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ORIGINAL ARTICLE

Identification of equol-7-glucuronide-4′-sulfate, monoglucuronides and monosulfates in human plasma of 2 equol producers after administration of *kinako* by LC-ESI-MS

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Abstract

Equol is a product formed during the biotransformation of the naturally occurring isoflavone daidzein by intestinal bacteria. The role of equol in the prevention of several hormone-dependent diseases such as prostate cancer and osteoporosis as well as vasomotor symptoms has been extensively investigated. Equol primarily occurs in the form of major metabolites such as glucuronides and sulfates, while intact equol has been detected at only ca. 1% in human plasma. However, to date, conjugated metabolites have been evaluated by measuring the free equol obtained after selective enzymatic hydrolysis. Thus, the precise types of conjugates circulating in vivo and the position(s) of the conjugation sites on the equol skeleton have yet to be clarified. Our study describes the identification of polar equol metabolites in the plasma of 2 equol-producers obtained at 8 hours after consuming 50 g of *kinako* (approximately 37 mg of daidzein). The structural identification of these conjugated metabolites in plasma was performed by comparison to the LC-ESI-MS^n and ^{1}H-NMR spectral data of the corresponding chemically synthesized compounds. The results of the LC-ESI-MS/MS analysis indicated that the main conjugated metabolite in plasma was (*S*)-equol-7-glucuronide-4′-sulfate along with lower amounts of 7- and 4′-monoglucuronides as well as 7- and 4′-monosulfates.

KEYWORDS
Equol, glucuronide, identification, LC-ESI-MS, sulfate

1 | INTRODUCTION

Equol [7-hydroxy-3-(4′-hydroxyphenyl)-chroman] was first isolated from the urine of pregnant mares in 1932,[1] and later identified in human urine by Axelson et al.[2] The compound is formed during the biotransformation of the isoflavone daidzein (Dein) by intestinal bacteria,[3] and possesses estrogenic activity owing to its affinity for estrogen receptors (ERs) of both the ERα and ERβ subtype.[4,5]

Abbreviations: DAD, diode-array detector; Dein, daidzein; Din, daidzin; DMSO, dimethyl sulfoxide; E-4′,7-diG, equol-4′,7-diglucuronide; E-4′,7-diS, equol-4′,7-disulfate; E-4′-G, equol-4′-glucuronide; E-4′-S, equol-4′-sulfate; E-7G-4′S, equol-7-glucuronide-4′-sulfate; E-7-G, equol-7-glucuronide; E-7S-4′G, equol-7-sulfate-4′-glucuronide; E-7-S, equol-7-sulfate; ER, estrogen receptor; ESI, electrospray ionization; Gein, genistein; Gin, genistin; HPLC, high-performance liquid chromatography; LC, liquid chromatography; MS, mass spectrometry; NMR, nuclear magnetic resonance; SULT, sulfotransferase; UGT, UDP-glucuronosyltransferase.

Several studies suggest that equol may play a crucial role in the prevention of several hormone-dependent diseases such as prostate cancer, breast cancer, and osteoporosis as well as vasomotor symptoms.[6-12] It is generally thought that the biological activity of isoflavonoids resides in their aglycone forms such as Dein and genistein (Gein), as well as the metabolite equol. However, conjugates of these compounds may also display biological activity, or may serve as precursors of biologically active species on or within the target cells. For example, Dein- and Gein-7-glucuronide exhibit weak estrogenic activity and can activate human natural killer cells at nutritionally relevant concentrations,[13] while equol-7-glucuronide (E-7-G) promotes osteoblastogenesis and inhibits osteoclast formation in MC3T3-E1 cells.[7] Dein- and Gein-7-glucuronide-4′-sulfate have a stimulatory effect on the growth of MCF-7 human breast cancer cells.[14] Additional observations of either the enhancing or reducing effects of Dein, Gein, and equol sulfates on the estrogen agonist activity in MCF-7 cells have demonstrated that the biological activity of isoflavones depends not only on their specific skeleton but also the sites of sulfation.[15] Therefore, establishing the exact types of conjugates circulating in the body will be important in evaluating their biological effects. Such a study will also help ascertain the metabolism and disposition of phase II metabolites.

We previously developed a HPLC-UV-DAD method for the simultaneous determination of Dein, Gein, and their 16 isoflavone metabolites in plasma.[16] By using this method, data on the entire profile, metabolism, and disposition of phase II metabolites in Japanese adults after consumption of *kinako* were obtained. It was found that the plasma profiles significantly varied between individuals.[17,18] Equol produced from Dein has mostly been found in metabolites such as glucuronides and sulfates, while intact equol was detected at only ca. 1% in human plasma.[19] Nonetheless, the precise types of conjugates circulating in vivo and the position(s) of the conjugation sites on the equol skeleton have yet to be defined. Indeed, the level of conjugated metabolites is estimated by measuring the free equol obtained after enzymatic hydrolysis. Therefore, information related to the parent compounds, such as glucuronides and sulfates, is lost using this protocol.

The aim of this study is to elucidate the profile of the conjugated metabolites of equol (Figure 1) in human plasma. The plasma extracts of 2 equol producers after oral administration of *kinako* were analyzed using LC-ESI-MS^n techniques. The major conjugated metabolites in plasma were semi-quantified by a standard addition method.

2 | MATERIALS AND METHODS

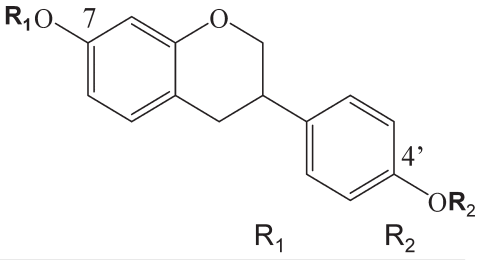
2.1 | Chemicals

Daidzein and (*R*, *S*)-equol were purchased from LC Laboratories (Woburn, MA). (*S*)- and (*R*)-equol were purchased from Cayman Chemical (Ann Arbor, MI). Phosphoric acid (99.999%) was purchased from Sigma-Aldrich (Milwaukee, WI), while *kinako* (baked soybean powder) was acquired from a retail store. All other chemicals and solvents were of analytical grade and used without further purification.

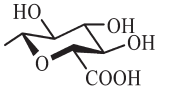
2.2 | Preparation of the reference substances

2.2.1 | Synthesis of equol-4′,7-diglucuronide (E-4′,7-diG)

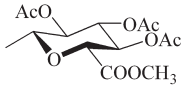
E-4′,7-diG was synthesized from equol-4′,7-yl di-[methyl(2″,3″,4″-tri-*O*-acetyl-β-D-glucopyranosiduronic acid)] (E-4′,7-diG′, Figure 1:



	R_1	R_2
Equol	H	H
E-4',7-diG	G	G
E-4',7-diS	S	S
E-7G-4'S	G	S
E-7S-4'G	S	G
E-4'-G	H	G
E-7-G	G	H
E-4'-S	H	S
E-7-S	S	H



G (glucuronide)



- G'

S (sulfate)

FIGURE 1 Structures of equol and its conjugated metabolites with glucuronic acid, sulfuric acid, or both

-G′) according to the method developed by Needs and Williamson[20] and Hosoda et al.[21] E-4′,7-diG′ was synthesized from equol (60 mg) and methyl-(2,3,4-tri-*O*-acetyl-α-D-glucopyranosyl trichloroacetimidate) uronate (230 mg) following a literature procedure.[21,22] ^{1}H-NMR δ (600 MHz, DMSO-d_6): 4.00 (dd, *J* = 9.6, 10.8 Hz, H-2), 4.20 (d, *J* = 9.6 Hz, H-2), 3.10 (m, H-3), 2.90 (m, H-4), 7.00 (d, *J* = 9.0 Hz, H-5), 6.54 (dd, *J* = 2.4, 7.8 Hz, H-6), 6.47 (dd, *J* = 2.4, 3.0 Hz, H-8), 7.23 (dd, *J* = 6.0, 8.4 Hz, H-2′, H-6′), 7.00 (d, *J* = 9.0 Hz, H-3′, H-5′); ESI-MS: *m/z* 612 $[M+NH_4]^+$, 243 [M-2 × glucuronic acid+H]$^+$, 123 $[C_7H_6O_2+H]^+$.

2.2.2 | Synthesis of equol-4′,7-disulfate (E-4′,7-diS)

E-4′,7-diS disodium salt was synthesized according to the method of Soidinsalo and Wähälä,[23] with the following minor modifications. ^{1}H-NMR δ (600 MHz, DMSO-d_6): 3.97 (dd, *J* = 10.5, 10.6 Hz, H-2), 4.22 (d, *J* = 10.4 Hz, H-2), 2.95 (m, H-3), 2.86 (m, H-4), 6.98 (d, *J* = 8.1 Hz, H-5), 6.66 (d, *J* = 2.3 Hz, H-6), 6.64 (m, H-8), 7.23 (d, *J* = 8.6 Hz, H-2′, H-6′), 7.12 (d, *J* = 8.6 Hz, H-3′, H-5′); ESI-MS: *m/z* 423 $[M+Na-2H]^-$, 343 $[M+Na-2H-SO_3]^-$, 263 $[M+Na-2H-2\times SO_3]^-$, 121 $[C_7H_6O_2-H]^-$.

2.2.3 | Synthesis of equol-4′-glucuronide (E-4′-G)

E-4′-G was obtained by catalytic reduction in Dein-4′-G′. By reacting Dein with methyl-(2,3,4-tri-*O*-acetyl-α-D-glucopyranosyl trichloroacetimidate) uronate, Dein-4′-G′ and Dein-4′,7-diG′ were obtained in a weight ratio of approximately 2:3. The respective compounds were purified by silica gel column chromatography using hexane-methyl acetate (2:3) followed by methanol-dichloromethane (2:98) as eluent. To a solution of Dein-4′-G′ (54.2 mg, 0.095 mmol) in 95% aqueous ethanol, 60 mg of 10% Pd/C was added. The suspended solution was stirred under a hydrogen atmosphere for 5 hours at room temperature. After filtration, the solution was evaporated to dryness. To a solution of the crude E-4′-G′ (10.4 mg, 0.0186 mmol) was deacetylated and desalted in a similar manner as our previous paper.[24] ^{1}H-NMR δ (600 MHz, DMSO-d_6): 3.94 (dd, *J* = 10.2, 10.8 Hz, H-2), 4.17 (d, *J* = 10.2 Hz, H-2), 3.08 (m, H-3), 2.84 (m, H-4), 6.87 (d, *J* = 8.4 Hz, H-5), 6.29 (dd, *J* = 2.4, 8.4 Hz, H-6), 6.19 (d, *J* = 2.4 Hz, H-8), 7.23 (d, *J* = 8.4 Hz, H-2′, H-6′), 6.98 (d, *J* = 8.4 Hz, H-3′, H-5′); ESI-MS: *m/z* 436 $[M+NH_4]^+$, 243 [M-glucuronic acid+H]$^+$, 123 $[C_7H_6O_2+H]^+$.

2.2.4 | Synthesis of equol-7-glucuronide (E-7-G)

E-monoG′ (12.8 mg, E-7-G′ and E-4′-G′), which was obtained as by-product from the diglucuronidation at the 4′ and 7 positions of equol, was deacetylated and desalted in a similar manner to that mentioned earlier. ^{1}H-NMR δ (600 MHz, DMSO-d_6): 3.94 (dd, *J* = 10.4, 10.8 Hz, H-2), 4.18 (d, *J* = 10.2 Hz, H-2), 3.04 (m, H-3), 2.86 (m, H-4), 7.00 (d, *J* = 8.5 Hz, H-5), 6.53 (d, *J* = 8.3 Hz, H-6), 6.46 (d, *J* = 2.0 Hz, H-8), 7.11 (dd, *J* = 1.7, 8.4 Hz, H-2′, H-6′), 6.72 (d, *J* = 7.6 Hz, H-3′, H-5′); ESI-MS: *m/z* 436 $[M+NH_4]^+$, 243 [M-glucuronic acid+H]$^+$, 123 $[C_7H_6O_2+H]^+$.

2.2.5 | Synthesis of equol-4′-sulfate (E-4′-S) and equol-7-sufate (E-7-S)

E-4′-S and E-7-S were synthesized according to their method.[23,25,26] Data for E-4′-S. ^{1}H-NMR δ (600 MHz, DMSO-d_6): 3.94 (dd, *J* = 10.2, 10.8 Hz, H-2), 4.18 (d, *J* = 10.8 Hz, H-2), 3.08 (m, H-3), 2.84 (m, H-4), 6.87 (d, *J* = 8.4 Hz, H-5), 6.29 (dd, *J* = 2.4, 8.4 Hz, H-6), 6.19 (d, *J* = 2.4 Hz, H-8), 7.22 (d, *J* = 8.4 Hz, H-2′, H-6′), 7.12 (d, *J* = 9.0 Hz, H-3′, H-5′); ESI-MS: *m/z* 321 $[M-H]^-$, 241 $[M-SO_3-H]^-$, 121 $[C_7H_6O_2-H]^-$.

Data for E-7-S. ^{1}H-NMR δ (600 MHz, DMSO-d_6): 3.93 (dd, *J* = 10.4, 10.5 Hz, H-2), 4.18 (d, *J* = 10.4 Hz, H-2), 3.04 (m, H-3), 2.85 (m, H-4), 6.96 (d, *J* = 8.2 Hz, H-5), 6.65 (dd, *J* = 2.4, 8.4 Hz, H-6), 6.63 (d, *J* = 2.4 Hz, H-8), 7.12 (d, *J* = 8.4 Hz, H-2′, H-6′), 6.73 (d, *J* = 9.0 Hz, H-3′, H-5′); ESI-MS: *m/z* 321 $[M-H]^-$, 241 $[M-SO_3-H]^-$, 121 $[C_7H_6O_2-H]^-$.

2.2.6 | Synthesis of equol-7-glucuronide-4'-sulfate (E-7G-4′S)

E-7G-4′S was synthesized from 4′-*O*-benzyldaidzein-7-*O*-triacetylglucuronide methyl ester according to a previously reported method[21,23] with the following minor modifications. 4′-*O*-benzyldaidzein-7-*O*-triacetylglucuronide methyl ester was converted to E-7-G′ derivatives by a similar method to that used for the catalytic reduction in Dein-4′-G′. After debenzylation, sulfonation, and deacylation, the crude E-7G-4′S was purified by using a preparative C_{18} (125 Å, 55-105 μm; Waters) column (120 mL of packing, 500 × 22 mm i.d.) with water as eluent. ^{1}H-NMR δ (500 MHz, DMSO-d_6): 4.00 (dd, *J* = 10.5, 10.5 Hz, H-2), 4.23 (d, *J* = 9.5, 10.5 Hz, H-2), 3.10 (m, H-3), 2.90 (m, H-4), 7.01 (d, *J* = 8.5 Hz, H-5), 6.56 (dd, *J* = 2.5, 8.5 Hz, H-6), 6.50 (d, *J* = 2.5 Hz, H-8), 7.24 (dd, *J* = 1.5, 8.5 Hz, H-2′, H-6′), 7.13 (d, *J* = 8.0 Hz, H-3′, H-5′); ESI-MS: *m/z* 497 $[M-H]^-$, 321 [M-glucuronic acid-H]$^-$, 241 [M-glucuronic acid-SO_3-H]$^-$, 121 $[C_7H_6O_2-H]^-$.

2.2.7 | Synthesis of equol-7-sulfate-4′-glucuronide (E-7S-4′G)

E-7S-4′G was synthesized by sulfonation of E-4′-G′, followed by deacylation according to the procedure used for the synthesis of E-7G-4′S described above. ^{1}H-NMR δ (600 MHz, DMSO-d_6): 3.96 (dd, *J* = 10.2, 10.8 Hz, H-2), 4.20 (d, *J* = 10.2, 10.8 Hz, H-2), 3.10 (m, H-3), 2.90 (m, H-4), 6.96 (d, *J* = 8.2 Hz, H-5), 6.65 (m, H-6), 6.65 (m, H-8), 7.23 (d, *J* = 8.7 Hz, H-2′, H-6′), 6.98 (d, *J* = 8.6 Hz, H-3′, H-5′); ESI-MS: *m/z* 497 $[M-H]^-$, 321 [M-glucuronic acid-H]$^-$, 241 [M-glucuronic acid-SO_3-H]$^-$, 121 $[C_7H_6O_2-H]^-$.

2.3 | ^{1}H-NMR analysis

Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker AV-500 or AV-600 spectrometer (500 and 600 MHz; Rheinstetten, Germany). Samples were analyzed as solutions in dimethyl sulfoxide-d_6. Chemical shifts are given in δ values (ppm) downfield to tetramethylsilane.

2.4 | LC-ESI-MS analysis

Chromatographic conditions were appropriately established for LC-ESI-MS^n analyses. The HPLC apparatus used in this study was a Nanospace SI-2 liquid chromatography system (Shiseido, Tokyo, Japan) equipped with a model 3201 dual pump, 3024 column oven, 3023 auto sampler, and 3002 UV-visible detector. The mobile phase was degassed using a Shiseido 3202 degasser. Mass spectral data were collected using a linear ion trap mass spectrometer (Finnigan LTQ XL; Thermo Fisher Scientific, Waltham, MA) equipped with a heated capillary electrospray interface. The ion polarity mode was set to either positive or negative for MS, MS/MS, MS/MS/MS, and MS/MS/MS/MS.

The structure of the 8 chemically synthesized metabolites E-4′, 7-diG, E-7G-4′S, E-7S-4′G, E-4′,7-diS, E-4′-G, E-7-G, E-4′-S, and E-7-S was confirmed by LC-ESI-MS^n analyses as well as ^{1}H-NMR experiments.

The chromatographic separation of these equol metabolites were performed on a Capcell Pak ADME column (150 × 2.1 mm i.d., particle size 3 μm; Osaka Soda, Osaka, Japan) and a guard cartridge (10 × 2.1 mm i.d.) made of the same material. These metabolites were eluted using a mixed solvent system composed of 10 mmol/L ammonium acetate solution (pH 6.8) (solvent A) and acetonitrile (solvent B) with a linear gradient from 98% to 60% solvent A (0-60 minutes). The flow rate was 0.2 mL/min at 50°C. Positive- or negative-ion electrospray mass spectrometric analyses were carried out under the following conditions: capillary temperature 200°C, sheath gas 50 arbitrary units (AU), and auxiliary gas 15 AU. Other conditions are given in Table 1. The relative collision energies for the MS/MS, MS/MS/MS, and MS/MS/MS/MS mode analyses were optimized at 35% (AU).

2.5 | Fractionation of the individual (*S*)- and (*R*)-diastereomers of E-7G-4′S and E-7S-4′G

E-7G-4′S (395.7 ng) and E-7S-4′G (393.2 ng) were separated into the respective diastereomers by preparative HPLC. The HPLC apparatus used in this study was a Jasco 2000 plus system (Jasco, Tokyo, Japan) equipped with a model PU-2089 gradient pump, CO-2067 column oven, AS-2059 auto sampler, and UV-2075 UV-visible detector. A Synergi Hydro-RP column was employed (150 × 2.0 mm i.d., particle size 4 μm; Phenomenex, Torrance, CA) along with a guard cartridge (AQC 18, 4 × 2.1 mm i.d.). Each diastereomer mixture composed of E-7G-4′S or E-7S-4′G was eluted using a solvent system comprising 10 mmol/L ammonium acetate solution and acetonitrile (99:1). The flow rate was 0.4 mL/min at 45°C. The UV detection wavelength was set at 280 nm. Each diastereomer showing as 2 peaks for E-7G-4′S and E-7S-4′G on the HPLC chromatogram was divided into 2 fractions. The individual eluted solution from the preparative HPLC was evaporated. The 4 individual residues were divided into halves. One half of each fraction was used for enzymatic hydrolysis, while the other half was used for identification of plasma E-7G-4′S or E-7S-4′G.

2.6 | Structural confirmation of each (*S*)- and (*R*)-diastereomers of E-7G-4′S and E-7S-4′G

The structure of the fractionated (*S*)- and (*R*)- diastereomers of E-7G-4′S and E-7S-4′G was confirmed by enzymatic hydrolysis using a combination of β-glucuronidase and sulfatase, followed by comparison between the obtained *S*-(−) and *R*-(+) equol with the reference *S*-(-) and *R*-(+) equol *via* LC-ESI-MS/MS analysis using a chiral column. To each of the 4 fractions, a solution (1.2 mL) of *H. pomatia* containing 1000 activity units of β-glucuronidase and 33 activity units of sulfatase in 0.2 mol/L acetate buffer solution (pH 4.5) was added. The reaction mixture was incubated at 37°C for 3 hours. Next, 5 mL of ethanol was added to the mixture, and the solution was vortexed for 30 seconds and centrifuged at 1800 ***g*** for 5 minutes prior to analysis by LC-ESI-MS/MS. A Chiralcel OJ-3R column (150 × 4.6 mm i.d., particle size 3 μm; Daicel Chemical, Tokyo, Japan) was used to separate *S*-(−) and *R*-(+) equol obtained by enzymatic hydrolysis. Elution was performed using a mobile phase consisting of water and methanol (20:80, v/v) at 40°C, while the flow-rate was set at 0.5 mL/min. ESI mass spectrometric analysis was carried out in negative mode according to the following conditions: capillary temperature 250°C, spray voltage 2.5 kV, sheath gas flow 50 AU, auxiliary gas flow 15 AU, and tube lens −59.23 V. The collision energies for the MS/MS mode analysis were optimized at 35% (AU).

TABLE 1 Summary of mass spectometric conditions

Compounds	Spray voltage (V)	Polarity	Parent mass (*m/z*)	Product mass (*m/z*)	Tube lens (V)
Equol	2500	−	241	121	−119.23
E-4′,7-diG	3000	+	612	243	95
E-4′,7-diS	2500	−	423	343	−259.23
E-7G-4′S E-7S-4′G	2500	−	497	321	−214.40
E-4′-G E-7-G	2500	+	436	243	85
E-4′-S E-7-S	2500	−	321	241	−174.23

2.7 | Sample collection

This study was approved by the Kyorin University, School of Health Sciences Human Subjects Review Board. Written informed consent was obtained from all the subjects. Two equol-producing volunteers[27] [28-year-old male (Subject 1) and 30-year-old female (Subject 2)] orally received 50 g of *kinako* containing Din 27.12 mg (65.2 μmol), Gin 40.59 mg (93.95 μmol), Dein 20.28 mg (79.85 μmol), and Gein 42.47 mg (157.3 μmol) suspended in 300 mL of cow's milk. The 2 volunteers (equol producers) did not ingest soy-containing foods from 1 week prior to the test period until completion of the study. Blood samples (7.0 mL) were collected from the median cubital vein into evacuated tubes containing Na_2EDTA just prior to ingestion of *kinako* and then 8 hours later (indicating the maximum blood level by a pilot study). The blood samples were centrifuged (1800 ***g***, 10 minutes) immediately after collection and the resulting plasma fractions stored at −20°C until analysis.

2.8 | Extraction of equol metabolites from plasma

Plasma samples (0.2 mL) were diluted with 2.0 mL of a 0.05 mol/L phosphoric acid solution prior to extraction using an Oasis® HLB cartridge (3 cc, 60 mg packing; Waters) according to a previously reported method.[16] The recovery of equol metabolites from plasma was determined by comparing the peak heights before and after the Oasis® HLB extraction procedure.

2.9 | Identification of E-7G-4′S in human plasma

For the separation of E-7G-4′S and E-7S-4′G into their individual diastereomers, the same column and guard cartridge (Capcell Pak ADME column and AQC 18 cartridge) used for studying their chromatographic behavior was utilized. Samples were eluted using a solvent system comprising 10 mmol/L ammonium acetate solution (solvent A) and acetonitrile (solvent B) with a linear gradient from 98% to 95% solvent A (0-35 minutes). The flow rate was 0.2 mL/min at 50°C. The conditions for the negative-ion electrospray mass spectrometric analyses of the 2 conjugated positional isomers were the same as those previously described (Table 1), except for a tube lens of −89.23 V.

The identification of these metabolites in human plasma was performed using LC-ESI-MS, MS/MS, MS/MS/MS, and MS/MS/MS/MS analyses by cochromatography with the chemically synthesized metabolites.

Peak identification was performed by spiking with the reference compounds of E-7G-4′S or E-7S-4′G on the LC-ESI-MS/MS chromatograms. Plasma extracts of Subject 1 and Subject 2 were spiked with half of the individual diastereomer fraction of E-7G-4′S or E-7S-4′G obtained by preparative HPLC.

2.10 | Semi-quantification

The determination of the equol metabolites in human plasma was performed by a standard addition method. Stock solutions of E-4′, 7-diG, E-4′,7-diS, E-4′-G, E-7-G, E-4′-S, E-7-S, and E-7G-4′S were prepared by dissolving these compounds in methanol-water (50:50, v/v). Calibration curves were prepared by adding the various reference compounds to 0.2 mL of a plasma sample for elucidation, in the following ranges: E-4′,7-diG (6.5-102.2 ng), E-7G-4′S (3.9-98.3 ng), E-4′,7-diS (6.0-115.0 ng), E-4′-G (4.6-114.0 ng), E-7-G (3.9-81.8 ng), E-4′-S (5.6-86.9 ng), E-7-S (3.4-105.8 ng), and equol (5.35-100.35 ng). Calibration curves were obtained by linear least squares fitting of the peak height on the LC-ESI-MS/MS chromatograms versus the amount of each equol metabolite added to the plasma.

3 | RESULTS

3.1 | Preparation of the reference compounds

The equol used for the chemical synthesis of equol conjugates was obtained by hydrogenation of daidzein over palladium charcoal.[28] All synthesized compounds (Figure 1) were obtained with more than 98% chemical purity, except for E-7S-4′G (96%). The structures of these compounds were confirmed by LC-ESI-MS as well as 500 and 600 MHz ^{1}H-NMR.

TABLE 2 MS data of the chemically synthesized metabolites of equol

		Production			
Compounds	Full MS	MS/MS	MS/MS/MS	MS/MS/MS/MS	(*m/z*)
E-4′,7-diG	612 $[M+NH_4]^+$	243 $[M-2\times G+H]^+$ 383 $[M-G-2H_2O+H]^+$	123 $[C_7H_6O_2+H]^+$	–	
E-4′,7-diS	423 $[M+Na-2H]^-$	343 $[M+Na-2H-S]^-$	263 $[M+Na-2H-2\times S]^-$	121 $[C_7H_6O_2-H]^-$	
E-7G-4′S E-7S-4′G	497 $[M-H]^-$	321 $[M-G-H]^-$	241 $[M-G-S-H]^-$	121 $[C_7H_6O_2-H]^-$	
E-4′-G	436 $[M+NH_4]^+$	243 $[M-G+H]^+$	123 $[C_7H_6O_2+H]^+$	–	
E-7-G	436 $[M+NH_4]^+$	383 $[M-2H_2O+H]^+$ 243 $[M-G+H]^+$	123 $[C_7H_6O_2+H]^+$	–	
E-4′-S E-7-S	321 $[M-H]^-$	241 $[M-S-H]^-$	121 $[C_7H_6O_2-H]^-$	–	

Table 2 summarizes the MS data of the chemically synthesized metabolites of equol. The corresponding mass spectral fragmentation patterns can be found in the Supporting Information (Figure S1).

The 500 and 600 MHz ^{1}H-NMR spectral data of all chemically synthesized compounds are shown in Table 3. A comparison of the ^{1}H-NMR spectral data of E-4′,7-diG, E-7G-4′S, and E-7-G with those of equol indicate a downfield shift of the C-6 proton and C-8 proton signals due to the glucuronyl group attached at the 7 position. For the other analogues (E-4′,7-diS, E-7S-4′G, and E-7-S), a downfield shift of the C-6 proton and C-8 proton signals was also observed due to the sulfate group attached at the 7 position. Furthermore, a comparison of the spectral data of E-4′,7-diG, E-7S-4′G, and E-4′-G with those of equol suggested a downfield shift of the C-3′,5′ proton signals due to the glucuronyl group attached at the 4′ position. However, the C-3′,5′ proton signals also shifted downfield due to the sulfate group attached at the 4′ position. The degree of contribution of different groups to the downfield shift can be explained on the basis of the corresponding electronegativity, as a sulfate group is larger than a glucuronyl moiety.

3.2 | Identification of E-4′-G, E-7-G, E-4′-S, and E-7-S in human plasma

Figure 2A (a)–(h) shows the MS/MS chromatograms of the chemically synthesized metabolites of equol and equol (i); (a) E-4′,7-diG, *m/z* 243 ([M-2 × glucuronic acid+H]$^+$); (b) E-7G-4′S, *m/z* 321 ([M-glucuronic acid-H]$^-$); (c) E-7S-4′G, *m/z* 321 ([M-glucuronic acid-H]$^-$); (d) E-4′, 7-diS, *m/z* 343 ([M+Na-2H-SO_3]$^-$); (e) E-4′-G, *m/z* 243 ([M-glucuronic acid+H]$^+$); (f) E-7-G, *m/z* 243 ([M-glucuronic acid+H]$^+$); (g) E-4′-S, *m/z* 241 ([M-SO_3-H]$^-$); (h) E-7-S, *m/z* 241 ([M-SO_3-H]$^-$); (i) equol, *m/z* 121 ([$C_7H_6O_2$-H]$^-$). Figure 2B also illustrates the MS/MS chromatograms at 321 for E-7G-4′S or E-7S-4′G (X), at 243 for E-4′-G or E-7-G (Y), and at 241 for E-4′-S or E-7-S (Z) in plasma extracts. The other MS/MS fragments corresponding to E-4′, 7-diG, E-4′, 7-diS, and equol on the MS/MS chromatograms could not be found. Because E-7G-4′S and E-7S-4′G had the same retention time (14.5 minutes, Figure 2B (X)) and gave similar spectra (MS/MS, MS/MS/MS, and MS/MS/MS/MS), it was not possible to identify these 2 compounds. The mass spectral analyses of the peaks at 24.8 or 25.3 minutes on the MS/MS chromatograms gave a parent peak of *m/z* 243 (a product ion of E-4′-G or E-7-G). The chromatographic characteristics and the MS, MS/MS, and MS/MS/MS spectra of the 2 monoglucuronides in human plasma matched those of the chemically synthesized monoglucuronides (Figure 2B (Y)). Similarly, the MS, MS/MS, and MS/MS/MS spectral data of the 2 peaks at 33.0 or 33.3 minutes in the MS/MS chromatograms (Figure 2B (Z)) of human plasma also coincided with those of the 2 authentic sulfates (E-4′-S or E-7-S), respectively.

3.3 | Fractionation and confirmation of the diastereomers of E-7G-4′S and E-7S-4′G

Chemically synthesized E-7G-4′S and E-7S-4′G were individually separated into 2 peaks by preparative HPLC chromatography using a Synergi Hydro-RP (Phenomenex) column. The compounds

TABLE 3 ^{1}H-NMR spectra of equol and its metabolites (δ in DMSO-d_6)

	Equol		E-4′,7-diG		E-4′,7-diS		E-7G-4′S		E-7S-4′G		E-4′-G		E-7-G		E-4′-S		E-7-S	
Proton	δ/ppm	J/Hz	δ/ppm	J/Hz	δ/ppm	J/Hz	δ/ppm	J/Hz	δ/ppm	J/Hz	δ/ppm	J/Hz	δ/ppm	J/Hz	δ/ppm	J/Hz	δ/ppm	J/Hz
2	3.89 (dd)	10.4, 10.5	4.00 (dd)	9.6, 10.8	3.97 (dd)	10.5, 10.6	4.00 (dd)	10.5, 10.5	3.96 (dd)	10.2, 10.8	3.94 (dd)	10.2, 10.8	3.94 (dd)	10.4, 10.8	3.94 (dd)	10.2, 10.8	3.93 (dd)	10.4, 10.5
	4.14 (d)	10.4, 10.5	4.20 (d)	9.6	4.22 (d)	10.4	4.23 (d)	9.5, 10.5	4.20 (d)	10.2, 10.8	4.17 (d)	10.2	4.18 (d)	10.2	4.18 (d)	10.8	4.18 (d)	10.4
3	3.00 (m)		3.10 (m)		2.95 (m)		3.10 (m)		3.10 (m)		3.08 (m)		3.04 (m)		3.08 (m)		3.04 (m)	
4	2.81 (m)		2.90 (m)		2.86 (m)		2.90 (m)		2.90 (m)		2.84 (m)		2.86 (m)		2.84 (m)		2.85 (m)	
5	6.86 (d)	8.2	7.00 (d)	9.0	6.98 (d)	8.1	7.01 (d)	8.5	6.96 (d)	8.2	6.87 (d)	8.4	7.00 (d)	8.5	6.87 (d)	8.4	6.96 (d)	8.2
6-H	6.28 (dd)	2.4, 8.2	6.54 (dd)	2.4, 7.8	6.66 (d)	2.3	6.56 (dd)	2.5, 8.5	6.65 (m)		6.29 (dd)	2.4, 8.4	6.53 (d)	8.3	6.29 (dd)	2.4, 8.4	6.65 (dd)	2.4, 8.4
8-H	6.18 (d)	2.5	6.47 (dd)	2.4, 3.0	6.64 (m)		6.50 (d)	2.5	6.65 (m)		6.19 (d)	2.4	6.46 (d)	2.0	6.19 (d)	2.4	6.63 (d)	2.4
2′, 6′-H	7.11 (d)	8.5	7.23 (dd)	6.0, 8.4	7.23 (d)	8.6	7.24 (dd)	1.5, 8.5	7.23 (d)	8.7	7.23 (d)	8.4	7.11 (dd)	1.7, 8.4	7.22 (d)	8.4	7.12 (d)	8.4
3′,5′-H	6.72 (d)	8.5	7.00 (d)	9.0	7.12 (d)	8.6	7.13 (d)	8.0	6.98 (d)	8.6	6.98 (d)	8.4	6.72 (d)	7.6	7.12 (d)	9.0	6.73 (d)	9.0

Abbreviations: d, doublet; dd, doublet of doublets; m, multilet.

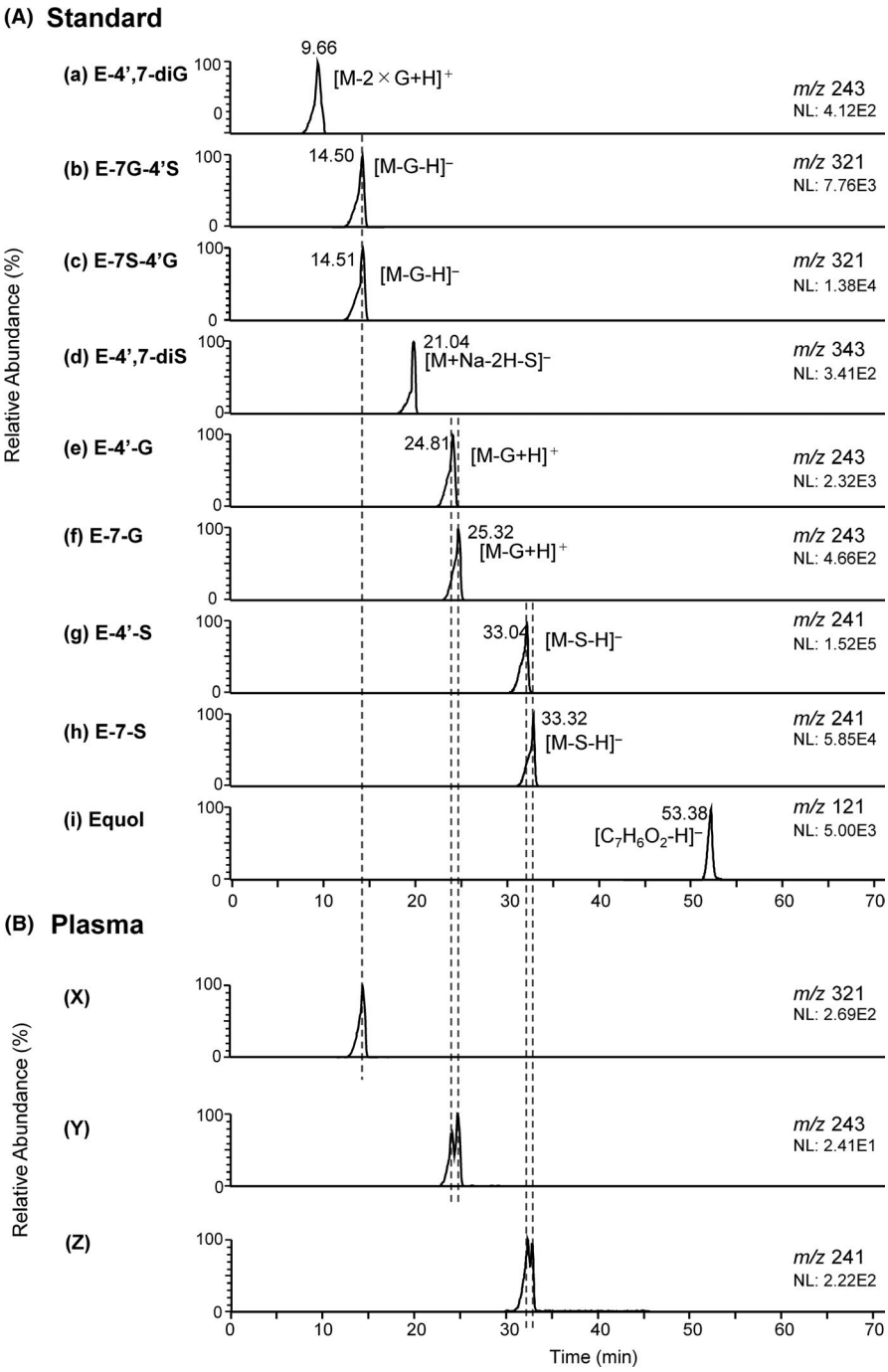


FIGURE 2 LC-ESI-MS/MS chromatograms of (A) chemically synthesized metabolites of equol (E-4′,7-diG, E-7G-4′S, E-7S-4′G, E-4′,7-diS, E-4′-G, E-7-G, E-4′-S, E-7-S) and equol, and (B) plasma extracts

corresponding to the respective peaks were fractionated and hydrolyzed using β-glucuronidase/sulfatase. The individual hydrolysate was confirmed as the (*S*)- or (*R*)- enantiomer by cochromatography with (*S*)- or (*R*)- equol using a chiral column. Based on the results of this analysis it was established that the compounds corresponding to 2 peaks were the (*S*)- and (*R*)- diastereomer of E-7G-4′S and E-7S-4′G, respectively.

3.4 | Identification of E-7G-4′S in human plasma

As shown in Figure 2A, E-7G-4′S and E-7S-4′G could not be separated by HPLC chromatography using a Capcell Pak ADME column. However, upon elution using a mixed solvent system comprising 10 mmol/L ammonium acetate solution (solvent A) and acetonitrile (solvent B) with a linear gradient from 98% to 95% of solvent A (0-35 minutes), the mixture of E-7G-4′S and E-7S-4′G could be separated into 3 partially overlapping peaks (Figure 3A (a)).

Figure 3A shows the MS/MS chromatograms of the (*R*)- and (*S*)-diastereomers of E-7G-4′S (b) and E-7S-4′G (c), respectively. Although it was possible to separate (*S*)-E-7G-4′S and (*R*)-E-7S-4′G, (*R*)-E-7G-4′S and (*S*)-E-7S-4′G could not be separated. Figure 3B illustrates the MS/MS chromatograms at *m/z* 321 for E-7G-4′S or E-7S-4′G in human plasma after dietary administration of *kinako* to 2 equol producers (Subject 1 and 2). It is obvious that the main peak in the MS/MS chromatograms of plasma taken from the 2 volunteers

corresponds to (*S*)-E-7G-4′S based on spiking experiments using an authentic sample of (*S*)-E-7G-4′S. However, it is uncertain whether or not the shoulder of the peak of (*S*)-E-7G-4′S was due to (*R*)-E-7G-4′S or (*S*)-E-7S-4′G. Nonetheless, the amount of ions in this shoulder peak was approximately 3% of the total ions of (*S*)-E-7G-4′S.

3.5 | Semi-quantification

Plasma samples (0.2 mL) were extracted using an Oasis® HLB cartridge (3 cc, 60 mg packing) (Waters) according to a previously reported method.[16] The recoveries (n = 3) of equol metabolites and equol in plasma using this cartridge varied between 94.0 and 114.0%. The main equol metabolites of both volunteers were (*S*)-E-7G-4′S [Sub 1, 28.9 ng/mL (35.8%); Sub 2, 10.9 ng/mL (57.4%)] (Figure 4). E-monoG (E-4′-G, E-7-G) and E-monoS (E-4′-S, E-7-S) were also found in both plasma samples [E-4′-G (Sub 1, 7.5 ng/mL; Sub 2, 0.6 ng/mL), E-7-G (Sub 1, 19.6 ng/mL; Sub 2, 6.4 ng/mL), E-4′-S (Sub 1, 7.6 ng/mL; Sub 2, 0.3 ng/mL), and E-7-S (Sub 1, 17.1 ng/mL; Sub 2, 0.8 ng/mL)].

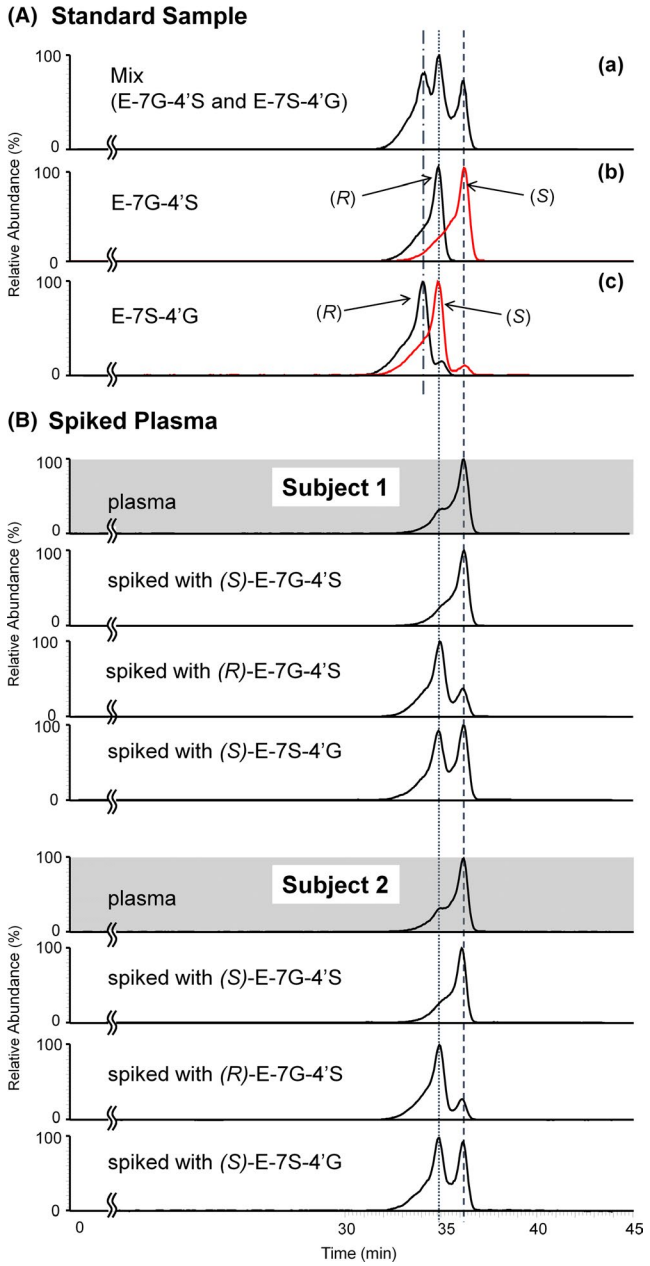


FIGURE 3 LC-ESI-MS/MS (*m/z* 321) chromatograms; (A) chemically synthesized metabolites of a mixture of E-7G-4′S and E-7S-4′G (a), (*R*)- or (*S*)- E-7G-4′S (b), and E-7S-4′G (c). (B) Spiked plasma (Subject 1 and 2): plasma and plasma spiked with (*S*)-E-7G-4′S, (*R*)-E-7G-4′S, and (*S*)-E-7S-4′G, respectively

4 | DISCUSSION

Equol mostly exists in human plasma as its conjugated metabolites such as glucuronides and sulfates. To date, these conjugated metabolites have been quantified by measuring free equol obtained after selective enzymatic hydrolysis. Consequently, the exact types of conjugates circulating in vivo and the position(s) of the conjugation sites on the equol skeleton have not been determined. This study aims to clarify the plasma profile of equol metabolites.

In order to further elucidate the plasma profile of equol metabolites, we chemically synthesized the 8 phase II metabolites of equol including E-4′,7-diG, E-4′,7-diS, E-7G-4′S, E-7S-4′G, E-4′-G, E-7-G, E-4′-S, and E-7-S. In a previous work, which aimed to understand the pharmacokinetics of isoflavone metabolites in human plasma, we chemically synthesized various isoflavone metabolites. However, because Dein-4′-G and Gein-4′-G could not be synthesized at the time, these metabolites were isolated from human urine.[24] In a later study, it was found that Dein-4′-G′ rather than Dein-7-G′ was selectively formed as a by-product of the reaction of Dein with methyl-(2,3,4-tri-*O*-acetyl-α-D-glucopyranosyl trichloroacetimidate) uronate toward the preparation of Dein-4′,7-diG′. In particular, Dein-4′-G′ was obtained in an approximate 2:5 in weight to Dein-4′,7-diG′. The stereospecificity of this reaction led to the successful syntheses of E-4′-G and E-7S-4′G by using Dein-4′-G′ as starting material.

As shown in Figure 2A and B, although E-4′-G, E-7-G, E-4′-S, and E-7-S could not be completely separated at the baseline of the MS/MS chromatogram, these metabolites were detected in plasma. The other metabolites could be readily separated on the MS/MS chromatogram except for E-7G-4′S and E-7S-4′G. However, E-4′,7-diG, E-4′,7-diS, and equol could not be identified in plasma. E-7G-4′S or E-7S-4′G was separated into the corresponding 2 sets of diastereomers (Figure 3A (b) and (c)). The peak corresponding to (*S*)-E-7G-4′S in the MS/MS chromatograms of the plasma extracts of Subject 1 and Subject 2 (Figure 3B) was spiked with an authentic standard sample of (*S*)-E-7G-4′S. Because labeled compounds were unavailable, determination of equol metabolites in the plasma of 2 equol-producers obtained 8 hours after consumption of 50 g of *kinako* were performed by a standard addition method. Results of the semi-quantification analysis showed the major metabolite for Subject 1 and Subject 2 was (*S*)-E-7G-4′S (36 and 57% of total metabolites, respectively). There were also lower amounts

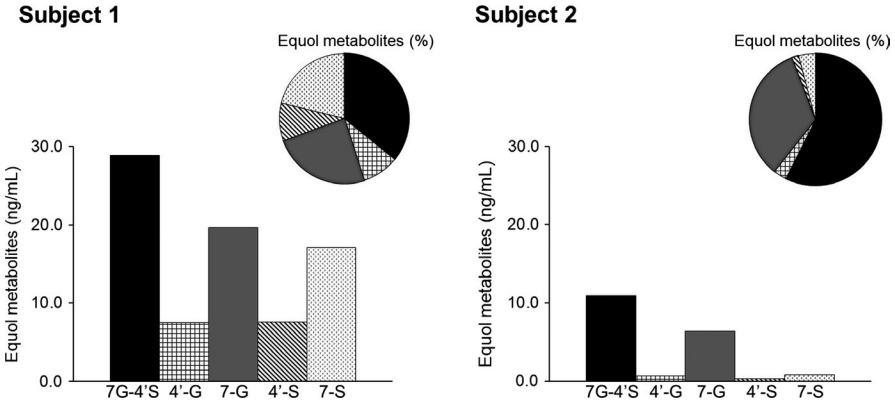


FIGURE 4 Percent contribution of each metabolite to the total concentration of equol metabolites in plasma (Subject 1 and Subject 2)

of E-7-G (24 and 34%), E-7-S (21 and 4%), E-4′-S (9 and 2%), and E-4′-G (9 and 3%) for Subjects 1 and 2, respectively (Figure 4). The difference in total amount of plasma metabolites between the 2 equol producers was significant. Specifically, the total amount of conjugated metabolites of equol in plasma samples from Subject 1 and Subject 2 were 0.33 and 0.08 μmol/L, respectively. This significant difference between the 2 equol producers may suggest a variation in the equol producing ability for biotransforming Dein into equol. Based on several previous reports, the main equol metabolite in human plasma is glucuronidated at the 7-position of the equol ring. The presence of several equol conjugated metabolites in human plasma has been reported in the literature. Gardana and Simonetti[29] identified the main metabolite of equol in the plasma of 12 female subjects as E-7-G by performing cochromatography with an authentic standard, while double conjugated metabolites such as diG, diS, and sulfate-glucuronide were not found. E-7-G was also described as the major metabolite produced by human pooled liver microsomes, while E-4′-G was not detected.[30] Our data also shows that E-7-G and E-7G-4′S, generated by sulfonation at the 4′ position of E-7-G, are the main metabolites. In this study, we investigated 2 healthy Japanese volunteers (aged 28 and 30 years), whereas Gardana and Simonetti[29] investigated Italian volunteers aged between 45 and 64 years. Therefore, the difference in the main equol metabolites by sulfonation at the 4′ position of E-7-G may be a result of the age difference and/or human race specificity. It should be noted that the 2 volunteers in this study were male (Subject 1) and female (Subject 2). Interestingly, the ratio of E-4′-G to E-4′-S in Subject 2 was very small (3 and 2%), while the corresponding ratio in Subject 1 was higher (9 and 9%). This observation suggests that the difference in the amount of metabolites might be gender related. Schwen et al[31] suggested that E-7S-4′G was one of the metabolites produced by human cryopreserved hepatocytes. Here, for the first time, intact (*S*)-E-7G-4′S was identified as the major metabolite in human plasma. Dein, Gein, and equol were conjugated by UDP-glucuronosyltransferase (UGTs)-mediated glucuronidation and sulfotransferase (SULTs)-mediated sulfonation.[32-34] UGTs and SULTs are present as multiple isoforms. There is a paucity of literature reports on the regioselective glucuronidation and sulfonation of equol. Nonetheless, the 4′-glucuronidation of Dein can be performed by UGT 1A10 contributing to the first-pass effect in the human small intestine.[35,36] However, in the human liver the 4′- and 7-glucuronidation are performed by UGT 1A1 and 1A9, respectively.[30,37] The SULTs responsible for the sulfonation of Dein and Gein are mainly SULT 1A1 for monosulfation at the 4′- or 7 -position and SULT 1E1 for disulfation at both positions.[26] Expression and function of these enzymes may be influenced by factors such as age, race, genetic polymorphisms, and gender.[38,39] Although the reason for the different occurrence of E-7G-4′S and E-7S-4′G as metabolites in human plasma is unclear, the data collected in this study on the plasma profile of equol producers will furnish useful information on the metabolism and disposition of phase II metabolites. As we investigated only 2 equol producers, therefore, a larger number of volunteers are needed to clarify the profile of the conjugated metabolites.

AUTHOR CONTRIBUTIONS

Participated in research design: Obara, Kinoshita, Hosoda, Yokokawa, Shibasaki, and Ishii. Conducted experiments: Obara, Kinoshita, Hosoda, Yokokawa, Shibasaki, and Ishii. Performed data analysis: Obara, Kinoshita, Hosoda, Yokokawa, Shibasaki, and Ishii. Wrote or contributed to the writing of the manuscript: Obara, Yokokawa, Shibasaki, and Ishii.

DISCLOSURES

None declared.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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