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Quercetin 3-O-β-Glucoside Is Better Absorbed than Other Quercetin Forms and Is Not Present in Rat Plasma

CHRISTINE MORAND*, CLAUDINE MANACH, VANESSA CRESPY and CHRISTIAN REMESY

Unité des Maladies Métaboliques et des Micronutriments, I.N.R.A. de Clermont-Ferrand/Theix, St Genès Champanelle, FRANCE

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The effect of the nature of the sugar moiety on quercetin absorption has been investigated in rats. Four groups of rats received an experimental meal containing 20 mg of quercetin equivalents, supplied as quercetin, quercetin 3-O-β-glucoside, quercetin 3-O-β-rhamnoside or rutin. Four hours after the meal, the metabolites identified in hydrolysed plasma were identical in all groups (3'- and 4'-methylquercetin). However, the total concentration of metabolites was markedly different: 11.2±1.8, 2.5±2.0 and 33.2±3.5 μM for the quercetin, rutin, and quercetin 3-glucoside meals respectively. After quercetin 3-rhamnoside consumption, we failed to detect any metabolites in the plasma. These data suggest that the 3-O-glucosylation improves the absorption of quercetin in the small intestine, whereas the binding of a rhamnose to the aglycone markedly depresses it. Additional experiments have shown that the higher plasma levels measured after quercetin 3-glucoside meal compared to the quercetin meal were maintained throughout the 24-hour period following the meal. Using a multi-electrode coulometric detection, together with suitable chromatographic conditions, we were able to distinguish between the conjugated and the glycosylated forms. Thus, we clearly showed the absence of quercetin 3-O-β-glucoside in the plasma from rats fed a diet containing this glucoside. This result suggests that quercetin 3-O-β-glucoside is hydrolysed before or during its intestinal absorption.

Keywords: quercetin; quercetin glycosides; flavonoid bioavailability; rat

INTRODUCTION

In the diet, most flavonoids, except catechins, are present not as aglycones but as different glycosides, bearing one or several sugars. There is much controversy as to whether natural flavonoid glycosides can be absorbed by the gastro intestinal tract, or whether they are hydrolysed in the intestine prior to absorption. Furthermore, the position and nature of the sugar residue may affect the glycoside absorption.

Enzymatic hydrolysis of β-glycosides in the stomach or small intestine, or hydrolysis by HCl in the stomach is highly improbable. Hertog et al.[1] showed that the optimum conditions to hydrolyse flavonol and flavone glycosides present in fruits and vegetables were a 2h incubation at 90 °C in 50% aqueous methanol containing 1.2M HCl. In addition, it has been reported that quercetin glycosides (3-O-glucoside, 3-O-galactoside, 3-O-rhamnoside, 4'-O-glucoside, 3-O-rutinoside, 3,4'-diglucoside) resisted conditions mimicking the stomach (prolonged acid treatment: 6h at 37°C, pH 2) and the small intestine (incubation for 5h at 37°C in the presence of amylases, proteases, and lipases).[2]

* Corresponding author: Christine MORAND Fax number: 04.73.62.46.38. email: cmorand@clermont.inra.fr

Griffiths and Barrow[3] reported that some glycosides, such as rutin and naringin, were recovered intact in the feces of germ-free rats. Other experiments showed that a crucial step was the hydrolysis of these glycosides by cecal microflora prior to their absorption.[4,5] Since the cecal microflora are also responsible for intensive degradation of the released aglycones into phenolic acids, and since the exchange area in the caecum is quite small compared to that of the small intestine, glycosides which must be hydrolysed by the cecal microflora are expected to to be less readily absorbed than their corresponding aglycones. We previously reported that in the rat, the absorption of rutin was delayed compared to that of quercetin, and that plasma concentrations were lower after consumption of rutin.[6] However, even if some glycosides such as rutin cannot be absorbed in the small intestine, it is very important not to generalize this finding to all the flavonol glycosides. Indeed, Hollman et al.[7] showed that after consumption of quercetin glucosides from onions, peak levels of quercetin were reached in human plasma in less than 0.7hr, demonstrating absorption from the stomach or the small intestine. Thus, the question of flavonol glycoside absorption must be re-examined.

Studies previously performed in our laboratory showed that, after the ingestion of quercetin or rutin by rats, the same metabolites were recovered in plasma, namely glucurono and glucuronosulfo-conjugates of quercetin and of 3'-methylquercetin.[6,8] In most studies dealing with flavonoid bioavailability, plasma analysis has been performed after acidic or enzymatic hydrolysis, so that the nature of the circulating metabolites, and especially the presence of intact glycosides, could not be determined. The aim of the present work was to compare the intestinal absorption and the nature of circulating metabolites in rats after the administration of quercetin, rutin, quercetin 3-glucoside and quercetin 3-rhamnoside.

MATERIALS AND METHODS

Animals and diets

Male Wistar rats, weighing about 170g, 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. The experimental protocols are described in Figure 1. Rats were adapted for 14 days to a control semi-purified diet containing: wheat starch (68%), casein (15%), peanut oil (5%), mineral mixture U.A.R. (6%) and vitamin mixture U.A.R. (1%). For the last meal, the control diet was supplemented with 0.1% quercetin, 0.15% quercetin 3-glucoside, 0.15% quercetin 3-rhamnoside or 0.2% rutin. In each case, animals entirely consumed the supply of 20 g of diet which corresponded to an intake of 20 mg of quercetin equivalents (or 83 mg/kg body weight). Animals were maintained and handled according to the recommendations of the Institutional Ethic Commitee (INRA), in accordance to the decree No. 87–848.

Sampling procedure

Rats were anesthetized with sodium pentobarbital (40 mg/kg b.wt.). Blood was drawn from the abdominal aorta into heparinized tubes. The plasma was immediately acidified with 10 mmol/l acetic acid to prevent flavonoid degradation. The resulting samples were analyzed immediately or stored at -80°C.

Sample preparation

For quantitative analysis, plasma was hydrolysed : 180 µl plasma was acidified to pH 4.9 with 20 µl of 0.58 M acetic acid solution, and incubated for 30 min at 37°C in the presence of 10 µl of an *Helix pomatia* extract (Sigma G-0876, containing $1x10^5$ units/ml β-glucuronidase and $5x10^3$ units/ml sulfatase). Aglycones were then

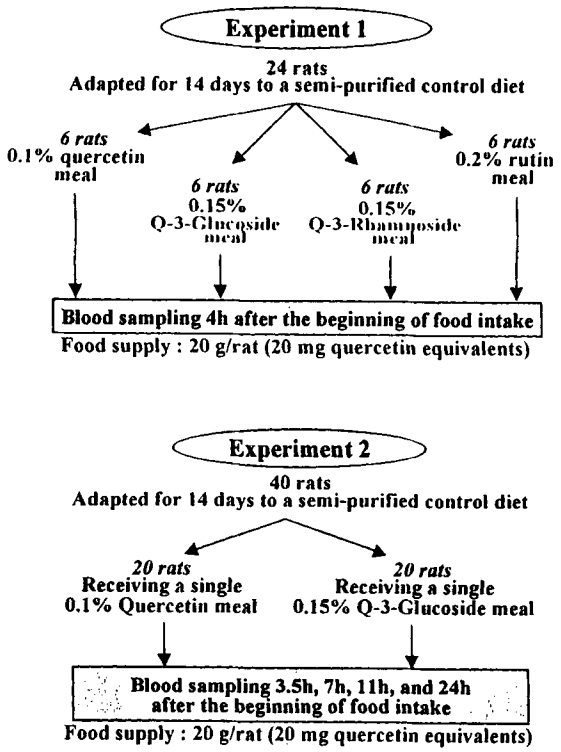


FIGURE 1 Experimental protocol

extracted with 500 µl methanol/HCl 200 mM. After centrifugation 4 min. at 14000xg, 20 µl of supernatant was injected for HPLC analysis.

For qualitative analysis of the circulating metabolites, 200 µl of plasma were extracted with 500 µl of methanol/HCl. After centrifugation for 4 min. at 14000xg, the supernatant was analysed by HPLC.

HPLC analysis

HPLC analysis of aglycones was performed using isocratic conditions with a 150x4.6 mm Hypersil BDS C18–5µ column (Life Science International, Cergy, France). The mobile phase consisted of 73% water-H_3PO_4 (99.5:0.5) and 27% acetonitrile, with a flow rate at 1.5 ml/min, and UV detection at 370 nM. To characterize the circulating metabolites of quercetin, detection was performed using an 8-electrode CoulArray Model 5600 system (Eurosep, France). The technique of multi-electrode coulometric detection (noted ECD) has been extensively described elsewhere[9,10] Briefly, when compounds pass through the detection system they are normally detected on several contiguous electrodes. For each compound, the ratio across these successive electrodes remains constant and is independent of concentration. With this technique compounds can be identified according to both their retention times and their electrochemical behaviours.

Mobile phases consisted of a 30 mM NaH_2PO_4 buffer (pH 3) containing 15% acetonitrile (A) or 40% acetonitrile (B). The column (150x4.6 mm Hypersil BDS C18–5µ) was eluted at 35°C with gradient conditions (0.8 ml/min): 0–13 min: 100% A to 100% B, 13–20 min: 100% B, 20–22 min: 100%B to 100%A, 22–32 min: 100% A. Potentials were set at 0, 50, 100, 150, 200, 250, 300, 350 mV and eight chromatograms were obtained simultaneously. These potentials are referred to a solid-state palladium reference electrode, their absolute value is about 250 mV lower than the corresponding potential measured by using an Ag/AgCl reference electrode.

RESULTS

Influence of the nature of the glycoside moiety on quercetin absorption

In order to study the absorption of the various glycosides in the small intestine, the plasma concentrations were measured four hours after the beginning of the experimental meal. When rats ingested 20 mg of quercetin supplied as aglycone or as three forms of its glycosides, marked differences were observed in the circulating levels of quercetin metabolites (Figure 2). The total concentration of metabolites, determined after plasma treatment with a β-glucuronidase/sulfatase, were 11.7±1.8 µM after ingestion of pure

quercetin, but was three fold higher when quercetin was supplied as quercetin 3-glucoside (33.2±3.5 μM). By contrast, the plasma concentrations of quercetin metabolites were quite low in rats fed the rutin diet (2.8±2.0 μM), and no metabolites were detected in the plasma of rats fed the quercetin 3-rhamnoside diet.

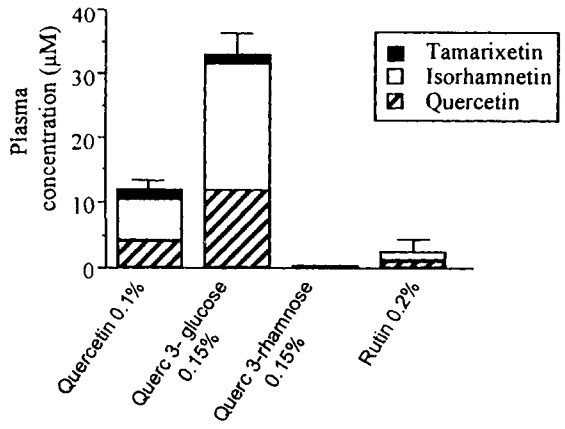


FIGURE 2 Influence of the nature of quercetin glycosylation on the efficiency of the absorption in the small intestine. Metabolites concentrations were measured in plasma 4h after the beginning of the experimental meal providing 20 mg of quercetin equivalents. Values are means ± SEM (n=6)

Whatever the quercetin source, the compounds recovered in hydrolysed plasmas were quercetin, 3′-methylquercetin (isorhamnetin) and 4′-methylquercetin (tamarixetin). The rate of methylation was quite similar in all groups (59% to 65%) and this process occured almost entirely at the 3′ position.

These data suggest that the 3-O-glucosylation improves the absorption of quercetin in the small intestine (+184%) whereas the binding of a rhamnose moiety to the aglycone markedly decreases it (from –75 to –100%).

Time curve analysis of quercetin plasma levels

To determine whether the bioavailability of quercetin was increased all along the light/dark cycle with a quercetin 3-glucoside supply, rats were killed 3.5, 7, 11, and 24 hrs after the beginning of a quercetin 3-glucoside or a quercetin meal. The shapes of the two kinetic curves are quite similar over a twenty four hour period (Figure 3), except that the plasma levels found in the quercetin 3-glucoside group were consistently higher (2.5 to 3 folds) than those determined in the quercetin group. Whatever the quercetin supply, the plasma metabolites were identified as quercetin, isorhamnetin and, for a minor part, tamarixetin. For a given time, the relative proportion of each of these metabolites are quite similar (Table I). The methylated forms of quercetin (isorhamnetin + tamarixetin) accounted for about 50% of the total metabolites concentration measured 3.5 hrs after the meal and ranged from 70 to 77% beyond 7hrs.

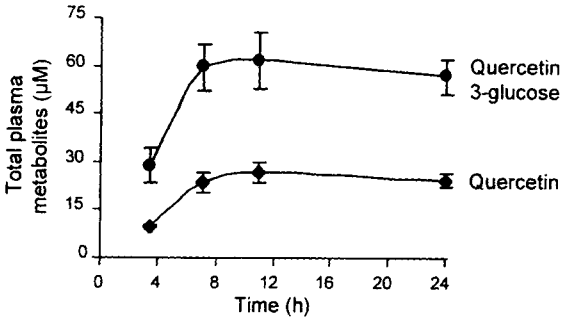


FIGURE 3 Evolution of the metabolite concentrations in the plasma of rats fed a 0.15% quercetin 3-glucose diet or a 0.1% quercetin diet over a 24h period. The total plasma metabolites corresponds to the sum of quercetin, isorhamnetin and tamarixetin liberated after hydrolysis by a β-glucuronidase/sulfatase. Values are means ± SEM (n=5 for each time point)

This experiment shows that the higher plasma levels after consumption of quercetin 3-glucoside compared to that of quercetin are not limited to the post-prandial period, but are maintained over a 24 hrs period following the meal.

Analysis of the circulating metabolites

We have compared the nature of the circulating metabolites in rats after ingestion of quercetin or quercetin 3-glucoside and we have looked for the presence of intact quercetin 3-glucoside in plasma of rats fed a meal containing this glycoside. HPLC analysis of non hydrolysed plasmas have been performed using a multi-electrode coulometric detection.

Figure 4 shows multichromatograms of a standard mix (A) and of non hydrolysed plasma from rats fed a quercetin (B) or a quercetin 3-glucoside meal (C). The gradient elution procedure has been chosen such that there is maximum separation of all compounds in the sample. The chromatogram from control plasma exhibited the same peaks as those present in the chromatograms of plasma from rats fed flavonoids for retention time lower than five minutes, and not any peak in the rest of the chromatogram (data not shown). It appears that the conjugated metabolites recovered in plasma from rats fed a quercetin or a quercetin 3-glucoside meal are identical, based on their retention times and electrochemical behaviours. In both cases the chromatograms present a predominant peak (noted 2, Tr = 7.3 min; maximal response at 200 mV) and five minor peaks, noted 1,3,4,5 and 6. The magnitude of each peak was different in the two groups in accordance with the better absorption of quercetin 3-glucoside reported above. No free quercetin or isorhamnetin were present in non hydrolysed plasma. In addition to their similar retention times, quercetin 3-glucoside and conjugated derivatives of quercetin also have close electrochemical behaviours. Thus, it was necessary to apply potentials separated from each other by small increments (+ 50 mV) to distinguish such compounds and especially quercetin 3-glucoside from the compound 4 possessing the same retention time but a lightly different electrochemical behaviour. In such analytical conditions, the absence of native quercetin 3-glucoside in plasma from rats receiving a meal containing this glucoside has been clearly confirmed by a direct supplementation of plasma with pure quercetin 3-glucoside (Figure 4D).

TABLE I Concentrations of quercetin and of its methylated forms in hydrolysed plasma from rats fed a 0.1% quercetin or a 0.15% quercetin 3-O glucose meal. Values are means ± SEM (n=5 for each time point)

		Quercetin	*Isorhamnetin*	*Tamarixetin*	*Methylated derivatives*
	(hours)		*(μM)*		*(%)*
Quercetin meal	3.5	5.2 ± 1.0	3.8 ± 0.7	0.7 ± 0.1	47
	7	5.5 ± 1.3	16.6 ± 0.9	1.3 ± 0.1	77
	10.5	7.6 ± 0.7	17.2 ± 2.3	1.7 ± 0.1	71
	24	6.1 ± 0.6	18.1 ± 2.0	ND	75
Quercetin 3-O glucose meal	3.5	13.7 + 2.4	12.9 ± 1.5	2.3 ± 0.6	53
	7	18.2 ± 2.0	38.5 ± 3.9	2.8 ± 0.4	69
	10.5	16.5 ± 2.6	40.3 ± 4.3	5.0 ± 1.3	73
	24	13.3 ± 1.0	43.8 ± 3.4	ND	77

ND: not detectable

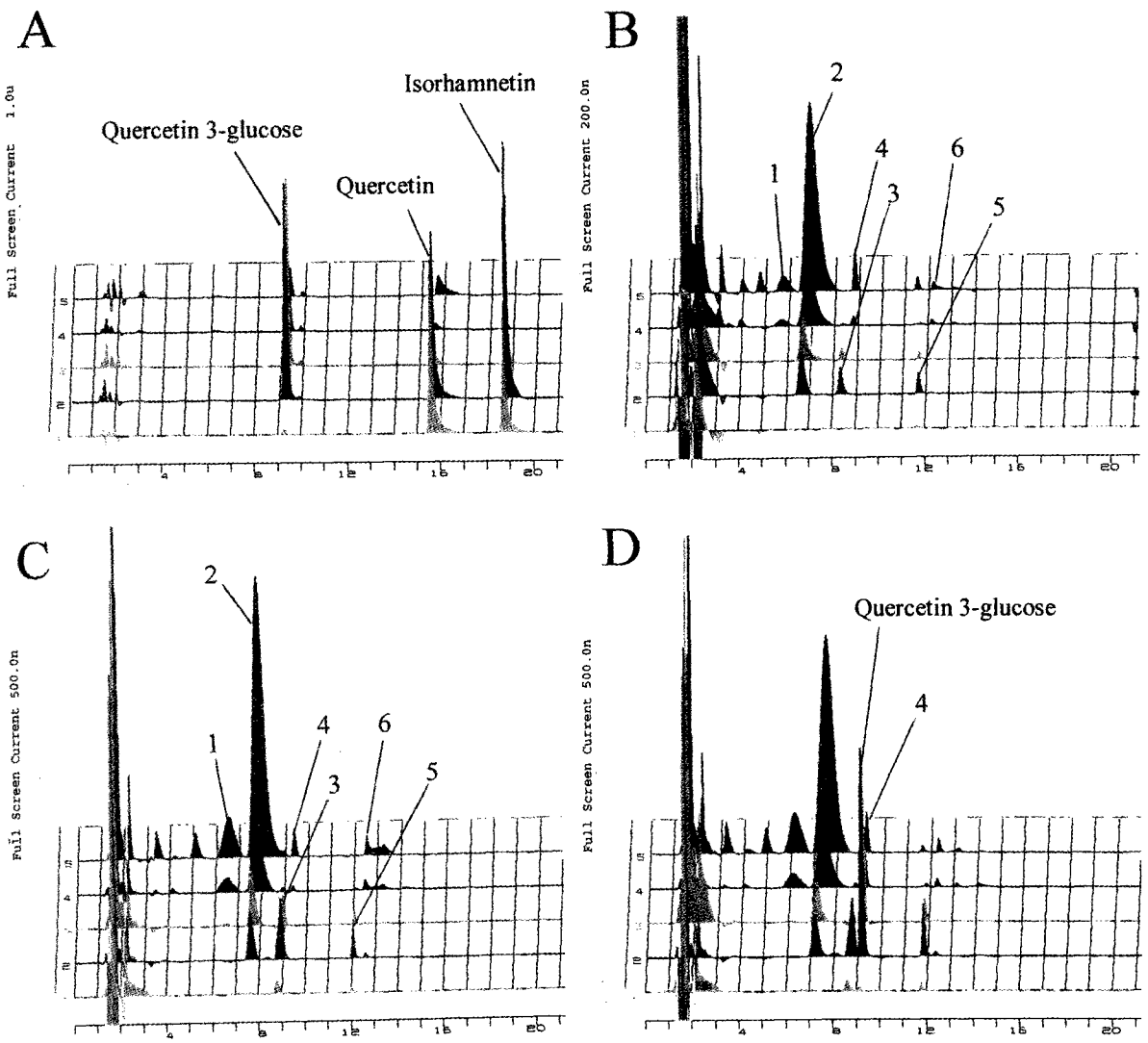
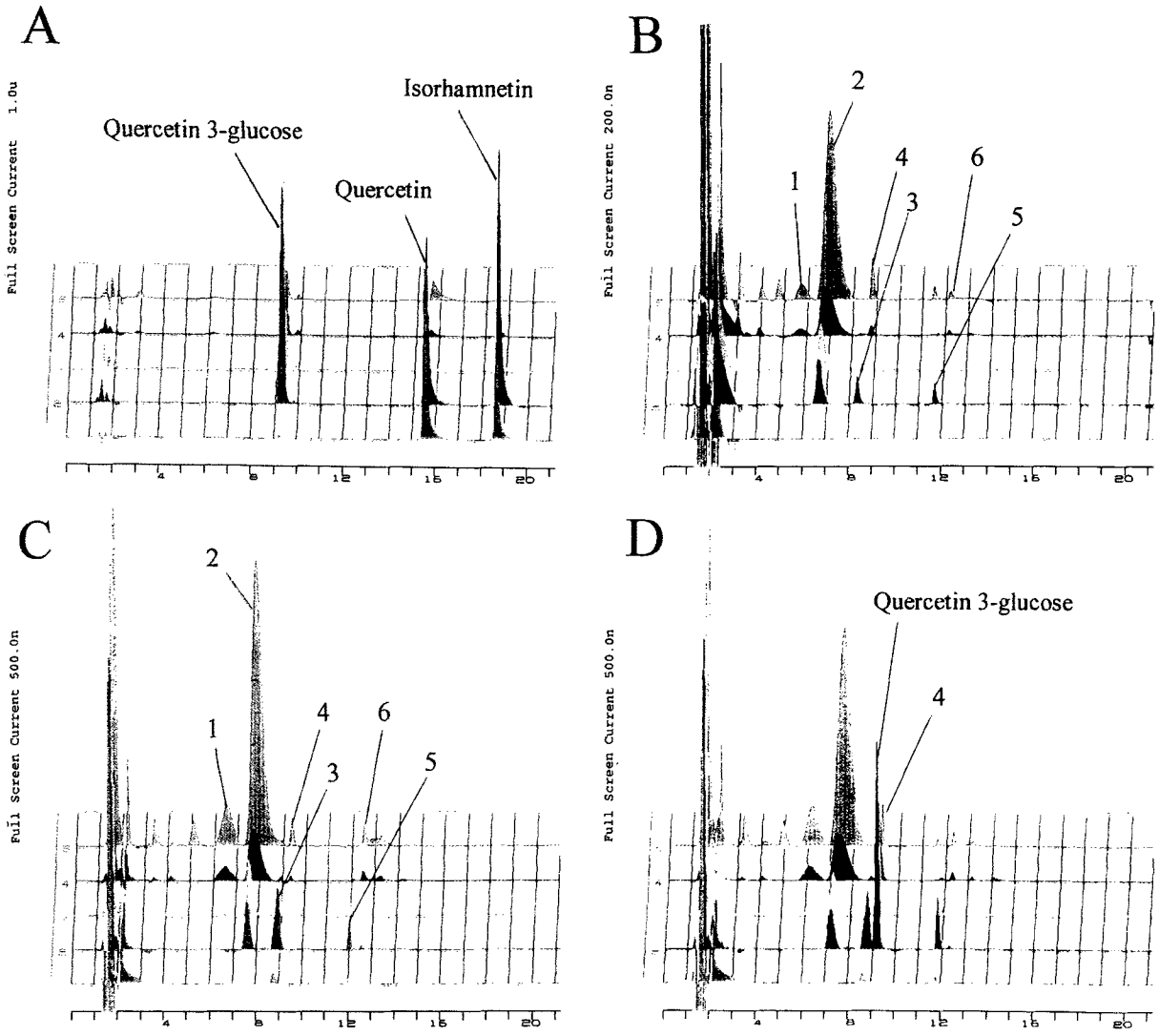


FIGURE 4 HPLC-ECD chromatograms representative of a standard mix (A) and of non hydrolysed plasma from rats fed a 0.1% quercetin (B) or a 0.15% quercetin 3-glucose meal (C). In (D) a non hydrolysed plasma from rat fed a quercetin 3-glucose meal was supplemented with a standard of this glucoside (See Color Plate I at the back of this issue)

DISCUSSION

The present study demonstrates the crucial role of the sugar moiety in the absorption efficiency of dietary quercetin in rats. So far, several experiments carried out on humans have shown that the absorption of flavonols may vary with the nature of the ingested glycosides. Hollman et al.[7] compared the absorption of about 100 mg of quercetin equivalents provided by onions, apples or pure rutin. Peak levels were reached 0.7 hrs after ingestion of onions (0.74 μM), 2.7 hrs after ingestion of apples (0.30 μM) and 9hrs after rutin (0.30 μM). A matrix effect could explain such differences, however these authors attributed them to the respective glycoside composi-



Color Plate I (See page 672, Figure 4) HPLC-ECD chromatograms representative of a standard mix (A) and of non hydrolysed plasma from rats fed a 0.1% quercetin (B) or a 0.15% quercetin 3-glucose meal (C). In (D) a non hydrolysed plasma from rat fed a quercetin 3-glucose meal was supplemented with a standard of this glucoside

tion of the supplements. Indeed, onions contained mainly glucosides of quercetin, whereas apples contained variety of quercetin glycosides including galactosides, arabinosides, rhamnosides, xylosides and glucosides. This study indicates that absorption of quercetin glucosides takes place in the stomach or small intestine, whereas other glycosides are likely absorbed from the colon. Furthermore, De Vries et al.[11] showed that absorption of quercetin from tea, rich in rutin, was about half that from onions. Hollman et al. have carried out additional experiments in which pure hydroalcoholic solutions of quercetin 4′-O-glucoside, quercetin 3-O-glucoside and rutin were administered to healthy subjects.[12,13] They confirmed that the two glucosides were much more rapidly and more efficiently absorbed than rutin. The position of the glucose molecule on the quercetin aglycone did not constitute an important factor in the absorption of glucosides.

The mechanisms involved in the absorption of flavonol glycosides at the intestinal level are still unknown. The main questions are listed in the figure 5. It is not known in which forms and by which mechanism glucosides enter the intestinal cells. Similar to sugars, diffusion of the hydrophilic polar glycosides across endothelial membranes is unlikely. Using differential scanning calorimetry, it has been shown that quercetin but not rutin was able to penetrate a dipalmitoylphosphatidylcholine vesicle as a biological model membrane.[14] Thus the aglycone could enter the cells passively or by using a transporter, whereas glycosides would inevitably use an active pathway. Hollman et al.[15]hypothesized that the active Na^+/glucose carrier, a transport protein complex located in the brush border membrane of the small intestine, is involved in the absorption of quercetin glycosides. The glucose carrier could absorb glucose even when attached to a bulky molecule such as quercetin, and thus enhance the efficiency of the flavonol absorption. Such a process has already been reported for naphthol glucosides.[16] However, there was no transepithelial flux of quercetin 4′-glucoside in the human colonic cell line Caco-2, an in vitro model reflecting human intestinal absorption.[17] Since these cells express the Na^+/glucose carrier, this result is inconsistent with the hypothesis proposed by Hollman and coworkers.[15] Although, in this study no attempt was made to look for the presence of glucuronidated and sulfated forms of quercetin. Thus, it can not be excluded that glucosides enter the Caco-2 cells, and are hydrolysed to quercetin, conjugated inside the cell, then released on the other side as conjugated forms of the aglycone.

Thus, the ability of flavonol glucosides to use the Na^+/glucose carrier should be further investigated. If the Na^+/glucose carrier is involved in the active absorption of quercetin glycosides then other quercetin glycosides (especially galactosides) would also have an efficient absorption, depending on their affinity for the carrier.

The second point which must be elucidated concerns the requirement of the glycosidic bond cleavage prior to absorption of flavonol glucosides (Figure 5). Ioku et al.[18] demonstrated a β-glucosidase activity in the duodenum, ileum and especially in the jejunum of rats. Quercetin glucosides and particularly quercetin 4′-glucoside were efficiently hydrolysed by mucosal extracts, whereas rutin constituted a poor substrate. Day et al.[19] showed that human cell-free extracts of small intestine exhibited a β-glucosidase activity towards flavonoid glycosides. Quercetin 4′-glucoside was easily hydrolysed, whereas quercetin 3-glucoside was poorly cleaved, and rutin and quercetin 3–4′-diglucoside were not deglycosylated. This activity was attributed to a broad-specificity cytosolic β-glucosidase. However, it can not be excluded from these data that the lactase phloridzin hydrolase (LPH) located in the intestinal mucosa may contribute to the deglycosylation. In an additionnal experiment, these authors showed that purified LPH from sheep small intestine was able to hydrolyse some quercetin glucosides but not

rutin.[20] Surprisingly, the majority of the glucosidase activity was from the lactase domain of LPH with only a small contribution from the phloridzin hydrolase site. Thus, the cytosolic and the membrane-bound glycosidases could both contribute to the hydrolytic activity towards flavonol glucosides. If LPH constitutes the main enzyme involved, the hydrolysis of glucosides at the membrane level could produce a concentration gradient leading to a better absorption of the aglycone moiety. The aglycone is conjugated, especially glucuronidated, inside the intestinal cells, and the glucuronidates formed are either absorbed or secreted in the lumen, as previously shown.[21]

The last question (figure 5) concerns the presence of intact glycosides in plasma. In most studies dealing with flavonoid bioavailability, plasma analysis has been performed after total hydrolysis, so that the nature of the circulating metabolites could not be determined. It must be noted that enzymatic or acidic treatment of plasma induces hydrolysis of both conjugated derivatives and glycosidic forms. In the present study, we used chromatographic conditions and multi-electrode coulometric detection suitable to distinguish glycosides from conjugates of quercetin in non-hydrolysed plasma. Using this procedure, we clearly showed that intact quercetin 3-glucoside was not present in the plasma of rats fed this glucoside. The circulating forms were identified as quercetin and methylquercetin conjugates. Similarly, Shimoi et al.[22] showed that no trace of luteolin 3-glucoside could be detected in rat plasma after oral administration of this glucoside. The main circulating forms were also conjugated forms of luteolin and methyl-luteolin. These authors also demonstrated that rat everted small intestine does not constitute a good model to investigate how glycosides are absorbed from the digestive tract. One study showed that after perfusion with quercetin 3-glucoside and rutin in an isolated rat intestine model, these glycosides were the sole or the main species recovered in serosal fluid.[23]In addition, an in situ perfusion model, which constitutes a more physiological model, has not confirmed these results.[21]

Rutin and flavonol glycosides have been detected in the plasma of two unsupplemented volunteers, using a diode array detection.[24] These authors reported concentrations ranging from 1.3 to 4.6 μM, which seems high for unsupplemented subjects. In this study, conjugated derivatives could have been mistaken for glycosides, as they exhibit very similar retention times and spectral profiles. In another study, the major metabolite present in plasma of volunteers who received 300 g of fried onions, was identified as isorhamnetin 4′-glucoside, using fluorimetric detection and co-chromatography with authentic standards.[25] However, such identification is questionable since the authors did not report a significant release of isorhamnetin after plasma hydrolysis. Furthermore, as in the previous study[24], the HPLC conditions and the detection system used were not adapted to distinguish glycosides from conjugated derivatives of quercetin. Consequently in humans, additional experiments using more discriminant techniques, such as multi-electrode coulometric or mass spectrometry detection, should be performed to unambiguously identify the circulating metabolites after ingestion of flavonol glycosides.

In conclusion, the present study, like previous studies in human, indicate that the nature of the sugar moiety markedly influences the bioavailability of dietary quercetin glycosides. In particular, glucosylation leads to a more rapid and more efficient absorption of flavonols. Thus, when a complex mixture of flavonol glycosides is present in the meal, it is likely that the absorption would be spread out over several hours after ingestion. Finally, the bioavailability of flavonoids depends on the glycoside composition of plant foods. Moreover, enrichment of food-stuffs or pharmacological preparations with glucosides might enhance the bioavailability of flavonols.

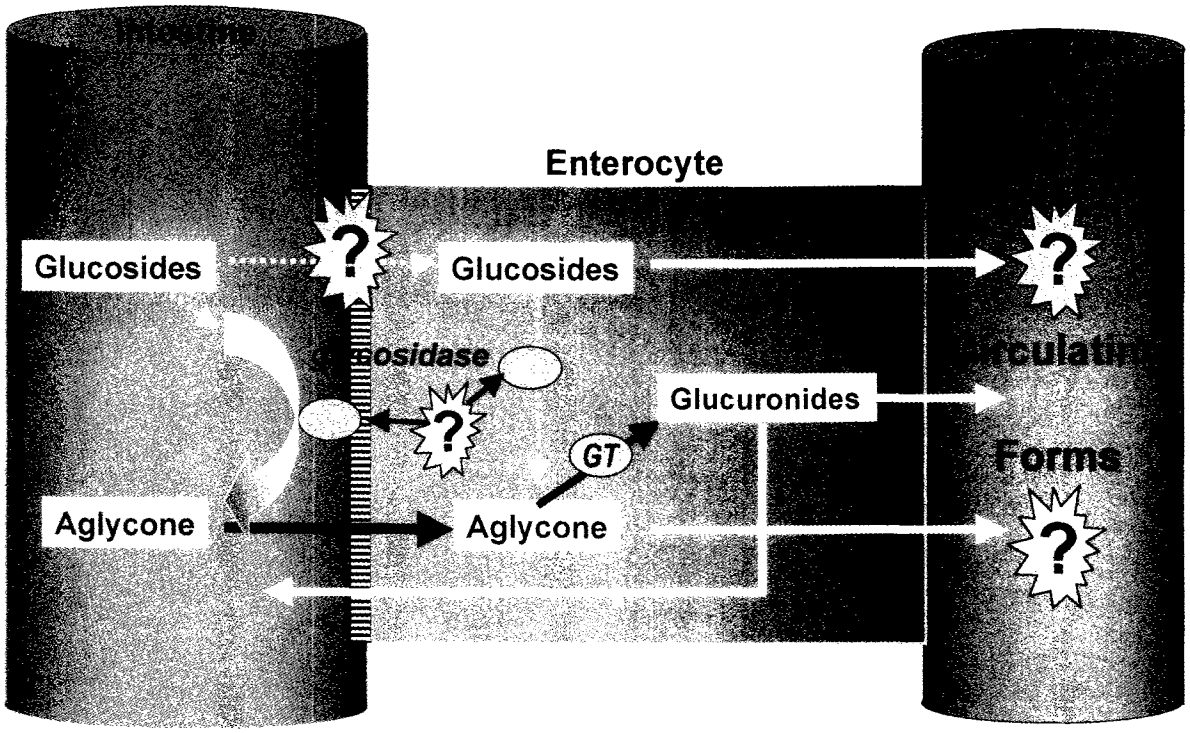


FIGURE 5 Representative diagram of quercetin glucosides metabolism at the intestinal level. Many questions must be resolved to understand the mechanisms involved in the intestinal absorption of flavonol glucosides: 1. How do glycosides enter the intestinal cell? 2. Do they have to be hydrolyzed prior to absorption? and by which enzymes? 3. How are they metabolized, especially hydrolysed and conjugated, inside the cell? 4. Are intact glycosides present in plasma? 5 How the conjugated forms synthetized by intestinal cells can be delivered in the lumen and in the blood? (See Color Plate II at the back of this issue)

Our results clearly demonstrate that intact quercetin glucosides are not present in rat plasma. However, additional studies with specific techniques must be carried out to resolve this issue in humans as it is essential to know the nature of the circulating metabolites to understand the biological effects of flavonoids.

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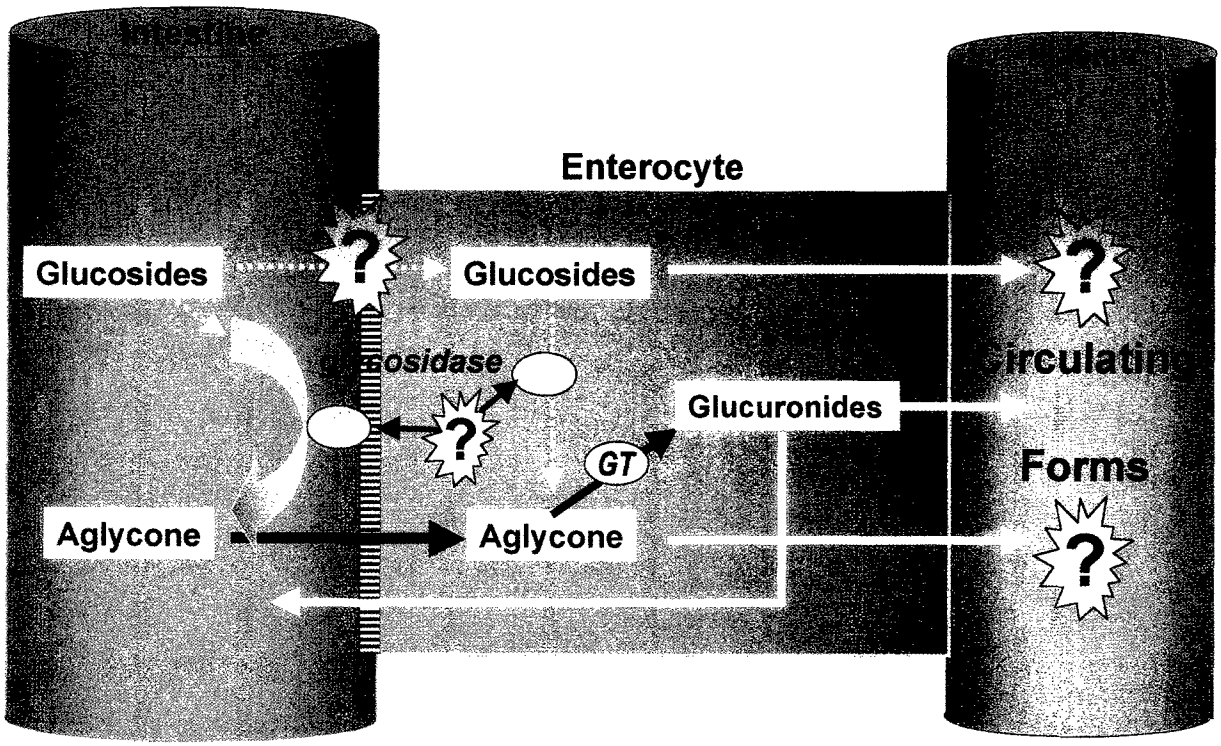

Color Plate II (See page 675, Figure 5) Representative diagram of quercetin glucosides metabolism at the intestinal level. Many questions must be resolved to understand the mechanisms involved in the intestinal absorption of flavonol glucosides: 1. How do glycosides enter the intestinal cell? 2. Do they have to be hydrolyzed prior to absorption? and by which enzymes? 3. How are they metabolized, especially hydrolysed and conjugated, inside the cell? 4. Are intact glycosides present in plasma? 5. How the conjugated forms synthetized by intestinal cells can be delivered in the lumen and in the blood?

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