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Absorption, excretion and metabolite profiling of methyl-, glucuronyl-, glucosyl- and sulpho-conjugates of quercetin in human plasma and urine after ingestion of onions

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It is essential to have a thorough knowledge of the bioavailability and metabolism of dietary flavonols to understand their role in disease prevention. Lightly fried onions containing 275 μmol flavonols, principally quercetin-4′-glucoside and quercetin-3,4′-diglucoside, were fed to healthy human volunteers and plasma and urine were collected over a 24 h period. Samples were analysed by HPLC with diode array and tandem mass spectrometric detection. Five flavonol metabolites, quercetin-3′-sulphate, quercetin-3-glucuronide, isorhamnetin-3-glucuronide, a quercetin diglucuronide and a quercetin glucuronide sulphate, were detected in plasma in quantifiable amounts with trace quantities of six additional quercetin metabolites. Sub-micromolar peak plasma concentrations (C_{max}) of quercetin-3′-sulphate, quercetin-3-glucuronide, isorhamnetin-3-glucuronide and quercetin diglucuronide were observed 0.6–0.8 h after ingestion. In contrast, the C_{max} of quercetin glucuronide sulphate was 2.5 h. The elimination half-lives ($t_{1/2}$) of quercetin-3′-sulphate, quercetin-3-glucuronide and quercetin diglucuronide were 1.71, 2.33 and 1.76 h respectively, while the $t_{1/2}$ of isorhamnetin-3-glucuronide was 5.34 h and that of quercetin glucuronide sulphate was 4.54 h. The profile of metabolites excreted in urine was markedly different to that of plasma with many of the major urinary components, including quercetin-3′-glucuronide, two quercetin glucoside sulphates and a methylquercetin diglucuronide, absent or present in only trace amounts in the bloodstream indicative of substantial phase II metabolism. Total urinary excretion of quercetin metabolites was 12·9 μmol, corresponding to 4·7 % of intake. If these samples had been subjected to hydrolysis, as in many previous studies, only quercetin and isorhamnetin would have been detected and quantified. The bioactivity of these metabolites should be considered.

Flavonols: Quercetin glucosides: Absorption: Metabolism: Excretion: Man: HPLC–MS^2

Flavonols are polyphenolic C6-C3-C6 compounds which, along with other flavonoids and phenolics, occur widely in plants and plant-derived foods and beverages (Crozier *et al.* 2006). They have several potential nutritional and health-promoting roles in the human body but there is still much to be learnt about their bioavailability and, in particular, which metabolites appear in plasma and in what amounts. This information is essential to understanding the potential role of these compounds in reducing CHD and cancer as it is likely that the metabolites do not have the same bioactivity as the parent compounds. To gain a full picture of the absorption and metabolism of flavonols it is essential to be able to detect and quantify all the major metabolites in plasma and urine and this requires the use of appropriate analytical methodology such as HPLC with tandem MS (MS^2).

Quercetin is the major flavonol in many foods including onions which consistently contain high levels of flavonols (Crozier *et al.* 1998) in the form of quercetin-3,4′-diglucoside (I in Fig. 1), quercetin-4′-glucoside (II in Fig. 1), and smaller amounts of other conjugates including isorhamnetin-4′-glucoside (III in Fig. 1) (Tsushida & Suzuki, 1995). It is now believed that absorption of quercetin glucosides from the gastrointestinal tract involves deglycosylation by luminal lactase phloridzin hydrolase and/or cleavage within the enterocyte by cytosolic β-glucosidase (Day *et al.* 2003). This is followed by metabolism of the aglycone which leads to the appearance of quercetin sulphate and glucuronide conjugates in the circulatory system (Day & Williamson, 2003). These metabolites are not available from commercial sources, which precludes their direct analysis. Thus, in initial studies on quercetin derivatives accumulating in plasma and urine, samples were treated with either acid or glucuronidase/sulphatase enzymes to release the parent aglycone prior to quantitative analysis by HPLC (Hollman *et al.* 1996, 1997; Aziz *et al.* 1998; Moon *et al.* 2000; Graefe *et al.* 2001).

More recently, the use of HPLC–MS has facilitated the analysis of flavonol metabolites without recourse to acid or enzyme treatment. An investigation using HPLC–MS^2 in

Abbreviations: C_{max}, maximum post-ingestion plasma concentration of quercetin metabolites; MS^2, tandem MS; PDA, photodiode array; t_{max}, time to reach C_{max}; t_R, retention time; $t_{1/2}$, the elimination half-life of the metabolites.

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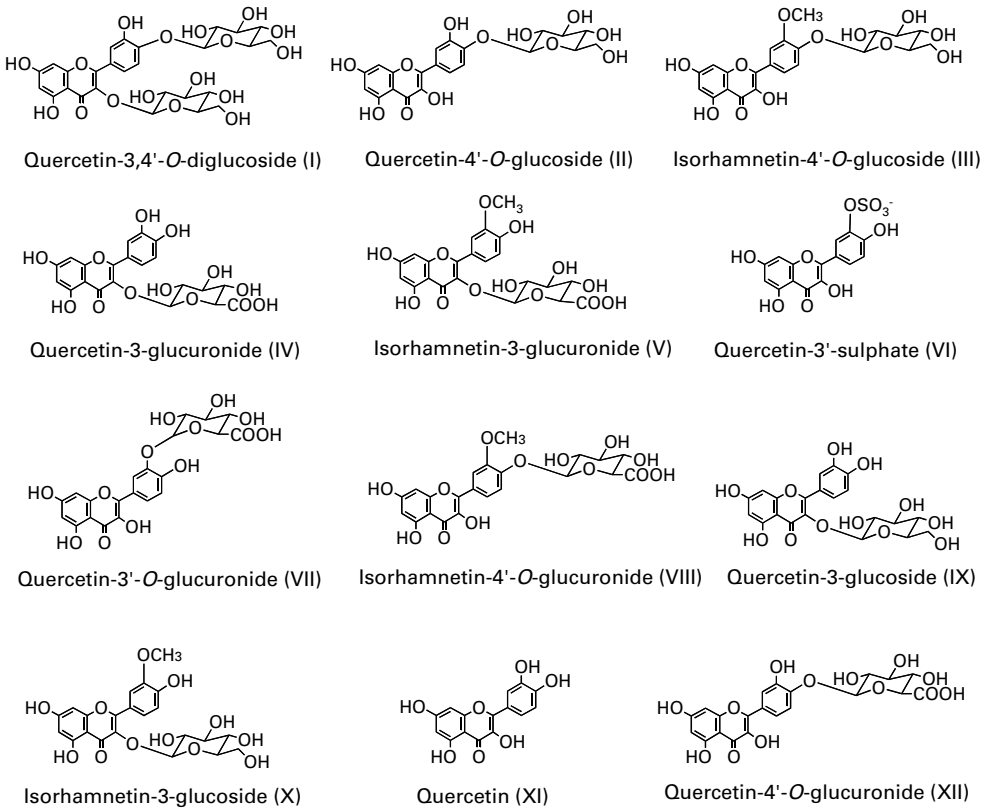

Fig. 1. Structures of flavonol glucosides and their metabolites.

the selected reaction monitoring mode detected five quercetin glucuronides in human plasma collected 1 h after ingestion of an 800 g onion supplement (Wittig *et al.* 2001). A further study in which plasma, collected 1.5 h post-ingestion of 200 g fried onions, was analysed by HPLC identified in total twelve putative quercetin metabolite peaks (Day *et al.* 2001). Identifications were based on chromatographic retention times (t_R) of absorbance peaks at 365 nm and enzyme hydrolysis data. Additional confirmation of metabolite identities was by MS analysis in selected ion monitoring mode which identified three of these metabolites as quercetin-3-glucuronide (IV in Fig. 1), isorhamnetin-3-glucuronide (V in Fig. 1) and quercetin-3′-sulphate (VI in Fig. 1) (Day *et al.* 2001). Subsequently, Mullen *et al.* (2004) fed 270 g lightly fried onions to human subjects and, using HPLC with photodiode array (PDA) and MS^2 detection, were able to identify twenty-three quercetin-based compounds in plasma and urine collected 1 and 0–4 h respectively, after ingestion. Here we report an extension of the earlier study in which plasma and urinary metabolites from six volunteers were analysed quantitatively in samples collected at a series of time-points over a 24 h period after supplementation.

Methods

Study design

Six volunteers (four males and two females), who were healthy, non-smokers and not on any medication, participated in the present study and gave their written consent. They were aged between 23 and 45 years and had a mean BMI of 23·7 (SE 1·2) (range 20·9–27·6). Subjects were required to follow a low flavonoid diet for 2 d and to fast overnight prior to supplementation. This diet excluded most fruits, vegetables and beverages including tea, coffee, fruit juices and wine. On the morning of the study, red onions (*Allium cepa*) were skinned, chopped into small slices, and fried for 4 min in margarine. Aliquots of the fried onions were taken for qualitative and quantitative analysis of their flavonol content.

All subjects consumed 270 g fried onions. Venous blood samples were taken before (0 h) and 0·5, 1, 2, 3, 6 and 24 h post-ingestion. Blood (12 ml) was collected in heparinised tubes at each time-point and immediately centrifuged at 4000***g*** for 10 min at 4°C. The plasma was separated from the erythrocytes and 500 μl aliquots were acidified to pH 3 with 15 μl 50 % aqueous formic acid and 50 μl ascorbic acid (10 mM) added to prevent oxidation. The plasma samples were then stored at −80°C prior to analysis. Urine was collected before and over 0–4, 4–8 and 8–24 h periods after the consumption of the fried onion supplement. The volume of each sample was recorded prior to acidification to pH 3·0 and the storage of aliquots at −80°C. The study protocol was approved by the Glasgow Royal Infirmary Local Research Ethics Committee.

Materials

Onions were purchased from a local supermarket (Sainsbury's, Glasgow, UK). HPLC-grade methanol and acetonitrile were obtained from Rathburn Chemicals (Walkerburn, UK).

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Formic acid was purchased from Riedel-DeHaen (Seeize, Germany) and acetic acid from BDH (Poole, UK). L-(+)-Ascorbic acid, quercetin and isorhamnetin-3-glucoside were purchased from Extrasynthese (Genay, France). AASC Ltd (Southampton, UK) supplied quercetin-3,4′-diglucoside, quercetin-4′-glucoside, quercetin-3-glucoside and isorhamnetin-4′-glucoside.

[2-^{14}C]Quercetin-4′-*O*-β-D-glucoside was synthesised in four steps from barium [^{14}C]carbonate (specific activity 3·75 mCi/mmol) by a method previously reported for the synthesis of [2-^{13}C]quercetin-4′-*O*-β-D-glucoside (Caldwell *et al.* 2000) except that the intermediate ester was not purified by filtration through alumina. The compound was pure by ^{1}H NMR spectroscopy and only one radioactive peak was detected by HPLC–radio counting.

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-3′-sulphate and isorhamnetin-3-glucuronide were kindly donated by Dr Paul Needs and Dr Paul Kroon (Institute of Food Research, Norwich, UK).

Extraction of onions

Aliquots of fried onions were taken for quantitative analysis of their flavonol content. Prior to the extraction, they were frozen in liquid nitrogen, lyophilised and powdered. Triplicate samples were extracted as follows: 35 mg dry powder were homogenised in 3 ml 70 % methanol in water for 1 min using an Ultra-Turrax T 25 (IKA^{R}-Werke, Staufen, Germany). During the homogenisation, the samples were kept on ice. The mixture was then centrifuged at 3000***g*** at 4°C for 15 min. The supernatant was collected and the pellet further extracted and centrifuged twice. The three supernatants were combined and reduced to dryness *in vacuo*. The dried extract was dissolved in 300 μl methanol and 1200 μl 5 % formic acid in water, before being centrifuged at 25 000***g*** at 4°C for 10 min. Aliquots (20 μl) of the supernatant were analysed by HPLC–PDA–MS^{2}.

Extraction of plasma

Triplicate samples of plasma were treated according to the method of Day *et al.* (2001). This involved adding 1·5 ml acetonitrile to 500 μl plasma. Samples were vortexed for 30 s every 2 min over a 10 min period, before centrifuging the mixture at 4000***g*** at 4°C for 10 min. The supernatant was collected and the pellet re-extracted as described earlier but with methanol instead of acetonitrile. Experiments with [^{14}C]quercetin-4′-glucoside, querctin-3-glucuronide and quercetin-3′-sulphate showed recoveries of about 75 % with the initial acetonitrile extraction which increased by a further 10–12 % with the second methanolic extraction. The acetonitrile and methanol supernatants were combined and reduced to dryness *in vacuo*. Extracts were then dissolved in 25 μl methanol plus 225 μl 1 % formic acid in water and centrifuged at 25 000***g*** at 4°C for 10 min prior to the analysis of 100 μl aliquots of the supernatant by HPLC–PDA–MS^{2} on the day of extraction. [2-^{14}C]Quercetin-4′-glucoside, used as an internal standard, was added to the plasma prior to extraction with acetonitrile. The level of radioactivity present in the sample prior to analysis was used to determine the extraction efficiency. Preliminary tests had shown no quercetin-4′-glucoside was present in the plasma samples.

Urine

The acidified frozen urine was defrosted, methanol was added to make the solution 5 % aqueous methanol, which resulted in any precipitated material being re-dissolved, and 100 μl aliquots were analysed directly by HPLC–PDA–MS^{2} without further processing.

HPLC with diode array and tandem MS detection

Samples were analysed on a Surveyor HPLC system comprising an HPLC pump, PDA detector, scanning from 250 to 700 nm and an autosampler cooled to 4°C (Thermo Finnigan, San Jose, CA, USA). Separation was carried out using a 250 × 4·6 mm i.d. 4 μm Synergi Max-RP column (Phenomenex, Macclesfield, UK) eluted with a 60 min gradient of 5–40 % acetonitrile in 1 % formic acid at a flow rate of 1 ml/min and maintained at 40°C. After passing through the flow cell of the diode array detector the column eluate was split and 0·3 ml/min was directed to a LCQ DecaXP ion trap mass spectrometer fitted with an electrospray interface (Thermo Finnigan). Analyses utilised the negative ion mode as this provided the best limit of detection for flavonols and their metabolites. Analysis was carried out using full-scan, data-dependent MS^{2} scanning from *m/z* 100 to 1000. Capillary temperature was 350°C, sheath gas and auxiliary gas were 60 and 10 units respectively, and the source voltage was 4 kV for negative ionisation and 1 kV for positive ionisation.

Quercetin, quercetin-3,4′-diglucoside, quercetin-4′-glucoside, quercetin-3-glucoside, isorhamnetin-4′-glucoside, quercetin-3-glucuronide and quercetin-3′-sulphate were all quantified by reference to standard calibration curves at 365 nm. Other flavonols were quantified in quercetin-4′-glucoside equivalents with the exception of a partially identified quercetin sulphate that was quantified in quercetin-3′-sulphate equivalents. In all instances peak identification was confirmed by HPLC retention times and MS^{2} fragmentation data.

Pharmacokinetic analysis of plasma metabolites

Maximum post-ingestion plasma concentration of quercetin 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: $t_{1/2} = 0{\cdot}693/K_e$ where K_e is the slope of the linear regression of the log of 0–24 h plasma metabolite concentrations.

Results

Analysis of fried onions

Gradient reverse-phase HPLC with absorbance detection and full-scan data-dependent MS^{2} was used to identify and quantify the flavonol content of fried onion meals. Absorbance at

365 nm and negative ionisation MS^2 were used for flavonol analysis. The total amount of flavonols in the 270 g onion meal was 275 (SE 8·8) μmol. In keeping with the data of Tsushida & Suzuki (1995), the major components were quercetin-3,4′-diglucoside (I; 107 (SE 1·4) μmol), quercetin-4′-glucoside (II; 143 (SE 12) μmol) and isorhamnetin-4′-glucoside (III; 11 (SE 1·4)) μmol) which accounted for 95 % of the 275 (SE 8·8) μmol flavonol intake.

Qualitative analysis of plasma and urine

Plasma and urine samples were analysed by HPLC with PDA and MS^2 detection. Flavonol metabolites were present in plasma and urine, corresponding to about 4 % of the intake, with a total of twenty-three quercetin-based compounds being detected. Typical HPLC traces obtained with absorbance at 365 nm are illustrated in Fig. 2 and the identifications based on MS^2 spectra and t_R data are summarised in Table 1. The use of HPLC–MS^2 to identify these quercetin metabolites has been discussed in detail in a publication by Mullen *et al.* (2004).

Quantitative analysis of flavonol metabolites in plasma

Eleven quercetin metabolites were detected in plasma in quantities that facilitated either their full or partial identification as outlined in Table 1. Those present in sufficient quantities to enable pharmacokinetic profiles to be obtained were a quercetin diglucuronide (peak 9), a quercetin glucuronide sulphate (peak 14), quercetin-3-glucuronide (IV), isorhamnetin-3-glucuronide (V) and quercetin-3′-sulphate (VI). Quercetin-3′-glucuronide (VII) and isorhamnetin-4′-glucuronide (VIII) was present in the plasma of all the human subjects in low, non-quantifiable, amounts while other flavonol derivatives, quercetin-3,4′-diglucoside, quercetin-3-glucoside (IX), isorhamnetin-3-glucoside (X) and the aglycone quercetin (XI), were detected, albeit in very small quantities, only in the plasma of volunteer 6 (Table 1).

The 0–6 h pharmacokinetic profiles of the five major plasma flavonol metabolites are illustrated in Fig. 3. No quercetin metabolites were present in plasma samples collected at either prior (0 h) or 24 h after supplementation. This was confirmed using the enhanced sensitivity and selectivity of MS^2 in the selected reaction monitoring mode. Pharmacokinetic analyses of the 0–24 h data-points are summarised in Table 3. The two main metabolites which accumulated in plasma were quercetin-3′-sulphate and quercetin-3-glucuronide. These compounds had a C_{max} of 665 (SE 82) and 351 (SE 27) nM respectively. In both instances t_{max} was less than 1 h after the ingestion of the onion supplement (Table 2). A quercetin diglucuronide (peak 9) had a similar t_{max} (0·80 (SE 0.12) h) but a lower C_{max} (62 (SE 12) nM) than the two main metabolites. The levels of all three metabolites declined after reaching C_{max} (Fig. 4) and they had a similar $t_{1/2}$ with values of 1.71–2.33 h (Table 3). The pharmacokinetic profiles of isorhamnetin-3-glucuronide and quercetin glucuronide sulphate (peak 14) were different to those of the other metabolites

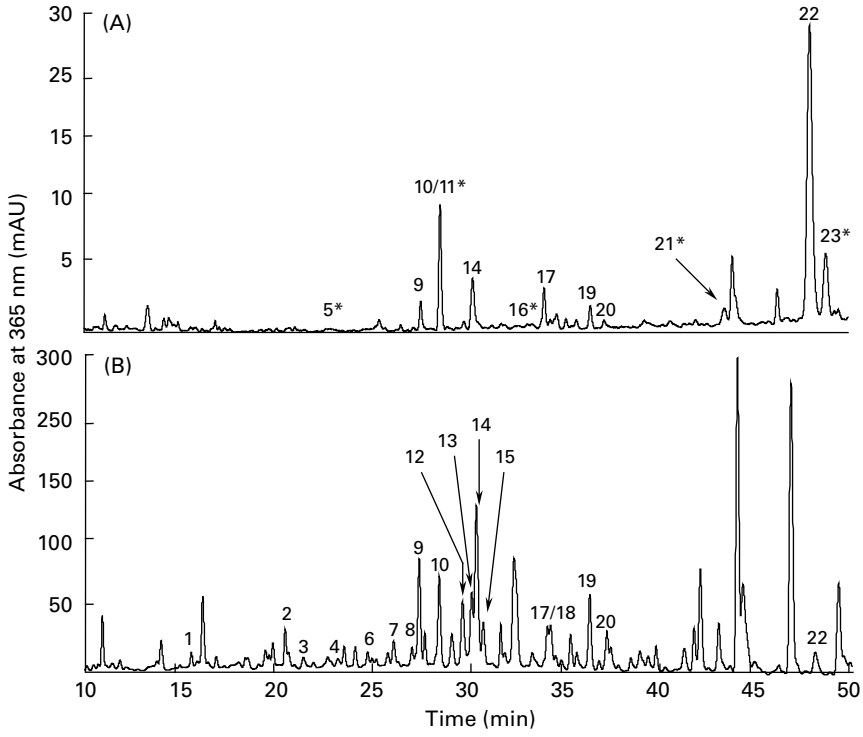

Fig. 2. Gradient reversed-phase HPLC with detection at 365 nm of quercetin metabolites in a plasma extract (A) and urine (B) obtained from a human subject after the consumption of 270 g of fried onions. Samples were analysed on a 250 × 4·6 mm i.d., 4 μm Synergi Max-RP column at 40°C and eluted at a flow rate of 1 ml/min with a 60 min gradient of 5–40 % acetonitrile in water containing 1 % formic acid. Detection was with a diode array detector operating at 365 nm. Peaks 1–23 represent components subsequently analysed by tandem MS with an electrospray interface with negative ionisation. For identity of peaks 1–23, see Table 1. *Peaks detected in samples from only one of the six human subjects. AU, arbitrary units.

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Table 1. HPLC–tandem MS (MS^2) identification of quercetin metabolites detected in plasma and urine of six human subjects after the consumption of 270 g fried onions*

Peak†	t_R (min)†	Compound	$[M-H]^-$ (m/z)	MS^2 fragments ions (m/z)	Location
1	15·6	Quercetin diglucuronide	653	477 ($[M-H]^-$–GlcUA), 301 ($[M-H]^-$-GlcUA-GlcUA)	Urine
2	20·4	Methylquercetin diglucuronide	667	491 ($[M-H]^-$-GlcUA), 315 ($[M-H]^-$-GlcUA-GlcUA)	Urine
3	21·5	Quercetin glucoside glucuronide	639	477 ($[M-H]^-$-Glc), 463 ($[M-H]^-$-GlcUA), 301 ($[M-H]^-$-GlcUA-Glc)	Urine
4	22·7	Methylquercetin diglucuronide	667	491 ($[M-H]^-$-GlcUA), 315 ($[M-H]^-$-GlcUA-GlcUA)	Urine
5	22·8	Quercetin-3,4′-diglucoside‡	625	463 ($[M-H]^-$-Glc), 301 ($[M-H]^-$-Glc-Glc)	Plasma
6	24·8	Quercetin diglucuronide	653	477 ($[M-H]^-$-GlcUA), 301 ($[M-H]^-$-GlcUA-GlcUA)	Urine
7	26·2	Quercetin glucoside glucuronide	639	477 ($[M-H]^-$-Glc), 463 ($[M-H]^-$-GlcUA), 301 ($[M-H]^-$-GlcUA-Glc)	Urine
8	27·0	Quercetin glucoside glucuronide	639	477 ($[M-H]^-$-Glc), 463 ($[M-H]^-$-GlcUA), 301 ($[M-H]^-$-Glc-GlcUA)	Urine
9	27·4	Quercetin diglucuronide	653	477 ($[M-H]^-$-GlcUA), 301 ($[M-H]^-$– GlcUA-GlcUA)	Urine, plasma
10	28·4	Quercetin-3-glucuronide	477	301 ($[M-H]^-$-GlcUA)	Urine, plasma
11	28·4	Quercetin-3-glucoside‡	463	301 ($[M-H]^-$-Glc)	Plasma
12	29·6	Quercetin glucoside sulphate	543	463 ($[M-H]^-$-SO_3), 381 ($[M-H]^-$-Glc), 301 ($[M-H]^-$-SO_3-Glc)	Urine
13	30·1	Quercetin glucuronide sulphate	557	477 ($[M-H]^-$-SO_3), 381 ($[M-H]^-$-GlcUA), 301 ($[M-H]^-$-SO_3-GlcUA)	Urine
14	30·3	Quercetin glucuronide sulphate	557	477 ($[M-H]^-$-SO_3), 381 ($[M-H]^-$-GlcAU), 301 ($[M-H]^-$-SO_3-GlcUA)	Urine, plasma
15	30·6	Quercetin glucoside sulphate	543	463 ($[M-H]^-$-SO_3), 381 ($[M-H]^-$-Glc), 301 ($[M-H]^-$-SO_3-Glc)	Urine
16	33·2	Isorhamnetin-3-glucoside‡	477	315 ($[M-H]^-$-Glc)	Plasma
17	34·1	Isorhamnetin-3-glucuronide	491	315 ($[M-H]^-$-GlcUA)	Urine, plasma
18	34·4	Quercetin-4′-glucuronide	477	301 ($[M-H]^-$-GlcUA)	Urine
19	36·3	Quercetin-3′-glucuronide	477	301 ($[M-H]^-$-GlcUA)	Urine, plasma
20	37·2	Isorhamnetin-4′-glucuronide	491	315 ($[M-H]^-$-GlcUA)	Urine, plasma
21	43·2	Quercetin‡	301	179, 151	Plasma
22	47·9	Quercetin-3′-sulphate	381	301 ($[M-H]^-$-SO_3)	Urine, plasma
23	48·3	Quercetin sulphate‡	381	301 ($[M-H]^-$-SO_3)	Plasma

Glc, glucosyl unit; GlcUA, glucuronyl unit; $[M-H]^-$, negatively charged molecular ion; t_R, retention time.
* For details of procedures, see p. 108.
† Peak numbers and HPLC retention times refer to HPLC traces in Fig. 2.
‡ Compounds detected only in the plasma of one of the six human subjects.

illustrated in Fig. 3. Isorhamnetin-3-glucuronide had a C_{max} of 112 (SE 18) nM and a t_{max} of 0.60 h and there was a slow rate of decline after C_{max} which is reflected in a $t_{1/2}$ of 5.34 h (Table 3). The C_{max} of the quercetin glucuronide sulphate (peak 14) was 123 (SE 26) nM while its t_{max} at 2.5 (SE 0·22) h was delayed compared to that of the other metabolites and the $t_{1/2}$ (4.54 h) was much slower than that observed with quercetin-3′-sulphate, quercetin-3-glucuronide and the quercetin diglucuronide (Table 3).

Quantitative analysis of flavonol metabolite excretion in urine

Eighteen flavonol metabolites were detected in urine samples collected 0–4, 4–8 and 8–24 h after the ingestion of red onions (Table 1). Six of these compounds, two quercetin diglucuronides (peaks 1 and 6), two quercetin glucoside sulphates (peaks 7 and 8), quercetin-4′-glucuronide (XII) and quercetin-3′-sulphate, were present in quantities insufficient for routine quantification.

Twelve urinary metabolites were detected in amounts that facilitated quantitative analysis (Table 4). These metabolites consisted of quercetin-3-glucuronide, quercetin-3′-glucuronide a quercetin diglucuronide, a quercetin glucuronide glucoside, two quercetin glucuronide sulphates, two quercetin glucoside sulphates, isorhamnetin-3-glucuronide, isorhamnetin-4′-glucuronide and two methylquercetin diglucuronides. The main urinary metabolite present was a quercetin diglucuronide (peak 9) with 2223 (SE 417) nmol being excreted over the 24 h period following ingestion of the onion supplement. Substantial amounts of quercetin-3′-glucuronide (1845 (SE 193) nmol), isorhamnetin-3-glucuronde (1789 (SE 27) nmol) and two quercetin glucuronide sulphates (peak 13, 1384 (SE 163) nmol; peak 14, 1229 (SE 190) nmol) were also detected.

Discussion

The results of the present study have provided, for the first time, detailed quantitative concentrations of metabolites of methyl-, glucuronyl- and sulpho-conjugates of quercetin in the plasma and urine of human subjects after ingestion of onions. The pharmacokinetics presented should allow better and more relevant studies of the bioactivity and role of dietary flavonols in disease prevention.

Quantitative analysis of flavonol absorption

The two major metabolites, quercetin-3′-sulphate and quercetin-3-glucuronide, appeared in plasma within 30 min of the ingestion of onions, both had t_{max} values of under 1 h and $t_{1/2}$ values of 1.71 and 2.33 h respectively (Fig. 3; Table 3). A quercetin diglucuronide (peak 9) with a lower C_{max} and similar t_{max} and $t_{1/2}$ values was also detected. The pharmacokinetic profiles of isorhamnetin-3-glucuronide and a quercetin glucuronide sulphate (peak 14) were somewhat different in that both had a much longer $t_{1/2}$ and the glucuronide sulphate also had a much delayed t_{max}. However, the total contribution of these two compounds to the overall absorption profile was minimal, having no effect on the t_{max} and only extending the $t_{1/2}$ to 2.61 h. This $t_{1/2}$ is much shorter than in similar absorption studies carried out previously (Hollman *et al.* 1996, 1997; Aziz *et al.* 1998; Graefe *et al.* 2001)

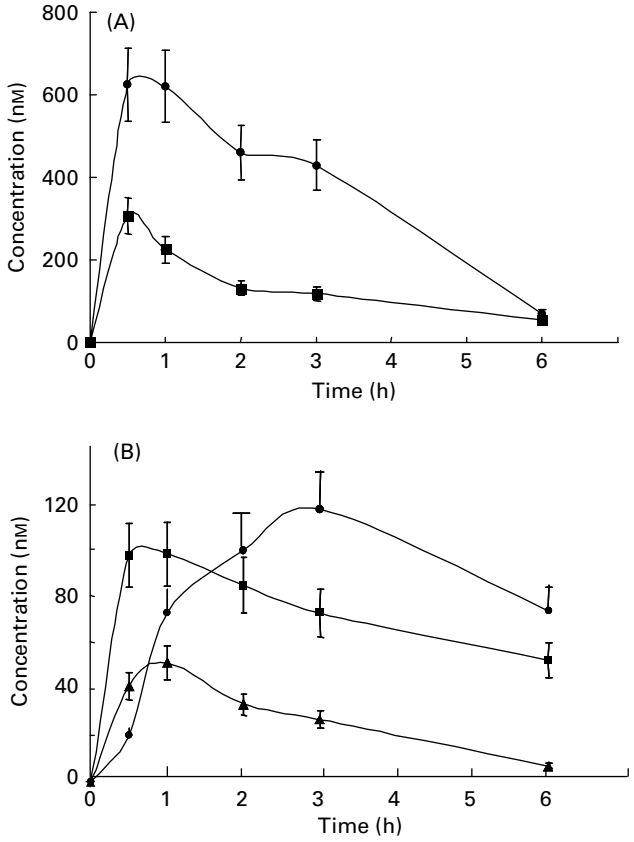

Fig. 3. (A), Concentration of quercetin-3′-sulphate (●) and quercetin-3-glucuronide (■); (B), concentration of a quercetin glucuronide sulphate (●), isorhamnetin-3-glucuronide (■) and a quercetin diglucuronide (▲), in plasma from six human subjects collected 0–6 h after the ingestion of 270 g fried onions. For details of procedures, see p. 108. Values are means with their standard errors depicted by vertical bars (*n* 6). Note that no quercetin metabolites were present in detectable amounts in plasma collected 24 h after supplementation.

which, arguably, is a consequence of the enhanced accuracy of analytical data obtained by HPLC–MS^2.

Confirming the validity of the short $t_{1/2}$ values presented in Table 3, 92 % of the urinary flavonol metabolites were excreted within the first 8 h after ingestion of onions (Table 4). Total 0–24 h flavonol metabolite excretion in urine for the individual subjects were 13·9, 13·7, 10·1, 16·4, 9·6 and 14·0 μmol and the mean value of 12·9 (SE 1·1) μmol corresponds to 4·7 % of intake. This is in agreement with the level of excretion of flavonols in urine after onion consumption by human subjects, reported by Graefe *et al.* (2001).

Qualitative analysis of flavonol absorption

The number and varieties of metabolites formed from the two main onion flavonols, quercetin-4′-glucoside and quercetin-3,4′-diglucoside, are shown in Table 1. The present study provides no information on the mechanisms involved or the efficiency with which these compounds enter the enterocyte and are hydrolysed. However, it is evident that following release of the aglycone, quercetin is subjected to glucuronidation, sulphation and/or methylation. The enzymes involved in the synthesis of these metabolites from quercetin, glucuronosyltransferase, sulphotransferase and *O*-methyltransferase, have been found in human intestine (Radominska-Pandya *et al.* 1998; De Santi *et al.* 2000; Chen *et al.* 2003; Murota & Terao, 2003). It is, therefore, feasible that after the initial deglycosylation of the onion quercetin glucosides, all the quercetin metabolites that appear in plasma are the result of conversions occurring in the lumen of the small intestine. The reason for the individual metabolites displaying different pharmacokinetic profiles could be due to differing enzyme specificities and/or varying rates of efflux from the enterocyte into the bloodstream although deposition in body tissues and a slow release in the bloodstream could also be factors of influence.

Another possibility is that the major plasma metabolites, quercetin-3′-sulphate and quercetin-3-glucuronide, are produced in the small intestine, pass into the portal vein and are further converted to the more minor components, the quercetin glucuronide sulphate, the quercetin diglucuronide and isorhamnetin-3-glucuronide in the liver as illustrated in Figs. 4 and 5. Human hepatocytes contain glucuronyl-, sulpho- and methyltransferases as well as β-glucuronidase activity (Boersma *et al.* 2002; O'Leary *et al.* 2003). *Ex vivo* incubation of quercetin-3-glucuronide with human hepG2 hepatonoma cells results in cleavage of the glucuronide moiety and the formation of quercetin-3′-sulphate (O'Leary *et al.* 2003). Further investigation is required to determine if this two-step pathway is the way in which the sulphate, the main quercetin plasma metabolite, is synthesised *in vivo*. A single-step sulphation of the aglycone in the enterocyte, as illustrated in Fig. 5,

Table 2. Pharmacokinetic parameters of quercetin metabolites in the plasma of six human subjects after the consumption of 270 g fried onions*

Metabolite	Peak number†	C_{max} (nM) Mean	C_{max} (nM) SE	t_{max} (h) Mean	t_{max} (h) SE	$t_{1/2}$ (h) Mean
Quercetin-3′-sulphate	22	665	82	0·75	0·12	1·71
Quercetin-3-glucuronide	10	351	27	0·60	0·10	2·33
Isorhamnetin-3-glucuronide	17	112	18	0·60	0·10	5·34
Quercetin diglucuronide	9	62	12	0·80	0·12	1·76
Quercetin glucuronide sulphate	14	123	26	2·5	0·22	4·54

C_{max}, maximum post-ingestion plasma concentration; t_{max}, time to reach C_{max}; $t_{1/2}$, the elimination half-life of the metabolites.
* For details of procedures, see p. 108.
† Peak numbers as in Fig. 2 and Table 1.

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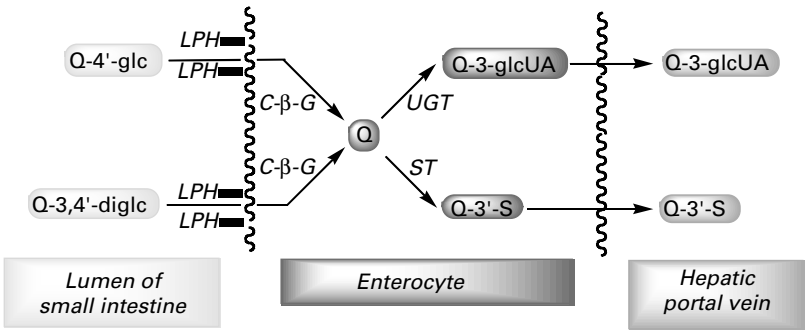

Fig. 4. Schematic of the proposed metabolic fate of quercetin-4′-glucoside and quercetin-3,4′-diglucoside as they pass from the lumen of the small intestine into the hepatic portal vein. C-β-G, cytosolic β-glucosidase; diglc, diglucoside; glc, glucoside; glcUA, glucuronide; Q, quercetin; LPH, lactase phlorizen hydrolase; S, sulphate; ST, sulphotransferase; UGT, glucuronyltransferase.

would appear to be a more straightforward, but not necessarily exclusive, route.

The C_{max} values of plasma metabolites and 24 h urinary excretion of the flavonol metabolites (Table 4) detected after consumption of onions presents clear evidence of substantial phase II metabolism with many of the major urinary metabolites either not being detected in plasma or being present in low concentrations. For instance, quercetin-3′-sulphate, the main plasma metabolite, was present in urine in only trace quantities while several quercetin glucoside glucuronides and quercetin glucoside sulphates, absent in plasma, were excreted in substantial amounts. The virtual absence of many of these urinary metabolites in plasma indicates that once released into the bloodstream they are rapidly removed by excretion via the kidneys. We assume that most of the observed metabolism occurs in the liver, which contains all the prerequisite enzymes, prior to transport to the kidneys. The exception, as illustrated in Fig. 5, may be the formation of the glucoside conjugates in the kidneys, which are known to possess β-glucosyltransferase activity (Matern & Matern, 1987).

The data obtained with volunteer 6 was of interest in that quercetin, quercetin-3,4′-diglucoside and other flavonol glucosides were detected in plasma (Table 1). However, the levels were extremely low and these compounds were not detected in the plasma of the other five subjects. It has previously been reported that quercetin-4′-glucoside and isorhamnetin-4′-glucoside appear in the bloodstream after ingestion of an onion meal by human volunteers (Aziz *et al.* 1998, 2003). These identifications were based on co-chromatography with authentic standards using a high resolution HPLC system with a post-column derivatisation procedure that produced fluorescent flavonol derivatives (Hollman *et al.* 1996). It has been suggested that the putative flavonol glucoside peaks were flavonol glucuronides which have very similar retention properties (Day & Williamson, 2001). The present study with HPLC using MS^2 detection indicates that this proposal is probably correct and that unmodified flavonol glucosides are not the main components to accumulate in plasma after the ingestion of onions. Similarly, reports on the occurrence of the disaccharide quercetin rutinoside in plasma (Paganga & Rice-Evans, 1997; Mauri *et al.* 1999) are likely to be inaccurate.

The 4·7 % recovery of the ingested flavonol glucosides as metabolites in urine leaves a large amount of the ingested dose unaccounted for. The most likely fate of these

Table 3. Concentration of quercetin metabolites (nmol) in the urine of six human subjects 0–24 h after the consumption of 270 g fried onions*

		0–4 h		4–8 h		8–24 h		Total	
Metabolites	Peak number†	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Quercetin-3-glucuronide	10	512	101	400	113	ND		912	149
Quercetin-3′-glucuronide	19	979	220	804	194	62	30	1845	193
Quercetin diglucuronide	9	1007	253	942	273	274	98	2223	417
Quercetin glucuronide glucoside	3	99	21	64	16	ND		163	23
Quercetin glucuronide sulphate	13	608	124	566	143	210	73	1384	163
Quercetin glucuronide sulphate	14	743	170	418	98	68	50	1229	190
Quercetin glucoside sulphate	12	226	73	130	34	35	26	392	60
Quercetin glucoside sulphate	15	538	127	257	98	26	11	821	156
Isorhamnetin-3-glucuronide	17	767	18	861	9	161	6	1789	239
Isorhamnetin-4′-glucuronide	20	451	11	249	2	ND		700	114
Methylquercetin diglucuronide	2	439	132	475	67	89	69	1003	156
Methylquercetin diglucuronide	4	189	49	163	41	74	36	426	99
Total		6558	1323	5329	1018	999	267	12 886	1038

ND, not detected.
* For details of procedures, see p. 108.
† Peak numbers as in Fig. 2 and Table 1.

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Table 4. Quercetin metabolites detected in plasma and urine of six human subjects after the consumption of 270 g fried onions*

Metabolite	Peak number†	Plasma‡ Mean	Plasma‡ SE	Urine§ Mean	Urine§ SE
Quercetin diglucuronide	1	ND		Trace‖	
Methylquercetin diglucuronide	2	ND		1003	156
Quercetin glucoside glucuronide	3	ND		163	23
Methylquercetin diglucuronide	4	ND		426	99
Quercetin diglucuronide	6	ND		Trace	
Quercetin glucoside glucuronide	7	ND		Trace	
Quercetin glucoside glucuronide	8	ND		Trace	
Quercetin diglucuronide	9	51	13	2223	417
Quercetin-3-glucuronide	10	306	42	912	149
Quercetin glucoside sulphate	12	ND		393	60
Quercetin glucuronide sulphate	13	ND		1384	163
Quercetin glucuronide sulphate	14	117	12	1229	190
Quercetin glucoside sulphate	15	ND		821	156
Isorhamnetin-3-glucuronide	17	98	17	1789	27
Quercetin-4′-glucuronide	18	ND		Trace	
Quercetin-3′-glucuronide	19	Trace		1845	193
Isorhamnetin-4′-glucuronide	20	Trace		700	11
Quercetin-3′-sulphate	22	539	46	Trace	

ND, not detected.
* For details of procedures, see p. 108.
† Peak numbers refer to HPLC traces in Fig. 2 and Table 1.
‡ Estimates expressed as nM at peak plasma concentration.
§ Amounts expressed as total amount excreted in nmol over a 24 h post-ingestion period.
‖ Trace: compound detected but not in sufficient amounts for routine quantification. Information on trace levels of metabolites detected exclusively in the plasma of volunteer 6 (see Table 1) are not presented.

compounds is that they are converted to low molecular weight phenolic acids (Depréz *et al.* 2000; Gonthier *et al.* 2003) most notably 3-hydroxyphenylpropionic acid, 3,4-dihydroxyphenylpropionic acid and 3-methoxy-4-hydroxyphenylpropionic acid (Olthof *et al.* 2003). These compounds were not analysed in the current study. They have a low extinction coefficient and a λ_{max} below 250 nm and as a result are not readily detected with a diode array detector and, in addition, they do not ionise readily when subjected to MS with an electrospray interface.

The data obtained in the present study reveal that extensive modification of quercetin glucosides occurs following ingestion of onions and the appearance of metabolites in the bloodstream and urine. The metabolic conversions involve a

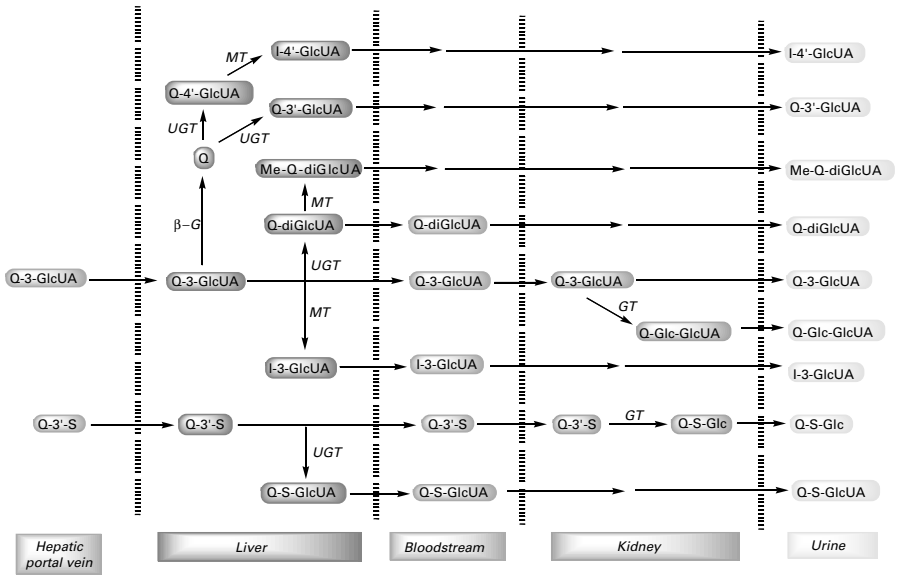

Fig. 5. Schematic of the proposed metabolic fate of quercetin-3-glucuronide and quercetin-3′-sulphate as they are transported from the small intestine to the liver where they are further metabolised before returning to the bloodstream and being excreted in urine via the kidneys. diglcUA, diglucuronide; β-G, β-glucosidase; glc, glucoside; glcUA, glucuronide; GT, glucosyltransferase; I, isorhamnetin; MT, methyltransferase; Q, quercetin; S, sulphate; UGT, glucuronyltransferase.

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complex combination of deglycosylation, glucuronidation, sulfation, methylation and possibly deglucuronidation steps. Where in the body these events take place and the sequence in which they occur after the initial deglycosylation, is a matter of speculation and a topic that requires further investigation. To this end, while experimentation with human subjects is useful it has it limitations as the deposition of flavonol metabolites in body tissues such as the liver, kidneys and brain is not possible for obvious reasons. *Ex vivo* studies with cultured cells and tissues have their place but it is open to doubt as to whether they reflect the true *in vivo* systems where the passage of metabolites into and out of cells and organs is likely to be subjected to refined controls. Animal test systems are, therefore, the only direct way in which the true bioavailability of flavonols and other dietary flavonoids and phenolics can be investigated. As demonstrated in recent studies with rats, this is best achieved using radiolabelled substrates as the accumulation of radioactivity in body fluids and tissues can be easily monitored by liquid scintillation counting and the compounds involved identified and quantified using HPLC–MS^n in combination with an on-line radioactivity monitor (Mullen *et al.* 2002, 2003).

There are several reasons why, in the present study, it was possible to obtain such a detailed insight into the fate of dietary quercetin glucosides following their ingestion. In the case of plasma samples, very clean extracts with high flavonol recoveries were obtained by using the extraction procedures of Day & Williamson (2001). Secondly, an earlier investigation, in which [2-^{14}C]quercetin-4′-glucoside was ingested by rats and radiolabelled metabolites were monitored, alerted us to the possibility that quercetin glucosides may be converted in man to a much larger number of metabolites than had previously been anticipated (Mullen *et al.* 2002). In addition, recent improvements in the sensitivity of PDA detectors, in terms of flow cell optics with increased path lengths, have lowered limits of detection. Also, negative ion MS using ion trap MS^n has made it much easier to identify metabolite peaks observed in the improved absorbance traces.

Conclusions

The present study with human subjects, in which unhydrolysed extracts were analysed by HPLC with PDA and full-scan data-dependent MS^n detection, provided a far more detailed picture of the fate of flavonol glucosides within the body than was possible in earlier investigations. In total, twenty-three metabolites were either identified or partially identified with five being quantified in plasma and twelve in urine. If these samples had been subjected to hydrolysis only quercetin and isorhamnetin would have been detected and quantified. These data are of great importance in understanding the role of dietary flavonols in the prevention of chronic disease. The bioactivity of these metabolites must be studied to confirm the extent of their bioactivity and mechanisms of action.

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