

Identification and quantification of daidzein-7-glucuronide-4'-sulfate, genistein-7-glucuronide-4'-sulfate and genistein-4',7-diglucuronide as major metabolites in human plasma after administration of *kinako*

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Abstract Much attention has been paid to the metabolism and disposition of isoflavones daidzein (Dein) and genistein (Gein) with regard to the prevention of several hormone-dependent diseases. Recent studies have reported that several conjugates as well as aglycones may be biologically active or may be activated within target cells. However, the disposition of Dein and Gein in plasma is still uncertain. This paper describes the identification and quantification of the highly polar metabolites, daidzein-7-glucuronide-4'-sulfate (D-7G-4'S), genistein-7-glucuronide-4'-sulfate (G-7G-4'S), daidzein-4',7-diglucuronide (D-4',7-diG), and genistein-4',7-diglucuronide (G-4',7-diG) in human plasma after dietary administration of *kinako* (baked soybean powder) to two healthy volunteers. The structure identification of these conjugated metabolites in plasma was performed in comparison to the LC-ESI-MS and 600 MHz ¹H-NMR spectral data of the chemically synthesized compounds. Furthermore, 16 isoflavone metabolites including D-7G-4'S, G-7G-4'S, D-4',7-diG, and G-4',7-diG in plasma were simultaneously measured by a high-performance liquid chromatography–UV-diode-array detector method combined with solid-phase extraction using an Oasis HLB cartridge. D-7G-4'S, G-7G-4'S and

G-4',7-diG were found to be major metabolites of Dein and Gein in plasma, while intact aglycones were detected to be only ca. 2% in both subjects. The findings suggest that the conjugated metabolites could be the key compounds responsible for pharmacological and medicinal properties of isoflavones.

Keywords Isoflavone · Sulfoglucuronide · Diglucuronide · High-performance liquid chromatography · Identification · Quantification

Introduction

Many epidemiological studies and in vitro experiments have indicated that isoflavones may play an important role in the prevention of several hormone-dependent diseases such as breast cancer, prostate cancer, ovarian cancer, and osteoporosis [1–4]. Isoflavonoid phytoestrogens are generally found as glycosides such as daidzin (Din) and genistin (Gin), and as their 6''-O-malonyl or 6''-O-acetic acid esters [5] in the plant (e.g., soybean and kudzu). Most glycosides are hydrolyzed to their aglycones and sugar moieties by endogenous microflora after administration [6]. The hydrolyzed aglycones are then subject to further metabolic processing [7, 8]. The absorbed aglycones and their non-conjugated metabolites are converted to either their glucuronide or sulfate conjugates, or both mixed conjugates as phase II metabolites. The conjugated metabolites are excreted in urine as mono- and diglucuronides, mono- and disulfates, and sulfoglucuronides at either or both 4' or 7 positions on the isoflavone ring [9]. Small amounts of the intact aglycones have been detected in plasma [10].

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The aglycones such as daidzein (Dein) and genistein (Gein), and equol formed from Dein in the gastrointestinal tract, are generally considered to be biologically active in humans. Recently, several investigators have reported that the conjugates may be biologically active or may be precursors of biologically active compounds for specific target cells. For example, a sulfoconjugate of Dein, daidzein-4',7-disulfate (D-4',7-diS), inhibits sterol sulfatase in hamster liver microsomes [11]. Dein and Gein glucuronides themselves have weak estrogenic activity and can activate human natural killer cells within the concentration range of 0.1 to 10 μM in vitro [12]. Therefore, it is important to clarify the disposition of the aglycones (Dein and Gein) and their conjugate metabolites in humans. However, the actual types of conjugates circulating in vivo and the position(s) of conjugation sites on the isoflavone skeleton have not yet been clarified.

In many studies, the conjugated compounds have generally been converted to free aglycones by selective enzymatic hydrolysis and then analyzed by high-performance liquid chromatography (HPLC) [13], gas chromatography–mass spectrometry (GC-MS) [14], and liquid chromatography–mass spectrometry (LC-MS) [15] because the standard conjugated compounds have not been available. These methods, however, only provide data on total glucuronides, sulfates, and free aglycones. No information is available regarding the type of conjugates or the conjugated position(s) on the isoflavone skeleton.

The objective of the present study was to clarify the disposition of aglycones and their conjugated metabolites in human plasma. We previously reported on a direct and simultaneous assay of isoflavone metabolites not requiring sample treatment processing that affected ratios of glycosides, their aglycones or conjugates, using an HPLC-UV-diode-array detector (DAD) combined with solid-phase extraction (SPE) [16]. We also identified plasma monoglu-

curonides and monosulfates at either the 4' or 7 position of Dein and Gein after administration of *kinako* (baked soybean powder) to two healthy volunteers. However, the positional elucidation of highly polar sulfoglucuronides of Dein and Gein could not be determined.

In the present study, the sites for sulfation and glucuronidation at the 4' and 7 positions of Dein and Gein were first identified in plasma by using the synthesized conjugates; daidzein-7-glucuronide-4'-sulfate (D-7G-4'S), genistein-7-glucuronide-4'-sulfate (G-7G-4'S), daidzein-4',7-diglucuronide (D-4',7-diG) and genistein-4',7-diglucuronide (G-4',7-diG). We then applied the previously developed method to the evaluation of plasma levels of 16 Dein and Gein metabolites, focusing on highly polar sulfoglucuronides and diglucuronides (Fig. 1), after dietary administration of *kinako*.

Materials and methods

Chemicals

Daidzein (7-hydroxy-3-(4-hydroxyphenyl)-4*H*-1-benzopyran-4-one; 4', 7-dihydroxyisoflavone) was purchased from LC Laboratories (Woburn, MA, USA). Genistein (5,7-dihydroxy-3-(4-hydroxyphenyl)-4*H*-1-benzopyran-4-one; 4',5,7-trihydroxyisoflavone) and luteolin-3',7-di-*O*-glucoside were purchased from Extrasynthese (Genay, France). Daidzein-7-glucuronide (D-7-G) and genistein-7-glucuronide (G-7-G) were synthesized in our laboratory [16, 17]. Daidzein-4'-glucuronide (D-4'-G) and genistein-4'-glucuronide (G-4'-G) were isolated from human urine [16]. Daidzein-7-sulfate (D-7-S), daidzein-4'-sulfate (D-4'-S), D-4',7-diS, genistein-7-sulfate (G-7-S), genistein-4'-sulfate (G-4'-S), and genistein-4',7-disulfate (G-4',7-diS) were synthesized in our laboratory [18]. D-7G-4'S, G-7G-4'S, D-4',7-diG, and G-4',7-diG were synthesized in this study

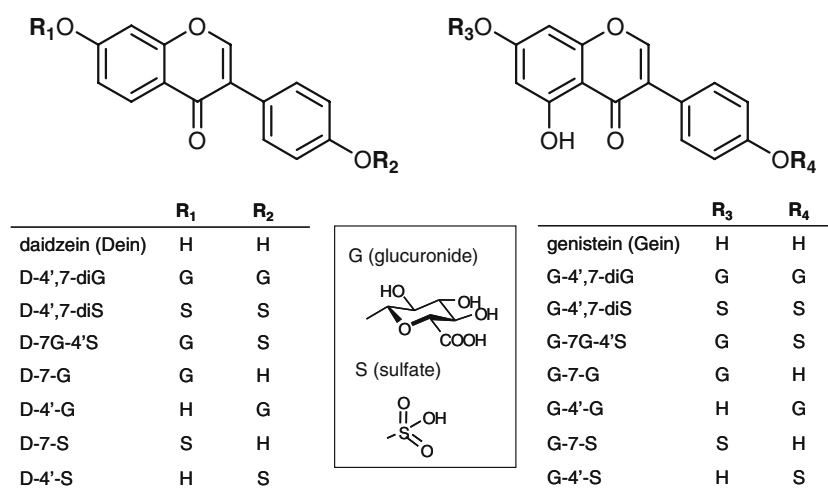


Fig. 1 Structures of isoflavones daidzein (*Dein*) and genistein (*Gein*) and their conjugated metabolites with glucuronic acid or sulfuric acid or both

(see following section). Phosphoric acid (99.999%) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Ammonia solution (28%) was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). *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. Stock standard solution of each compound were prepared by dissolving the appropriate amount of each compound in methanol followed by dilution with methanol–water (50:50, *v/v*) as working standard solutions except for Dein. Stock standard solution of Dein was prepared by dissolving the appropriate amount of Dein in ethanol followed by dilution with ethanol–water (50:50, *v/v*).

Syntheses

Synthesis of D-7G-4'S

D-7G-4'S was synthesized from 4'-*O*-benzyl daidzein according to the method of Soidinsalo and Wähälä [19], with the following minor modifications. 4'-*O*-Benzyl daidzein and 4'-*O*-benzyl daidzein 7-*O*-triacetylglucuronide methyl ester were purified by silica gel (particle size 75–150 μm) column chromatography using dichloromethane–ethyl acetate (80:20) and dichloromethane–ethyl acetate (90:10) as an eluant, respectively. The solution containing crude daidzein 7-*O*- β -D-glucuronide-4'-sulfate disodium salt was neutralized with 10% HCl and the solution was evaporated. The crude product was purified by use of a Preparative C18 (125 \AA , 55–105 μm ; Waters, Milford, MA, USA) column (120 ml of packing, 500 \times 22 mm i.d.) and water as an eluant. $^1\text{H-NMR}$ δ (600 MHz, $\text{DMSO-}d_6$): 8.45 (s, H-2), 8.07 (d, $J=8.89$ Hz, H-5), 7.16 (dd, $J=8.90, 2.24, 2.30$ Hz, H-6), 7.27 (d, $J=2.24$ Hz, H-8), 7.49 (d, $J=9.01$ Hz, H-2',6'), 7.22 (d, $J=9.02$ Hz, H-3',5'), 5.16 (s, H-1''), 5.48 (s, H-5''). ESI-MS: m/z 511 $[\text{M}+\text{H}]^+$, 431 $[\text{M}-\text{SO}_3+\text{H}]^+$, 335 $[\text{M-dehydroglucuronic acid}+\text{H}]^+$, 255 $[\text{M-dehydroglucuronic acid-SO}_3+\text{H}]^+$.

Synthesis of G-7G-4'S

G-7G-4'S was synthesized from 4'-*O*-benzylgenistein according to the procedure for D-7G-4'S. $^1\text{H-NMR}$ δ (600 MHz, $\text{DMSO-}d_6$): 8.51 (s, H-2), 12.87 (s, H-5), (broad s, H-6), (broad s, H-8), 7.42 (broad s, H-2',6'), 7.20 (d, $J=7.78$ Hz, H-3',5'), 5.00 (s, H-1''). ESI-MS: m/z 527 $[\text{M}+\text{H}]^+$, 447 $[\text{M-SO}_3+\text{H}]^+$, 351 $[\text{M-dehydroglucuronic acid}+\text{H}]^+$, 271 $[\text{M-dehydroglucuronic acid-SO}_3+\text{H}]^+$.

Synthesis of D-4',7-diG

D-4',7-diG was synthesized from daidzein-4',7-yl di-[methyl(2'',3'',4''-tri-*O*-acetyl- β -D-glucopyranosiduronic

acid)] according to the method of Needs and Williamson [17]. Daidzein-4',7-yl di-[methyl(2'',3'',4''-tri-*O*-acetyl- β -D-glucopyranosiduronic acid)] was synthesized from Dein and methyl (2,3,4-tri-*O*-acetyl- α -D-glucopyranosyl trichloroacetimidate) uronate prepared by the method of Jacquinet [20]. Daidzein-4',7-yl di-[methyl(2'',3'',4''-tri-*O*-acetyl- β -D-glucopyranosiduronic acid)] was purified on a PLC Silica gel 60 F₂₅₄ plate (1 mm; Merck, Darmstadt, Germany) using dichloromethane–methanol (95:5) as an eluant. D-4',7-diG was purified on a Preparative C18 column using water as an eluant in a similar manner to the purification of D-7G-4'S. ESI-MS: m/z 607 $[\text{M}+\text{H}]^+$, 431 $[\text{M-dehydroglucuronic acid}+\text{H}]^+$, 255 $[\text{M-2}\times\text{dehydroglucuronic acid}+\text{H}]^+$.

Synthesis of G-4',7-diG

G-4',7-diG was synthesized from genistein-4',7-yl di-[methyl(2'',3'',4''-tri-*O*-acetyl- β -D-glucopyranosiduronic acid)] according to the procedure for D-4',7-diG. $^1\text{H-NMR}$ δ (600 MHz, $\text{DMSO-}d_6$): 8.49 (s, H-2), 6.73 (d, $J=1.99$ Hz, H-6), 6.48 (d, $J=1.93$ Hz, H-8), 7.52 (d, $J=8.64$ Hz, H-2',6'), 7.09 (d, $J=8.75$ Hz, H-3',5'). ESI-MS: m/z 623 $[\text{M}+\text{H}]^+$, 447 $[\text{M-dehydroglucuronic acid}+\text{H}]^+$, 271 $[\text{M-2}\times\text{dehydroglucuronic acid}+\text{H}]^+$.

Sample collection

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 [a 51-year-old male (subject 1) and a 26-year-old female (subject 2)] were given a 50-g oral dose 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's 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 Na₂EDTA just before and at 1, 2, 4, 6, 8, 10, 12, 14, 16, 20, and 24 h after administration and were immediately centrifuged (2,500 rpm, 10 min). The plasma samples were stored at -20 °C until analysis.

Sample extraction

Dein, Gein, and their conjugated metabolites in plasma (1 ml) were extracted as described previously [21]. Luteolin-3',7-di-*O*-glucoside was used as an internal standard. SPE was performed on an Oasis HLB cartridge (3 cm^3 , 60 mg of packing; Waters, Milford, MA, USA) with a recovery of >ca. 80%.

Identification

The identification of D-7G-4'S, G-7G-4'S, D-4',7-diG, and G-4',7-diG in human plasma was performed by LC-ESI-MS analyses by comparison with the chemically synthesized metabolites. For detection of these four metabolites in human plasma by LC-ESI-MS, plasma samples (3 ml) were collected at 6 h after oral administration of 50 g of *kinako* to subject 1 and extracted according to the method described above. The structures of chemically synthesized D-7G-4'S, G-7G-4'S, and G-4',7-diG were confirmed by LC-ESI-MS and ¹H-NMR analyses. The structure of chemically synthesized D-4',7-diG was confirmed by LC-ESI-MS analysis.

LC-ESI-MS

LC-ESI-MS, MS/MS, and MS/MS/MS analyses were performed using a LCQ Fleet ion trap mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) equipped with a U-HPLC Accela pump, U-HPLC Accela autosampler, and U-HPLC Accela PDA detector. Positive-ion electrospray mass spectrometric analysis was carried out under the following conditions; capillary temperature 350 °C, capillary voltage 30 V, tube lens voltage 105 V, sheath gas 60 arbitrary units (AU) and auxiliary gas 20 AU. Data analyses were performed under Xcalibur, a software system (Thermo Fisher Scientific, Waltham, MA, USA).

Chromatographic separation was performed on a Cadenza CD-C18 column (100×4.6 mm i.d., particle size 3 μm; Imtakt, Kyoto, Japan) and a guard cartridge (5×2.0 mm i. d.) of the same material. Samples were eluted using a solvent system comprising 10 mM ammonium acetate solution (solvent A) and acetonitrile mixed using a linear gradient, held at 96.0% solvent A for 1.5 min and then decreasing linearly to 80.0% solvent A at 20 min. The flow rate was 0.5 ml/min at room temperature. The UV detection wavelength was set at 250 nm.

¹H-NMR analysis

NMR spectra were recorded on an NMR AV-600 spectrometer (Bruker, Rheinstetten, Germany; 600 MHz in the dimethyl sulfoxide-*d*₆ solutions). Chemical shifts are given in δ values (in parts per million) downfield from tetramethylsilane.

Quantification

For the simultaneous quantification of Dein, Gein, and their conjugated metabolites, HPLC-UV-DAD analyses were performed on a Nanospace SI-2 liquid chromatograph system (Shiseido, Tokyo, Japan) under conditions

reported previously [21]. The HPLC assay was performed on a Hydrosphere C18 column (100×4.6 mm i.d., particle size 3 μm; YMC Co. Ltd., Kyoto, Japan). The mobile phase consisted of a mixture of 10 mM ammonium acetate solution and acetonitrile run under gradient mode at a flow rate of 1.5 ml/min. The UV detection wavelength was set at 250 nm. For UV spectral analysis, the diode-array detection wavelength was set at 220 to 360 nm. All HPLC analyses were performed at 45 °C. Each calibration for the quantification of 16 metabolites gave a linear signal ($r > 0.997$) over a concentration range of 5–5,000 ng/ml. The lower limits of quantification of these metabolites were 21.1–23.4 ng/ml and the lower limits of detection were 7.9–9.4 ng/ml.

Results and discussion

Reference compounds

In our previous study [16], we detected sulfoglucuronides of Dein and Gein. However, positional elucidation of the highly polar sulfoglucuronides of Dein and Gein was not achieved. We also detected another metabolite with UV spectrum similar to that of Gein. Furthermore, the question has been raised of whether the highly polar diglucuronides of Dein and Gein (analogous to the sulfoglucuronides) actually exist or not in human plasma. In the present study, authentic D-7G-4'S, G-7G-4'S, D-4',7-diG, and G-4',7-diG were synthesized. The structures of these compounds except for D-4',7-diG were confirmed by LC-ESI-MS and 600 MHz ¹H-NMR. The structure of D-4',7-diG with only two positions as the conjugated sites was confirmed by the LC-ESI-MS.

Figures 2A and B show MS data of chemically synthesized D-7G-4'S and G-7G-4'S. MS/MS analyses of the m/z 511 or 527 precursor ion gave a base peak of m/z 335 or 351 ([M-dehydroglucuronic acid +H]⁺), and the MS/MS/MS analyses of the m/z 511 or 527 ion then produced a single peak at m/z 255 or 271 ([M-dehydroglucuronic acid-SO₃+H]⁺). Figures 2C and D also show MS data of D-4',7-diG (C) and G-4',7-diG (D). By full mass analyses, parent peaks ([M+H]⁺) of D-4',7-diG or G-4',7-diG were observed at m/z 607 or 623 [Figs. 2C(a) and D(a)]. MS/MS analyses of the m/z 607 or 623 precursor ion gave a base peak at m/z 431 or 447 ([M-dehydroglucuronic acid +H]⁺) [Figs. 2C(b) and D(b)], and the MS/MS/MS analyses of the m/z 607 or 623 ion then produced a single peak at m/z 255 or 271 ([M-2×dehydroglucuronic acid +H]⁺) [Figs. 2C(c) and D(c)].

The 600 MHz ¹H-NMR spectral data for D-7G-4'S, G-7G-4'S and G-4',7-diG are shown in Table 1. Comparison of ¹H-NMR spectral data for D-7G-4'S, G-7G-4'S and G-

Fig. 2 MS, MS/MS, and MS/MS/MS spectra of chemically synthesized metabolites of Dein and Gein; D-7G-4'S (A), G-7G-4'S (B), D-4',7-diG (C), and G-4',7-diG (D)

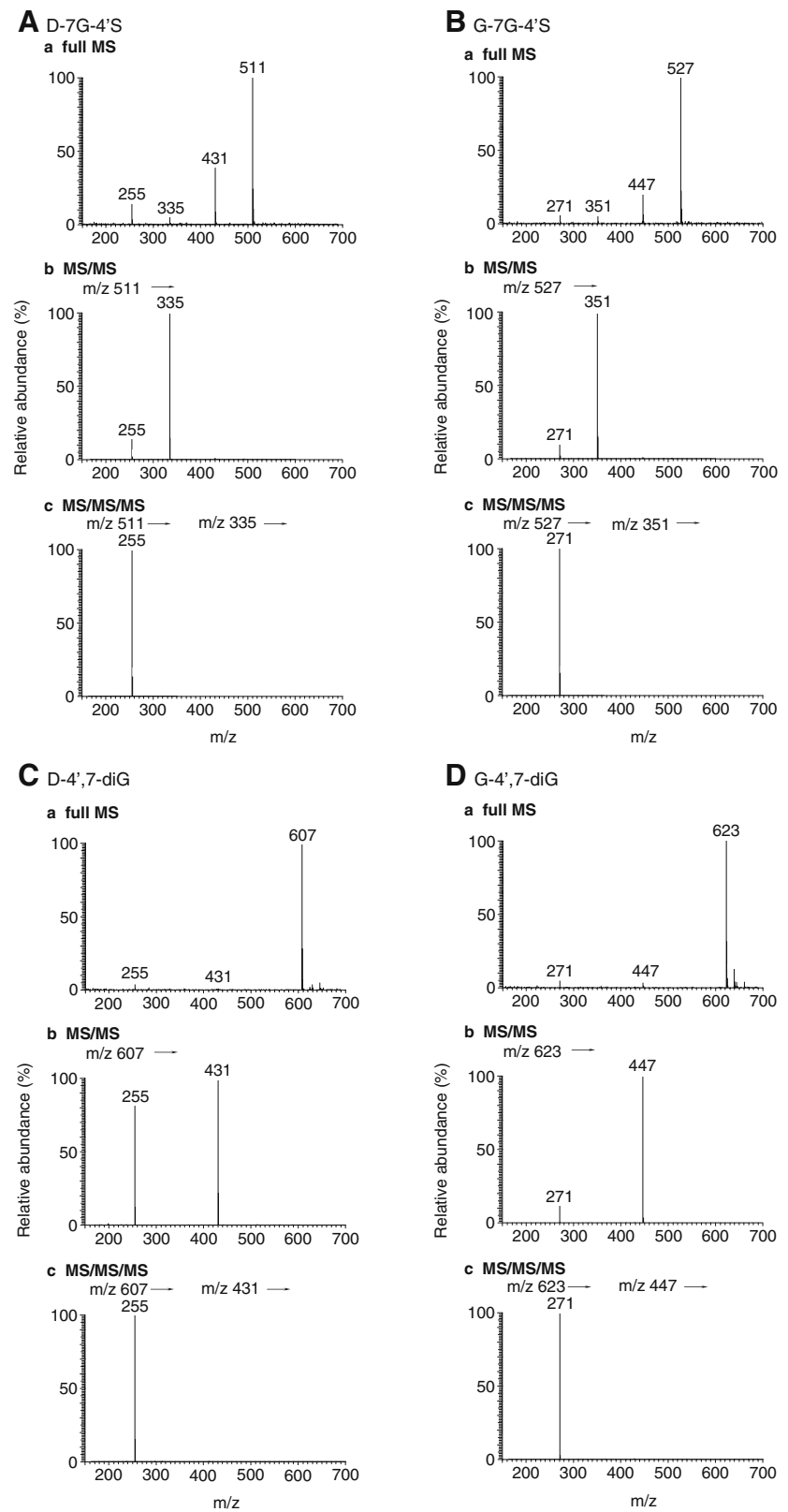


Table 1 $^1\text{H-NMR}$ spectra of aglycones and their metabolites (δ in $\text{DMSO-}d_6$)

Proton	Dein ^a		D-7G-4'S		Gein ^a		G-7G-4'S		G-4',7-diG	
	δ /ppm	<i>J</i> /Hz	δ /ppm	<i>J</i> /Hz	δ /ppm	<i>J</i> /Hz	δ /ppm	<i>J</i> /Hz	δ /ppm	<i>J</i> /Hz
2	8.28 (s)		8.45 (s)		8.32 (s)		8.51 (s)		8.49 (s)	
5	7.96 (d)	8.75	8.07 (d)	8.89	12.96 (s)		12.87 (s)			
6	6.93 (dd)	8.77, 2.17, 2.18	7.16 (dd)	8.90, 2.24, 2.30	6.22 (d)	2.04			6.73 (d)	1.99
7	–		–		10.89 (s)		–			
8	6.86 (d)	2.17	7.27 (d)	2.24	6.38 (d)	2.06			6.48 (d)	1.93
2',6'	7.38 (d)	8.52	7.49 (d)	9.01	7.37 (d)	8.57	7.42 (broad s)		7.52 (d)	8.64
3',5'	6.81 (d)	8.56	7.22 (d)	9.02	6.82 (d)	8.56	7.20 (d)	7.78	7.09 (d)	8.75
4'	–				9.60 (s)					
1''	–		5.16 (s)		–		5.00 (s)		–	
5''	–		5.48 (s)		–				–	

s singlet, *d* doublet, *dd* doublet of doublets

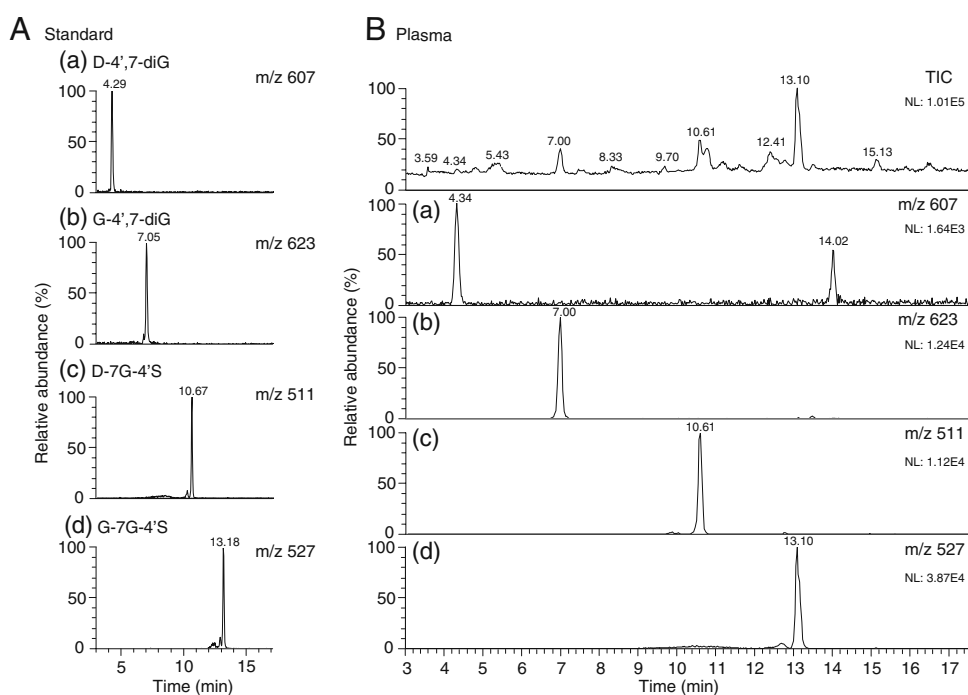
^a Hosoda et al. [16]

4',7-diG with those of the corresponding aglycones indicate a downfield shift of C-6 proton (0.23 ppm for D-7G-4'S and 0.51 ppm for G-4',7-diG) and C-8 proton signals (0.41 ppm for D-7G-4'S and 0.10 ppm for G-4',7-diG) due to the glucuronyl group attached to the C-7 hydroxyl group. Furthermore, downfield shifts of C-2',6' proton signals (0.11 ppm for D-7G-4'S, 0.05 ppm for G-7G-4'S and 0.15 ppm for G-4',7-diG) and C-3',5' proton signals (0.41 ppm for D-7G-4'S, 0.38 ppm for G-7G-4'S and 0.27 ppm for G-4',7-diG) due to the glucuronyl group or sulfuric group attached to the C-4' hydroxyl group were observed. The $^1\text{H-NMR}$ spectral data for these sulfoglu-

curonides were consistent with the data reported by Soidinsalo and Wähälä [19] and Yasuda et al. [22]. The chemical shifts of C-2',6', C-3',5', C-6, and C-8 proton signals in the $^1\text{H-NMR}$ spectrum of G-4',7-diG were nearly consistent with those at C-2',6' and C-3',5' proton signals of G-4'-G and at C-6 and C-8 proton signals of G-7-G reported in our previous study [16], indicating the presence of two glucuronyl group at the C-4' and C-7 positions.

The purities of chemically synthesized D-7G-4'S, G-7G-4'S, D-4',7-diG, and G-4',7-diG were determined to be more than 98.5% by HPLC.

Fig. 3 LC-ESI-MS chromatograms of (A) chemically synthesized metabolites of Dein and Gein; D-4',7-diG, G-4',7-diG, D-7G-4'S, and G-7G-4'S, and (B) plasma extracts



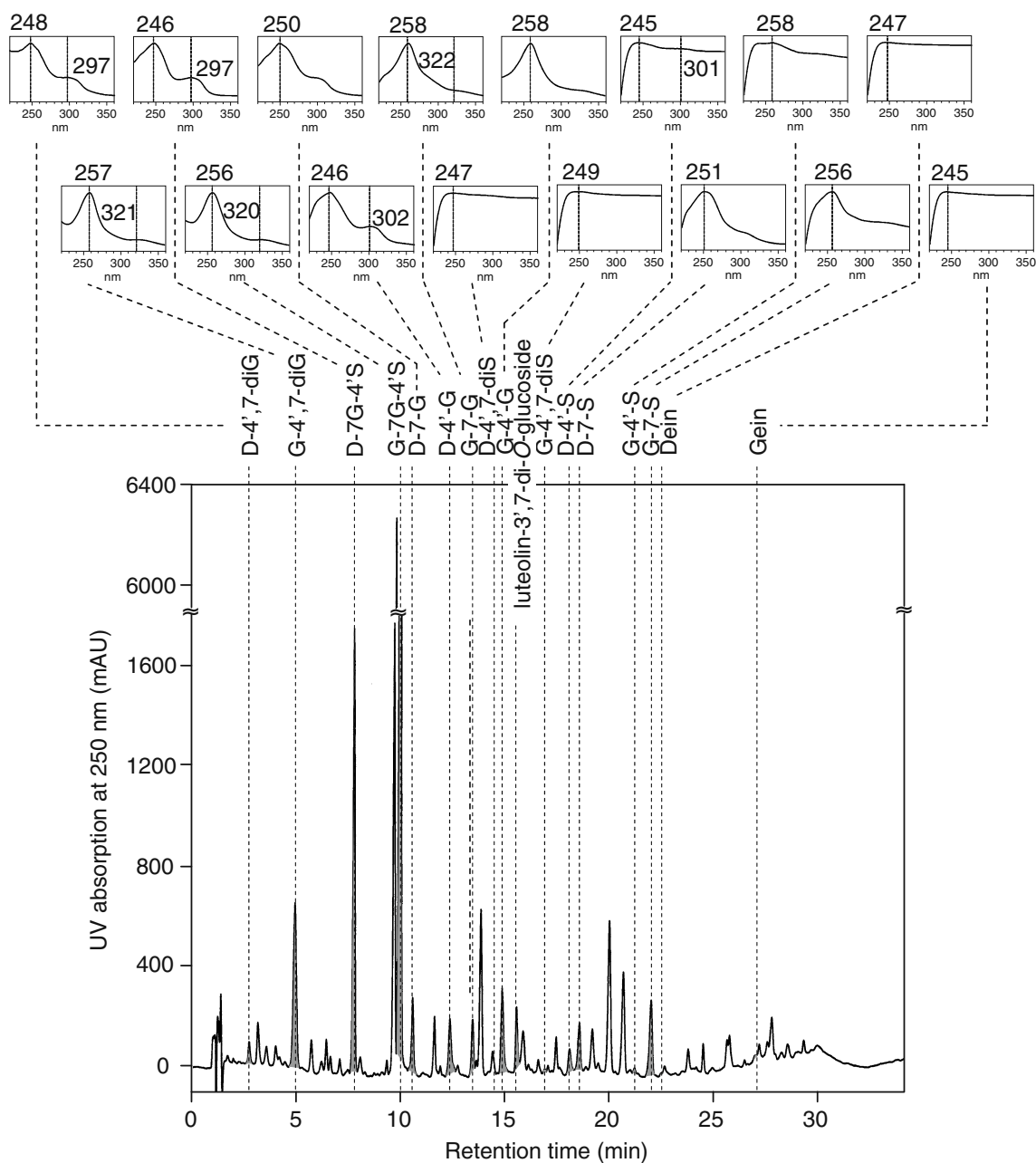


Fig. 4 HPLC chromatogram and UV spectra of the plasma extract obtained at 8 h after administration to subject 1

Identification of D-7G-4'S, G-7G-4'S, D-4',7-diG, and G-4',7-diG in human plasma

D-7G-4'S, G-7G-4'S, D-4',7-diG, and G-4',7-diG in plasma were identified by comparing with mass spectral data for the reference compounds. Figure 3A shows the extracted ion chromatograms of the reference compounds. The total ion chromatogram of the plasma extract is shown at the top of Fig. 3B. Figure 3B also shows mass chromatograms at m/z 607 (a proton adduct ion of D-

4',7-diG; (a)), m/z 623 (a proton adduct ion of G-4',7-diG; (b)), m/z 511 (a proton adduct ion of D-7G-4'S; (c)), and m/z 527 (a proton adduct ion of G-7G-4'S; (d)). The mass spectral analyses of the peaks which eluted at 4.34 min or 7.00 min on the mass chromatograms gave a parent peak of m/z 607 or 623 ($[M+H]^+$), respectively. The chromatographic behavior and the MS, MS/MS, and MS/MS/MS spectra of the two diglucuronides in human plasma coincided with the chemically synthesized diglucuronides (D-4',7-diG and G-4',7-diG).

Fig. 5 Time courses of plasma concentrations of isoflavone metabolites

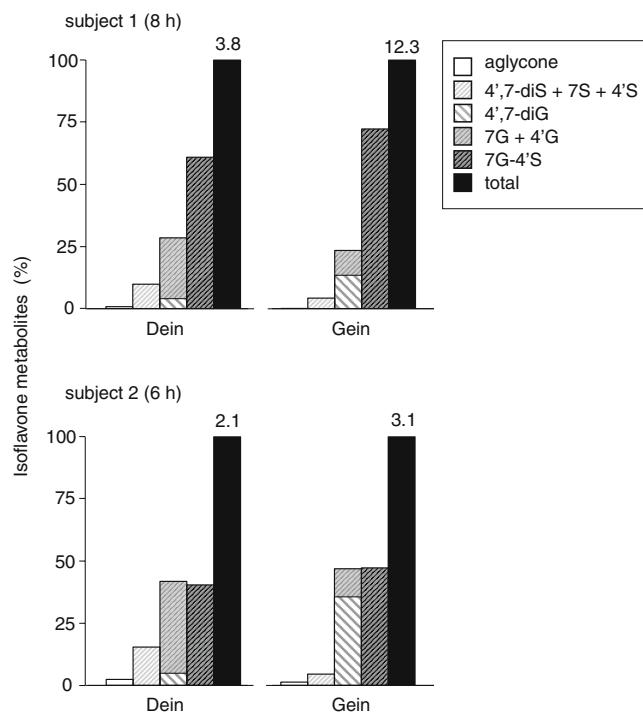
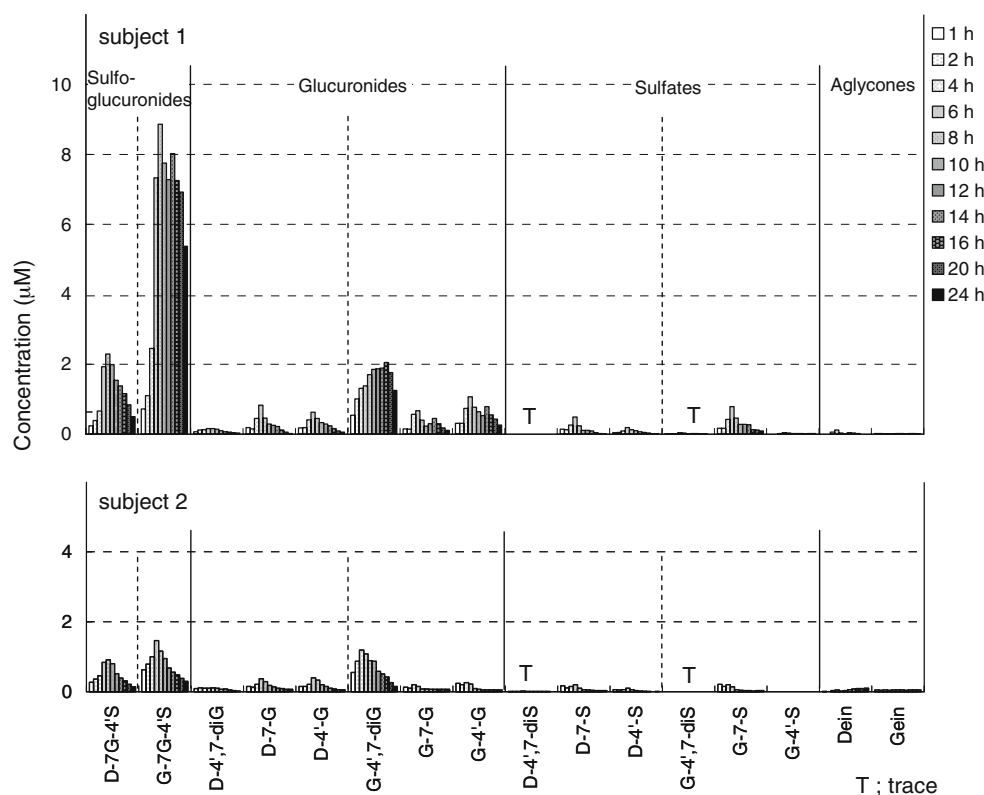


Fig. 6 Percent contribution of each metabolite to the total concentration of isoflavone metabolites in plasma at 8 h (subject 1) and 6 h (subject 2). The values shown in the total bars (black bars) are the total concentration of isoflavone metabolites (micromolar in plasma) at the indicated time

The HPLC retention times and the MS spectral data of the two sulfolglucuronides in human plasma (10.61 min; m/z 511 and 13.10 min; m/z 527) coincided with those of the two authentic sulfolglucuronides (D-7G-4'S and G-7G-4'S) and our previously reported data (Dein-sulfolglucuronide and Gein-sulfolglucuronide) [16].

Quantification of isoflavone metabolites in human plasma

Quantification of isoflavone metabolites in plasma after oral administration of *kinako* to two healthy volunteers was carried out by HPLC-UV-DAD analysis. As an example, Fig. 4 shows the HPLC chromatogram and UV spectra at 8 h after oral administration to subject 1. The spectral data of 12 isoflavone metabolites coincided with those of the spectral library [21]. However, we failed to evaluate the spectral data of D-4',7-diS, G-4',7-diS, Dein, and Gein, because of low concentration (< LOD) in plasma. Figure 5 (subjects 1 and 2) shows plasma concentrations (μM) of the various isoflavone metabolites at 1, 2, 4, 6, 8, 10, 12, 14, 16, 20, and 24 h after oral administration. The concentrations of D-7G-4'S, G-7G-4'S and G-4',7-diG exceeded those of the other metabolites in subjects 1 and 2. In particular, the concentration of G-7G-4'S in subject 1 was very high at each time point. On the other hand, only trace amounts of D-4',7-diS and G-4',7-diS were found in both subjects.

The peak concentrations of the total isoflavone metabolites determined in plasma were observed at 8 h (subject 1) and 6 h (subject 2). Figure 6 shows the percentage of each isoflavone metabolite relative to total metabolites in plasma at each time point. The values shown in the total bars (black bars) are the total concentration of isoflavone metabolites (micromolar in plasma) at the indicated time. The major metabolites of Dein and Gein at 8 h (subject 1) or 6 h (subject 2) were D-7G-4'S (61%; 2.3 μ M in subject 1 and 40%; 0.8 μ M in subject 2), G-7G-4'S (72%; 8.9 μ M in subject 1 and 47%; 1.5 μ M in subject 2) and G-4',7-diG (14%; 1.7 μ M in subject 1 and 35%; 1.1 μ M in subject 2), whereas Shelnutt et al. [23] reported that approximately 30% of the total amount of Dein or Gein metabolites comprised sulfoglucuronides at 4 h after intake of a soy beverage. However, the conjugation positions of these metabolites were not reported. It has been suggested that the major metabolites of isoflavones are 7- or 4'-monoglucuronides, accompanied by small proportions of sulfates [10, 23, 24]. Intact aglycones also were reported to be low in plasma, e.g., <1% at 4 h after administration of soy beverage [25] and negligible for calculation purposes [23]. In our study, in subject 1, we found that the total concentration of 7- or 4'- monoglucuronides was only 24% of total Dein metabolites and 10% of total Gein metabolites at 8 h plasma, respectively. Similarly, in subject 2, the total concentration of 7- or 4'- monoglucuronides was 37% and 12% of the total at 6 h plasma, respectively. Intact aglycones detected were ca. 2% in both subjects, consistent with other reports.

In human urine, monoglucuronides are the most abundant conjugates, whereas sulfoglucuronides are minor species [9, 26, 27]. In the present study, D-7G-4'S, G-7G-4'S, and G-4',7-diG were found to be major Dein and Gein metabolites by profiling the isoflavone conjugates in plasma. Renal excretion of sulfoglucuronides may involve further metabolic processing including desulfation and deglucuronidation in vivo. Metabolism of isoflavones is expected to effect isoflavone activity. It will be required to determine precisely the intact 16 isoflavone metabolites in human urine after oral administration of *kinako* for pharmacokinetic investigation of isoflavones. The application of the developed method to quantitative determination of potentially pharmacologically active metabolites in plasma and urine is suitable for a better understanding of the metabolic pathway of isoflavones.

Conclusion

In the present study, 16 isoflavone metabolites in plasma were simultaneously measured by an HPLC-UV-DAD method combined with SPE. Conjugation at both 4' and 7 positions on the isoflavone ring with glucuronic or sulfuric acid or both (D-7G-4'S, G-7G-4'S, and G-4',7-diG) was

found to be the major metabolic transformations of Dein and Gein in plasma after oral administration of *kinako* to two healthy volunteers, while intact aglycones were detected to be only ca. 2% in both subjects. These findings indicate that the conjugated metabolites could be the key compounds responsible for pharmacological and medicinal properties of isoflavones. The sites for sulfation and glucuronidation at the 4' and 7 positions of Dein and Gein in plasma were identified, based on the LC-ESI-MS and 600 MHz ¹H-NMR spectral data for chemically synthesized authentic compounds (D-7G-4'S, G-7G-4'S, D-4',7-diG, and G-4',7-diG). Taking into consideration the previous reports that the monoglucuronides of Dein and Gein were major urinary metabolites, and that excretion of sulfoglucuronides was minor, the present plasma profiling of 16 metabolites of Dein and Gein suggest possible desulfation or deglucuronidation of the conjugated metabolites (D-7G-4'S, G-7G-4'S, and G-4',7-diG) during renal excretion. Further studies will be required to determine precisely the intact isoflavone sulfate and glucuronide conjugated metabolites in human urine after oral administration of *kinako*.

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