Strawberry pelargonidin glycosides are excreted in urine as intact glycosides and glucuronidated pelargonidin derivatives in rats

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Anthocyanins are natural dietary pigments with a wide array of biological properties that are possibly involved in the prevention of various diseases. These properties depend on their absorption and metabolism in the body. In the present study we first examined the gastric and intestinal absorption of pelargonidin 3-glucoside (Pg 3-glc) using rat in situ models. A high proportion of Pg 3-glc was rapidly absorbed from both the stomach (23 %) and small intestine (24 %). Its metabolism was further studied by feeding rats during 8 d with a diet enriched in freeze-dried strawberries. Only low amounts of total anthocyanins were recovered in 24 h urine (0.163 (SEM 0.013) % of ingested anthocyanins; n 8). Strawberry anthocyanins were analysed in urine by HPLC-electrospray ionisation-tandem MS. Similar proportions of intact glycosides (about 53 %) and glucuronidated metabolites (about 47 %) were found. Pg 3-glc was thus glucuronidated to a larger extent than cyanidin 3-glucoside. These results highlight the influence of the aglycone structure on anthocyanin metabolism.

Absorption: Anthocyanins: Metabolism: Pelargonidin: Strawberries

Anthocyanins are natural dietary pigments widely distributed in fruits and especially in berries1. Their average daily intake was estimated for a long time at about 200 mg/d in the USA2, but much lower intake values were determined by others (6.6 mg/d in Germany and 12.5 mg/d in the USA)3,4. However, intakes higher than 100 mg/d could be easily achieved with the regular consumption of red fruits or berries. Also the contribution of natural food colorants rich in anthocyanins to the total intake of anthocyanins has never been estimated. It may significantly contribute to the total anthocyanin dietary intake.

Anthocyanins are implicated in many biological activities that may impact positively on health5.6. They may reduce the risk of CHD, exert anticarcinogenic and neuroprotective activities, reduce inflammatory insult and modulate the immune response7–11. These actions might be mediated by their antioxidant activities5,12,13.

In view of these multiple biological activities, the bioavailability and metabolism of anthocyanins are important issues. We observed in a previous human study that the urinary excretion of anthocyanin metabolites differs according to the anthocyanins ingested14,15. This excretion was about 10-fold lower after consumption of blackberries (cyanidin 3-glucoside; Cy 3-glc) than after ingestion of strawberries (pelargonidin 3-glucoside; Pg 3-glc) using the same study design. So, it seems that the metabolic fate of anthocyanins may differ according to their aglycone structure. The reason for this difference between Pg 3-glc and Cy 3-glc urinary excretion has not been elucidated to date. It may be linked to different metabolic pathways and/or from different absorption rates. Thus, the aim of the present study was to study the metabolism of strawberry anthocyanins (Pg 3-glc) and to determine Pg 3-glc absorption along the digestive tract in the rat. For this purpose, the urinary excretion and metabolism of strawberry anthocyanins were evaluated in rats fed a diet enriched with freeze-dried strawberries. The gastric and intestinal absorption of Pg 3-glc was also studied using in situ rat models.

Materials and methods

Chemicals

Pg 3-glc and Cy 3-glc were purchased from Extrasynthese (Genay, France).

Animals and diets

’Adapted rat’ study. Eight male Wistar rats (Iffa-Credo, L’Arbresle, France) weighing approximately 200 g were housed two per cage in temperature-controlled rooms.

Abbreviations: Cy 3-glc, cyanidin 3-glucoside; HPLC-ESI-MS/MS, HPLC-electrospray ionisation-tandem MS; Pg, pelargonidin; Pg 3-glc, pelargonidin 3-glucoside; Pg 3-malglc, pelargonidin 3-malonylglucoside.

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(22°C), with a controlled dark period from 08.00 to 20.00 hours and access to food from 08.00 to 16.00 hours. They were fed a semi-purified control diet (755 g wheat starch, 150 g casein, 50 g groundnut oil, 35 g AIN-93M mineral mixture, 10 g AIN-76A vitamin mixture per kg) for 6 d\(^\text{16}\). They were then individually housed in metabolism cages fitted with urine and faeces separators and received the control diet supplemented with strawberry powder (200 g/kg diet) for 8 d. Rats ate about 19.3 g diet/d, i.e. about 3.85 g strawberry powder/d. Strawberry powder resulted from frozen strawberries that were lyophilised, pulvèrised and then sieved to eliminate achenes. To quantify anthocyanins administered to rats, strawberry powder (0.5 g) was treated for 30 min under agitation with 95 ml methanol. After filtration, the volume of solution was adjusted to 100 ml, and this solution was 5-fold-diluted with 0.12 M-HCl before HPLC analysis (20 \(\mu\)l) as described later.

In situ experiments. Twelve male Wistar rats (Iffa-Credo) weighing approximately 200 g were housed two per cage in temperature-controlled rooms (22°C), with a controlled dark period from 20.00 to 08.00 hours and access to food from 16.00 to 08.00 hours. They were fed the semi-purified control diet for 2 weeks.

All animals were maintained and handled according to the recommendations of the Institutional Ethics Committee (INRA), in accordance with French decree no. 87–848.

Sampling procedure during ‘adapted rat’ study

Rats were killed at 3 h after the beginning of the last experimental meal (i.e. at 11.00 hours) after being anaesthetised with sodium pentobarbital (40 mg/kg body weight). Blood was withdrawn from the abdominal aorta into heparinised tubes and urine present in the bladder was collected. Plasma was withdrawn from the abdominal aorta into heparinised tubes. Urine present in the bladder was also withdrawn into heparinised tubes. Bile was collected throughout the 30 min of the experiment. Urine present in the bladder was also collected. Plasma, bile and urine samples were acidified with 240 mM-HCl while stomach contents were acidified with 40 mM-HCl. All samples were stored at 2°C until analysis. We have controlled that Pg 3-gluc was stable under the experimental conditions, i.e. incubated for 30 min at 37°C in the gastric buffer (pH 3).

In situ intestinal perfusion. After cannulation of the bile duct, a perfusion of the jejuno-ileal segment of the intestine (from 5 cm distal from the duodeno-jejunal flexure up to the ileo-caecal valve) was prepared by installing cannulas at each extremity. This segment was continuously perfused in situ for 45 min at a flow rate of 0.75 ml/min with a buffer containing KH\(_2\)PO\(_4\) (5 mM), K\(_2\)HPO\(_4\) (2.5 mM), NaHCO\(_3\) (5 mM), NaCl (50 mM), KCl (40 mM), CaCl\(_2\) (2 mM), MgSO\(_4\) (1 mM), K\(_2\)C\(_6\)H\(_5\)O\(_7\) (10 mM), glucose (12 mM), glutamine (2 mM) and taurocholic acid (1 mM) (pH 6.6) at 37°C\(^\text{18}\). The buffer was supplemented with about 12 \(\mu\)M-Pg 3-gluc. The intestine was washed of its contents during the first 25 min. Effluents were directly collected at the exit of the ileum during the last 5 min of perfusion. Effluent volume was estimated by weighing. Bile was collected throughout the 45 min of the experiment. At the end of the experiment, blood samples were withdrawn from the mesenteric vein and abdominal aorta into heparinised tubes. Urine present in the bladder was also collected. Perfused solution, effluent, bile, plasma and urine samples were rapidly acidified with 240 mM-HCl and stored at 2°C until analysis.

To determine the stability of Pg 3-gluc throughout the in situ perfusion experiment (at 37°C, pH 6.6), a sample of the perfused buffer maintained at 37°C was collected at the beginning (t = 0), at t = 25 min and at the end of the perfusion period (t = 45 min), and Pg 3-gluc was analysed by HPLC after acidification with 240 mM-HCl, as described later. The overall percentage of degradation (2.26 (SEM 0.53) %; n = 6) was calculated by the decrease in Pg 3-gluc concentrations between 0 and 45 min. Moreover, anthocyanin degradation was a linear function of time. Thus, the amounts of Pg 3-gluc perfused were determined from the mean of Pg 3-gluc concentrations in the perfused buffer at t = 0 and t = 45 min.

Sample preparation

Urine samples were centrifuged at 12000 \(g\) for 5 min and the supernatant fraction (80 \(\mu\)l) was injected and analysed by HPLC as described later.

Anthocyanins present in plasma samples were extracted with a Sep-Pak C\(_\text{8}\) Plus solid-phase extraction cartridge (Waters, Milford, MA, USA), using Cy 3-gluc as internal standard as previously described\(^\text{17}\), and then analysed by HPLC (60 \(\mu\)l).

Cecal contents (0.2 g) were extracted with 18 ml water–acetone (1 : 1, v/v) containing 500 mM-HCl, then briefly sonicated and centrifuged for 5 min at 12000 \(g\) at room temperature. Supernatant fractions were evaporated under an N\(_\text{2}\) stream to half their initial volume to eliminate the acetone. Finally, aqueous extracts (60 \(\mu\)l) were analysed for anthocyanin content as described later.

Stomach contents were centrifuged for 8 min at 12000 \(g\) at room temperature, then analysed (20 \(\mu\)l) by HPLC. Absorption
through the gastric wall was estimated by the difference between the amount of Pg 3-glc administered into the stomach and the amount recovered at the end of incubation.

After centrifugation for 8 min at 12 000 g, the supernatant fractions of intestinal effluents were analysed (20 μl) by HPLC as described later. All the concentrations measured in the effluent samples were corrected by taking into account the intestinal absorption of water. Water absorption was estimated by calculating the difference between effluent flow (estimated by effluent weighing) and perfusion flow (0.75 ml/min). Absorption through the intestinal barrier was estimated by calculating the difference between the amount of Pg 3-glc administered through the intestinal segment and the amount recovered at the end of the ileal segment. These amounts were determined for the last 5 min of perfusion. Anthocyanin stability was also taken into account in evaluating intestinal absorption.

High-performance liquid chromatography analysis

Quantification of anthocyanins was performed by HPLC using a photodiode array detector (DAD 200; Perkin Elmer, Courtaboeuf, France) and a UV-visible detector (785 A; Perkin Elmer) at 524 nm. Samples were loaded onto a 150 × 4.6 mm Hypersil C18-5 μm column protected by a 10 × 4 mm Hypersil C18-5 μm guard column (Interchim, Montluçon, France) and analysed as previously described10. Strawberry anthocyanins and anthocyanin metabolites were identified by HPLC-electrospray ionisation-tandem MS (HPLC-ESI-MS/MS) analysis. These analyses were performed on a Hewlett-Packard HPLC system equipped with MS/MS detection (API 2000; Applied Biosystem, Les Ulis, France) as previously described14. The MS data were collected in multiple reaction monitoring mode by monitoring the transition of parent and product ions specific for each compound, using a dwell time of 0.5 s. Anthocyanins were detected according to the respective m/z values of their parent and product ions: pelargonidin (Pg) (271/121); Pg 3-glc (433/271); pelargonidin 3-malonylglucoside (Pg 3-malglc) (519/271); Pg glucuronide (447/271); Pg 3-glc glucuronide (609/271); Cy 3-glc (519/271) and accounted for 23.6% of total anthocyanins. Peak 2 was identified as Cy 3-glucuronide (m/z 449/287), accounting for only 3.7% of total anthocyanins. The concentrations of Pg 3-glc, Pg 3-malglc and Cy 3-glc in the diet were respectively 1.11, 0.36 and 0.06 mmol/kg and their consumption 2.1, 6.9 and 1.1 μmol/d.

Anthocyanins were analysed in the urine of strawberry-fed rats (Fig. 1(b)). Urine collected directly in the bladder or in the metabolism cages (24 h urine) both presented the same HPLC profile. The urinary HPLC profile showed strawberry anthocyanins as well as metabolites. The presence of intact strawberry anthocyanins, Cy 3-glc (peak 2), Pg 3-glc (peak 3) and Pg 3-malglc (peak 5) was confirmed by HPLC-ESI-MS/MS analysis (specific parent and product ions at m/z 449/287, 433/271 and 519/271, respectively). Peak 4 was identified as Pg monoglucuronide according to its parent and product ion pair (447/271). Peak 1 displayed a parent and product ion pair at m/z 609/271, suggesting the presence of Pg 3-glc glucuronide. Evaluation of anthocyanin urinary excretion was only based on Pg derivatives since Cy 3-glucuronide concentration was too low to allow accurate evaluation of its bioavailability. The mean urinary excretion of Pg derivatives over a 24 h period is presented in Table 1. A total of about 0.16% of ingested Pg glycosides was recovered in urine.

Polyethylene glycol measurements

Polyethylene glycol, a compound that is not absorbed by the stomach, was added to the gastric buffer. Its concentration in the gastric buffer was determined by the method of Powell & Malawer19. The ratio between the initial concentration and that measured at the end of the experiment reflected the intensity of gastric secretion (gastric volume at the end of the experiment was about 6 ml). This parameter has to be taken into account in order to obtain the correct concentration of anthocyanins at the end of the experiment.

Data analysis

Values are given as means with their standard errors. When appropriate, significance of differences between values was determined by an unpaired t test (GraphPad; Instat, San Diego, CA, USA). Values of P<0.05 were considered significant.

Results

‘Adapted rat’ study

The strawberry powder used in the present work showed by HPLC analysis three peaks (Fig. 1(a)). Pg 3-glc (peak 3) was the major anthocyanin (72.8%). Two other anthocyanins were detected and identified by HPLC-ESI-MS/MS. Peak 5 was identified as Pg 3-malglc by detection of the respective parent and product ion pair (m/z 519/271) and accounted for 23.6% of total anthocyanins. Peak 2 was identified as Cy 3-glucuronide (m/z 449/287), accounting for only 3.7% of total anthocyanins. The concentrations of Pg 3-glc, Pg 3-malglc and Cy 3-glc in the diet were respectively 1.11, 0.36 and 0.06 mmol/kg and their consumption 2.1, 6.9 and 1.1 μmol/d.

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The HPLC analysis of plasma from strawberry-fed rats showed the presence of two anthocyanins identified as Pg 3-glc and Pg monoglucuronide by HPLC-ESI-MS/MS (m/z 433/271 and 447/271, respectively). At the time of killing, i.e. 3 h after the beginning of the last meal, total plasma anthocyanin concentration was low (48.1 (SEM 5.1) nM). Plasma concentration of Pg glucuronide was significantly higher than plasma concentration of Pg 3-glc (respectively 29.0 (SEM 3.5) nM and 19.1 (SEM 1.9) nM; \( P, 0.01 \)).

Analysis of the caecal contents collected 3 h after the beginning of the last meal showed the presence of the three strawberry anthocyanins (Cy 3-glc, Pg 3-glc and Pg 3-malglc) as well as that of low amounts of Pg (Fig. 2).

In situ gastric administration
Pg 3-glc (61.0 (SEM 0.4) nmol; \( n = 6 \)) was infused in situ in the rat stomach. Analysis of the stomach content 30 min after infusion showed an absorption of 14.1 (SEM 1.0) nmol (23.1 (SEM 1.6) %). No metabolites of Pg 3-glc were observed in the stomach contents. HPLC analysis of pooled urine samples treated by solid-phase extraction revealed the presence of small amounts of Pg 3-glc (Fig. 3(a)).

In situ intestinal perfusion
Pg 3-glc was also perfused in situ in the small intestine of rats. The HPLC profile of effluents was similar to that of the perfused solution. After perfusion of 50.6 (SEM 4.3) nmol Pg 3-glc (\( n = 6 \)), 11.9 (SEM 1.5) nmol (23.5 (SEM 2.1) %) Pg 3-glc was absorbed from the intestine during the last 5 min of perfusion. HPLC analysis of urine (Fig. 3(b)) and of plasma collected from the mesenteric vein revealed the presence of Pg 3-glc and Pg monoglucuronide.

Discussion
The aim of the present study was to evaluate the absorption, metabolism and urinary excretion of strawberry anthocyanins (Pg 3-glc) and compare the results with those previously obtained on blackberry anthocyanins (Cy 3-glc)\(^1\) in order to better understand the influence of aglycone structure on anthocyanin urinary excretion. Metabolism and urinary excretion of strawberry anthocyanins were studied in rats fed for 8 d with a diet enriched with a lyophilised strawberry powder. The strawberry powder contained mainly Pg 3-glc (about 73 %). An acylated derivative of Pg 3-glc (Pg 3-malglc, about 24 %) as well as a small amount of Cy 3-glc (< 4 %) were also present as previously reported\(^2,22\). Rats ingested about 14 mg anthocyanins/d. This intake corresponds to about a 280 mg/d intake in human consumers, an amount corresponding to one to two servings of red fruits or berries\(^5\).

Intact Pg glycosides (Pg 3-glc and Pg 3-malglc) were recovered in urine together with glucuronidated metabolites such as Pg monoglucuronide and Pg 3-glc monoglucuronide.
The amount of Pg glucuronide excreted was close to that of Pg 3-glc. This relatively high proportion of glucuronidated anthocyanidin seems to be characteristic of Pg. Indeed, in rats, glucuronide metabolites were only detected in very small amounts in urine after Cy 3-glc feeding and no glucuronides were produced after delphinidin 3-glucoside administration. Pg glucosides were more glucuronidated than cyanidin glycosides in rats, pigs and human subjects. El Mohsen et al. have recently identified Pg glucuronide in plasma and tissues after oral administration into rats with the aglycone Pg. In previous human studies, we have shown that Pg glucuronides were the main strawberry anthocyanin metabolites in urine (Pg glucuronide urinary excretion was about 25-fold higher than that of Pg 3-glc) whereas anthocyanidin glucuronide urinary excretion was only 5-fold higher than that of Cy 3-glc after blackberry feeding. Pg has only one hydroxyl group on the B ring. It thus cannot be methylated and may be more available for glucuronidation than other anthocyanins such as Cy 3-glc.

The level of urinary excretion of strawberry anthocyanin metabolites (0-16 % of the amount ingested) was of the same order of magnitude as previously reported in rats for other anthocyanins and lower than reported in human subjects or weanling pigs after Pg 3-glc ingestion. On the other hand, in human subjects, urinary excretion of anthocyanin metabolites was higher after Pg 3-glc ingestion (1-8 %) than after that of Cy 3-glc (0-16 %). This 10-fold higher urinary excretion of Pg derivatives as compared with those of cyanidins observed in human subjects may result in part from a very high level of Pg anthocyanin glucuronidation since the main strawberry anthocyanin metabolite in human urine was a Pg glucuronide that accounted for 83 % of total urinary metabolites. Moreover the extent of anthocyanin glucuronidation seems higher in man than in rats.

At 3 h after the beginning of the last meal, plasma anthocyanin levels were very low. Food consumption was spread over a period of 8 h and anthocyanins were thus slowly ingested. Such a slow ingestion as well as a rapid elimination probably resulted in low plasma concentrations. Moreover, plasma Pg glucuronide concentration was significantly higher (about 23 %) of Pg 3-glc than that of Cy 3-glc (about 22 %), as previously reported using the same model. We had showed that the aglycone structure influences the anthocyanin intestinal absorption, the methylated anthocyanin glucosides (malvidin 3-glucoside, petunidin 3-glucoside) being less easily absorbed than the non-methylated Cy 3-glc. However, the number of free hydroxyl groups on the B ring did not seem to influence intestinal absorption. The absorption and further elimination of Pg 3-glc occurred very quickly since it was recovered in urine only 30 min after the beginning of the experiment.

Intestinal anthocyanin absorption was also investigated using in situ intestinal (jejuno-ileal) perfusion. A high proportion of Pg 3-glc (about 24 %) is also absorbed from the small intestine. The extent of intestinal absorption is similar to that of Cy 3-glc (about 22 %), as previously reported using the same model. We had showed that the aglycone structure influences the anthocyanin intestinal absorption, the methylated anthocyanin glucosides (malvidin 3-glucoside, petunidin 3-glucoside) being less easily absorbed than the non-methylated Cy 3-glc. However, the number of free hydroxyl groups on the B ring did not seem to influence intestinal absorption. The absorption and further metabolism of Pg 3-glc occurred very quickly, since native glucoside as well as glucuronidated Pg were recovered in plasma and urine only 45 min after the beginning of the experiment. Pg glucuronide could be formed during the intestinal absorption process. Indeed, UDP-glucuronosyltransferase activity is present in the small intestine and intestinal formation of cyanidin glucuronides has been recently reported. Taken as a whole, these results raise the question of anthocyanin tissue distribution and their possible transformation after absorption. Indeed, despite a significant absorption in both the stomach and small intestine, only a very small proportion of ingested anthocyanins was recovered in urine and plasma. The present study focused only on anthocyanin metabolites having an anthocyanin skeleton and being thus detected at 524 nm. However, anthocyanin chemistry is complex, and a large part of the absorbed anthocyanins could thus be metabolised to non-coloured forms and/or degradation products due to their chemical instability at physiological pH, and thereby escape detection under usual conditions.
Strawberry anthocyanin metabolism

In conclusion, the present study showed that significant amounts of Pg 3-glc were rapidly absorbed from both stomach and small intestine, similarly to Cy 3-glc. However, Pg 3-glc was glucuronidated to a larger extent than Cy 3-glc. These results highlight the influence of the aglycone structure on anthocyanin metabolism.

References