Chapter 21

Urinary Pharmacokinetics of Queen Garnet Plum Anthocyanins in Healthy Human Subjects

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A new variety of *Prunus salicina*, Queen Garnet plum (QGP), was developed as a high anthocyanin, high antioxidant plum, in a Queensland, Australia, Government breeding program. In this manuscript, we are presenting for the first time data about the urinary pharmacokinetics of QGP anthocyanins and derived metabolites in healthy humans. Following consumption of 400 mL QGP juice (QGPJ; 2.49 mmol anthocyanins) by two male subjects, QGP anthocyanins (cyanidin-3-glucoside and cyanidin-3-rutinoside) were excreted mainly as methylated glycosides, glucuronides, and sulfoconjugated metabolites in urine. The cumulative excretion of anthocyanins could be fitted to a one-compartment pharmacokinetic model with instantaneous, parallel excretion of anthocyanin metabolites. The usefulness of this non-linear modeling statistical

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technique for characterizing the urine excretion-time profiles and estimating relevant PK parameters is demonstrated. Results from this pilot study indicate that methylation and glucuronidation are significant metabolic routes when cyanidin-based anthocyanins are consumed in QGPJ. Future studies investigating the benefits of the consumption of anthocyanin-rich Queen Garnet plums and/or derived products should therefore focus on identifying anthocyanin metabolites and include their putative colonic degradation products. This will assist in evaluating the biological relevance of these compounds to health and disease prevention.

Introduction

Anthocyanins, a polyphenol subclass, are one of the most abundant phenolic compounds in nature and are responsible for the red, purple, and blue colours of many fruits and vegetables, including plums. Approximately 640 individual anthocyanins have been identified to date (*1*). Anthocyanins also provide the food industry with natural alternatives for some synthetic food colourants to address the increasing public concern about synthetic food dyes (*1–3*). The six anthocyanidins (aglycons) commonly found in plants are classified according to the number and position of hydroxyl and methoxyl groups on the flavan nucleus and are named pelargonidin, cyanidin, delphinidin, peonidin, petunidin, and malvidin (*4*). Numerous studies, mostly *in vitro,* and some animal experiments, have demonstrated a broad range of biological properties for anthocyanins, including antioxidant, anti-inflammatory, antimicrobial, and anti-carcinogenic activities (*4*). Their daily intake has been estimated at between 12.5 mg in the United States (*5*) and 83 mg in Finland (*6*) which is considerably higher than the intake estimated for many other polyphenols. The evaluation of the bioavailability and pharmacokinetics (PK) of anthocyanins has recently been gaining significant interest as consumers seek foods with potential disease prevention benefits in addition to their nutritional value ("functional food") (*1*, *7*). However, detailed information on the absorption and metabolism of anthocyanins from raw and processed food is still limited. Several studies have found that anthocyanins are absorbed as intact glycosides and appear in blood and urine, whereas some other studies have found anthocyanins to be present mainly as metabolites (*4*, *8*, *9*).

Studies on anthocyanin bioavailability, as measured by absorption and excretion of intact anthocyanins and their main metabolites, appear to be very low and usually much less than 1% (*10*). The identification of the metabolic fate of these plant pigments, including their biotransformation by intestinal bacteria, as well as establishing their PK, is a basic requirement to evaluate their biological relevance to health and disease prevention. Furthermore, there is now emerging evidence that *in vivo* metabolites of dietary anthocyanins and other polyphenols, which appear in the circulatory system in a nanomolar to low micromolar range, can exert modulatory effects in cells through several effects on the intracellular signalling cascades (*11*).

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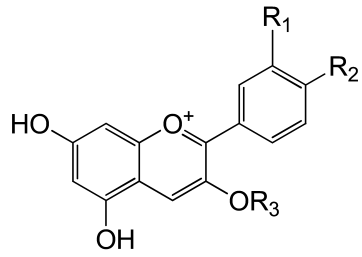
A new variety of the Japanese plum *Prunus salicina* Lindl., named Queen Garnet plum (QGP), was developed within a Queensland, Australia, Government breeding program (*12*). The strategy of this breeding program was to produce a high yielding plum with an outstanding anthocyanin content and antioxidant capacity. The anthocyanins present in QGP and QGP juice (QGPJ) were identified previously as cyanidin-3-glucoside and cyanidin-3-rutinoside (Figure 1) (*13*).



Anthocyanin	R1	R2	R3
cyanidin 3-glucoside	OH	OH	Glucose
cyanidin 3-rutinoside	OH	OH	Rutinose

Figure 1. Chemical structures of anthocyanins identified in QGPJ; Rutinose is 6-O-α-L-rhamnosyl-D-glucose.

In contrast to the development of synthetic drugs, there is no regulatory need to study the PK of food ingredients. Nonetheless, most pharmacokinetic principles of synthetic drugs are considered to be applicable to phenolic compounds from raw or processed food (*14*). Metabolism of xenobiotics including anthocyanins has been regarded as one of the most important and complex processes in the body, leading to the excretion of those substances. To optimize the use of anthocyanins from raw or processed food (e.g. dosage, regimen and administration route), knowledge on their biological fate including the disposition pathways and kinetics in the human body is needed to establish potential dose-effect and dose-concentration relationships. At a later stage this knowledge would allow for conducting proper therapeutic monitoring for anthocyanins. Pharmacokinetic studies of anthocyanins from food also provide information of potential food-drug interactions.

Although the typical pharmacokinetic analysis is based on data from blood samples, modeling of urine samples is seen as an important area (*15*). Urine collection is less invasive than blood sampling and does not require subject presence at the clinic site at the time of sample collection as voids can be collected and retained under appropriate storage conditions for later delivery to the clinic site or laboratories. The objective of the present study was (i) to

investigate the absorption, metabolism, and excretion of QGP anthocyanins and derived metabolites in two healthy human subjects, and (ii) to characterize the concentration profiles of anthocyanins and metabolites in urine as a basis for improving and simplifying bioavailability studies, as urine collection can be made at an optimal time to capture the exposure to anthocyanins.

Material and Methods

Chemicals

Unless otherwise stated, all chemicals were purchased from Merck (Kilsyth, VIC, Australia) or Sigma-Aldrich (Castle Hill, NSW, Australia), and were of analytical or HPLC grade. De-ionized water was used throughout. Cyanidin-3-glucoside and cyanidin-3-rutinoside were purchased from ChromaDex (Irvine, California, U.S.A.).

QGPJ Preparation

QGP which had been stored at -20°C was thawed and manually halved and de-pitted. QGP was then placed into a bowl chopper for 30 s and heated in a steam vat to 50°C before enzyme treatment with a commercial pectinase (PectinexMash, 200ppm, Novozymes, Sydney, Australia). The QGP puree was incubated at 50°C for 1 h before being juiced in a screw press (Brown International Corporation, Model 3600). Collected QGPJ was then pasteurized (by heating to 80°C and holding for 5 min) and stored at -10°C prior to ingestion. Microbial analysis (plate count) was undertaken on QGPJ to ensure juice was fit for human consumption.

Pilot Study with Healthy Human Subjects

Ethical permission was obtained from the CSIRO Human Experimentation Ethics Committee (code 08/22). Two healthy, non-smoking male volunteers were recruited; subject 1: 26 years, body weight 80 kg, body height 1.88 m, subject 2: 41 years, 74 kg, 1.70 m. Participants adhered to their usual diet, but abstained from food and beverages rich in polyphenols and ascorbic acid from 24 h prior to treatment. Alcohol and medication, including over the counter drugs, were refrained from consumption during the course of the pilot study. Both subjects had the following experimental treatments by which each subject served as his own control: 400 mL of QGPJ (containing 2.49 mmol of anthocyanins expressed as cyanidin-3-glucoside equivalents) or 400 mL of water as an anthocyanin-free control beverage separated by a one week wash-out phase. At 8.00 a.m. after an overnight fast, volunteers took 400 mL of one of the two beverages, respectively, together with white bread rolls. During the experimental periods, only the consumption of water and of three further standardised meals (white bread rolls with cheese for lunch, afternoon snack, and dinner) was allowed. Urine samples were collected pre-dose and quantitatively in six intervals up to 24 h after dosing (0-2, 2-4, 4-6, 6-8, 8-10, and 10-24 h). Aliquots of 7 mL were acidified and stabilised with conc. formic acid (1:1.3) and stored frozen at -80°C until analysed.

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Analysis of Anthocyanins

Anthocyanins in QGPJ

QGPJ was diluted 1:100 with HPLC mobile phase A (water/formic acid/acetonitrile, 87:10:3, v/v/v) and filtered through a 0.45-µm GHP Acrodisc Minispike filter (Pall Life Sciences, MI, U.S.A.) before injection into the HPLC. The juice was analysed in triplicate with the HPLC method and system described below.

Anthocyanins and Derived Metabolites in Urine

Urinary anthocyanins and metabolites were extracted with a solid-phase extraction (SPE) cartridge (Sep-Pak C18; Waters, Milford, MA) according to Felgines *et al.* (*16*) with slight modifications. Acidified urine samples were thawed and maintained for 60 min at room temperature before SPE extraction to obtain the maximal yield of the coloured flavylium cations. The SPE cartridge was activated with 10 mL of methanol and equilibrated with 10 mL of 12 mM aqueous HCl before use. Subsequently, 9 mL of acidified urine was applied to the equilibrated cartridge. The cartridge was then washed with 10 mL of 12 mM aqueous HCl, and anthocyanins were eluted with 15 mL of 12 mM HCl in methanol. The methanolic extract was evaporated under nitrogen to a volume of 1 mL by use of a Rotavapor R-210 (Buchi, Flawil, Switzerland) at 35°C. Aliquots of 1 µL (for identification of anthocyanins and anthocyanin metabolites) and of 50 µL (for quantification) were used for HPLC.

Analysis of anthocyanins and anthocyanin metabolites was carried out by HPLC-PDA according to the method of Kammerer *et al.* (*17*) with slight modifications. The HPLC system consisted of a 600E multisolvent delivery system (pump), in line-degasser AF, 2996 photodiode array detector (PDA), 717 plus auto-injector and 600 system controller (Waters Corporation, Milford Massachusetts, U.S.A.) equipped with an Aqua Luna C18(2) (250 x 4.6 mm i.d.) reversed phase column with a particle size of 5 µm (Phenomenex, Lane Cove, NSW, Australia) protected by a Phenomenex 4.0 x 3.0 mm i.d. C18 ODS guard column. The column was operated at a temperature of 25°C. The mobile phase consisted of water/formic acid/acetonitrile (87:10:3, v/v/v; eluent A) and of acetonitrile/water/formic acid (50:40:10, v/v/v; eluent B). The gradient program was as follows: 10% B to 25% B (10 min), 25% B to 31% B (5 min), 31% B to 40% B (5 min), 40% B to 50% B (10 min), 50% B to 100% B (10 min), 100% B (5 min. isocratic), 100% to 10% B (1 min), and 10% B for 5 min before injecting the next sample. The injection volume was 50 µL and samples were filtered through a 0.45-µm GHP Acrodisc Minispike filter prior to injection. Detection was carried out at 520 nm at a flow rate of 0.8 mL/min. Anthocyanins and individual anthocyanin metabolites were quantified by comparison of their peak areas with an external calibration curve obtained using known concentrations of cyanidin-3-glucoside. The cumulative amount of compound excreted into urine within 0 to 24 hours ($A_{e(0\text{-}24)}$) was calculated according to Eq. (1).

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$$A_{e(0\text{-}24)} = \Sigma(C \times V) \quad (1)$$

where C is the concentration in the collection interval and V the fractional urine volume. The fraction of orally administered compound excreted into urine within 24 hours (f_e/f) was calculated according to Eq. (2).

$$f_e/f\,(\%) = A_{e(0\text{-}24)}/Dose \times 100\% \quad (2)$$

Results were expressed as micromoles (μmol) or nanomoles (nmol) of cyanidin-3-glucoside equivalents. It should be noted that the present study focused on only anthocyanins (unchanged glycosides) and anthocyanin metabolites having an intact flavylium skeleton and being thus detected at 520 nm.

LC-PDA-MS analysis was carried out on a Quantum triple stage quadrupole (TSQ) mass spectrometer (ThermoFinnigan, NSW, Australia) equipped with a quaternary solvent delivery system, a column oven, a photo-diode array detector and an autosampler. An aliquot (20 μL) of a methanolic solution (250 μg/mL) was chromatographed on a Luna C18(2) column (150 x 2.1 mm, 5 μm particle size), (Phenomenex, NSW, Australia) which was maintained at 25 °C. The mobile phase consisted of 1% formic acid in water (A) and 1% formic acid in acetonitrile (B) at the rate of 0.3 mL/min (no splitting). A linear gradient was used (10% B to 100% B over 40 min). Ions were generated using an electrospray source in the positive mode under conditions set following optimisation using solutions of cyanidin-3-glucoside. MS experiments in the full scan (parent and product-specific) and the selected reaction monitoring (SRM) mode were carried out to confirm the identity of components.

All analysis and determinations were performed in duplicate unless otherwise stated.

The non-linear modeling approach wherein estimated PK parameters derived from fitting an appropriate PK model was used to determine the PK of intact QGP anthocyanins (cyanidin-3-glucoside and cyanidin-3-rutinoside) and their metabolites (sum of cyanidin monoglucuronide, cyanidin monosulfate, peonidin monoglucuronide, peonidin-3-glucoside, and peonidin-3-rutinoside).

The cumulative urine excretion of intact QGP anthocyanins and their metabolites was evaluated by using the WinNonlin Professional 5.2.1 program (Pharsight Corporation, Mountain View, CA, U.S.A.). A one-compartment model with parallel linear elimination kinetics and first-order input (Figure 2) was fitted to the experimental cumulative excreted amount-time data for each subject.

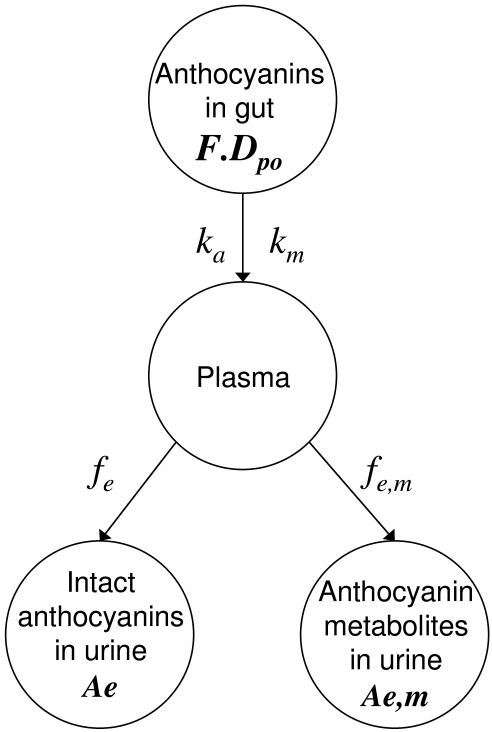

Figure 2. Schematic illustration of the one-compartment model with parallel elimination of intact anthocyanins and their metabolites into urine. A fraction of the anthocyanin is converted to a metabolite, which is then instantaneously excreted via the kidneys. Further explanation is given in the text.

A_e is the measured cumulative amount of intact anthocyanins excreted to the urine, and $A_{e,m}$ is the measured cumulative amount of the sum of anthocyanin metabolites (Eqs. 3 and 4).

$$A_e = f_e \cdot k_a \cdot FD_{po}\left[\frac{1}{k_a} + \frac{e^{-k\cdot t}}{k-k_a} - \frac{k\cdot e^{-k_a\cdot t}}{k_a\cdot\left(k-k_a\right)}\right] \tag{3}$$

$$A_{e,m} = f_{e,m} \cdot k_m \cdot FD_{po}\left[\frac{1}{k_m} + \frac{e^{-k\cdot t}}{k-k_m} - \frac{k\cdot e^{-k_m\cdot t}}{k_m\cdot\left(k-k_m\right)}\right] \tag{4}$$

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In Eq. (3) f_e is the fraction of absorbed dose excreted into urine, D_{po} the administered dose, k_a the first-order absorption rate constant, k the first-order elimination rate constant and F the unknown bioavailability (assumed to be 1). The model modification arose from switching k_a to k_m, the metabolism rate constant of the sum of metabolites, and $f_{e,m}$ as the fraction of metabolites formed and excreted into urine (Eq. 4).

Using this first-order rate model, the maximum likelihood equation for time of maximum excretion of intact anthocyanins (T_{max}) and sum of metabolites ($T_{max,m}$) may be calculated using Eq. (5) and (6), resp.

$$T_{\max} = \frac{\ln(k_a / k)}{(k_a - k)} \tag{5}$$

$$T_{\max,m} = \frac{\ln(k_m / k)}{(k_m - k)} \tag{6}$$

The elimination half-life for anthocyanins and metabolites is calculated using Eq. (7)

$$t_{1/2} = \frac{0.693}{k} \tag{7}$$

The metabolite ratio R_{met} is calculated using Eq. (8).

$$R_{met} = \frac{A_{e,m}}{A_e} \tag{8}$$

Ranges of starting values were evaluated using results from a preceding non-compartmental PK analysis. The estimated standard errors in each nonlinear regression were determined by the WinNonlin program.

Results and Discussion

The bioanalytical determination of the native anthocyanin content of the QGPJ yielded the administered doses that are summarized in Table I. The ingestion of QGPJ resulted in the appearance of both native QGP anthocyanins and at least five identified anthocyanin metabolites in the volunteers' urine (Figure 1 and 3).

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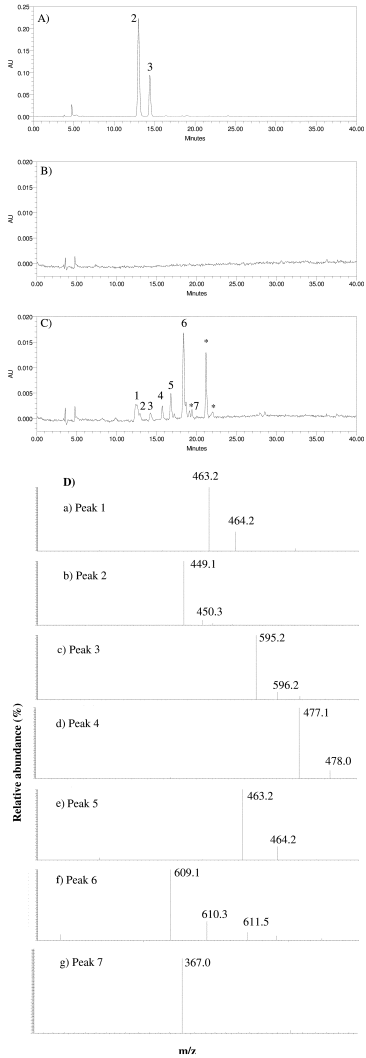

Figure 3. Representative HPLC chromatograms (A-C) and fragmentation patterns (D) of QGPJ (A) and of human urine (one subject) before (B) and 4 h after (C) the consumption of 400 mL QGPJ. Detection was performed at 520 nm. Urine was treated by solid phase extraction. Peaks: (1) cyanidin monoglucuronide, (2) cyanidin-3-glucoside, (3) cyanidin-3-rutinoside, (4) peonidin monoglucuronide, (5) peonidin-3-glucoside, (6) peonidin-3-rutinoside, (7) cyanidin monosulfate, and (*) unknown anthocyanin metabolites. (D) HPLC-ESI-MS precursor scans for product m/z 287 (a-c, g) and m/z 301 (d-f) showing native QGP anthocyanins and metabolites with intact flavylium skeleton. Components are numbered according to (C).

Table I. Administered doses of intact anthocyanins via QGPJ by analysis

	Anthocyanins (mmol)
Cyanidin-3-glucoside	1.84
Cyanidin-3-rutinoside	0.646
Total anthocyanins	2.49

Anthocyanins are calculated as cyanidin-3-glucoside equivalents.

The results are presented in Table II. Subject 1 excreted 9.5 μmol anthocyanins plus metabolites or 0.38% of the ingested dose, and subject 2 excreted 15.9 μmol or 0.64% of the ingested dose. The two subjects differed in their excretion of intact anthocyanins by 25% (1.8 μmol versus 2.3 μmol), but by 78% in the excretion of anthocyanin metabolites (7.6 μmol versus 13.6 μmol). The first difference could be explained by a difference in bioavailability with an extra variability that was most probably added by different metabolic capacities or physiological state. Cyanidin monoglucuronide and peonidin-3-rutinoside were the primary anthocyanin metabolites identified in the urine samples accounting for 19% (subject 1)/19.6% (subject 2) and 40% (subject 1)/42.5% (subject 2) of the identified anthocyanins and metabolites, respectively. Intact cyanidin-3-glucoside and cyanidin-3-rutinoside accounted for 19.5% (subject 1)/14.6% (subject 2), whereas the sum of the five identified metabolites (cyanidin monoglucuronide, cyanidin monosulfate, peonidin monoglucuronide, peonidin-3-glucoside, and peonidin-3-rutinoside) accounted for 80% (subject 1)/85% (subject 2) of the total anthocyanin excretion.

The metabolite ratio R_{met} (Table III) indicates that the sum of anthocyanin metabolites exceeded the excreted amount of intact anthocyanins 4-fold (subject 1) or 5.7-fold (subject 2).

These results, methylated and glucuronidated derivatives of cyanidin as the main urinary metabolites, are in agreement with several animal and human studies carried out with purified cyanidin compounds, fruit extracts or fruits rich in cyanidins (*8*, *18*). Furthermore, Felgines *et al.* (*16*) demonstrated that with six healthy volunteers after consumption of 200 g of strawberries (with pelargonidin-3-glucoside as the main anthocyanin) more than 96% of the excreted anthocyanins were related to pelargonidin metabolites (predominantly glucuronides). In the present study, the urinary recovery of intact anthocyanins and metabolites was 9.5 to 15.9 μmol corresponding to 0.38 to 0.64% of the administered anthocyanin dose. These data are within the range as reported in the literature for urinary excretion of anthocyanins and metabolites after ingestion of anthocyanin-rich food (reported range: 0.004-5%) (*10*, *18*, *19*).

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Table II. Urinary excretion of anthocyanins and anthocyanin metabolites following ingestion of a single oral dose of 400 ml QGPJ containing 2.49 mmol anthocyanins in two healthy male subjects

Anthrocyanin compounds	*Subject 1*		*Subject 2*	
	$A_{e(0-24)}$ *(nmol)*	f_e/f *(%)*	$A_{e(0-24)}$ *(nmol)*	f_e/f *(%)*
Cyanidin-3-glucoside	1095	0.0595	1468	0.0797
Cyanidin-3-rutinoside	758	0.117	854	0.132
Intact anthocyanins1	**1854**	**0.0745**	**2322**	**0.0933**
Cyanidin monoglu-curonide	1824	0.0733	3112	0.125
Peonidin monoglu-curonide	1504	0.0605	2467	0.0991
Peonidin-3-glucoside	226	0.0091	1058	0.0425
Peonidin-3-rutinoside	3772	0.152	6742	0.271
Cyanidin monosulfite	279	0.0112	176	0.0071
Sum metabolites2	**7606**	**0.306**	**13555**	**0.545**
Sum intact anthocyanins and metabolites	9460	0.38	15877	0.64

Results are expressed as cyanidin-3-glucoside equivalents. $A_{e(0-24)}$: amount of compound excreted into urine within 0 to 24 hours, f_e/f: fraction of orally administered compound excreted into urine within 24 hours (for the glucuronides, sulfates, peonidin glycosides, and the sums f_e/f is related to 2.49 mmol intact anthocyanins). [1] Sum of cyanidin-3-glucoside and cyanidin-3-rutinoside [2] Sum of cyanidin monoglucuronide, peonidin monoglucuronide, peonidin-3-glucoside, peonidin-3-rutinoside and cyanidin monosulfate

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Table III. Primary model parameter estimates and derived, secondary PK parameter

Model parameter	*Subject 1*		*Subject 2*	
	Estimate	*Standard error*	*Estimate*	*Standard error*
Intact anthocyanins				
k_a (h^{-1})	14.5	96.3	1.89	6.98
k (h^{-1})	0.42	0.092	0.48	0.64
f_e	0.000754	0.00002	0.000957	0.0001
T_{max} (h)	0.25	1.24	0.97	1.74
$T_{1/2}$ (h)	1.67	0.37	1.45	1.93
A_e (nmol)	1876	48.5	2382	329
r[1]	0.9996		0.9987	
Sum metabolites				
k_m (h^{-1})	1.58	0.208	0.55	1.94
k (h^{-1})	0.28	0.012	0.60	2.21
$f_{e,m}$	0.00303	0.00002	0.00549	0.00016
$A_{e,m}$ (nmol)	7547	59.9	13658	396
T_{max}	1.33	0.095	1.74	0.26
$T_{1/2}$ (h)	2.46	0.102	1.16	4.27
R_{met}	4.02	0.109	5.73	0.808
r	0.9996		0.9959	

Secondary parameters are A_e, $A_{e,m}$, T_{max}, $T_{1/2}$ and R_{met}. A_e, and $A_{e,m}$ are predicted amounts because they are calculated from the estimated model parameter f_e ($f_{e,m}$) × Dose. [1] Correlation coefficient between observed and predicted amounts.

The cumulative excretion of anthocyanins could be fitted to a one-compartment model with instantaneous, parallel excretion of anthocyanin metabolites (Figure 4).

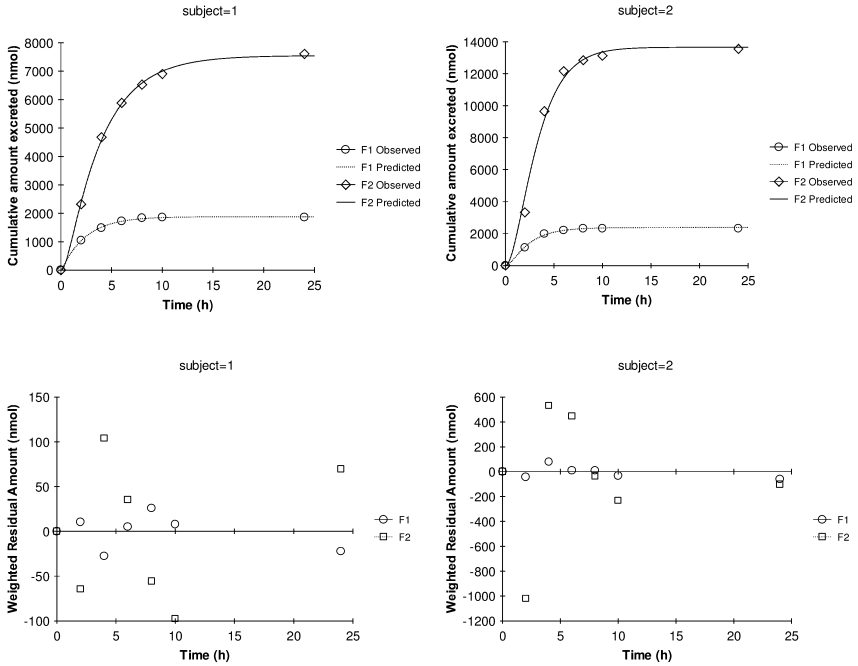

Figure 4. Cumulative amount of intact anthocyanins (F1) and sum of anthocyanin metabolites (F2) excreted by the urine and the predicted cumulative renal excretion. A one-compartment model with parallel (parent/metabolite) first-order renal elimination was fitted to data. Lower panel: goodness-of-fit plots indicating a satisfying fit, i.e., residuals are evenly spread around the zero value over time, of intact anthocyanins (F1), but a problematic fit of the sum of anthocyanin metabolites (F2), especially for subject 2.

T_{max} of intact anthocyanins was 0.25 h (subject 1) and 0.97 h (subject 2, Table III). However, the absorption rate constant k_a, and consequently T_{max} for intact anthocyanins, could only poorly be determined as the large standard error indicates. T_{max} of the metabolites was 1.33 h (subject 1) and 1.74 h (subject 2), respectively. The rapid peak excretion of the native cyanidin glycosides within 2 h after QGPJ consumption appears to confirm the important role of gastric absorption. In addition, the formation of anthocyanin metabolites is characterised as a rapid process. The metabolism rate constant k_m and the elimination rate constant of the metabolites k could be determined precisely in subject 1, but less precisely in subject 2. The reason is a large correlation of k and k_m (>0.95) in subject 2 (results not shown) which simply reflects that these rate constants are very close in magnitude.

The inspection of the plots of the fitted curve superimposed on the observed data and the residuals over time indicates that the consistency was more or less satisfying between observed and predicted data for the proposed model (Figure 4). In addition, the correlation coefficient *r* reached more than 0.995 for both subjects (Table III).

The half-life of both intact anthocyanins and metabolites is short (<2.5 hours). Under the assumption of the high k_a (rapid absorption) and short half-life of anthocyanins the poor precision of the estimates can also be caused by inappropriate design, i.e., urine collection intervals. Study design should be optimized such that the bin width of urine collection intervals is smaller than 2 hours at early post-dose time points. The non-linear modeling analysis is suitable to use spot urine samples collected at irregular, but early time points (according to the natural urinating scheme) in a more precise estimation of pharmacokinetic parameters.

The maximal excretion of cyanidin-3-glucoside and cyanidin-3-rutinoside was observed during the first 2 h (~52% of total anthocyanin excretion) whereas the metabolites' excretion was maximal between 2 and 4 h (~41% of total metabolite excretion) after QGPJ consumption. It is apparent that the urinary excretion of peonidin-3-rutinoside continued until the end of the experimental treatment (24 h post ingestion). A similar excretion pattern was observed by Felgines et al. (*16*) after the consumption of strawberries rich in pelargonidin-3-glucoside by six healthy volunteers: the maximal excretion of intact pelargonidin-3-glucoside was observed during the first 2 h, whereas the maximal excretion of pelargonidin metabolites was measured between 2 and 4 h after the strawberry breakfast. The rapid peak excretion of the native cyanidin glycosides within 2 h after QGPJ consumption appears to confirm the important role of gastric absorption. After absorption from the stomach into the bloodstream the native QGP anthocyanins are glucuronidated and methylated through the activities of UDP-glucuronyl-transferase and catechol-O-methyltransferase, respectively, mostly in the liver and kidney which are the major sites of glucuronidation and methylation *in vivo* (*18*). Besides glucuronidated and methylated derivatives, Felgines and colleagues could also detect sulfoconjugates of pelargonidin and cyanidin in the volunteers' urine after the consumption of strawberries and blackberries, respectively (*16*, *20*). The urinary recovery of cyanidin monosulfate in the blackberry study was too low for an unambiguous identification on the HPLC-DAD chromatogram and subsequent quantification (*20*); whereas in the strawberry study, pelargonidin monosulfate could be quantified in the volunteers' 24 h urine in the same concentration range (>100 nmol) as pelargondin-3-glucoside (*16*). The results of the latter study are in agreement with our findings: both subjects excreted significant amounts of cyanidin monosulfate (279 nmol (subject 1)/176 nmol (subject 2)) after QGPJ ingestion. It is remarkable, that cyanidin monosulfate is the only analyte that subject 2 excreted less than subject 1 (Table I). Sulfoconjugate formation requires hydrolysis (e.g. by lactase phloridzin hydrolase) of the glycosides to the respective aglycons (anthocyanidins) and then sulfoconjugation of the aglycons by sulfotransferases which is present in the intestine, liver, and other tissues (*11*, *20*). However, no aglycons could be detected in the present study with QGPJ

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whereas Felgines and colleagues reported significant amounts of pelargonidin as well as cyanidin and peonidin in the volunteers' 24 h urine after the ingestion of strawberries and blackberries, respectively (*16*, *20*). Due to the instability of anthocyanin-derived aglycons at physiological pH, the authors suggested that the detected aglycons are most likely the product of β-glucosidases and sulfatases present in kidney tissue and urine (*21*, *22*) and do not arise from the small intestine. But several other bioavailability studies failed to detect aglycons in plasma and/or urine of human subjects after the consumption of anthocyanin-rich food, which is consistent with our results (*8*, *9*, *23–25*).

At baseline, and in the control treatment, all urine samples did not contain any native anthocyanins, aglycons or metabolites with intact flavylium skeleton.

The degradation of dietary anthocyanins to several phenolic acids, such as homovanillic, vanillic, protocatechuic, p-hydoxybenzoic, and syringic acids by *in vitro* models mimicking fecal fermentation as well as in *in vivo* studies with healthy human subjects has been demonstrated recently (*1*, *18*, *26–30*). Furthermore, Woodward and colleagues showed that anthocyanins rapidly degraded to their respective phenolic acid and aldehyde constituents under simulated physiological conditions (*31*). A single anthocyanin may generate several main and minor metabolites, perhaps as many as 20 as in the case of quercetin glycosides (*32*) or even more. It is obvious that the compounds that reach the cells and tissues are chemically and biologically different from the dietary forms in the plants/plant food and that such features may have a significant effect on their potential bioactivity *in vivo*.

The anti-thrombotic activities of dihydroferulic acid and 3-(3-hydroxyphenyl)propionic acid, both putative colonic metabolites of cyanidin and delphinidin based anthocyanins, are an example for the biological significance of these catabolites (*33*). Although the identification and quantification of anthocyanin metabolites represents an emerging field of research since the interest about the potential impact of these plant pigments on health and disease prevention has significantly increased over the past decade, the potential biological activity of anthocyanin metabolites needs to be better investigated (*1*, *7*).

Consequently, it would be highly desirable in future *in vivo* studies with animals and/or human subjects to utilize labelled anthocyanins for the identification of all major metabolites which should include conjugates with intact flavylium skeleton as well as catabolites produced by the intestinal microflora. Otherwise, the 'true' bioavailability of anthocyanins and their relevance for human health and disease prevention will remain a 'Black-Box'.

Conclusion

This is the first published report about the urinary pharmacokinetics of QGP anthocyanins and metabolites in healthy human subjects. The urinary excretion of QGP anthocyanins, mainly as glucuronidated and methylated metabolites within 24 h after QGP juice consumption by two healthy male subjects, indicates an extensive metabolism of the native QGP anthocyanins.

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The usefulness of non-linear modeling statistical techniques for characterizing the urine excretion-time profiles and estimating relevant PK parameters has been shown. This statistical technique, coupled with experimental designs in which multiple spot urine voids are collected for a specified period after a short-term (peak or spike) exposure, is useful for the determination of maximum excretion time, a potentially important parameter in bio-monitoring under non-steady-state conditions. Blood samples should be used in future studies, as the benefits of simultaneously utilizing two different sources of data for modelling in regards to the increase in parameter precision has been demonstrated repeatedly. Thus, the potential biological activity of the metabolites derived from QGP anthocyanins (including catabolites produced by the intestinal microflora) needs to be better investigated in the future. Therefore, additional human studies with QGP and/or derived products including blood plasma, urine and fecal samples, as well as a larger number of subjects, are warranted to identify the complete spectra of *in vivo* metabolites and to investigate their interaction with chronic disease processes by using appropriate cell-based assays and animal models.

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