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Extended Glucuronidation Is Another Major Path of Cyanidin 3-*O*-*β*-D-Glucopyranoside Metabolism in Rats

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We previously determined that five rather hydrophobic metabolites appeared in blood plasma after oral administration of cyanidin 3-*O*-*β*-D-glucopyranoside, but a group of hydrophilic metabolites still remained unidentified. In the present study, 12 hydrophilic metabolites found were collected from urine and plasma samples by high-performance liquid chromatography (HPLC) and then analyzed by tandem MS spectrometry. From the MS spectra, four metabolites out of 12 were assigned as glucuronides of cyanidin 3-*O*-*β*-D-glucopyranoside and six out of 12 were glucuronides of the primary metabolites of cyanidin 3-*O*-*β*-D-glucopyranoside (*O*-methyl cyanidin 3-*O*-*β*-D-glucopyranoside). Extended glucuronides of cyanidin 3-*O*-*β*-D-glucopyranoside and *O*-methyl cyanidin 3-*O*-*β*-D-glucopyranoside showed their maximum plasma concentrations at 15 and 60 min (or 30 min) after oral administration, respectively. Their maximum plasma concentrations ranged from 15 to 70 nM. From the profile of urinary-excreted anthocyanins after intravenous administration, it was deduced that extended glucuronides of cyanidin 3-*O*-*β*-D-glucopyranoside and *O*-methyl cyanidin 3-*O*-*β*-D-glucopyranoside were mainly produced in the liver rather than by intestinal flora. The area under the plasma concentration curve was 0.25 *μ*mol min/L for extended glucuronides of cyanidin 3-*O*-*β*-D-glucopyranoside and 0.14 *μ*mol min/L for *O*-methyl cyanidin 3-*O*-*β*-D-glucopyranoside, respectively, when evaluated as cyanidin 3-*O*-*β*-D-glucopyranoside equivalent, indicating that extended glucuronidation is a critical pathway in cyanidin 3-*O*-*β*-D-glucopyranoside metabolism in rats.

KEYWORDS: Cyanidin 3-*O*-*β*-D-glucopyranoside; *O*-methyl cyanidin 3-*O*-*β*-D-glucopyranoside; glucoside-glucuronide; metabolic pathway; enterohepatic circulation

INTRODUCTION

The health benefits of anthocyanins [antioxidant (*1*−*7*) and anticancer (*8*, *9*) properties, improvement of vision (*10*, *11*), and induction of apoptosis (*12*)] have been widely discussed. Recently, the suppression of NFk-B activation by anthocyanins was also reported (*13*). However, for better understanding of these in vivo functions, studies on uptake and biotransformation of anthocyanins are important. Gastrointestinal absorption of anthocyanins has been reported in both experimental animals and human subjects (*14*−*19*). Several metabolic studies on anthocyanins have also been reported (*20*−*25*). Recently, we studied the metabolic pathway of anthocyanins using purified cyanidin 3-*O*-*β*-D-glucopyranoside and delphinidin 3-*O*-*β*-D-glucopyranoside (**Figure 1**) and discussed how the structural diversity changes the metabolic fate (*23*−*25*). We revealed that *O*-methylation is one of the major and common metabolic pathways of anthocyanin and thus concluded that B ring structure is a major determinant of anthocyanin metabolism.

On the other hand, it is known that glucuronyl or sulfate conjugation is another metabolic pathway of other flavonoids (*26*−*28*). Although many human studies have been reported on urinary metabolites of anthocyanins, the glucuronyl or sulfate conjugates of anthocyanidins have not been reported (*14*−*19*, *22*). Felgines et al. (*21*) found glucuronides and sulfate of pelargonidin excreted in human urine after ingestion of strawberry, which contains pelargonidin 3-*O*-*β*-D-glucopyranoside (**Figure 1**) as the major ingredient. They determined at least four types of pelargonidin-glucuronides in the urine after oral ingestion of freeze-dried strawberry, although the amounts of these metabolites differed greatly.

Recently, we also determined glucuronides of cyanidin and *O*-methyl cyanidin in rat plasma after oral administration of cyanidin 3-*O*-*β*-D-glucopyranoside, although the amount of cyanidin and *O*-methyl cyanidin glucuronides produced was small as compared to that of other flavonoids and indicated that both aglycone structure and the type of sugar moiety affect the production of glucuronides of anthocyanidins (*24*). At the same

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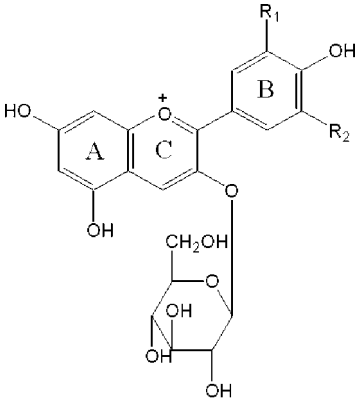

	R_1	R_2
1	OH	H
2	OH	OH
3	H	H

Figure 1. Chemical structures of cyanidin 3-*O*-*β*-D-glucopyranoside (**1**), delphinidin 3-*O*-*β*-D-glucopyranoside (**2**), and pelargonidin 3-*O*-*β*-D-glucopyranoside (**3**).

time, we observed that several hydrophilic metabolites eluted faster than cyanidin 3-*O*-*β*-D-glucopyranoside by reverse phase high-performance liquid chromatography (HPLC), but they remained unidentified. We expected that they were extended glucuronides of cyanidin 3-*O*-*β*-D-glucopyranoside and its *O*-methyl metabolites (*24*). Wu et al. (*20*) determined an extended glucuronide of cyanidin 3-*O*-*β*-D-glucopyranoside by liquid chromatography−electrospray ionization−tandem mass spectrometry (LC-ESI-MS-MS) after ingestion of elderberry, but the profile of metabolites was quite different from what we observed. From the HPLC chromatogram, it was clear that the amounts of these metabolites in blood plasma were several times higher than hydrophobic metabolites of cyanidin 3-*O*-*β*-D-glucopyranoside, indicating that these metabolites will contribute to the health benefits of anthocyanins in vivo. Thus, in the present study, we focused on clarifying the structures of hydrophilic metabolites detected after oral administration of cyanidin 3-*O*-*β*-D-glucopyranoside to investigate the metabolism of cyanidin 3-*O*-*β*-D-glucopyranoside in rats.

MATERIALS AND METHODS

Chemicals. Cyanidin 3-*O*-*β*-D-glucopyranoside (**Figure 1**) was purified from purple black rice (PBR) according to our previously described method with a slight modification (*4*). Briefly, the PBR was peeled and the anthocyanins were extracted from the peel by methanol containing 3% trifluoroacetic acid (TFA). The methanolic extract was evaporated to dryness in vacuo and redissolved in distilled water containing 1% TFA. The extract was washed with ethyl acetate to recover the anthocyanins in the water fraction. After removal of residual ethyl acetate by evaporation, the water fraction was further separated by a 30 cm × 5.7 cm open column packed with poly(vinylpyrrolidone) (ISP, Japan). The column was washed with 1% aqueous TFA solution for the elution of water soluble components, and furthermore, cyanidin 3-*O*-*β*-D-glucopyranoside was eluted by 30% methanol containing 1% TFA. The cyanidin 3-*O*-*β*-D-glucopyranoside rich fraction was collected and evaporated to dryness in vacuo under 40 °C. The cyanidin 3-*O*-*β*-D-glucopyranoside recovered was dissolved in a small amount of methanol containing 0.1% hydrochloric acid, and diethyl ether was added in order to precipitate it as a chloride salt. The purity of cyanidin 3-*O*-*β*-D-glucopyranoside was checked by tandem MS and NMR as described in our previous report (*29*). All other reagents including methanol were purchased from Wako Pure Chemical Industries and used without further purification.

Animals and Diets. SPF male Wistar ST rats (6 weeks old, 160 g body weight) purchased from Japan SLC Inc. (Hamamatsu, Japan) were 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 control diet for 7 days before the experiment. Rats were treated in accordance with the Guidelines of Niigata University of Pharmacy and Applied Life Sciences.

Experimental Design and Plasma Preparation. The experimental design and preparation of plasma were carried out according to our previously described method (*24*). Briefly, four rats were cannulated with a polyethylene tube into a neck vein under anesthesia with diethyl ether. The neck vein was isolated, and a small hole was made by scissors to insert the polyethylene tube (PE-50). After the tube was inserted, the vein and tube were occluded and the tube was guided out from the back of rats. After starvation for 24 h, cyanidin 3-*O*-*β*-D-glucopyranoside (100 mg/kg body weight) dissolved in 0.1% citric acid was orally administered. During the experiment, the rats were allowed to move freely in the cages. The cyanidin 3-*O*-*β*-D-glucopyranoside dose administered was based on the orally administered experiment as described previously (*24*).

Donor blood was obtained from the inferior vena cava of other healthy rats under anesthesia with diethyl ether using a syringe containing 500 μL of 10% sodium citrate for 8 mL of blood. After withdrawal of the blood sample (600 μL), the same volume of donor blood was injected through the cannulated vein tube. Each blood sample was immediately centrifuged at 3000*g* for 5 min at 4 °C for HPLC analysis.

For intravenous administration, four rats were cannulated into a neck vein with a polyethylene tube (PE-50) before the experiment under anesthesia described in the section of oral administration study. After 24 h of starvation, the bile duct was isolated and a small hole was made for the insertion of the polyethylene tube (PE-20) under anesthesia with diethyl ether for bile collection and the rats were set in cages. Then, cyanidin 3-*O*-*β*-D-glucopyranoside (2 mg/kg body weight) dissolved in saline was directly injected via the polyethylene tube into the vein and both bile and urine were collected.

Urine samples were collected during 4 h of both experiments, and the urine remaining in the bladder was further collected by a syringe after sacrifice. Blood samples were collected via the cannulated tube using a heparinized syringe at 15, 30, 60, 120, and 240 min after the cyanidin 3-*O*-*β*-D-glucopyranoside administration.

Determination of Anthocyanin Metabolites in Rat Plasma and Urine. Extraction of anthocyanins and its metabolites from plasma and urine was carried out according to our previous report using a Sep-Pak C_{18} cartridge light (Waters, United States) (*24*). Briefly, the collected plasma (300 μL) and urine (sum of 4 h) samples were applied to Sep-Pak C_{18} cartridges conditioned with methanol (2 mL) and 3% TFA aqueous solution (2 mL). After the sample application, the cartridges were washed successively with 2 mL of 3% TFA aqueous solution, dichloromethane, benzene, and cyanidin 3-*O*-*β*-D-glucopyranoside and its metabolites were eluted with 50% acetonitrile containing 1% TFA aqueous solution (1 mL). The eluent was evaporated to dryness in vacuo and dissolved in 150 μL of distilled water containing 0.5% TFA. The TFA solution was passed through 0.45 μm Centricut (Kurabou Co. Ltd., Japan) before HPLC injection. HPLC was performed according to our previous report with modification (*24*, *30*). Briefly, aliquots (100 μL) of the solutions were injected into an HPLC system (Hitachi 7200) equipped with a 150 mm × 1.0 mm i.d. Develosil ODS HG-5 column (Nomura Chemical Co., Ltd., Aichi, Japan). The elution was performed using a solvent gradient system consisting of solvent A (0.5% aqueous TFA) and solvent B (methanol containing 0.5% TFA). The gradient condition was as follows: 75% A 0−15 min then linear gradient from 75 to 60% A for 40 min, and then held for 10 min at a flow rate of 0.1 mL/min. The elution peaks were monitored at 520 nm with a UV/vis detector (Hitachi, Japan). The recovery of cyanidin 3-*O*-*β*-D-glucopyranoside in this method was checked using authentic cyanidin 3-*O*-*β*-D-glucopyranoside and found to be 82.3%.

Isolation and Identification of Anthocyanin Metabolites. Cyanidin 3-*O*-*β*-D-glucopyranoside metabolites were separated and recovered by HPLC using a 150 mm × 4.6 mm i.d. column. The separation condition was 15% MeOH containing 0.5% TFA solution at a flow rate of 2

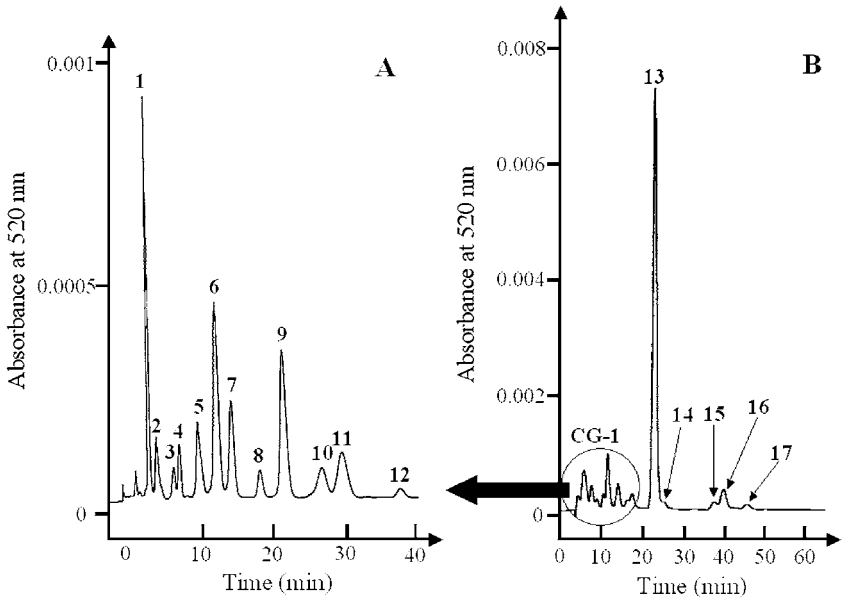

Figure 2. Typical HPLC chromatogram of cyanidin 3-*O*-*β*-D-glucopyranoside administrated rat plasma. (**A**) Hydrophilic fraction (CG-1) and (**B**) 15 min after oral administration. Peak identification is shown in **Table 1**.

mL/min. The purity of each metabolite recovered was checked by the semimicro-HPLC system described above. Identification of anthocyanin metabolites was performed by tandem MS. Each metabolite sample was dissolved in methanol, and 20 μL was subjected to mass spectrometry performed with a quantitative time-of-flight (Q-TOF) Ultima (Waters, Manchester, United Kingdom). The conditions for TOF MS-MS were as follows: A syringe pump (KD Science Inc., United States) was used to provide a constant infusion (300 μL/h) of the sample into the MS ion source. MS parameters used were as follows: 3.2 kV for capillary; 9.1 kV for reflection. Argon gas was used for collision at a pressure of 11 psi, and the applied voltage was 24 V.

RESULTS

Cyanidin 3-*O*-*β*-D-glucopyranoside and its metabolites were detected in rat plasma and urine after the oral administration. The typical HPLC chromatogram of cyanidin 3-*O*-*β*-D-glucopyranoside administrated rat plasma was shown in **Figure 2B**. In addition to the original cyanidin 3-*O*-*β*-D-glucopyranoside and the four metabolites that we identified previously (*24*), several hydrophilic metabolite peaks were observed. To analyze the structures of these hydrophilic metabolites, the separation condition for the hydrophilic fraction (CG-1) was further examined by HPLC. As shown in **Figure 2A**, CG-1 collected from blood plasma was found to contain 12 peaks. Using this HPLC condition, these metabolites were isolated and the purity of each metabolite was checked by semimicro-HPLC. Although the data are not shown, all metabolites were highly purified enough for tandem MS analysis (>98% at 520 nm absorbance). The MS data obtained for the metabolites were as follows: *m*/*z* 625/463/449/287 for peaks 1, 5, 6, and 8, indicating that the metabolites are the glucuronides of cyanidin 3-*O*-*β*-D-glucopyranoside, and *m*/*z* 639/477/463/301 for peaks 2, 3, 7, 9, 10, and 11, indicating that the metabolites are the glucuronides of *O*-methyl cyanidin 3-*O*-*β*-D-glucopyranoside, the primary metabolites of cyanidin 3-*O*-*β*-D-glucopyranoside (**Figure 3** and **Table 1**), respectively.

The average plasma concentration profiles of these metabolites are given in **Figure 4**. As in **Figure 4A**, all cyanidin 3-*O*-*β*-D-glucopyranoside-glucuronides showed their maximum plasma concentration 15 min after oral administration whereas *O*-methyl cyanidin 3-*O*-*β*-D-glucopyranoside-glucuronides showed their maximum concentration at 60 min (except for peak 2) (**Figure 4B**), although the maximum plasma levels of these metabolites were varied, such as from 10 to 70 nM for cyanidin 3-*O*-*β*-D-glucopyranoside-glucuronides and 5 to 30 nM for *O*-methyl cyanidin 3-*O*-*β*-D-glucopyranoside-glucuronides. Both cyanidin 3-*O*-*β*-D-glucopyranoside-glucuronides and *O*-methyl cyanidin 3-*O*-*β*-D-glucopyranoside-glucuronides were not detected 240 min after oral administration of cyanidin 3-*O*-*β*-D-glucopyranoside.

Figure 5 shows the area under the plasma concentration curve (AUC) of cyanidin 3-*O*-*β*-D-glucopyranoside and its metabolites during the first 4 h of oral administration. The major anthocyanin in the blood plasma was the original cyanidin 3-*O*-*β*-D-glucopyranoside (0.64 μmol min/L). The AUCs of extended glucuronides were 0.246 and 0.144 μmol min/L for cyanidin 3-*O*-*β*-D-glucopyranoside-glucuronides and *O*-methyl cyanidin 3-*O*-*β*-D-glucopyranoside-glucuronides, respectively. The AUC of cyanidin-glucuronide and *O*-methyl cyanidin-glucuronide, which are the metabolites produced via the trans-glucosidation process, was quite small as compared to those for glucoside-glucuronides, and it was only 0.099 μmol min/L. Furthermore, the AUC of *O*-methyl cyanidin 3-*O*-*β*-D-glucopyranoside (sum of 3′- and 4′-*O*-methyl analogues) was smaller than any other metabolites and it was only 0.084 μmol min/L.

On the other hand, the total amount of anthocyanins, original cyanidin 3-*O*-*β*-D-glucopyranoside, and its metabolites recovered in the urine was approximately 0.005% of the orally administered dose (100 mg/kg body weight). Original cyanidin 3-*O*-*β*-D-glucopyranoside (0.578 nmol) and two *O*-methyl cyanidin 3-*O*-*β*-D-glucopyranoside (0.501 nmol) were the major anthocyanins recovered in the urine (**Figure 6**). The recovered amounts of extended glucuronides in the urine were 0.445 nmol for cyanidin 3-*O*-*β*-D-glucopyranoside-glucuronides and 0.386 nmol for and *O*-methyl cyanidin 3-*O*-*β*-D-glucopyranoside-glucuronides, respectively. However, the urinary excretion of cyanidin-glucuronide and *O*-methyl cyanidin-glucuronide was only 0.091 nmol.

To investigate the effect of enterohepatic circulation on anthocyanin absorption and metabolism, anthocyanins (cyanidin 3-*O*-*β*-D-glucopyranoside and its metabolites) excreted in bile

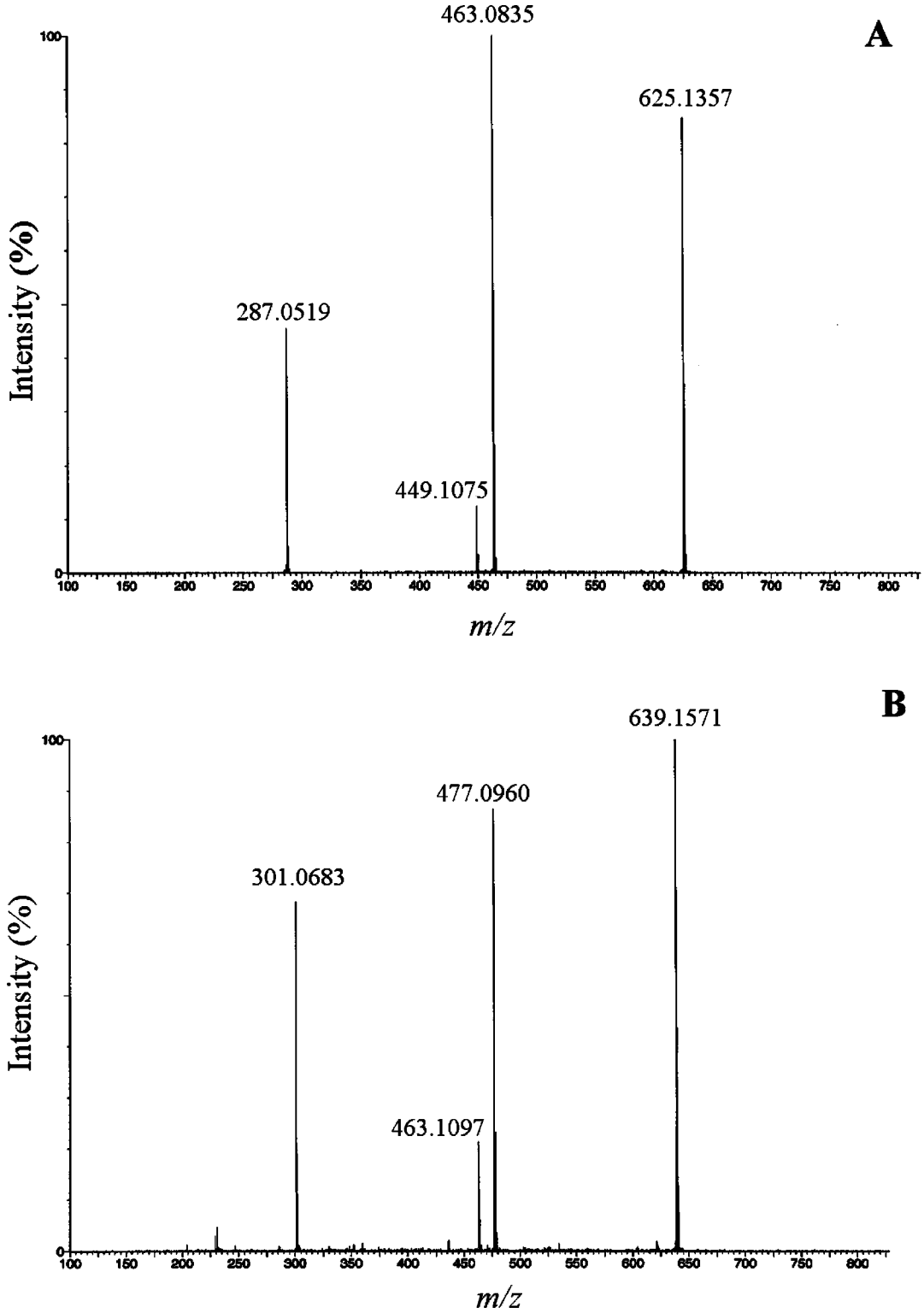

Figure 3. Tandem MS spectra of hydrophilic metabolites. (**A**) Peak 1 and (**B**) peak 2.

after intravenous administration were also analyzed together with urinary-excreted anthocyanins. It was found that cyanidin 3-*O*-*β*-D-glucopyranoside and its metabolites recovered as totals were 36% in urine and 12% in bile during the first 4 h of intravenous administration.

DISCUSSION

We previously studied the metabolism of several anthocyanins in rats and showed that their metabolic fates were quite different dependent on the chemical structure (*23*−*25*). In those studies, we showed that *O*-methylation is the major reaction that occurred in anthocyanins and the site of methylation differed according to the B ring structure. That is, delphinidin 3-*O*-*β*-D-glucopyranoside exclusively produced 4′-*O*-methyl metabolite, but both 3′-*O*- and 4′-*O*-methyl analogues were formed in the case of cyanidin 3-*O*-*β*-D-glucopyranoside. The *O*-methylation reaction is observed in other flavonoids, but phase II metabolism also occurred. For example, glucuronides of quercetin and epicatechin were reported (*26*−*29*). The phase II metabolism

Table 1. Identification of Cyandin 3-*O*-β-D-Glucopyranoside Metabolites

peak no.	type of metabolite	MS-MS fragment
1	cyanidin 3-*O*-β-D-glucopyranoside-glucuronide	625/463/449/287
2	*O*-methyl cyanidin 3-*O*-β-D-glucopyranoside-glucuronide	639/477/463/301
3	*O*-methyl cyanidin 3-*O*-β-D-glucopyranoside-glucuronide	639/477/463/301
4	unknown	
5	cyanidin 3-*O*-β-D-glucopyranoside-glucuronide	625/463/449/287
6	cyanidin 3-*O*-β-D-glucopyranoside-glucuronide	625/463/449/287
7	*O*-methyl cyanidin 3-*O*-β-D-glucopyranoside-glucuronide	639/477/463/301
8	cyanidin 3-*O*-β-D-glucopyranoside-glucuronide	625/463/449/287
9	*O*-methyl cyanidin 3-*O*-β-D-glucopyranoside-glucuronide	639/477/463/301
10	*O*-methyl cyanidin 3-*O*-β-D-glucopyranoside-glucuronide	639/477/463/301
11	*O*-methyl cyanidin 3-*O*-β-D-glucopyranoside-glucuronide	639/477/463/301
12	unknown	
13	cyanidin 3-*O*-β-D-glucopyranoside	449/287
14	cyanidin-glucuronide	449/287
15	4′-*O*-methyl cyanidin 3-*O*-β-D-glucopyranoside	463/301
16	3′-*O*-methyl cyanidin 3-*O*-β-D-glucopyranoside	463/301
17	*O*-methyl cyanidin-glucuronide	477/301

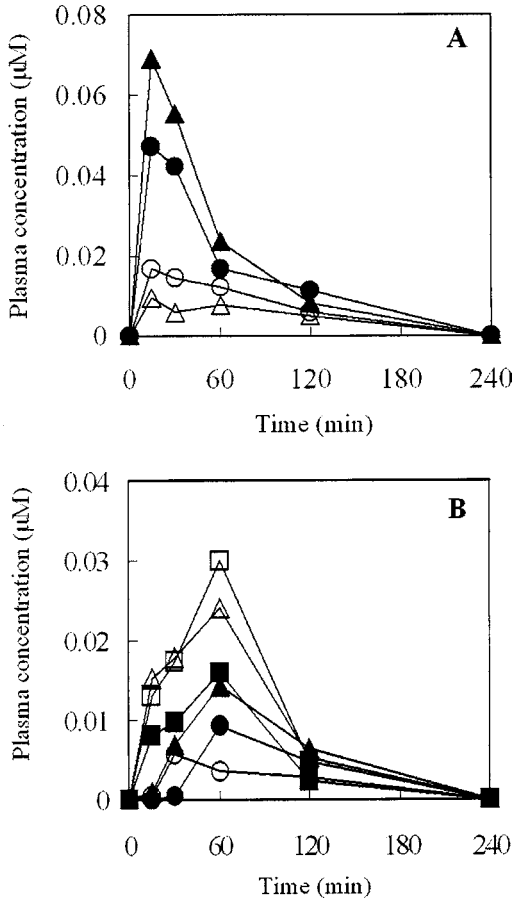

Figure 4. Plasma concentration profiles of cyanidin 3-*O*-β-D-glucopyranoside-glucuronides and *O*-methyl cyanidin 3-*O*-β-D-glucopyranoside-glucuronides. (**A**) Cyanidin 3-*O*-β-D-glucopyranoside-glucuronides. Symbols: ●, peak 1; ○, peak 5; ▲, peak 6; and △, peak 8. Values are means of four rats. (**B**) *O*-Methyl cyanidin 3-*O*-β-D-glucopyranoside-glucuronides. Symbols: ○, peak 2; ●, peak 3; ▲, peak 7; △, peak 9; ■, peak 10; and □, peak 11. Values are means of four rats.

might also be important for anthocyanin metabolism. Felgines et al. (*21*) reported glucuronides and a sulfate of pelargonidin excreted in the urine of humans after ingestion of freeze-dried strawberry. They determined at least four types of pelargonidin-glucuronides in the urine that were all predicted from the number of free hydroxyl groups in the aglycone (pelargonidin), although the amounts of these metabolites differed greatly.

We also reported the production of glucuronides of cyanidin and *O*-methyl cyanidin in rat plasma, although the site of methylation and glucuronidation was not assigned (*24*). Interestingly, delphinidin did not produce any glucuronide in our previous study (*23*) indicating that the glucuronidation is also dependent on the type of anthocyanidin (aglycone) structures. We further studied the metabolism of cyanidin 3-*O*-rutinoside and delphinidin 3-*O*-rutinoside in rats and observed that the same type of methylation occurred as cyanidin 3-*O*-β-D-glucopyranoside and delphinidin 3-*O*-β-D-glucopyranoside, but no glucuronyl conjugate was detected in plasma for the rutinosides (unpublished results). In the urine, however, glucuronides of cyanidin 3-*O*-rutinoside and delphinidin 3-*O*-rutinoside were detected after oral administration, although the amounts were much smaller than cyanidin 3-*O*-β-D-glucopyranoside. We thus tentatively concluded that both aglycone structure and conjugated sugar moiety are the determinants of the metabolic fate of anthocyanins.

In the previous study, we observed several hydrophilic metabolites in both plasma and urine, which eluted faster than cyanidin 3-*O*-β-D-glucopyranoside by reverse phase HPLC, but they remained unidentified. We expected that they were extended glucuronides of cyanidin 3-*O*-β-D-glucopyranoside and its metabolites (*24*). Because the amounts of these metabolites were larger than that of cyanidin-glucuronide and *O*-methyl cyanidin 3-*O*-β-D-glucopyranoside assigned in our previous study (*24*), in the present study, we focused our attention on these hydrophilic metabolites to determine their chemical structures to understand the metabolism of cyanidin 3-*O*-β-D-glucopyranoside in rats. Ten hydrophilic metabolites out of 12 observed in the HPLC chromatogram were assigned as glucuronides of cyanidin 3-*O*-β-D-glucopyranoside and glucuronideds of *O*-methyl cyanidin 3-*O*-β-D-glucopyranoside by tandem MS. As shown in **Figure 3**, fragments with *m*/*z* 463 and 477 observed in the MS spectra corresponded to cyanidin-glucuronide and *O*-methyl cyanidin-glucuronide, respectively. Therefore, the glucuronic acid was directly attached to a phenolic group on anthocyanidin. The numbers and the types of these metabolites were consistent with those predicted from the numbers of free hydroxyl groups in the aglycone structure of cyanidin 3-*O*-β-D-glucopyranoside and its *O*-methyl metabolites.

It should be noted that the maximum concentration of glucuronides in the plasma differed widely among metabolites as shown in **Figure 4**. All cyanidin 3-*O*-β-D-glucopyranoside-glucuronides reached their maximum at 15 min after oral administration but *O*-methyl cyanidin 3-*O*-β-D-glucopyranoside-

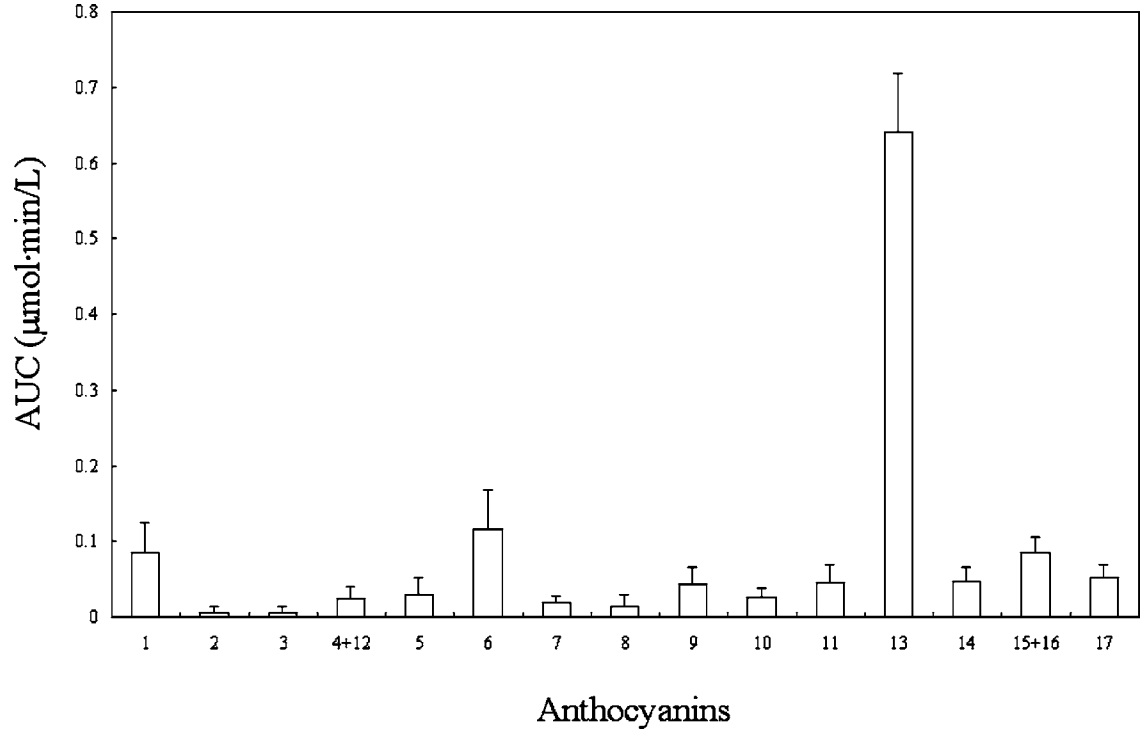

Figure 5. Plasma level (AUC) of anthocyanins during first 4 h. Values are means ± SEM of four rats.

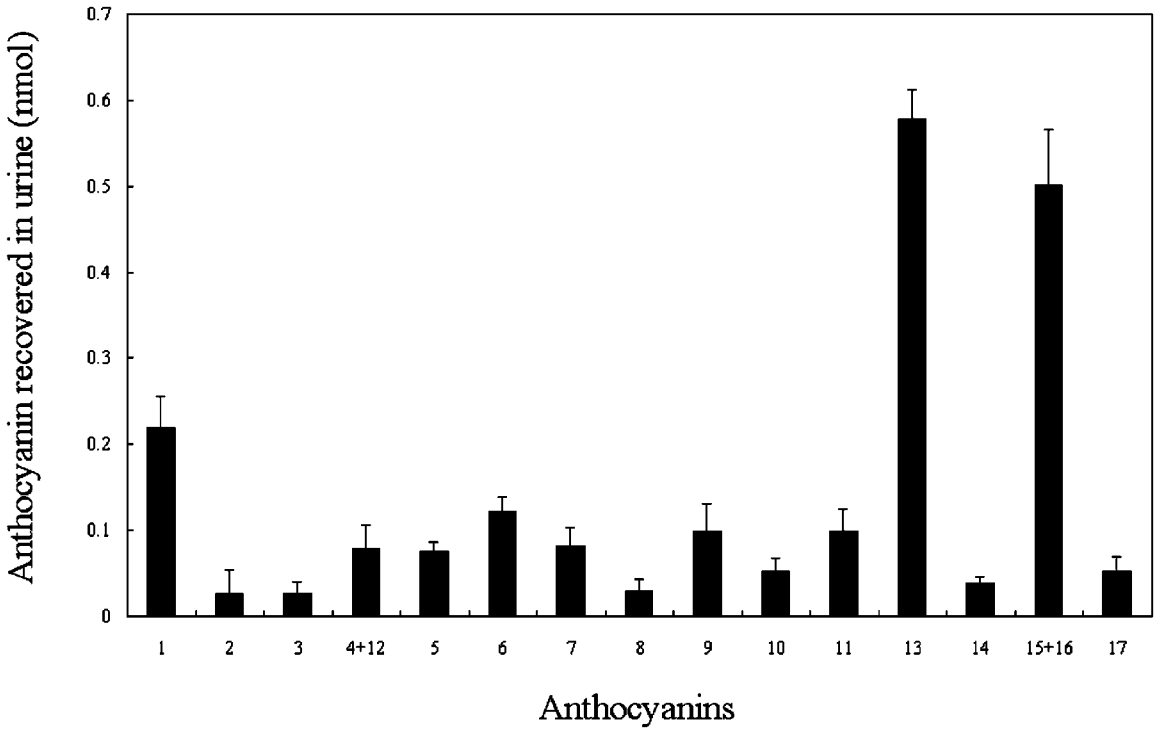

Figure 6. Urinary excretion amounts of anthocyanins during first 4 h. Values are means ± SEM of four rats.

glucuronides at 60 min; except for peak 2, the maximum appeared at 30 min.

Although the data are not shown, cyanidin 3-*O*-*β*-D-glucopyranoside-glucuronides and *O*-methyl cyanidin 3-*O*-*β*-D-glucopyranoside-glucuronides were also detected in blood plasma and urine after intravenous administration of cyanidin 3-*O*-*β*-D-glucopyranoside (2 mg/kg) indicating that the extended glucuronidation occurred in the liver rather than in the small intestine. The slower appearance of *O*-methyl cyanidin 3-*O*-*β*-D-glucopyranoside-glucuronides in the blood plasma (the maximum plasma level appeared at 30−60 min) as compared to cyanidin 3-*O*-*β*-D-glucopyranoside-glucuronides suggested enterohepatic circulation of the metabolites. Thus, to clear the effect of bile excretion on anthocyanin metabolism, bile was collected after intravenous administration of cyanidin 3-*O*-*β*-D-glucopyranoside. Cyanidin 3-*O*-*β*-D-glucopyranoside and its metabolites recovered in bile and urine were about 12 and 36% of intravenously administered dose, respectively. The low recovery of cyanidin 3-*O*-*β*-D-glucopyranoside in urine and bile after intravenous administration might be explained by tissue uptake and degradation, although we do not have any conclusive data at present. Furthermore, studies are needed to clarify this point. On the

other hand, urinary recovery of anthocyanins after oral administration was approximately 0.005% during the first 4 h. From those values, we can estimate the recirculation of cyanidin 3-*O*-*β*-D-glucopyranoside and the metabolites by enterohepatic circulation are not greater than 0.0017% of orally administered dose (0.34 *μ*g was excreted in small intestine via bile solution when 20 mg of cyanidin 3-*O*-*β*-D-glucopyranoside was orally administered). Therefore, it was concluded that the effect of enterohepatic circulation on anthocyanin metabolism is negligible. It was thus concluded that *O*-methyl cyanidin 3-*O*-*β*-D-glucopyranoside-glucuronides were produced in the liver rather than in the intestine. Miyazawa et al. (*22*) also reported two *O*-methyl cyanidin 3-*O*-*β*-D-glucopyranoside distributed mainly in liver. Thus, cyanidin 3-*O*-*β*-D-glucopyranoside uptaken in liver was primarily *O*-methylated and successively glucuronidated to be washed out into the blood circulation.

It is interesting to note that there is a large difference between the AUC and the urinary-excreted anthocyanin metabolites profile as shown in **Figures 5** and **6**. The AUCs of intact cyanidin 3-*O*-*β*-D-glucopyranoside and *O*-methyl cyanidin 3-*O*-*β*-D-glucopyranoside were 0.64 and 0.084 *μ*mol min/L, respectively. On the other hand, the urinary excretions of cyanidin 3-*O*-*β*-D-glucopyranoside and *O*-methyl cyanidin 3-*O*-*β*-D-glucopyranoside were 0.578 and 0.501 nmol, respectively. The ratio of cyanidin 3-*O*-*β*-D-glucopyranoside vs *O*-methyl cyanidin 3-*O*-*β*-D-glucopyranoside was 7.61 for the AUC and 1.16 for urinary recovered amounts (see compounds **13** and **15** + **16** in **Figures 5** and **6**). This indicates that *O*-methylation occurred in the kidney. Anthocyanin metabolism was usually studied for urine samples, especially in human (*20*, *21*), but our data suggested that it is important to know the plasma anthocyanin profile and its metabolites for the understanding of physiological functions.

It was also noted that the AUCs of extended glucuronides were 0.246 *μ*mol min/L for cyanidin 3-*O*-*β*-D-glucopyranoside-glucuronides and 0.144 *μ*mol min/L for *O*-methyl cyanidin 3-*O*-*β*-D-glucopyranoside-glucuronides, respectively; therefore, extended glucuronidation is also the major metabolic pathway of cyanidin 3-*O*-*β*-D-glucopyranoside. On the other hand, the AUC of glucuronides (sum of cyanidin-glucuronide and *O*-methyl cyanidin-glucuronide) was only 0.099 *μ*mol min/L.

In the case of other flavonoids such as quercetin and epicatechin, the glucuronide of the aglycone is the major excretion form in urine (*26*−*28*), and no glucoside glucuronide was reported. It is thus indicated that the glucuronidation reaction is variable, dependent on the type of flavonoid aglycone. It is interesting to note that the production of glucuronides of glucosylated anthocyanidin is more marked in anthocyanins than other flavonoids. Flavonoids such as epicatechin and quercetin have stable aglycones as compared to anthocyanins as they occur in free aglycone form in nature. In contrast, anthocyanidins (aglycones) are quite unstable and quickly degraded under physiological conditions, although anthocyanins (glycosides of aglycones) are relatively stable as compared to the aglycone. It is thus reasonable to consider that the production of glucoside-glucuronide is more preferable for anthocyanins rather than anthocyanidins as compared to other flavonoids. In other words, glucuronidation of anthocyanins competes with the decomposition process. In conclusion, the present study revealed that the phase II metabolism also plays an important part in the metabolic fate of cyanidin 3-*O*-*β*-D-glucopyranoside in addition to the primary metabolic modification on the aglycone B ring (*O*-methylation). Further studies on the physiological function of these metabolites are needed to clarify anthocyanin function in vivo.

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