Bioavailability of the flavanone naringenin and its glycosides in rats

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Flavonoids are a widely distributed group of polyphenolic compounds characterized by a common benzo-γ-pyrone structure. Over 4,000 different flavonoids have been described, and they are categorized into flavonols, flavones, flavanones, isoflavones, catechins, and anthocyanidins. They occur naturally in fruits and vegetables, mainly as flavonoid glycosides, and are thus important constituents of the human diet. The daily Western intake of mixed flavonoids has been estimated to be in the range of 0.5 to 1 g (20), although recent investigations have reported that the actual daily intake is frequently lower (5, 14, 16). Flavonoids have been reported to exhibit a wide range of biological effects, and they may play a dietary role in reducing the risk of chronic diseases such as cardiovascular diseases and cancer (5, 13, 16).

Flavanones, such as hesperidin and naringin, have a more restricted distribution than other flavonoid compounds and are specific to citrus fruits. Citrus flavonoids have been investigated for biological activity, and anti-inflammatory, anticarcinogenic, and antitumor activities have been reported (2, 24, 26). Naringin (4’,5,7-trihydroxyflavanone-7-rhamnoglucoside or naringenin-7-rhamnoglucoside) is the predominant flavanone in grapefruit (Citrus paradisi) (up to 10% of the dry weight) and is responsible for the bitterness of grapefruit juices (29).

Although there have been some recent studies on flavone and flavanol bioavailability, data on flavanone metabolism are still scarce. Thus the aim of this work was to evaluate and compare the kinetics of absorption of the aglycone naringenin and of two of its glycosides (naringenin-7-glucoside and naringenin-7-rhamnoglucoside) in rats after a single meal. Moreover, the influence of adaptation to a diet containing naringenin or naringenin-7-rhamnoglucoside on its absorption and metabolism was also investigated.

Materials and Methods

Chemicals. Naringenin, naringenin-7-rhamnoglucoside, β-glucuronidase/sulfatase from Helix pomatia, and β-glucuronidase type VII-A from Escherichia coli were purchased from Sigma Chemical (Saint-Quentin-Fallavier, France). Naringenin-7-glucoside and daidzein were purchased from Extrasyntèse (Genay, France).

Animals and diets. Male Wistar rats (Ifa-Credo, L’Arbresle, France) weighing ~170 g were housed two per cage in temperature-controlled rooms (22°C), with a dark period from 0800 to 2000 and access to food from 0800 to 1600. They were randomly divided into two groups: nonadapted and adapted rats. Nonadapted rats were fed a semi-purified control diet for 14 days (75% wheat starch, 15%...
casein, 5% corn oil, 3.5% mineral mixture, and 1% vitamin mixture). Nonadapted rats were then further divided into four groups and received a single experimental meal (25 g/rat) of a control diet (control rats) or of a control diet supplemented with 0.25% naringenin, 0.38% naringenin-7-glucoside, or 0.5% naringenin-7-rhamnoglucoside. These amounts correspond to an equivalent supply of aglycone (9.2 mmol/kg diet). Adapted rats were divided into three groups and received either the control diet (control rats) or the control diet supplemented with 0.25% naringenin or 0.5% naringenin-7-rhamnoglucoside for 14 days. Animals were maintained and handled according to the recommendations of the Institutional Ethics Committee (Institut National de la Recherche Agronomique), in accordance with decree no. 87-848.

**Sampling procedure.** Rats were killed at 3, 6, 10, and 24 h after the beginning of the last experimental meal after being anesthetized with pentobarbital sodium (40 mg/kg body wt). Blood was withdrawn from the abdominal aorta into heparinized tubes. Plasma was acidified with 10 mM acetic acid to prevent losses of flavonoids because the pH of plasma increases with time, due to the decomposition of bicarbonates, and flavonoids are unstable at a pH higher than 7.4. For all rats, urine present in the bladder was collected. Urine from rats housed in metabolic cages was collected during the last 24 h. Cecal contents were drained by finger pressure into microfuge tubes and immediately frozen. All samples were stored at −20°C until analysis.

**HPLC analysis.** Plasma was spiked with daidzein (21.8 μM final concentration) as an internal standard and acidified to pH 4.9 with 0.1 volume of 0.58 M acetic acid solution. In the case of enzymatic hydrolysis, samples were incubated for 1 h at 37°C with 0.1 volume of 0.58 M acetic acid solution. In m final concentration) as an internal standard and acidified

H. pomatia

12,000

E. coli

mL

acetone, and 10 μL of this mixture was injected into a HPLC column and eluted as previously described. In the analysis conditions previously described, the detection limits for flavanones (naringenin, naringenin-7-glucoside, and naringenin-7-rhamnoglucoside) were 3 μM in plasma and urine and 0.15 μmol/g cecal contents.

**Results.**

HPLC analysis of standard solutions of naringenin, naringenin-7-glucoside, and naringenin-7-rhamnoglucoside indicated that, in our chromatographic conditions, naringenin-7-rhamnoglucoside and naringenin-7-glucoside had similar retention times (~22.5 min), whereas naringenin eluted at 30.8 min (Fig. 1, A and B). Representative chromatograms of plasma from control rats and from rats receiving a flavanone are presented in Fig. 1, C and D. Whatever the flavanone in the diet, the HPLC profiles of plasma shared five unidentified peaks, referred to as 1, 2, 3, 4, and 5 (Fig. 1D), corresponding to conjugated metabolites of naringenin. Neither free flavanone nor aglycone was recovered in rat plasma. The HPLC profile for control rats did not show any trace of these peaks. To confirm that the five unidentified peaks were conjugated forms of naringenin, plasma was treated by β-glucuronidase/sulfatase before HPLC analysis. The five peaks present in the nonhydrolyzed plasma disappeared after enzymatic treatment, as shown in Fig. 1E, and naringenin was released. Treatment of plasma from rats fed flavanone by a purified β-glucuronidase released ~5–10% of total plasma naringenin. The remaining part of naringenin was thus present as sulfo- or glucuronosulfonfocoujugated derivatives. Furthermore, phenolic acids, such as p-hydroxyphenylpropionic acid (p-HPPA), p-coumaric acid (p-CA), and p-hydroxybenzoic acid (p-HBA), were detected in some plasma samples, but in most samples concentrations were below the limit of quantification.

Regardless of the flavanone diet, HPLC analysis of urine collected in the bladder revealed the presence of similar glucuronosulfocoujugated derivatives as those found in plasma, as well as of some traces of phenolic acids (p-HPPA, p-CA, p-HBA). These conjugates completely disappeared after β-glucuronidase/sulfatase hydrolysis as the levels of free phenolic acids and naringenin markedly increased. No naringenin-7-glucoside or naringenin-7-rhamnoglucoside could be detected in urine.

In rats receiving a single experimental meal (nonadapted rats), the appearance of naringenin in plasma was delayed in naringenin-7-rhamnoglucoside-fed rats compared with naringenin- or naringenin-7-glucoside-fed rats (Fig. 2). Three hours after the beginning of the meal, a significant concentration of naringenin was recorded in plasma from rats fed naringenin (37.0 ± 6.2 μM) and naringenin-7-glucoside (32.9 ± 4.3 μM). In contrast, no naringenin was detected at this time in the plasma from rats fed naringenin-7-rhamnoglucoside. At 10 h, plasma concentrations of naringenin were significantly increased and similar in rats fed naringen-
nin (128 ± 2 μM), naringenin-7-glucoside (144 ± 8 μM), or naringenin-7-rhamnoglucoside (139 ± 15 μM). Twenty-four hours after the beginning of the meal, high levels of naringenin (~68 μM) were still present in plasma after all flavanone diets. The urinary excretion of naringenin over a 24-h period was significantly higher (P < 0.001) in rats fed naringenin or naringenin-7-glucoside than in rats fed naringenin-7-rhamnoglucoside (Fig. 3).

The presence of flavanones was also studied in cecal contents. Ten hours after the beginning of the experimental meal, naringenin was detected in the cecal contents of rats receiving aglycone or glycoside forms (Fig. 4). No naringenin-7-glucoside was detected in cecal contents from rats fed this glucoside. However, a
large amount of naringenin-7-rhamnoglucoside (23.2 ± 5.4 μmol) was detected in naringenin-7-rhamnoglucoside-fed rats. The identity of naringenin-7-rhamnoglucoside was verified by using chromatographic conditions that allowed separation of naringenin-7-rhamnoglucoside and naringenin-7-glucoside. In naringenin-7-rhamnoglucoside-fed rats, total flavanone accumulation in the cecum was significantly higher (P < 0.01) than in rats fed the two other diets.

Figure 5 presents the evolution of plasma naringenin concentration in nonadapted rats or rats adapted to a diet supplemented with naringenin or naringenin-7-rhamnoglucoside. In nonadapted rats, there was a progressive increase of naringenin in the plasma until 10 h, followed by a marked decrease between 10 and 24 h. At 3 and 6 h after the beginning of the single meal, plasma naringenin concentration was significantly higher (P < 0.05) after naringenin feeding than after naringenin-7-rhamnoglucoside feeding. A similar evolution of plasma naringenin concentration was observed in adapted rats. In the latter, plasma naringenin concentrations were not significantly different between the two diets except 3 h after the beginning of the last meal. Moreover, the plasma concentrations of naringenin were significantly higher (P < 0.05) after 3 h and lower (P < 0.05) after 24 h than in the nonadapted rats after the same diet. The highest plasma naringenin concentrations were recorded 10 h after the beginning of the meal for all the groups and reached ~140 μM.

Twenty-four-hour urine excretion of naringenin was not significantly different between nonadapted and adapted rats whatever the diet (Table 1). However, it was nearly twofold higher in naringenin-fed rats than in naringenin-7-rhamnoglucoside-fed ones (P < 0.001). Urinary excretion of phenolic acids (p-HPPA, p-CA, p-HBA) was not significantly different between the groups (Table 1). p-HPPA was the major phenolic acid excreted in urine after flavanone feeding, followed by p-CA and p-HBA.

Accumulation of flavanones in the cecum is depicted in Fig. 6. After a single meal, cecal naringenin content increased between 3 and 10 h and then decreased until 24 h for all of the diets. Naringenin-7-rhamnoglucoside feeding resulted in accumulation of naringenin-7-rhamnoglucoside in cecal contents from 3 to 10 h, but no more naringenin-7-rhamnoglucoside was detected 24 h after the beginning of the meal. In adapted rats, significantly lower quantities of flavanones were detected.

Table 1. Twenty-four-hour urinary excretion of naringenin and phenolic acids

<table>
<thead>
<tr>
<th>Urinary Excretion, amol/24 h</th>
<th>Single Meal</th>
<th>14-Day Adaptation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naringenin</td>
<td>N7RG</td>
<td>Naringenin</td>
</tr>
<tr>
<td>N</td>
<td>66.2 ± 3.1</td>
<td>32.0 ± 5.4*</td>
</tr>
<tr>
<td>p-HPPA</td>
<td>16.9 ± 2.2</td>
<td>21.9 ± 1.7</td>
</tr>
<tr>
<td>p-CA</td>
<td>4.01 ± 0.49</td>
<td>5.02 ± 0.64</td>
</tr>
<tr>
<td>p-HBA</td>
<td>1.24 ± 0.55</td>
<td>1.78 ± 0.34</td>
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</tbody>
</table>

Values are means ± SE; n = 4–6 rats. Rats received a single meal containing 0.25% naringenin (N) or 0.5% naringenin-7-rhamnoglucoside (N7RG) or were adapted to the same diets for 14 days. p-HPPA, p-hydroxyphenylpropionic acid; p-CA, p-coumaric acid; p-HBA, p-hydroxybenzoic acid. *P < 0.001 vs. N diet (single meal); †P < 0.001 vs. N diet (14-day adaptation).
Flavanone metabolites in plasma and urine, the impact of the glycosylation of flavanones on their bioavailability, and the influence of adaptation to the flavanone diets on bioavailability. The structure of the metabolites was observed between the different flavanone diets. Glycosides were not detected in plasma and urine, and the circulating metabolites are glucurono- and/or sulfoconjugated derivatives. Chromatographic profiles show that the same conjugated derivatives are present in plasma and urine. Naringenin was largely esterified to sulfate groups (~90%). Previous studies on rats fed quercetin or catechin have indicated that the main circulating metabolites of quercetin were sulfo and/or glucuronosulfo derivatives, whereas metabolites of catechin were only glucuronidated (22, 27). However, catechin sulfates have been reported in the plasma of volunteers given red wine (8). In contrast to catechin and quercetin (8, 22), no methylation of naringenin was observed, which was expected because naringenin contains no catechol groups and therefore is not a substrate for catechol-O-methyltransferase (25).

Naringenin was also metabolized into phenolic acids. The same compounds, namely p-HPPA, p-CA, and p-HBA, were detected in plasma and urine as previously reported (3, 11), mainly as glucurono or sulfo conjugates. These phenolic acids were formed by the intestinal microflora, as established by previous studies in germ-free or normal rats after parenteral administration of flavonoids (10–12), although it has been suggested that p-HBA is essentially a tissue metabolite, probably derived from p-CA that was metabolized to p-HBA by a mitochondrial enzyme of rat liver (11, 30).

In rats fed a single meal, the rate of naringenin appearance in plasma differed between the diets providing aglycone, glucoside, or rhamnoglucoside. Three hours after the beginning of the meal, ~35 μM naringenin was found in plasma from rats fed naringenin or its 7-glucoside, whereas at this time naringenin was not detected in any plasma samples from naringenin-7-rhamnoglucoside-fed rats. However, at maximal plasma concentrations (at 10 h), the plasma concentration of naringenin was of the same order of magnitude regardless of the diet. This underlines the influence of the glycoside moiety on the delay of intestinal absorption of naringenin-7-rhamnoglucoside. These results are in accordance with previous work demonstrating that rutin (quercetin-3-rutinoside) was absorbed more slowly than its aglycone quercetin in rats (21). Studies in humans with various glycosides of quercetin have also demonstrated that rutinoside absorption was delayed compared with the glucoside or aglycone (15, 17). Our results thus suggest that absorption of naringenin and naringenin-7-glucoside takes place early in the digestive tract (stomach or small intestine), as previously reported by Choudhury et al. (4). On the other hand, the prolonged time needed for absorption of the rhamnoglucoside suggests that it transits the small intestine and is absorbed from the cecum. The hydrolysis of naringenin-7-rhamnoglucoside suggests that it transits the small intestine and is absorbed from the cecum. The hydrolysis of naringenin-7-rhamnoglucoside may also be necessary before absorption can occur. Colonic microflora can mediate hydrolysis but are likely to degrade a portion of the liberated aglycone in the process (20). The delay in the intestinal absorption of naringenin from naringenin-7-rhamnoglucoside resulted in a marked decrease of naringenin urinary excretion. Thus, despite similar plasma concentrations of naringenin at 10 and 24 h after all three diets, the bioavailability of naringenin-7-rhamnoglucoside is lower than that for naringenin-7-glucoside or naringenin. Accordingly, biological activity of the glycoside will be lower than that of the aglycone. However, despite a lower
area of exchange in cecum than in small intestine, absorption of the aglycone from the rhamnoglucoside in the cecum seems to be quantitatively of some importance.

Examination of cecal contents has shown that no naringenin-7-glucoside was found in cecum after naringenin-7-glucoside feeding, thus indicating that the glucoside moiety of naringenin-7-glucoside had been hydrolyzed in the proximal part of the intestine or by the cecal microflora. On the other hand, after naringenin-7-rhamnoglucoside feeding, this glycoside accumulated in the cecum in significant amounts and was accompanied by some naringenin, but no glucoside had been detected. This suggests that a small part of this glycoside had been hydrolyzed to its corresponding aglycone by glycosidases produced by intestinal bacteria (11, 19). All of these results are in accordance with a recent work showing that the human small intestine possesses a cytosolic β-glucosidase capable of efficiently hydrolyzing naringenin-7-glucoside but not naringenin-7-rhamnoglucoside (7). Moreover, it has been shown in rats that the hydrolysis of flavonoid glucosides occurs mainly in the jejunum (18). In the same way, it has been reported that quercetin was absorbed after in situ perfusion of rat small intestine, whereas rutin was not (6).

Adaptation to naringenin- or naringenin-7-rhamnoglucoside-containing diets for 14 days did not result in a marked accumulation of plasma naringenin. Adapted animals showed a higher plasma naringenin concentration 3 h after the beginning of the final meal compared with nonadapted rats; however, the plasma concentration also declined faster between 10 and 24 h. This result contrasts with previous studies on flavonols showing that, after adaptation of rats to a 0.2% quercetin diet, plasma concentration of quercetin metabolites was quite constant along the nycthemere (~100 μM), and this concentration was higher than those obtained after a single quercetin meal (21). Twenty-four-hour urinary excretion of naringenin and phenolic acids was similar in adapted and nonadapted rats. This means that, during a 24-h period, similar amounts of flavanone were absorbed along the digestive tract as well as hydrolyzed in cecum. However, adaptation to the flavanone diet did affect flavanone cecal content. Indeed, in contrast to what happened in nonadapted rats, almost no flavanone accumulated into the cecum in adapted rats. In these rats, cecal microflora have adapted and thus hydrolyzed flavanones that reach the large intestine into aglycone and phenolic acids more rapidly than in nonadapted rats. Aglycone and phenolic acids were then absorbed. This explains why, despite similar amounts of flavanones that enter the cecum, few intact flavanones were recovered into cecal contents in adapted rats.

Overall, our results have demonstrated that flavanones were efficiently absorbed after being fed to rats. After a single meal, the percentage of naringenin recovered in urine (calculated as the ratio of naringenin excreted to naringenin ingested) was ~28, 31, and 14% after aglycone, glucoside, or rhamnoglucoside intake, respectively. A recent study (4) has reported that total urinary recovery of naringenin after gavage with naringenin or naringenin-7-glucoside was ~10%. Discrepancies between these results could be related to the mode of flavanone administration. Indeed, feeding rats with a semipurified diet supplemented with flavanone allows an increasing duration of intestinal absorption, whereas after gavage with a flavanone solution in overnight-fasted rats (4), the bolus of flavanone transits more rapidly along the digestive tract. Moreover, in humans fed naringenin-7-rhamnoglucoside or grapefruit juices, naringenin-7-rhamnoglucoside bioavailability was between 5 and 9% (1, 9). In our study, the magnitude of urinary excretion was similar with naringenin and naringenin-7-glucoside, suggesting that the glucose moiety did not affect the bioavailability. It should be noted that the delayed absorption of the rhamnoglucoside affects the urinary excretion, whereas the plasma concentrations were of the same magnitude during the postabsorptive period.

Compared with studies dealing with the bioavailability of flavonols in rats (21, 23), our data show that flavanones are more efficiently absorbed. Since naringenin-7-rhamnoglucoside is present in grapefruit and grapefruit juices in high amounts (from 100 to 500 mg/l in juices, depending on grapefruit variety) (28), its bioavailability in humans would be of some interest.

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