Chlorogenic Acid Is Absorbed in Its Intact Form in the Stomach of Rats

Sophie Lafay, Angel Gil-Izquierdo, Claudine Manach, Christine Morand, Catherine Besson, and Augustin Scalbert

Unité des Maladies Météaboliques et Micronutriments, INRA de Clermont-Ferrand/Theix, 63122 Saint Genès Champanelle, France

ABSTRACT The bioavailability of chlorogenic acid, a major polyphenol of the human diet that is particularly abundant in coffee and various fruits, was explored in rats. To identify the form under which it is absorbed through the gut mucosa and the site of absorption along the gastrointestinal tract, rats were fed a diet supplemented with chlorogenic acid (0.25%, wt:wt). Chlorogenic acid and its metabolites were estimated in the stomach, small intestine and cecal contents as well as in bladder urine and plasma by HPLC with coulometric detection at several time points (1.5, 3, 4.5, and 7 h) after the beginning of the meal. Minor hydrolysis of chlorogenic acid (<1%) occurred in the stomach and small intestine contents, whereas 15–32% of ingested chlorogenic acid was hydrolyzed into caffeic acid in the cecum. Chlorogenic acid and caffeic acid appeared early (at 1.5 h) in plasma and urine, suggesting an absorption of chlorogenic acid into the upper part of the gastrointestinal tract. Gastric absorption of chlorogenic acid was further examined by infusing chlorogenic acid in the ligated stomach of food-deprived rats. After 30 min of infusion, intact chlorogenic acid was found in the gastric vein and aorta. No other metabolites could be detected by HPLC-electrospray ionization-MS-MS. These results show for the first time that chlorogenic acid is quickly absorbed in the rat stomach in its intact form.


KEY WORDS: • chlorogenic acid • absorption • gastrointestinal tract • stomach • rats

Chlorogenic acid, an ester of caffeic acid, is found with quinic acid in a wide range of fruits and vegetables and is particularly abundant in coffee (1). Both chlorogenic and caffeic acid possess antioxidant properties in vivo (2–4). Chlorogenic acid and caffeic acid were reported to prevent different cancers and cardiovascular diseases in several experimental studies in animal models (5–9). The biological properties of hydroxycinnamic acids depend on their absorption in the gut and on their metabolism. The intestinal absorption of caffeic acid is well characterized in both experimental animals and human subjects (10–13). The absorption and metabolism of chlorogenic acid are less studied. Caffeic acid appeared rapidly in plasma after chlorogenic acid ingestion in both rats and human subjects, suggesting that chlorogenic acid is hydrolyzed in the upper part of the gastrointestinal tract (13,14). Using an in situ intestinal perfusion model, we could confirm that chlorogenic acid was effectively absorbed in the small intestine of rats, hydrolyzed in the mucosa, and recovered as free phenolic acid in the plasma (15). However, other authors also identified intact chlorogenic acid in human urine after ingestion of chlorogenic acid, chlorogenic acid-containing coffee, or prune, with recovery yields varying from 0.3 to 2.3%, suggesting that chlorogenic acid is also absorbed without hydrolysis (12,16–18). More recently, chlorogenic acid was also identified in rabbit plasma shortly after oral administration of a honeysuckle extract (19).

The aim of this work was to explore the stability of chlorogenic acid in the gut, the form under which it is absorbed through the gut mucosa and the site of absorption along the gastrointestinal tract. Rats were fed a diet supplemented with chlorogenic acid, and phenolic acids were estimated in the stomach, small intestine, and cecal contents at different time points during the meal as well as in bladder urine and plasma. Absorption of chlorogenic acid through the stomach mucosa was also examined by infusing chlorogenic acid into the stomach of food-deprived rats.

MATERIALS AND METHODS

Chemicals. Chlorogenic acid (5-cafeoylquinic acid according to the IUPAC numbering system), caffeic acid, ferulic acid, sinapic acid and β-glucuronidase from Escherichia coli type XA were purchased from Sigma. Isoferulic acid was purchased from Extrasynthese. The sulfate ester of ferulic acid and p-coumaric acid, glucuronide of ferulic and p-coumaric acid were donated by O. Dangles and S. Galland, INRA Avignon, France.

Animals and diets. Male Wistar rats, weighing ~180 g were caged singly in temperature-controlled rooms (22°C), with a dark period from 0800 to 2000 and with access to food from 0800 to 1600. They were fed a semipurified control diet for 7 d (Table 1). Rats were...
Table 1

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Control diet (g/kg dry feed)</th>
<th>Chlorogenic diet (g/kg dry feed)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wheat starch</td>
<td>749.5</td>
<td>747</td>
</tr>
<tr>
<td>Casein</td>
<td>150</td>
<td>150</td>
</tr>
<tr>
<td>Peanut oil</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Mineral mixture ¹</td>
<td>35</td>
<td>35</td>
</tr>
<tr>
<td>Vitamin mixture ¹</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>L-Cystine</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Choline</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>Chlorogenic acid</td>
<td>—</td>
<td>2.5</td>
</tr>
</tbody>
</table>

¹ Mineral mixture AIN-93-M and Vitamin mixture AIN-93-VX described in (34) and (35).

maintained and handled according to the recommendations of the National Institute for Agricultural Research Ethics Committee, in accordance with Decree 87-848. For 3 d, 16 rats were then fed the same diet supplemented with chlorogenic acid (0.25% wt/wt) (Table 1). These quantities of chlorogenic acid in the diet correspond to a consumption of ~7 cups of coffee/d for humans [for a diet consumption of 500 g dry matter/d, an amount of chlorogenic acid in coffee of 750 mg/100 L and a cup volume of 200 mL (1)]. Diet intake was measured on the last day when each rat was killed and chlorogenic acid consumption was calculated accordingly. Six other rats were deprived of food for 24 h before the stomach infusion experiment.

On d 3 of chlorogenic acid supplementation, rats were anaesthetised with sodium pentobarbital (40 mg/kg body, i.p.) 1.5, 3, 4.5, and 7 h (n = 4 per sampling time) after the beginning of the meal. Stomach, small intestine, and cecal contents, aortic blood and bladder urine were collected at each time. The gastric and intestinal contents were weighed separately and immediately frozen at −20°C until analysis. Blood was drawn into heparinized tubes. Plasma was obtained by centrifugation (10,000 × g for 2 min). Urine and plasma were acidified with 10 mmol/L acetic acid. All samples were stored at −20°C until analysis.

For gastric infusion, rats (n = 4) were anaesthetized with sodium pentobarbital (40 mg/kg body weight) and kept alive under anesthesia throughout the experiment. After cannulation of the biliary duct, the pylorus was ligated and the stomach was filled in situ through the cardia with a 5-μL chlorogenic acid solution buffered to mimic the osmotic and pH conditions found in the stomach during a meal. This buffer (pH 3) maintained at 37°C, contained KH₂PO₄ (7.5 mmol/L), NaCl (50 mmol/L), KCl (50 mmol/L), CaCl₂ (2 mmol/L), acetic acid (25 mmol/L), lactic acid (25 mmol/L), MgSO₄ (1 mmol/L), polyethylene glycol (PEG)³ 6000 (5 g/L), and chlorogenic acid (7 mmol/L). Chlorogenic acid was stable in buffer under the experimental conditions (data not shown). Thus, the stomach contained 35 μmol of chlorogenic acid. The cardia sphincter was ligated to prevent any gastroesophageal reflux. The amount of chlorogenic acid infused into the stomach was established to fit in the quantities found in the stomach during the chlorogenic acid feeding experiment. The stomach contents were collected 30 min later; blood was withdrawn from the gastric vein and abdominal aorta into heparinized tubes and centrifuged (10,000 × g; 2 min). Two more rats were used as control, infused with the same buffer without chlorogenic acid, and their blood treated under identical conditions. Plasma samples were acidified with 10 mmol/L acetic acid. All samples were stored at −20°C before analysis.

Treatment of gut content samples. The contents of the stomach, small intestine, and cecum collected from rats fed the chlorogenic acid–supplemented diet were spiked with sinapic acid as an internal standard. Then 9 volumes of methanol:H₂O:concentrated HCl (70:28:2, by vol) containing sinapic acid was added to 1 volume of each content, mixed on a vortex for 30 s, sonicated at 0°C, and centrifuged for 10 min at 4°C and 3000 × g. The supernatant was diluted with water (1:1, v/v), 1 mL of hexane was added, and vortex mixing took place. After centrifugation (3000 × g; 10 min), the upper organic phase was removed and the lower one was diluted 25 times in methanol:H₂O:concentrated HCl (70:28:2, by vol) except for cecal samples, which were analysed directly.

For the gastric infusion experiment, the stomach contents were centrifuged (3000 × g; 10 min), filtered through glass, and analysed directly. Phenolic acid concentrations in the stomach lumen were corrected for variations in volume due to gastric secretion. PEG, a compound not absorbed in the stomach, was added to the gastric buffer, and its concentration in the gastric buffer determined at the beginning and at the end of the experiment (20). The ratio between these 2 concentrations reflected the intensity of the gastric secretion.

Treatment of plasma and urine samples. Before analysis, urine samples were diluted with 0.5 mol/L sodium acetate containing 2 g/L ascorbic acid to reach pH 6.8. Plasma samples were adjusted to pH 6.8 with 0.1 volume of 0.5 mol/L sodium acetate containing 2 g/L ascorbic acid. Urine and plasma samples were incubated for 4 h at 37°C in the presence of 10 μL β-glucuronidase (2 × 10⁸ U/L) from Escherichia coli. This enzyme was selected rather than the Helix pomatia enzyme preparation because we noticed that chlorogenic acid was partially hydrolyzed by this last enzyme mixture (18) but was stable when treated in this enzyme preparation of esterase activity able to hydrolyze chlorogenic acid. The presence of sulfatase activity in the E. coli enzyme preparation was verified using a rat plasma sample containing known sulfates esters of quecetin (data not shown). Phenolic acids in treated samples were extracted by adding 2.85 volumes of methanol:H₂O:concentrated HCl (70:28:2, by vol) and centrifuged for 4 min at 12,000 × g. The resulting supernatant was analyzed by HPLC as described below.

Phenolic acid analyses. Phenolic acids in gut contents and plasma and urine were analysed by HPLC coupled to an 8-electrode CoulArray Model 5600 detector (Eurosep) with potentials at 25, 100, 250, 325, 360, 400, 475, and 550 mV. The system was fitted with a 5μm-C-18 Hypersil BDS analytic column (150 × 4.6 mm; Life Sciences International). Mobile phase A was 5% acetonitrile in 30 mmol/L NaH₂PO₄ at pH 3, and mobile phase B, 50% acetonitrile in 30 mmol/L NaH₂PO₄ at pH 3. The separation was performed at 35°C. The flow rate was 0.8 mL/min. Two different gradients were applied for content analysis and fluid analysis. For content analysis, the gradient was as follows: linear from 0 to 60% B from 0 to 45 min; linear from 60 to 100% B from 45 to 46 min; 100% B from 46 to 50 min. For plasma and urine analyses, the gradient was as follows: linear from 0 to 22% B from 0 to 21 min; linear from 22 to 29% B from 21 to 41 min; 100% B from 41 to 45 min. In plasma, recoveries ranged from 75–85% according to the nature of the phenolic acids. The precision and accuracy for each of the standard curve detection concentration points after back-calculation were 1.93–3.89% and −0.11–2.07%, respectively. The limits of detection were 0.06 μmol/L for caffeic and ferulic acids, and 0.08μmol/L for chlorogenic and isoferic acids.

Plasma samples, collected in the gastric vein and abdominal aorta after the gastric infusion experiment, were analysed by HPLC-electrospray ionization-tandem mass spectrometry (HPLC-ESI-MS/ MS; API 2000, Applied Biosystem, Canada). Plasma was treated as above and injected directly (40 μL) into the LC-ESI-MS-MS system, fitted with a YMC-Pack ODS-AM column (250 × 3.0 mm I.D., 5-μm particle size, YMC Europe) and a YMC-Pack ODS-AM guard column (10 × 4 mm I.D., 5-μm particle size, YMC Europe). The mobile phase consisted of water:acetonitrile/formic acid (94.5:5.5:1, by vol) (solvent A) and water:acetonitrile/formic acid (49.5:50.5:1, by vol) (solvent B). The eluent was delivered at a 200 μL/min flow rate as follows: 5% solvent B from 0 to 10 min, 10% solvent B from 10 to 12 min, 10–27% solvent B from 12 to 14 min, 27–29% solvent B from 14 to 19 min, and 29% solvent B up to 30 min. The ionization conditions were adjusted at 400°C and 5000 V for drying gas temperature and voltage, respectively. The other ionization parameters were as follows: nebulizer gas (N₂) 70 arbitrary units (au), drying gas (N₂) 30 au, and curtain gas (N₂) 20 au. The common nitrogen flow for collision active dissociation was set at 1.2 au. The particular MS voltages and collision
energies for chlorogenic, caffeic, ferulic, and isoflavonic acids were optimized (data not shown). The analyses were performed in the negative and multiple reaction monitoring (MRM) modes. The selected MS transitions were 353/191, 179/135, and 193/134 for chlorogenic, caffeic and ferulic/isoferulic acids, respectively. The phenolic acids were identified by comparison of the signals and retention times to those of corresponding standards. Conjugations of chlorogenic, caffeic, ferulic/isoferulic acids, and p-coumaric acid with glucuronic acid (m/z, 529/353, 355/179, 369/193, respectively), sulfate groups (m/z, 433/353, 259/179, 273/193, respectively), glycine (m/z, 410/353, 236/179, 250/193, respectively), glutathione (m/z, 642/353, 468/179, 482/193, respectively) and with both glucuronide and sulfate groups (m/z, 609/353, 435/179, 449/193, respectively) were also investigated in the MRM mode using the same MS voltages as applied to the corresponding free phenolic acids. MS transitions were determined from the analysis of appropriate standards (sulfate ester of ferulic acid and p-coumaric acid, glucuronide of ferulic and p-coumaric acid) or calculated (loss of conjugated group) when the standard was missing.

Data analysis. Numerical values are means ± SEM (n = 4 for bioavailability study or 6 for gastric infusion study). Data were analyzed using XLStat version 7.5 Addinsoft 2004. Significant differences were determined by 1-way ANOVA (gastric infusion experiment) or by 2-way ANOVA. Data were log-transformed when non-Gaussian distribution occurred. When significant differences were detected Tukey’s Multiple Comparison Test was used to assess differences among the means. Differences with P < 0.05 were considered significant.

RESULTS

Dietary supplementation of chlorogenic acid in rats

Diet consumption and chlorogenic acid intake. Chlorogenic acid was administered with the diet (0.25%) from the beginning of the dark period and its consumption was determined for the next 7 h. The total amounts of chlorogenic acid consumed were as follows: 145.4 ± 16.7 μmol from 0 to 1.5 h, 37.3 ± 7.4 μmol from 1.5 to 3 h, 25.6 ± 5.6 μmol from 3 to 4.5 h, and 31.4 ± 7.5 μmol from 4.5 to 7 h.

The major part of the meal and of chlorogenic acid (60.5%) was ingested during the first 90 min of this period. Rats ingested lower amounts of chlorogenic acid during the next 3 periods (15.6, 10.7 and 13.1%, respectively). The amount of chlorogenic acid ingested during the first 90 min was greater than during the other periods (P < 0.001).

Chlorogenic and caffeic acid concentrations in gastrointestinal contents. In the stomach, similar quantities of chlorogenic acid were found at the end of each time period. In the small intestine, the chlorogenic acid content increased significantly after 3 h. The contents at 4.5 and 7 h did not differ from that at 3 h (Fig. 1). Only traces of caffeic acid (1.0 ± 0.1% of total phenolic acids) could be detected in either the stomach or the small intestine.

The contents of chlorogenic acid were much lower in the cecum. The maximal value was observed 4.5 h after the beginning of the meal. It was significantly higher than the contents found at the other time points (0.13 ± 0.02 μmol). There was also a significant amount of caffeic acid in the cecum, accounting for 15, 32, 21, and 26%, respectively, of total phenolic acids measured at each time point.

Plasma kinetics of chlorogenic acid and its metabolites. No chlorogenic acid or other phenolic acids were detected in the aortic plasma of the control group. In the supplemented group, both chlorogenic and caffeic acids were detected in similar concentrations early (1.5 h) after the beginning of the meal (Table 2). The concentrations did not differ during the meal.

Concentrations of phenolic acids in urine taken directly into the bladder and repartition of excreted metabolites. No chlorogenic acid or other phenolic acids were detected in urine of the control group. In the supplemented group, chlorogenic acid was detected in urine from 1.5 h after the beginning of the meal together with caffeic, ferulic, and isoflavonic acids (Fig. 2). Chlorogenic acid excretion tends to decrease over time in proportion to the amount ingested. Caffeic acid was also excreted early after the beginning of the meal; its relative excretion over other phenolic acids increased at 3 h compared with 1.5 h (P < 0.001). The early appearance of chlorogenic acid in plasma and urine suggested a possible absorption in the stomach.

In situ gastric infusion of chlorogenic acid in rats. A significant proportion of chlorogenic acid was absorbed as indicated by its disappearance in the stomach after a 30-min infusion (Table 3). There were only traces of caffeic acid in the stomach contents after incubation. The plasma collected from the gastric vein and aorta by LC-ESI-MS/MS after the 30-min infusion period was examined for the presence of phenolic acids and their metabolites. There were 2 peaks with the same m/z transition as chlorogenic acid (Fig. 3). They were absent in plasma when rats were infused with control buffer. One of the 2 peaks has a retention time identical to that of the
Ferulic and isoferulic acids were not detected at 0.06 and 0.08 μmol/L,

These values are means ± SEM, n = 4.

Values are means ± SEM; n = 4 per time point.

Chlorogenic acid appeared early in both plasma and urine, 1.5 h after the beginning of the meal. A similar fast absorption was also reported in rabbits with a maximal absorption peak of chlorogenic acid observed 35 min after administration (19). This rapid appearance of the intact ester in plasma or urine suggests a direct absorption in the stomach or in the small intestine. The latter is unlikely as suggested by our previous intestinal perfusion experiments with chlorogenic acid, in which no chlorogenic acid but only free phenolic acids could be detected in the mesenteric vein (15). We therefore explored chlorogenic acid absorption in the ligated stomach of rats. Chlorogenic acid was absorbed in the stomach and was identified in both the gastric vein and aorta in its intact form. Using a similar model, several polyphenols such as daidzein, genistein, quercetin, rutin, anthocyanins or ferulic acid were shown to be absorbed in the stomach (24–29). The direct absorption of chlorogenic acid shows that esterification with quinic acid does not prevent its absorption in the stomach. In contrast to our previous experiments with perfused small intestine, the absence of caffeic acid or of its conjugated forms in the gastric vein and aorta shows that the absorption of chlorogenic acid in the stomach follows different mechanisms. Chlorogenic acid could be absorbed through the bilitranslocase, which is involved in the transport of anthocyanins in the stomach (28,30) or through other unidentified organic anion transporters. Passive transport of chlorogenic acid was also described in Caco-2 cell cultures (31).

Caffeic acid also appears early in plasma and urine (Table 2, Figure 3). Its O-methylated derivatives, ferulic and isofurulic

DISCUSSION

The aim of this study was to explore the fate and metabolism of chlorogenic acid in the gastrointestinal tract of rats over the postprandial period and to determine the form under which chlorogenic acid is absorbed through the different parts of the gut barrier. Chlorogenic acid and its metabolites were estimated in the gut contents, plasma, and urine of rats fed a diet supplemented with chlorogenic acid. Chlorogenic acid was the major phenolic acid present in the gut for the 7 h of the experiment. Only traces of caffeic acid were detected in the stomach and small intestine, showing the absence of significant esterase activity in the gut lumen. Chlorogenic acid absorption in the ligated stomach of rats. Chlorogenic acid was absorbed in the stomach and was identified in both the gastric vein and aorta in its intact form. Using a similar model, several polyphenols such as daidzein, genistein, quercetin, rutin, anthocyanins or ferulic acid were shown to be absorbed in the stomach (24–29). The direct absorption of chlorogenic acid shows that esterification with quinic acid does not prevent its absorption in the stomach. In contrast to our previous experiments with perfused small intestine, the absence of caffeic acid or of its conjugated forms in the gastric vein and aorta shows that the absorption of chlorogenic acid in the stomach follows different mechanisms. Chlorogenic acid could be absorbed through the bilitranslocase, which is involved in the transport of anthocyanins in the stomach (28,30) or through other unidentified organic anion transporters. Passive transport of chlorogenic acid was also described in Caco-2 cell cultures (31).

Caffeic acid also appears early in plasma and urine (Table 2, Figure 3). Its O-methylated derivatives, ferulic and isofurulic

Aorta, 16.3 ± 1.9
Gastric vein, μmol/L 3.3 ± 1.3
Aorta, μmol/L 1.6 ± 0.3

1 Values are mean ± SEM, n = 6. *Different from rats injected in the gastric lumen, P < 0.001.
acids, were also present in urine. They most likely arose from the absorption and hydrolysis of chlorogenic acid in the mucosa of the small intestine as suggested previously (15,26). Other authors reported that caffeic acid was detected early in plasma, 30 to 60 min after consumption of pure chlorogenic acid or coffee by rats or humans (13,14). These authors suggested that chlorogenic acid was hydrolyzed in the upper digestive tract because they could not detect any chlorogenic acid in plasma. The absence of chlorogenic acid would most likely be explained by a too rapid transit through the empty stomach in these 2 studies carried out with chlorogenic acid solutions or brewed coffee in food-deprived rats or fasting humans. In contrast, when volunteers consumed coffee with a whole breakfast, chlorogenic acid was present in urine (18).

The fraction of chlorogenic acid that is not absorbed in the upper intestinal tract reaches the cecum. The low amounts of chlorogenic acid estimated in the cecal content suggest an intense microbial metabolism. Chlorogenic acid is hydrolyzed into caffeic acid and further degraded to low-molecular-weight phenolic acids such as m-coumaric acid, 3-hydroxyphenylproponic acid, or 3-hydroxybenzoic acid (32,33). These metabolites were shown to be the main metabolites identified in rat urine, together with much lower amounts of intact caffeic acid and its O-methylated metabolites (33).

In conclusion, this study shows for the first time that chlorogenic acid is not hydrolyzed in the stomach and the small intestine, but absorbed in the stomach in its intact form and as hydrolyzed forms such as caffeic and (iso)ferulic acids in the small intestine. Once reaching the cecum, chlorogenic acid is hydrolyzed into caffeic acid and further metabolized into other aromatic acids. Further studies will be required to understand the exact mechanisms of absorption and identify the transporters involved in the different parts of the gastrointestinal tract. Moreover, the role of the stomach in polyphenol absorption and the influence of the food matrix on absorption should be investigated further.

ACKNOWLEDGMENTS

The authors are grateful for the expert assistance of J. F. Martin for statistical analyses.

LITERATURE CITED