.0 was incubated with UGT1A1 (final protein concentration of 0.25 mg/ml) in the presence of 10 mM MgCl2 for 2 h at 37°C. The reaction was initiated by the addition of UDP-glucuronic acid. A 4-fold volume of ethanol was added to the samples after incubation, and the mixture was vortexed for 30 s, centrifuged at 3500 rpm for 10 min, and then analyzed using HPLC.

Isolation of D-4'-G and G-4'-G from human urine. Urine samples obtained at 4 to 7 h from a human volunteer (subject 1) after oral administration of 50 g of kinako were used to isolate isoflavone metabolites. Urine (650 ml) was molecular sieves (1.2 g) under argon atmosphere at room temperature. To this mixture, 0.9 ml of collidine (6.8 mmol) was added dropwise over 10 min. The mixture was stirred in the dark for 7 days. After filtration with celite, the solution was washed sequentially with 10% aqueous acetic acid, water, 0.1 mol/l Na2S2O3, water, saturated NaHCO3, and water, and then dried with MgSO4. After evaporation under reduced pressure, the residue (1.5 g) was purified by silica gel column chromatography and eluted with hexane-ethyl acetate (1:1). Then 376 mg of purified methyl (4'-O-acetyldaidzein-7-yl β-D-α-D-glucopyranosyluronate was suspended in 30 ml of methanol and cooled to 1°C. To this suspension was added 30 ml of 0.5 mol/l LiOH precooled to 1°C. The mixture was then stirred at 1°C for 16 h. A Dowex 50WX2–100 ion-exchange resin (H+ form, Aldrich Chemical Co.) of 20-ml volume was added to this solution. The reaction mixture was eluted through a 50 × 22 mm i.d. column of Dowex, and the latter was eluted with methanol. The combined eluate was evaporated to dryness (yield, 148 mg). The crude D-7-G was dissolved in 1 ml of methanol and purified by preparative HPLC.

Synthesis of G-7-G. G-7-G was synthesized from 4',5-di-O-acetylgensiestein according to the preparation procedure for D-7-G. 1H-NMR δ (600 MHz, DMSO-d6): 8.43 (s, H-2), 12.95 (s, H-5), 6.75 (d, J = 1.93 Hz, H-6), 6.50 (d, J = 1.99, H-8), 7.40 (d, J = 8.43, H-2', 6'), 6.83 (d, J = 8.47, H-3', 5'), 9.62 (s, H-4'), 5.55 (s, H-1'), 4.05 (s, H-5'). ESI-MS: m/z 447 [M + H]+, 271 [M – glucuronic acid + H]+.

Fig. 3. Preparative HPLC chromatogram of the glucuronidation fraction extracted from human urine.

A 230°C

B 270°C

FIG. 4. MS spectra of chemically synthesized D-7-G and G-7-G and of D-4'-G and G-4'-G isolated from human urine. The temperatures of the heated capillary were set at 290°C (A) and at 270°C (B).
applied to a Dowex 50WX2–100 ion-exchange resin (Aldrich Chemical Co.) column (H\(^+\) form, 150 ml of packing, 500 × 22 mm i.d.), which was previously activated with 1000 ml of methanol followed by 500 ml of water. The elution rate from the column was 2.5 ml/min. The column was washed with 1000 ml of water followed by 570 ml of methanol. After evaporating the eluate containing the glucuronides of Dein and Gein at 40°C in vacuo, the residue was dissolved in 800 ml of methanol with vortex mixing for 30 s followed by 3200 ml of water with vortex mixing for 30 s. A 200-μl portion of the solution was subjected to preparative HPLC 20 times.

Preparative chromatography. The preparative HPLC was performed on a liquid chromatograph (HPLC) system (Jasco, Tokyo, Japan) equipped with a model PU-2089 Plus pump and a model UV-2075 Plus UV detector. Data processing was performed with a model Sic chromatocorder 21 (System Instruments, Tokyo, Japan). The HPLC system consisted of an Inertsil ODS-3 column (250 × 20 mm i.d., particle size 5 μm; GL Sciences, Tokyo, Japan). The mobile phase was acetonitrile-0.1% trifluoroacetic acid (23:77, v/v), and the flow rate was 10.0 ml/min. The UV detection wavelength was set at 250 nm. All HPLC analyses were performed at room temperature. Fractions that eluted during the time corresponding to the peak half-width were collected (D-4'-G, RT 15.7 min; approximately 15.4–16.2 min; G-4'-G, RT 31.1 min; approximately 30.6–32.0 min). After evaporation of the eluates, D-4'-G and G-4'-G fractions were obtained, respectively, D-4'-G: \(^1\)H-NMR \(^{600}\text{MHz, DMSO-}d_6\): 8.35 (s, H-2), 7.97 (d, \(J = 8.74, H-5\)), 6.94 (d, \(J = 8.76 Hz, H-6\)), 6.88 (s, H-8), 7.49 (d, \(J = 8.54, H-2',6'\), 7.08 (d, \(J = 8.54, H-3',5'\)). ESI-MS: \(m/z\) 431 [M + H]\(^+\), 448 [M + NH\(_4\)]\(^+\), 255 [M – glucuronic acid + H]\(^+\), G-4'-G: \(^1\)H-NMR \(^{600}\text{MHz, DMSO-}d_6\): 8.39 (s, H-2), 6.24 (s, H-6), 6.41 (s, H-8), 7.49 (d, \(J = 8.41, H-2',6'\)), 7.09 (d, \(J = 8.63, H-3',5'\)). ESI-MS: \(m/z\) 447 [M + H]\(^+\), 464 [M + NH\(_4\)]\(^+\), 271 [M – glucuronic acid + H]\(^+\).

Analytical Chromatography. Sample collections. This study was approved by Kyorin University, School of Health Sciences Human Subjects Review Board. Written informed consent was obtained from all subjects. Two healthy volunteers [50-year-old male (subject 1) and 25-year-old female (subject 2)] orally received 50 g of kinako containing 27.4 mg (65.9 μmol) of Din, 45.8 mg (106.0 μmol) of Gin, 30.4 mg (119.7 μmol) of Dein and 55.3 mg (204.8 μmol) of Gein suspended in 300 ml of cow milk. The volunteers did not ingest soy-containing foods from 1 week before the test until completion of the study. Blood samples (7.0 ml each) were collected from a medial cubital vein into evacuated tubes containing Na\(_2\)EDTA just before and at 1, 2, 4, and 7 h after administration and were immediately centrifuged (2500 rpm, 10 min). The plasma fractions were removed and stored at −80°C until analysis.

Extraction of isoflavone metabolites from plasma. Plasma samples (1.0 ml) were diluted with 2.0 ml of 0.01 mol/l phosphoric acid solution and were then subjected to an Oasis HLB cartridge (3 cc, 60 mg of packing; Waters, Milford, MA). The cartridge was placed on a vacuum manifold and activated with 2.5 ml of methanol followed by 2.5 ml of 0.01 mol/l phosphoric acid solution. The sample was then loaded at a flow rate of 0.2 ml/min. The cartridge was washed.

FIG. 5. HPLC chromatogram and UV spectra of a standard mixture of Din, Gin, and their conjugated metabolites: D-7-G, 15.8 ng; D-4'-G, 18.1 ng; G-7-G, 16.6 ng; G-4'-G, 18.2 ng; D-7-S, 8.4 ng; D-4'-S, 7.6 ng; G-7-S, 11.7 ng; G-4'-S, 7.8 ng; Din, 11.8 ng; Gin, 13.7 ng; Dein, 23.1 ng; and Gein, 28.6 ng.
with 5.0 ml of 10% methanol. After purging with air, the cartridge was eluted with 5.0 ml of methanol at a flow rate of 0.2 ml/min. After the eluate was evaporated at 40°C in vacuo, the residue was dissolved in 1.0 ml of methanol and the solution was filtered through an HPLC disk filter (pore size 0.45 μm) (Kanto Chemical, Tokyo, Japan). The filtrate was transferred to a spitz tube with a ground-glass joint and evaporated to dryness at 40°C in vacuo. The residue was dissolved in 50 μl of methanol with vortex mixing for 30 s and then 150 μl of 10 mM ammonium acetate solution with vortex mixing for 30 s. A 20-μl portion of the solution was subjected to HPLC. The recoveries of isoflavones from plasma were calculated by comparing the peak heights before and after the Oasis HLB extraction procedure.

**HPLC-UV-DAD analysis of isoflavone metabolites.** HPLC-UV-DAD analyses were performed on a Nanospace SI-2 liquid chromatograph system (Shiseido, Tokyo, Japan) equipped with two model 3001 pumps, a model 3004 column oven, and a model 3002 UV-visible detector. A model UV6000LP diode-array detector (Thermo Fisher Scientific, Waltham, MA) was added to carry out spectral analysis in series. The mobile phase was degassed with a model 3009 degasser. The system was controlled by EZChrom Elite, a software system designed for HPLC. The HPLC system consisted of a Cadenza CD-C18 column (100 × 4.6 mm i.d., particle size 3 μm; Imtak, Kyoto, Japan) and a guard cartridge (5 × 2.0 mm i.d.) of the same material. Elution was performed using a solvent system comprising 10 mM ammonium acetate solution (solvent A) and acetonitrile mixed using a linear gradient, held at 93.3% solvent A for 1.5 min and then decreasing linearly to 72.5% solvent A at 15 min, increasing back to 93.3% solvent A at 22 min, and held for 3.0 min. The flow rate was 1.5 ml/min. The UV detection wavelength was set at 250 nm. For spectral analysis, the diode-array detection wavelength was set at 220 to 360 nm. All HPLC analyses were performed at 60°C. The purities of isolated D-4′-G (>99%) and G-4′-G (98.6%) were also estimated by this method.

**Partial Purification of Din and Gin from Plasma Extracts.** Isoflavone glycosides Din and Gin were partially purified from plasma samples (~10 ml each) collected 1 h after oral administration of 50 g of kinako to subjects 1 and 2, using the preparative HPLC system used to isolate D-4′-G and G-4′-G. The column used was an Inertsil PREP-ODS (250 × 10 mm i.d., particle size 10 μm). The flow rate of the mobile phase consisting of acetonitrile-10 mM ammonium acetate (23:77, v/v) was 3.0 ml/min at room temperature. By using the mobile phase, Gin (RT 19.1 min; approximately 18.8–19.6 min) was initially fractionated from the plasma extract. After removal of the Gin fraction, the plasma extract was then used for the isolation of Din. By using the mobile phase consisting of acetonitrile-10 mM ammonium acetate (15:85, v/v), the Din fraction was obtained (RT 19.7 min; approximately 19.5–20.0 min).

**LC-ESI-MS Analysis.** The HPLC apparatus and chromatographic conditions were appropriately designed for LC-ESI-MS, MS/MS, and MS/MS/MS analyses. The HPLC apparatus used was a liquid chromatograph (Waters) controlled by a model 600S controller equipped with a model 616 pump, a

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**Figure 6.** Typical HPLC chromatograms of plasma extracts (1 ml) without spiking of the various isoflavones (blank) and spiked with low and high amounts of the isoflavones standard mixture (spiked 1 and 2).
model 486-MS tunable absorbance detector, and a model Sic chromatocorder 21 (System Instruments, Tokyo, Japan) as a data processor. The mobile phase was degassed with an in-line degasser (Waters). The detection wavelength was set at 250 nm. The mass spectral data were collected with a quadrupole ion trap mass spectrometer (Finnigan LCQ; Thermo Fisher Scientific) equipped with a heated capillary electrospray interface. The ion polarity mode was set to the positive MS, MS/MS, and MS/MS/MS mode. The sprayer needle voltage was 4.5 kV with a nebulizer gas flow set at 75% of the maximum. Capillary and tube lens voltages were 3 and 10 V, respectively. Two microscans were carried out. The collision gas pressure was \(10^{-3}\) Torr (1 Torr = 133.322 Pa). The relative collision energies for the MS/MS and MS/MS/MS mode analyses were optimized at 75% of the maximum setting (5 V). The MS/MS and MS/MS/MS spectra were obtained with a precursor ion isolation width of 5 \( m/z \).

Identification of D-4'-G, G-4'-G, Din, and Gin by mass spectrometry. For the mass spectral analyses of D-4'-G, G-4'-G, Din, and Gin, a Luna C18(2) column (150 × 2.0 mm i.d., particle size 5 \( \mu \text{m} \)) (Phenomenex, Torrance, CA) was used. The mobile phase for the analyses of D-4'-G and G-4'-G was acetonitrile-10 mM ammonium acetate (10:90, v/v) at a flow rate of 0.4 ml/min. The mobile phase for Din and Gin was acetonitrile-10 mM ammonium acetate (15:85, v/v) at a flow rate of 0.3 ml/min. The temperatures of the heated capillary were 230 or 270°C for D-4'-G and G-4'-G and 240°C for Din and Gin.

Mass spectral analyses of unknown metabolites (U1, U2, and U3). For the unknown compounds (U1, U2, and U3), a Cadenza CD-C18 column (100 × 4.6 mm i.d., particle size 3 \( \mu \text{m} \)) was used. A plasma sample (4 ml) collected 7 h after oral administration of 50 g of kinako to subject 1 was used to extract the unknown metabolites. The mobile phase was 10 mM ammonium acetate solution (solvent A) and acetonitrile mixed using a linear gradient held at 92% solvent A for 1.5 min and then decreasing linearly to 77% solvent A at 20 min, and the total flow rate was 0.5 ml/min. The temperature of the heated capillary was 260°C.

Semiquantification. Stock solutions of Din, Gin, and Dein were prepared by dissolving these compounds with ethanol-water (50:50, v/v). Stock solutions of Gein, D-7-G, D-4'-G, G-7-G, G-4'-G, D-7-S, D-4'-S, G-7-S, and
G-4"/H11032-S were prepared by dissolving these compounds with methanol-water (50:50, v/v). Calibration curves were prepared in 1.0 ml of blank plasma by a standard addition method, in the following ranges: D-7-G (31.5–394.1 ng), D-4"/H11032-G (36.1–464.4 ng), G-7-G (33.1–394.1 ng), G-4"/H11032-G (36.4–507.6 ng), D-7-S (15.1–141.6 ng), D-4"/H11032-S (16.8–168.0 ng), G-7-S (15.6–204.8 ng), G-4"/H11032-S (23.4–175.4 ng), Din (23.7–222.0 ng), Gin (27.4–246.6 ng), Dein (46.1–403.5 ng), and Gein (57.1–471.1 ng). After determining the absolute peak height on the HPLC chromatograms, calibration graphs were obtained by least-squares linear fitting of the absolute peak height versus the absolute amount of the each isoflavone added to the blank plasma.

**1H-NMR Analysis.** NMR spectra were recorded on an NMR AV-600 spectrometer (Bruker, Rheinstetten, Germany) (600 MHz in the dimethyl sulfoxide-d₆ solutions). Chemical shifts are given in δ values (parts per million) downfield from tetramethylsilane.

**Results**

**Reference Compounds.** D-7-G and G-7-G were synthesized in four steps from Dein or Gein as a starting material. D-4"/H11032-G and G-4"/H11032-G were isolated from human urine after intake of kinako (subject 1). The purities of D-7-G and G-7-G (chemically synthesized) and of D-4"/H11032-G and G-4"/H11032-G purified by preparative HPLC were determined to be more than 98% by HPLC. D-7-S, D-4"/H11032-S, G-7-S, and G-4"/H11032-S were previously synthesized in our laboratory (Nakano et al., 2004). A mixture of the 7- and 4"/H11032-glucuronide conjugates of Dein and Gein was also prepared on a micro scale using the microsomal recombinant human UGT1A1 according to the method reported by Doerge et al. (2000). Figure 2 shows the HPLC chromatogram of enzymatically obtained D-7-G, D-4"/H11032-G (Fig. 2A), G-7-G, and G-4"/H11032-G (Fig. 2B). The preparative HPLC chromatogram of the glucuronide fraction extracted from urine by ion-exchange chromatography is shown in Fig. 3. Identification of the respective glucuronides was achieved by cochromatography with the compounds obtained by enzymatic synthesis and by comparing their mass spectra. The mass spectra of the chemically synthesized D-7-G and G-7-G and of D-4"/H11032-G and G-4"/H11032-G purified from urine were obtained using the ESI-MS apparatus equipped with a heated capillary electrospray interface at 230°C (Fig. 4A) and 270°C (Fig. 4B). The positive ion ESI mass spectra of these glucuronides (Fig. 4, A and B) showed the proton adduct ions at m/z 431 (M + H⁺) for D-7-G and D-4"/H11032-G and at m/z 447 (M + H⁺) for G-7-G and G-4"/H11032-G as the principal ion and the proton adduct ions of the respective aglycones at m/z 255 (M + H⁺) for Dein and at m/z 271 for Gein. Furthermore, the MS spectra of 4"/H11032-glucuronides obtained at 230°C (Fig. 4A) showed a relatively abundant ion peak at m/z 448 for D-4"/H11032-G (relative abundance ~40%) or at m/z 464 for G-4"/H11032-G (relative abundance ~85%). These were the ammonium adduct ions of the respective 4"/H11032-glucuronides ([M + NH₄⁺]). However, the ammonium adduct ions of 7-glucuronides were not observed at 230°C (Fig. 4A). When the temperature of the heated capillary was raised from 230 to 270°C, the relative intensity of the ammonium adduct ions of 4"/H11032-glucuronides decreased to approximately 10%, and the proton adduct ions were obtained as the principal ion.

A comparison of 600 MHz 1H-NMR spectral data for two 7-glucuronides (D-7-G and G-7-G) with those of the corresponding aglycones indicated there was a downfield shift of C-6 proton signals (0.22 ppm for D-7-G and 0.53 ppm for G-7-G) and C-8 proton signals (0.41 ppm for D-7-G and 0.12 ppm for G-7-G) because of the glucuronyl group attached to the C-7 hydroxy group. On the other hand, a comparison of spectral data obtained for two 4"/H11032-glucuronides (D-4"/H11032-G and G-4"/H11032-G) with data for the corresponding aglycones indicated that...
there was a downfield shift of C-2'/6' signals (0.11 ppm for D-4'-G and 0.12 ppm for G-4'-G) and C-3',5' signals (0.27 ppm for D-4'-G and G-4'-G, respectively) because of the glucuronyl group attached to the C-4' hydroxyl group.

HPLC Behavior and UV Spectra of Reference Compounds. An HPLC chromatogram and UV spectra for a standard mixture containing the 12 isoflavones considered in this study are shown in Fig. 5. The peaks of D-4'-S and D-7-S were not completely separated from each other on the HPLC chromatogram baseline. However, all other peaks were well separated within 17 min on the HPLC chromatogram. From the absorption spectral data for isoflavones in the mobile phase (10 mM ammonium acetate-acetonitrile system), the maximal absorption wavelengths for Din and its related compounds were found to be 245 to 249 nm (band II) and a discernible shoulder was found at 303 nm (band I). On the other hand, Gin and its related compounds had maximal absorption wavelengths at 259 to 260 nm (band II) and a shoulder at 327 nm (band I). Gin and its related compounds with a shoulder at 327 nm possessed a hydroxyl group at the 5-position of the isoflavone skeleton.

Figure 6 (blank) shows a typical HPLC profile of human plasma without spiking of the various isoflavones. Figure 6 (spiked 1 and spiked 2) illustrates a chromatogram of an extract of human plasma spiked with small amounts (spiked 1, low) and large amounts (spiked 2, high) of the isoflavone standard mixture. Comparison of chromatograms obtained from plasma samples (blank and spiked 1) in Fig. 6 indicated that several small peaks (peaks a–d) derived from the endogenous compounds were observed near at the retention time of D-4'-S, D-7-S, G-4'-S, and G-7-S. However, the retention times of these peaks differed from those of the respective authentic standards.

HPLC Profile of the Isoflavone Metabolites. Figure 7 showed HPLC profiles of the isoflavone metabolites in plasma samples collected from two healthy volunteers (subjects 1 and 2) 1 to 7 h after oral ingestion of 50 g of kinako. UV absorption spectra of the respective peaks at 1 to 7 h that had the maximum peak intensity on the HPLC chromatogram are also shown at the top of Fig. 7. Isoflavone glycosides and their metabolites were not detected in the plasma sample collected just before ingestion of 50 g of kinako. For subject 1, the peak heights of D-7-G, D-4'-G, G-7-G, G-4'-G, D-4'-S, D-7-S, G-7-S, D, and Gein rose relatively slowly on the HPLC chromatogram, whereas for subject 2 a peak was reached 4 h after oral administration, except for G-4'-S.

Standard curves were generated using the method of standard addition, following the extraction and HPLC analyses of the spiked plasma samples, and the various isoflavones were quantitated.
recovery of isoflavone metabolites from plasma were calculated by comparing the peak heights before and after the Oasis HLB extraction procedure. The recovery range was in the range of approximately 70.0 to 104.5% (except for 63.6% recovery of D-7-S). The calibration curve coefficients (r) for the metabolite standards used were 0.9274 to 0.9991. The amounts of the respective metabolites were calculated by subtracting the amounts of the respective interference compounds.

Figure 8 (subjects 1 and 2) showed plasma concentrations (nanomoles per millilitre) of the various isoflavone metabolites at 1, 2, 4, and 7 h after oral administration. The concentrations of glucuronides generally exceeded those of the sulfates at different time points after oral administration. There were also more Gein glucuronides than Dein glucuronides. Conjugation of the isoflavone skeleton at the 7-position was more prevalent, but G-4'-G levels exceeded G-7-G levels in subject 1.

Detection of the Isoflavone Glycosides Din and Gin in Plasma. Figure 9 (subjects 1 and 2) is an enlargement of the region corresponding to Din or Gin on the HPLC chromatograms in Fig. 7 (indicated by circles) for the blank and for the 1-h plasma. Because the Din and Gin peaks are too small on the HPLC chromatograms, partial purification of trace amounts of Din and Gin in the plasma extract was performed by the preparative HPLC method. Mass chromatograms and MS/MS spectra of the concentrated Din or Gin fractions are shown in Fig. 10 (subjects 1 and 2), respectively. The positive ion ESI mass chromatograms showed 1) the total ion chromatogram, 2) a proton adduct ion of Din or Gin by MS analysis, 3) a proton adduct ion of Dein or Gein by the MS/MS analysis of Din (precursor ion; [M + H]+, m/z 417) or Gin (precursor ion; [M + H]+, m/z 433), and 4) MS/MS spectra of Din and Gin.

Mass Spectrometric Analysis of Unknown Compounds (U1, U2, and U3). HPLC chromatograms of the unknown compounds (U1, U2, and U3) are shown in Fig. 7. The wavelengths of maximal absorption for U1, U2, and U3 were 258, 248, and 257 nm (band II) and discernible shoulders were found at 327, 302, and 326 nm, respectively (Fig. 11). The structure of U1 could not be identified by the mass spectrometric data. The mass chromatogram of U2 (Fig. 12A) showed 1) the total ion chromatogram, 2) an abundant ion peak at m/z 511 for a proton adduct ion ([M + H]+) of Dein-monoG-monoS, and 3) a peak at m/z 255 for a proton adduct ion of Dein. The mass chromatogram of U3 (Fig. 12B) showed ion peaks at m/z 527 for a proton adduct ion ([M + H]+) of Gein-monoG-monoS and at m/z 271 for a proton adduct ion of Gein. The structures of U2 (Dein-monoG-monoS) and U3 (Gein-monoG-monoS) were confirmed by positive ion LC-ESI-MS/MS and MS/MS analyses. MS/MS analyses of the m/z 511 or 527 precursor ion gave a base peak of m/z 335 or 351 ([M - glucuronic acid + H]+) [Fig. 12A, d) or Fig. 12B, d), and the MS/MS/MS analyses of the m/z 511 or 527 ion then produced a single peak at m/z 255 or 327 [Fig. 12A, e) or Fig. 12B, e]) of Gein-monoG-monoS and at m/z 511 or 527 ion then produced a single peak at m/z 255 or 327 [Fig. 12A, e) or Fig. 12B, e]) of Gein-monoG-monoS.

Discussion

Several studies have shown that the main metabolites of isoflavone are 7-glucuronides followed by 4'-glucuronides and that there are also small proportions of sulfate esters (Doerge et al., 2000; Clarke et al., 2003). The aim of this study was to directly measure the isoflavone metabolites simultaneously, without any sample treatment process affecting the ratios of glycosides and their aglycones and conjugates using an HPLC-UV-DAD method combined with solid-phase extraction. The 7-glucuronides, 7-sulfates, and 4'-sulfates of Dein or Gein were prepared for use as reference compounds for isoflavone metabolites. 4'-Glucuronides were isolated from human urine after oral administration of kinako. Comparison of 600 MHz 1H-NMR spectral data for two 7-glucuronides (D-7-G and G-7-G) and their correspond-
intake of kinako. G-7G-4’S, the major biliary metabolite in rat, has been isolated and identified from rat urine (Yasuda et al., 1996). Tsuchihashi et al. (2004) isolated and identified D-7G-4’S and G-7G-4’S from human urine. D-7G-4’S had a stimulatory effect on the growth of MCF-7 cells and bound to human estrogen receptors (Kinjo et al., 2004). Recently, D-7G-4’S was chemically synthesized by Soidinsalo and Wåhälä (2007). It is possible that U₃ and U₄ in plasma, as the main metabolites of soybean flour kinako, are binary metabolites such as D-7G-4’S and G-7G-4’S. Our semiquantified data for the metabolites plasma of human subjects who ingested kinako indicated that conjugation of the principal metabolites was at the 7-position, similar to data reported previously for urine (Clarke et al., 2002). However, in Gein glucuronides isolated from 1- to 7-h samples from subject 1, the principal glucuronide was 4-glucuronide.

Despite numerous reports of hydrolysis of flavonoid glycosides by intestinal microflora and brush border enzymes (Day et al., 2000; Nielsen and Williamson, 2007) and of glycoside uptake by an intestinal glucose transporter (Walgren et al., 2000), the question of whether or not flavonoid glycosides are absorbed from the intestine in human in vivo remains open. We have already reported that a flavanone glycoside, naringin, and a flavonol glycoside, rutin, were partly absorbed from human gut. In this study, intact Din and Gin were found in human plasma. Furthermore, Din and Gin were also found in 2- to 4-h urine, as confirmed by the positive ion ESI mass spectra that were similar to those for plasma (data not shown). Although the mechanism of intestinal absorption of isoflavone glycosides is unclear, the present study provides evidence that intact isoflavone glycosides can be directly absorbed in humans.

In the present study we generated HPLC profiles of the intact isoflavone metabolites obtained by HPLC-UV-DAD with solid-phase extraction. The plasma profiling of isoflavone glycosides (Din and Gin), aglycones, and their conjugated metabolites (glucuronides and

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**FIG. 12.** Mass chromatograms (a–c), and MS/MS (d) and MS/MS/MS (e) spectra of U₂ (A) and U₃ (B) in human plasma extracts.
TABLE 1

\[1H\]-NMR spectra of aglycones and glucuronides (\(s = 6\) in DSMO-d6)

<table>
<thead>
<tr>
<th>Proton</th>
<th>Dein</th>
<th>D-7-G</th>
<th>D-4'-G</th>
<th>Geni</th>
<th>G-7-G</th>
<th>G-4'-G</th>
</tr>
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<tr>
<td></td>
<td>(\delta)</td>
<td>Hz</td>
<td>(\delta)</td>
<td>Hz</td>
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<td>Hz</td>
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<td>2</td>
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<td>8.32 (s)</td>
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</tr>
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<td>7.96 (d)</td>
<td>8.75</td>
<td>8.05 (d)</td>
<td>8.72</td>
<td>7.97 (d)</td>
<td>8.74</td>
</tr>
<tr>
<td>6</td>
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<td>8.77, 2.17, 2.18</td>
<td>7.15 (d)</td>
<td>8.35</td>
<td>6.94 (d)</td>
<td>8.76</td>
</tr>
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</tr>
<tr>
<td>8</td>
<td>6.86 (d)</td>
<td>2.17</td>
<td>7.27 (s)</td>
<td></td>
<td>6.88 (s)</td>
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<td>2', 6'</td>
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<td>8.52</td>
<td>7.40 (d)</td>
<td>8.04</td>
<td>7.49 (d)</td>
<td>8.54</td>
</tr>
<tr>
<td>3', 5'</td>
<td>6.81 (d)</td>
<td>8.64</td>
<td>6.82 (d)</td>
<td>8.05</td>
<td>7.08 (d)</td>
<td>8.54</td>
</tr>
<tr>
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<td>9.57 (s)</td>
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<td>4.01 (s)</td>
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</tr>
</tbody>
</table>

s, singlet; d, doublet; dd, doublet of doublets.

References


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sulfates) should provide useful information for pharmacokinetic and pharmacodynamic investigations of isoflavones in humans. Structure elucidation of the unknown metabolites (U1, U2, and U3) is now in progress and should be reported in due course.

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