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Ingested Delphinidin-3-rutinoside Is Primarily Excreted to Urine as the Intact Form and to Bile as the Methylated Form in Rats

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Many reports have described the bioavailability of anthocyanins; however, most of these reports investigated only the amount of anthocyanins excreted in urine. In the present study, we calculated the pharmacokinetic bioavailability of anthocyanins in rats by measuring the plasma concentration of delphinidin-3-rutinoside that had been administered orally or intravenously. Delphinidin-3-rutinoside was primarily absorbed in the blood and excreted into urine as unmetabolized forms with a T_{max} of 26.3 min and a C_{max} of 0.285 ± 0.071 μmol/L. We detected small amounts of the metabolite 4′-*O*-methyl-delphinidin-3-rutinoside in the plasma, but we detected neither anthocyanidin (aglycone) nor glucuro- or sulfoconjugates. For the 8 h period after intake, delphinidin-3-rutinoside and 4′-*O*-methyl-delphinidin-3-rutinoside were excreted to urine at 795 ± 375 and 12.3 ± 2.91 nmol, respectively. Relative to intravenous injection, oral administration of delphinidin-3-rutinoside resulted in complete bioavailability (0.49 ± 0.06%). Analysis of delphinidin-3-rutinoside plasma concentrations in bile cannulated rats revealed that, for the 8-h period after intake, the intact delphinidin-3-rutinoside excretion ratio in bile was 11% of the excretion ratio of 4′-*O*-methyl-delphinidin-3-rutinoside, 1.91 ± 0.35 nmol versus 17.4 ± 8.67 nmol, respectively. Setting the bile duct cannulation in a Bollman-type cage, however, significantly increased the bioavailability of orally administered delphinidin-3-rutinoside (18.14 ± 6.24%). This effect appears to stem immobilization stress by reducing gastrointestinal motility. The cumulative excretion of delphinidin-3-rutinoside and 4′-*O*-methyl-delphinidin-3-rutinoside in urine and bile was 2.67 ± 1.24% (w/w) of the dose ingested. Studies report that several metabolites are formed after oral ingestion of anthocyanins. Examples include glucuronyl from cyanidin-3-glucoside and both glucuronyl and sulfate conjugates from pelargonidin-3-glucoside. Our results indicate that delphinidin-3-rutinoside might be metabolized differently from cyanidin-3-glucoside and pelargonidin-3-glucoside.

KEYWORDS: Delphinidin-3-rutinoside; bioavailability; anthocyanin; plasma; urine; bile

INTRODUCTION

Anthocyanins are a group of naturally occurring phenolic compounds responsible for the color of many flowers, fruits (particularly berries), and vegetables. Dietary anthocyanins have attracted considerable interest because of their health benefits which include reducing the risk of coronary heart disease and preventing several chronic diseases (*1*). Black currant (*Ribes nigrum* L.) berries and juice are rich in anthocyanins and are consumed throughout the world. We previously reported the development of a powdered concentrate of black currant anthocyanins (BCAs) from a commercial source (*2*). BCAs consist of four anthocyanins: delphinidin-3-rutinoside (**Figure 1**), delphinidin-3-glucoside, cyanidin-3-rutinoside, and cyanidin-3-glucoside. In previous studies we demonstrated that oral intake of BCAs has several beneficial effects, including antioxidant effects (*3*), improved vision (*4*), and improved blood circulation (*5*). As a primary component of black currant anthocyanin, delphinidin-3-rutinoside also has unique physiological effects that include relaxation of ciliary smooth muscle (*6*) and reduction of peripheral vascular resistance (unpublished results). We previously demonstrated in humans and rats that BCAs are absorbed in the gastrointestinal tract and can be detected in blood as unmetabolized forms (*7*). Plasma concentrations of anthocyanins were very low (10−50 nmol/L), and relative urinary excretion was also low at approximately 0.1% of intake.

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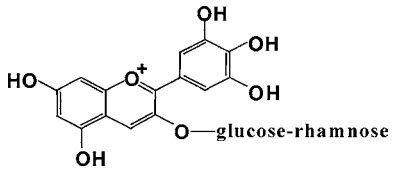

Figure 1. Chemical structure of delphinidin-3-rutinoside.

Recently, several metabolites were reported to be formed after oral ingestion of anthocyanins. These metabolites include a glucuronyl conjugate from cyanidin-3-glucoside and both glucuronyl and sulfate conjugates from pelargonidin-3-glucoside (*8*, *9*). After ingestion of delphinidin-3-glucoside, the intact glucoside and its metabolite, 4′-*O*-methyl-delphinidin-3-glucoside (*10*), were detected in rat plasma, although the concentration of the latter was less. Anthocyanins are known to change to five different equilibrium forms in response to changes in pH (*11*). It is well-known that the quinoidal base form is unstable at neutral pH such as that of blood plasma (pH 7.4). A study by Lietti et al. (*12*) reported that anthocyanins have a greater affinity for tissues such as kidney and skin than for plasma, but this study did not directly assess the bioavailability of anthocyanin. In the present study, we calculated the pharmacokinetic bioavailability of anthocyanins in rats by measuring the plasma concentration of delphinidin-3-rutinoside that had been administered orally or intravenously. Additionally, we examined and compared urine and bile excretion profiles of delphinidin-3-rutinoside.

MATERIALS AND METHODS

Chemicals. We used crystal form of delphinidin-3-rutinoside·HCl·$^1/_2H_2O$ (MW 656.47) prepared from commercial black currant extract as previously described (*3*). 4′-*O*-methyl-delphinidin-3-rutinoside was purified and collected as previously described (*14*). All other nutrients, reagents, and chemicals used were purchased from commercial sources.

Animals and Diets. SPF male Wistar ST rats (6 weeks of age, about 160 g body weight) 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 controlled diet for 7 days before the experiment. All rats were handled in accordance with the NIH standards (*13*).

Sample Preparation. The preparation of plasma was carried out as previously described (*14*). For HPLC analysis, blood samples were immediately centrifuged at 3000*g* for 5 min at 4 °C. Urine remaining in the bladder was further collected by a syringe after sacrifice. Extraction of delphinidin-3-rutinoside and 4′-*O*-methyl-delphinidin-3-rutinoside was carried out essentially according to a previous report using Sep-Pak C_{18} light cartridges (Waters, Milford, MA) (*14*). Briefly, the collected plasma (200 μL), bile (100 μL), and urine (100 μL) 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, and benzene, followed by elution of delphinidin-3-rutinoside and 4′-*O*-methyl-delphinidin-3-rutinoside with 50% acetonitrile containing 1% TFA aqueous solution (1 mL). The eluents were evaporated to dryness in vacuo and dissolved in 150 μL of distilled water containing 0.5% TFA. The TFA solution was passed through a 0.45 μm Centricut filter (Kurabou Co. Ltd., Osaka, Japan) before HPLC injection.

Identification of Anthocyanins. HPLC was performed as previously described (*14*). Briefly, aliquots (100 μL) of these solutions were injected into a model 7200 HPLC system (Hitachi, Tokyo, Japan) equipped with a 150 × 1.0 mm i.d. Develosil ODS-HG5 column (Nomura Chemical Co., Ltd., Aichi, Japan). The elution was performed using a solvent gradient system consisting of solvent A (0.5% TFA aqueous solution) and solvent B (0.5% TFA containing methanol solution). The gradient condition was as follows: 75% A 0−15 min and then linear gradient from 75% A to 60% for 15 to 40 min, and then held 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). These peaks were identified by comparing the retention time on the HPLC chromatogram with delphinidin-3-rutinoside and 4′-*O*-methyl-delphinidin-3-rutinoside standards, which were identified by a HPLC-ESI-MS spectrum.

Design for Bioavailability (BA) Calculation. The experimental design was carried out as previously described at cyanidin-3-glucosidestudy (*14*). Briefly, four rats were cannulated with polyethylene tubes (PE-50) in a neck vein under anesthesia with diethyl ether. After 24 h of starvation, delphinidin-3-rutinoside (152 μmol/kg of body weight) dissolved in 0.1% citric acid was orally administered. During the experiment, the rats were allowed to move freely in the cages to avoid excess stress. Blood samples were collected via the cannulated tube using a heparinized syringe at 15, 30, 60, 120, 240, 360, and 480 min after delphinidin-3-rutinoside administration. Then 8 mL of donor blood was obtained from the inferior vena cava of other healthy rats using a syringe containing 500 μL of 10% sodium citrate 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 intravenous administration, four anesthetized rats were cannulated into a neck vein with a polyethylene tube (PE-50) before the experiment as described above. On the day of the experiment, rats were set in Bollman-type cages, and delphinidin-3-rutinoside (7.62 μmol/kg of body weight) was dissolved in saline and directly injected into the vein via the polyethylene tube. Blood samples were then collected via the cannulated tube using a heparinized syringe at 1, 5, 15, 30, 60, and 120 min after injection.

Design for Excretion Analysis. Prior to the experiment, four anesthetized rats were each cannulated into a neck vein with a polyethylene tube (PE-50) as described above. After 24 h starvation, a polyethylene tube (PE-20) was cannulated into the bile duct for bile collection, and rats were set in Bollman-type cages. Then, delphinidin-3-rutinoside (152 μmol/kg of body weight), dissolved in 0.1% citric acid, was orally administrated. Blood samples were collected via the cannulated tube using a heparinized syringe at 15, 30, 60, 120, 240, and 480 min after the delphinidin-3-rutinoside administration. Bile and urine were collected 0−2, 2−4, and 4−8 h in test tube and 1 mL of 3% TFA was added.

Pharmacokinetic Analysis. Analysis of the blood concentration−time data was performed by noncompartment model analysis using WinNonlin Professional (version 3.1) (Pharsight Co., Mountain View, CA). The delphinidin-3-rutinoside maximum plasma concentration (C_{max}) and the time to reach the maximum plasma concentration (T_{max}) were calculated from the observed values. The total area under the concentration time curve AUC_{0-inf} was calculated by the trapezoidal rule based on the plasma concentrations up to the time of final measurement. BA (%) was calculated by the indicated formula. BA = (AUC_{0-inf}, p.o.)/(dose, p.o.) ÷ (AUC_{0-inf}, i.v.)/(dose, i.v.) × 100.

RESULTS

Absorption and Metabolism of Delphinidin-3-rutinoside in Rats. To characterize the absorption and metabolism of anthocyanins in rats, we orally administered purified delphinidin-3-rutinoside (152 μmol/kg body weight) and subsequently measured plasma concentrations by HPLC. No anthocyanins were detected in the plasma before we administered delphinidin-3-rutinoside. **Figure 2** shows a typical high-performance liquid chromatogram obtained from the plasma of a rat administered delphinidin-3-rutinoside. When the absorbance of the eluate was monitored at 520 nm, the only peaks we observed were a delphinidin-3-rutinoside peak and a slight 4′-*O*-methyl-delphinidin-3-rutinoside peak (**Figure 2**). Delphinidin-3-rutinoside and 4′-*O*-methyl-delphinidin-3-rutinoside were identified by comparing the respective retention times on HPLC with those of a standard (unpublished data). Plasma concentrations of delphinidin-3-rutinoside rapidly increased to 250 ± 10 nmol at 15 min

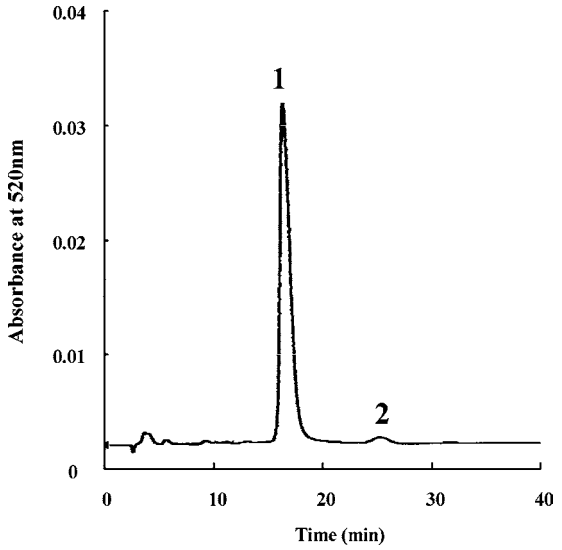

Figure 2. HPLC chromatogram of rat plasma. Peaks are as follows: (1) delphinidin-3-rutinoside; (2) 4′-*O*-methyl-delphinidin-3-rutinoside.

postadministration (**Figure 3A**), reaching a maximum of 270 ± 70 nmol at 30 min postadministration before decreasing over time. Plasma concentrations of 4′-*O*-methyl-delphinidin-3-rutinoside (calculated as delphinidin-3-rutinoside form) peaked at 6.5 ± 3.0 nmol at 120 min postadministration before decreasing over time (**Figure 3B**).

We next monitored the temporal concentration changes after directly injecting delphinidin-3-rutinoside into the neck vein (the dosage 7.62 μmol/kg body weight). Following intravenous injection, the plasma concentration of delphinidin-3-rutinoside reached a maximum of 25.7 ± 4.0 μmol at 1 min postinjection (**Figure 4**). Thereafter, the decline was rapid so that by 120 min delphinidin-3-rutinoside was not detected in plasma. The terminal half-life was approximately 73 min. Using the plasma concentration data, we next calculated the pharmacokinetic parameters of delphinidin-3-rutinoside using a noncompartment model. We observed a T_{max} of 26.3 min and a C_{max} of 0.285 ± 0.071 μmol (**Table 1**). The bioavailability of delphinidin-3-rutinoside was 0.49% for oral and intravenous administration.

Determination of Excretion Route. We next determined the excretion of delphinidin-3-rutinoside and 4′-*O*-methyl-delphinidin-3-rutinoside in urine and bile using HPLC. For the 8 h period after intake, delphinidin-3-rutinoside and 4′-*O*-methyl-delphinidin-3-rutinoside urinary excretion was 795 ± 375 and 12.3 ± 2.91 nmol, respectively, while delphinidin-3-rutinoside and 4′-*O*-methyl-delphinidin-3-rutinoside excretion into the bile was 1.91 ± 0.35 and 17.4 ± 8.67 nmol, respectively. The intact delphinidin-3-rutinoside excretion ratio in urine was 60 times the excretion ratio of 4′-*O*-methyl-delphinidin-3-rutinoside, and in bile it was $^1/_9$ that of 4′-*O*-methyl-delphinidin-3-rutinoside.

Pharmacokinetic Parameters and Recovery Rate in Bile Cannulated Rats. We next used plasma concentration data to analyze the pharmacokinetic parameters of delphinidin-3-rutinoside from bile cannulated rats. We observed a T_{max} of 30 min and a C_{max} of 9.255 ± 5.472 μmol/L. For oral and intravenous administration of delphinidin-3-rutinoside, we calculated the systemic bioavailability to be 18.14 ± 6.24%. The cumulative excretion of delphinidin-3-rutinoside and 4′-*O*-methyl-delphinidin-3-rutinoside in urine and bile was 2.67 ± 1.24% (w/w) of the dose ingested.

DISCUSSION

In the past few years, several human studies have reported that anthocyanins are recovered in urine as the intact glycosidic forms, whereas neither anthocyanidin (aglycone) nor glucuro- or sulfoconjugates are detected (*15*, *16*). Recently, several metabolites excreted in urine were reported to form after oral ingestion of anthocyanins. These metabolites include a glucuronyl conjugate from cyanidin-3-glucoside and both glucuronyl conjugate (*8*) and sulfate conjugate from pelargonidin-3-glucoside (*9*). Previously we demonstrated in humans and rats that BCAs are absorbed through the gastrointestinal tracts and can be detected in the blood as unmetabolized forms (*7*). Plasma concentration of anthocyanins was very low (10−50 nmol/L), and relative urinary excretion was also low, approximately 0.1% of the intake. Furthermore, we found that, although delphinidin-

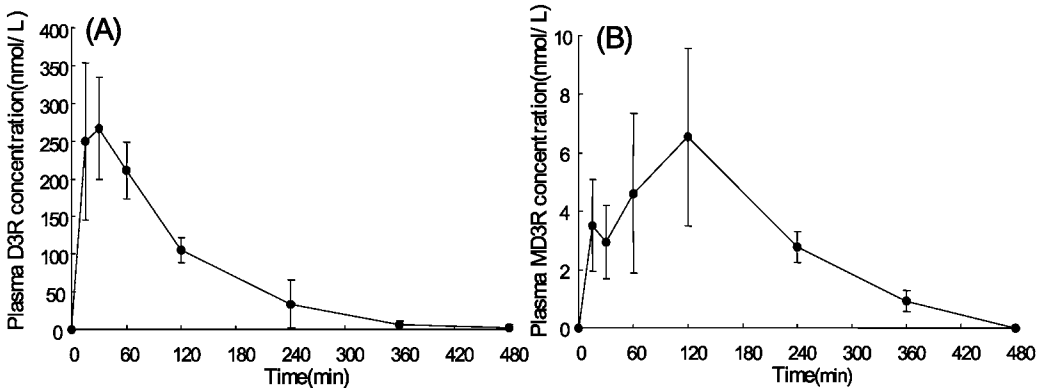

Figure 3. Plasma concentration profiles of delphinidin-3-rutinoside after oral administration: (**A**) delphinidin-3-rutinoside; (**B**) 4′-*O*-methyl-delphinidin-3-rutinoside. Values are means ± SD of four rats.

Table 1. Pharmacokinetic Parameters of Delphinidin-3-rutinoside[a]

group	C_{max} (μmol/L)	T_{max} (min)	$T_{1/2}$ (min)	$AUC_{0-480min}$ (μmol·min/L)	AUC_{0-inf} (μmol·min/L)	BA (%)
bile uncannulated rats	0.285 ± 0.071	26.3 ± 7.5	73.1 ± 53.5	33.7 ± 4.9	34.2 ± 4.1	0.492 ± 0.059
bile cannulated rats	9.255 ± 5.472	26.3 ± 7.5	30.0 ± 26.0	1254.6 ± 431.4	1261.8 ± 434.1	18.14 ± 6.24

[a] Values are means ± SD of four rats.

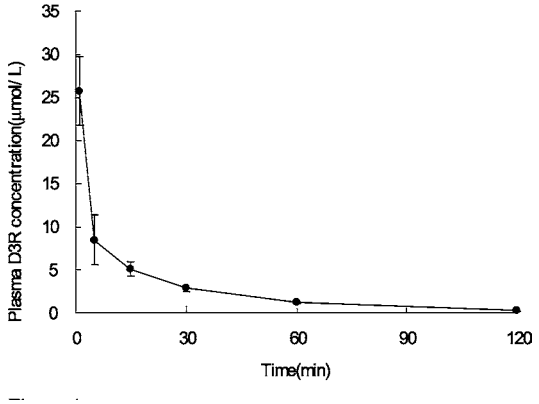

Figure 4. Plasma concentration profiles of delphinidin-3-rutinoside after intravenous injection. Values are means ± SD of four rats.

3-rutinoside was metabolized to 4′-*O*-methylated delphinidin-3-rutinoside, neither glucuro- or sulfoconjugates were detected.

Previously, we reported that oral intake of BCAs has many beneficial physiological effects (*2*−*6*). Despite the interest in the effects of BCAs, however, the understanding of BCA metabolism has remained unclear partly because of a lack of good information on the bioavailability of BCAs. The true bioavailability of anthocyanins has been difficult to determine because they are unstable at neutral pH. Thus, to better understand the bioavailability of BCA, in the present study we have attempted to evaluate both the true bioavailability of delphinidin-3-rutinoside and the rate at which it is converted to metabolites in vivo.

Our results demonstrate that, upon oral administration, delphinidin-3-rutinoside was primarily absorbed in the blood and excreted into urine as unmetabolized forms, with 4′-*O*-methyl-delphinidin-3-rutinoside being detected as a minor metabolite (**Table 1** and **Figure 3A,B**). Compared to intravenous injection, the extent of delphinidin-3-rutinoside bioavailability following oral administration is complete (0.49 ± 0.06%). It demonstrated that other 99.5% is not absorbed from the digestive system and evacuated to feces. Interestingly, delphinidin-3-rutinoside bioavailability was markedly increased by setting the bile duct cannulation in Bollman-type cages (18.14 ± 6.24%). Similarly, Ruiz-Garcia et al. (*17*) reported that plasma levels and AUC values of Flumequine were increased when biliary circulation was interrupted. Reduced gastric emptying could explain these increases in bioavailability. Delphinidin-3-rutinoside was not significantly excreted in bile (0.01% of the oral administration dose), and the plasma levels and AUC values observed in animals with interrupted bile flow always surpassed those found in animals with intact enterohepatic circulation. These observations might be explained by biases in delphinidin-3-rutinoside plasma concentrations that could occur if biliary extraction reduces the volume and alters the homeostatic equilibrium of the animal. Watanabe et al. (*18*) reported that immobilization stress increased the T_{max} of omeprazole by reducing gastrointestinal motility. Nieuwenhuijs et al. (*19*) also reported that disruption of bile flow affects interdigestive small bowel motility in rats. Thus, previous work suggests that although bioavailability of delphinidin-3-rutinoside is normally low, it could potentially be increased by improving delphinidin-3-rutinoside absorption through manipulation of gastrointestinal motility. With intravenous (7.62 μmol/kg of body weight) administration, the average recovery of delphinidin-3-rutinoside in excreta was 13.5% up to 8 h postadministration, and almost all urinary anthocyanin was the intact form. As anthocyanin also has a strong affinity for kidneys and the skin (*12*), this suggests that the residual delphinidin-3-rutinoside may have binding to these organs. Another possibility of this low recovery is that delphinidin-3-rutinoside might be converted and decomposed into currently unidentified substances because it is well-known that the quinoidal base form of anthocyanin is unstable at neutral pH (*11*). The elimination half-life of delphinidin-3-rutinoside in the blood is 4 h. Upon oral administration of BCA, delphinidin-3-rutinoside plasma concentrations did not show biphasic absorption kinetics (**Figure 3A**). The excretion rates of delphinidin-3-rutinoside and 4′-*O*-methyl-delphinidin-3-rutinoside into bile were small, suggesting that enterohepatic circulation is not important for the absorption and metabolism of delphinidin-3-rutinoside. For the 8 h period postadministration, we detected only small amounts of 4′-*O*-methyl-delphinidin-3-rutinoside in the urine and bile. We found more 4′-*O*-methyl-delphinidin-3-rutinoside than delphinidin-3-rutinoside in the bile. These results suggest that delphinidin-3-rutinoside is absorbed, appears in the plasma, and is then metabolized to 4′-*O*-methyl-delphinidin-3-rutinoside in the liver, probably by catechol *O*-methyltransferase (COMT). 4′-*O*-methyl-delphinidin-3-rutinoside is then likely secreted with bile into the intestine, reabsorbed into the blood through enterohepatic circulation, and excreted into the urine. The significance of 4′-*O*-methyl-delphinidin-3-rutinoside in humans is unclear, however, because the extent of bile reabsorption in humans is not large when compared to rats.

Upon intravenous administration, the bioavailability in nonbile cannulated free-moving rats was 0.5%, compared to 18.14% in bile cannulated rats in Bollman-type cages. For the latter, we calculated that the cumulative delphinidin-3-rutinoside excretion in urine and bile (including 4′-*O*-methyl-delphinidin-3-rutinoside) was 2.67 ± 1.24% (w/w) of the dose ingested. These results suggest that although absorbed delphinidin-3-rutinoside is excreted in small amounts as the intact form and as 4′-*O*-methyl-delphinidin-3-rutinoside, it is primarily degraded into unknown substances. Our results further indicate that delphinidin-3-rutinoside might be metabolized differently from cyanidin-3-glucoside and pelargonidin-3-glucoside.

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