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The relative contribution of the small and large intestine to the absorption and metabolism of rutin in man

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Abstract

Tomato juice containing rutin (quercetin-3-rutinoside) was ingested by healthy volunteers and ileostomists. Blood and urine collected over 24 h were analysed by HPLC with photodiode array (PDA) and tandem mass spectrometric detection. Low concentrations of isorhamnetin-3-glucuronide ($C_{max} = 4.3 \pm 1.5$ nmoles/l) and quercetin-3-glucuronide ($C_{max} = 12 \pm 2$ nmoles/l) were detected in plasma of healthy subjects. Metabolites appeared in blood after 4 h indicating absorption from the large intestine. Nine metabolites of rutin were detected in urine but with considerable variation in total amount (40 ± 1–4981 ± 115 nmoles over 24 h). No metabolites were detected in plasma or urine of ileostomists and $86 \pm 3\%$ of the ingested rutin was recovered in ileal fluid. In subjects with an intact large intestine, but not ileostomists, rutin was catabolised with the appearance of 3,4-dihydroxyphenylacetic acid, 3-methoxy-4-hydroxyphenylacetic acid and 3-hydroxyphenylacetic acid in urine accounting for 22% of rutin intake.

Keywords: *Tomato juice, rutin, ileostomy, absorption, metabolism, catabolism*

Abbreviations: *HPLC, high performance liquid chromatography; PDA, photodiode array; MS^2, tandem mass spectrometry; LPH, lactase phlorizin hydrolase; CBG, cytosolic β-glucosidase; ESI, electrospray ionisation; APCI, atmospheric pressure chemical ionization*

Introduction

The ubiquitous nature of flavonoids, phenolics and other secondary metabolites in plants ensures that they are found in substantial quantities in the human diet [1]. However, their potential health benefits are very much dependent upon their absorption and disposition in target tissues and cells [2]. For certain flavonol glycosides, such as quercetin-4′-glucoside (I in Figure 1), post-ingestion metabolism in the small intestine involves deglycosylation by luminal lactase phlorizin hydrolase (LPH) and/or cleavage within the enterocyte by cytosolic β-glucosidase (CBG) [3,4]. This is followed by metabolism of the released aglycone, which leads to the appearance of quercetin sulfate and glucuronide conjugates in the circulatory system [3,5–7]. Other flavonols, such as

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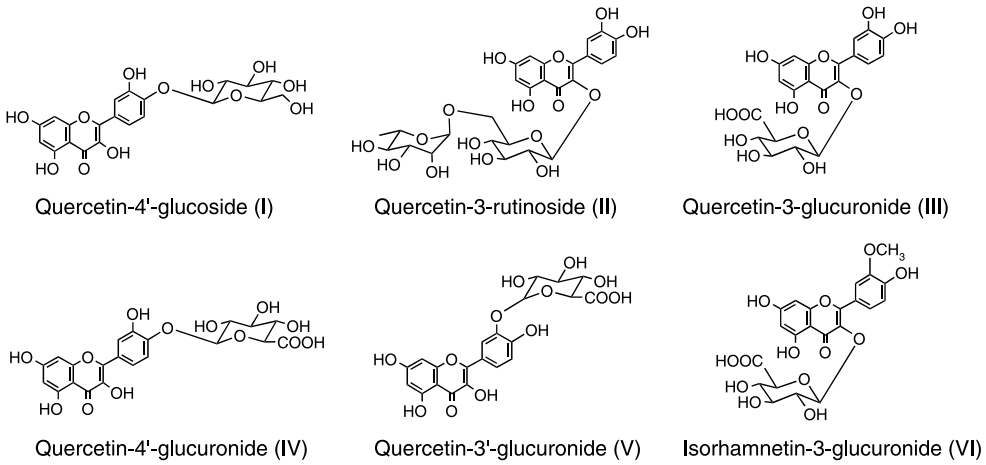

Figure 1. Structures of rutin and its metabolites detected in plasma and urine.

quercetin-3-rutinoside (rutin) (II) where the conjugating moiety is a rhamnose-glucose disaccharide, are not cleaved by either LPH or CBG and they seemingly pass intact from the small intestine to the large intestine where they are hydrolyzed and possibly degraded by the colonic microflora [8,9]. At least part of the released quercetin is absorbed [10]. This was indicated, albeit indirectly, by the appearance of rutin metabolites in plasma, 4–8 h after intake, which implies absorption in the colon rather than the small intestine [11–13]. A more direct approach to identify the colon as the major site of rutin absorption involves the use of human subjects with an ileostomy. Hollman et al. [8] fed rutin to ileostomists and samples of ileal fluid were analysed after acid hydrolysis. The released quercetin corresponded to 83% of the ingested rutin. The exact form in which rutin reached the colon could not be determined by this method. The fate of the unabsorbed rutin and it metabolites in the large intestine is therefore crucial to an understanding of the bioavailability of the quercetin disaccharide.

In addition to the formation of phase II metabolites, phenolic acids may also be formed through the degradation of the flavonoid ring by bacteria in the large intestine [9]. This paper reports on the use of HPLC with photodiode array (PDA) and tandem mass spectrometry (MS^2) detection to analyse human plasma, urine and ileal fluid collected from healthy human subjects and volunteers with an ileostomy after the consumption of tomato juice fortified with rutin. The comparison of the fate of rutin in humans with and without a colon enables the role of the colon in rutin absorption and the appearance of metabolites in plasma and urine to be determined. The analytical methods employed allowed identification and quantification of rutin metabolites and catabolites in the circulatory and excretory system and also enabled pharmacokinetic data to be obtained on these compounds, something which was not possible in previous studies.

Materials and methods

Materials

The Del Monte tomato juice was purchased from a local supermarket (Safeway Stores, Byres Road, Glasgow). HPLC grade methanol and acetonitrile were obtained from Rathburn Chemicals (Walkerburn, Scotland). Formic acid was purchased from Riedel-DeHaen (Seeize, Germany) and acetic acid from BDH (Poole, UK). 4-Hydroxyhippuric acid was obtained from Bachem (UK) Ltd, (St Helens, UK). Quercetin-3-glucuronide was extracted from French beans (Phaseolus vulgaris) and purified by partitioning against ethyl acetate and fractionation using preparative reversed phase HPLC. Quercetin-3′-glucuronide, quercetin-4′-glucuronide, quercetin-7-glucuronide, quercetin-3′-sulfate and isorhamnetin-3-glucuronide were donated by Dr Paul Needs and Dr Paul Kroon (Institute of Food Research, Norwich, UK). All other chemicals and reagents were obtained from Sigma-Aldrich (Poole, Dorset, UK).

Study design

Six healthy human subjects, (five females, one male), and five volunteers with an ileostomy, and hence no colon (four males, one female), participated in this study. They were non-smokers and not on any medication, aged between 22 and 48 years and had a body mass index of 24.5 ± 1. All the ileostomy volunteers had their operation at least 5 years prior to the study and had minimal resection of the small intestine. Subjects were required to follow a diet low in flavonoids and phenolics for two days prior to the study; avoiding most fruits, vegetables and beverages such as tea, coffee, fruit juices and wine. On the morning of the study, the volunteers were asked to consume 300 ml of tomato juice. The tomato juice had been fortified with rutin to bring the concentration to a standard 176 μmoles. Ten millilitre of

blood were collected in heparinised tubes at 0, 1, 2, 3, 4, 5, 6, 7, 8 and 24 h post-ingestion of the tomato juice and immediately centrifuged at 4000*g* for 10 min at 4°C. One millilitre aliquots of plasma were acidified with 30 μl of 50% aqueous formic acid and 100 μl of ascorbic acid (10 mM) was added to prevent oxidation. The plasma samples were then stored at −80°C prior to analysis. Urine and ileal fluid were collected prior to supplementation and over 0–2, 2–5, 5–8 and 8–24 h periods after the consumption of rutin-fortified tomato juice. The study protocol was approved by the Glasgow Royal Infirmary Local Research Ethics Committee and all subjects gave written informed consent.

Extraction of tomato juice

Aliquots of tomato juice were taken for quantitative analysis of their flavonol content. Triplicate samples of 5 ml of tomato juice were extracted with 5 ml of 50% methanol containing 1% formic acid and 20 mM sodium diethyldithiocarbamate. (+)-Catechin was used as an internal standard. This mixture was placed on a basic orbital IKA (KS 130) shaker at 350 rpm for 30 min after which it was centrifuged at 4000*g* at 4°C for 10 min. The supernatant was collected and the pellet further extracted twice with 5 ml methanol. The three supernatants were combined and reduced to dryness *in vacuo*. The dried extract was dissolved in 125 μl methanol to which 2375 μl of 1% formic acid was added and 10 μl aliquots were analysed by HPLC–PDA–MS^2.The extraction recovery for the internal standard was 94 ± 4.5%.

Extraction of flavonoids from plasma

Triplicate samples of plasma were extracted using the method by Day et al. [5] with slight modifications. Two hundred and fifty microlitre of plasma was added dropwise to 600 μl of acetonitrile to precipitate the proteins. The mixture was vortexed for 30 s every 2 min over a 10 min period, before centrifuging at 13,000*g* at 4°C for 20 min. The supernatant was retained and the pellet re-extracted as described above but with methanol instead of acetonitrile. Experiments with quercetin-3-glucuronide and quercetin-3′-sulphate showed recoveries of ca. 75% with the initial acetonitrile extraction which increased by a further 10–12% with the second methanolic extraction. The two supernatants were combined and reduced to dryness *in vacuo*. Extracts were then dissolved in 25 μl of methanol plus 225 μl of 1% formic acid in water and centrifuged at 16,000*g* at 4°C for 2 min prior to the analysis of 250 μl aliquots of the supernatant by HPLC–PDA–MS^2.(+)-Catechin was used as an internal standard with 625 ng being added to acetonitrile prior to the addition of the plasma. The extraction recovery for the internal standard from the spiked samples was 85 ± 4.9%.

Extraction of flavonoids from ileal fluid

Ileal fluid was collected at the specified time points where all the contents of the pouch were emptied and stored at −20°C before transfer to −80°C within 24 h. Prior to extraction, the ileal fluid was defrosted and thoroughly mixed. Triplicate 0.5 g samples were extracted with 3 ml of 95% methanol containing 1% formic acid and 20 mM sodium diethyldithiocarabamate. Five microgram (+)-catechin was added to the extraction mixture to act as an internal standard. Samples were homogenised for 5 min (Disruptor Genie, Scientific Industries) and then centrifuged at 16,000*g* for 15 min. The supernatant was collected and the pellet re-extracted twice as described above. The three supernatants were combined and reduced to dryness *in vacuo*. Extracts were then made up to 1 ml with 50 μl methanol and 950 μl 1% formic acid. Aliquots of the centrifuged supernatant 20–100 μl were analysed by HPLC–PDA–MS^2.

Preparation of urine

Urine samples were defrosted, thoroughly mixed, centrifuged at 16,000*g* at 4°C for 2 min prior to injection of 200 μl aliquots of the supernatant into the HPLC–PDA–MS^2 for analysis of flavonoids and phenolic acids.

HPLC with diode array and MS^2 detection

Samples were analysed on a Surveyor HPLC system comprising of a HPLC pump, PDA detector, scanning from 250 to 700 nm and an autosampler cooled to 4°C. (Thermo Finnigan, San Jose, USA). Separation was carried out using a 250 × 4.6 mm I.D. 4 μm Synergi Max-RP column for flavonoids and (Phenomenex, Macclesfield, UK) eluted with a 65 min gradient of 5–43% acetonitrile in 1% formic acid at a flow rate of 1 ml min^{-1} and maintained at 40°C. Similar conditions were adopted for phenolic acid detection, but being less polar, a 250 × 4.6 mm I.D. 4 μm Synergi Hydro-RP column and a 60 min gradient of 2–20% acetonitrile in 0.25% acetic acid was used. After passing through the flow cell of the diode array detector, the column eluate was split and 0.3 ml min^{-1} was directed to a LCQ DecaXP ion trap mass spectrometer fitted with either an electrospray ionisation (ESI) or an atmospheric pressure chemical ionisation (APCI) interface (Thermo Finnigan). Analysis was carried out using full scan, data dependant MS^2 scanning from *m/z* 100 to 1000. For the ESI interface, the capillary temperature was

250°C, sheath gas and auxiliary gas were 40 and 5 units, respectively, and the source voltage was 5 kV for negative ionisation. For the APCI interface, the capillary temperature was 130°C, vaporizer temperature was 350°C, sheath gas and auxiliary gas were 80 and 60 units, respectively, and the source voltage was 6 kV for negative ionisation. Rutin, quercetin-3-glucuronide and isorhamnetin-3-glucuronide were all quantified by reference to standard calibration curves at 365 nm. Other flavonols were quantified in quercetin-3-glucuronide equivalents. 3-Hydroxyphenylacetic acid, 3,4-dihydroxyphenylacetic acid and 4-hydroxy-3-methoxyphenylacetic acid were quantified by reference to standard calibration curves at 280 nm. Other phenolic acids were quantified in 2-hydroxyhippuric acid equivalents. Peak identification was confirmed by co-chromatography and/or MS^2 fragmentation data.

Pharmacokinetic analysis of plasma metabolites

Maximum post-ingestion plasma concentration of rutin metabolites was defined as C_{max}. The time to reach maximum plasma concentration (T_{max}) was defined as the time in hours at which C_{max} was reached. The elimination half-life for the metabolites in hours was computed by using the following formula $T_{1/2} = 0.693/K_e$, where K_e is the slope of the linear regression of the log of the plasma metabolite concentrations during the elimination phase. The data points used in this calculation were 4, 5, 6, 7 and 8 h for quercetin-3-glucuronide and 6, 7 and 8 h or isorhamnetin-3-glucuronide.

Statistical analyses

Each sample was analysed in triplicate and data were presented as mean values ± standard error ($n = 3$).

Results

HPLC-tandem mass spectrometry analysis of tomato juice

In total, nine phenolic compounds were identified in the unfortified tomato juice based on PDA and ESI–MS^2 data. These being 3-caffeoylquinic acid, caffeic acid hexose conjugate, 5-caffeoylquinic acid, quercetin-3-rutinoside-7-glucoside, quercetin-rutinoside-pentoside, rutin, kaempferol-3-rutinoside, 3,4-dicaffeoylquinic acid and naringenin. In keeping with the data of Woeldecke and Herrmann [14], the major component was rutin with 300 ml of juice containing 7.3 ± 0.9 mg (12.0 ± 1.4 μmoles). In an earlier study, lightly fried onions containing 275 μmoles of quercetin glucosides were ingested by human volunteers [15]. In order that the amounts fed in the two studies would be broadly comparable, tomato juice was spiked with rutin so that the 300 ml supplement contained a total of 176 μmoles of the quercetin disaccharide.

Identification and quantification of conjugated metabolites in the plasma and urine of volunteers with a colon

Plasma and urine collected at the different time points over a 24 h period from the healthy volunteers with a colon after ingestion of the rutin-spiked tomato juice were analysed by HPLC–PDA–ESI–MS^2. In the 365 nm traces obtained with plasma at 0, 2 and 3 h samples and with urine at 0, 0–2 h samples no peaks corresponding to any flavonol-based compounds were detected. However, in the 4–8 h plasma samples and the 2–5 h urine samples onwards, small quantities of quercetin-based compounds were detected. A total of nine conjugated quercetin metabolites were detected in urine and two in plasma. Typical HPLC traces obtained at $A_{365\,nm}$ are shown in Figure 2 and identifications based on MS^2 spectra are summarised in Table I. The different classes of metabolites that were detected can be summarised as follows:

Quercetin monoglucuronides. Three quercetin monoglucuronides were detected, each being characterised by a negatively charged molecular ion ($[M - H]^-$) at m/z 477, which on MS^2 fragments with a loss of 176 amu, corresponding to the cleavage of a glucuronide unit, to produce an ion at m/z 301 from quercetin. Co-chromatography with reference compounds facilitated the identification of quercetin-3-glucuronide (III) (*peak 5*), quercetin-4′-glucuronide (IV) (*peak 8*) and quercetin-3′-glucuronide (V) (*peak 9*).

Quercetin diglucuronides. *Peak 6* was present only in urine of subjects from volunteers 1 and 2. It had a $[M - H]^-$ at m/z at 653 which yielded MS^2 fragments at m/z 477 ($[M - H]^- - 176$) and m/z 301 ($[M - H]^- - 352$), loss of two glucuronide units, indicating the presence of a quercetin diglucuronide.

Isorhamnetin-3-glucuronide. This compound was detected in both urine and plasma and corresponded to *peak 7*, which was characterised by a mass spectrum with fragment ions at m/z values 14 amu higher than obtained with quercetin glucuronides. *Peak 7* co-chromatographed with an authentic isorhamnetin-3-glucuronide (VI) standard.

Methylquercetin diglucuronides. *Peak 1*, which yielded an MS^2 spectrum with fragment ions 14 amu higher than that of *peak 6*, was a methyl quercetin diglucuronide. It was present only in the urine of volunteers 1 and 2.

Quercetin glucoside glucuronides. *Peaks 2–4* which were detected in the urine had a $[M - H]^-$ at m/z 639 which on MS^2 yielded ions at m/z 477 ($[M - H]^- - 162$, loss of glucose), m/z 463 ($[M - H]^- - 176$, loss of a

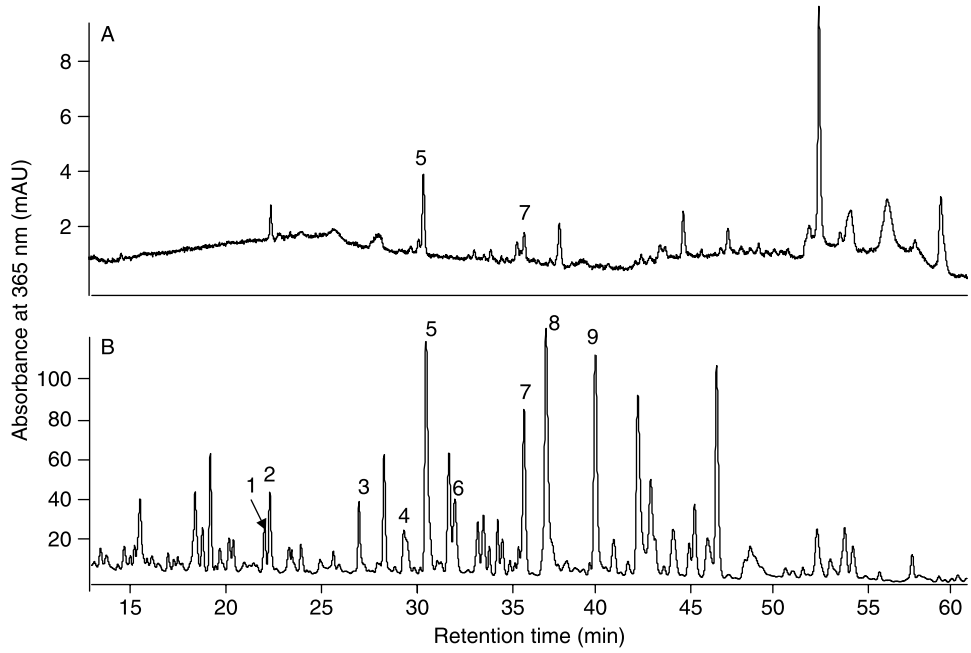

Figure 2. Gradient reversed-phase HPLC with detection at 365 nm of quercetin metabolites in (A) a plasma extract and (B) urine obtained from volunteer 1 after consumption of tomato juice containing with 176 μmoles of rutin. For identification of peaks refer to Table I.

glucuronide unit) and *m/z* 301 indicating that these compounds are quercetin glucoside glucuronides.

Of these nine quercetin metabolites, only two, quercetin-3-glucuronide and isorhamnetin-3-glucuronide, were detected in the plasma (Table I). The 0–24 h pharmacokinetic profiles of these two metabolites are shown in Figure 3. Neither compound was present in plasma collected either immediately before ingestion (0 h), or at 2, 3 and 24 h time points. It is evident from the data in Table II that the extent to which the two metabolites accumulated in plasma varied between the volunteers with the highest concentrations being found in samples from volunteer 1 and the lowest in plasma from volunteers 4–6. A pharmacokinetic analysis of the data is summarised in Table III. The major component was quercetin-3-glucuronide with a C_{max} of 12 ± 2 nmoles/l while isorhamnetin-3-glucuronide had a C_{max} of 4.3 ± 1.5 nmoles/l. The T_{max} of quercetin-3-glucuronide was 4.7 ± 0.3 h and that of isorhamnetin-3-glucuronide was 5.4 ± 0.2 h. The plasma concentration of quercetin-3-glucuronide returned to baseline levels 8 h after ingestion of the rutin supplement and the $T_{1/2}$ value was 5.7 ± 0.6 h. The $T_{1/2}$ of isorhamnetin-3-glucuronide was estimated to be 6.9 ± 0.3 h (Table III). However, in the case of two of the six volunteers, the trace levels of this metabolite did not go back to baseline values after 8 h and as a result its mean concentration at 8 h was still ca. 20% of the C_{max} (Table II). To draw a more accurate picture of the $T_{1/2}$

Table I. HPLC–MS^2 identification of quercetin metabolites detected in plasma and urine from human volunteers with a colon post-consumption of 300 ml tomato juice containing 176 μmoles rutin*.

Peak	t_R (min)	Compound	$[M-H]^-$ (*m/z*)	MS^2 fragments ions (*m/z*)	Location
1	21.5	Methylquercetin diglucuronide	667	491([M − H]$^-$-GlcUA), 315([M − H]$^-$-GlcUA-GlcUA)	Urine
2	21.9	Quercetin glucoside glucuronide	639	477([M-H]$^-$-Glc), 463([M-H]$^-$-GlcUA), 301([M-H]$^-$-GlcUA-Glc)	Urine
3	26.8	Quercetin glucoside glucuronide	639	477([M − H]$^-$-Glc), 463([M − H]$^-$-GlcUA), 301([M − H]$^-$-GlcUA-Glc)	Urine
4	29.1	Quercetin glucoside glucuronide	639	477([M − H]$^-$-Glc), 463([M − H]$^-$-GlcUA), 301([M − H]$^-$-Glc-GlcUA)	Urine
5	30.5	Quercetin-3-glucuronide	477	301 ([M − H]$^-$-GlcUA)	Urine, plasma
6	31.9	Quercetin diglucuronide	653	477([M − H]$^-$-GlcUA), 301([M − H]$^-$-GlcUA-GlcUA)	Urine
7	35.7	Isorhamnetin-3-glucuronide	491	315([M − H]$^-$-GlcUA)	Urine, plasma
8	36.7	Quercetin-4′-glucuronide	477	301([M − H]$^-$-GlcUA)	Urine
9	39.8	Quercetin-3′-glucuronide	477	301([M − H]$^-$-GlcUA)	Urine

* Peak numbers and HPLC retention times refer to HPLC trace in Figure 1. t_R, retention time; $[M-H]^-$, negatively charged molecular ion; Glc, glucosyl unit; GlcUA, glucuronyl unit.

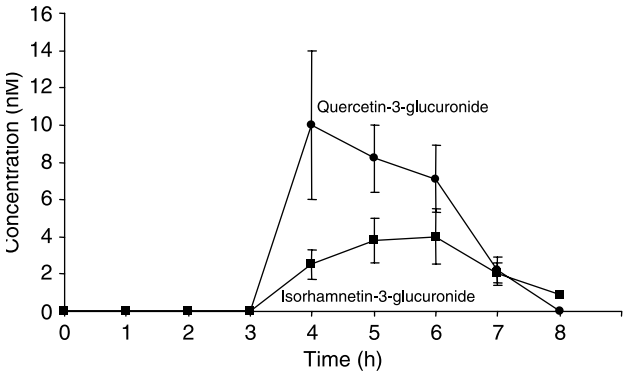

Figure 3. Mean concentration of quercetin-3-glucuronide and isorhamnetin-3-glucuronide in the plasma of six healthy human subjects 0–8 h after the consumption of tomato juice containing 176 μmoles of rutin. Error bars indicate standard error. Neither metabolite was detected in plasma collected 24 h after ingestion.

of isorhamnetin-3-glucuronide, time points later than 8 h would have to be analysed. Nevertheless, based on the behaviour of quercetin-3-glucuronide we would anticipate a rapid decrease to 0 h levels which would have only a limited effect in extending the T_{max}.

There were considerable inter-individual variations in the excretion of the urinary metabolites (Table IV). Over a 24 h period, volunteer 1 excreted a total of 4981 nmoles of metabolites which corresponds to 2.8% of the ingested rutin. In contrast, excretion by the other subjected ranged from 40 to 608 nmoles, equivalent to 0.02–0.35% of intake. A total of nine metabolites were detected in the urine of volunteers 1 and 2, namely quercetin diglucuronide, methyl-quercetin diglucuronide, three isomers of quercetin glucoside glucuronide, quercetin-3-glucuronide, quercetin-4′-glucuronide, quercetin-3′-glucuronide and isorhamnetin-3-glucuronide. The lower level of excretion of rutin metabolites by volunteers 3–6, was limited to isorhamnetin-3-glucuronide and the 3-, 3′- and 4′-glucuronides of quercetin (Table IV). The main urinary metabolites present in all the volunteers, with the exception of volunteer 4, were the three quercetin glucuronides with quantities excreted over the 24 h period ranging from 29 to 3521 nmoles. Isorhamnetin-3-glucuronide was also a significant metabolite with the amount excreted ranging from 11 to 686 nmoles.

Identification and quantification of rutin metabolites in the ileal fluid, plasma and urine of ileostomy volunteers

The plasma and urine from volunteers with an ileostomy contained none of the quercetin metabolites that were present in samples from the subjects with an intact colon. However, ileal fluid collected over a 24 h period was found to contain 151 ± 5 μmoles of unmetabolised rutin which corresponds to 86 ± 3% of the amount ingested. Most of the rutin was excreted in the ileal fluid 2–5 h post ingestion of tomato juice (Table V).

Identification of rutin catabolites in urine

On the basis of HPLC–PDA and MS^2 obtained with an APCI interface, six phenolic acids were identified in urine of healthy volunteers with a colon and three in volunteers without a colon. The identification of the phenolic compounds is based on the evidence discussed below, which is summarised in Table VI.

Peak 1 ($t_R = 13.6$ min, $\lambda_{max} = 260$ nm) had a $[M - H]^-$ at *m/z* 194 and MS^2 yielded a major ion at *m/z* 100 and a smaller fragment at *m/z* 93. The mass spectrometric data and co-chromatography with an authentic standard identified this compound as 4-hydroxyhippuric acid.

Peak 2 ($t_R = 15.9$ min, $\lambda_{max} = 280$ nm) had a $[M - H]^-$ at *m/z* 167 with MS^2 producing an ion

Table II. Concentration of quercetin metabolites in the plasma of six human subjects 0–24 h after the consumption 300 ml of tomato juice containing 176 μmoles of rutin*.

Metabolite (peak number)	Subject	4 h	5 h	6 h	7 h	8 h
Queercetin-3-glucuronide (5)	1	22 ± 6	14 ± 1	14 ± 1	4.6 ± 0.3	n.d.
	2	24 ± 2	10 ± 1	10 ± 2	2.8 ± 0.7	n.d.
	3	11 ± 0	13 ± 1	7.6 ± 0.5	2.7 ± 0.5	n.d.
	4	5.2 ± 1.5	4.4 ± 0.6	2.0 ± 0.2	n.d.	n.d.
	5	n.d.	3.9 ± 1.0	3.7 ± 0.9	2.9 ± 0.7	n.d.
	6	n.d.	4.6 ± 0.7	5.2 ± 0.4	n.d.	n.d.
	Mean	10 ± 4	8.2 ± 1.8	7.1 ± 1.8	2.2 ± 0.7	n.d.
Isorhamnetin-3-glucuronide (7)	1	4.3 ± 0.1	8.2 ± 0.4	10 ± 0	4.0 ± 0.2	n.d.
	2	5.7 ± 0.7	5.9 ± 0.5	7.7 ± 2.3	2.9 ± 0.2	3.2 ± 0.2
	3	1.2 ± 0.1	3.8 ± 0.3	3.3 ± 0.2	2.7 ± 0.5	2.0 ± 0.2
	4	2.2 ± 0.2	2.9 ± 0.2	1.7 ± 0.2	1.2 ± 0.2	n.d.
	5	1.8 ± 0.1	2.1 ± 0.0	1.2 ± 0.0	0.9 ± 0.1	n.d.
	6	n.d.	n.d.	n.d.	n.d.	n.d.
	Mean	2.5 ± 0.8	3.8 ± 1.2	4.0 ± 1.5	2.0 ± 0.6	0.9 ± 0.6

* Data for the individual subjects are expressed as nmoles/l ± standard error ($n = 3$). Mean values are expressed as nmoles/l ± standard error ($n = 6$). No metabolites are detected in 0, 2, 3 and 24 h plasma samples. n.d., not detected. For MS^2 data and identification of peaks, refer to Table I and Figure 1.

Table III. Pharmacokinetic parameters of quercetin metabolites in the plasma of human volunteers after the consumption 300 ml of tomato juice containing 176 μmoles of rutin*.

Metabolite	C_{max}	T_{max}	$T_{1/2}$
Quercetin-3-glucuronide	12 ± 2	4.7 ± 0.3	5.7 ± 0.6
Isorhamnetin-3-glucuronide	4.3 ± 1.5	5.4 ± 0.2	6.9 ± 0.3

* C_{max}, maximum concentration in plasma expressed in nmoles/l; T_{max}, time to reach C_{max} expressed in hours; $T_{1/2}$, the elimination half-life of metabolites in hours. Data presented as mean values ± standard error ($n = 6$). n.d., not detected.

at *m/z* 123 and a smaller fragment at *m/z* 108. The mass spectrometric data and co-chromatography with an authentic standard indicate that this compound is 3,4-dihydroxyphenylacetic acid.

Peak 3 ($t_R = 26.8$ min, $\lambda_{max} = 270$ nm) had a $[M - H]^-$ at *m/z* 151 which on MS^2 produced three charged fragments, the main one at *m/z* 107 with less intense ions at *m/z* 93 and 121. The mass spectrometric data and co-chromatography with an authentic standard demonstrated that this compound is 3-hydroxyphenylacetic acid.

Peak 4 ($t_R = 29.1$ min, $\lambda_{max} = 280$ nm) had a $[M - H]^-$ at *m/z* 181 with MS^2 yielding an ion at *m/z* 137. The mass spectrometric data and co-chromatography with an authentic standard identified this compound is 3-methoxy-4-hydroxyphenylacetic acid.

Peak 5 ($t_R = 33.2$ min, $\lambda_{max} = 300$ nm) had a $[M - H]^-$ at *m/z* 194 which yielded an MS^2 ion at *m/z* 150. This data and co-chromatography with an authentic standard demonstrate the presence of 2-hydroxyhippuric acid.

Peak 6 ($t_R = 39.1$ min, $\lambda_{max} = 300$ nm), like *peak 5*, had a $[M - H]^-$ at *m/z* 194 with MS^2 yielding a

Table IV. Quantity of metabolites in urine of six healthy human subjects 0–24 h after consumption of tomato juice containing 176 μmoles of rutin*.

Subject	Metabolites (peak number)	2–5 h	5–8 h	8–24 h	Total	(% of intake)
1	Methylquercetin diglucuronide (*1*)	94 ± 5	35 ± 4	n.d.	129 ± 9	
	Quercetin glucoside glucuronide (*2*)	216 ± 30	n.d.	n.d.	216 ± 30	
	Quercetin glucoside glucuronide (*3*)	131 ± 26	n.d.	n.d.	131 ± 26	
	Quercetin glucoside glucuronide (*4*)	112 ± 10	9 ± 0	n.d.	121 ± 9	
	Quercetin-3-glucuronide (*5*)	1122 ± 90	70 ± 26	n.d.	1192 ± 104	
	Quercetin diglucuronide (*6*)	147 ± 27	28 ± 4	n.d.	176 ± 23	
	Isorhamnetin-3-glucuronide (*7*)	547 ± 5	140 ± 5	n.d.	686 ± 5	
	Quercetin-4′-glucuronide (*8*)	1198 ± 8	23 ± 3	n.d.	1221 ± 10	
	Quercetin-3′-glucuronide (*9*)	1108 ± 62	n.d.	n.d.	1108 ± 62	
	Total metabolites	4675 ± 90	306 ± 30	n.d.	4981 ± 115	2.8%
2	Methylquercetin diglucuronide (*1*)	6 ± 0	15 ± 2	n.d.	21 ± 2	
	Quercetin glucoside glucuronide (*2*)	11 ± 0	5 ± 1	n.d.	16 ± 1	
	Quercetin glucoside glucuronide (*3*)	2 ± 1	n.d.	n.d.	2 ± 1	
	Quercetin-3-glucuronide (*5*)	71 ± 1	67 ± 2	4 ± 1	142 ± 2	
	Quercetin diglucuronide (*6*)	7 ± 1	12 ± 1	n.d.	18 ± 2	
	Isorhamnetin-3-glucuronide (*7*)	30 ± 3	40 ± 1	60 ± 2	129 ± 3	
	Quercetin-4′-glucuronide (*8*)	87 ± 2	35 ± 1	n.d.	122 ± 3	
	Quercetin-3′-glucuronide (*9*)	92 ± 1	46 ± 0	19 ± 1	157 ± 62	
	Total metabolites	305 ± 3	219 ± 3	82 ± 1	608 ± 4	0.35%
3	Quercetin-3-glucuronide (*5*)	81 ± 6	43 ± 2	51 ± 19	175 ± 14	
	Isorhamnetin-3-glucuronide (*7*)	10 ± 0	20 ± 1	±	64 ± 2	
	Quercetin-4′-glucuronide (*8*)	18 ± 1	18 ± 1	n.d.	36 ± 0	
	Quercetin-3′-glucuronide (*9*)	10 ± 1	17 ± 1	10 ± 1	37 ± 1	
	Total metabolites	118 ± 6	97 ± 1	95 ± 19	310 ± 14	0.18%
4	Quercetin-3-glucuronide (*5*)	16 ± 1	7 ± 0	n.d.	23 ± 1	
	Isorhamnetin-3-glucuronide (*7*)	21 ± 0	16 ± 0	82 ± 1	120 ± 0	
	Quercetin-4′-glucuronide (*8*)	41 ± 1	n.d.	n.d.	41 ± 1	
	Quercetin-3′-glucuronide (*9*)	4 ± 0	n.d.	n.d.	4 ± 0	
	Total metabolites	82 ± 2	23 ± 1	82 ± 1	187 ± 2	0.11%
5	Quercetin-3-glucuronide (*5*)	6 ± 2	12 ± 2	46 ± 3	64 ± 5	
	Isorhamnetin-3-glucuronide (*7*)	21 ± 0	17 ± 1	27 ± 0	65 ± 0	
	Quercetin-4′-glucuronide (*8*)	1 ± 0	1 ± 0	n.d.	2 ± 0	
	Quercetin-3′-glucuronide (*9*)	2 ± 0	6 ± 0	2 ± 0	10 ± 1	
	Total metabolites	30 ± 1	36 ± 2	75 ± 3	141 ± 5	0.07%
6	Quercetin-3-glucuronide (*5*)	n.d.	3 ± 0	24 ± 1	27 ± 1	
	Isorhamnetin-3-glucuronide (*7*)	4 ± 0	7 ± 0	n.d.	11 ± 0	
	Quercetin-3′-glucuronide (*9*)	n.d.	n.d.	2 ± 0	2 ± 0	
	Total metabolites	4 ± 0	10 ± 0	26 ± 1	40 ± 1	0.02%

* Data for the individual subjects are expressed as nmoles ± standard error ($n = 3$). n.d., not detected. For identification of peaks refer to Table I and Figure 1.

Table V. Amount of rutin in the ileal fluid of five ileostomy subjects collected 0–24 h after consumption of tomato juice containing 176 μmoles of rutin*.

Subject	0–2 h	2–5 h	5–24 h	Total
1	21 ± 1	132 ± 1	4.7 ± 0.0	158 ± 1
2	6 ± 0	109 ± 7	40 ± 1	155 ± 6
3	11 ± 1	118 ± 3	6 ± 1	135 ± 1
4	1 ± 0	100 ± 6	43 ± 0	149 ± 1
5	n.d.	119 ± 2	37 ± 1	156 ± 3
Mean	8 ± 5	115 ± 7	26 ± 11	151 ± 5
% of intake	5 ± 1	65 ± 4	15 ± 7	86 ± 3

* Data for the individual subjects are expressed as μmoles ± standard error ($n = 3$). Mean values are expressed as μmoles ± standard error ($n = 5$). No rutin was detected at 0 h time point. n.d., not detected.

charged fragment at *m/z* 150. This is in keeping with the presence of a hydroxyhippuric acid. Although no reference compound was available, *peak 6* is probably 3-hydroxyhippuric acid, as the 2- and 4-isomers were detected in peaks 1 and 5, respectively.

Quantitative analysis of urinary phenolic acids

All of the above six urinary phenolic acids were detected in healthy human subjects with a colon (Table VII). The main urinary metabolite excreted was 4-hydroxyhippuric acid with a mean excretion of 62 ± 6 μmoles, 0–24 h after ingestion of rutin. An average of 31 ± 7 μmoles was excreted by subjects with an ileostomy over the same period. Smaller quantities of 2- and 3-hydroxyhippuric acid were excreted and in the case of the 3-isomer, the ileal volunteers excreted more than the healthy subjects, 21 ± 11 compared to 5.7 ± 1.6 μmoles. In contrast to the hydroxyhippuric acids, 3-hydroxyphenylacetic acid, 3,4-dihydroxyphenylacetic acid and 3-methoxy-4-hydroxyphenylacetic acid were excreted only by volunteers with a colon (Table VII). In keeping with absorption in the colon, only trace amounts of these compounds were excreted in the first 5 h after ingestion of the rutin supplement. The overall quantity of the three phenylacetic acids excreted by the healthy volunteers, 39.4 μmoles, corresponds to 22% of the 176 μmoles of rutin intake.

Discussion

Most of the earlier studies on the bioavailability of flavonoids either used enzyme or acid hydrolysis of samples to release aglycones, prior to HPLC to determine levels in plasma and urine. However, the current study with human volunteers in which unhydrolysed extracts were analysed by HPLC with PDA and full scan data dependent MS^2 detection, provided a far more detailed and realistic picture of the fate of rutin within the body than was possible in earlier investigations. In the present study, a total of 15 metabolites and catabolites were detected and quantified in plasma and urine following consumption of tomato juice containing 176 μmoles of rutin. From the metabolite profiles in the plasma and urine, it is clear that rutin is extensively metabolised and made bioavailable. Following the release of the aglycone, quercetin is subjected to glucuronidation and methylation with quercetin-3-glucuronide and isorhamnetin-3-glucuronide appearing in circulatory and the excretory system. Phase II metabolism then takes place and, as a consequence, an additional seven metabolites are excreted in urine (Table I).

The metabolites quercetin-3-glucuronide and isorhamnetin-3-glucuronide appeared in plasma after ingestion of rutin with C_{max} values of 12 ± 2 and 4.3 ± 1.5 nmoles/l and T_{max} s of 4.7 ± 0.3 and 5.4 ± 0.2 h, respectively (Table III). These values differ markedly from those reported for the same metabolites by Mullen et al. [15] after feeding onions containing quercetin-4′-glucoside and quercetin-3,4′-diglucoside to human volunteers as they obtained C_{max} values more than 25-fold higher and T_{max} values of less than 1 h. The difference in the post-absorption kinetic behaviour of the quercetin glucosides and rutin is believed to stem from the sugar moiety attached to quercetin. In contrast to quercetin glucosides, the rutinose moiety of rutin is not cleaved by either LPH or CBG associated with the wall of the gastrointestinal tract [6]. As a consequence, most of the rutin passes intact through the small intestine without being absorbed and enters the colon of healthy subjects or emerges in the ileal fluid in the case of subjects with an ileostomy. There are several pieces of evidence to

Table VI. HPLC–MS^2 identification of phenolic acids detected in urine samples of volunteers with and without a colon*.

Peak	t_R (min)	Compound	$[M - H]^-$ (*m/z*)	MS^2 fragments ions (*m/z*)
1	13.6	4-Hydroxyhippuric acid	194	100, 93
2	15.9	3,4-Dihydroxyphenylacetic acid	167	123, 108
3	26.8	3-Hydroxyphenylacetic acid	151	107, 93, 121
4	29.5	3-Methoxy-4-hydroxyphenylacetic acid	181	137
5	32.2	2-Hydroxyhippuric acid	194	150
6	38.1	3-Hydroxyhippuric acid	194	150

* Peak numbers and HPLC retention times and the peaks refer to HPLC trace in Figure 3; t_R, retention time; $[M - H]^-$, negatively charged molecular ion.

Table VII. Excretion of phenolic acids in the urine of human subjects with and without a colon 0–24 h after consumption of rutin supplemented tomato juice*.

Phenolic acids	Colon	0–2 h	2–5 h	5–8 h	8–24 h	Total
2-Hydroxyhippuric acid	w	1.2 ± 0.3	5.3 ± 2.1	2.5 ± 0.7	8.7 ± 2.5	18 ± 5
	w/o	1.7 ± 0.7	1.1 ± 0.6	3.4 ± 1.4	3.9 ± 2.2	10 ± 3
3-Hydroxyhippuric acid	w	0.3 ± 0.1	0.8 ± 0.2	2.0 ± 0.5	2.7 ± 1.2	5.8 ± 1.6
	w/o	4.5 ± 3.6	1.8 ± 1.1	7.6 ± 4.7	6.8 ± 2.1	21 ± 11
4-Hydroxyhippuric acid	w	2.8 ± 0.6	5.8 ± 1.6	18 ± 4	35 ± 4	62 ± 6
	w/o	2.8 ± 0.9	2.9 ± 1.0	6.0 ± 1.1	19 ± 5	31 ± 7
3-Hydroxyphenylacetic acid	w	0.2 ± 0.1	0.4 ± 0.1	1.1 ± 0.3	2.7 ± 0.9	4.4 ± 1.1
	w/o	n.d.	n.d.	n.d.	n.d.	n.d.
3,4-Dihydroxyphenylacetic acid	w	n.d.	0.9 ± 0.6	3.1 ± 0.7	12 ± 3	16 ± 3
	w/o	n.d.	n.d.	n.d.	n.d.	n.d
3-Methoxy-4-hydroxyphenylacetic acid	w	0.9 ± 0.5	2.4 ± 0.8	6.1 ± 1.4	10 ± 4	19 ± 6
	w/o	n.d.	n.d.	n.d.	n.d.	n.d.

* Mean values expressed as μmoles ± standard error ($n = 6$). For MS^2 data and identification of peaks, refer to Table VI; n.d., not detected. w, with, w/o, without.

support this conclusion (i) the absence of rutin metabolites in the plasma and urine of ileostomy volunteers; (ii) the recovery of 86 ± 3% of the ingested rutin in ileal fluid (Table V) and (iii) the fact that quercetin-3-glucuronide and isorhamnetin-3-glucuronide do not appear in plasma of healthy volunteers until 4 h after rutin ingestion (Figure 3) and have respective T_{max} values of 4.7 and 5.4 h (Table III).

In the large intestine, rutinose is cleaved by the gut microflora. The microbial enzymes involved in the deglycolysation of the rutinoside moiety from quercetin, such as α-L -rhamnosidases and β-glucosidase have been detected in the human colon [16]. However, the low concentrations of quercetin metabolites detected in the plasma after ingestion of rutin could be due, in part, to the relatively low activity of α-L-rhamnosidase compared to that of β-glucosidase [13]. In contrast to the metabolism in the small intestine, the extent of quercetin absorption and the further breakdown into ring fission products depend largely on the composition of the colonic microflora of individual subjects and their intestinal transit times. The dependency of the metabolism of rutin on the composition of the microflora in the large intestine also probably explains the large inter-individual variations in the level of metabolites in plasma (Table II). Similarly, it could also be responsible for the very large quantitative and qualitative variations in the urinary excretion of quercetin metabolites by the six healthy volunteers (Table IV).

Further metabolism of the isorhamnetin-3-glucuronide and quercetin-3-glucuronide that appear in the circulatory system, probably in the liver and/or kidneys, results in the formation of quercetin glucoside glucuronides, a quercetin diglucuronide, a methylquercetin diglucuronide, quercetin-4′-glucuronide and quercetin-3′-glucuronide which, although not detected in plasma, were excreted in the urine after the consumption of rutin. The absence of these urinary metabolites in plasma suggests they are rapidly removed from the blood stream.

From the data presented in Table IV it is evident that 94% of the urinary metabolites of rutin were excreted in the 2–8 h period after ingestion of the tomato juice supplement. This is in keeping with the profile of plasma metabolites in Figure 3 and the estimated $T_{1/2}$ values for the plasma metabolites (Table III).

No evidence of sulfation of quercetin was detected in the present investigation, which is in marked contrast to studies on bioavailability of quercetin glucosides where quercetin-3′-sulfate was the major plasma metabolite and quercetin glucoside and glucuronide sulfates were the main urinary metabolites after consumption of onions [15]. This indicates that the sulfation step occurs principally, if not exclusively, in the small intestine and is in keeping with the report of van der Woude et al. [17], based on *in vitro* incubations, suggesting that in humans sulfation of quercetin is restricted to the small intestine. The human colon does contain sulfotransferase activity [18] but the absence of sulfated quercetin metabolites derived from rutin indicates that they do not have an affinity for quercetin-based compounds. Likewise, although *in vitro* studies have demonstrated sulfation of quercetin metabolites by liver cell-free preparations [19], the absence of sulfated quercetin metabolites following absorption in the large intestine indicates that *in vivo* sulfation does not occur in the liver.

The 0.02–2.8% urinary recovery of the ingested rutin as glucuronide, glucoside and methylated metabolites of quercetin (Table IV) does not account for the vast majority of the flavonol intake. The probable fate of most of the rutin is that it is broken down to low molecular weight phenolic acids by the colonic microflora [9]. Under strictly controlled dietary conditions, a total of six potential rutin

catabolites, hydroxyhippuric and hydroxyphenylacetic acids, were detected in the urine of healthy volunteers (Table VI). These catabolites were excreted in substantial amounts post-supplementation of rutin although the hydroxyphenylacetic acids, in contrast to the hydroxyhippuric acids, accumulated only in the urine of subjects with an intact colon (Table VII). This is in keeping with the data of Olthof et al. [9] who proposed that the urinary hydroxyphenylacetic acids originate in the colon through hydrolysis of rutin releasing quercetin which undergoes colonic bacterial mediated-fission of ring B producing 3,4-dihydroxyphenylacetic acid which is partially converted to 3-hydroxyphenylacetic acid. The two phenylacetic acids then pass into the bloodstream and 3,4-dihydroxyphenylacetic acid in methylated in the liver yielding 3-methoxy-4-hydroxyphenylacetic acid (Figure 4) which along with 3-hydroxyphenylacetic acid and 3,4-dihydroxyphenylacetic acid, is excreted in urine. In contrast, the production of hydroxyhippuric acids is not restricted to the colon and, indeed, may not necessarily be associated with rutin. Hippuric acid is, for instance, known to be a product of oxidative attack on exogenous benzenoid compounds by detoxifying enzymes system of the endoplasmic reticulum and microsomes [20]. Excretion of 2-hydroxhippuric acid is a marker of gastro-intestinal dysfunction in children [21] and 4-hydroxyhippuric acid is a marker of chronic renal failure [22].

In previous investigations in which plasma quercetin metabolites were monitored after the ingestion of rutin [12,13,23] somewhat different pharmacokinetic profiles were obtained to those presented in Figure 3 and summarised in Table III. With doses of 100–200 mg (88–176 μmoles) of rutin, these studies, which analysed the amount of quercetin in hydrolysed plasma extracts rather than individual rutin metabolites, reported plasma $t_{1/2}$ values of 11.8 h [13], 28.1 h [23] and beyond [12]. This compares with figures of 5.6 h and 6.9 h for quercetin-3-glucuronide and isorhamnetin-3-glucuronide, respectively, obtained in the present investigation (Table III). In the case of the studies by Erlund et al. [12] and Hollman et al. [23] one reason for the extended $t_{1/2}$ values may be that they are due to quercetin levels being determined by isocratic HPLC with short retention times which offer only limited resolution. In such circumstances, the quercetin peak is eluted against a declining baseline (see Figure 2 in Hollman et al. [24] and in Erlund et al. [25]) and, as a consequence of co-eluting impurities, an inaccurate over-estimate of the quercetin content is obtained. This results in quercetin appearing to be present in plasma at the 0 h time point at levels of ca. 20 nmoles/l and, after supplementation with rutin or quercetin glucosides, in elevated C_{max} values which take 24 h or more to decline to the, albeit high, 0 h level [12,23]. In contrast, in our studies, in which much better chromatographic resolution was achieved, almost all the plasma metabolites are detected against a flat, stable baseline (Figure 2). Using these procedures with feeds of rutin, as well as quercetin glucosides [15], no metabolites were detectable at 0 h and, after supplementation, metabolites fell to baseline before 12 h. In the present study, it is of note that 95% of the quercetin-3-glucuronide and isorhamnetin-3-glucuronide were excreted in urine within the first 8 h after ingestion of the rutin

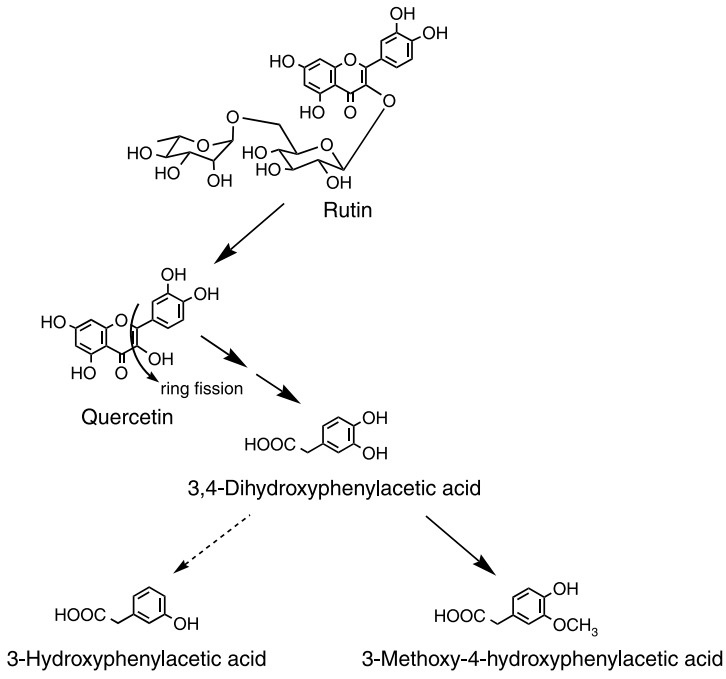

Figure 4. Proposed pathway for the catabolism of rutin in the large intestine. Dotted arrows indicate a minor route.

supplement (Table IV). This is in keeping with the plasma $t_{1/2}$ values presented in Table III but not the much longer 24+h values reported in the earlier studies [12,23].

In summary, the results obtained in the current study confirmed the colon is the major site of rutin metabolism and absorption, principally towards catabolic pathways, with the production of conjugated quercetin metabolites being a minor route. Following deglycosylation, only a small percentage of quercetin, 0.02–0.35% of intake in five of the six volunteers (Table IV) appears to be absorbed and proceed to the production of phase II methyl, glucuronyl and glucosyl, but not sulfated, metabolites. A substantially larger percentage of the released quercetin, corresponding to 22% intake, is acted upon by the colonic microflora leading to production of the phenolic catabolites 3-hydroxyphenylacetic acid, 3,4-dihydroxyphenylacetic acid and 3-methoxy-4-hydroxyphenylacetic acid probably via the pathway shown in Figure 4. This data should help greater understanding of the role of dietary rutin in the prevention of disease. The factors influencing variability in the appearance of metabolites in urine, which may be related to other dietary components such as non-digestible carbohydrates, should be explored along with the actions of the absorbed metabolites.

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