Anthocyanins Exist in the Circulation Primarily as Metabolites in Adult Men

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ABSTRACT Anthocyanins are reported to have many “health promoting” properties; however, despite numerous reports of their bioactivities, their absorption and metabolism in humans are poorly understood. The objective of this research was to detail the pharmacokinetic parameters of anthocyanins and glucuronide conjugates in the human subjects. Solid-phase extraction, preparative-HPLC, preparative-TLC, HPLC-diode array detection, HPLC-MS, and NMR were utilized to isolate, identify, and quantify anthocyanins in 0- to 7-h (0, 1, 2, 3, 4, 5, 6, 7 h) serum and 0- to 24-h urine samples (total individual urine voids over 24 h). The cumulative concentration of total anthocyanins (parent and metabolites) detected in the serum (0–7 h) was 376.65 ± 16.20 (nmol·h)/L (area under the concentration time curve), reaching a maximum concentration ($C_{\text{max}} = 96.08 ± 6.04$ nmol/L) within 2.8 h. The parent anthocyanins represented only 32.0% [120.63 ± 2.85 (nmol·h)/L] of the total anthocyanins detected with 68.0% [256.02 ± 5.23 (nmol·h)] identified as conjugated metabolites. Additionally, the total urinary excretion of anthocyanins over 24 h was 1071.54 ± 375.46 μg, reaching a maximal rate of excretion ($R_{\text{max}} = 202.74 ± 85.06$ μg/h) at 3.72 ± 0.83 h. Parallel to the serum data, only 32.5% (347.85 ± 60.61 μg) of the anthocyanins excreted in the urine (total 24 h) were the parent compounds with 67.5% (723.69 ± 92.59 μg) occurring as conjugated metabolites. The metabolites were identified as glucuronidated and methylated derivatives of the parent cyanidin-3-glycosides. The above results indicate that cyanidin-3-glycosides are rapidly absorbed and metabolized extensively following a moderate-to-high oral dose in humans. J. Nutr. 135: 2582–2588, 2005.

KEY WORDS: • anthocyanins • cyanidin 3-glycosides • pharmacokinetics • metabolites

Within the last decade, many studies have focused on the potential biological activities or health effects of anthocyanins in humans (1–3). Although there is a great deal of evidence indicating the bioactivity of anthocyanins, very little progress has been made in determining the pharmacokinetics of these compounds, with aspects such as absorption and metabolism left essentially unstudied. Previously, it was reported that anthocyanins were poorly absorbed and circulated in the blood exclusively as unmetabolized parent glycosides (4–6). It is only recently that researchers have begun to suggest that anthocyanins are metabolized; however, the identification of derived metabolites has been limited as a result of their diversity and low concentrations in the blood.

In our previous investigation focusing on identifying anthocyanin metabolites in human serum and urine, subjects were fed ~1.2 g of cyanidin 3-glycosides from chokeberries, which resulted in the identification of glucuronide and methyl derivatives. The aim of the present investigation was to determine the pharmacokinetics of the cyanidin-3-glycosides in humans as well as to establish the extent of their metabolic fate after a lower, more realistic anthocyanin dose (721 mg). Specifically, the chokeberry extract was chosen because it contained exclusively cyanidin 3-glycosides, thereby permitting the monitoring of its metabolites and their pharmacokinetics. Subsequent investigations will be required to identify the biological activity of these metabolites.

SUBJECTS AND METHODS

Subjects. Healthy male volunteers (n = 3; 40 ± 14.2 y old) participated in the cyanidin-3-glycoside consumption intervention. Subjects had a mean BMI of 28.3 ± 1.6 kg/m² and had no clinical disease as determined using a medical history questionnaire. Subjects were instructed to consume an essentially anthocyanin-free diet (no fruit or vegetables, including foods colored with red or blue dyes) for 2 d before the study; they were also asked to avoid taking aspirin or anti-inflammatory medications, and antioxidant or herbal supplements for 2 wk before the investigation. The major constituents of the anthocyanin-free washout diet were milk, tuna, white bread, chicken, and white rice. Compliance with the anthocyanin-free diet was monitored using food diaries and confirmed in baseline samples via reverse-phase HPLC (RP-HPLC) with diode array detection.

3 Abbreviations used: C-3-ara, cyanidin 3-arabinoside; C-3-gal, cyanidin 3-galactoside; C-3-glu, cyanidin 3-glucoside; C-3-xyl, cyanidin 3-xyloside; CD3OD, Methanol-d4; CF3COOD, trifluoroacetic acid-d; Cmax, maximum concentration; DAD, diode array detector; E440/Emax, ratio of the absorbance intensity at 440 nm vs. the maximum absorbance intensity; ESI-MS, electrospray ionization MS; m/z, mass to charge ratio; P, peak; PCA, perchloric acid; P-3-gal, peonidin 3-galactoside; P-3-xyl, peonidin 3-xyloside; Prep-HPLC, preparative HPLC; RF, reference value; Rmax, maximum rate of urinary excretion; RP-HPLC, reverse phase HPLC; Rt, retention time;
Identification of anthocyanins and anthocyanin metabolites in human urine and serum after the consumption of 721 mg of cyanidin 3-glycosides

**Study design.** Subjects were admitted to the clinic (Okanagan Clinical Laboratory; Penticton, BC) on the morning of the study dates after fasting (12 h, 24 h no alcohol). Baseline urine samples (first void, t = 0) were taken in the morning of each study date along with individual urine voids (total volume) over the next 24 h (t = 0, 2, 4, 6, 8, 10, 12, and 24 h). Immediately after baseline (t = 0) blood sampling, the volunteers consumed 250 mL of water (gel caps) every hour for 5 h with subsequent ad libitum consumption. An anthocyanin-free lunch and dinner was provided for the subjects at 4 and 8 h postconsumption of the extract. The experiment was repeated at a later date (30 d wash-out) with individual urine voids (total volume) over the next 24 h (levels 0, 1, 2, 3, 4, 5, 6, 7). The sampling regimen was necessary to acquire the volume of blood needed for the analysis. Individual urine samples were also collected over 24 h (levels = 0, 2, 4, 6, 8, 10, 12, 24) at each visit (n = 3 × 3 repetitions; y = 9 replicates). After consumption of the extract, subjects were instructed to consume 250 mL of water every hour for 5 h with subsequent ad libitum consumption. An anthocyanin-free lunch and dinner was provided for the subjects at 4 and 8 h postconsumption of the extract.

Blood samples (~20 mL) were drawn by venipuncture from a brachial vein into 10-mL evacuated glass tubes (2 tubes/time point) (Vacutainer; Becton Dickinson). The blood samples were allowed to clot at room temperature for 30 min. Samples were then immediately centrifuged (1000 × g) for 15 min at 5°C to recover the serum. Urine samples were acidified with 20 mL of 12 mol/L HCl/mL urine upon collection, to a pH of 1.2, maintained at 5°C, and analyzed within 12 h of collection.

**TABLE 1**

<table>
<thead>
<tr>
<th>Peak</th>
<th>Anthocyanin/identity</th>
<th>Rt</th>
<th>MW parent/daughter fragment</th>
<th>Absorption spectra</th>
<th>Characteristics of anthocyanin standards</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>λ&lt;sub&gt;max&lt;/sub&gt;, E&lt;sub&gt;λmax&lt;/sub&gt;/E&lt;sub&gt;max&lt;/sub&gt; (as %)</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Cyanidin 3-galactoside&lt;sup&gt;4&lt;/sup&gt;</td>
<td>23.8</td>
<td>449/287</td>
<td>280, 517</td>
<td>31</td>
</tr>
<tr>
<td>2</td>
<td>Cyanidin glucuronide</td>
<td>27.6</td>
<td>463/287</td>
<td>280, 517</td>
<td>31</td>
</tr>
<tr>
<td>3</td>
<td>Cyanidin 3-arabinoside&lt;sup&gt;4&lt;/sup&gt;</td>
<td>29.6</td>
<td>419/287</td>
<td>280, 517</td>
<td>31</td>
</tr>
<tr>
<td>4</td>
<td>Peonidin 3-galactoside&lt;sup&gt;4&lt;/sup&gt;</td>
<td>33.4</td>
<td>463/301</td>
<td>280, 517</td>
<td>31</td>
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<td>5</td>
<td>Methylated cyanidin glucuronide</td>
<td>36.3</td>
<td>477/301</td>
<td>280, 514</td>
<td>37</td>
</tr>
<tr>
<td>6</td>
<td>Methylated cyanidin glucuronide</td>
<td>37.0</td>
<td>477/301</td>
<td>280, 515</td>
<td>32</td>
</tr>
</tbody>
</table>

**Figure 1** Chromatograms of anthocyanins in baseline human urine sample (A), chokeberry extract (B), typical serum sample (C), and typical urine sample (D). HPLC analysis as outlined in the methods. The identification of each peak represented above (peaks 1–6) is given in Table 1. Urine and serum pharmacokinetic data of each compound represented by peaks 1–6 are given in Tables 2 and 3, respectively.

**Notes:**
- **SPE:** solid phase extraction; t<sub>0</sub>, baseline; t<sub>1/2a</sub>, elimination half-life; t<sub>1/2b</sub>, absorption half-life; t<sub>max</sub>, time point at which maximal serum concentration occurs; t<sub>maxR</sub>, time point at which maximal rate of urinary excretion occurs; TFA, trifluoroacetic acid; UV-vis, UV-visible.
- **MW:** molecular weight.
- **E<sub>λmax</sub>/E<sub>max</sub> (as %): relative peak height.
- **Rt:** retention time.
- **TLC (R<sub>f</sub>):** thin layer chromatography (R<sub>f</sub>).

**Absorption spectra:**
- **λ<sub>max</sub>:** maximum wavelength Absorption spectra for each compound.
- **E<sub>λmax</sub>/E<sub>max</sub> (as %):** relative peak height at λ<sub>max</sub>.

**Characteristics of anthocyanin standards:**
- **MW:** molecular weight.
- **Rt:** retention time.
- **λ<sub>max</sub>:** maximum wavelength Absorption spectra for each compound.
- **E<sub>λmax</sub>/E<sub>max</sub> (as %):** relative peak height at λ<sub>max</sub>.

**Identification of anthocyanin standards based on HPLC-DAD:**
- **1 Identification (retention time and absorption spectra) based on Agilent HPLC-DAD.
- **2 Identification (MW) based on Waters microcapillary HPLC-MS.
- **3 Comparisons made between serum and urinary samples using HPLC-DAD (Fig. 1, C and D) indicated that the serum peaks matched both retention time and UV-vis spectrum with the peaks identified as anthocyanins in the urine, and were therefore regarded as the same compounds.
- **4 Data based on analysis of purified anthocyanin from chokeberry extract.
- **5 Data based on analysis of purchased standard (Extrasynthese).**
Pharmacokinetic parameters of cyanidin 3-glycosides and corresponding metabolites in human urine after the consumption of 721 mg of cyanidin 3-glycosides

<table>
<thead>
<tr>
<th>Peak</th>
<th>Anthocyanin/identity</th>
<th>Quantity</th>
<th>$R_{\max}$</th>
<th>$t_{\max}$</th>
<th>$t_{1/2}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Cyanidin 3-galactoside</td>
<td>267.55 ± 90.82</td>
<td>48.74 ± 14.80</td>
<td>3.39 ± 1.14</td>
<td>3.72 ± 0.42</td>
</tr>
<tr>
<td>2</td>
<td>Cyanidin glucuronide</td>
<td>173.25 ± 86.46</td>
<td>39.47 ± 20.77</td>
<td>3.17 ± 0.86</td>
<td>3.63 ± 0.56</td>
</tr>
<tr>
<td>3</td>
<td>Cyanidin 3-arabinoside</td>
<td>80.30 ± 31.30</td>
<td>13.76 ± 4.68</td>
<td>2.94 ± 1.39</td>
<td>4.05 ± 0.82</td>
</tr>
<tr>
<td>4</td>
<td>Peonidin 3-galactoside</td>
<td>103.88 ± 35.63</td>
<td>19.27 ± 7.74</td>
<td>3.93 ± 1.14</td>
<td>4.28 ± 0.59</td>
</tr>
<tr>
<td>5</td>
<td>Methylated cyanidin glucuronide</td>
<td>111.06 ± 60.61</td>
<td>19.61 ± 10.51</td>
<td>4.05 ± 1.21</td>
<td>4.53 ± 0.92</td>
</tr>
<tr>
<td>6</td>
<td>Methylated cyanidin glucuronide</td>
<td>335.50 ± 187.66</td>
<td>66.63 ± 37.13</td>
<td>4.05 ± 1.21</td>
<td>4.39 ± 0.83</td>
</tr>
</tbody>
</table>

1 Pharmacokinetic data are means ± SD, n = 9, over 24 h as quantified via HPLC-DAD (Table 1).

<table>
<thead>
<tr>
<th>Peak</th>
<th>Anthocyanin/identity</th>
<th>Quantity</th>
<th>$C_{\text{max}}$</th>
<th>$T_{\text{max}}$</th>
<th>AUC</th>
<th>$t_{1/2}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Cyanidin 3-galactoside</td>
<td>23.36 ± 2.33</td>
<td>2.5 (2–3)</td>
<td>66.64 ± 3.27</td>
<td>&lt;1.35</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Cyanidin glucuronide</td>
<td>14.51 ± 4.04</td>
<td>2.0 (2)</td>
<td>90.93 ± 4.08</td>
<td>&lt;1.67</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Cyanidin 3-arabinoside</td>
<td>8.85 ± 0.50</td>
<td>3.5 (3–4)</td>
<td>53.89 ± 2.43</td>
<td>&lt;1.67</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Peonidin 3-galactoside</td>
<td>3.76 ± 0.78</td>
<td>4.0 (4)</td>
<td>30.67 ± 1.12</td>
<td>&lt;1.67</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Methylated cyanidin glucuronide</td>
<td>12.81 ± 0.42</td>
<td>2.5 (2–3)</td>
<td>34.42 ± 2.77</td>
<td>&lt;1.67</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Methylated cyanidin glucuronide</td>
<td>32.79 ± 10.88</td>
<td>2.5 (2–3)</td>
<td>100.00 ± 12.85</td>
<td>&lt;1.67</td>
<td></td>
</tr>
</tbody>
</table>

1 Pharmacokinetic data are means ± SD, n = 3, over 7 h as quantified via HPLC-DAD (Table 1).
2 Values are medians (range).
3 $t_{1/2}$: precise absorption half-lives for 2 of the 3 subjects could not be determined because there was only one serum sample (t = 1 h) during the absorption phase. Therefore, as a result of the rapid absorption phase (<1 h) for 2 of the 3 subjects, SDs cannot be specified. The absorption half-life values above are from 1 subject only and it can be assumed that the mean absorption half-life for the 3 subjects would be <1 h. For a more accurate estimate of absorption additional half-life samples are required before the end of h 1. The $t_{1/2}$ value was determined using the method of residuals (14).

**Materials/reagents.** The chokeberry extract (no. 74190, lot L18010) was purchased from Artemis International. The anthocyanin standards, cyanidin 3-glucoside chloride, cyanidin 3-galactoside chloride (ideacloride), peonidin 3-glucoside chloride, cyanidin chloride, and peonidin chloride were purchased from Extrasynthese. Formic acid (Fisher Scientific), hydrochloric acid (HCl), trifluoroacetic acid (TFA), and glacial acetic acid (DH) were all reagent grade; all solvents used for HPLC analysis were HPLC grade. The anthocyanins were extracted from biological fluids before HPLC analysis using disposable SPE C18 cartridges (Supelclean ENVII-16 6 mL 2000 mg; Sigma; lot # SP2419C). Unfiltered blood serum (4 mL) or 2–10 mL of unfiltered acidified urine (2 mL, t = 0–9 h; or 10 mL, t = 10–24 h) were utilized for the extraction. Individual blood and urine samples (nonpooled) were extracted in duplicate, and each extract was injected into the HPLC column in duplicate (total of 4 injections/sample) for quantitative HPLC analysis.

**Pharmacokinetic analysis.** The chokeberry extract, serum, and urine anthocyanins were quantified via HPLC-DAD. Individual serum samples were collected every hour for 7 h (n = 3) and individual urine voids were collected (separately) over a 24-h period (n = 3 × 3 repetitions) as detailed in the study design. Every serum and urine sample was analyzed and quantified individually via HPLC-DAD before pooling for identification purposes. After quantification, urine samples were pooled for purification (XAD adsorption chromatography), isolation (preparative) HPLC and prep-TLC, and identification (HPLC-MS; HPLC-DAD; TLC; NMR). Identification of the anthocyanins was based on the matching of molecular weight (parent and daughter fragments), retention time (Rt), $\lambda_{\text{max}}$, $E_{\text{max}}$, and reference (Rf) values with those of available anthocyanin standards as well as isolated chokeberry anthocyanins (Table 1). TLC data (post-acid hydrolysis) were used for additional confirmation when considered necessary. Peaks lacking absorption maxima in the 280 and 520 nm range were not considered anthocyanin metabolites, and no attempt was made to identify these unknown compounds. Selected pharmacokinetic variables were determined for the identified compounds from the initial HPLC quantitative results (Table 2 and 3; Figs. 2 and 3).

**Methods of extraction [C18 solid-phase extraction (SPE)].** Unfiltered whole serum (4 mL) or 2–10 mL of unfiltered acidified urine (2 mL, t = 0–9 h; or 10 mL, t = 10–24 h) were utilized for the extraction. Individual blood and urine samples (nonpooled) were extracted in duplicate, and each extract was injected into the HPLC column in duplicate (total of 4 injections/sample) for quantitative HPLC analysis.

**Purification of anthocyanins in pooled human urine samples (postquantitative HPLC analysis).** The procedure was based on general methods as described by Markham (9). The column (50 × 3.0 cm) was filled with prewashed (24 h) Sigma XAD-7 polymeric adsorbent (Sigma; Lot # 77H0157) before isolation of individual anthocyanin/metabolite peaks using prep-HPLC. The procedure was based on general methods as described by Markham (9). The column (50 × 3.0 cm) was filled with prewashed (24 h) Sigma XAD-7 polymeric adsorbent (Sigma; Lot # 77H0157) before isolation of individual anthocyanin/metabolite peaks using prep-HPLC. The procedure was based on general methods as described by Markham (9). The column (50 × 3.0 cm) was filled with prewashed (24 h) Sigma XAD-7 polymeric adsorbent (Sigma; Lot # 77H0157) before isolation of individual anthocyanin/metabolite peaks using prep-HPLC.

**Analytical HPLC analysis.** The chroomanin metabolites were analyzed using a Zorbax SB C18 RP column (5 μm, 4.6 × 250 mm) with a Supelguard LC-18 guard.
column (C_{18}, 5 \mu m, 4.6 \times 20 \text{ mm}; \text{Supelco, Sigma-Aldrich}). The following procedure was modified from previously published methods (7,10).

Prep-HPLC separation of individual anthocyanins from pooled urine samples was performed on a Waters Chromatographic system (Waters) comprised of 3 Model 510 pumps, and a Model 490 programmable multiwavelength detector set at 525nm. The preparative column system (Waters PrepPak) consisted of 2 Nova-Pak HR C_{18} radial compression cartridges (25 \times 100 \text{ mm}; 6 \mu m, 60A; PrepPak Cartridge; Waters) with a Nova-Pak HR C_{18} guard insert (Waters). Injections were carried out on a manual injection port (Rheodyne) equipped with a 500-\mu L injection loop. The column and injector were kept at ambient temperature, with an injection volume of 250 –500 \mu L. The mobile phase consisted of 0.1% TFA in water (solvent A) and 100% MeOH (solvent B). The flow rate was 15 mL/min with an isocratic run of 80% A and 20% B. Peaks on the chromatogram corresponding to anthocyanins, as identified by spectral analysis (peaks detected at 525 nm with \lambda_{\text{max}} 250–300 and 500–550 nm), were collected manually from the prep-HPLC column and concentrated using a rotary evaporator. The remaining extract was removed in a freeze-dryer, and brought to dryness in a freeze-dryer. The remaining extract was sealed under nitrogen gas and stored at −80°C until further analysis.

For the postacid hydrolysis of anthocyanins for verification of aglycones, 0.20-mm silica gel 60 analytical TLC plates (Macherey-Nagel; Batch 901/021) containing a fluorescent indicator (UV 254) were used. Acid hydrolysis of the anthocyanin glycosides was achieved by dissolving a portion of the dry anthocyanin extracts in 200 \mu L of 2 mol/L HCl. The solution was then sealed under nitrogen gas and heated to 100°C for 1.5 h. The samples were then cooled immediately in an ice bath and plated using the above solvent system.

MS identification of individual compounds was conducted postseparation via prep-HPLC and prep-TLC (as outlined above). The analysis was carried out on a Waters Alliance 2695 HPLC coupled serially with a Waters 2996 photodiode array detector and a Waters ZQ 2000 quadrupole analyzer utilizing the electrospray ionization interface (ESI-MS) (Waters). The chromatographic separation was performed on a 250 \times 20 \text{ mm Synergy 4-\mu m Max-RP 80Å column (Phenomenex) with a 4 \times 2 mm Phenomenex Max RP guard cartridge (Phenomenex). Injection volumes were 2 \mu L. The mobile phase consisted of an acidified (0.18% v:v acetic acid) water:acetonitrile mixture (95:5) (solvent A) and 100% acetonitrile (solvent B). The flow rate was 130 \mu L/min; the solvent gradient program used 100% A at 0–2 min and was ramped to 100% B at 60 min. The instrument was operated in electrospray positive ion mode (ES+). Micromass ZQ single quadrupole MS with electrospray interface and MassLynx 4.0 software (Micromass) was used for data acquisition. The MS parameters were loosely based on methods previously published by Felgines et al. (12) and García-Beneytez et al. (13).

NMR spectra were obtained on a Bruker Avance DRX 500 MHz spectrometer (Bruker Biospin), equipped with a cryoprobe, at 300 K. For 1H (500 MHz) NMR, a solvent mixture of methanol-d4 to trifluoroacetic acid-d (CD_{3}OD:CF_{3}COOD) (98:2, v:v, 200 \mu L) was used and 8 values were referenced to CD_{3}OD (CHD_{2}OD at 3.30 ppm). Analysis of the 1H NMR spectra was based on the comparison of the chemical shift and relative intensity of the signals with those of standard compounds.

**Statistical analysis.** The primary analyses were performed on urinary values (urinary values only) using the mixed models procedure (PROC MIXED) in SAS (version 9.1; SAS Institute). The data are presented as means ± SD unless otherwise stated. The distributions of anthocyanins were corrected with natural log transforma-

![FIGURE 2](image-url)  
**FIGURE 2** Time course of total, parent, and metabolized anthocyanins in human urine (A) and serum (B) of subjects after the consumption of 721 mg of cyanidin 3-glycosides. For urinary (A; n = 9 replicates) and serum (B; n = 3) data, values are means ± SD as represented by vertical bars. (A) Different letters for the points indicate that the concentrations of total urinary anthocyanins differed across time, P < 0.05. Identification of each peak as labeled in the legend (as peaks 1–6) is given in Table 1. Urinary and serum pharmacokinetic data of each compound represented by peaks 1–6, is given in Tables 2 and 3, respectively.

![FIGURE 3](image-url)  
**FIGURE 3** Cumulative time course of individual anthocyanins excreted in human urine after the consumption of 721 mg of cyanidin 3-glycosides. Values are means ± SD (n = 9 replicates) as represented by vertical bars. Different letters for the lines indicate that the concentrations differed at the level of the individual anthocyanin, P < 0.05. For peak identities, refer to Table 1. For urinary and serum pharmacokinetic data of each compound represented by peaks 1–6, refer to Tables 2 and 3, respectively.
RESULTS

Serum and urine samples collected before the administration of the chokeberry extract (baseline, \( t = 0 \)) contained no detectable anthocyanins (Fig. 1A). Postconsumption, both cyanidin 3-galactoside and cyanidin 3-arabinoside [peak (P1,3; Fig. 1) were present in the serum and urine. Both glucuronidated (loss \( m/z = 176 \) upon fragmentation) and methylated (\( m + 14 \)) derivatives of cyanidin were also present. In total, 4 derivatives/metabolites were isolated from the urine in sufficient quantities for structural identification. One metabolite (P2; Fig. 1C and D) was identified as a cyanidin glucuronide, as indicated by its molecular ion at \( m/z \) 463 and fragment at \( m/z \) 287, indicating a loss of \( m/z 176 \) upon fragmentation (representing a glucuronide residue; \( m/z = 176 \); Table 1). Additionally, the hydrolysis of the compound resulted in an aglycone with the same HPLC Rt, UV-vis spectral data, and TLC Rf value as the purchased cyanidin standard. There was insufficient evidence to determine the exact position of the glucuronide residue because the NMR spectra were uninterpretable (result of insufficient quantity and poor solubility). The second identified metabolite (P4; Fig. 1C and D) had the chemical characteristics of a methylated derivative of C-3-gal, having a parent ion of \( m/z 463 \), daughter fragment of \( m/z 301 \) (consistent with methylation; \( m/z 287 + 14 = 301 \)), and loss of \( m/z \) 162 upon fragmentation (indicative of a hexose sugar; \( m/z = 162 \); Table 1). The hydrolysis of the compound resulted in an aglycone with similar HPLC Rt, UV-vis spectral data, and TLC Rf value as the purchased peonidin standard. There was insufficient evidence to determine the exact position of the methylation and glucuronidation because the NMR spectra were uninterpretable (result of insufficient quantity and poor solubility). Two other metabolites (P5,6; Fig. 1, C and D) were identified as methylated derivatives of cyanidin glucuronide, having parent ions of \( m/z 477 \) and daughter fragments of \( m/z 301 \), indicating a loss of \( m/z 176 \) upon fragmentation (consistent with glucuronic acid residue; \( m/z = 176 \); Table 1). The derivatives were dissimilar to peonidin, having different HPLC and TLC characteristics. Comparisons made between serum and urinary samples using HPLC-DAD (Fig. 1, C and D; Table 1) indicated that the serum peaks matched both retention time and UV-vis spectrum of the peaks identified as anthocyanins in the urine, and were therefore regarded as the same compounds.

As a result of the sampling regimen, the mixed models procedure was performed on urinary variables only. There was a significant main effect of time \( (P < 0.0001) \) for the total (P1–6) and individual level of anthocyanins (P1,2,3,4,5,6), as well as a significant interaction between time and anthocyanin species \( (P = 0.0016) \). Additionally, the level of total anthocyanins did not differ among the 3 subjects \( (P = 0.30) \). Serum variables were utilized only for pharmacokinetic analyses. The pharmacokinetic analysis of urine and serum variables (Tables 2 and 3) and their graphical representations (Figures 2 and 3) utilized untransformed data as was previously described (14). The results of both urinary (Table 2) and serum (Table 3) analyses indicated that parent compounds and their metabolites had similar pharmacokinetic profiles \( (T_{\text{max}} \text{ and } T_{1/2}) \).

DISCUSSION

In previous investigations, it was questioned whether the concentration of anthocyanins observed in the blood was sufficient to yield biological activity. We hypothesized that unidentified anthocyanin metabolites may contribute to the reported effects of anthocyanins. A previous investigation by our group identified anthocyanin metabolites in human serum and urine after the consumption of cyanidin 3-glycosides in chokeberries (7). Subjects were fed \( \sim 1.2 \text{ g of cyanidin 3-glycosides, leading to the identification of glucuronide and methylated derivatives in the serum and urine. The aim of the present investigation was to identify metabolites and their time course (pharmacokinetics) after a lower, more realistic anthocyanin dose. The present investigation involved a 721-mg oral dose of cyanidin 3-glycosides with the subsequent collection of serum over 7 h and urine over 24 h. This dose is equivalent to \( \sim 120\text{ to } 230 \text{ g of whole berries (fresh weight) (15). Additionally, a 721-mg dose is roughly the median dose of 12 reviewed anthocyanin human consumption trials in the literature (788 \pm 833 \text{ mg}) (5,10,12,16–24). The chokeberry extract as utilized in the present investigation was chosen for its simplistic anthocyanin profile, consisting of only cyanidin 3-glycosides. The use of a fruit extract containing only one anthocyanidin species (cyanidin) was crucial for establishing the origin of methylated cyanidin derivatives. Further investigations are required to identify the biological activity of these metabolites.

In the present investigation, no anthocyanins were identified in the serum or urine of fasting subjects suggesting that the washout phase and prestudy dietary exclusion of anthocyanins was sufficient. Glucuronidation was the major metabolic pathway observed for anthocyanin metabolism in the present investigation, representing 59.8 and 57.8% of the total anthocyanins detected in the blood and urine, respectively. Methylation was the second most commonly observed metabolic transformation for anthocyanins, representing 43.8 and 51.4% of the total anthocyanins detected in the serum and urine, respectively. Even though only a few researchers have reported glucuronidated and methylated anthocyanins in the urine and blood of humans and animals (7,8,12), methylated and glucuronidated derivatives of the flavonoid quercetin are well documented (18,25,26). Although some recent investigations described the detection of anthocyanin metabolites in urine, this is the first study to give detailed pharmacokinetic parameters for anthocyanin metabolites.

In the present investigation, no attempt was made to identify HPLC peaks lacking characteristic anthocyanin profiles (maxima in the 280 and 520 nm range). There are likely other anthocyanin metabolites (breakdown products) present in the serum and urine with absorbance outside the 240- to 525-nm range and that go beyond the scope of this investigation.
range; however, identifying these compounds is beyond the scope of this investigation. Further studies involving labeled anthocyanins are necessary for the complete characterization of metabolites.

The appearance of anthocyanins (parent compounds) in the blood (t1/2C = 1.51 ± 0.2 4 h, tmax = 3.0 h) (Table 3; Fig. 2B) as well as their subsequent elimination in the urine was rapid (t1/2G of 3.88 ± 0.62 h, tmaxG at 3.16 ± 1.26 h; Table 2; Fig. 2A). Additionally, the proportion of parent anthocyanins excreted in the urine was ~0.048% of the total ingested dose, which is consistent with the literature (4,5,19–23,27,28). The low plasma and urinary concentrations of parent anthocyanins, as well as their rapid absorption and elimination, suggest either a low absorption or a rapid and efficient metabolism.

The maximum serum concentration of total parent and metabolized anthocyanins was observed at 2.8 h, with the maximum rate of urinary excretion (tmaxR) at 3.72 ± 0.83 h and an elimination half-life of 4.12 ± 0.4 h (Tables 2 and 3). Similar elimination kinetics are reported in the literature (6,21). The data presented in this manuscript suggest that the elimination of parent anthocyanins from the body follows a first-order model. This elimination kinetic model was also reported by Cao et al. (5), after the consumption of an anthocyanin mixture derived from elderberries.

Glucuronidated and methylated anthocyanin metabolites were observed in the present investigation at levels twice that of the parent (intact) compounds. Only 32% of the total anthocyanins detected in the serum were the parent compounds (P1,3) with 68% identified as conjugated metabolites (P2,4,5,6; Table 1). Similarly, only 32.5% of the anthocyanins excreted in the urine were the parent compounds (P1,3) with 67.5% occurring as conjugated metabolites (P2,4,5,6; Fig. 1D; Table 2). The elimination of the metabolites in this investigation mirrored that of the parent compounds having similar tmax, t1/2, and t1/2 (Table 2 and Fig. 2A) suggesting that the metabolites and parent compounds follow the same metabolic pathway in the body.

The total urinary excretion of metabolites and parent compounds over 24 h was 1071.54 ± 375.46 μg, accounting for 0.15% of the initial dose. Several studies reported that the excretion of anthocyanins accounted for as little as 0.05% of the initial dose (5,6,10,21,27); however, many of these studies were unable to identify anthocyanin metabolites in biological fluids. This shortfall is likely the consequence of insufficient extraction procedures as well as the limited sensitivity of using UV-vis HPLC alone to distinguish between anthocyanin glycosides and glucuronides. Additionally, studies unable to identify metabolites of anthocyanins in biological fluids undoubtedly underestimated their absorption. Furthermore, the metabolism of anthocyanins may also result in the formation of phenolic acids, phenolic acid residues, H, or CO2 (8,18,26), which augments their underestimation in biological fluids. In this investigation, both parent glycoside and metabolized derivatives were identified in the urine and serum, and the excretion of metabolites was 2 times that of the parent glycosides. Recent studies focusing pharmacological or megadoses of anthocyanins [1.2–3.5 g (7,24), respectively] reported that the parent glycosides were the major circulating compounds. This is likely the result of the saturation of metabolic pathways and would not be expected to occur after “typical” or “normal” dietary consumption of fruits, vegetables, juices, or wines. It is therefore clear that further research is required to establish a more comprehensive understanding of anthocyanin absorption and metabolism in humans.

To conclude, the above results indicate that cyanidin 3-glycosides are absorbed and transported in human serum and urine primarily as glucuronide and methyl-glucuronide derivatives after a moderate-to-high oral dose. Future research focused on identifying the physiological effects of these compounds should therefore be conducted using anthocyanins as they exist in the circulation in both forms (i.e., parent and metabolite) and concentrations.

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LITERATURE CITED


