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

William Mullen1, Christine A. Edwards2 and Alan Crozier1*

1Plant Products and Human Nutrition Group, Graham Kerr Building, Division of Biochemistry and Molecular Biology, Institute of Biomedical and Life Sciences, University of Glasgow, Glasgow G12 8QQ, UK
2Human Nutrition Section, University of Glasgow Division of Developmental Medicine, Yorkhill Hospital, Glasgow G3 8SJ, UK

(Received 19 September 2005 – Revised 13 January 2006 – Accepted 21 February 2006)

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 (Cmax) 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 Cmax of quercetin glucuronide sulphate was 2.5 h. The elimination half-lives (t1/2) of quercetin-3'-sulphate, quercetin-3-glucuronide and quercetin diglucuronide were 1.71, 2.33 and 1.76 h respectively, while the t1/2 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–MS²

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 (MS²). 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'-diglucoside (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–MS² in

Abbreviations: Cmax, maximum post-ingestion plasma concentration of quercetin metabolites; MS², tandem MS; PDA, photodiode array; tmax, time to reach Cmax; tR, retention time; t1/2, the elimination half-life of the metabolites.

* Corresponding author: Professor Alan Crozier, fax +44 (0)141 330 5364, email a.crozier@bio.gla.ac.uk
the selected reaction monitoring mode detected five 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 photo-diode array (PDA) and MS² 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).
Formic acid was purchased from Riedel-DeHaen (Seelze, 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.

[2-14C]Quercetin-4′-O-β-d-glucoside was synthesised in four steps from barium [14C]carbonate (specific activity 3·75 mCi/mmole) by a method previously reported for the synthesis of [2-13C]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 1H 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 Kroom (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®-Werke, Staufen, Germany). During the homogenisation, the samples were kept on ice. The mixture was then centrifuged at 3000 g 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 for 10 min. Aliquots (20 µl) of the supernatant were analysed by HPLC–PDA–MS2.

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 for 10 min. The supernatant was collected and the pellet re-extracted as described earlier but with methanol instead of acetonitrile. Experiments with [14C]quercetin-4′-glucoside, quercetin-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–MS2 on the day of extraction. [2-14C]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–MS2 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 MS2 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′-gluco 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 MS2 fragmentation data.

Pharmacokinetic analysis of plasma metabolites

Maximum post-ingestion plasma concentration of quercetin metabolites was defined as Cmax. The time to reach maximum plasma concentration (tmax) was defined as the time in hours at which Cmax 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 0–24 h plasma metabolite concentrations.

Results

Analysis of fried onions

Gradient reverse-phase HPLC with absorbance detection and full-scan data-dependent MS2 was used to identify and quantify the flavonol content of fried onion meals. Absorbance at
365 nm and negative ionisation MS² 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-3,4⁰-diglucoside (I; 107 (SE 1·4) μmol), quercetin-4⁰-glucoside (II; 143 (SE 12) μmol) and isorhamnetin-4⁰-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 MS² 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 MS² spectra and tR data are summarised in Table 1. The use of HPLC–MS² 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-3,4⁰-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 MS² 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-3,3³-sulphate and quercetin-3-glucuronide. These compounds had a Cmax of 665 (SE 82) and 351 (SE 27) nM respectively. In both instances tmax was less than 1 h after the ingestion of the onion supplement (Table 2). A quercetin diglucuronide (peak 9) had a similar tmax (0·80 ( SE 0.12) h) but a lower Cmax (62 (SE 12) nM) than the two main metabolites. The levels of all three metabolites declined after reaching Cmax (Fig. 4) and they had a similar t1/2 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 x 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.
Two urinary metabolites were detected in amounts that illustrated in Fig. 3. Isorhamnetin-3-glucuronide had a $C_{\text{max}}$ of 112 (SE 18) nmol and a $t_{\text{max}}$ of 0.60 h and there was a slow rate of decline after $C_{\text{max}}$ which is reflected in a $t_{1/2}$ of 5.34 h (Table 3). The $C_{\text{max}}$ of the quercetin glucuronide sulphate (peak 14) was 123 (SE 26) nmol while its $t_{\text{max}}$ 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'-glucoside and quercetin-4'-sulphate, 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'-glucuronide (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 $t_{\text{max}}$ 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 $C_{\text{max}}$ and similar $t_{\text{max}}$ 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 $t_{\text{max}}$. However, the total contribution of these two compounds to the overall absorption profile was minimal, having no effect on the $t_{\text{max}}$ 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)

---

### Table 1. HPLC–tandem MS (MS²) identification of quercetin metabolites detected in plasma and urine of six human subjects after the consumption of 270 g fried onions

<table>
<thead>
<tr>
<th>Peak†</th>
<th>$t_{\text{max}}$ (min)†</th>
<th>Compound</th>
<th>[M-H]⁻ (m/z)</th>
<th>MS² fragments ions (m/z)</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>22-8</td>
<td>Quercetin-3,4'-diglucoside†</td>
<td>625</td>
<td>463 ([M-H]⁻ – GlicUA), 301 ([M-H]⁻ – GlicUA-GlicUA)</td>
<td>Plasma</td>
</tr>
<tr>
<td>6</td>
<td>24-8</td>
<td>Quercetin diglucuronide</td>
<td>653</td>
<td>477 ([M-H]⁻ – GlicUA), 301 ([M-H]⁻ – GlicUA-GlicUA)</td>
<td>Urine</td>
</tr>
<tr>
<td>8</td>
<td>27-0</td>
<td>Quercetin glucoside glucuronide</td>
<td>639</td>
<td>477 ([M-H]⁻ – GlicUA), 301 ([M-H]⁻ – GlicUA-GlicUA)</td>
<td>Urine</td>
</tr>
<tr>
<td>10</td>
<td>28-4</td>
<td>Quercetin-3-glucuronide</td>
<td>477</td>
<td>301 ([M-H]⁻ – GlicUA)</td>
<td>Urine, plasma</td>
</tr>
<tr>
<td>11</td>
<td>28-4</td>
<td>Quercetin-3-glucoside†</td>
<td>463</td>
<td>301 ([M-H]⁻ – GlicUA)</td>
<td>Plasma</td>
</tr>
<tr>
<td>12</td>
<td>29-6</td>
<td>Quercetin glucoside sulphate</td>
<td>543</td>
<td>463 ([M-H]⁻ – SO₃)</td>
<td>Urine</td>
</tr>
<tr>
<td>15</td>
<td>30-6</td>
<td>Quercetin glucoside sulphate</td>
<td>543</td>
<td>463 ([M-H]⁻ – SO₃-GlcUA), 301 ([M-H]⁻ – SO₃-GlicUA)</td>
<td>Urine</td>
</tr>
<tr>
<td>16</td>
<td>33-2</td>
<td>Isorhamnetin-3-glucoside‡</td>
<td>477</td>
<td>315 ([M-H]⁻ – GlicUA)</td>
<td>Plasma</td>
</tr>
<tr>
<td>17</td>
<td>34-1</td>
<td>Isorhamnetin-3-glucuronide</td>
<td>491</td>
<td>315 ([M-H]⁻ – GlicUA)</td>
<td>Urine, plasma</td>
</tr>
<tr>
<td>18</td>
<td>34-4</td>
<td>Quercetin-4'-glucuronide</td>
<td>477</td>
<td>301 ([M-H]⁻ – GlicUA)</td>
<td>Plasma</td>
</tr>
<tr>
<td>19</td>
<td>36-3</td>
<td>Quercetin-3'-glucuronide</td>
<td>477</td>
<td>301 ([M-H]⁻ – GlicUA)</td>
<td>Urine, plasma</td>
</tr>
<tr>
<td>20</td>
<td>37-2</td>
<td>Isorhamnetin-4'-glucuronide</td>
<td>491</td>
<td>315 ([M-H]⁻ – GlicUA)</td>
<td>Urine, plasma</td>
</tr>
<tr>
<td>21</td>
<td>43-2</td>
<td>Quercetin-2'</td>
<td>301</td>
<td>179, 151</td>
<td>Plasma</td>
</tr>
<tr>
<td>22</td>
<td>47-9</td>
<td>Quercetin-3'-sulphate</td>
<td>381</td>
<td>301 ([M-H]⁻ – SO₃)</td>
<td>Urine, plasma</td>
</tr>
<tr>
<td>23</td>
<td>48-3</td>
<td>Quercetin sulphate‡</td>
<td>381</td>
<td>301 ([M-H]⁻ – SO₃)</td>
<td>Plasma</td>
</tr>
</tbody>
</table>

Glc, glucosyl unit; GlicUA, glucuronyl unit; [M-H]⁻, negatively charged molecular ion; $t_{\text{max}}$, retention time.

† 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.

Downloaded from https://www.cambridge.org/core. IP address: 54.70.40.11, on 27 Sep 2018 at 20:17:20, subject to the Cambridge Core terms of use, available at https://www.cambridge.org/core/terms . https://doi.org/10.1079/BJN20061809
Supplementation.

Onions were present in detectable amounts in plasma collected 24 h after ingestion of 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–MS².

Confirming the validity of the short t₁/₂ 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 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 hepatoma 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 sulphonation of the aglycone in the enterocyte, as illustrated in Fig. 5,

Figure 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).

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

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Peak number†</th>
<th>Cmax (nM)</th>
<th>tmax (h)</th>
<th>t₁/₂ (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SE</td>
<td>Mean</td>
<td>SE</td>
</tr>
<tr>
<td>Quercetin-3'-sulphate</td>
<td>22</td>
<td>665</td>
<td>0·75</td>
<td>0·12</td>
</tr>
<tr>
<td>Quercetin-3-glucuronide</td>
<td>10</td>
<td>351</td>
<td>0·60</td>
<td>0·10</td>
</tr>
<tr>
<td>Isorhamnetin-3-glucuronide</td>
<td>17</td>
<td>112</td>
<td>0·60</td>
<td>0·10</td>
</tr>
<tr>
<td>Quercetin diglucuronide</td>
<td>9</td>
<td>62</td>
<td>0·80</td>
<td>0·12</td>
</tr>
<tr>
<td>Quercetin glucuronide sulphate</td>
<td>14</td>
<td>123</td>
<td>2·5</td>
<td>0·22</td>
</tr>
</tbody>
</table>

Cmax, maximum post-ingestion plasma concentration; tmax, time to reach Cmax; t₁/₂, the elimination half-life of the metabolites.

* For details of procedures, see p. 108.

† Peak numbers as in Fig. 2 and Table 1.
would appear to be a more straightforward, but not necessarily exclusive, route.

The $C_{\text{max}}$ 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-glucuronide, 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 $\beta$-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 MS² 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

<table>
<thead>
<tr>
<th>Metabolites</th>
<th>Peak number</th>
<th>0–4 h</th>
<th>4–8 h</th>
<th>8–24 h</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean</td>
<td>SE</td>
<td>Mean</td>
<td>SE</td>
</tr>
<tr>
<td>Quercetin-3-glucuronide</td>
<td>10</td>
<td>512</td>
<td>101</td>
<td>400</td>
<td>113</td>
</tr>
<tr>
<td>Quercetin-3'-glucuronide</td>
<td>19</td>
<td>979</td>
<td>220</td>
<td>804</td>
<td>194</td>
</tr>
<tr>
<td>Quercetin diglucuronide</td>
<td>9</td>
<td>1007</td>
<td>253</td>
<td>942</td>
<td>273</td>
</tr>
<tr>
<td>Quercetin glucuronide glucose</td>
<td>3</td>
<td>99</td>
<td>21</td>
<td>64</td>
<td>16</td>
</tr>
<tr>
<td>Quercetin glucuronide sulphate</td>
<td>13</td>
<td>608</td>
<td>124</td>
<td>566</td>
<td>143</td>
</tr>
<tr>
<td>Quercetin glucoside sulphate</td>
<td>14</td>
<td>743</td>
<td>170</td>
<td>418</td>
<td>98</td>
</tr>
<tr>
<td>Quercetin glucuronide sulphate</td>
<td>12</td>
<td>225</td>
<td>73</td>
<td>130</td>
<td>34</td>
</tr>
<tr>
<td>Quercetin glucuronide sulphate</td>
<td>15</td>
<td>538</td>
<td>127</td>
<td>257</td>
<td>98</td>
</tr>
<tr>
<td>Isorhamnetin-3-glucuronide</td>
<td>17</td>
<td>767</td>
<td>18</td>
<td>861</td>
<td>9</td>
</tr>
<tr>
<td>Isorhamnetin-4'-glucuronide</td>
<td>20</td>
<td>451</td>
<td>11</td>
<td>249</td>
<td>2</td>
</tr>
<tr>
<td>Methylquercetin diglucuronide</td>
<td>2</td>
<td>439</td>
<td>132</td>
<td>475</td>
<td>67</td>
</tr>
<tr>
<td>Methylquercetin diglucuronide</td>
<td>4</td>
<td>189</td>
<td>49</td>
<td>163</td>
<td>41</td>
</tr>
</tbody>
</table>

ND, not detected.

* For details of procedures, see p. 108.
† Peak numbers as in Fig. 2 and Table 1.
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<sub>max</sub> 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.](https://doi.org/10.1079/BJN20061809)
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 flavonoids 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 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 [2-14C]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 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 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.

Acknowledgements

The authors would like to thank the volunteers who participated in this study and also Drs Paul Needs and Paul Kroon, Food Research Institute, Norwich, UK for generously supplying us with samples of quercetin metabolites. We would also like to thank Aurélie Boitier for her skilful assistance and Alison Sutcliffe who isolated quercetin-3-glucuronide from her home-grown French beans. The HPLC–MS system used in this study was purchased with a BBSRC grant to A. Crozier and J. R. Coggins.

References


