Human metabolism and elimination of the anthocyanin, cyanidin-3-glucoside: a $^{13}$C-tracer study$^{1-3}$

Charles Czank, Aedín Cassidy, Qingzhi Zhang, Douglas J Morrison, Tom Preston, Paul A Kroon, Nigel P Botting, and Colin D Kay

ABSTRACT
Background: Evidence suggests that the consumption of anthocyanin-rich foods beneficially affects cardiovascular health; however, the absorption, distribution, metabolism, and elimination (ADME) of anthocyanin-rich foods are relatively unknown.

Objective: We investigated the ADME of a $^{13}$C$_5$-labeled anthocyanin in humans.

Design: Eight male participants consumed 500 mg isotopically labeled cyanidin-3-glucoside (6,8,10,3’5’$^{13}$C$_5$-C$_3$G). Biological samples were collected over 48 h, and $^{13}$C and $^{13}$C-labeled metabolite concentrations were measured by using isotope-ratio mass spectrometry and liquid chromatography–tandem mass spectrometry.

Results: The mean ± SE percentage of $^{13}$C recovered in urine, breath, and feces was 43.9 ± 25.9% (range: 15.1–99.3% across participants). The relative bioavailability was 12.38 ± 1.38% (5.37 ± 0.67% excreted in urine and 6.91 ± 1.59% in breath). Maximum rates of $^{13}$C elimination were achieved 30 min after ingestion (32.53 ± 14.24 µg$^{13}$C/h), whereas $^{13}$C-labeled metabolites peaked (maximum serum concentration: 5.97 ± 2.14 µmol/L) at 10.25 ± 4.14 h. The half-life for $^{13}$C-labeled metabolites ranged between 12.44 ± 4.22 and 51.6 ± 22.55 h. $^{13}$C elimination was greatest between 0 and 1 h for urine (90.3 ± 15.28 µg/L), at 6 h for breath (132.87 ± 32.23 µg/L), and between 6 and 24 h for feces (557.28 ± 247.88 µg/L), whereas the highest concentrations of $^{13}$C-labeled metabolites were identified in urine (10.77 ± 4.52 µmol/L) and fecal samples (43.16 ± 18.00 µmol/L) collected between 6 and 24 h. Metabolites were identified as degradation products, phenolic, hippuric, phenylactic, and phenylpropenoic acids.

Conclusion: Anthocyanins are more bioavailable than previously perceived, and their metabolites are present in the circulation for ≥48 h after ingestion. This trial was registered at clinicaltrials.gov as NCT01106729.


INTRODUCTION
Interest in dietary anthocyanins continues to increase as evidence of their vascular bioactivity emerges from epidemiologic, clinical (randomized controlled trials), and in vitro studies (1–5). However, over the past decade, there has been ongoing speculation regarding the absorption, distribution, metabolism, and elimination (ADME)$^4$ and mechanisms of action of anthocyanins (6–8). Currently available ADME data from human-intervention studies have suggested maximal serum concentrations of anthocyanins and anthocyanidin phase II conjugates are, on average, reached by 1.5 h at amounts of ~100 nmol/L after doses of ≥500 mg anthocyanins (6, 9–13). These findings have led to the supposition that anthocyanins are considerably less bioavailable than other flavonoid subclasses. Thus, epidemiologic observations that suggested that anthocyanin consumption is associated with reduced cardiovascular disease risk (1, 14) appear ambiguous because the average consumption of anthocyanins in the diet has been reported to be relatively low (12 mg/d in the United States) (15). Either anthocyanins are extremely potent and, therefore, active at low serum concentrations (ie, low nanomolar concentrations) or their dietary occurrence or bioavailability has been underestimated.

Evidence based on in vitro gastric (16) and microbial fermentation studies (17, 18) suggested that, after ingestion, anthocyanins are likely to be broken down (either spontaneously or enzymatically) into phenolic degradation products, which are then further metabolized. Similar findings were observed in a berry-extract human-consumption study, although because the intervention contained a complex profile of dietary phenolics, the metabolites could not be traced back conclusively to the anthocyanins over other phenolics within the extract (19). Isotope-tracer studies are required to conclusively establish the extent to which anthocyanins are metabolized to phenolic acid derivatives and the relative contribution of these metabolites to the absorption, distribution, and elimination of ingested anthocyanins.

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2 Supported by funding from the UK Biotechnology and Biological Sciences Research Council Diet and Health Research Industry Club (BB/H004963/1; principal investigator: CDK).
3 Address correspondence to CD Kay, Department of Nutrition, Norwich Medical School, University of East Anglia, Norwich Research Park, Norwich NR4 7TJ, United Kingdom. E-mail: colin.kay@uea.ac.uk.
4 Abbreviations used: ADME, absorption, distribution, metabolism, and elimination; C$_3$G, cyanidin-3-glucoside; HPLC-MS/MS, HPLC–tandem mass spectrometry; IRMS, isotope-ratio mass spectrometry; PCA, protec-echuic acid; SPE, solid-phase extraction; $^{13}$C$_5$-C$_3$G, 6,8,10,3’5’$^{13}$C$_5$-cyanidin-3-glucoside.

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The aim of the current study was to trace metabolites derived from both phenolic rings (Figure 1, A and B rings) of the parent-anthocyanin structure, to establish a complete profile of their absorption and elimination kinetics. To our knowledge, this is the first study to feed a chemically synthesized multistable-isotope labeled anthocyanin [cyanidin-3-glucoside (C3G)] to establish bioavailability and identify metabolites in humans.

SUBJECTS AND METHODS

Isotopically labeled anthocyanin tracer

Isotopically labeled C3G that contained three $^{13}$C atoms on the A ring and 2 $^{13}$C atoms on the B ring [6,8,10,3',5'-$^{13}$C$_5$-C3G ($^{13}$C$_5$-C3G); Figure 1] was synthesized as previously described (20) [with an enrichment of 99 atom percent at each position and established as 99.8% pure $^{13}$C$_5$-C3G by using HPLC–tandem mass spectrometry (HPLC-MS/MS)] and was accurately weighed and encapsulated (250-mg gelatin capsules) at the Ipswich Hospital Pharmaceutical Manufacturing unit.

Subjects

A classic single-bolus oral pharmacokinetic study design was carried out in 8 healthy male participants recruited from the University of East Anglia and local community of Norwich, United Kingdom. The healthy participants were chosen to maintain a homogeneous study population and minimize metabolic variation. Participants were nonsmokers, had BMI (kg/m$^2$) in the range from 18.5 to 30.5, and were aged 18–45 y, moderate drinkers, and not taking dietary supplements. The protocol was explained to participants, and they provided informed consent. The study was conducted at the Clinical Research and Trials Unit at the University of East Anglia according to the principles expressed in the Declaration of Helsinki and was approved by the local Research Ethics Committee (ref 10/H0306/42) and registered at clinicaltrials.gov as NCT01106729.

TABLE 1

<table>
<thead>
<tr>
<th>Sample</th>
<th>Maximum rate of elimination $\mu$/h</th>
<th>Time of maximum rate of elimination h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>32.53 ± 14.24</td>
<td>6</td>
</tr>
<tr>
<td>Urine</td>
<td>90.30 ± 15.28</td>
<td>0–1</td>
</tr>
<tr>
<td>Breath</td>
<td>132.87 ± 32.23</td>
<td>6</td>
</tr>
<tr>
<td>Feces</td>
<td>557.28 ± 247.88</td>
<td>6–24</td>
</tr>
</tbody>
</table>

$^1$Concentration of $^{13}$C was quantified by using liquid chromatography–isotope-ratio mass spectrometry (mean ± SE; n = 8). C3G, cyanidin-3-glucoside.
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Study design

After fasting overnight, participants provided blood (30 min and 1, 2, 4, 6, 24, and 48 h), urine (individual voids between 0 and 6 h; total voids between 6 and 24 and 24 and 48 h), breath (30 min and 1, 2, 4, 6, 24, and 48 h), and fecal (all voids between 0 and 6, 6 and 24, and 24 and 48 h) samples after the consumption of a 500-mg (as two 250-mg capsules) single oral bolus dose of $^{13}$C$_5$-C3G (see Figure 1 under “Supplemental data” in the online issue). A standardized breakfast was provided 30 min postbolus and a standardized lunch between the 2- and 4-h sample-collection time points. Standardized breakfasts were also provided on each of the follow-up days (for 24 and 48 h).

Dietary intake

Participants were asked to avoid anthocyanin-rich foods and foods that contained a higher natural abundance of $^{13}$C (21) over the 7-d period (washout period) before the administration of the capsule and during the intervention (a list of foods to avoid was provided). Completed food diaries during the washout period (3 d), study day, and 4-h follow-up were monitored for compliance. Macronutrient and micronutrient intakes were calculated with the WISP software package (version 3; Tinuviel Software), and polyphenol intake was determined with the Phenol Explorer V2.0 database (http://www.phenol-explorer.eu) (22).

Biological sample collection

Blood was collected via a cannula for the first 6 h and via venepuncture at 24 and 48 h into untreated 10-mL evacuated tubes. For isotope-ratio mass-spectrometry (IRMS) analysis, whole blood samples were transferred into cryovials (Sigma Aldrich) and frozen immediately in liquid nitrogen, whereas for the HPLC-MS/MS analysis, blood samples were allowed to clot for 1 h followed by centrifugation at 3000 $g$ for 10 min. The serum was acidified to pH 2.4 with formic acid by using a pH meter (Omega) to prevent degradation of anthocyanins (16) and transferred into cryovials for storage. Breath samples were collected simultaneously (with blood samples) by using the Alveosampler system (QuinTrony) and stored in 10-mL evacuated tubes at room temperature for IRMS. Individual and complete urine voids were collected into Urisafe collection containers (VWR International) with 100 mg ascorbate added per 500 mL urine and were acidified manually to pH 2.4 with formic acid. Individual fecal voids were collected into custom-made stool-collection kits, weighed, and transferred to ultracold storage containers (Nalgene). All blood, serum, urine, and feces samples were stored at $-80^\circ$C until analysis.

Analytic methods

Chemicals and materials

C3G was obtained from Extrasynthese, whereas phase II conjugates of phenolic acids (protocatechuic acid (PCA))-3-glucuronide, PCA-4-glucuronide, vanillic acid-4-glucuronide, benzoic acid-4-glucuronide, isoferulic acid-3-glucuronide, isoferulic acid-3-sulfate, PCA-4-sulfate, vanillic acid-4-sulfate, PCA-3-sulfate, and benzoic acid-4-sulfate were synthesized as previously described (23). HPLC-MS/MS–grade methanol and acetonitrile were purchased from Fisher Scientific. Strata-X solid-phase extraction (SPE) columns (6 mL, 500 mg), a Kinetex polyfluorophenol reverse-phase–HPLC column (2.6 $\mu$m; 100 × 4.6 mm), and SecurityGuard cartridges (polyfluorophenol; 4 × 2.0 mm) were purchased from Phenomenex. Bond Elute C18 (20 mL; 5 g) SPE columns were purchased from Agilent. Discovery DSC-18 SPE columns (6 mL; 1 g) and Acrodisc 13-mm, 0.45-$\mu$m polytetrafluoroethylene syringe filters and all other chemicals were purchased from Sigma-Aldrich.

IRMS

A total of 0.5 mL whole blood (diluted 1:1 with 0.5 mL 4 M NaCl) or 1 mL urine samples was transferred to ultrafiltration cartridges (Amicon Ultra 2; Millipore) (30,000 molecular weight cutoff) and centrifuged at 6000 $g$ for 20 min. Ultrafiltered blood and urine were subsequently analyzed by using liquid chromatography-IRMS (IsoPrime) via a direct-injection mode using previously described methods (24, 25). Breath samples were analyzed by using continuous-flow IRMS (AP 2003; IsoPrime). Aliquots (4 mg) of each fecal sample were weighed into tin capsules, which yielded ~400 $\mu$g carbon for elemental analysis IRMS (Sercon). In each IRMS analysis, the CO$_2$ generated was calibrated against Vienna Pee Dee Belemnite by

### TABLE 2

Pharmacokinetic characteristics of metabolites in serum over 48 h in 8 healthy adult males after consumption of 500 mg $^{13}$C$_5$-C3G

<table>
<thead>
<tr>
<th>Metabolites</th>
<th>$C_{max}$ (µmol/L)</th>
<th>$t_{max}$ (h)</th>
<th>AUC$_{0-48}$ (µmol · h/L)</th>
<th>$t_{1/2}$ (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C3G</td>
<td>0.14 ± 0.05</td>
<td>1.81 ± 0.16</td>
<td>0.31 ± 0.13</td>
<td>ND</td>
</tr>
<tr>
<td>Degradants</td>
<td>0.72 ± 0.23</td>
<td>6.06 ± 0.75</td>
<td>9.09 ± 3.01</td>
<td>12.44 ± 4.22</td>
</tr>
<tr>
<td>Phase II conjugates</td>
<td>2.35 ± 0.15</td>
<td>13.44 ± 2.46</td>
<td>43.92 ± 5.05</td>
<td>29.32 ± 8.95</td>
</tr>
<tr>
<td>Ferulic acid</td>
<td>0.94 ± 0.37</td>
<td>11.29 ± 4.23</td>
<td>21.22 ± 10.99</td>
<td>51.62 ± 22.55</td>
</tr>
<tr>
<td>Hippuric acid</td>
<td>1.96 ± 1.39</td>
<td>15.69 ± 4.07</td>
<td>46.42 ± 30.31</td>
<td>21.69 ± 4.56</td>
</tr>
</tbody>
</table>

All values are means ± SEs. Serum concentrations of metabolites were quantified by using HPLC–tandem mass spectrometry ($n$ = 8). AUC$_{0-48}$, AUC (0–48 h); C3G, cyanidin-3-glucoside; $C_{max}$, maximum serum concentration; degrada-

tants, sum of protocatechuic acid and phloroglucinaldehyde; ND, analyte identified in too few time points to model pharmacokinetics (≥3); PCA, protocatechuic acid; phase II conjugates of PCA, sum of phase II conjugates of PCA (including PCA-3-glucuronide, PCA-4-glucuronide, PCA-3-sulfate, PCA-4-sulfate, vanillic acid, isoferulic acid, vanillic acid-glucuronide, isoferulic acid-glucuronide, vanillic acid-sulfate, isoferulic acid-sulfate, methyl 3,4-dihydroxybenzoate, and 2-hydroxy-4-methoxybenzoic acid); $t_{max}$, time to reach maximum serum concentration; $t_{1/2}$, elimination half-life.
using calibrated laboratory standards as previously described (26).

**HPLC-MS/MS**

Serum, urine, and fecal samples were extracted by SPE with extraction efficiencies of 84.5 ± 6.15% for serum, 84.0 ± 9.5% for urine, and 78.8 ± 1.3% for feces. Fecal samples were homogenized, freeze dried, and alcohol extracted (50% methanol in phosphate-buffered saline; pH 2.4) before SPE. Sample extracts (5 μL) were injected onto a Kinetix polyfluorophenol column (Phenomenex) and separated with an Agilent 1200 HPLC by using 0.1% formic acid (vol:vol) in water (solvent A) and 0.1% formic acid (vol:vol) in acetonitrile (solvent B) at a flow rate of 1.5 mL/min and gradient that consisted of 1% B at 0 min and was ramped to 30% B over 32.5 min. Metabolite identification was performed on a QTrap 4000 linear ion-trap mass spectrometer (ABSciex) by using multireaction monitoring optimized for the detection of pure standards with a m/z of the parent and daughter fragments adjusted to +2 or +3 m/z units, which allowed for the identification of 13C-labeled metabolites. Metabolites were confirmed on the basis of the retention time and ≥3 parent-daughter ion-fragmentation transitions.

**Data processing and statistical analysis**

IRMS data were collected and expressed as δ13C (relative to Vienna Pee Dee Belemnite) and, where appropriate, converted to parts per million13C. Absolute tracer excretion in the breath was calculated by estimating the CO2 production rate (27) from the body surface area (28) by using an estimated correction of physical activity levels (of 1.3) appropriate for sedentary behavior (26). Absolute tracer excretion was determined in urine and feces as the product of the total amount of carbon excreted calculated from the area ratio of the major ion beam (m/z 44) of the sample compared with the laboratory standard of known carbon content and the 13C enrichment of the sample. The amount of the tracer excreted in each pool was calculated by summation of the tracer excreted in the study period and expressed as percentage of the administered dose of 13C. A similar calculation was undertaken for blood to determine the instantaneous percentage of the dose transiting the serum pool.

HPLC-MS/MS data were collected with Analyst 1.5.1 software (ABSciex, California), and the peak-area data for the most abundant ion-pair transition for each compound was exported to the Excel 2007 program (Microsoft). The peak area of 13C metabolites was quantified against the slope of the fitted regression line of pure standards (which had been subtracted for the baseline ionization signal to account for differences in background ionization between 13C and 12C compounds). Data from all individual urine samples were pooled to the nearest 1-h period during the first 6 h. Fecal concentrations were expressed relative to 1 g wet weight. The proportion of 13C-labeled metabolites relative to total 13C was established by calculating the fractional molar contribution of 13C atoms in the metabolites and the total 13C concentration (μmol 13C/L) by using the volume for the urine and fecal water content. Pharmacokinetic modeling of metabolite concentrations was performed with the program PKSolver (29) for Excel 2007 (Microsoft) by using noncompartmental analysis. All values are presented as means ± SEs (n = 8) unless otherwise stated.

**RESULTS**

**Study participants**

Eight male participants, with a mean age of 27.8 ± 8.1 y and BMI of 23.2 ± 1.5, completed the study. Anthocyanin consumption (mean ± SD) during the washout period was 3.3 ± 3.5 mg/d (see Table 1 under “Supplemental data” in the online issue for mean baseline characteristics) and intake was <0.5 mg on
the study day and follow-up days. The mean basal breath CO₂ natural abundance of δ¹³C was $-22.85 \pm 0.68$ per mil, which indicated a low natural abundance $^{13}$C had been achieved across participants. Therefore, all participants complied with the study protocol, and no adverse effects were associated with the single dose of $^{13}$C₅-C₃G.

Elimination of $^{13}$C

The maximal rate of $^{13}$C elimination (32.53 ± 14.24 μg/h) was observed at 30 min after ingestion (Table 1), with blood $^{13}$C content that ranged between 8.91 ± 3.99 and 23.47 ± 12.05 μg over the first 24 h (Figure 2A), and a peak $^{13}$C content was reached at 48 h (33.40 ± 13.90 μg/mL). The sum of all $^{13}$C-labeled metabolites reached a total peak concentration of 5.97 ± 2.14 μmol/L in serum at 10.25 ± 4.14 h on the basis of pharmacokinetic modeling (Table 2; Figure 3A). The half-life of $^{13}$C-labeled metabolites ranged from 12.44 ± 4.22 h for degradation products (PCA and phloroglucinaldehyde) to $\approx 51.62 \pm 22.55$ h for ferulic acid (Table 2).

Maximum elimination rates for $^{13}$C (Table 1) in urine (90.30 ± 15.28 μg/h) occurred between 0 and 1 h after ingestion (Figure 2B), in breath occurred at 6 h (132.87 ± 32.23 μg/h) and in feces occurred between 6 and 24 h (557.28 ± 247.88 μg/h). The highest concentration of $^{13}$C was observed in urine (1.55 ± 0.43 μg/mL) during the 6–24 h (Figure 2B) time period postingestion, in breath (2341.62 ± 1010.22 μg/mL) at 24 h, and in feces (105.69 ± 20.68 μg/g) at 24–48 h (Figure 4A). In relation to metabolites (sum of all degradants and their metabolites), greatest concentrations were shown in 6–24-h urine samples (10.77 ± 4.52 μmol/L; Figure 3B) and 6–24-h fecal samples (43.16 ± 18.00 μmol/L; Figure 4B).

Urine was the primary route of elimination over the first 6 h (Figure 5A) and represented 66.33 ± 13.22% of the total $^{13}$C recovery followed by that in breath (28.94 ± 6.89%). Feces represented the major route of elimination over the 6–24- and 24–48-h time periods (Figure 5A). A similar pattern was observed for metabolites, with highest concentrations of $^{13}$C-labeled metabolites in urine in the first 6 h (5.57 ± 2.56 μmol/L; Figure 5B), whereas feces was the predominant compartment thereafter (Figure 5B).

A total of 43.9 ± 25.9% of the bolus dose of $^{13}$C was recovered in urine, breath, and feces, with a wide interindividual variability (15.1–99.3%) exhibited by participants. Maximum observed quantities of $^{13}$C in blood accounted for 0.18 ± 0.11% of the ingested dose, whereas mean recoveries of $^{13}$C were 5.37 ± 0.67%, 6.91 ± 1.59%, and 32.13 ± 6.13% for urine, breath, and feces, respectively (Figure 5A). Together, these data suggest a relative bioavailability of $\approx 12.38 \pm 1.38$% calculated from the combined elimination via urine and breath.

Characteristics of $^{13}$C-labeled metabolites

A total of 25 $^{13}$C-labeled compounds that consisted of $^{13}$C₅-C₃G and 24 labeled metabolites were identified. Metabolites included phase II conjugates of C₃G and cyanidin (cyanidin-glucuronide, methyl cyanidin-glucuronide, and methyl C₃G-glucuronide), degradants (PCA, phloroglucinaldehyde and phloroglucinaldehyde), phase II conjugates of PCA (including PCA-3-glucuronide, PCA-4-glucuronide, PCA-3-sulfate, PCA-4-sulfate, vanillic acid, isovanillic acid-sulfate, vanillic acid-sulfate, vanillic acid-sulfate, vanillic acid-sulfate, methyl 3,4-dihydroxybenzoate, 2-hydroxy-4-methoxybenzoic acid, and methyl vanillate), phenylacetic acids (3,4-dihydroxyphenyl acetic acid and 4-hydroxyphenylacetic acid), phenylpropenoic acids (caffeic acid and ferulic acid), and hippuric acid.

In serum, C₃G reached a peak concentration of 0.14 ± 0.05 μmol/L at 1.81 ± 0.16 h postconsumption, whereas its degradation products peaked at 0.72 ± 0.23 μmol/L (Table 2). The major metabolites of C₃G in serum were phase II conjugates of PCA, ferulic acid, and hippuric acid, which peaked between 6- and 24-h collection time points at concentrations between 0.94 ± 0.37 and 2.35 ± 0.15 μmol/L. The serum concentration AUC ranged from 0.31 ± 0.13 μmol · h/L for C₃G to 46.42 ± 30.31 μmol · h/L (Table 2) for hippuric acid.

![Figure 4. Mean (±SE) concentrations of $^{13}$C tracer (A) and $^{13}$C-labeled metabolites (B) in feces after the consumption of a 500-mg bolus dose of $^{13}$C₅-C₃G by 8 healthy male participants. Eight participant samples were collected for the 6–24- and 24–48-h time points, whereas 2 participants provided samples within the first 6 h of sampling. C₃G, cyanidin-3-glucoside; degradants, sum of PCA and phloroglucinaldehyde; PCA, protocatechuic acid; phase II conjugates, sum of phase II conjugates of PCA (including PCA-3-glucuronide, PCA-4-glucuronide, PCA-3-sulfate, PCA-4-sulfate, vanillic acid, isovanillic acid, vanillic acid-glucuronide, vanillic acid-glucuronide, vanillic acid-sulfate, isovanillic acid-sulfate, methyl 3,4-dihydroxybenzoate, 2-hydroxy-4-methoxybenzoic acid, and methyl vanillate); phenylacetic/phenylpropenoic, sum of ferulic acid, caffeic acid, 3,4-dihydroxyphenylacetic acid, and 4-hydroxyphenylacetic acid.](image-url)
C3G and its degradants reached a peak in urine at 2 h post-consumption (Table 3) at 1.78 ± 0.18 and 0.48 ± 0.10 μmol/L, respectively (Figure 3B). Also, phase II conjugates of C3G and cyanidin were identified in the urine at a maximum concentration of 1.17 ± 0.52 μmol/L at 1 h (collectively) and consisted of cyanidin-glucuronide (m/z 468/454/292), methylated cyanidin-glucuronide (m/z 482/468/292), and methylated C3G-glucuronide (m/z 644/482/468/292) on the basis of tandem mass spectrometry fragmentation patterns and quantified relative to C3G (because analytic standards were not available). Phase II conjugates of PCA peaked in the urine at 24 h (5.54 ± 0.49 μmol/L), phenylacetic and phenylpropenoic acids at 48 h (0.72 ± 0.23 μmol/L) (Table 2) at 6.06 ± 0.75 h postbolus, which, to our knowledge, has not been previously reported.

C3G was established to have a minimum relative bioavailability of 12.38 ± 1.38% on the basis of the total elimination of the absorbed 13C dose via urine and breath. In samples collected, a maximum of 0.18 ± 0.11% of the 13C dose could be recovered from blood, 5.37 ± 0.67% of the 13C dose could be recovered from urine, 6.91 ± 1.59% of the 13C dose could be recovered from breath, and 32.13 ± 6.13% of the 13C dose could be recovered from feces. Previously, anthocyanins have been reported to have one of the lowest bioavailabilities of all of the dietary flavonoid subclasses (~0.4%) (7, 30). However, our data suggested that anthocyanins are as bioavailable as other flavonoid subclasses, such as flavan-3-ols and flavones, which have relative bioavailabilities between 2.5% and 18.5% (7, 30).

There was also considerable interindividual variability in the recovery of the 13C tracer in the current study that ranged from 15.1% to 99.3%, probably as a result of a high variation in gastric and intestinal transit times, composition, and catabolic activity of colonic flora and the ability to take up and excrete catabolites and metabolites (6).

A total of 43.9 ± 25.9% of the dose of 13C was recovered in urine, breath, and feces; however the fate of the remaining ingested 13C remains unknown. The recovery of flavonoids similar in structure to C3G (such as 14C-labeled quercetin) has been reported to be between 58.5% and 96% (31, 32). Furthermore, of the total 13C recovered, only 36.72 ± 14.39% and 2.01 ± 0.01% of the tracer was recovered as 13C-labeled phenolic metabolites in the urine and feces respectively, which suggested that there are many metabolites still undetected by current HPLC-MS/MS techniques. On the basis of the instability of anthocyanins and appearance of a relatively large number of diverse breakdown products and metabolites, other metabolites might exist at lower concentrations and, therefore, escape detection.

Serum concentrations of C3G that we observed were similar to those previously reported (6, 7); however, degradation products of C3G (PCA and phloroglucinaldehyde) reached collective peak serum concentrations of 0.72 ± 0.23 μmol/L (Table 2) at 6.06 ± 0.75 h postbolus, which, to our knowledge, has not been previously studied.
HUMAN METABOLISM OF $^{13}$C-LABELED ANTHOCYANINS

TABLE 3

<table>
<thead>
<tr>
<th>Metabolites</th>
<th>Maximum concentration</th>
<th>Time of maximum concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\mu$mol/L</td>
<td>h</td>
</tr>
<tr>
<td>C3G</td>
<td>1.78 ± 0.18</td>
<td>2</td>
</tr>
<tr>
<td>Phase II conjugates of C3G and cyanidin</td>
<td>1.17 ± 0.52</td>
<td>1</td>
</tr>
<tr>
<td>Degradants</td>
<td>0.48 ± 0.10</td>
<td>2</td>
</tr>
<tr>
<td>Phase II conjugates of PCA</td>
<td>5.54 ± 0.49</td>
<td>24</td>
</tr>
<tr>
<td>Phenylpropenoic and phenylacetic acids</td>
<td>1.51 ± 0.75</td>
<td>48</td>
</tr>
<tr>
<td>Hippuric acid</td>
<td>5.21 ± 3.43</td>
<td>24</td>
</tr>
</tbody>
</table>

$^1$ Urinary concentrations of metabolites quantified by using HPLC–tandem mass spectrometry ($n=8$). C3G, cyanidin-3-glucoside; degradants, sum of protocatechuic acid and phloroglucinaldehyde; PCA, protocatechuic acid; phase II conjugates of C3G and cyanidin, sum of cyanidin-glucuronide, methylated cyanidin-glucuronide, and methylated C3G-glucuronide; phase II conjugates of PCA, sum of phase II conjugates of PCA (including PCA-3-glucuronide, PCA-4-glucuronide, PCA-3-sulfate, PCA-4-sulfate, vanillic acid, isovanillic acid, vanillic acid-glucuronide, isovanillic acid-glucuronide, vanillic acid-sulfate, isovanillic acid-sulfate, methyl 3,4-dihydroxybenzoate, and 2-hydroxy-4-methoxybenzoic acid); phenylpropanoic and phenylacetic acids, sum of ferulic acid, 3,4-dihydroxyphenylacetic acid, and 4-hydroxyphenylacetic acid.

$^2$ All values are means ± SEs.

reported. On the basis of the rapid appearance of C3G degradation products and their phase II conjugates within the serum, some degradation likely occurred in the small intestine (either preabsorption or postabsorption), which suggested that anthocyanin C ring cleavage may not require the action of colonic microflora as reported for other flavonoids such as quercetin and catechin (18).

A total of 24 metabolites were identified at concentrations that ranged from 0.1 to 42.2 $\mu$mol/L, whereas a total of 51 putative metabolites of C3G were initially explored. Seven hydroxybenzoic acids, 10 methoxybenzoic acids, 3 phenylpropenoic acids, 2 isomers of methylvippuric acid, homovanillic acid, hydroxybenzylalcohol, benzoic acid-4-sulfate, and hydroxybenzaldehydes were either not present or only present in trace amounts. Although many of the metabolites identified in the current study have been identified in animal studies (18, 33, 34), to our knowledge, this is the first study to show this diversity of metabolites in humans by using a stable-isotope–labeled single-compound approach.

Estimates of the half-lives of elimination were between 12.44 ± 4.22 h and 51.62 ± 22.55 h for $^{13}$C-labeled metabolites (Table 2), which suggested a relatively slow urinary clearance of some metabolites. Long elimination half-lives may be the result of a combination of hepatic recycling, enterohepatic circulation, and prolonged colonic production and absorption. Indeed, the biliary excretion of C3G (35) and other similarly structured flavonoids (quercetin and catechin) (31, 32, 36, 37) has previously been reported in animal studies.

The urinary recovery of $^{13}$C reached a maximal concentration in the pooled 6–24-h collections, which represented a peak $^{13}$C-labeled metabolite concentration of 10.77 ± 4.52 $\mu$mol/L (Figure 3B). These concentrations were considerably higher than those previously published (0.02–0.592 $\mu$mol/L) (6, 7). In the breath, peak concentrations of the $^{13}$C tracer were detected at 24 h as $^{13}$CO₂ (Figure 5). To our knowledge, this is the first human study to report the breath as a route for the clearance of anthocyanin-derived carbon from the body. The sustained excretion of the label as $^{13}$CO₂ over 48 h suggested the prolonged absorption of low molecular weight fecal metabolites from the colon, perhaps derived from labeled-aliphatic compounds or carboxylic acids or via direct fermentation to $^{13}$CO₂. Studies in

TABLE 4

<table>
<thead>
<tr>
<th>Metabolites</th>
<th>Serum$^1$</th>
<th>Urine$^1$</th>
<th>Feces$^1$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ng</td>
<td>ng</td>
<td>ng</td>
</tr>
<tr>
<td>C3G</td>
<td>9.99 ± 3.90</td>
<td>36.47 ± 20.27</td>
<td>0.44$^4$</td>
</tr>
<tr>
<td>Degradants</td>
<td>149.88 ± 93.20</td>
<td>60.00 ± 23.24</td>
<td>7.40 ± 1.81</td>
</tr>
<tr>
<td>Phase II conjugates of PCA</td>
<td>321.73 ± 130.89</td>
<td>1124.89 ± 830.70</td>
<td>30.82 ± 11.39</td>
</tr>
<tr>
<td>Phenylpropanoic and phenylacetic acids</td>
<td>153.32 ± 60.95$^5$</td>
<td>248.59 ± 85.28</td>
<td>56.00 ± 34.64</td>
</tr>
<tr>
<td>Hippuric acid</td>
<td>384.04 ± 246.32</td>
<td>309.82 ± 261.92</td>
<td>4.26 ± 2.29</td>
</tr>
</tbody>
</table>

$^1$ All values are means ± SEs. Metabolite recovery determined by using HPLC–tandem mass spectrometry ($n=8$). C3G, cyanidin-3-glucoside; degradants, sum of protocatechuic acid and phloroglucinaldehyde; PCA, protocatechuic acid; phase II conjugates of PCA, sum of phase II conjugates of PCA (including PCA-3-glucuronide, PCA-4-glucuronide, PCA-3-sulfate, PCA-4-sulfate, vanillic acid, isovanillic acid, vanillic acid-glucuronide, isovanillic acid-glucuronide, vanillic acid-sulfate, isovanillic acid-sulfate, methyl 3,4-dihydroxybenzoate, and 2-hydroxy-4-methoxybenzoic acid); phenylpropanoic and phenylacetic acids, sum of ferulic acid, 3,4-dihydroxyphenylacetic acid, and 4-hydroxyphenylacetic acid.

$^2$ Recovery at maximum serum concentration.

$^3$ Total recovery.

$^4$ C3G was detected in feces of one participant only.

$^5$ Ferulic acid was the only phenylpropanoic/phenylacetic acid recovered in serum.
which $^{14}$C-labeled flavonoids were fed to rodents reported between 8% and 17.5% excretion as $^{14}$CO$_2$ (35, 38), in addition to the presence of $^{13}$C-labeled aliphatic intermediates (41). The greatest proportion of the $^{13}$C dose was excreted in feces (32.13 ± 6.13%). A previous study reported 44.5% recovery of an oral bolus dose of $^{13}$C-labeled C3G in mice (35), and fecal recoveries of other flavonoids have been reported between 1.9% and 30% (33, 34).

In the current study, there was a 42-fold higher abundance of $^{13}$C-labeled metabolites relative to $^{13}$C$_3$-C3G at their respective maximum serum concentration. Previous studies reported a 6-fold higher proportion of $^{13}$C metabolites relative to the parent anthocyanin in mice fed $^{13}$C-labeled C3G (35). Phase II conjugates of C3G could only be detected in the urine and reached peak concentrations of 1.17 ± 0.52 μmol/L at 1 h (collectively). Previous studies in which similarly structured isotope-labeled flavonoids (quercetin and catechin) were fed have also reported glucuronide, methyl, or sulfate derivatives of the parent flavonoid structure (36–38). Phase II metabolites of PCA were the primary metabolites identified during the first 6 h in serum and urine (Figure 3), whereas hippuric, phenylacetic, and phenylpropenoic acids and phase II conjugates of PCA were the predominant metabolites at 24 h in serum. In the 48-h samples, a mixture of hippuric acid, ferulic acid, and phase II conjugates of PCA were present (Figure 3).

Although the study was limited to men only, there was a high variation in ADME observed, which suggested an extremely high variability in ADME should be expected across mixed-sex populations as a result of differences in absorption, metabolism, and, perhaps more importantly, the gut microbiome (18); all of which should be considered when associations in epidemiologic studies on the health effects of flavonoid consumption are interpreted.

A limitation of the current study was the inability to recover all of $^{13}$C label or $^{13}$C-labeled metabolites, for which there were many possible reasons. Primarily, a considerable proportion of the unrecovered label likely remained in feces >48 h because concentrations of $^{13}$C did not reach their peak by this time (Figure 5A), which suggested that a longer fecal sampling period (perhaps ≥72 h) would have yielded a greater recovery. Extraction and quantification methods used were also optimized for 51 analytes, and therefore, the methods may be less sensitive in the detection or quantification of other novel metabolites. Furthermore, extraction efficiencies of the methods used were 75–98% efficient across matrices, which potentially accounted for ≥25% of identified metabolites having been quantitatively underestimated. Although we did not fully recover the $^{13}$C dose (via IRMS) or the relative concentration of $^{13}$C-labeled metabolites (via HPLC-MS/MS), the current results improved on those of previous studies in which the recovery of metabolites rarely exceeded 1% of the dose (7, 10, 11, 19, 39–43). In addition, previous studies have been unable to trace the kinetics of the A ring metabolites as a result of the localization of previously used isotope labels to the C and B rings of the flavonoid backbone (36, 38). The synthesizing of a labeled C3G with a different number of $^{13}$C atoms on the A and B rings was a major advantage in the current study.

In conclusion, the current study indicates that anthocyanins have a minimum relative bioavailability of 12.3 ± 1.3%, and their metabolites reach a 42-fold higher peak serum concentration that occurred much later than that of the parent anthocyanin. Overall, the present study indicated a much higher relative bioavailability and greater diversity of circulating metabolites than previously reported. The mechanisms of action and relative importance of these metabolites on health outcomes should be the focus of future anthocyanin research.

We dedicate this article to NPB, who died on 4 June 2011. NPD devised the synthetic process and labeling strategy for the $^{13}$C-labeled anthocyanin used in this study, and without his expertise and dedication to this project, this work would not have been possible. We also thank the participants for taking part in the study, Rachel de Ferrars for her assistance with method development and sample processing, Hiren Amin, Mark Philo, Shikha Saha, Sandra Small, and Eleanor McKay for their contributions to sample collection and analysis and K Saki Raheem and David O’Hagan for their work on the chemical synthesis of phenolic conjugate standards.

The authors’ responsibilities were as follows—CDK, AC, and NPB: conceived the project; CDK, AC, PAK, and NPB: designed the study; CDK and CC: addressed research-governance issues, sought ethical approval, conducted the feeding study, and analyzed and interpreted data; NPB and QZ: developed the synthesis strategy and synthesized the $^{13}$C$_3$-C3G; CDK and PAK: managed the analytical work; CC: processed the samples, performed the HPLC-MS/MS analysis and pharmacokinetic modeling, and compiled and analyzed raw data; DJM and TP: jointly developed the $^{13}$C-isotope analysis methodology, oversaw analyses, and conducted the data processing; CDK, CC, AC, and DJM: contributed to the development of the manuscript; TP, QZ, and PAK: contributed to the critical review of the manuscript; CDK: had primary responsibility for the final content or the manuscript; and all authors: contributed to the manuscript and agreed on the final version of the manuscript. CDK and AC have received research funding from GlaxoSmithKline for UK Biotechnology and Biological Sciences Research Council CASE studentships, and CDK has performed a small amount of consultancy work for GlaxoSmithKline. CC, QZ, DJM, TP, PAK, and NPB had no conflicts of interest.

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