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Simultaneous determination of glucuronic acid and sulfuric acid conjugated metabolites of daidzein and genistein in human plasma by high-performance liquid chromatography

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ABSTRACT

Isoflavone aglycones daidzein (Dein) and genistein (Gein) are mainly present as glucuronides and sulfates in human plasma, and small amounts of the intact aglycones are also detected. In the present study, we have developed a high-performance liquid chromatography (HPLC)-UV-diode-array detector (DAD) method for the determination of intact 16 metabolites of Dein and Gein in plasma, especially focusing on highly polar conjugated metabolites at both 4′ and 7 positions on the isoflavone ring with glucuronic acid and/or sulfuric acid (7-glucuronide-4′-sulfates and 4′,7-diglucuronides). Luteolin-3′,7-di-*O*-glucoside was used as an internal standard. Solid-phase extraction was performed on an Oasis® HLB cartridge (60 mg, 3 cm^3) with a recovery of >ca. 80%. The HPLC assay was performed on a Hydrosphere C18 column (100 mm × 4.6 mm I.D., particle size 3 μm). 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 220–360 nm. All HPLC analyses were performed at 45 °C. Each calibration for the determination of 16 metabolites gave a linear signal ($r > 0.997$) over a concentration range of 5–5000 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. This method was used in a preliminary experiment to determine the plasma concentration of intact 16 metabolites after oral administration of *kinako* (baked soybean powder) to a healthy volunteer. The present HPLC-UV-DAD method should be useful for the metabolic and pharmacokinetic investigations of isoflavones in humans.

1. Introduction

Isoflavonoid phytoestrogens occur naturally in the plant kingdom, particular in soybean as a rich source. These compounds generally are present as glycosides in the common human diet [1]. Flavonoid glycosides are known to be biotransformed to the corresponding aglycones and sugar moieties by enteral microorganisms [2]. The absorbed isoflavones are then circulated in body and excreted as glucuronide, sulfate and sulfoglucuronide conjugates in human urine [3,4]. It is known that isoflavonoid phytoestrogens could play a role in a protecting effect against the several hormone-dependent diseases such as breast cancer, prostate cancer and osteoporosis [5–8]. The aglycones such as daidzein (Dein) and genistein (Gein), and their unconjugated metabolites have been generally considered as biologically active compounds [7]. Recently, several investigators reported that the conjugates such as sulfoconjugate of Dein, Dein-4′,7-disulfate (D-4′,7-diS), and Gein glucuronides may either have biological activity themselves or serve as the precursors of biologically active compounds at or within target cells [9,10]. Pugazhendhi et al. [11] reported the enhancing or the reducing effects of Dein, Gein, and equol sulfates on the oestrogen agonist activity in MCF-7 human breast cancer cells, and demonstrated that the biological effects depend upon not only the isoflavone skeletons but also the positioning sites of sulfation. Therefore, the information regarding the actual type and concentration of conjugates circulating in the body is important in estimation of the biological effects of isoflavones.

The plasma or urine concentrations of the conjugated compounds are usually estimated by measuring the free aglycones obtained after the selective enzymatic hydrolysis, followed by high-performance liquid chromatography (HPLC) [12–18], gas chromatography–mass spectrometry (GC–MS) [19–24] and liquid chromatography (LC)–MS methods [4,25–32]. Adlercreutz et al. [33] quantified mono- and disulfates separately from mono- and diglucuronides by using ion-exchange solid-phase extraction (SPE).

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The conjugated metabolites were hydrolyzed and then quantified by GC–MS. The estimation of the enzymatically or chemically released aglycones do not provide the structural information on the conjugated flavone metabolites circulating in plasma, requiring the direct and simultaneous determination of all intact conjugates.

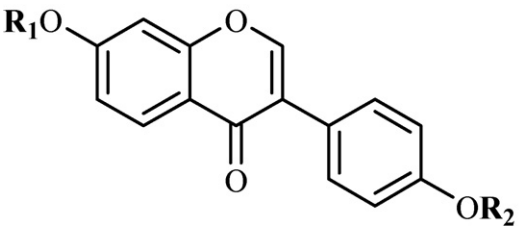
We previously reported the HPLC chromatographic behaviors of the authentic standards of intact isoflavone metabolites, Dein, Gein, Dein-7-glucuronide (D-7-G), D-4′-G, Gein-7-glucuronides (G-7-G), G-4′-G, Dein-7-sufates (D-7-S), D-4′-S, Gein-7-sulfate (G-7-S) and G-4′-S by using HPLC-UV-diode-array detector (DAD) [34]. We also investigated the profile of these intact isoflavone metabolites in human plasma after intake of *kinako*. However, highly polar conjugates such as 4′,7-sulfoglucuronides and 4′,7-diglucuronides of Dein and Gein in plasma were not yet analyzed, because of difficulty obtaining standard compounds available for the analysis.

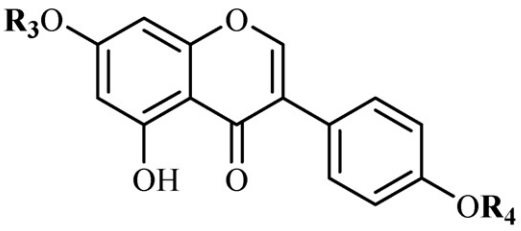
In the present study, we developed an HPLC-UV-DAD method for the simultaneous determination of the 16 isoflavone metabolites (Fig. 1) in plasma, especially focusing on 4′,7-sulfoglucuronides and 4′,7-diglucuronides of Dein and Gein (Dein-7-glucuronide-4′-sulfate, D-7G-4′S; Gein-7-glucuronide-4′-sulfate, G-7G-4′S; Dein-4′,7-diglucuronide, D-4′,7-diG; Gein-4′,7-diglucuronide, G-4′,7-diG).

2. Experimental

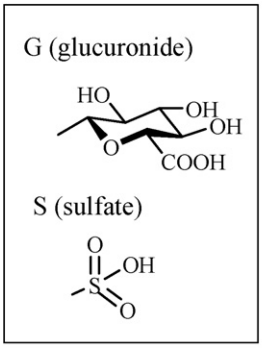
2.1. Chemicals and reagents

Daidzein (7-hydroxy-3-(4-hydroxyphenyl)-4*H*-1-benzopyran-4-one, 4′,7-dihydroxyisoflavone) was purchased from LC Laboratories (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 according to the method of Needs and Williamson [35] in our laboratory [34]. Daidzein-4′-glucuronide (D-4′-G) and genistein-4′-glucuronide (G-4′-G) were isolated from human urine [34]. Daidzein-7-sulfate (D-7-S), daidzein-4′-sulfate (D-4′-S), daidzein-4′,7-disulfate (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 [36]. Daidzein-7-glucuronide-4′-sulfate (D-7G-4′S) and genistein-7-glucuronide-4′-sulfate (G-7G-4′S) were synthesized according to the method of Soidinsalo and Wähälä [37]. Daidzein-4′,7-diglucuronide (D-4′,7-diG) and genistein-4′,7-diglucuronide (G-4′,7-diG) were synthesized according to the method of Needs

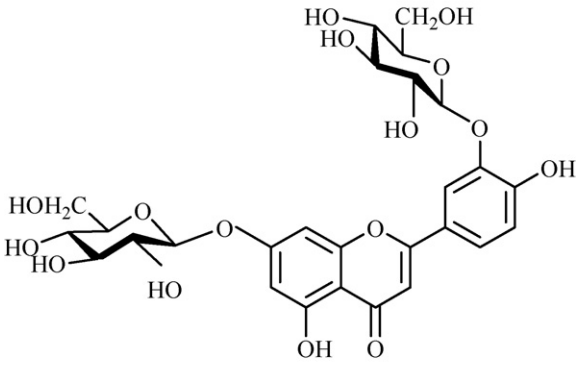




	R_1	R_2
daidzein (Dein)	H	H
D-4',7-diG	G	G
D-4',7-diS	S	S
D-7G-4'S	G	S
D-7-G	G	H
D-4'-G	H	G
D-7-S	S	H
D-4'-S	H	S



	R_3	R_4
genistein (Gein)	H	H
G-4',7-diG	G	G
G-4',7-diS	S	S
G-7G-4'S	G	S
G-7-G	G	H
G-4'-G	H	G
G-7-S	S	H
G-4'-S	H	S



luteolin-3',7-di-*O*-glucoside

Fig. 1. Structures of the relevant isoflavones, their conjugated metabolites and luteolin-3′,7-di-*O*-glucoside.

and Williamson [35]. The structures of synthesized compounds were confirmed by LC–ESI-MS and/or ^{1}H NMR analyses (data not shown). The purities of these compounds were determined to be more than 98.5% by HPLC.

Stock solution of Dein was prepared by dissolving this compound in ethanol followed by dilution with ethanol-water (50:50, v/v) as working solution. Stock solutions of D-4′,7-diG, D-4′,7-diS, D-7G-4′S, D-7-G, D-4′-G, D-7-S, D-4′-S, G-4′,7-diG, G-4′,7-diS, G-7G-4′S, G-7-G, G-4′-G, G-7-S, G-4′-S, Gein and luteolin-3′,7-di-*O*-glucoside were prepared by dissolving these compounds in methanol followed by dilution with methanol-water (50:50, v/v) as working solutions.

Phosphoric acid (99.999%) was purchased from Aldrich Chemical Co. (WI, 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.

2.2. Sample preparation

Blood sample was collected from a medial cubital vein into evacuated tube containing Na_2EDTA and was immediately centrifuged (2500 rpm, 10 min). The plasma fraction was separated and luteolin-3′,7-di-*O*-glucoside (510.0 ng) was added as internal standard to plasma sample (1.0 ml). The plasma sample was diluted with 2.0 ml of 50 mM phosphoric acid solution (pH 2.0). An Oasis® HLB cartridge (3 cm^3, 60 mg of packing; Waters, MA, USA) was placed on a vacuum manifold and activated with 2.5 ml of methanol, followed by 2.5 ml of 50 mM phosphoric acid solution. The plasma sample was then loaded onto the cartridge at a flow rate of 0.2 ml/min. The cartridge was washed with 5.0 ml of methanol–50 mM phosphoric acid solution (20:80, v/v, pH 2.0). After purging with air, the cartridge was eluted with 2.5 ml of methanol–28% ammonia solution (95:5, v/v, pH 10.0) 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 HLC-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 were added with vortex mixing for 30 s. A 20-μl portion of the solution was subjected to HPLC.

Blank plasma was obtained from a healthy volunteer (53-year-old male) who avoided consumption of the phytoestrogen-containing foods for 1 week before the study.

2.3. HPLC apparatus and conditions

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 DAD (Thermo Fisher Scientific, MA, USA) 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 Hydrosphere C18 column (100 mm × 4.6 mm I.D., particle size 3 μm; YMC Co. Ltd., Kyoto, Japan) and a guard cartridge (23 mm × 4.0 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 95.3% solvent A for 1.5 min and then decreasing linearly to 68.0% solvent A at 27 min, increasing back to 95.3% solvent A at 34 min, and then held for 10 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 220–360 nm. All HPLC analyses were performed at 45 °C.

2.4. Recovery

The extraction recoveries of isoflavone metabolites from plasma were assessed at two concentration levels (low and high). The samples were prepared by adding appropriate amounts of the working solutions to blank plasma. After sample treatment by an SPE cartridge (Section 2.2) without internal standard, to the eluting solution an external standard compound (luteolin-3′,7-di-*O*-glucoside; 510.0 ng) was added. The mixed solution was evaporated and analyzed by HPLC. The extraction recoveries were calculated by comparing the peak height ratios (isoflavone metabolites to luteolin-3′,7-di-*O*-glucoside as external standard) of the HPLC chromatograms with those of reference compounds at two concentration levels as follows: Dein (33.8 and 269.0 ng/ml), Gein (39.4 and 314.1 ng/ml), D-4′,7-diG (32.0 and 256.2 ng/ml), G-4′,7-diG (34.3 and 274.5 ng/ml), D-4′,7-diS (11.3 and 90.3 ng/ml), G-4′,7-diS (20.6 and 162.8 ng/ml), D-7G-4′S (31.2 and 249.6 ng/ml), G-7G-4′S (31.0 and 248.0 ng/ml), D-7-G (33.1 and 262.8 ng/ml), D-4′-G (38.7 and 309.6 ng/ml), G-7-G (33.1 and 264.8 ng/ml), G-4′-G (42.3 and 338.4 ng/ml), D-7-S (11.7 and 94.4 ng/ml), D-4′-S (14.0 and 112.0 ng/ml), G-7-S (17.2 and 136.6 ng/ml), and G-4′-S (14.6 and 116.9 ng/ml).

2.5. Calibration

Seven point calibration curves were prepared in 1.0 ml of blank plasma by an internal standard method, in the following ranges: Dein (8.4, 16.8, 33.6, 67.3, 134.5, 269.0 and 538.0 ng), Gein (8.0, 16.1, 32.1, 64.2, 128.5, 257.0 and 513.9 ng), D-4′,7-diG (8.0, 16.0, 32.0, 64.1, 128.1, 256.2 and 512.4 ng), G-4′,7-diG (9.2, 22.9, 54.9, 137.3, 320.3, 823.5 and 2013.0 ng), D-4′,7-diS (4.0, 7.9, 15.8, 31.6, 63.2, 126.4 and 252.8 ng), G-4′,7-diS (4.3, 8.5, 17.0, 33.9, 67.8, 135.6 and 271.3 ng), D-7G-4′S (9.4, 21.8, 56.2, 140.4, 343.2, 873.6 and 2184.0 ng), G-7G-4′S (9.3, 26.4, 77.5, 217.0, 620.0, 1783.0 and 5115.0 ng), D-7-G (8.2, 16.4, 32.8, 65.7, 131.4, 262.8 and 525.5 ng), D-4′-G (8.1, 16.1, 32.3, 64.5, 129.0, 258.0 and 516.0 ng), G-7-G (8.3, 16.6, 33.1, 66.2, 132.4, 264.8 and 529.6 ng), G-4′-G (8.6, 17.2, 34.4, 68.7, 137.5, 275.0 and 549.9 ng), D-7-S (8.3, 16.5, 33.0, 66.1, 132.2, 264.3 and 528.6 ng), D-4′-S (7.9, 15.8, 31.5, 63.0, 126.0, 252.0 and 504.0 ng), G-7-S (8.5, 17.1, 34.1, 68.3, 136.6, 273.1 and 546.2 ng) and G-4′-S (8.2, 16.4, 32.9, 65.8, 131.5, 263.1 and 526.1 ng). The standard samples were prepared in duplicate. After determining the peak height ratios (isoflavone metabolites to luteolin-3′,7-di-*O*-glucoside) of the HPLC chromatograms, the calibration graphs were obtained by least-squares linear fitting of the peak height ratios versus the mixed mass ratios of the each isoflavone metabolite to the internal standard.

2.6. Accuracy and precision

Quality control solutions were prepared by adding working solutions containing appropriate amounts of isoflavone conjugates to blank plasma. Accuracy and precision were determined by assaying in duplicate six preparations of 1.0-ml aliquots of human blank plasma containing three different amounts (high, middle, and low amounts, Table 3) of isoflavone metabolites and a fixed amount (510.0 ng) of luteolin-3′,7-di-*O*-glucoside as the internal standard.

2.7. Stability

For examination of stability of 16 isoflavone metabolites in plasma at −20 °C, a healthy volunteer (53-year-old male) orally

received 50 g of *kinako* and plasma sample (6 ml) was collected at 4 h after administration. Half of the plasma sample (1 ml) ($n = 3$) was determined immediately after collection. The remainder of the plasma (1 ml) ($n = 3$) was determined after stored at −20 °C for 6 months.

2.8. Application

A healthy volunteer (53-year-old male) orally received 50 g of *kinako* (baked soybean powder) 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 malonyl and acetyl glycosides were not contained in *kinako powder*. The volunteer did not ingest soy-containing foods for 1 week before the administration and during the 24-h test. Plasma samples (3 ml each) were collected from a medial cubital vein into evacuated tubes containing Na_2EDTA just before and at 2, 4, 7 and 24 h after administration. This study was approved by Kyorin University, School of Health Sciences Human Subject Review Board. Written informed consent was obtained from the volunteer.

3. Results

3.1. HPLC chromatographic behavior

Structures of 16 isoflavone metabolites and luteolin-3′,7-di-*O*-glucoside as internal standard are shown in Fig. 1. An HPLC chromatogram and UV spectra for a standard mixture containing the 16 isoflavone metabolites are shown in Fig. 2. The metabolites were well separated from each other within 28 min on the HPLC chromatogram.

Fig. 3 (blank) shows a typical HPLC profile of human plasma without spiking the various isoflavone metabolites and the internal standard. Fig. 3 (spiked) illustrates a chromatogram of an extract of human plasma spiked with small amounts of the isoflavone standard mixture and an internal standard. There is no significant interference from endogenous components in the analysis of plasma isoflavone metabolites except for G-7G-4′S and D-4′-G.

3.2. Recovery

The extraction recoveries of isoflavone metabolites from plasma ranged between 76.6% and 109.4% (Table 1). The absolute recovery of internal standard (luteolin-3′,7-di-*O*-glucoside) from plasma was 97.0%.

3.3. Calibration

Calibration graphs were prepared by using 1.0-ml aliquots of the blank plasma spiked with different amounts of isoflavone metabolites and luteolin-3′,7-di-*O*-glucoside as internal standard. The peak height ratios were plotted against the mixed mass ratios of isoflavone metabolites to the internal standard. Linear calibration curves were obtained for each of the metabolites (Table 2). There are linear responses for the isoflavone metabolites with correlation coefficients ranging from 0.9976 to 0.9999 in plasma.

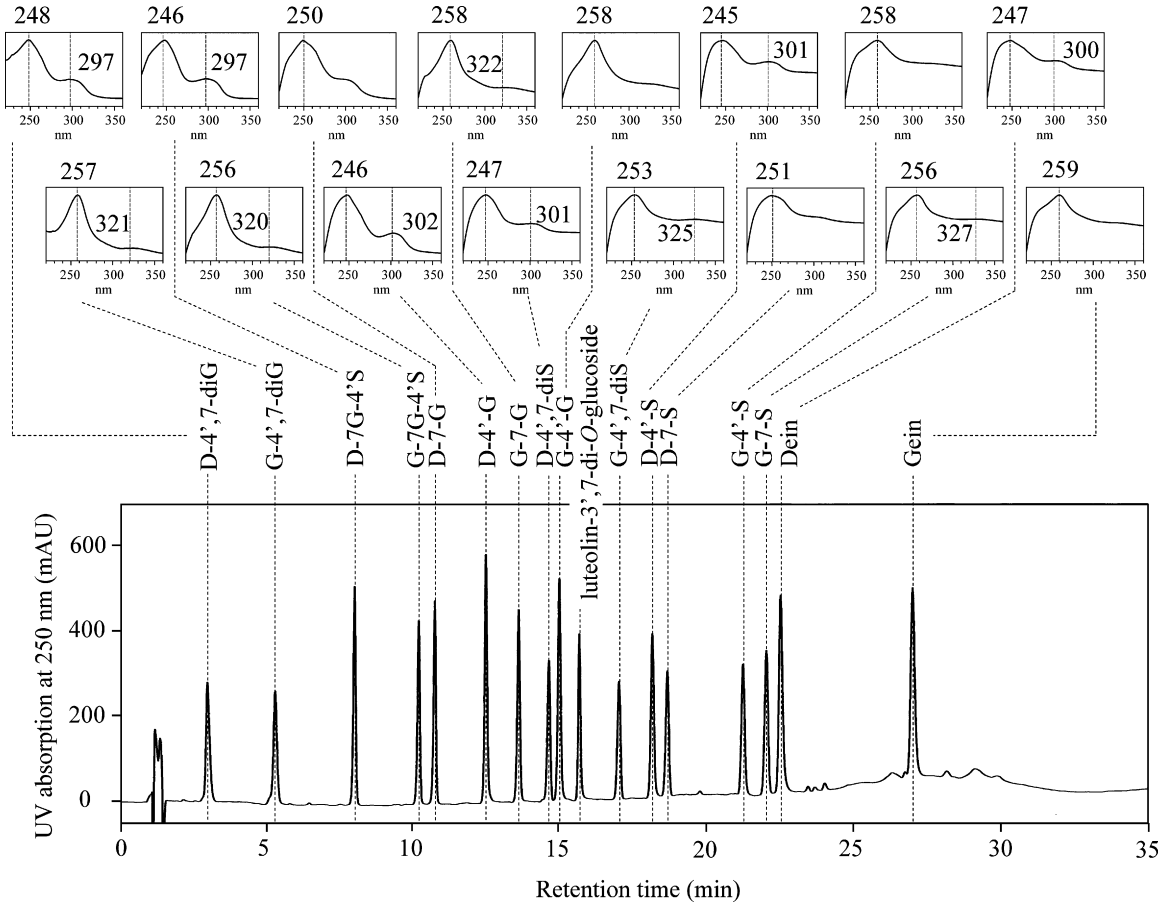


Fig. 2. HPLC chromatogram and UV spectra of a standard mixture of Dein, Gein and their conjugated metabolites, and luteolin-3′,7-di-*O*-glucoside as an internal standard: D-4′,7-diG, 256 ng/ml; G-4′,7-diG, 275 ng/ml; D-7G-4′S, 250 ng/ml; G-7G-4′S, 248 ng/ml; D-7-G, 263 ng/ml; D-4′-G, 310 ng/ml; G-7-G, 265 ng/ml; G-4′-G, 338 ng/ml; D-4′,7-diS, 90 ng/ml; G-4′,7-diS, 163 ng/ml; D-7-S, 94 ng/ml; D-4′-S, 112 ng/ml; G-7-S, 137 ng/ml; G-4′-S, 117 ng/ml; Dein, 269 ng/ml; Gein, 314 ng/ml; luteolin-3′,7-di-*O*-glucoside, 510 ng/ml. As shown in Section 2.2, after sample treatment, a 20-μl portion (1/10 of total amounts) of the sample solution was subjected to HPLC.

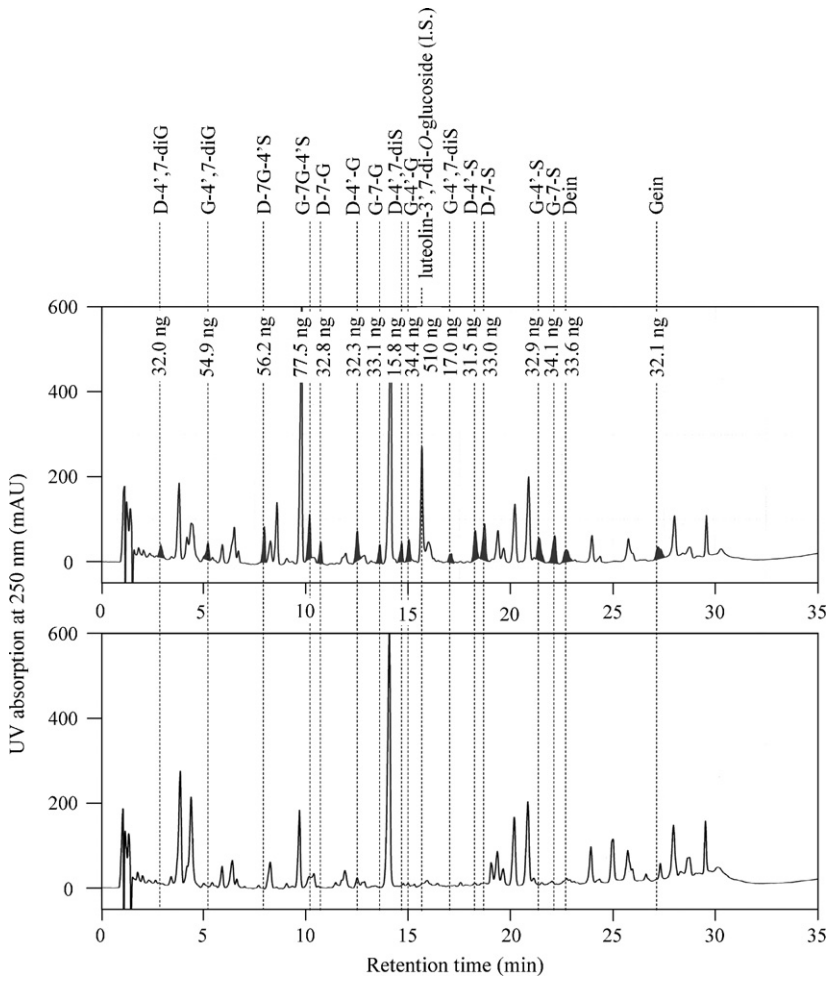


Fig. 3. Typical HPLC chromatograms of plasma extracts (1 ml) without spiking of the various isoflavones and luteolin-3′,7-di-*O*-glucoside (blank), and spiked with small amounts of the isoflavones and luteolin-3′,7-di-*O*-glucoside (spiked).

3.4. Accuracy and precision

The inter-assay accuracy and precision ($n = 6$) determined at three different concentrations are shown in Table 3. The amounts of isoflavone metabolites added were in good agreement with the amounts of the metabolites determined, the relative errors being less than ±5% (each metabolite in the midranges and at the high end of the ranges, except in the midranges of D-7-G, D-4′-G and G-4′-G). Large relative errors were observed at the near end of range (11.6%, D-4′,7-diG; −19.4%, D-7-G; −15.8%, D-7-S; −12.3%,

Table 1
Extraction recoveries of isoflavone metabolites from human plasma.

Isoflavone metabolites	Added (ng/ml)	Recovery (%) Mean ± SD ($n = 3$)	Isoflavone metabolites	Added (ng/ml)	Recovery (%) Mean ± SD ($n = 3$)
Dein	33.8	95.4 ± 3.9	Gein	39.4	90.3 ± 5.2
	269.0	104.1 ± 3.1		314.1	104.1 ± 5.6
D-4′,7-diG	32.0	85.1 ± 7.4	G-4′,7-diG	34.3	92.2 ± 4.6
	256.2	80.9 ± 3.0		274.5	98.6 ± 1.3
D-4′,7-diS	11.3	95.8 ± 4.0	G-4′,7-diS	20.6	109.4 ± 5.8
	90.3	100.3 ± 1.3		162.8	93.4 ± 3.5
D-7G-4′S	31.2	97.8 ± 5.7	G-7G-4′S	31.0	103.0 ± 9.3
	249.6	98.5 ± 2.5		248.0	98.1 ± 3.6
D-7-G	33.1	95.5 ± 2.2	G-7-G	33.1	94.6 ± 0.3
	262.8	97.4 ± 1.7		264.8	97.4 ± 1.8
D-4′-G	38.7	88.8 ± 0.6	G-4′-G	42.3	92.1 ± 0.6
	309.6	97.1 ± 1.6		338.4	96.0 ± 1.6
D-7-S	11.7	90.5 ± 6.2	G-7-S	17.2	83.7 ± 2.3
	94.4	101.7 ± 2.3		136.6	100.0 ± 2.5
D-4′-S	14.0	86.2 ± 4.3	G-4′-S	14.6	76.6 ± 3.2
	112.0	100.1 ± 2.3		116.9	97.6 ± 1.2

Table 2
Linear regression analyses of calibration plots for isoflavone metabolites ($n = 3$).

Isoflavone metabolites[a]	Range of concentration (ng/ml)	Slope	*y*-Intercept	Correlation coefficient (*r*)
Dein	8.4–538.0	0.0031 ± 0.0002	0.0208 ± 0.0152	0.9990 ± 0.0010
D-4′,7-diG	8.0–512.4	0.0034 ± 0.0004	−0.0223 ± 0.0212	0.9981 ± 0.0019
D-4′,7-diS	4.0–252.8	0.0095 ± 0.0026	0.0087 ± 0.0156	0.9976 ± 0.0039
D-7G-4′S	9.4–2184.0	0.0059 ± 0.0006	−0.0950 ± 0.1453	0.9999 ± 0.0001
D-7-G	8.2–525.5	0.0055 ± 0.0004	−0.0413 ± 0.1091	0.9997 ± 0.0002
D-4′-G	8.1–516.0	0.0042 ± 0.0002	−0.0077 ± 0.1266	0.9995 ± 0.0004
D-7-S	8.3–528.6	0.0093 ± 0.0007	0.0221 ± 0.0665	0.9996 ± 0.0001
D-4′-S	7.9–504.0	0.0080 ± 0.0004	0.0134 ± 0.0198	0.9997 ± 0.0001
Gein	8.0–513.9	0.0031 ± 0.0003	0.0041 ± 0.0225	0.9988 ± 0.0014
G-4′,7-diG	9.2–2013.0	0.0026 ± 0.0004	−0.0048 ± 0.0339	0.9993 ± 0.0005
G-4′,7-diS	4.3–271.3	0.0049 ± 0.0004	−0.0010 ± 0.0036	0.9991 ± 0.0008
G-7G-4′S	9.3–5115.0	0.0052 ± 0.0005	−0.2117 ± 0.3971	0.9999 ± 0.0001
G-7-G	8.3–529.6	0.0050 ± 0.0004	−0.0438 ± 0.1003	0.9989 ± 0.0016
G-4′-G	8.6–549.9	0.0037 ± 0.0002	−0.0065 ± 0.0947	0.9997 ± 0.0002
G-7-S	8.5–546.2	0.0072 ± 0.0005	−0.0047 ± 0.0602	0.9996 ± 0.0002
G-4′-S	8.2–526.1	0.0064 ± 0.0002	0.0187 ± 0.0301	0.9988 ± 0.0013

[a] Values are means ± SD.

G-7G-4′S; −14.4%, G-4′-G and −14.4%, G-7-S). The relative errors of the other metabolites were within ±10% at the end of the range. The inter-assay relative standard deviations (RSDs) ($n = 6$) were less than 4% of each metabolite in the midranges and at the high end of the range. The RSDs at the near end of each metabolite were less than 10%, except for 12.1% of D-4′,7-diG, 17.6% of D-4′-G and 11.2% of D-7-S.

3.5. Stability

For the examination of stability of 16 isoflavone metabolites in plasma at −20 °C for 6 months, 4 h plasma after intake of *kinako* was used. The concentrations (original amounts) of isoflavone metabolites determined immediately after collection were as follows; D-4′,7-diG, 51.5 ng/ml: D-7G-4′S, 771.3 ng/ml: D-7-G, 507.2 ng/ml: D-4′-G, 427.6 ng/ml: D-7-S, 194.5 ng/ml: D-4′-S, 70.0 ng/ml: G-4′,7-diG, 845.5 ng/ml: G-7G-4′S, 1441.6 ng/ml: G-7-G, 232.3 ng/ml: G-4′-G, 425.4 ng/ml: G-7-S, 219.3 ng/ml. The amounts of Dein, D-4′,7-diS, Gein, G-4′,7-diS and G-4′-S were under the LOQ. The concentrations of isoflavone metabolites in the plasma samples stored at −20 °C for 6 months were determined and compared to the original amounts (%; means ± SD, $n = 3$): D-4′,7-diG, 100.5 ± 5.7: D-7G-4′S, 92.1 ± 4.7: D-7-G, 103.8 ± 4.1: D-4′-G, 95.2 ± 4.8: D-7-S, 104.8 ± 2.4: D-4′-S, 100.2 ± 5.8: G-4′,7-diG, 102.8 ± 11.5: G-7G-4′S, 89.7 ± 1.4: G-7-G, 99.9 ± 6.8: G-4′-G, 109.1 ± 8.3: G-7-S, 94.0 ± 4.1.

3.6. Application

As an example of the application of this method, the plasma concentrations of 16 isoflavone metabolites were measured in a healthy volunteer who received orally 50 g of *kinako*. Fig. 4 (4 h) illustrates a chromatogram of plasma extract at 4 h after oral administration of *kinako*. Several metabolites peaks except for D-4′,7-diS and G-4′,7-diS were observed. Fig. 5 shows the plasma

Table 3
Accuracy and precision of HPLC determination of isoflavone metabolites in human plasma.

Isoflavone metabolites	Added (ng/ml)	Determined (ng/ml) Mean ± SD ($n = 6$)	Relative error (%)	RSD (%)	Isoflavone metabolites	Added (ng/ml)	Determined (ng/ml) Mean ± SD ($n = 6$)	Relative error (%)	RSD (%)
	23.1	24.8 ± 2.0	7.4	8.0		22.8	21.0 ± 1.5	−8.0	7.1
Dein	230.6	236.9 ± 4.5	2.7	1.9	Gein	228.4	229.1 ± 5.8	0.3	2.5
	461.1	459.9 ± 9.6	−0.3	2.1		456.8	453.3 ± 10.4	−0.8	2.3
	21.4	23.9 ± 2.9	11.6	12.1		21.5	20.5 ± 1.0	−4.8	4.9
D-4′,7-diG	234.9	231.4 ± 5.3	−1.5	2.3	G-4′,7-diG	915.0	929.1 ± 20.6	1.5	2.2
	469.7	469.9 ± 6.0	0.1	1.3		1830.0	1806.4 ± 28.2	−1.3	1.6
	21.7	19.7 ± 1.5	−9.4	7.6		21.7	20.5 ± 1.1	−5.5	5.3
D-4′,7-diS	108.4	112.4 ± 4.1	3.7	3.7	G-4′,7-diS	108.5	105.4 ± 3.8	−2.8	3.6
	216.7	224.5 ± 3.2	3.6	1.4		217.0	217.2 ± 5.4	0.1	2.5
	23.4	23.2 ± 1.0	−0.7	4.4		21.7	19.0 ± 1.4	−12.3	7.1
D-7G-4′S	1014.0	1009.0 ± 16.6	−0.5	1.6	G-7G-4′S	2325.0	2334.5 ± 33.1	0.4	1.4
	2028.0	1948.2 ± 28.9	−2.2	1.5		4650.0	4652.1 ± 71.7	0.1	1.5
	21.1	17.0 ± 0.9	−19.4	5.4		23.2	22.8 ± 0.6	−1.5	2.7
D-7-G	236.5	248.8 ± 4.0	5.2	1.6	G-7-G	231.7	234.6 ± 6.8	1.3	2.9
	473.0	449.8 ± 8.6	−4.9	1.9		463.4	465.1 ± 6.3	0.4	1.4
	21.9	21.6 ± 3.8	−1.4	17.6		21.2	18.1 ± 1.6	−14.4	9.0
D-4′-G	232.2	247.6 ± 3.8	6.6	1.5	G-4′-G	232.7	247.6 ± 3.1	6.4	1.2
	464.4	464.9 ± 6.2	0.1	1.3		465.3	472.9 ± 8.9	1.6	1.9
	22.7	19.1 ± 2.1	−15.8	11.2		23.4	20.0 ± 1.5	−14.4	7.5
D-7-S	226.7	233.2 ± 7.0	2.9	3.0	G-7-S	234.1	230.2 ± 8.3	−1.7	3.6
	453.3	456.6 ± 8.4	0.7	1.8		468.2	482.7 ± 10.5	3.1	2.2
	22.4	21.8 ± 1.9	−2.9	8.9		23.4	22.5 ± 1.6	−3.9	7.1
D-4′-S	224.0	228.8 ± 1.6	2.1	0.7	G-4′-S	233.8	238.5 ± 4.8	2.0	2.0
	448.0	457.9 ± 6.7	2.2	1.5		467.6	476.4 ± 15.1	1.9	3.2

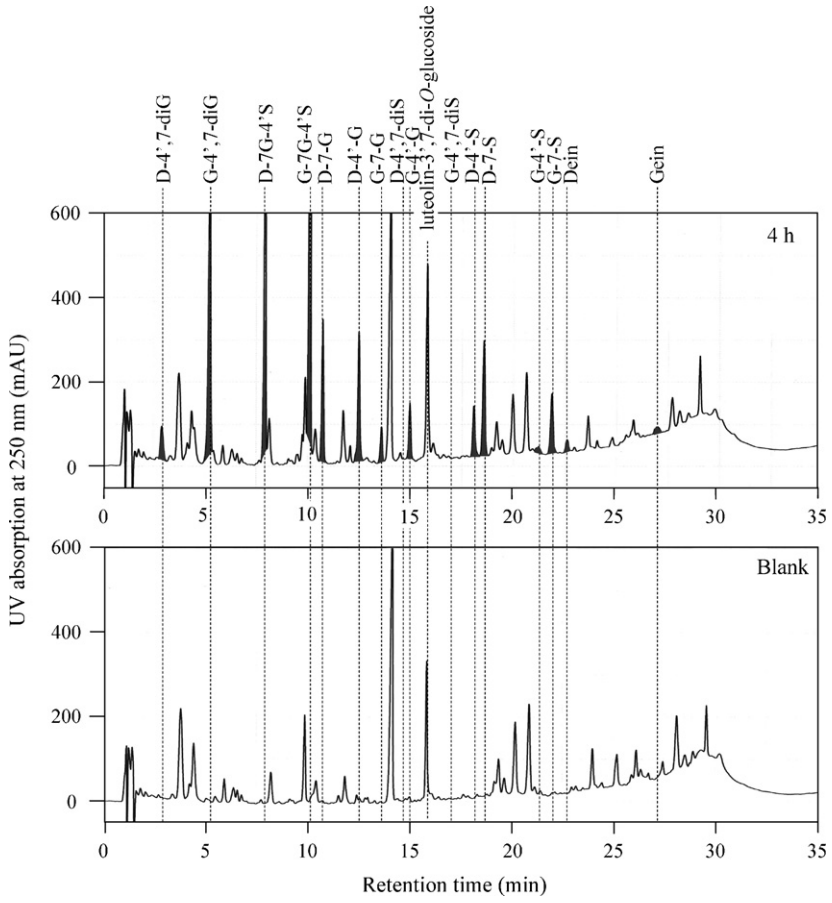


Fig. 4. HPLC chromatograms of plasma extracts obtained just before (spiked with I.S., blank) and at 4 h after administration (spiked with I.S., 4 h).

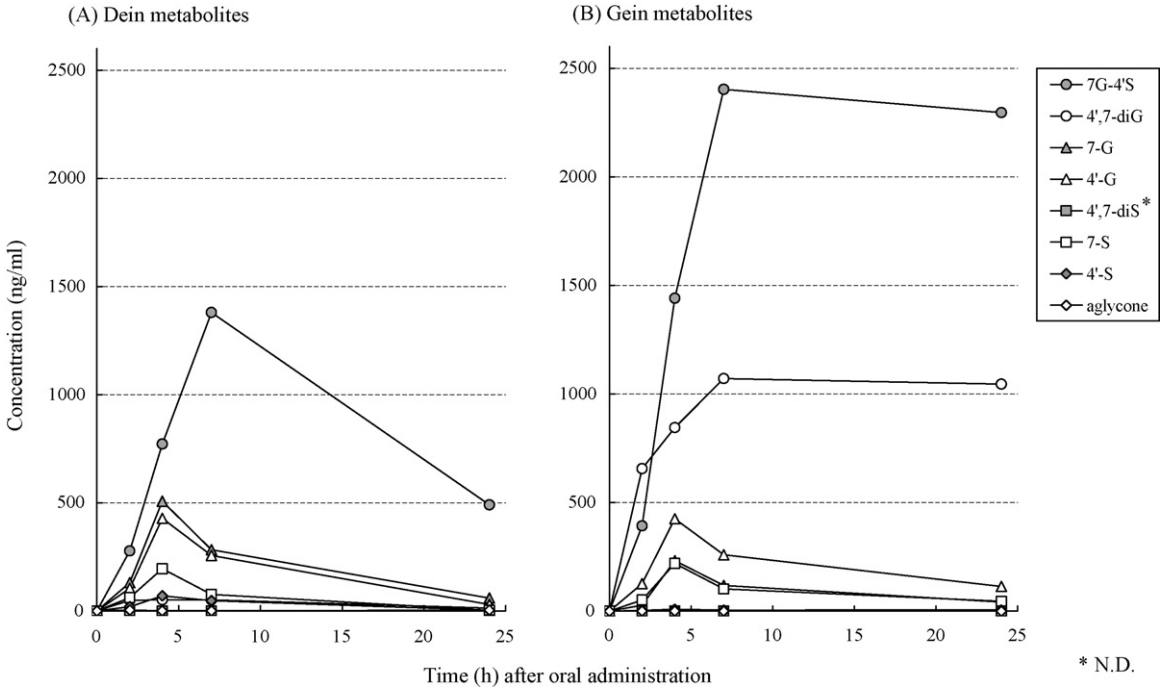


Fig. 5. Time courses of plasma concentrations of isoflavone metabolites. (A) Dein metabolites; (B) Gein metabolites.

concentration of (A) Dein metabolites and (B) Gein metabolites at 2, 4, 7 and 24 h after oral administration. The peak concentrations of the several Dein metabolites were observed at 4 h, except for D-7G-4′S (7 h). Similarly, those of G-7G-4′S and G-4′,7-diG were observed at 7 h. However, D-4′,7-diS and G-4′,7-diS could not be detected at any time after the administration.

4. Discussion

Isoflavone are mainly found as glycosides such as Din and Gin in the plant (e.g., soybean and kudzu). Most of glycosides are then hydrolyzed to their aglycones and sugar moieties by enteral microorganisms [2]. A large part of the absorbed isoflavone aglycones are converted to either their glucuronide or sulfate conjugates or both as the phase II metabolites. Intact aglycones are still detected in small proportions in plasma [38]. In numerous studies on absorption or bioavailability, metabolism and excretion, the metabolites containing the phase II conjugates have been analyzed by HPLC [12–18], GC–MS [19–24] and LC–MS [4,25–32] after enzymatic hydrolysis. These procedures 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. We synthesized the standard compounds of Dein- and Gein-sulfoglucuronides and diglucuronides in order to directly measure all conjugates simultaneously without any sample treatment processes affecting the ratios of its aglycones and conjugates.

We previously reported the plasma profile of intact isoflavone metabolites by HPLC [34]. By using HPLC-UV-DAD combined SPE, the isoflavone metabolites were simultaneously analyzed. However, an internal standard for quantification of these conjugated metabolites could not be found and the effective extracting method also could not be developed. In particular, the highly polar acidic compounds such as Dein- and Gein-monoglucuronide-monosulfate were poorly recovered (<5%). Therefore we developed the extraction method using an Oasis® HLB cartridge which is a *N*-vinylpyrrolidone/divinylbenzene copolymer stationary phase possessing both hydrophilic and lipophilic properties. Retaining reversed phase property to interact with the flavone ring, the cartridge also strongly held the hydrophilic sites of the conjugates. The strongly hydrophilic interaction of the copolymer stationary phase and conjugate site was able to be dissociated by methanol–28% ammonia solution. The use of a cartridge effectively eliminated the interfering material in plasma with efficient extraction (extraction recovered >97%, Table 1) of Dein- and Gein-monoglucuronide-monosulfates emerging at short retention time (0–10 min) on the HPLC chromatogram. In the process, we identified not only Dein- and Gein-monoglucuronide-monosulfate (as Dein- and Gein-7G-4′S), but also Dein- and Gein-4′,7-diG at the shorter retention time than Dein- and Gein-7G-4′S on the plasma HPLC chromatogram. The details of the identification of these compounds in plasma will be reported elsewhere. This SPE method using an Oasis® HLB cartridge enabled effective simultaneous extraction of 16 metabolites containing the highly polar phase II double conjugates with the recoveries of >ca. 80%.

The HPLC behavior of 16 metabolites extracted from human plasma was examined by using a Hydrosphere C18 column. The use of the linear gradient system consisting of 10 mM ammonium acetate solution and acetonitrile was found to provide good chromatographic profiles for the isoflavone metabolites and luteolin-3′,7-di-*O*-glucoside for the determination of plasma isoflavone metabolites. In Fig. 3 (blank), a small peaks were observed near at the retention time of G-7G-4′S and D-4′-G on the HPLC chromatogram. However, the retention times of these peaks differed from those of the respective authentic standards. These peaks were co-eluting interferences with an equivalent UV response to 6.0 ng/ml of G-7G-4′S and 7.3 ng/ml of D-4′-G, respectively, and were not significant when determining human plasma G-7G-4′S and D-4′-G concentrations higher than 30 ng/ml.

Calibration graphs were prepared by using 1.0-ml aliquots of the blank pooled plasma with different amounts of the various metabolites ranging from ca. 5 to 5000 ng and 510.0 ng of luteolin-3′,7-di-*O*-glucoside as internal standard. A good correlation was found between the observed peak height ratios (y) and mixed mass ratios (x) for each metabolite. Each least-squares regression analysis gave a typical regression line with good correlation coefficient (0.9976–0.9999).

The accuracy of measurements was determined in duplicate by adding the various amounts of isoflavone metabolites to 1.0-ml aliquots of blank plasma with a fixed amount of luteolin-3′,7-di-*O*-glucoside (510.0 ng). Table 3 shows that the various amounts of isoflavone metabolites added were in good agreement with the amounts of the metabolites determined. The relative errors of each metabolite were within ±20% and the RSDs ($n = 6$) were less than 20% at the near end of each metabolite. The acceptance criteria are not more than 15% RSD for precision and not more than 15% deviation from the nominal value for accuracy. However, at the limit of quantification (LOQ), 20% is acceptable for both precision and accuracy in view of the international recommendations [39]. Therefore, the LOQ of this method was defined as the lowest concentration of isoflavone metabolites with 20% of accuracy (relative error) and precision (RSDs) (21.1–23.4 ng/ml plasma). The lower limit of detection (LOD) was approximately 8 ng/ml defined as signal-to-noise ratio of about 3. Furthermore, isoflavone metabolites (D-4′,7-diG, D-7G-4′S, D-7-G, D-4′-G, D-7-S, D-4′-S, G-4′,7-diG, G-7G-4′S, G-7-G, G-4′-G and G-7-S) were found to be stable for 6 months at −20 °C as determined ranging from 89.7% to109.1% of the original amounts. We failed to examine the stability of Dein, D-4′,7-diS, Gein, G-4′,7-diS and G-4′-S.

It has been suggested that the major metabolites of isoflavones are 7- or 4′-monoglucuronides, accompanied by small proportions of sulfates [13,32,38]. Shelnutt et al. [4] reported that approximately 30% of the total amounts of Dein or Gein metabolites comprised sulfoglucuronides at 4 h after intake of a soy beverage. These data were obtained by the selective enzymatic hydrolysis (β-glucuronidase and sulfatase) and HPLC analysis of the converted aglycones. Therefore, the actual types of conjugates circulating in body and the position(s) of conjugation sites on the isoflavone skeleton have not yet been clarified. As an example of the preliminary application of this method, the isoflavone conjugates were analyzed in the plasma from a healthy volunteer who orally received 50 g of *kinako*. A time course of plasma concentrations of the isoflavone metabolites measured by HPLC is shown in Fig. 5. The isoflavone metabolites were not detected in the blood sample collected just before the intake of 50 g *kinako*. There was a relatively slow rise in the plasma concentration of the isoflavone metabolites to LOQs (ca. 25 ng/ml)–650 ng/ml at the first blood sampling time of 2 h after oral administration, and a peak level of 1380 ng/ml for D-7G-4′S, 2400 ng/ml for G-7G-4′S and 1070 ng/ml for G-4′,7-diG were observed at 7 h.

5. Conclusions

We have successfully developed a method for the determination of intact 16 isoflavone metabolites using HPLC-UV-DAD combined with SPE in human plasma. The present method provides a sensitive (generally LOD ca. 10 ng/ml; LOQ ca. 25 ng/ml) and reliable technique for the determination of plasma concentration of isoflavone metabolites. Each of these metabolites is significant as the circulating compounds in plasma. The application of this method on

the analysis of the isoflavone metabolites in plasma and urine will contribute to depict the detailed pharmacokinetics of isoflavone in humans.

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