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Structural Elucidation and Biological Fate of Two Glucuronyl Metabolites of Pelargonidin 3-*O*-β-D-Glucopyranoside in Rats

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ABSTRACT: A high proportion of pelargonidin 3-*O*-β-D-glucopyranoside (Pg3G) is metabolized to glucuronides and excreted in mammal urine after ingestion of strawberry fruit, suggesting that these metabolites play important functional roles in vivo. The aim of the present study was to elucidate the structures and determine the biological fate of the two dominant metabolites of Pg3G in rats to enable an accurate discussion of the biological properties of anthocyanins. Authentic Pg3G was orally administered to rats. One pelargonidin monoglucuronide and three Pg3G-monoglucuronides (glucuronides of the glucoside) were identified together with intact Pg3G in both blood plasma and urine samples. The structures of the two dominant metabolites were elucidated as pelargonidin 3-*O*-β-D-glucuronide (Pg3GlcA) and pelargonidin 3-*O*-β-D-glucuronyl-(1→2)-β-D-glucoside by means of ^{1}H and ^{13}C nuclear magnetic resonance spectroscopy and heteronuclear multiple-bond connective spectroscopy. The bioavailability of Pg3G in its intact form was 0.31% of the orally administered dose, and 0.65% was absorbed in the Pg3GlcA form.

KEYWORDS: *anthocyanin, bioavailability, metabolic fate, pelargonidin 3-O-β-D-glucopyranoside, pelargonidin 3-O-β-D-glucuronide*

INTRODUCTION

Epidemiological studies have indicated that eating fruits and vegetables is effective for prevention of lifestyle-related diseases,[1−3] and various phytochemicals have been identified as active components in dietary food.[4−6] Colored flavonoids, which are anthocyanins present in fruits[7,8] and vegetables,[9,10] have attracted much attention because of their high intake from daily diets,[11] and numerous studies have demonstrated their multiple biological activities, including anticancer activity,[12,13] improvement of vision,[14] and neuroprotective effects.[15] Recently, the antiobesity activity of anthocyanins was reported in an animal model.[16] A possible molecular mechanism underlying the antidiabetic effect has been investigated in detail in a cell culture system that used authentic samples.[17] However, the resulting biological activities of ingested phytochemicals are obviously dependent on their amounts and the molecular structure present in target tissues.

Absorption of anthocyanins from the gastrointestinal tract has been evaluated several times in experimental animals[18,19] and humans.[20,21] Typically, approximately 0.1% of ingested anthocyanins, and sometimes even less, has been detected in human urine in their intact forms. Thus, the metabolism of anthocyanins has been investigated for further understanding of their function in vivo.[22,23] O-Methylation is one of the major metabolic pathways of various flavonoids.[24] We used ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy and heteronuclear multiple-bond connective (HMBC) spectroscopy to determine that 4′-*O*-methyldelphinidin 3-*O*-β-D-glucopyranoside was dominantly distributed in tissues as the metabolite of orally administered delphinidin 3-*O*-β-D-glucopyranoside (Dp3G; Figure 1).[25] Similar metabolites were reported for other anthocyanins also, although their sites of O-methylation were not determined.[26]

On the other hand, other flavonoids are dominantly present in blood plasma as sulfo- and glucuroconjugated forms.[27,28] In contrast, only a trace of cyanidin monoglucuronide (Cy-monoglucuronide) [26] could be detected in both rat blood plasma and urine as a metabolite of cyanidin 3-*O*-β-D-glucopyranoside (Cy3G), carrying a 3′,4′-dihydroxyl group on the B ring of aglycone (Figure 1). Alternatively, glucuronides of anthocyanidin glycosides have been identified as specific metabolites of Cy3G,[29] although their amounts detected in blood plasma were at low levels compared with those of the conjugated metabolites of other flavonoids. Thus, glucuronidation was, in general, believed to be a minor route for metabolism of anthocyanins. However, several studies demonstrated relatively high excretion of pelargonidin monoglucuronide (Pg-monoglucuronide) in human urine (approximately 2% of the orally ingested amount of Pg3G) after ingestion of freeze-dried strawberries.[30−32] Similar metabolites have also been reported in rat urine after 1 week of feeding of a Pg3G-rich fraction from strawberry fruits.[33] This suggests the possibility that Pg-monoglucuronide might positively affect various biological functions in humans because of its relatively high level after consumption of Pg3G-rich food. Therefore,

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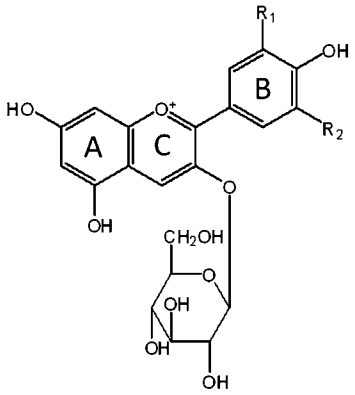
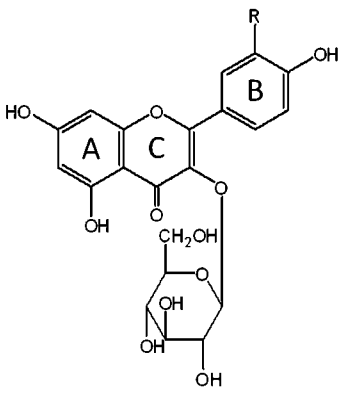


	R_1	R_2
pelargonidin 3-*O*-β-D-glucoside	H	H
cyanidin 3-*O*-β-D-glucoside	OH	H
delphinidin 3-*O*-β-D-glucoside	OH	OH



	R
kaempherol 3-*O*-β-D-glucoside	H
quercetin 3-*O*-β-D-glucoside	OH

Figure 1. Structures of anthocyanin and related flavonols.

structural elucidation of glucuronyl metabolites of Pg is a critical issue for understanding the biological properties of anthocyanins in vivo. In the present study, two dominant metabolites of Pg3G, the glucuronide of Pg aglycone (Pg-monoglucuronide) and the glucuronides of the glucoside (Pg3G-monoglucuronide), were isolated from the urine and bile of rats, and their structures were elucidated by two-dimensional NMR spectroscopy (2D-NMR spectroscopy) and tandem time-of-flight mass spectrometry (tandem TOF-MS). The biological fates of the detectable metabolites together with that of the intact Pg3G have also been discussed in detail.

MATERIALS AND METHODS

Chemicals. All chemicals, including trifluoroacetic acid (TFA), were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan) and used without further purification. MCI gel CHP 20P (70−150 μm) was purchased from Mitsubishi Chemical Industries Ltd. (Tokyo, Japan). Strawberry fruits were a kind gift from Sekiguchi Farm, Gunma, Japan.

Isolation of Pg3G from Strawberry Fruit. Pg3G (Figure 1) was isolated from strawberry fruit according to a previously described method with a slight modification.[8,34] Briefly, 13 kg of fresh strawberry fruit was immersed in 20 L of acetone for 3 h and filtered. This procedure was repeated three times. The filtered acetone fruit mixture was evaporated in vacuo at 40 °C, and the residual water fraction was applied onto an HP 20 liquid chromatography column (100 cm × 7 cm; Mitsubishi Chemical Industries Ltd.). The column was washed well with distilled water to remove water-soluble components, and the anthocyanins were successively eluted with 50% aqueous methanol solution. The fraction containing the anthocyanins was evaporated to dryness in vacuo, redissolved in distilled water, and applied onto an MCI column chromatography (50 cm × 4.5 cm). The fraction containing anthocyanins was obtained by stepwise elution under gravity flow with an increasing concentration of methanol in distilled water (10−100%). The eluted fraction was analyzed by high-performance liquid chromatography (HPLC) (Hitachi L-2000; Hitachi Ltd., Tokyo, Japan) using a Develosil ODS HG-5 column (150 mm × 4.6 mm i.d.; Nomura Chemical, Aichi, Japan) at 40 °C. The compounds were eluted with a 20% aqueous methanol solution containing 0.5% TFA at a flow rate of 1.0 mL/min, and the eluent was monitored at 520 nm with an ultraviolet−visible detector. The Pg3G-rich fraction was evaporated, redissolved in distilled water, and further separated by LH-20 column chromatography (35 cm × 3.5 cm; Amersham Biosciences, Inc., USA) with distilled water containing an increasing concentration of methanol as described above. The obtained Pg3G-rich fraction was finally purified by using semi-preparative HPLC with a Develosil ODS HG-5 column (250 mm × 20 mm i.d.; Nomura Chemical). The compounds were separated at room temperature with a mobile phase of 13% aqueous acetonitrile solution containing 0.5% TFA. The peak fraction was collected and evaporated to dryness in vacuo and stored at −80 °C until analysis. The purity of Pg3G was determined to be 98% by using tandem TOF-MS and 2D-NMR spectroscopy following the same methods used for identification of the metabolites described below.

Animals and Diets. SPF male Wistar ST-strain rats (age, 5 weeks; body weight, 120 g) were purchased from Japan SLC Inc. (Hamamatsu, Japan) and individually housed in stainless steel wire-mesh cages at 23 ± 1 °C for conditioning under a 12 h light/dark cycle. The rats were allowed free access to tap water and a control diet for 7 days before the experiment. The animals were maintained according to the Guidelines for Animal Experimentation of Niigata University of Pharmacy and Applied Life Sciences, and the protocol was approved by the Ethical Committee at Niigata University of Pharmacy and Applied Life Sciences.

Experimental Design and Plasma Preparation. After the 7 day conditioning period, 10 rats were cannulated with a polyethylene tube (PE-50) into a neck vein under anesthesia with diethyl ether, according to a previously described method.[29] The neck vein was isolated, and a small hole was made using scissors to insert the polyethylene tube. After insertion of the tube, the vein was occluded, and the tube was penetrated through the skin and guided out from the back of the rat. After fasting for 16 h, the rats were randomly assigned to two groups. The administered dose of Pg3G was determined on the basis of our previous reports for the comparison.[25,26,29] For oral administration (po), Pg3G dissolved in an aqueous solution containing 0.1% citric acid was administered to five rats by direct stomach intubation at a dose of 100 mg/kg body weight. For intravenous administration (iv), Pg3G (2 mg/kg body weight) was freshly prepared in a physiological saline solution and immediately injected into a separate group of five rats via the polyethylene tube. During the experiment, the rats were allowed to move freely in the stainless steel wire-mesh cages.

Blood samples were collected through the cannulated tube by using a heparinized syringe before administration, at 15, 30, 60, 120, 240, and 480 min after oral administration of Pg3G and at 5, 15, 30, 60, 120, 240, and 480 min after intravenous administration. Donor blood was obtained from the inferior vena cava of other healthy rats by using a needle and syringe containing sodium citrate (500 μL of 10% sodium citrate for 8 mL of whole blood) under anesthesia with diethyl ether. After withdrawal of the blood sample (600 μL), the same volume of donor blood was injected through the cannulated vein tube. For

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HPLC analysis, each blood sample was immediately centrifuged at 3000*g* for 5 min at 4 °C to obtain blood plasma. The urine was collected from a stainless plate under the wire-mesh cages immediately after excretion and stored in a bottle with an appropriate volume of 3% TFA aqueous solution on ice.

Determination of Pg3G and Its Metabolites in Plasma and Urine. Extraction of Pg3G and its metabolites from plasma samples was performed by using the Sep-Pak Light C_{18} cartridge (Waters Corp., Milford, MA, USA), according to our previously described method.[29] Briefly, the plasma samples (300 μL) were applied to Sep-Pak C_{18} cartridges conditioned with methanol (2 mL) and 3% TFA aqueous solution (2 mL). After sample application, the cartridges were washed with 2 mL of 3% TFA aqueous solution, dichloromethane, and benzene. Pg3G and its metabolites were then successively eluted with 50% aqueous acetonitrile solution containing 1% TFA (1 mL). The sample fractions were evaporated to dryness in vacuo and then dissolved in 150 μL of 0.5% TFA aqueous solution. Urine samples were prepared as described above by using a Sep-Pak environmental C_{18} cartridge (Waters Corp.) and diluted appropriately with 0.5% TFA aqueous solution. Each sample was passed through a Centricut filter (0.45 μm pore size; Kurabou Co. Ltd., Japan), and aliquots (100 μL) of the solutions were analyzed using a semimicro HPLC system (Hitachi L-7000; Hitachi Ltd.) equipped with a Develosil ODS HG-5 column (Nomura Chemical; 1.0 mm × 150 mm) at 40 °C with a mobile phase of 18% aqueous methanol solution containing 0.5% TFA at a flow rate of 0.1 mL/min. The elution peaks were monitored at 520 nm with an ultraviolet−visible detector (Hitachi L-2000; Hitachi Ltd.). The recovery of Pg3G by using this method was checked by blood plasma spiked with authentic Pg3G and found to be 96.5% at the detection wavelength (520 nm).

Isolation of Pg3G Metabolites. Two dominant metabolites of Pg3G were isolated from the urine and bile of the rats described below. For the collection of Pg-monoglucuronide, five male Wistar ST-strain rats (8 weeks) were fasted for 16 h in stainless steel wire-mesh cages. Pg3G dissolved in an aqueous solution containing 0.1% citric acid (20 mg/mL) was then orally administered to each rat at a dose of 100 mg/kg, and urine was immediately collected from a stainless steel plate under the wire-mesh cages and stored in a bottle with an appropriate volume of 3% TFA aqueous solution on ice to maintain the metabolites of anthocyanin in their stable flavylium forms. The administration of Pg3G was repeated three times at 6 h intervals for each rat.

For the collection of the Pg3G-monoglucuronides, another five male Wistar ST-strain rats (8 weeks) were cannulated in their neck veins and fasted for 16 h in stainless steel wire-mesh cages. Pg3G dissolved in physiological saline (20 mg/mL) was intravenously injected (2 mg/kg body weight) through the cannulated tube. The injection was repeated five times at 2 h intervals for each rat. Urine was collected in a bottle with appropriate volumes of 3% TFA aqueous solution on ice.

The urine samples collected in the above-mentioned steps were centrifuged, and the supernatants were separately applied onto an MCI gel CHP-20 column (20 cm × 2.5 cm; Mitsubishi Chemical Industries Ltd.) for chromatography. The column was washed well with distilled water to remove hydrophilic components from the biological samples. Next, Pg3G metabolites were obtained by stepwise elution under gravity flow with an increasing concentration of methanol in distilled water (10−100%). The metabolite-rich fraction was collected, evaporated to dryness in vacuo, and dissolved in 1% TFA aqueous solution for further purification by semipreparative HPLC under the same separation conditions described under Isolation of Pg3G from Strawberry Fruit. Peak fractions corresponding to metabolites were collected, dried in vacuo, and stored at −80 °C until analysis.

Structural Elucidation. Aliquots of the original Pg3G and isolated metabolites from the biological samples were dissolved in methanol and analyzed by tandem TOF-MS performed using a Q-Tof Ultima (Waters Corp.). A syringe pump (single syringe pump; KD Science Inc., USA) was used to provide constant infusion (300 μL/h) of the sample into the MS ion source. The MS parameters used were as follows: 3.2 kV, capillary; 9.1 kV, reflection; argon gas at 11 psi; applied voltage, 24 kV. ^{1}H and ^{13}C NMR spectroscopy were performed by using a JEOL-ECA-500 NMR spectrometer (JEOL Ltd., Tokyo, Japan) at magnetic field strengths of 500 or 125 MHz, respectively, in DMSO-d_6/TFA-*d* (9:1), with tetramethylsilane as the internal reference.

Pharmacokinetic Evaluation of Pg3G and Its Metabolites. Pharmacokinetic parameters of Pg3G and its metabolites were evaluated. The bioavailability (BA) of Pg3G in its intact form was calculated according to the pharmacologically defined equation

$$\mathrm{BA}\,(\%) = (\mathrm{AUC}_{\mathrm{po,Pg3G}}/\mathrm{dose}_{\mathrm{po,Pg3G}}) \times 100/(\mathrm{AUC}_{\mathrm{iv,Pg3G}}/\mathrm{dose}_{\mathrm{iv,Pg3G}}) \quad (1)$$

where $\mathrm{AUC}_{\mathrm{po,Pg3G}}$ and $\mathrm{dose}_{\mathrm{po,Pg3G}}$ are the AUC values and doses for oral administration of Pg3G, respectively, and $\mathrm{AUC}_{\mathrm{iv,Pg3G}}$ and $\mathrm{dose}_{\mathrm{iv,Pg3G}}$ are the AUC values and doses for intravenous administration of Pg3G, respectively. Similarly, the plasma presence amount (PPA) of each metabolite was calculated according to the equation

$$\mathrm{PPA}\,(\%) = (\mathrm{AUC}_{\mathrm{po,metabolites}}/\mathrm{AUC}_{\mathrm{po,Pg3G}}) \times \mathrm{BA} \quad (2)$$

where $\mathrm{AUC}_{\mathrm{po,metabolites}}$ is the AUC value of each metabolite for oral administration.

To evaluate the effect of hepatic first-pass metabolism, the extent of glucuronidation of the glucoside in both oral and intravenous studies was calculated from the ratio of the AUC of the Pg3G-monoglucuronides to that of the intact Pg3G, as follows:

$$\text{extent of glucuronidation of the glucoside for oraladministration}\,(\%) = (\mathrm{AUC}_{\mathrm{po,Pg3G\text{-}monoglucuronides}}/\mathrm{AUC}_{\mathrm{po,Pg3G}}) \times 100 \quad (3)$$

$$\text{extent of glucuronidation of the glucoside for intravenous administration}\,(\%) = (\mathrm{AUC}_{\mathrm{iv,Pg3G\text{-}monoglucuronides}}/\mathrm{AUC}_{\mathrm{iv,Pg3G}}) \times 100 \quad (4)$$

$$\text{extent of first-pass metabolism}\,(\%) = (\text{eq 3}) - (\text{eq 4}) \quad (5)$$

$$\text{PPA of the Pg3G-monoglucuronides produced by hepatic first-pass metabolism}\,(\%) = \mathrm{AUC}_{\mathrm{po,Pg3G\text{-}monoglucuronides}} \times (\text{eq 5}) \quad (6)$$

$\mathrm{AUC}_{\mathrm{po,Pg3G\text{-}monoglucuronides}}$ and $\mathrm{AUC}_{\mathrm{iv,Pg3G\text{-}monoglucuronides}}$ are the AUC values for oral and intravenous administration of Pg3G-monoglucuronides (sum of M1−M3), respectively.

RESULTS

Absorption and Metabolism of Pg3G in Rats. Figure 2 shows typical HPLC chromatograms of authentic Pg3G isolated from strawberry (A) and rat blood plasma 15 min after oral administration of Pg3G (B) detected at 520 nm. No peaks other than the one for Pg3G were observed at 520 nm in the purified Pg3G sample (Figure 2A). Five peaks (M1−M5) presumably due to the metabolites of Pg3G were detected in the rat blood plasma together with intact Pg3G (Figure 2B). In contrast, only three metabolites (M1, M2, and M3) were observed in the rat blood plasma after intravenous administration of Pg3G (Figure 2C), suggesting that both M4 and M5 were produced during the process of absorption from the gastrointestinal tract. The Pg3G metabolites (M1−M5) detected in rat blood plasma were isolated from the urine of rats by semipreparative HPLC and were successively analyzed by tandem TOF-MS to obtain molecular information. Table 1

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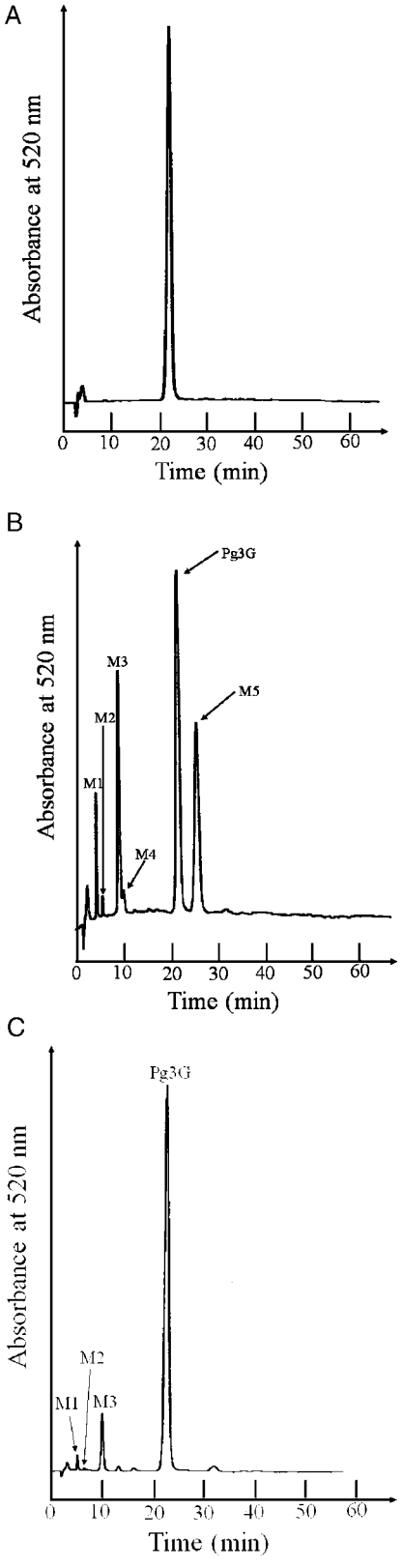

Figure 2. Typical HPLC chromatogram of rat blood plasma after administration of Pg3G: (A) authentic Pg3G purified from strawberry fruits; (B) blood plasma 15 min after oral administration of Pg3G; (C) blood plasma 15 min after intravenous administration of Pg3G.

shows a summary of the results from the tandem MS analysis of authentic Pg3G and its five metabolites, expressed as the parent and product ion pairs. The parent and product ion pairs for M1, M2, and M3 were m/z 609/447/433/271, suggesting the presence of a Pg3G-monoglucuronide (the glucuronide of Pg3G). On the other hand, M5 was presumed to be Pg-monoglucuronide (the glucuronide of Pg aglycone) on the basis of its parent and product ion pair (m/z 447/271). However, results could not be obtained for M4 because of its poor resolution from the abundant metabolite M3. Figure 3 shows the time-dependent plasma concentration profile of Pg3G and its metabolites after oral administration of Pg3G. Table 1 also summarizes the results for the maximum plasma concentration (C_{max}), the time required to reach maximum plasma concentration (t_{max}), and the area under the plasma concentration curve (AUC) of Pg3G and its metabolites. Pg3G rapidly reached its C_{max} (1.49 μM) at 15 min after oral administration, whereas M5 reached its maximum of 1.79 μM as Pg3G equivalent at 30 min (Figure 3A and Table 1). Furthermore, M5 showed a 2-fold higher AUC level than the intact Pg3G, indicating that M5 was the dominant form in blood circulation after ingestion of Pg3G. The C_{max} values of the four hydrophilic metabolites M1, M2, M3, and M4 as Pg3G equivalents were 0.13, 0.03, 0.55, and 0.11 μM, respectively. Most of the hydrophilic metabolites (M1, M2, and M3) had t_{max} values of 30 min, whereas M4, the unknown metabolite, had a t_{max} of 60 min. When the AUC values were compared among the hydrophilic metabolites, M3 was dominant and showed a 4−10-fold higher level than the other metabolites.

The BA of Pg3G in its intact form was calculated to be 0.31% using eq 1. Similarly, the PPA of each metabolite was calculated using eq 2. As shown in Table 1, the PPAs of the two dominant metabolites M3 and M5 were 0.12 and 0.65% of the orally administered doses, respectively. On the other hand, the urinary excretion of intact Pg3G was only 0.067% of the ingested dose, whereas 0.074% was recovered for the most abundant metabolite M5. In total, the urinary recovery of Pg3G including detectable metabolites was 0.167% of the ingested dose of Pg3G.

To evaluate the effect of hepatic first-pass metabolism, the extent of glucuronidation of the glucoside in both oral and intravenous studies was calculated from the ratio of the AUC of the Pg3G-monoglucuronides (sum of M1−M3) to that of the intact Pg3G, and the results are presented in Table 2. The extent of glucuronidation of the glucoside was finally calculated to be 4.26% for intravenous administration and 50.69% for oral administration. The PPA of the Pg3G-monoglucuronides produced by hepatic first-pass metabolism was finally calculated as 0.14% of the ingested dose of Pg3G.

Structural Elucidation of Pg3G Metabolites in the Rat. To determine the site of glucuronidation, two (M3 and M5) of five metabolites were further isolated from the urine of rats and analyzed by means of ^{1}H and ^{13}C NMR spectroscopy. The NMR data of M3 and M5 are summarized in Table 3 together with those of authentic Pg3G. The ^{1}H and ^{13}C NMR spectra of M5 were similar to those of Pg3G except for the signals arising from the sugar moiety. In the ^{13}C NMR spectrum, the observation of a signal assignable to the carboxylic acid group at δ_C 170.2 instead of to the hydroxymethyl carbon due to a glucosyl C-6 suggested that M5 was a Pg-monoglucuronide. The proton signals of the sugar moiety, which had large coupling constants, were in agreement with the presence of a glucuronic acid moiety. The location of the glucuronic acid moiety was determined by HMBC correlation spectroscopy; the selected HMBC correlations are shown in Figure 4. The anomeric signal at δ_H 5.55 showed an HMBC correlation with

Table 1. Parent and Product Ion Pairs of Pg3G Metabolites and Their Biological Parameters in Plasma and Urine[a]

metabolite	parent and product ion pairs (m/z)	C_{max} (μM)	t_{max} (min)	AUC (μmol min L^{-1})	plasma presence amount (%)	urinary excreted amount (nmol)	urinary recovery (%)
Pg3G	433/271	1.492 ± 0.172	13	104.47 ± 5.61	0.31[b]	23.19 ± 1.85	0.0670 ± 0.0053
Pg3G-monoglucuronide (M1)	609/447/433/271	0.126 ± 0.024	30	9.76 ± 0.47	0.03	1.10 ± 0.25	0.0318 ± 0.0007
Pg3G-monoglucuronide (M2)	609/447/433/271	0.027 ± 0.001	30	2.41 ± 0.55	0.01	0.36 ± 0.08	0.0011 ± 0.0002
Pg3G-monoglucuronide (M3)	609/447/433/271	0.553 ± 0.071	30	39.71 ± 1.70	0.12	5.89 ± 0.36	0.0170 ± 0.0010
unknown (M4)		0.112 ± 0.033	60	10.78 ± 1.72	0.03	1.65 ± 0.15	0.0048 ± 0.0004
Pg-monoglucuronide (M5)	447/271	1.793 ± 0.175	30	219.58 ± 33.40	0.65	25.69 ± 3.67	0.0743 ± 0.0106

[a]Values are the mean ± SEM of five rats. [b]Bioavariability of Pg3G.

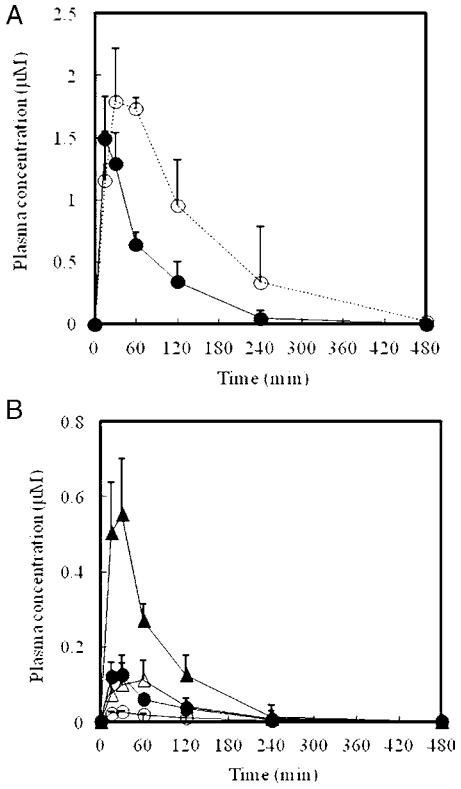

Figure 3. Time-dependent plasma concentration profile of Pg3G and its metabolites: (A) ○ = Pg3G, ● = M5; (B) ● = M1, ○ = M2, ▲ = M3, △ = M4. Values are mean + SEM of five rats.

Table 2. Extent of Glucuronidation of the Glucoside in Different Administered Routes and Effect of First-Pass Metabolism[a]

	M1	M2	M3	total
extent of glucuronidation of the glucoside in intravenous administration (%)	0.56	0.05	3.65	4.26
extent of glucuronidation of the glucoside in oral administration (%)	9.51	2.47	38.71	50.69
extent of first-pass metabolism (%)	8.95	2.42	35.06	46.42
PPA of Pg3G-monoglucuronides produced by hepatic first-pass metabolism (%)	0.027	0.007	0.106	0.140

[a]Values are the mean of five rats.

an oxygen-bearing aromatic carbon signal at δ_c 144.1 through a three-bond coupling. Furthermore, this carbon resonance also showed an HMBC correlation with H-4 at δ_H 8.95. This spectral observation clearly showed that the glucuronic acid moiety is attached at the C-3 hydroxyl group of the pelargonidin moiety. The β-linkage of the glucuronic acid moiety was deduced from the coupling constant value (J = 7.7 Hz) of the anomeric proton signal in the ^{1}H NMR spectrum. On the basis of the spectral examination described above, the structure of M5 was established as pelargonidin 3-*O*-β-D-glucuronide (Pg3GlcA).

On the other hand, the ^{1}H and ^{13}C NMR spectra of M3 showed two anomeric signals [δ_H 5.57 (d, J = 7.8 Hz) and 4.78 (d, J = 8 Hz); δ_C 103.9 and 100.2], indicating a diglycosidic structure. The ^{13}C NMR spectrum exhibited, together with 14 carbon signals arising from a pelargonidin moiety, 12 carbon resonances including 2 anomeric carbons, 9 oxygen-bearing carbons, and a carboxyl carbon; 6 of these were in good accord with the signals for the glucuronic acid moiety of Pg3GlcA. This observation suggested that M3 contains a glucuronic acid moiety as a terminal sugar. Detailed analysis of the 2D-NMR data revealed the presence of a glucosyl moiety because all of the proton signals due to the other hexosyl moiety had large coupling constants. The connections of the two sugar moieties were deduced from the HMBC correlations shown in Figure 5. Thus, the HMBC correlation of H-1″ with C-3 indicated that the location of the glucosyl moiety was at C-3 of pelargonidin. Furthermore, the glucuronic acid moiety was shown to be attached to the glucosyl C-2 from the HMBC cross-peaks of H-1‴ with C-2″. The β-linkages for both sugars were deduced from the anomeric coupling constant values (J = 7.8 and 8.0 Hz). On the basis of the spectroscopic evidence described above, the structure of M3 was characterized as pelargonidin 3-*O*-β-D-glucuronyl-(1→2)-β-D-glucoside (Pg3G-GlcA).

■ DISCUSSION

Anthocyanins have attracted much attention because of their various biological activities that have positive influence on health.[12−16] To clarify the molecular mechanism underlying these multiple functions in vivo, determination of the BA and elucidation of the metabolism of anthocyanins are critical. The available data suggested that absorption and metabolism of anthocyanins were influenced by not only the nature of the sugar moiety but also the structure of the anthocyanidin aglycone.[18−26] Although the exact site of glucuronidation has not been clarified to date, several studies have reported that a high proportion of Pg-monoglucuronides was excreted in human and rat urine after ingestion of Pg3G.[30−33] This suggests the possibility that the biological function of orally ingested Pg3G was in part related to these metabolites. The aim of the present study was to determine the two dominant metabolites

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Table 3. ^{1}H and ^{13}C NMR Data for Pg3G, M5, and M3[a]

	Pg3G		M5		M3	
	δH	δC	δH	δC	δH	δC
2		162.4		162.7		162.4
3		144.5		144.1		144.0
4	9.01 (s)	136.2	8.95 (s)	136.2	8.97 (s)	135.9
5		156.4		156.5		156.4
6	7.00 (d, 2)	102.7	7.00 (d, 2)	102.3	6.98 (d, 2)	102.7
7		169		169.0		168.9
8	6.75 (d, 2)	94.6	6.77 (d, 2)	94.8	6.73 (d, 2)	94.7
4a		112.5		112.4		
8a		158		157.8		158.4
1′		119.7		119.7		119.7
2′	8.62 (d, 9.2 Hz)	135	8.61 (d, 9.2 Hz)	135.0	8.55 (d, 9.2)	135.1
3′	7.10 (d, 9.2 Hz)	117.2	7.10 (d, 9.2 Hz)	117.2	7.13 (d, 9.2)	117.5
4′		165.1		165.1		165.1
5′						
6′						
glucosyl						
1	5.37 (d, 7.5)	102.7			5.57 (d, 7.8)	103.9
2	3.50 (dd, 7.5, 9.2)	73.5			3.85 (dd, 7.8,9)	81.1
3	3.28 (t, 9.2)	78.1			3.63 (t, 9)	76.6
4	3.42 (t, 9.2)	70			3.32(t, 9)	69.6
5	3.56 (m)	76.8			3.54 (m)	77.8
6	3.53, 3.77 (brd, 10.5)	61.1			3.51 (dd, 5,11), 3.70 (br d, 11)	61.0
glucuronyl						
1			5.55 (d, 7.7)	102.3	4.78 (d, 8)	100.2
2			3.55 (dd, 7.7, 8.8)	73.2	3.03 (dd, 8,9)	74.2
3			3.45 (t, 8.8)	76.0	3.15 (t, 9)	76.2
4			3.53 (t, 8.8)	71.6	3.32 (t, 9)	71.8
5			4.11 (d, 8.8)	76.0	3.39 (d, 9)	76.2
6				170.2		170.5

[a]Measured at 500 MHz (^{1}H) and 125 MHz (^{13}C) in DMSO-d_6 + TFA-d (9:1).

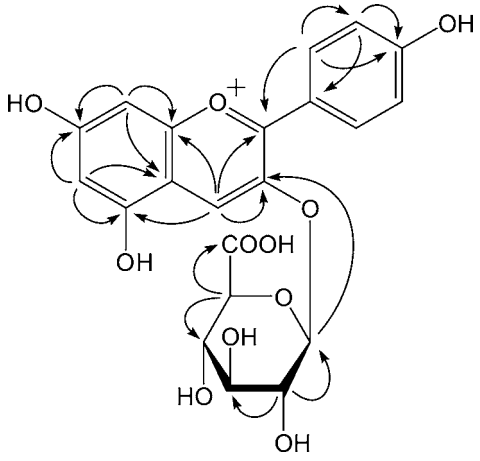

Figure 4. Selected HMBC correlation of M5.

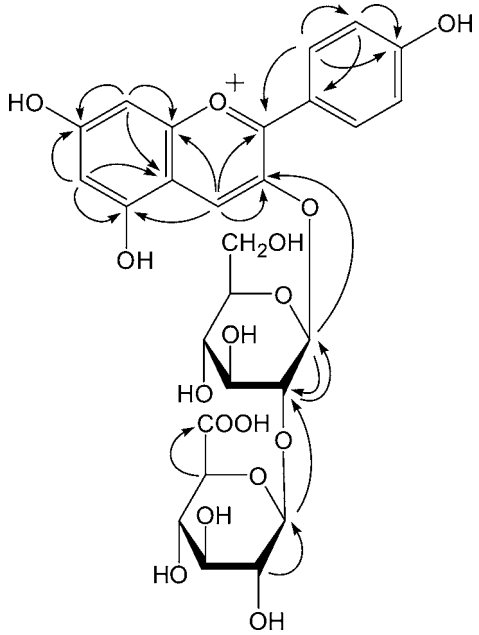

Figure 5. Selected HMBC correlation of M3.

of Pg3G isolated from rat urine and bile to enable an accurate discussion of the biological properties of anthocyanins.

In the present study, only one Pg-monoglucuronide that eluted later than the intact Pg3G was detected in the HPLC chromatogram of the rat blood plasma after oral administration of purified Pg3G, and its structure was elucidated, for the first time, as Pg3GlcA. The AUC levels of Pg3GlcA and original Pg3G obtained from rat blood plasma were 219.57 μmol·min/L and 104.47 μmol·min/L, respectively. In contrast, the urinary excretions of both the anthocyanins were similar, with 25.69 nmol for Pg3GlcA and 23.19 nmol for intact Pg3G. Felgines et al. reported results similar to ours,[33] where only one Pg-monoglucuronide was detected in rat urine together with intact Pg3G after feeding food containing strawberry extract, whereas

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three different Pg-monoglucuronides were observed in human urine together with a trace amount of intact Pg3G.[30] However, in humans, one of three Pg-monoglucuronides was dominant, and the amounts reached 83% of monoglucuronides in urine.[30] The HPLC retention times of the most abundant Pg-monoglucuronides in previous human and rat studies were identical when analyzed under exactly the same analytical conditions.[30,33] Moreover, the elution patterns on the HPLC chromatograms in the present study and those in previous human and rat studies clearly demonstrated that the most abundant Pg-monoglucuronide in each study was eluted just after intact Pg3G; therefore, the dominant Pg-monoglucuronides detected in three different studies were considered to be identical.

Limited studies on structural elucidation of glucuronyl metabolites of various flavonoids have indicated that the sites of glucuronidation were mostly different, depending on the structure of the original flavonoids ingested orally. For example, quercetin 3-*O*-glucuronide (Q3GlcA) was identified as the dominant metabolite of quercetin 3-*O*-*β*-D-glucopyranoside (Q3G) (flavonol),[35] whereas glucuronidation of the hydroxyl group occurred at the 3′-position on the B ring or at the 7-position on the A ring for (−)-epicatechin (flavan-3-ol).[36] In the present study, we determined that glucuronidation of the hydroxyl group occurred at the 3-position on the C ring for the most abundant Pg-monoglucuronide. This suggested that the oxidation level of the central pyran ring (C ring) probably determined the site of glucuronidation. On the other hand, the amount of glucuronides produced in vivo was also different when the same flavonoid categories were compared. Relatively high production of Pg-monoglucuronides seems to be characteristic of Pg3G, whereas only a trace of Cy-monoglucuronide was detected in both human and rat urine as a metabolite after oral administration of Cy3G.[26] In addition, our previous work clearly demonstrated that Dp-monoglucuronide was rarely detected in rat blood plasma after ingestion of Dp3G carrying three hydroxyl groups on the B ring of aglycone.[37] Similarly, DuPont et al.[38] reported that urinary excretion of kaempferol 3-*O*-glucuronide was 1.9% of the ingested dose of kaempferol 3-*O*-*β*-D-glucopyranoside. This level was much higher than that for Q3GlcA in a case when metabolism of flavonol was studied in humans.[38] These results clearly demonstrated that flavonoids carrying one hydroxyl group on the aglycone B ring were easily glucuronidated in the gastrointestinal tract regardless of the C-ring structure. Taken together, these observations lead to the general conclusion that the amount of glucuronides was mainly determined by the B-ring pattern in the same flavonoid categories, whereas the oxidation level of the C ring possibly modulates the site of glucuronidation among the different types of flavonoids.

Pg3GlcA could not be detected in rat blood plasma after intravenous administration of Pg3G, which supports the fact that it was formed in the gastrointestinal tract by intestinal first-pass metabolism during the absorption process as reported for other flavonoid glycosides.[27] Generally, two possible pathways could explain the formation of Pg-monoglucuronide. A possible pathway reported in the metabolism of various flavonoid glycosides is that Pg3G was hydrolyzed to aglycone and then rapidly glucuronidated in the intestine. As has been suggested by Wu et al., another possible pathway is that Pg3G could serve as a substrate for uridine diphosphate glucose dehydrogenase, which is present in both the small intestine and liver in various animal species, to form Pg-monoglucuronides.[21] The latter hypothesis does not require hydrolysis of Pg3G to aglycone Pg, which is unstable at the physiological pH. Furthermore, the structure of Pg3GlcA elucidated in the present study might support the latter hypothesis. However, Felgines et al.[33] revealed that Pg (anthocyanidin) was released from Pg3G in rat cecal content after oral administration of strawberry extract. Because Pg aglycone was not present in the original strawberry extract, they concluded that Pg detected in the cecum was a result of glycolytic cleavage of Pg3G by microflora glycosidase. From previous human studies, the findings that the presence of three different Pg-monoglucuronides required release of Pg also support the former pathway.[30−32] Therefore, there is strong support for the former pathway as one that leads to the production of Pg3GlcA in the small intestine.

Glucuronides of glycoside are specific metabolites of anthocyanins that are mainly absorbed in their intact glycosidic forms. We previously identified 10 individual glucuronides of the glucoside for Cy3G and its *O*-methyl metabolites in rats,[29] although their sites of glucuronidation were not determined. Similar metabolites were also observed in a previous human study.[39] The amounts of glucuronides of glycoside were largely influenced by both types of the attached sugar moiety and aglycone structure, such that it was the dominant route for Cy3G[29] but a minor one for Dp3G or anthocyanidin 3-rutinosides.[25,37] In addition, accumulation of glucuronides of glycoside was observed in rat peripheral tissues despite increasing polarization by the attached glucuronyl moiety.[19] In this work, several hydrophilic metabolites (M1, M2, M3, and M4) were also detected in rat blood plasma and urine after oral and intravenous administration of Pg3G. Three of four hydrophilic metabolites (M1, M2, and M3) were identified as Pg3G-monoglucuronides according to tandem TOF-MS analysis (m/z 609/447/271). The structure of M3, the most abundant of the hydrophilic metabolites, was elucidated as Pg3G-GlcA by 2D-NMR analysis. Despite our previous prediction that glucuronidation of glycoside may occur on the phenolic group of aglycone,[29] it actually occurred on the hydroxyl group of the attached glucosyl moiety. Pg3G-GlcA elucidated in the present study retained a free hydroxyl group at the 4′-position, which has been reported as a critical site for various biological activities of anthocyanins.[39,40] Moreover, the C_{max} of Pg3G-GlcA was several fold higher than that of other typical anthocyanins that mainly presented in their intact forms.[18,25,26,37] Therefore, Pg3G-GlcA elucidated in the present study was believed to have remained bioactive and to have an effect in vivo. Furthermore, the results obtained here provide information useful for prediction of the structures of Cy3G-monoglucuronides, which were previously reported in humans.[41]

In the present study, the time-dependent plasma concentration profiles of Pg3G and its metabolites were evaluated to determine the biological fate of Pg3G. The plasma level of orally administered Pg3G in its intact form reached its C_{max} of 1.49 μM 15 min after oral administration. This value was 5−7-fold higher than that in our previous Dp3G[25] and Cy3G[26] studies, in which similar doses of anthocyanin (100 mg/kg) were administered to rats. Here we expressed the BA of Pg3G from AUC values obtained after both oral and intravenous administration of Pg3G according to the pharmacological definition of eq 1, and it was found to be 0.31% (Table 1). Several studies have reported that various food components enhanced or suppressed gastrointestinal absorption of anthocyanins.[31,42] Especially, absorption of anthocyanin was

enhanced >10-fold compared with the control levels in both humans and rats when 2.5% of phytic acid (inositol 6-phosphate) was simultaneously administered with anthocyanins from black currant.[42] However, the present results clearly demonstrated that enhanced absorption of Pg3G was not due to the effect of other components present in fruits or food additives; therefore, we concluded that the monohydroxyl group on the B ring of anthocyanidin is a critical factor for enhancement of gastrointestinal absorption of anthocyanins in their intact glycosidic forms.

On the other hand, the C_{max} of the most dominant metabolite, Pg3GlcA, reached 1.8 μM as Pg3G equivalent 30 min after oral administration. The relatively slow t_{max} of Pg3GlcA supports the fact that the upper part of the intestine is the major site of glucuronidation of Pg. AUC values for Pg3GlcA were 2-fold higher than those for intact Pg3G. The PPA of Pg3GlcA was expressed according to eq 2 and was found to be 0.65% of orally administered Pg3G. As discussed in the previous section, Pg3GlcA detected in blood plasma was produced by intestinal first-pass metabolism during the absorption process. Therefore, 0.65% of orally administered Pg3G was absorbed from the gastrointestinal tract in the Pg3GlcA form. Similar to Pg3GlcA, M4 was determined to be produced by intestinal first-pass metabolism during the absorption process from the comparison of HPLC chromatograms following both oral and intravenous administration, although its molecular information could not be obtained. In contrast, Pg3G-monoglucuronides were determined to be derived from Pg3G by hepatic metabolism after entering the systematic blood circulation as the glucuronides were also observed in blood plasma after bolus intravenous administration. However, as shown in Figure 2B,C, the amounts of the Pg3G-monoglucuronides (M1−M3) were mostly different depending on the administered route (oral or intravenous), which indicated hepatic first-pass metabolism. Thus, the extent of glucuronidation of the glucoside for both routes was calculated from the AUC ratio of the Pg3G-monoglucuronides to that of the intact Pg3G to clarify the effect of hepatic first-pass metabolism (Table 2). After intravenous administration, only 4.26% of Pg3G in blood circulation underwent glucuronidation of the glucoside, whereas more than half of Pg3G (50.69%) was metabolized to Pg3G-monoglucuronides after oral administration. This indicates that the high proportion of Pg3G-monoglucuronides observed in the blood plasma after oral administration was produced from Pg3G in portal vein blood by hepatic first-pass metabolism during the absorption process before entering systematic blood circulation. The amount of Pg3G-monoglucuronides produced during the absorption process by hepatic first-pass metabolism totaled 0.14% as the PPA value (sum of M1, M2, and M3), although 0.16% was present in blood plasma. In total, 1.11% of orally administered Pg3G was absorbed from the gastrointestinal tract as its intact and various metabolized forms.

We previously reported that only 30% of anthocyanins were recovered in rat urine after bolus intravenous administration of bilberry anthocyanins,[19] which suggested that analysis of urine is not suitable for determination of the absorbed amounts of anthocyanins. In contrast to the PPA of Pg3G and its metabolites (1.11%) calculated above, only 0.167% was recovered in rat urine in intact and metabolized forms during the first 8 h after oral administration of Pg3G (100 mg/kg). This was the same order of magnitude reported from a previous rat study (0.16%), where a similar dose of Pg3G (approximately 14 mg for each rat) was administered.[33] These results indicated that a large proportion of Pg3G and its metabolites present in blood circulation could not be recovered in urine. On the other hand, several studies reported high excretion of Pg3G metabolites in human urine (0.75−2.2% of the ingested dose).[30,32] These values were 4−13-fold higher than those observed in the rats in the present study. Although there have been only a limited number of studies that have conducted pharmacokinetic evaluations of Pg3G in humans, Mullen et al.[31] reported pharmacokinetic parameters of Pg-monoglucuronide in human blood plasma as 274 ± 24 nM for C_{max} and 856 ± 42 nmol·h/L for AUC following ingestion of 222 μmol of Pg3G. These levels were 5−6-fold higher for C_{max} and 8−11-fold higher for AUC than the levels obtained for rats when the values in humans were normalized by the orally administered dose. Taken together, the PPA of Pg3G and its metabolites in humans was estimated to be much higher than the urinary excreted amount (0.75−2.2%) and presumed to be 9−13% of the orally ingested amount of Pg3G. As discussed above, Pg3GlcA elucidated in the present study is expected to be the dominant metabolite of Pg3G in humans and to have various biological activities that are beneficial to human health.

Several studies reported that flavonoids exhibit various biological functions in their metabolized forms.[43,44] For example, 3′-*O*-methylepicatechin expressed caspase-3 activation in neuronal cells, but epicatechin glucuronide did not.[43] Although Pg3G has shown poor biological activity among various anthocyanins in vitro,[39,40] the present study clearly demonstrated that the PPAs of Pg3G and its metabolites were much higher than those of other anthocyanins. Furthermore, recent studies reported that ingestion of Pg3G was associated with attenuation of postprandial inflammation and insulin response in vivo.[45] However, it was not determined whether the anthocyanin glucuronides reported in the present study showed such an activity. Further studies are required to determine the biological functions of these metabolites, including signal transduction and gene expression.

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ABBREVIATIONS USED

Pg3G, pelargonidin 3-*O*-β-D-glucopyranoside; Cy3G, cyanidin 3-*O*-β-D-glucopyranoside; Dp3G, delphinidin 3-*O*-β-D-glucopyranoside; Q3G, quercetin 3-*O*-β-D-glucopyranoside; Pg3GlcA, pelargonidin 3-*O*-β-D-glucuronide; Q3GlcA, quercetin 3-*O*-glucuronide; BA, bioavailability; PPA, plasma presence amount; AUC, area under the plasma concentration curve; 2D-

NMR spectroscopy, two-dimensional nuclear magnetic resonance spectroscopy; tandem TOF-MS, tandem time-of-flight mass spectrometry; TFA, trifluoroacetic acid; C_{max}, maximum plasma concentration; t_{max}, time required to reach maximum plasma concentration; HMBC, heteronuclear multiple-bond connective

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