Anthocyanins are Bioavailable in Humans following an Acute Dose of Cranberry Juice

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Abstract

Research suggests that anthocyanins from berry fruit may affect a variety of physiological responses, including endothelial function, but little information is available regarding the pharmacokinetics of these flavonoids in humans. To determine the pharmacokinetics of cranberry anthocyanins, a study was undertaken in 15 participants (age: 62 ± 8 y) with coronary artery disease. Blood and urine samples were collected between baseline (0 h) and 4 h after consumption of 480 mL cranberry juice (64% juice; 835 mg total polyphenols; 94.47 mg anthocyanins). Marked inter-individual differences in plasma anthocyanin pharmacokinetics were observed with maximum anthocyanin concentrations detected between 1 and 3 h. Cranberry anthocyanins were bioavailable but with notable differences in the maximum concentration and area under the curve between individual participants. The pattern of anthocyanin glucosides observed in plasma and urine generally reflected the relative concentration determined in the juice. Plasma concentrations of the individual anthocyanins ranged between 0.56 and 4.64 nmol/L. Total recovery of urinary anthocyanin was 0.79 ± 0.090% of the dose delivered. These data are in agreement with the pharmacokinetics of anthocyanins from other foods suggesting that cranberry anthocyanins are poorly absorbed and rapidly removed from plasma. Observed concentrations of plasma anthocyanins appear insufficient to alter radical load or redox potential but may be adequate to affect signal transduction and/or gene expression.

Introduction

The common American cranberry, Vaccinium oxycoccos macrocarpon, and the small cranberry, Vaccinium oxycoccos microcarpus, were used by Native Americans and early Europeans to flavor food, dye materials, and as a traditional medicine. The putative health benefits have recently emerged from study of Vaccinium species include cardioprotective effects (1), anticancer properties (2), and reversal of age-related motor behavioral deficits (3). The compounds responsible for this bioactivity have not been fully elucidated; however, the focus of research has centered on classes of polyphenols abundant in berries, especially anthocyanins (4). Cranberries contain appreciable quantities of anthocyanins (5,6). Studies of individual anthocyanins reveal their bioavailability to plasma is generally <1% of consumed quantities. Only recently have studies been conducted to determine tissue concentrations of anthocyanins (7) and their fate in the lower intestinal tract is unknown. Despite low bioavailability, plasma concentrations of anthocyanins appear sufficient to induce changes in signal transduction and gene expression in vivo (8,9) in a manner that suggests their putative role in physiological functions and health outcomes.

To date, there have been no detailed pharmacokinetic studies, to our knowledge, of anthocyanins after acute cranberry juice consumption. Although cranberry anthocyanins decrease oxidation of human LDL in vitro and lower total and LDL cholesterol in animals (10–12), to elucidate other possible cardiovascular protective mechanisms, cranberry anthocyanin bioavailability and pharmacokinetics must be more clearly established in humans.

Using a single acute dose of 12 g elderberry extract containing 720 mg anthocyanins, Milbury et al. (13) detected unmetabolized anthocyanins in human plasma and urine and found the time to maximal plasma concentrations was reached within 72 min. The elimination of plasma anthocyanins appeared to follow first-order kinetics and the elimination half-life of plasma total anthocyanins was calculated as between 133 and 68.9 min, depending on the anthocyanin. Stoner et al. (14) conducted a study in which humans consumed 45 g of freeze-dried black raspberries daily for 7 d. Analysis of samples collected on d 1 and 7 showed that the pharmacokinetic
parameters for anthocyanins and ellagic acid did not differ between these time points. Anthocyanin and ellagic acid in urine were <1% of the amount consumed in the black raspberries and concentrations of their glucuronide metabolites were insubstantial, suggesting they either passed through the gastrointestinal tract or were extensively metabolized by microflora in situ or by tissue metabolism in vivo. Kay et al. (15) conducted a similar study in 2 men using 1.34 mg of a chokecherry extract containing primarily cyanidin-glycosides. They found cyanidin-glycosides were absorbed and circulated as both glycosides and glucuronides; the presence of methylated products in plasma was considered a result of the high dose of the extract used. In a subsequent study, these same investigators confirmed their earlier findings using a 721-mg oral dose of purified cyanidin-3-glycosides from chokecherry extract (16).

Zhang and Zu (17) determined the levels of phenolic compounds in plasma from a fasting healthy volunteer after drinking 1800 mL cranberry juice cocktail (containing 27% cranberry juice). They measured 16 phenolic compounds, including myricetin, quercetin, resveratrol, and benzoic and phe- nolic acids but did not investigate anthocyanin bioavailability. In healthy females consuming 750 mL/d cranberry juice for 2 wk, Duthie et al. (18) were unable to detect anthocyanins or catechins in plasma or urine samples of either the control or cranberry-supplemented participants, possibly due to the fact that the volunteers fasted overnight prior to sample collection.

No pharmacokinetic studies have been undertaken after consumption of anthocyanins from usual dietary amounts of cranberry juice. Thus, the objective of this study was to collect absorption and pharmacokinetic data on cranberry anthocyanins after consumption of a single acute dose of cranberry juice.

Materials and Methods

Chemicals. HPLC grade water was produced via a Modulab MLU filter unit (U.S. Filter). Methanol was obtained from Fisher Scientific. Formic acid and trifluoroacetic acid were obtained from Sigma Chemical. Authentic anthocyanin standards were obtained either from Polyphenols AS or Extrasynthése. They included cyanidin-3-glucoside (cyan-glu), cyaniding-3-galactoside (cyan-gal), cyanidin-3-arabinoside (cyan-ara), peonidin-3-glucoside (peo-glu), peonidin-3-galactoside (peo-gal), peonidin-3-arabinoside (peo-ara), malvidin-3-glucoside (mal-glu), and malvidin-di-glucoside. Double-strength cranberry juice (54% juice) was provided by Ocean Spray Cranberries.

Study volunteers. Fifteen volunteers from the Boston, MA area were recruited to participate in a pilot study of flow mediated dilation in patients with established coronary artery disease. The blood and urine samples were made available to us for anthocyanin analysis. Two of the volunteers were female. Their age was (mean ± SD) 62 ± 8 y with a range of 45–73 y. All participants were free of any symptoms of gastrointestinal dysfunction. Additional cohort biometrics are available online in Supplemental Table 1. Glucose, total cholesterol, HDL, triglycerides, and creatinine were determined in freshly collected serum samples by an automated Bayer Advia 1640 analyzer (Bayer Diagnostics). LDL cholesterol was calculated using the Friedewald formula. Volunteers gave written informed consent prior to participation. All procedures were approved by the Institutional Review Boards at Tufts Medical Center and Tufts University Health Sciences Campus and at Boston University Medical Center.

Sample collection and preparation. The total of soluble phenolics in the cranberry juice was determined using an acetonitrile extract and Folin-Ciocalteu reagent according to Singleton and Rossi (19) using gallic acid as a standard. Study volunteers arrived at Boston University Medical Center after an overnight fast and were instructed not to take their medications until after the 4-h collection period. Volunteers consumed 480 mL of a double-strength cranberry juice (54% juice containing 835 mg total polyphenols). Commercial cranberry juice products typically contain 25–27% juice. Plasma samples were collected from volunteers at 0, 1, 2, 3, and 4 h after consumption of a single dose of the juice, and urine was collected at baseline and for 4 h thereafter. Blood samples were immediately centrifuged at 1000 × g for 15 min at 5°C to recover the plasma. Unfiltered blood plasma (3 mL) or unfiltered urine (10 mL) was acidified by addition of 40 μL 6 mol/L HCl and all samples were then stored at −80°C until analysis.

Anthocyanins were extracted from the samples using disposable solid-phase extraction C18 cartridges (Waters Sep-Pak Vac 12 cc C18-2 g.). Cartridges were preconditioned with 7 mL acidified methanol (0.1% trifluoroacetic acid, pH 2.1) followed by 7 mL acidified water (10 mmol/L citric acid). Samples were thawed and diluted with 10 mmol/L citric acid (1:1, v/v), vortexed, and loaded directly onto the cartridge. The samples were drained under gravity (~1 drop/s) and washed with 2 vol acidified water (10 mmol/L citric acid, ~12 mL). Anthocyanins were eluted from the cartridge with 6 mL acidified methanol (0.1% trifluoroacetic acid). The eluent was evaporated at ambient temperature in a SpeedVac Plus-S SCI10A condenser (Savant Instruments) to 0.1–0.5 mL and the residual eluent brought to dryness under nitrogen gas at room temperature. The residue was dissolved in 300 μL of 5% acetonitrile in a 1% aqueous formic acid solution for LC-MS/MS analysis. Malvidin-di-glucoside, which is not found in inappreciable quantities in cranberry, was used as an internal standard to correct for losses in sample preparation and daily differences in MS performance. Recovery of malvidin-di-glucoside was 91 ± 5%.

HPLC/diode array/ion-trap MS/MS analysis of anthocyanins. Analysis was accomplished using an Agilent 1100 HPLC system (Agilent Technologies) fitted with a Phenomenex Synergy Max-RP C18 analytical column, 250 × 4.6 mm, 4-μm particle size by gradient and column modifications of the method described by Milbury et al. (20,7). Chromatographic separation of anthocyanins was achieved using a gradient between 4.5% formic acid in water (mobile phase A) to 4.5% formic acid in 100% acetonitrile (mobile phase B) over an 80-min analytical run at a flow rate of 0.3 mL/min. Detection was achieved using an Agilent UV G1315A diode array detector monitoring absorbance between 250 and 700 nm. Anthocyanin glycosides and the transitions to anthocyanidins (loss of sugars) were monitored according to the parent glycoside molecule retention time and UV absorbance and the mass charge ratio (m/z) values for the parent glycosides and aglycone fragmentation products parameters (Supplemental Table 2) using a Bruker Esquire ion trap MS/MS (Bruker Daltonics) fitted with an electrospray interface operated in the positive ion mode with alternating MS and MS/MS scans from m/z 150 to 1000. Observed UV-vis spectra patterns and λmax absorances of anthocyanins were the same as reported by Wulf and Nagel (21).

Calculations and statistics. MS/MS scans of the anthocyanin aglycone fragments were analyzed and compared with those of authentic anthocyanin standards using Bruker Daltonics Esquire LC 4.5 (Build 21) and data analysis software version 3.0 (Build 49). An equation for the standard curves was calculated by fitting a linear line using the least squares method. The resulting equation was used to calculate plasma and urine anthocyanin values.

Based on individual anthocyanin plasma concentration compared with time data, calculations were made to determine maximal plasma concentration (Cmax), elimination half-life, elimination rate constant, area under the plasma concentration curve from 0 to 4 h (AUCo-4), and from zero to infinity (AUC∞) for cyan-glu, cyan-ar-ara, peo-gal, peo-ara, and mal-glu. AUCo-4 was determined by the linear trapezoidal rule. AUC was extrapolated to infinity (AUC∞) by adding the last quantifiable concentration divided by the elimination rate
constant. These parameters were determined using the pharmacokinetic functions for the Microsoft Excel computer program (22). Values in the text and plots are means ± SD. Data were analyzed by paired t-tests using SPSS 16.0 for Windows statistical analysis software and P < 0.05 was considered significant.

Results

Anthocyanins in cranberry drink. Anthocyanins in the juice were extracted by the same procedure used to extract anthocyanins from plasma. Anthocyanins were identified and quantified using the aglycone fragment ion retention time and MS intensity and comparing them to the UV traces and retention times for the parent glucosides (Supplemental Fig. 1). Sixteen ounces (480 mL) of the 54% cranberry juice contained 94.47 mg of anthocyanins composed predominantly of: cyn-gal, 18.7 mg; cyn-glu, 1.58 mg; cyn-ara, 16.47 mg; peo-gal, 30.83 mg; peo-glu, 5.85 mg; and peo-ara, 21.03 mg. The anthocyanin concentration profile (expressed as μmol/L) determined by LC-MS/MS analysis showed higher amounts of peo-glu and peo-ara than was found using HPLC with UV detection (Fig. 1). The difference between results obtained with UV compared with MS/MS in the same analytic run for peonidin may be due to the fact that it is unique among anthocyanins with regard to its metabolism by catechol-O-methyl transferase (EC 2.1.1.6). Wu et al. (23) identified 2 forms of peo-glu, one with the methoxyl group in the 3' position in the B-ring and another with the methoxyl group in the peonidin 4' position (isopeonidin) (24). The same structures may exist for peo-ara; these isoforms are difficult to resolve chromatographically. Whereas the isoforms produce the same m/z fragments in MS/MS analysis, it is possible that the location of the methoxyl group on the B ring may affect the UV spectral characteristics of these isoforms of peonidin. Peo-gal was the major anthocyanin detected in the cranberry juice at ~32% of the total anthocyanins measured. Cyn-gal and peo-ara were the next most prevalent anthocyanins in the juice at 23 and 18%, respectively.

Anthocyanins in human plasma and urine. Of the 15 anthocyanins detected in the cranberry juice, only the 6 most prominent ones (cyn-gal, cyn-glu, cyn-ara, peo-gal, peo-glu, peo-ara) and mal-glu (a minor anthocyanin in cranberry) were measured at baseline and monitored in the volunteers over 4 h after consumption of the juice. The other anthocyanins were either below instrument detection limits or not reliably observed in all volunteers at signal levels 3-times greater than the background signal noise. Peo-gal was the major anthocyanin detected in the plasma. The patterns of mean anthocyanin Cmax (Fig. 2A) and AUC0–4 (Fig. 2B) after cranberry juice consumption reflected the patterns of anthocyanins observed in cranberry juice. The calculated plasma pharmacokinetic data (Table 1) revealed that all anthocyanins measured reached their highest concentration in plasma within 1.5 h after consumption. Although the anthocyanin with the highest concentration in cranberry juice (peo-gal) reached the highest concentration in the blood, the presence of a glucose moiety on the anthocyanindins cyanidin and peonidin appear to make them more bioavailable as a percentage of the delivered dose than the same anthocyanindins carrying either a galactoside or an arabinoside. All individuals had increased concentrations of each anthocyanin in their 4-h urine samples with the exception of cyn-glu (Fig. 3A). The pattern of recovered urinary anthocyanin concentrations in 0- to 4-h urine samples (Fig. 3A) and the total recovered urinary anthocyanins (Fig. 3B) reflected the pattern of anthocyanin concentrations of the juice. However, the percent of the original dose of individual anthocyanins from the juice recovered in the urine was very low (Table 2). The overall total of

FIGURE 1  Concentrations of anthocyanins in 57% cranberry juice measured by HPLC with UV detection at 560 nm and by LC-MS/MS. Values are means ± SD, n = 3 (LC-MS/MS) or 5 (HPLC/UV). *Different from HPLC/UV, P < 0.05.

FIGURE 2  Scatter plot of individual anthocyanin Cmax in plasma (A) and AUC0–4 (B) after acute intake of 480 mL of 57% cranberry juice. Means (n = 15) are depicted as solid black bars.
anthocyanins recovered in urine was 0.79 ± 0.90% of the administered dose with a range of 0.078–3.2%.

**Discussion**

We analyzed the parent anthocyanin glycosides found in cranberry juice, absorbed into plasma, and excreted into urine. Generally, flavonoids are metabolized to glucuronidated and/or sulfated derivatives, although most studies of anthocyanins have reported detection of only native glycosylated forms (25). Glucuronidated, methylated, and/or sulfated forms have been identified after feeding berry fruit juice (26,27); however, looking exclusively at urinary anthocyanin metabolites after cranberry juice consumption, Ohnishi et al. (28) found no anthocyanidin glucuronides in urine.

We were able to reliably detect in plasma and urine 7 of the 15 anthocyanins that have been measured in cranberry juice. Reports from feeding studies of berries show that the majority of anthocyanins are excreted in the first 4 h (29), with recovery from elderberry, blackcurrant, boysenberry, and blueberry ranging from 0.02 to 0.37% (13,23,30–32). However, studies of anthocyanins in urine after strawberry consumption have found; 1.8 and 2% recovery (33,34), and 1 study found 5% of the dose of administered anthocyanins in urine after cranberry juice consumption (28). We found total urinary anthocyanin recovery from cranberry juice was highly variable between individuals, ranging between 0.078 and 3.2% of the administered dose, a finding consistent with, although slightly higher than, the mean of values reported in other berry anthocyanin bioavailability studies (35). We observed a high degree of inter-individual variability in the uptake of anthocyanins. Nonetheless, there was consistency in relative bioavailability within individuals, i.e. a person showing a greater absorption of one anthocyanin generally had higher plasma levels of all the anthocyanins and a higher amount recovery in the urine. The high degree of inter-individual variability in anthocyanin bioavailability may result from differences in xenobiotic metabolism in the gastrointestinal tract, liver, and other tissues. Indeed, human polymorphisms have been reported in the genes for catechol-O-methyltransferase, glutathione S-transferases, and UDP glucuronosyl transferase (36).

The higher level of recovery in urine of anthocyanin from cranberry juice reported here and by Ohnishi et al. (28) compared with the same compounds delivered either alone or from other berries suggests these flavonoids may be absorbed and metabolized differently from various food matrices. Additional studies are needed on the effects of food matrix in absorption efficiency and stability of anthocyanins.

The concentration of peo-gal in cranberry juice is higher than that of other anthocyanidins and this relationship is reflected in the bioavailability patterns of plasma and urine. The Cmax:dose ratio for galactosides and arabinosides of cyanidin and peonidin were considerably lower than that for the glucosides. A similar pattern was observed with the AUC0–4:dose ratio calculations. These results suggest that cyn-glu and peo-glu are more

<table>
<thead>
<tr>
<th>Compound</th>
<th>Cmax</th>
<th>Cmax:M (pmol/L)/nmol</th>
<th>Cmax:mg (pmol/L)/100 mg</th>
<th>Time to maximal plasma concentration</th>
<th>AUC0–4 (nmol * h/L)</th>
<th>AUC0–4:M (pmol * h/L)/100 mg</th>
<th>AUC0–4:mg (pmol * h/L)/100 mg</th>
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</thead>
<tbody>
<tr>
<td>cyn-gal</td>
<td>1.38</td>
<td>1.16</td>
<td>0.033</td>
<td>1.27 ± 0.59</td>
<td>3.91 ± 3.32</td>
<td>0.094</td>
<td>6.89 ± 6.69</td>
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<tr>
<td>cyn-glu</td>
<td>0.93</td>
<td>1.04</td>
<td>0.833</td>
<td>1.13 ± 0.83</td>
<td>1.99 ± 1.48</td>
<td>1.794</td>
<td>3.66 ± 3.62</td>
</tr>
<tr>
<td>cyn-ara</td>
<td>3.61</td>
<td>2.87</td>
<td>0.097</td>
<td>1.47 ± 0.64</td>
<td>9.16 ± 6.99</td>
<td>0.247</td>
<td>13.50 ± 11.04</td>
</tr>
<tr>
<td>peo-gal</td>
<td>4.64</td>
<td>3.62</td>
<td>0.073</td>
<td>1.47 ± 0.64</td>
<td>12.00 ± 10.17</td>
<td>0.189</td>
<td>18.68 ± 19.46</td>
</tr>
<tr>
<td>peo-glu</td>
<td>0.71</td>
<td>0.42</td>
<td>0.117</td>
<td>1.40 ± 0.83</td>
<td>1.85 ± 1.12</td>
<td>0.304</td>
<td>3.72 ± 3.19</td>
</tr>
<tr>
<td>peo-ara</td>
<td>1.78</td>
<td>1.29</td>
<td>0.052</td>
<td>1.27 ± 0.59</td>
<td>4.13 ± 3.37</td>
<td>0.121</td>
<td>5.35 ± 5.33</td>
</tr>
<tr>
<td>mal-glu</td>
<td>0.56</td>
<td>0.98</td>
<td>0.033</td>
<td>0.93 ± 1.10</td>
<td>1.25 ± 1.61</td>
<td>0.094</td>
<td>2.55 ± 1.80</td>
</tr>
</tbody>
</table>

1 Values are means ± SD, n = 15.

**FIGURE 3** Human urine anthocyanin concentrations relative to creatinine at baseline and in the 0- to 4-h pooled sample (A) and total anthocyanin excretion in 4 h (B). Values are means ± SD, n = 15. *Different from baseline, P < 0.05.
bioavailable, more stable, and/or cleared more slowly than either the galactosides or arabinosides of cyanidin and peonidin.

We found anthocyanins from cranberry juice are bioavailable with 7 predominate anthocyanins detected and quantified in the plasma and urine of 15 study volunteers. The concentration of cranberry anthocyanins achieved in plasma appear too low to compete effectively with antioxidants like ascorbate and glutathione in quenching free radicals. We have demonstrated in pigs that dietary anthocyanins accumulate in tissues during long-term feeding and have a longer residence time in tissues than in plasma (7). It is not known whether these flavonoids accumulate in cardiac or vascular tissues during long-term cranberry consumption, although Vaccinium anthocyanins have been shown to affect vascular reactivity in animal studies (37). Vascular endothelial cells increase their expression of tumor necrosis factor-α and production of proinflammatory cytokines and adhesion molecules when damaged or under oxidative stress. When fed 4% freeze-dried whole blueberries in the diet, tumor necrosis factor-α, monocyte chemoattractant protein-1, and interleukin-10 were decreased in rats fed a high-fat diet but not in those fed a low-fat diet (36), suggesting that Vaccinium berries may be helpful in reducing inflammatory and oxidative stress in the vascular system.

Ruel et al. (38) found that a 12-wk supplementation with low-calorie cranberry juice cocktail increased HDL-cholesterol, reduced plasma oxidized LDL, and decreased circulating soluble intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 in men between 18 and 70 y (39). However, in a study of healthy females aged 18–40 y, Duthie et al. (15) found consumption of 750 mL/d cranberry juice for 2 wk for did not affect plasma total cholesterol, triglycerides, HDL, or LDL. The divergent results regarding the effect of cranberry in these trials may be related to their inclusion criteria for volunteers and underscores the need for additional studies of the effects of age, gender, genetics, and health status on the bioavailability of anthocyanins. More complete information on the pharmacokinetic and dose-response relationships as well as the food matrix characteristics of anthocyanins will help inform the design of new human studies of the bioactivity, mechanisms of action, and functional outcomes of consuming Vaccinium berries and other foods rich in these flavonoids. Our study examined the bioavailability and pharmacokinetics of cranberry anthocyanins and so does not directly address mechanisms of action or associations with health outcomes. However, these findings do suggest that anthocyanin concentrations are too low to directly contribute to in vivo quenching of reactive oxygen species but may be adequate to influence signal transduction and gene expression pathways.

**TABLE 2 Anthocyanins recovered in human urine after acute cranberry juice consumption**

<table>
<thead>
<tr>
<th>Anthocyanin</th>
<th>Total dose in juice</th>
<th>Total recovered in urine</th>
<th>Percent of original dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>cyn-gal</td>
<td>41,648</td>
<td>2.8 ± 3.6</td>
<td>0.007 ± 0.008</td>
</tr>
<tr>
<td>cyn-glu</td>
<td>1114</td>
<td>0.077 ± 0.045</td>
<td>0.007 ± 0.004</td>
</tr>
<tr>
<td>cyn-ara</td>
<td>37,088</td>
<td>3.6 ± 5.2</td>
<td>0.010 ± 0.014</td>
</tr>
<tr>
<td>peo-gal</td>
<td>63,542</td>
<td>9.7 ± 6.3</td>
<td>0.015 ± 0.010</td>
</tr>
<tr>
<td>peo-glu</td>
<td>8901</td>
<td>1.9 ± 3.6</td>
<td>0.029 ± 0.059</td>
</tr>
<tr>
<td>peo-ara</td>
<td>33,995</td>
<td>3.3 ± 3.3</td>
<td>0.010 ± 0.010</td>
</tr>
</tbody>
</table>

1 Values are means ± SD, n = 15.

**ACKNOWLEDGMENTS**

P.E.M. was the principal investigator responsible for the anthocyanin analyses, collection of data, pharmacokinetic and statistical calculations, data interpretation, and manuscript preparation. J.A.V. was responsible for the design and conduct of the clinical trial from which the study blood and urine samples were obtained. P.E.M. and J.B.B. designed the study, interpreted the data, and prepared the manuscript. All authors read and approved the final manuscript.

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