Anthocyanin Excretion by Humans Increases Linearly with Increasing Strawberry Dose

Colleen Carkeet, Beverly A. Clevidence, and Janet A. Novotny*

Food Components and Health Laboratory, Beltsville Human Nutrition Research Center, Agricultural Research Service, USDA, Beltsville, MD 20705

Abstract

A clinical study was conducted to investigate the dose response and metabolism of strawberry anthocyanins. In a crossover study design, 12 healthy adults consumed each of 3 strawberry treatments. The treatments were 100 g, 200 g, and 400 g of pureed strawberries, delivering 15 μmol, 30 μmol, and 60 μmol anthocyanin, respectively. Urine samples were collected for 24 h after each dose and samples were analyzed by HPLC with diode array detection and ion trap MS. Pelargonidin 3-glucoside was the major anthocyanin form in the treatments, and pelargonidin 3-glucoside and 3 metabolites of pelargonidin 3-glucoside (detected as monoglucuronides) were excreted in urine after ingestion. One predominant monoglucuronide form was detected in urine in masses 10-fold higher than the other 2 monoglucuronide forms. Increasing dose resulted in increasing appearance of anthocyanins in urine, and the mass of each pelargonidin monoglucuronide increased in urine with increasing dose. These results suggest that pelargonidin 3-glucoside absorption and metabolism are not saturated at masses ≤ 60 μmol, thus showing that more strawberry anthocyanin can be absorbed with increasing dose. J. Nutr. 138: 897–902, 2008.

Introduction

Anthocyanins are highly pigmented polyphenolic compounds found in many red, purple, and blue fruits and vegetables. These anthocyanins are glycoside and acylglucoside derivatives of 6 common anthocyanidins: pelargonidin, cyanidin, delphinidin, peonidin, petunidin, and malvidin. Studies with anthocyanins have suggested that these brightly colored compounds are associated with a wide variety of health benefits, including decreased risk of cardiovascular disease (1–4), reduced risk of cancer (2,5–10), reversal of age-related neurodegenerative declines (11–15), improved glucoregulation (16,17), and protection of brain tissue from hypoxia (18,19).

Strawberries, a staple fruit of the American diet, are naturally high in anthocyanins (20). Marketed year round, strawberries are the 5th highest consumed fresh fruit in the United States, and consumption rate is on the rise, having increased ≈50% in the previous decade (21). Therefore, strawberries offer an important vehicle for delivery of dietary anthocyanins. Strawberries and radishes are the most abundant sources of pelargonidin-based anthocyanins in the diet (22) and pelargonidin-based anthocyanins provide ≈3% of total anthocyanin intake (22).

Because of the potential importance of anthocyanins to the promotion of health and the prevention of chronic disease, it is important to understand factors, such as dose-response, that affect their bioavailability. Two studies have been published previously addressing the effect of dose on anthocyanin absorption. The first study showed that blood and urinary recovery of purple carrot anthocyanins was not different for a 250-g dose compared with a 500-g dose (23). The other study showed that urinary recovery of red cabbage anthocyanins increased linearly with increasing dose, although the increase in urinary recovery was not of the same magnitude as the increase in dose size (24). Both purple carrot anthocyanins and red cabbage anthocyanins are cyanidin-based. In contrast, strawberry anthocyanins are primarily pelargonidin-based. Wu et al. (25) showed that pelargonidin-based anthocyanins are absorbed and metabolized differently from cyanidin-based anthocyanins. No studies to date to our knowledge have investigated the dose response of strawberry anthocyanins.

The objective of this study was to determine the dose response of anthocyanin absorption and metabolism after consumption of strawberries. To meet this objective, a clinical feeding study was conducted in which subjects consumed pureed strawberries at 3 dose levels in a crossover study design. We analyzed urine for anthocyanin appearance as an index of anthocyanin absorption to determine whether dose size influences anthocyanin absorption and metabolism.

Materials and Methods

Chemicals. HPLC grade water and methanol were obtained from Fisher Scientific. Formic acid and trifluoroacetic acid were obtained from Sigma Chemical. Cyanidin-3-glucose and malvidin-3-galactoside were purchased from Indofine Chemical and pelargonidin-3-glucose was purchased from ChromaDex.

Subjects. Twelve healthy, nonsmoking volunteers from the Beltsville, MD area participated in this study. The 6 males and 6 females had a
Treatments, study design, sample collection, and diet. Strawberries were blended with a commercial mixer to obtain a homogeneous product. Blended berries were stored at $-20\degree C$ until the day of treatment. Treatments consisted of the following: 1) 100 g (3.5 oz.) of blended berries; 2) 200 g (7 oz.) of blended berries; and 3) 400 g (14 oz.) of blended berries. These masses of blended berries are approximately equivalent to eating 8, 16, and 32 medium strawberries, respectively. All treatments were prepared simultaneously as a single batch. On the day of treatment, the 100-g and 200-g treatments of blended berries were mixed with 300 g and 200 g water, respectively, so that the final masses of the 3 treatments were the same. To improve palatability, 1 packet of noncaloric sweetener (Equal) was added to each treatment immediately prior to consumption. Each dose was served to subjects with 50 g of water to rinse the remaining dose from the container.

The 3 strawberry treatments were administered to subjects in a crossover experimental design. All subjects received each of the treatments in random order, with the treatment periods being separated by 1-wk breaks. During the study, subjects were instructed to avoid anthocyanin-containing foods and were provided a list of these foods. On d 1 of each treatment period, subjects consumed anthocyanin-free meals provided by the Beltsville Human Nutrition Research Center and continued to eat only foods provided by the Nutrition Research Center for the remainder of the treatment period. Following an overnight fast, subjects consumed 1 of the 3 strawberry treatments on the 2nd morning. Subjects were offered anthocyanin-free meals and snacks starting 4 h after the dose. Vitamins and supplements were prohibited throughout the study.

Subjects collected a baseline urine sample immediately prior to consuming the strawberry treatments. Additionally, subjects collected all urine produced during the 24 h following the treatment meal, with collections occurring at 2-h intervals for the first 12 h and a pooled collection from 12 to 24 h.

Extraction of anthocyanins from strawberries. Three aliquots of blended strawberries were extracted and analyzed for anthocyanin content. First, samples were freeze-dried over 72 h. Two milliliters of 10% formic acid in methanol (v:v) and 1.0 mL of malvidin 3-galactoside (10.88 \mu mol/L) as an internal standard were added to 75 mg of freeze-dried sample. The mixture was sonicated for 10 min, then centrifuged at 3000 \times g; 10 min at 20\degree C. The supernatant was transferred into a 10-mL volumetric flask and the extraction repeated 3 times with 2 mL of 10% formic acid in methanol each time. All supernatants were combined in a 100-mL volumetric flask and the final volume was adjusted to 10 mL with 10% formic acid in methanol. Immediately prior to sample analysis, the strawberry extract was dried under nitrogen and resuspended in 10% formic acid in water.

Biological sample preparation. The total volume of each urine collection was weighed and recorded and 10-mL aliquots were stored in vials containing 2 mL of 0.44 mol/L trifluoroacetic acid and 2 mL of malvidin 3-galactoside (10.88 \mu mol/L) as an internal standard. All samples were stored at $-80\degree C$ until analysis. Prior to HPLC quantification, samples were thawed and passed through C$_{18}$-solid phase extraction cartridges to isolate and concentrate the anthocyanins. Sep-pak cartridges (Waters Associates) were conditioned with 5 mL of methanol and then 5 mL of 0.44 mol/L aqueous trifluoroacetic acid prior to adding sample. After the sample was added, water soluble compounds were eluted with 5 mL of 0.44 mol/L aqueous trifluoroacetic acid, followed by the elution of anthocyanins with 5 mL of 10% formic acid in methanol. Recovery of the internal standard was 84%. Samples were dried under nitrogen and reconstituted in methanol:10% aqueous formic acid (1:9).

HPLC/ESI ion-trap MS analysis of anthocyanins. We analyzed the anthocyanins on an Agilent MSD SL ion trap mass spectrometer equipped with an electrospray ionization interface, an 1100 HPLC, a diode array detector (DAD), and ChemStation software. The column used was a Luna C18 column (5 \mu m, 250 mm \times 4.6 mm) and a Fusion-RP guard column (Phenomenex). The HPLC utilized a binary solvent system with solvent A as 89:10:1 water:formic acid:methanol and solvent B as 10% formic acid in methanol. For strawberry anthocyanin analysis, the solvent system was isocratic at 84% solvent A and 16% solvent B and the system used a flow rate of 0.8 mL/min. For identification of urinary anthocyanins and metabolites, the solvents were held at 83% solvent A and 17% solvent B for 30 min. From 30 min to 60 min, there was a linear ramp to 100% solvent B. For 5 min, the solvents were held at 100% solvent B, followed by a return to starting conditions over 5 min. For quantitative analysis of anthocyanins and metabolites in urine, the starting conditions were 90% solvent A and 10% solvent B. There was a linear ramp to 86% solvent A from 0 to 5 min, which was held from 5 to 36 min. From 36 to 41 min, there was a linear ramp to 100% solvent B and this was held for 4 min, followed by a linear gradient back to initial conditions. For all analyses, the DAD was monitored at 520 nm and the injection volume was 50 \mu L. MS and electrospray ionization interface, conditions were as follows: nebulizer, 60 psi; dry gas (N$_2$), 11 L/min; dry temperature, 350\degree C; trap drive, 66; skimmer, 40 V; octopole RF amplitude, 200 Vpp; capillary exit, 102.7 V. The ion trap mass spectrometer was operated in positive ion mode scanning from 100 to 800 mass-to-charge ratio (m/z). Trap ICC was 30,000 and maximal accumulation time was 200 ms.

Calculations and statistics. Malvidin 3-galactoside was used as an internal standard to account for sample loss during extraction. Molar concentrations of individual strawberry anthocyanins in treatments and urine were calculated using external standard curves of pelargonidin 3-glucoside for pelargonidin-based anthocyanins and cyanidin 3-glucoside for cyanidin-based anthocyanins.

The data were tested for normality (using the Kolmogorov-Smirnov test) and equal variance (using the Levene Median test), then we used a 1-way repeated measures ANOVA to compare urine responses among treatments, with $P < 0.05$ considered significant. The Tukey method was used for pairwise comparisons. Statistical analysis was performed using the SigmaStat Software Package (version 3.0, SPSS). Linear regression analysis of mean anthocyanin excretion for the 3 doses was performed using Microsoft Excel 2003.

Results
All 12 subjects completed the study. One female subject forgot to collect urine samples after 10 h for the 200-g treatment, so her data for the 200-g treatment were incomplete and thus excluded from the analysis.

Anthocyanins in strawberry treatments. Four peaks were detected in the strawberry treatment extract in addition to the internal standard, as determined by ion trap MS. Anthocyanins detected in the strawberry treatments were cyanidin-3-glucoside, pelargonidin-3-glucoside, pelargonidin-3-rutinoside, and pelargonidin-3-(6'-acetoxy)glucoside, which were detected previously in...
strawberries by Wu et al. (20). Pelargonidin-3-glucoside was the major anthocyanin in the strawberry treatment, comprising 88% of total anthocyanin content, with a concentration of 133.4 nmol/g of blended berries. Cyanidin-3-glucoside and pelargonidin-3-rutinoside were present in concentrations of 11.6 nmol/g and 6.8 nmol/g of blended berries, respectively. The 4th anthocyanin peak, pelargonidin-3-(6''-acetoyl)glucoside, was below the quantification limit. The structures of pelargonidin aglycone and cyanidin aglycone are shown in Figure 1. Total anthocyanin contents of treatments were 15 μmol (6.7 mg), 30 μmol (13.4 mg), and 60 μmol (26.8 mg) of anthocyanins for the 100-g, 200-g, and 400-g treatments, respectively. Note that the concentration of pelargonidin 3-rutinoside is based on pelargonidin 3-glucoside molar equivalents.

Four anthocyanin-related peaks, in addition to the internal standard, were identified in urine of subjects who consumed strawberries (Fig. 2). All 4 peaks were derivatives of pelargonidin, with 1 being pelargonidin-3-glucoside and 3 others being pelargonidin monoglucuronides. One of those peaks was dominant and accounted for 80% of the total anthocyanin recovered. Mass chromatograms are shown in Figure 3. Pelargonidin monoglucuronides presented molecular ions with m/z of 447 atomic mass units (amu), and tandem MS resulted in a loss of 176 amu (indicating a glucuronide residue), leaving a fragment with m/z of 271 (characteristic of pelargonidin aglycone). The MS was unable to indicate the location of the monoglucuronidation. The pelargonidin-3-glucoside displayed a molecular ion with m/z of 433 and tandem MS resulted in the loss of 162 amu (characteristic of a hexose group), leaving a fragment with m/z of 271 (characteristic of pelargonidin aglycone).

Maximum output of urinary anthocyanins occurred at 2 h after treatment (Fig. 4). More than 50% of the recovered anthocyanins were collected by 4 h, and >90% of the recovered anthocyanins were collected by 10 h.

Total urinary anthocyanin excretion increased linearly with dose (Table 1). For each dose level, ~2% of the dose was recovered during the 24-h collection period. Regression analysis of mean anthocyanin excretion for the 3 doses showed that the relationship between anthocyanin dose and urinary recovery was linear with an R value of 0.99.

The linear dose response occurred for the parent compound pelargonidin 3-glucoside as well as the 3 monoglucuronides (Table 2). The mass of each compound excreted in 24 h

FIGURE 2 Chromatogram of anthocyanins excreted in urine of volunteers after consumption of strawberry treatments (UV signal collected at 520 nm).

FIGURE 3 Mass chromatograms of anthocyanin peaks excreted in urine after volunteers consumed strawberry treatments.
increased significantly with dose, except the difference in pelargonidin 3-glucoside excretion between the 200-g and 400-g strawberry doses was not significant ($P = 0.09$).

The fraction of dose recovered as each analyte was dependent on treatment for pelargonidin 3-glucoside and the major pelargonidin 3-monoglucuronide (Table 3). Percent recovery as pelargonidin 3-glucoside decreased with increasing dose size, whereas percent recovery of the major monoglucuronide increased with increasing dose size.

Discussion

The emerging health benefits of anthocyanins combined with the popularity of strawberries justifies research into the absorption and metabolism of strawberry anthocyanins. The strawberries used in this study contained primarily pelargonidin 3-glucoside, as has been observed by researchers in other laboratories (20,26,27). The strawberries used in this study were slightly lower in anthocyanin content than previously reported and the lower pigment content was apparent visually before processing. These strawberries contained 15.2 μmol/100 g (6.7 mg/100 g). In comparison, Wu et al. (22) reported 21–41 mg/100 g total anthocyanins in strawberries, and Felgines et al. (26) reported 89.5 μmol/100 g.

Generally, flavonoids are metabolized to glucuronidated and/or sulfated derivatives, although most anthocyanin studies have reported detection of only native glycosylated forms (28). However, recent studies have reported detection of glucuronidated, methylated, and/or sulfated forms (25–27,29). We observed 4 anthocyanin peaks in urine originating from the strawberry dose. One peak represented the parent compound pelargonidin 3-glucoside and the remaining 3 peaks were monoglucuronides. Felgines et al. (26) observed these peaks, in addition to 1 sulfoconjugate and pelargonidin aglycone, after humans consumed 200 g strawberries. Our anthocyanin doses were all lower (15, 30, and 60 μmol) than those given in the study by Felgines et al. (26) (179 μmol), which may explain why we observed fewer compounds in the urine. Similar to our results, a glucuronide derivative of pelargonidin was also found to be the major metabolite of pelargonidin 3-glucoside in rat plasma and urine (27,29) and in pigs (25).

In this study, anthocyanin recovery in urine increased linearly with increasing dose and the increase in anthocyanin recovery was of the same magnitude as the increase in dose. Two studies addressing anthocyanin dose response have been published previously. In one study, volunteers consumed 100, 200, or 300 g red cabbage, which is high in cyanidin-based anthocyanins, many of which are acylated. Urinary recovery of anthocyanins increased linearly with dose, although the magnitude of the increase was only ~50% the increase in anthocyanin dose (24).

The following study of purple carrots, urinary recovery of anthocyanins (again, predominantly cyanidin-based and largely acylated) did not differ for a 250-g vs. a 500-g cooked dose. Total anthocyanin dose for the red cabbage and carrot studies were 138–414 μmol and 357–714 μmol, respectively. The differing results among these studies may be related to anthocyanin backbone, food matrix, or dose sizes used.

## TABLE 3 Percent of anthocyanin dose excreted in 24 h after human consumption of 100, 200, or 400 g pureed strawberries

<table>
<thead>
<tr>
<th>Anthocyanins fed, nmol</th>
<th>100-g dose</th>
<th>200-g dose</th>
<th>400-g dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pg-3-Glucoside</td>
<td>1.7 ± 0.8*</td>
<td>4.9 ± 0.8*</td>
<td>3.6 ± 0.8*</td>
</tr>
<tr>
<td>Pg-Monoglucuride A</td>
<td>8.3 ± 0.5*</td>
<td>8.2 ± 0.5*</td>
<td>7.6 ± 0.5*</td>
</tr>
<tr>
<td>Pg-Monoglucuride B</td>
<td>77.9 ± 1.6*</td>
<td>80.0 ± 1.7*</td>
<td>82.3 ± 1.1*</td>
</tr>
<tr>
<td>Pg-Monoglucuride C</td>
<td>6.5 ± 0.4*</td>
<td>6.9 ± 0.6*</td>
<td>6.4 ± 0.4*</td>
</tr>
</tbody>
</table>

1 Values are means ± SEM, $n = 12$ for 100 g and 400 g; $n = 11$ for 200 g. Means in a row with superscripts without a common letter differ, $P < 0.05$.
dose of anthocyanins (purple carrot study) showed no increase in urinary anthocyanin recovery with increasing dose, suggesting saturation of absorption mechanisms. It is possible that reduced absorption efficiency would be observed for strawberry anthocyanins consumed at higher doses, but higher doses would not represent normal intake levels.

Anthocyanin recoveries over 24 h of urine collection for this study were ~2% of the anthocyanins dose administered. Generally, previous reports show anthocyanin with other aglycon backbones exhibit much lower recoveries, and in most cases, only native anthocyanin forms were detected. Most reports of anthocyanin recoveries in urine refer to collection periods of 5–8 h (instead of 24 h, as in this study). For comparison, anthocyanin excretions are >50% in the first 4 h, as observed in this study and others (23,24). Some of the previously reported anthocyanin recoveries include 0.077% in 4 h for elderberry (30), 0.053% in 5 h for elderberry (31), 0.003–0.012% in 6 h for elderberry (32), 0.37% in 7 h for elderberry (31), 0.004% in 6 h for blueberry (30), 0.02–0.05% in 5 h for blackcurrant juice (33), 0.04% in 7 h for blackcurrant (31), <0.03% in 6 h for red wine or grape juice (34), 0.18% in 7 h for red wine (35), 0.23% in 7 h for red grape juice (35), 0.20% in 7 h for a mixed berry extract of blackcurrant, boysenberry, and blueberry (36), 0.02–0.038% in 24 h for purple carrots (23), and 0.036–0.073% in 24 h for red cabbage (24). In contrast, anthocyanins from strawberries appear to be more effectively absorbed, showing 2% urinary recovery after 24 h in this study and 1.8% urinary recovery after 24 h in a study by Felgines et al. (26). Further, when pigs were administered marionberry containing cyanidin and pelargonidin derivatives, urinary recoveries were 0.084% for cyanidin 3-rutinoside, 0.087% for cyanidin 3-glucoside, and 0.58% for pelargonidin 3-glucoside, again suggesting that pelargonidin-based anthocyanins may be more effectively absorbed than other anthocyanins (25).

Approximately 98% of the anthocyanin dose was not recovered in urine. It is unknown what portion of this was deposited into tissues and what portion was excreted into feces. Regardless of the apparently low absorption efficiency of anthocyanins, the portion that is absorbed may be very biologically active. In addition, the portion that remains in the gastrointestinal tract may convey important health benefits to intestinal tissue. Therefore, the apparently low absorption efficiency does not imply low potential for health benefits.

In summary, anthocyanins from strawberries exhibit a linear dose response over ranges of 15–60 μmol. In addition, 24-h urinary recoveries observed in this study were substantially higher than those reported for most other anthocyanin bioavailability studies, suggesting that pelargonidin-based anthocyanins may be more efficiently absorbed than other anthocyanins. However, these higher recoveries may be at least partially related to the lower doses administered in this study compared with others.

**Literature Cited**


