Urinary Excretion of Anthocyanins in Humans after Cranberry Juice Ingestion

Ryoko Ohnishi,1 Hideyuki Ito,1,2 Naoki Kasajima,2 Miyuki Kaneda,2 Reiko Kariyama,3
Hiromi Kumon,3 Tsutomu Hatano,1 and Takashi Yoshida4

1Department of Pharmacognosy, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Tsushima, Okayama 700-8530, Japan
2School of Pharmacy, Shujitsu University, Nishigawara, Okayama 703-8516, Japan
3Department of Urology, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Shikata, Okayama 700-8558, Japan
4Matsuyama University, Bunkyo-cho, Matsuyama 790-8578, Japan

Received January 16, 2006; Accepted February 25, 2006; Online Publication, July 23, 2006
[doi:10.1271/bbb.60023]

Cranberry, which is rich in polyphenols, including anthocyanins and proanthocyanidins, has been found to have various effects beneficial to human health, including prevention of urinary tract infections. These effects have been associated with polyphenols in the fruit. We investigated the excretion of anthocyanins in human urine after ingestion of cranberry juice. Eleven healthy volunteers consumed 200 ml of cranberry juice containing 650.8 mg total anthocyanins. Urine samples were collected within 24 h before and after consumption. Six of 12 anthocyanins identified in cranberry were quantified in human urine by HPLC coupled with electrospray ionization and tandem mass spectrometry (HPLC–ESI–MS–MS). Among these, peonidin 3-O-galactoside, the second most plentiful anthocyanin in the juice, was found most abundantly in urine within 24 h, corresponding to 41.5 nmol (56.1% of total anthocyanins). The urinary levels of anthocyanins reached a maximum between 3 and 6 h after ingestion, and the recovery of total anthocyanins in the urine over 24 h was estimated to be 5.0% of the amount consumed. This study found high absorption and excretion of cranberry anthocyanins in human urine.

Key words: anthocyanins; cranberry juice; human urine; HPLC–ESI/MS; MS–MS

Cranberry (Vaccinium macrocarpon Ait., Ericaceae), distributed in North America, has traditionally been used in the treatment and prevention of urinary tract infections, and has been reported to exhibit various biological properties, including inhibition of Helicobacter pylori adhesion to human gastric mucus,1) protection against lipoprotein,2) and in vitro anticancer activity.3) Reduction of urinary tract infections in women by drinking cranberry juice was also proved in randomized, double-blind placebo-controlled trials.4,5) Among the reported ingredients of cranberry, including proanthocyanidins,6,7) anthocyanins,8–10) flavonoids,11,12) triterpenoids,13) iridoids,14) and organic acids,15,16) the proanthocyanidins, fructose, and macromolecules17) have been found to inhibit the adherence of uropathogenic P-fimbriated Escherichia coli to eucaryotic cells, and this might be associated with the prevention of urinary infection by cranberry.6,7) On the other hand, the preventive effect on urinary infection has also been accounted for by the acidification of urine with hippuric acid (glycine-conjugate of benzoic acid), produced by the metabolism of quinic acid through benzoic acid.18,19) Thus the in vivo urinary metabolites of the other polyphenolic ingredients, such as proanthocyanidins and anthocyanins, might also play an important role in the prevention of urinary tract infections.

Anthocyanins are widely distributed in fruits and vegetables such as blueberries, strawberries, cherries, plums, grapes, and red cabbage. The daily consumption of anthocyanins by humans has been estimated to be 180–215 mg/d in the United States,20) much higher than that of other flavonoids such as quercetin, kaempferol, and myricetin in the Dutch diet (23 mg/d).21) Nevertheless, information about the rate and extent of absorption, metabolism, and excretion of cranberry anthocyanins in the human body is to date quite limited. The aim of the present study was to investigate the urinary excretion of anthocyanins in cranberry juice through the identification of metabolites in human urine.

1 To whom correspondence should be addressed. Fax: +81-86-251-7926; E-mail: hiro@cc.okayama-u.ac.jp

Abbreviations: HPLC–ESI–MS–MS, HPLC coupled with electrospray ionization and tandem mass spectrometry; DAD, diode array detector; TFA, trifluoroacetic acid
Materials and Methods

Materials and reagents. The cranberry juice (Cranberry UR-100, Kikkoman Corporation, Chiba, Japan) used in this study was prepared by concentration of fresh juice to about 1/5 volume, followed by adjustment with water and tasting agents (sugar and organic acid) to contain 100% solid body of the fresh juice. Cyanidins 3-O-galactoside chloride (ideain chloride), cyanidin 3-O-glucoside chloride (kuromanine chloride), and 6-hydroxyflavone were purchased from Extrasynthese (Lyon, France). All solvents used for HPLC analysis were of HPLC grade.

Study design. After the study was approved by the ethical committee for human experimentation of Faculty of Pharmaceutical Sciences, Okayama University (ethics reference no. 1), all subjects provided written informed consent prior to participation. Eleven healthy volunteers in total (nine men and two women) aged 25.5 ± 4.9 years (mean ± SD) followed an anthocyanin-free diet containing no fruits, vegetables, coffee, or tea, and were allowed to drink water from half a day before test ingestion to the next morning. All subjects consumed 200 ml cranberry juice at 9 AM. Blank urine samples were collected before dosing, along with individual urine over the next 24 h, in plastic bottles containing no fruits, vegetables, coffee, or tea, and were immediately stored in a freezer until analysis. The subjects were under their own control except for restriction of diet and drink throughout the study periods. They did not use any medications.

Analysis of anthocyanins in cranberry juice and urine. Anthocyanins in cranberry juice (Cranberry UR-100) and urine were quantified by HPLC–ESI–MS–MS–MS. Urine samples (500 µl) acidified with 1 M HCl (20 µl) and 25% trifluoroacetic acid (TFA) (10 µl), and spiked with 6-hydroxyflavone (50 µM) in 0.58 M acetic acid (40 µl) as an internal standard, were extracted with solid-phase extraction (SPE) cartridges (Bond-Elut C18, 50 mg/1 ml; Varian, CA, USA), which were washed with 0.5% TFA–methanol and equilibrated with 0.5% aqueous TFA before use. Urine samples loaded onto the cartridge were washed with 0.5% aqueous TFA, and anthocyanins were eluted with 0.5% TFA–methanol. The methanolic eluate was evaporated to dryness under nitrogen gas at ambient temperature. The residue was dissolved in 25% aqueous methanol containing 0.5% TFA (200 µl), and filtered. An aliquot (20 µl) of this solution was injected into an HPLC–ESI–MS–MS system and an HPLC equipped with diode array detector (DAD).

HPLC–ESI–MS–MS analysis was performed on a Hewlett-Packard 1100 HPLC system equipped with a triple-stage quadrupole mass spectrometer, API 4000 (Applied Biosystems, Tokyo, Japan). The chromatographic column was a Nucleosil 100-5C18 (4.6 mm i.d. × 150 mm, GL Sciences, Tokyo, Japan) maintained at 40°C, and the mobile phase consisted of acetonitrile–water–TFA (20:80:0.5, by vol.) (solvent A) and acetonitrile–water–TFA (95:5:0.5 by vol.) (solvent B). A gradient system was applied as follows: The proportion of solvent B in the eluent initialized at 0% (t = 10 min), increased from 0% to 100% (t = 15 min) and 100% (t = 20 min), and decreased back to 0% (t = 20.1 min) until the next injection (t = 30 min). The flow rate was 0.8 ml/min with 0.2 ml/min split directed to the mass spectrometer. Mass detection was carried out using an electrospray interface operating in positive-ion mode at 450°C, with nebulizer pressure of 90 pounds per square inch, a drying nitrogen gas flow of 111/min, a fragmentor voltage of 20 V, and capillary voltage of 4,000 V. Ionization and fragmentation were optimized for authentic anthocyanins by direct infusion of a standard solution (0.2 µM in 50% aqueous acetonitrile containing 0.5% TFA solution). The mass data were collected in multiple reaction monitoring (MRM) mode by the transition of parent and product ions specific for each anthocyanin at a dwell time of 80 ms. HPLC–DAD analysis was performed on a Hitachi diode array detector L-7455 monitoring absorbance at 524 nm, and a Hitachi L-2130 pump. Other HPLC methodology was as mentioned above.

Identification of the anthocyanins was based on the matching of molecular weight (parent and product ions) and retention time with those of available anthocyanin standards and a comparison of those reported HPLC–MS–MS data. Anthocyanins were quantified by comparison with a standard curve obtained using known concentrations of an available standard, cyanidin 3-O-galactoside. The urinary concentrations of anthocyanins were determined based on the corrected peak area divided by the peak area ratios shown in Table 1. Calibration curves were prepared by spiking blank urine with solution at deferent concentrations (0.01–100 µM), with duplicate injections at each level.

Statistical analysis. Values are given as means with standard errors.

Results and Discussion

Anthocyanins in cranberry juice
Anthocyanins constitute a large group of plant pigments distributed mainly in flowers, fruits, and vegetables. Cranberry contains large amounts of sugars, proanthocyanidins, flavonoids, and organic acids as well as anthocyanins. The structures of aglycone of 12 analyzed anthocyanins are depicted in Fig. 1. The ion chromatograms of cranberry juice analyzed by HPLC–ESI–MS–MS are shown in Fig. 2. By the HPLC–ESI–MS–MS method, 12 anthocyanins were identified by
Table 1. Parent and Product Ions, Retention Times, and Peak Area Ratios of Anthocyanidins in Cranberry and Internal Standard

<table>
<thead>
<tr>
<th>Anthocyanin</th>
<th>Parent ion ( m/z )</th>
<th>Product ion ( m/z )</th>
<th>Retention time (min)</th>
<th>Peak area ratio$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cy 3-O-gal</td>
<td>449</td>
<td>287</td>
<td>4.53</td>
<td>1.0</td>
</tr>
<tr>
<td>Cy 3-O-glc</td>
<td>449</td>
<td>287</td>
<td>4.81</td>
<td>1.0</td>
</tr>
<tr>
<td>Cy 3-O-ara</td>
<td>419</td>
<td>287</td>
<td>5.79</td>
<td>1.5</td>
</tr>
<tr>
<td>Pn 3-O-gal</td>
<td>463</td>
<td>301</td>
<td>6.35</td>
<td>1.6</td>
</tr>
<tr>
<td>Pn 3-O-glc</td>
<td>463</td>
<td>301</td>
<td>7.07</td>
<td>2.6</td>
</tr>
<tr>
<td>Pn 3-O-ara</td>
<td>433</td>
<td>301</td>
<td>8.75</td>
<td>3.8</td>
</tr>
<tr>
<td>Dp 3-O-ara</td>
<td>435</td>
<td>303</td>
<td>4.60</td>
<td>—</td>
</tr>
<tr>
<td>Pg 3-O-gal</td>
<td>433</td>
<td>271</td>
<td>5.58</td>
<td>—</td>
</tr>
<tr>
<td>Pg 3-O-ara</td>
<td>403</td>
<td>271</td>
<td>7.52</td>
<td>—</td>
</tr>
<tr>
<td>Pt 3-O-gal</td>
<td>479</td>
<td>317</td>
<td>4.98</td>
<td>—</td>
</tr>
<tr>
<td>Mv 3-O-gal</td>
<td>493</td>
<td>331</td>
<td>7.16</td>
<td>—</td>
</tr>
<tr>
<td>Mv 3-O-ara</td>
<td>463</td>
<td>331</td>
<td>10.03</td>
<td>—</td>
</tr>
<tr>
<td>6-Hydroxyflavone</td>
<td>239</td>
<td>137</td>
<td>16.99</td>
<td>—</td>
</tr>
</tbody>
</table>

$^a$Peak area ratio calculated by HPLC–ESI–MS–MS versus HPLC–DAD at the same concentration of individual anthocyanins.

$^b$Not detected by HPLC–DAD.

Cy, cyanidin; Pn, peonidin; Dp, delphinidin; Pg, pelargonidin; Pt, petunidin; Mv, malvidin; gal, galactoside; glc, glucoside; ara, arabinoside

Fig. 1. Chemical Structures of Aglycones of Anthocyanins Used in the HPLC–ESI–MS–MS Assay.

Fig. 2. Total Ion Chromatogram (TIC) and Extracted Single Ion Chromatograms of Cranberry Juice Obtained by the HPLC–ESI–MS–MS Method in Multiple Reaction Monitoring Mode with Positive Ionization.

$^a$The \( m/z \) values of parent/product ions.
Cranberry juice was estimated at 650.8 μg/200 ml ingested in the present study. Among these, peonidin 3-O-arabinoside, at a concentration of 230.0 μg/200 ml juice, was the major anthocyanin in the juice, accounting for 35.3% of total quantifiable anthocyanins. Subsequently, the other anthocyanins, cyanidins 3-O-galactoside and 3-O-arabinoside, and peonidin 3-O-galactoside, at 19.7, 16.1, and 26.8% in total respectively, were predominant in the juice. The cranberry juice contained six types of anthocyanidin (cyanidin, peonidin, pelargonidin, malvidin, delphinidin, and petunidin), with two types of sugar form (galactoside and arabinoside). Furthermore, glucosides of cyanidin and peonidin were also present in the juice.

### Human Urine

The purpose of the present study was to identify potential metabolites of anthocyanins in human urine for a better understanding of the active principles in the prevention of urinary tract infection. Several human studies have reported that anthocyanins are recovered in urine in intact or conjugated forms. A significant difference was observed in the peak areas calculated by MS and UV for individual anthocyanins. In order to calculate a more accurate quantity value (Table 1), we took the peak area ratio of ionization by MS and UV absorbance at 524 nm by DAD of individual detectable anthocyanins into consideration. The concentrations of quantifiable anthocyanins in cranberry juice and urine, which were defined as cyanidin 3-O-galactoside equivalents, were determined by relating their peak areas to that of 6-hydroxyflavone as internal standard. The calibration curves was nicely linear over the concentration range (0.01–100 μM) studied, with correlation coefficients \( r^2 > 0.995 \). The limit of quantification (\( S/N > 10 \)) was determined for all samples.

The total amount of quantifiable anthocyanins in cranberry juice was estimated at 650.8 μg/200 ml ingested in the present study. Among these, peonidin 3-O-arabinoside, at a concentration of 230.0 μg/200 ml juice, was the major anthocyanin in the juice, accounting for 35.3% of total quantifiable anthocyanins. Subsequently, the other anthocyanins, cyanidins 3-O-galactoside and 3-O-arabinoside, and peonidin 3-O-galactoside, at 19.7, 16.1, and 26.8% in total respectively, were predominant in the juice. The cranberry juice contained six types of anthocyanidin (cyanidin, peonidin, pelargonidin, malvidin, delphinidin, and petunidin), with two types of sugar form (galactoside and arabinoside). Furthermore, glucosides of cyanidin and peonidin were also present in the juice.

### Human Urine

The purpose of the present study was to identify potential metabolites of anthocyanins in human urine for a better understanding of the active principles in the prevention of urinary tract infection. Several human studies have reported that anthocyanins are recovered in urine in intact or conjugated forms. In the present study, we found a higher excretion of several anthocyanin metabolites in human urine after a single dose of cranberry juice.

Figure 3 presents typical chromatograms of urine samples after consumption of cranberry juice, obtained by the HPLC–ESI–MS–MS method. The representative anthocyanins in the juice, peonidin 3-O-arabinoside, peonidin 3-O-galactoside, cyanidin 3-O-galactoside, and cyanidin 3-O-arabinoside, were observed in the urine of all subjects. Excretion of peonidin 3-O-glucoside, pelargonidin 3-O-arabinoside, and petunidin 3-O-galactoside among the minor pigments in the juice was also detected and identified in the urine of most of the subjects. Malvidin 3-O-arabinoside was not detected in any sample.

Table 2 gives the urinary excretion of individual cranberry anthocyanins after cranberry juice intake in the present study. The total excretion of six main cranberry anthocyanins in urine over 24 h reached 5.0% of the amount consumed. Most studies have reported that relative urinary excretion of anthocyanins was very low, less than 0.1% of ingested pure anthocyanin or the foods containing it, indicating poor absorption and excretion of these compounds compared with other polyphenols, but there are a few reports indicating higher anthocyanin levels in urine (up to a few percentage points) after red wine or strawberry consumption. The above data suggest that the absorption of anthocyanins is accelerated by other components in cranberry. It might also be due to a sufficient extraction procedure as well as high selectivity and sensitivity by the HPLC–ESI–MS–MS method. Following cranberry juice ingestion, almost all the urinary anthocyanins over 24 h were excreted as respective galactoside and arabinoside of cyanidin and peonidin, accounting for more than 90% of total anthocyanin excretion. Among these, the urinary concentration of peonidin 3-O-galactoside reached a level of 41.5 ± 6.2 nmol/24 h (56.1% of total anthocyanin excretion). Up to 80% of the total anthocyanin dose and the main urinary anthocyanin, peonidin 3-O-galactoside, were excreted within 6 h post-consumption. The urinary excretion of all anthocyanins was maximal between 3 and 6 h after ingestion, and they were mostly exhausted in urine within 12 h, indicating that a major proportion of anthocyanins was quickly excreted in the urine (Fig. 4). Additionally, the urinary level (up to 11% of the dose) of peonidin glycosides over 24 h was remarkably higher than that of other glycosides (a few percentage points), except for peonidin arabinoside. The methylation of cyanidin glycosides into peonidin glycosides by catechol O-methyltransferase in the liver has been suggested in human and animal studies. The high excretion of peonidin glycosides in urine might have resulted partly from methylation of cyanidin glycosides. Although there is insufficient evidence for a metabolic difference among cyanidin glycosides, metabolic methylation for anthocyanins might occur preferably in the galactosides and glucosides over the arabinosides.

Moreover, some researchers have reported that the major metabolites of anthocyanins were recovered as their glucuronide conjugated forms in human urine following anthocyanin-rich food consumption. We evaluated for glucuronide conjugations by detection of their parent and product ion pairs due to the anthocyanidin glucuronides and anthocyanidins respectively. The peaks corresponding to the glucuronides of cyanidin and peonidin were not detectable in the extended single-ion chromatograms of human urine samples after cranberry juice intake, suggesting an absence of these glucuronides in the urine samples or degradation of conjugates during sample processing owing to instability.

In conclusion, 12 anthocyanins, including cyanidin, peonidin, delphinidins, pelargonidin, malvidin, and
Table 2. Concentrations of Anthocyanins in Cranberry Juice and in Human Urine after Juice Consumption by 11 Healthy Volunteers

<table>
<thead>
<tr>
<th>Anthocyanin</th>
<th>Cranberry juice Concentration (µg/200 ml)</th>
<th>% of total anthocyanins&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Concentration&lt;sup&gt;b,c&lt;/sup&gt; (nmol/24 h)</th>
<th>% of total anthocyanins&lt;sup&gt;d&lt;/sup&gt;</th>
<th>% of dose&lt;sup&gt;b,e&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cy 3-O-gal</td>
<td>128.4</td>
<td>19.7</td>
<td>10.5 ± 1.7</td>
<td>14.3</td>
<td>3.7 ± 0.6</td>
</tr>
<tr>
<td>Cy 3-O-glc</td>
<td>3.6</td>
<td>0.6</td>
<td>0.11 ± 0.06</td>
<td>0.2</td>
<td>1.4 ± 0.7</td>
</tr>
<tr>
<td>Cy 3-O-ara</td>
<td>104.6</td>
<td>16.1</td>
<td>8.7 ± 1.2</td>
<td>12.0</td>
<td>3.6 ± 0.5</td>
</tr>
<tr>
<td>Pn 3-O-gal</td>
<td>174.3</td>
<td>26.8</td>
<td>41.5 ± 6.2</td>
<td>56.1</td>
<td>11.0 ± 1.6</td>
</tr>
<tr>
<td>Pn 3-O-glc</td>
<td>9.9</td>
<td>1.5</td>
<td>2.4 ± 0.6</td>
<td>3.3</td>
<td>11.3 ± 2.6</td>
</tr>
<tr>
<td>Pn 3-O-ara</td>
<td>230.0</td>
<td>35.3</td>
<td>10.4 ± 1.7</td>
<td>14.1</td>
<td>2.0 ± 0.3</td>
</tr>
<tr>
<td>total</td>
<td>650.8</td>
<td>100</td>
<td>73.6 ± 11.0</td>
<td>100</td>
<td>5.0 ± 0.8</td>
</tr>
</tbody>
</table>

<sup>a</sup>Percentages of total quantifiable anthocyanins in cranberry juice.
<sup>b</sup>Values are means ± SEM.
<sup>c</sup>Urinary concentrations during 24 h following ingestion were determined using HPLC–ESI–MS–MS and HPLC–DAD, and were defined as cyanidin 3-O-galactoside equivalents.
<sup>d</sup>Percentages of the total anthocyanins excreted in urine.
<sup>e</sup>Percentages of ingested individual anthocyanin amounts.

Fig. 3. Typical Extracted Single Ion Chromatograms of Human Urine Samples after Consumption of Cranberry Juice Obtained by the HPLC–ESI–MS–MS Method in Multiple Reaction Monitoring Mode with Positive Ionization.

IS, internal standard. *The m/z values of parent/product ions.
petunidin glycosides have been identified in cranberry juice. Six of them, viz. galactosides, glucosides, and arabinosides of cyanidin and peonidin, have also been quantified in urine after consumption of cranberry juice. These results show that the main anthocyanins in cranberry juice were absorbed into the human circulatory system and transported in the urine in intact form, which might contribute to the health benefits of cranberry. Identification and quantification of other polyphenols and their metabolites in human urine after cranberry juice intake are under investigation to clarify the active principles against urinary tract infection.

Acknowledgments

This study was supported in part by the Agricultural Chemical Research Foundation (no. 1-264 to H.I.), and by a Grant-in-Aid for Scientific Research (no. 17604005 to H.I.) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan. The authors thank Kikkoman Corporation for supplying cranberry juice.

References