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Identification and Quantification of the Conjugated Metabolites Derived from Orally Administered Hesperidin in Rat Plasma

HIKARU MATSUMOTO,* YOSHINORI IKOMA, MINORU SUGIURA, MASAMICHI YANO, AND YOSHINORI HASEGAWA

Department of Citrus Research, National Institute of Fruit Tree Science, Shimizu-okitsunakacho, Shizuoka, Shizuoka 424-0292, Japan

Hesperidin is a biologically effective flavonoid. Several studies have reported that dietary hesperidin was converted to conjugated metabolites, such as hesperetin-glucuronides and sulfoglucuronides, during absorption and metabolism. However, the chemical structures of the conjugated metabolites, especially the sites of glucuronidation and sulfoglucuronidation in plasma, were unconfirmed. Therefore, the concentrations of the metabolites conjugated at various sites in plasma could not be individually quantified. In the present study, we identified the chemical structures and concentrations of the major conjugated metabolites in rat plasma after oral administration of hesperidin. Two hesperetin-glucuronides were prepared and identified as hesperetin-7-*O*-*β*-D-glucuronide and hesperetin-3′-*O*-*β*-D-glucuronide. Using these authentic compounds, the concentrations of hesperetin-7-*O*-*β*-D-glucuronide and hesperetin-3′-*O*-*β*-D-glucuronide in rat plasma were individually determined by liquid chromatography−mass spectrometry. In rat plasma, hesperetin-glucuronides were primarily comprised of hesperetin-7-*O*-*β*-D-glucuronide and hesperetin-3′-*O*-*β*-D-glucuronide. The concentration of hesperetin-7-*O*-*β*-D-glucuronide was slightly higher than that of hesperetin-3′-*O*-*β*-D-glucuronide. Furthermore, not only hesperetin conjugates but also homoeriodictyol conjugates were observed in rat plasma. The present study is the first report elucidating the chemical structures and changes in individual concentrations in rat plasma of glucuronides derived from orally administered hesperidin.

KEYWORDS: Hesperidin; metabolite; hesperetin-glucuronide; homoeriodictyol-glucuronide; rats

INTRODUCTION

Hesperidin (**Figure 1**), a flavanone type flavonoid abundant in citrus fruit, has biologically beneficial effects, such as cholesterol-lowering and anticarcinogenic activities (*1*−*4*). Several studies with animals have investigated the absorption, bioavailability, and pharmacokinetics of citrus flavanones, such as hesperidin and naringin (*5*−*8*). These studies suggested that orally administered hesperidin and naringin are absorbed as aglycones (hesperetin and naringenin) after the removal of disaccharide by intestinal bacteria and that they are immediately metabolized to glucuronides in the intestinal epithelium and liver. Thus, hesperidin, naringin, and their aglycones have not been observed in plasma, bile, and urine (*5*−*8*). Several reports in studies with humans have noted that the major metabolites of hesperidin and naringin in urine and plasma were the glucuronides of hesperetin and naringenin (*9*−*12*). In these studies, the conjugated metabolites, such as glucuronides and sulfoglucuronides in plasma, were quantified from amounts of aglycone liberated from conjugated metabolites by hydrolysis with deconjugation enzymes, *β*-glucuronidase, and sulfatase. In these studies, the chemical structures of conjugated metabolites, especially the sites of glucuronidation and sulfoglucuronidation, were unconfirmed. Therefore, metabolites conjugated at various sites could not be individually quantified.

To investigate the biological activities of hesperidin in vitro, most studies used hesperidin and/or hesperetin, which are not present in plasma (*3*, *4*). These results may not appropriately indicate the biological effects of orally ingested hesperidin because conjugated metabolites, rather than hesperidin and hesperetin, exist in plasma. To understand the precise mechanisms showing the biological activities of orally ingested hesperidin, it is important to identify the really active chemical structure and site of glucuronidation and sulfoglucuronidation in conjugated metabolites and to prepare the really active compounds for assays in vitro.

Recently, several reports on flavonoids, such as (+)-catechin and quercetin, noted that flavonoid metabolites in plasma have biological activities (*13*−*16*). In some cases, the activities of the metabolites in vitro were different from those of intact forms in food. Koga et al. (*13*) reported that (+)-catechin metabolites extracted from plasma inhibited the adhesion of monocyte cells to human aortic endothelial cells (HAEC) stimulated with interleukin-1b and the generation of reactive oxygen species in HAEC, whereas intact (+)-catechin had no effect. Day et al. (*14*) reported that quercetin glucuronides had inhibitory effects

* To whom correspondence should be addressed. Tel: 81-543-69-7100. Fax: 81-543-69-2115. E-mail: hikaruoo@affrc.go.jp.

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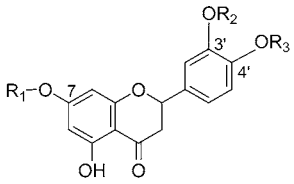

Compound Name	R_1	R_2	R_3
Hesperidin	Rhamonosylglucose	H	CH_3
Hesperetin-7-*O*-β-D-glucuronide	Glucuronic acid	H	CH_3
Hesperetin-3'-*O*-β-D-glucuronide	H	Glucuronic acid	CH_3
Homoeriodictyol-glucuronide	(Glucuronic acid or H)*	CH_3	(Glucuronic acid or H)*
Hesperetin	H	H	CH_3
Homoerioictyol	H	CH_3	H
Eriodictyol	H	H	H

Figure 1. Chemical structures of compounds analyzed in this study. *The site of monoglucuronidation was not identified in the present study. Either R_1 or R_2 is glucuronidated.

on the activities of xanthine oxidase and lipoxygenase and that their effects depended on conjugated sites. Proteggente et al. (*16*) reported that hesperetin-glucuronides, but not hesperetin, prevented UV-A-induced necrotic cell death. These findings suggest that the conjugated metabolites derived from hesperidin, rather than hesperidin itself, the intact form in food, may possess an important biological activity.

In the present study, to determine the chemical structures of conjugated metabolites derived from hesperidin, we prepared authentic compounds, several hesperetin-glucuronides synthesized chemically and isolated from rat urine. By using these authentic compounds and liquid chromatography−mass spectrometry (LC-MS), two hesperetin-glucuronides, hesperetin-7-*O*-β-D-glucuronide and hesperetin-3′-*O*-β-D-glucuronide (**Figure 1**), were identified as conjugated metabolites in rat plasma after oral administration of hesperidin. We also demonstrated that hesperetin-glucuronides were comprised primarily of hesperetin-7-*O*-β-D-glucuronide and hesperetin-3′-*O*-β-D-glucuronide. The concentration of hesperetin-7-*O*-β-D-glucuronide was slightly higher than that of hesperetin-3′-*O*-β-D-glucuronide. Furthermore, not only hesperetin conjugates but also homoeriodictyol conjugates were observed in rat plasma.

MATERIALS AND METHODS

Chemicals. Hesperidin, hesperetin, uridine-5′-diphospho (UDP)-glucuronic acid, UDP-*N*-acetylglucosamine, β-glucuronidase type VII-A (EC 3.2.1.31, from *Escherichia coli*), sulfatase type H-5 (EC 3.1.6.1, from *Helix pomatia*), sulfatase type VIII (EC 3.1.6.1, from *Abalone entrails*), and UDP-glucuronosyltransferase 1A6 isozyme human (EC 2.4.1.17, recombinant) were purchased from Sigma Chemical Co. (St. Louis, MO). Apigenin and homoeriodictyol were obtained from Extrasynthese (Genay, France). Other chemicals and solvents were obtained from Wako Pure Chemical Ind., Ltd. (Osaka, Japan) and Aldrich Chemical Co. Inc. (Milwaukee, WI) and used without further purification.

Chemical Synthesis of Hesperetin-glucuronide. Acetobromo-α-D-glucuronic acid methyl ester (0.70 mmol), silver carbonate (0.70 mmol), and molecular sieves 4A (metal aluminosilicates having many pores, which absorb H_2O) were added into a solution of hesperetin (0.3 mmol in 5 mL of anhydrous chloroform−pyridine) at 0 °C. The mixture was stirred for 36 h in dark at room temperature under an atmosphere of argon. After the mixture was filtered, the filtrate was dissolved in 30 mL of ethyl acetate (EtOAc). The EtOAc phase was washed with 10% citric acid and saturated NaCl and subsequently dehydrated with anhydrous Na_2SO_4 and concentrated under vacuum. The concentrate was applied to a silica gel column, 150 mm × 25 mm i.d., 63−210 μm, neutral silica gel 50N (Kanto Chemical, Tokyo, Japan), conditioned with chloroform, and eluted with chloroform−methanol (MeOH) (40:1). After the fractions were concentrated, 79.4 mg of the crude product was obtained as a colorless oil. The crude product was dissolved in 5 mL of anhydrous MeOH. The solution was stirred for 30 min at 0 °C after adding 28% sodium methoxide in MeOH (150 μL). The solution was then stirred for 1 h at room temperature after adding 300 μL of distilled water. The solution was neutralized with Amberlite IR-120 (H^+) resin (ICN Biochemicals Inc., United States) and concentrated under vacuum. The residue was applied to a silica gel column and eluted by EtOAc−MeOH−H_2O (80:15:10). The eluent was applied to a HP 1100 series high-performance liquid chromatographer (HPLC) (Agilent Technologies Inc., Waldbronn, Germany) equipped with a C18 reverse-phase column, 150 mm × 20 mm i.d., 5 μm, YMC Pack ODS-A (YMC Co., Ltd., Kyoto, Japan) and a photodiode array detector (Agilent Technologies Inc.). The mobile phase consisted of two eluents, 0.1% aqueous trifluoroacetic acid (TFA) (solvent A) and acetonitrile (solvent B). The gradient program was as follows: the initial composition consisted of 80% A and 20% B for 2 min, followed by a linear gradient to 80% B for 60 min. The single peak separated by the HPLC was collected. From the peak, 32 mg of the product was obtained as white foam by lyophilization. The product was analyzed by LC-MS and NMR and identified as highly pure hesperetin-7-*O*-β-D-glucuronide.

Isolation of Hesperetin-glucuronide from Rat Urine. Animals were acclimated as explained in the section concerning animals and their diets. After acclimation, rat urine (500 mL) from 15 rats was collected in metabolic cages (Natsume Seisakusho, Tokyo, Japan) for 18 h after the administration of hesperidin (100 mg/kg in 10 mL of 0.5% carboxymethyl cellulose). After filtration, 450 mL of the urine was acidified with the same volume of 0.01 mol/L oxalic acid. The diluted urine was applied to a column, 150 mm × 25 mm i.d., 140 μm, Cosmosil 140 C18-PREP (Nacalai Tesque, Kyoto, Japan). After the column was washed with 5% aqueous MeOH, the metabolites from the urine were eluted with MeOH. The eluents were evaporated. The eluents were applied to the HPLC equipped as above and eluted by 0.1% aqueous TFA (solvent A) and acetonitrile (solvent B) in a gradient manner. The gradient program was as follows: the initial composition consisted of 90% A and 10% B for 5 min, followed by a linear gradient to 20% B for 5 min, 45% B for 25 min, and 80% B for 15 min. The eluents were then applied to another HPLC equipped with a C18 reverse-phase column, 250 mm × 10 mm i.d., 5 μm, GL-Pak Hypersil ODS (GL Science Inc., Tokyo, Japan), and a photodiode array detector. The mobile phase consisted of two eluents, 0.1% aqueous acetic acid (solvent A) and acetonitrile (solvent B). The gradient program was as follows: the initial composition consisted of 80% A and 20% B for 2

min, followed by a linear gradient to 45% B for 20 min, and 70% B for 11 min. The single peak separated by the HPLC was collected. From the peak, 3 mg of the metabolite was obtained as white foam by lyophilization. The metabolite was analyzed by LC-MS and NMR and identified as hesperetin-3′-*O*-*β*-D-glucuronide.

Enzymatic Synthesis of Homoeriodictyol-glucuronide. Homoeriodictyol dissolved in dimethyl sulfoxide (DMSO) (10 mmol/L, 2.5 μL) was mixed with 497.5 μL of a buffer containing 25 mmol/L HEPES (pH 7.4), 4 mmol/L UDP-glucuronic acid, 2 mmol/L UDP-*N*-acetylglucosamine, 10 mmol/L magnesium chloride, and 0.13 unit/mL UDP-glucuronosyltransferase and subsequently incubated for 18 h at 37 °C. The reaction mixture was applied to a BondElut C18 cartridge (Varian, Harbor City, CA). After the cartridge was washed with 0.01 mol/L oxalic acid and subsequently with distilled water, the product was eluted with 1 mL of MeOH. The eluent was evaporated to dryness and dissolved in 50 μL of MeOH. After filtration, the filtrate (5 μL) was analyzed by LC-MS and identified as homoeriodictyol-glucuronide.

LC-MS Analysis. LC-MS was performed on a HP 1100 series HPLC (Agilent Technologies, Inc.) equipped with a JEOL (JMS-700, Tokyo, Japan) Mstation-700, double-focusing magnetic sector mass spectrometer. An aliquot (5 μL) of a sample was injected into the LC-MS and separated by a C18 reverse-phase column, 75 mm × 2.0 mm i.d., 5 μm, YMC-Pack Pro ODS, at a flow rate of 0.2 mL/min. The column temperature was maintained at 35 °C. The mobile phase consisted of solvents A (0.1% aqueous TFA) and B (acetonitrile). The gradient program was as follows: the initial composition consisted of 80% A and 20% B for 2 min, followed by a linear gradient to 45% B for 18 min, 65% B for 10 min, and 100% B for 5 min. The eluent was ionized by negative electrospray ionization. The electrospray needle voltage was set to 2.04 kV, the ring lens voltage was set to 31.65 V, and the ion guide voltage was set to 3.0 V. The desolvation temperature was 250 °C. The nebulizing gas was nitrogen, and it was used at 7 kg/cm^2 and 5 L/min. Negative ions (m/z = 269, 301, 477, and 609) were monitored individually by the selected ion monitoring mode (SIM) with an ion accelerating voltage of 5 kV and a dwell time of 100 ms. These ions were the molecular ions $[M - H]^-$ derived from internal standard (apigenin, m/z 269), hesperetin (m/z 301), homoeriodictyol (m/z 301), hesperetin-monoglucuronides (m/z 477), and hesperidin (m/z 609).

The quantifications of hesperetin-7-*O*-*β*-D-glucuronide, hesperetin-3′-*O*-*β*-D-glucuronide, hesperetin, and homoeriodictyol were performed according to an internal standard method. Apigenin was used as an internal standard. For the preparation of standard solutions, hesperetin, homoeriodictyol, hesperetin-7-*O*-*β*-D-glucuronide, and hesperetin-3′-*O*-*β*-D-glucuronide dissolved in DMSO were added into blank plasma. After apigenin was added into the standard solutions, a five-point calibration curve (0.05, 0.1, 1.0, 5.0, and 10.0 μmol/L) was constructed for each compound according to an internal standard method. The calibration curve of each compound showed excellent linearity in the range of 0.05−10 μmol/L. The quantification of plasma samples was performed within the linear range. The limits of quantitation in plasma for these compounds were 0.05 μmol/L. The recoveries of these compounds from plasma were >95%.

NMR Spectroscopy. NMR spectra were recorded on a Bruker DPX-400 spectrometer (Bruker BioSpin, Rheinstetten, Germany). Samples were dissolved in DMSO (containing 99.6% DMSO-d_6 and 0.4% DMSO). Chemical shifts were expressed in ppm relative to DMSO (δ 2.50) and DMSO-d_6 (δ 39.52) as internal standards.

Animals and Diets. Male Wistar rats (200−220 g) were obtained from Clea Japan, Inc. (Tokyo, Japan). The animals were acclimated in an air-conditioned room at 23 °C under a 12 h light/dark cycle, with free access to tap water and a standard MF diet (Oriental Yeast Co., Ltd., Tokyo, Japan). The experiments were started after acclimating the animals for at least 1 week. Animals were maintained and handled according to the National Institutes of Health Guide for Care and Use of Laboratory Animals (*17*).

Preparation of Rat Plasma. The acclimated rats (n = 5) were fasted overnight and placed in metabolic cages. Fifty mg/kg of hesperidin in 10 mL of 0.5% carboxymethyl cellulose was orally administered to rats by gastric intubation. At 0, 4, 6, 8, 10, 12, 15, 18, and 24 h after the administration of hesperidin, 100 μL of blood was withdrawn from the heart under anesthetization with ether. The sampled blood was immediately mixed into the same volume of phosphate-buffered saline containing heparin (2 units /mL). After centrifugation (1000*g*) of the blood samples at 4 °C for 10 min, rat plasma was isolated. The plasma was divided into four aliquots (30 μL). One microliter of DMSO containing apigenin (50 μmol/L), an internal standard, was added into an aliquot of plasma. One aliquot of plasma was used for direct quantification of the hesperidin metabolites. Other aliquots of plasma were used for the quantification of metabolites after a treatment with deconjugation enzymes.

Direct Quantification of Hesperetin-7-*O*-*β*-D-glucuronide and Hesperetin-3′-*O*-*β*-D-glucuronide in Rat Plasma. The concentrations of hesperetin-7-*O*-*β*-D-glucuronide and hesperetin-3′-*O*-*β*-D-glucuronide in plasma were directly quantified using an authentic sample. An aliquot of plasma was acidified with 100 μL of 0.01 mol/L oxalic acid. The solution was applied to BondElut C18 cartridge conditioned with MeOH and subsequently with distilled water. After the cartridge was washed with 0.01 mol/L oxalic acid and subsequently with distilled water, the metabolites were eluted with 1 mL of MeOH. The eluent was evaporated to dryness and dissolved in 50 μL of MeOH. After centrifugal filtration (0.22 μm filter unit), the filtrate (5 μL) was analyzed using LC-MS.

Indirect Quantification of Conjugated Metabolites by Hydrolysis with Enzymes. The concentrations of total hesperetin conjugates and total homoeriodictyol conjugates in plasma were quantified from the amount of corresponding aglycone (hesperetin or homoeriodictyol) liberated from the conjugated metabolites by hydrolysis with *β*-glucuronidase and sulfatase. The concentration of glucuronides was quantified from the amount of corresponding aglycone (hesperetin or homoeriodictyol) liberated from the conjugated metabolites by hydrolysis with *β*-glucuronidase. The concentration of sulfates was quantified from the amount of corresponding aglycone (hesperetin or homoeriodictyol) liberated from the conjugated metabolites by hydrolysis with sulfatase.

The conditions of hydrolysis are described in the following: for hydrolysis by *β*-glucuronidase, an aliquot of plasma was incubated with 30 μL of a 0.1 mol/L sodium acetate buffer (pH 6.8) containing *β*-glucuronidase type VII-A (30 units) for 90 min at 37 °C; for hydrolysis by sulfatase, an aliquot of plasma was incubated with 30 μL of a 0.1 mol/L sodium acetate buffer (pH 5.0) containing sulfatase type VIII (15 units) and 10 μL of a 0.1 mol/L sodium acetate buffer (pH 5.0) containing saccharic acid 1,4-lactone (30 mmol/L) for 2 h at 37 °C (*18*); for hydrolysis by *β*-glucuronidase and sulfatase, an aliquot of plasma was incubated with 30 μL of a 0.1 mol/L sodium acetate buffer (pH 6.8) containing *β*-glucuronidase type VII-A (30 units) for 90 min at 37 °C and subsequently with 60 μL of a 0.1 mol/L sodium acetate buffer (pH 5.0) containing sulfatase type H-5 (30 units) for 1 h at 37 °C. To stop the reaction of enzymes, 0.01 mol/L oxalic acid (100 μL) was added into the reaction mixture. The reaction mixtures were purified with a BondElut C18 cartridge as described above and analyzed by LC-MS.

RESULTS

Chemical Structure Determination of Hesperetin-glucuronide Synthesized Chemically. To synthesize hesperetin-glucuronide, the Koenigs−Knorr reaction was applied (*19*, *20*). Hesperetin was combined with acetobromo-α-D-glucuronic acid methyl ester in the presence of silver carbonate and subsequently deacetylated and saponified. By silica gel chromatography and HPLC, compound **1** was purified. These experimental procedures gave compound **1** with an overall yield of 20.2%.

The chemical structure of compound **1** was elucidated by LC-MS and NMR. The LC-MS spectrum of compound **1** showed a prominent peak at m/z 477 corresponding to the molecular ion $[M - H]^-$ of hesperetin-monoglucuronide (data not shown). ^{1}H NMR, ^{13}C NMR, ^{1}H−^{1}H COSY (correlation spectrometry), and HMBC (heteronuclear multiple bond correlation) experiments were performed. The assignments of ^{1}H NMR and ^{13}C NMR signals for compound **1** are summarized in **Table 1**. The

Table 1. Assignment of ^{1}H NMR and ^{13}C NMR Signals for Compounds **1** and **2**

	chemical shift of **1**		chemical shift of **2**	
position	^{1}H NMR (*J*; Hz)	^{13}C NMR	^{1}H NMR (*J*; Hz)	^{13}C NMR
2	5.50 dd (12.3, 3.1)	78.5	5.47 dd (12.4, 3.0)	78.2
3 (axial)	3.30 dd (17.2, 12.3)	42.2	3.28 dd (17.1, 12.4)	41.9
3 (equatorial)	2.75 dd (17.2, 3.1)		2.73 dd (17.1, 3.0)	
4		197.0		196.1
5		162.9		163.4
6	6.14 d (2.0)	96.4	5.88 d (2.0)	95.9
7		165.0		166.8
8	6.19 d (2.0)	95.3	5.91 d (2.0)	95.1
9		162.6		162.8
10		103.3		101.7
1′		130.9		130.8
2′	6.94 d (2.2)	114.1	7.25 d (1.7)	114.1
3′		146.5		146.0
4′		148.0		149.3
5′	6.93 m	112.1	7.02 d (8.4)	112.5
6′	6.89 dd (8.1, 2.2)	117.8	7.09 m	120.7
O-Me	3.77 s	55.7	3.77 s	55.8
		glucuronide		
1″	5.11 d (7.1)	99.0	5.09 d (7.2)	99.7
2″	3.23 m	72.8	3.31	72.9
3″, 4″	3.31–3.28 m	71.4, 75.8	3.38–3.32 m	71.4, 76.2
5″	3.82–3.80 m	74.9	3.82–3.80 m	75.2
6″		170.4		170.4

^{13}C NMR spectrum of compound **1** showed six signals assigned to a glucuronide moiety, including one anomeric carbon (δ 99.0) and one carboxyl carbon (δ 170.4), in addition to those assigned to a hesperetin moiety. In the ^{1}H NMR spectrum of compound **1**, two proton signals, which possessed the same coupling constant ($J = 2.0$ Hz), were observed at δ 6.14 and 6.19. These two signals were assigned to the A ring of hesperetin. Three proton signals at δ 6.89 (1H, dd, $J = 8.1$, 2.2 Hz), 6.93 (1H, m), and 6.94 (1H, d, $J = 2.2$ Hz) were assigned to the B ring of hesperetin. Three proton signals at δ 5.50, 3.30, and 2.75 were assigned to the C ring of hesperetin. The proton signal at δ 5.50 (1H, dd, $J = 12.3$, 3.1 Hz) was assigned to H-2 in the C ring of hesperetin. The proton signal at δ 3.30 (1H, dd, $J = 17.2$, 12.3 Hz) was assigned to axial H-3. The proton signal at δ 2.75 (1H, dd, $J = 17.2$, 3.1 Hz) was assigned to equatorial H-3 in the C ring of hesperetin. These data indicated the presence of hesperetin moiety. The signals of H-1″−H-5″ indicated the presence of a glucuronide moiety. In addition, the coupling constant for the anomeric proton (H-1″) of glucuronide moiety showed the characteristics of the β-amoner (δ 5.11, d, $J = 7.1$ Hz). In the HMBC spectrum, the cross-peak from H-1″ (δ 5.11) of the glucuronide moiety to C-7 (δ 165.0) of the hesperetin moiety was observed. In addition, the cross-peak from the proton (δ 3.77) of the methoxy group to C′-4 (δ 148.0) of hesperetin was detected in the HMBC spectrum. The cross-peak observed in the HMBC spectrum showed that the C-7 of hesperetin was *O*-glucuronidated. From these results, the structure of compound **1** was identified as hesperetin-7-*O*-β-D-glucuronide (**Figure 1**). Therefore, compound **1** was used as an authentic sample for the identification and quantification of hesperetin-7-*O*-β-D-glucuronide in rat plasma.

Chemical Structure Determination of Hesperetin-glucuronide Isolated from Rat Urine. Compound **2** was isolated from rat urine by silica gel chromatography and HPLC. These experimental procedures gave 3 mg of compound **2**.

The chemical structure was characterized by procedures described for compound **1**. The LC-MS spectrum of compound **2** showed a prominent peak at *m*/*z* 477 corresponding to the molecular ion [M − H]$^-$ of hesperetin-monoglucuronide (data not shown). The assignments of ^{1}H NMR and ^{13}C NMR signals for compound **2** are summarized in **Table 1**. The characteristics of signals for ^{1}H NMR and ^{13}C NMR in compound **2** were similar to those in compound **1**, indicating the presence of hesperetin and glucuronide moiety. In the HMBC spectrum, the cross-peak from H-1″ (δ 5.09) of the glucuronide moiety to C-3′ (δ 146.0) of the hesperetin moiety was observed. In addition, the cross-peak from the proton (δ 3.77) of the methoxy group to C′-4 (δ 149.3) of hesperetin was detected in the HMBC spectrum. The cross-peak observed in the HMBC spectrum showed that the C-3′ of hesperetin was *O*-glucuronidated. From these results, the structure of compound **2** was identified as hesperetin-3′-*O*-β-D-glucuronide (**Figure 1**). Therefore, compound **2** was used as an authentic sample for the identification and quantification of hesperetin-3′-*O*-β-D-glucuronide in rat plasma.

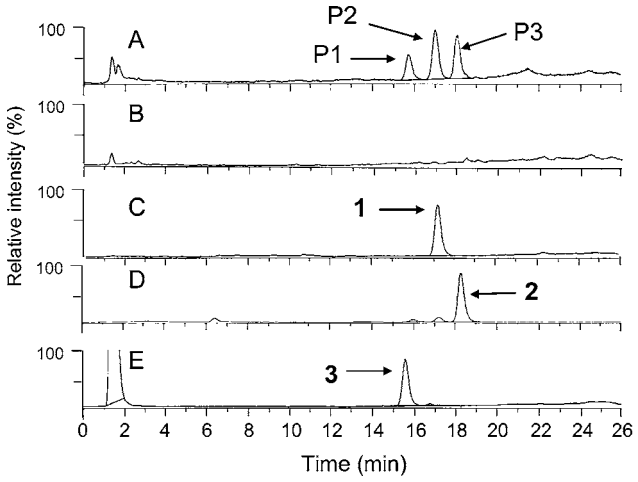

Figure 2. LC-MS chromatograms monitored at *m*/*z* 477 for hesperetin and homoeriodictyol-monoglucuronide analyses. An ion of *m*/*z* 477 corresponds to the molecular ion [M − H]$^-$ of hesperetin and homoeriodictyol-monoglucuronide. After oral administration, three peaks (P1, P2, and P3) were detected in rat plasma without hydrolysis. (**A**) Rat plasma without hydrolysis, (**B**) rat plasma hydrolyzed with β-glucuronidase, (**C**) compound **1** identified as hesperetin-7-*O*-β-D-glucuronide, (**D**) compound **2** identified as hesperetin-3′-*O*-β-D-glucuronide, and (**E**) compound **3** identified as homoeriodictyol-glucuronide.

Synthesis of Homoeriodictyol-monoglucuronide. Homoeriodictyol-monoglucuronide was enzymatically synthesized, and the reaction mixture was analyzed by LC-MS. In the LC-MS chromatogram monitored at *m*/*z* 477, which corresponds to the molecular ion [M − H]$^-$ of homoeriodictyol-monoglucuronide, a peak was observed at a retention time (R_t) of 15.7 min (**Figure 2E**). The LC-MS spectrum of the peak showed a prominent peak at *m*/*z* 477 (data not shown). The LC-MS spectrum suggested that compound **3** was identified as homoeriodictyol-monoglucuronide, although the conjugated position was unclear (**Figure 1**). Actually, as shown in **Figure 2**, the retention time of compound **3** ($R_t = 15.7$ min) was different from those of **1** ($R_t = 17.2$ min, **Figure 2C**) and **2** ($R_t = 18.3$ min, **Figure 2D**).

Identification of Hesperetin-monoglucuronides and Homoeriodictyol-monoglucuronides in Rat Plasma. In rat plasma, hesperidin and its metabolites were identified by LC-MS with the SIM mode. Hesperidin (monitored at *m*/*z* 609) was not observed in rat plasma after oral administration of hesperidin (data not shown).

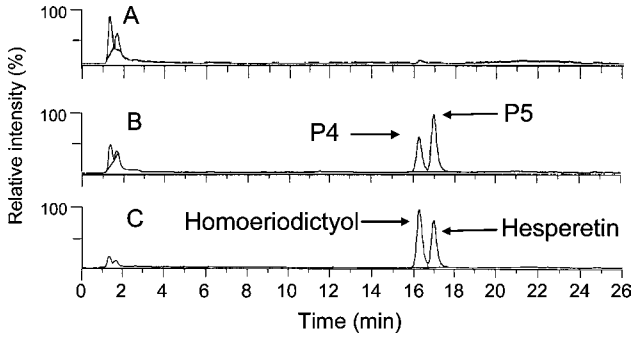

Figure 3. LC-MS chromatograms monitored at *m*/*z* 301 for hesperetin and homoeriodictyol analyses. An ion of *m*/*z* 301 corresponds to the molecular ion $[M - H]^-$ of hesperetin and homoeriodictyol. After oral administration, two peaks (P4 and P5) were detected in rat plasma hydrolyzed with β-glucuronidase. (**A**) Rat plasma without hydrolysis, (**B**) rat plasma hydrolyzed with β-glucuronidase, and (**C**) authentic hesperetin and homoeriodictyol.

For aglycone identification, negative ions were monitored at *m*/*z* 301 (**Figure 3**). Before hydrolysis with β-glucuronidase, a negative ion (*m*/*z* 301) corresponding to hesperetin, the aglycone of hesperidin, could not be observed in rat plasma (**Figure 3A**). These results suggested that the aglycone of hesperidin, hesperetin, did not exist in rat plasma. After the hydrolysis of the plasma with β-glucuronidase, two peaks (P4 and P5) were observed (**Figure 3B**). The retention time of P5 was consistent with that of authentic hesperetin, suggesting that P5 was hesperetin (**Figure 3B**,**C**). The retention time of P4 was consistent with that of authentic homoeriodictyol (3′,5,7-trihydroxy-3-methoxyflavanone), which is the isomer of hesperetin (3′,5,7-trihydroxy-4-methoxyflavanone), suggesting that P4 was homoeriodictyol (**Figure 3B**,**C**). These results suggested that not only hesperetin-glucuronide but also homoeriodictyol-glucuronide existed in rat plasma after oral administration of hesperidin. Therefore, we prepared compounds **1**, **2**, and **3** in the present study.

For hesperetin and homoeriodictyol-monoglucuronide identification, negative ions were monitored at *m*/*z* 477 corresponding to the molecular ion $[M - H]^-$ of hesperetin and homoeriodictyol-monoglucuronides (**Figure 2**). Before hydrolysis with β-glucuronidase, three peaks (P1, P2, and P3) were observed in rat plasma (**Figure 2A**). After the hydrolysis of the plasma with β-glucuronidase, these peaks disappeared completely (**Figure 2B**). These results suggested that these peaks (P1, P2, and P3) were glucuronides. The retention time of P1 was consistent with those of compound **3** (identified as homoeriodictyol-monoglucuronide), suggesting that P1 was homoeriodictyol-monoglucuronide (**Figure 2A**,**E**). The retention time of P2 was consistent with those of compound **1** (identified as hesperetin-7-*O*-β-D-glucuronide), suggesting that P2 was hesperetin-7-*O*-β-D-glucuronide (**Figure 2A**,**C**). The retention time of P3 was consistent with those of compound **2** (identified as hesperetin-3′-*O*-β-D-glucuronide), suggesting that P3 was hesperetin-3′-*O*-β-D-glucuronide (**Figure 2A**,**D**).

Quantification of Conjugated Metabolites Derived from Hesperidin in Rat Plasma. After hydrolysis with sulfatase alone, the aglycones, hesperetin and homoeriodictyol, were not detected in the plasma of hesperidin-administered rat throughout the experimental period (**Figure 4A**,**B**). This result suggested that the levels of the concentrations of hesperetin and homoeriodictyol sulfates were undetectable. After hydrolysis with β-glucuronidase alone and both β-glucuronidase and sulfatase, hesperetin and homoeriodictyol were detected. The concentrations of total hesperetin and homoeriodictyol conjugates (aglycones released by hydrolysis with β-glucuronidase and sulfatase) were higher than those of hesperetin and homoeriodictyol glucuronides (aglycones released by hydrolysis with β-glucuronidase) throughout the experimental period (**Figure 4A**,**B**). It was estimated that the difference in concentration between total conjugates and glucuronides was the concentration of sulfoglucuronide, since the concentration of sulfates was at an undetectable level in plasma. These results suggested that the glucuronides and sulfoglucuronides of hesperetin and homoeriodictyol were major conjugates derived from hesperidin in rat plasma. Moreover, it was estimated that most conjugates were glucuronides, with a composition of glucuronides to total conjugates: 66.4% at 4 h in hesperetin conjugates and 80.5% at 6 h in homoeriodictyol conjugates.

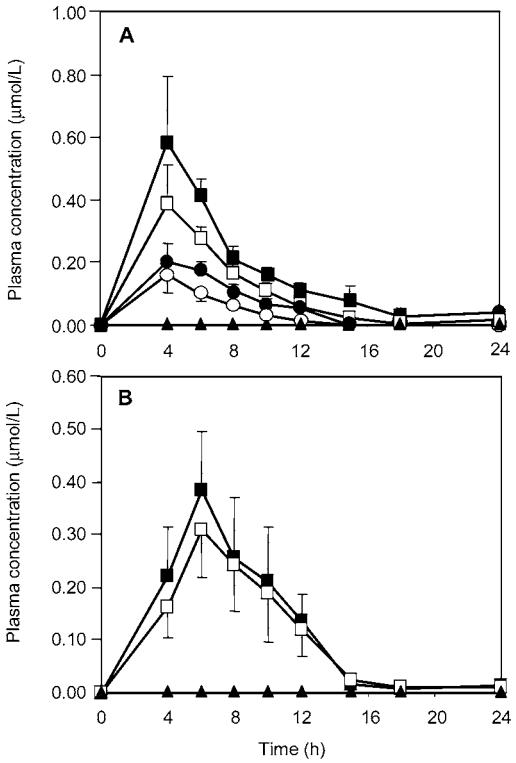

Figure 4. Time course of the concentrations of conjugated metabolites in rat plasma derived from hesperidin after oral administration. (**A**) Concentration of hesperetin conjugates. The total hesperetin conjugates (■), hesperetin-glucuronides (□), hesperetin-sulfates (▲), hesperetin-7-*O*-β-D-glucuronide (●), and hesperetin-3′-*O*-β-D-glucuronide (○) are plotted. (**B**) Concentrations of homoeriodictyol conjugates. The total homoeriodictyol conjugates (■), homoeriodictyol-glucuronides (□), and homoeriodictyol-sulfates (▲) are plotted. Values are the means ± SEM ($n = 3$).

The concentration of hesperetin-glucuronides reached a peak at 4 h (**Figure 4A**). In contrast, that of homoeriodictyol-glucuronides reached a peak at 6 h (**Figure 4B**). The time to reach a maximum concentration in homoeriodictyol-glucuronides was about 2 h later than that in hesperetin-glucuronides. In the 4 h following administration, the concentration of hesperetin-glucuronides in plasma was higher than that of homoeriodictyol-glucuronides. However, from 6 to 12 h after administration, the concentration of homoeriodictyol-glucuronides in plasma was comparable to that of hesperetin-glucuronides. The concentrations of both hesperetin-glucuronides and homoeriodictyol-glucuronides decreased to nearly undetectable levels at 24 h after administration.

The time course of the concentrations of hesperetin-7-*O*-*β*-D-glucuronide and hesperetin-3′-*O*-*β*-D-glucuronide in plasma was individually detected for 24 h after administration (**Figure 4A**). The level of the concentration of hesperetin-7-*O*-*β*-D-glucuronide in plasma was slightly higher than that of hesperetin-3′-*O*-*β*-D-glucuronide during the experimental period, with a composition to hesperetin-glucuronides: 51.6% in hesperetin-7-*O*-*β*-D-glucuronide and 41.4% in hesperetin-3′-*O*-*β*-D-glucuronide at 4 h, 62.3% in hesperetin-7-*O*-*β*-D-glucuronide and 34.9% in hesperetin-3′-*O*-*β*-D-glucuronide at 6 h). The sum of the concentrations of hesperetin-7-*O*-*β*-D-glucuronide and hesperetin-3′-*O*-*β*-D-glucuronide in plasma was almost consistent with the concentration of hesperetin-glucuronides at any time point. These results suggested that hesperetin-glucuronides in rat plasma were comprised of hesperetin-7-*O*-*β*-D-glucuronide and hesperetin-3′-*O*-*β*-D-glucuronide.

DISCUSSION

Chemical Structures and Individual Concentration of Hesperetin-Glucuronides. In the present study, we prepared authentic hesperetin-glucuronides. By LC-MS and NMR, hesperetin-glucuronides synthesized chemically and isolated from rat urine were identified as hesperetin-7-*O*-*β*-D-glucuronide and hesperetin-3′-*O*-*β*-D-glucuronide, respectively. Moreover, the time courses of the concentrations of hesperetin-7-*O*-*β*-D-glucuronide and hesperetin-3′-*O*-*β*-D-glucuronide in plasma were individually determined after oral administration. These results are, to our knowledge, the first reported to identify the chemical structure of hesperetin-glucuronides and determine the concentrations of each hesperetin-glucuronide (hesperetin-7-*O*-*β*-D-glucuronide and hesperetin-3′-*O*-*β*-D-glucuronide) individually.

From the chemical structure of hesperetin, it was thought that the 3′-hydroxyl group of hesperetin is sterically hindered by the 4′-methoxy group of hesperetin when the glucuronide moiety is introduced into the 3′-hydroxyl group. In contrast, the 7-hydroxyl group is free from any steric hindrance. Therefore, the efficiency of glucuronidation at the 3′-hydroxyl group was expected to be lower than that at the 7-hydroxyl group because of the effect of steric hindrance on the 3′-hydroxyl group. Actually, glucuronidation at the 3′-hydroxyl group did not occur in a chemical synthesis experiment. The present study showed that in rat plasma, the level of concentration of hesperetin-3′-*O*-*β*-D-glucuronide was lower than that of hesperetin-7-*O*-*β*-D-glucuronide. Thus, the difference in synthesizing efficiency derived from the steric hindrance may cause the difference in concentration between these glucuronides in rat plasma.

The present study also indicated that the sum of the concentrations of hesperetin-7-*O*-*β*-D-glucuronide and hesperetin-3′-*O*-*β*-D-glucuronide in plasma was almost consistent with the concentration of hesperetin-glucuronides at any time point. To our knowledge, this is the first report suggesting that hesperetin-glucuronides were primarily comprised of hesperetin-7-*O*-*β*-D-glucuronide and hesperetin-3′-*O*-*β*-D-glucuronide in rat plasma. Therefore, we thought that the investigation of the biological activities of hesperetin-7-*O*-*β*-D-glucuronide and hesperetin-3′-*O*-*β*-D-glucuronide was important to understand the precise mechanisms showing the biological effects of orally ingested hesperidin in the rat.

Metabolism from Hesperidin to Homoeriodictyol-Glucuronide in the Rat. We showed that not only hesperetin conjugates but also homoeriodictyol conjugates existed in rat plasma. Previously, Nielsen et al. (*21*) reported the in vitro biotransformation of flavonoids by rat liver microsomes. Their study demonstrated that the C4′ methoxy group of hesperetin was demethylated and, consequently, converted to eriodictyol (**Figure 1**) with the formation of catechol moiety. Miyake et al. (*22*) reported that the catechol moiety of eriodictyol was methylated and, consequently, transformed to hesperetin and homoeriodictyol in the rat. On the basis of these results, it seemed that in the present study, hesperetin derived from hesperidin was demethylated by rat liver and, consequently, transformed to eriodictyol. Subsequently, the catechol moiety of eriodictyol was methylated and, as a result, transformed to homoeriodictyol (3′-*O*-methylated compound) or hesperetin (4′-*O*-methylated compound). Thus, we thought that homoeriodictyol was converted in rat liver from hesperidin via eriodictyol by demethylation and remethylation. In the present study, we also monitored eriodictyol (the intermediate from hesperetin to homoeriodictyol) and its conjugates (glucuronides, sulfates, and sulfoglucuronides) in rat plasma by LC-MS (data not shown). However, we could not detect the corresponding peaks of eriodictyol and its conjugates. From these results, it seemed that the eriodictyol was immediately remethylated in rat liver.

Previous studies have shown that in the rat, hesperidin was absorbed as hesperetin from the intestine after the removal of disaccharide by intestinal bacteria and that hesperetin was immediately glucuronidated in the intestinal epithelium and, consequently, transformed to hesperetin-glucuronides (*1*). The present study showed that the time to reach a maximum concentration in hesperetin-glucuronides was about 2 h earlier than that in homoeriodictyol-glucuronides. We thought that the immediate synthesis of hesperetin-glucuronides in the intestinal epithelium contributed to an early increase in the concentration of hesperetin-glucuronides in rat plasma. After absorption from the intestine, it was assumed that the hesperetin-glucuronides reaching the liver were deglucuronidated, demethylated, remethylated, and reglucuronidated, and, consequently, transformed to hesperetin-glucuronides or homoeriodictyol-glucuronides by hepatic enzymes. Thus, we hypothesized that the metabolism from hesperetin-glucuronides to hesperetin-glucuronides or homoeriodictyol-glucuronides in rat liver played an important role in the comparable concentrations of hesperetin-glucuronides and homoeriodictyol-glucuronides from 6 to 12 h after administration.

Composition of Glucuronides and Sulfoglucuronides to Total Conjugates. The present study did not analyze hesperetin-sulfoglucuronides directly; however, it did show the presence of hesperetin-sulfoglucuronides on the basis of the difference in concentration between total conjugates and glucuronides. Therefore, we thought that glucuronides and sulfoglucuronides were major hesperetin conjugates in rat plasma, since hesperetin sulfates were not detected. Several reports on animals and humans have noted that the major metabolites of orally administered hesperidin were hesperetin-glucuronides and hesperetin-sulfoglucuronides (*5*−*12*). The present study also suggested that the concentration of hesperetin-sulfoglucuronides was much lower than that of hesperetin-glucuronides.

In conclusion, the chemical structures and concentrations of conjugated metabolites derived from hesperidin in rat plasma were studied after oral administration of hesperidin. Two hesperetin-glucuronides were prepared and identified as hesperetin-7-*O*-*β*-D-glucuronide and hesperetin-3′-*O*-*β*-D-glucuronide. Hesperetin-glucuronides were primarily comprised of hesperetin-7-*O*-*β*-D-glucuronide and hesperetin-3′-*O*-*β*-D-glucuronide in rat plasma. The concentration of hesperetin-7-*O*-*β*-D-glucuronide was slightly higher than that of hesperetin-3′-*O*-*β*-D-glucuronide. We elucidated that not only hesperetin conjugates but also homoeriodictyol conjugates exist in rat

plasma. The concentration of homoeriodictyol-glucuronides was comparable to that of hesperetin-glucuronides from 6 to 12 h after oral administration.

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