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HPLC-MS² METABOLITE PROFILING OF QUERCETIN METHYL, GLUCURONYL, GLUCOSYL AND SULPHO-CONJUGATES IN PLASMA AND URINE AFTER THE INGESTION OF ONIONS BY HUMAN VOLUNTEERS

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ABSTRACT

Lightly fried onions containing 275 µmoles of flavonols, principally quercetin-4′-glucoside and quercetin-3,4′-diglucoside, were fed to healthy human volunteers with plasma and urine collected over a 24 hr 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-µM peak plasma concentrations (C_{max}) of quercetin-3′-sulphate, quercetin-3-glucuronide, isorhamnetin-3-glucuronide and quercetin diglucuronide were observed 0.6-0.8 hr after ingestion. In contrast, the C_{max} of quercetin glucuronide sulphate was 2.5 hr. 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 hr respectively while the $T_{1/2}$ of isorhamnetin-3-glucuronide was 5.34 hr and that of quercetin glucuronide sulphate was 4.54 hr. The profile of metabolites excreted in urine was markedly different to that of plasma with many of the major urinary components 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 µmoles corresponding to 4.7% of intake. The bioactivity of these metabolites should be considered.

Keywords: quercetin glucosides, absorption, metabolism, excretion, humans.

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INTRODUCTION

Flavonols are polyphenolic C_6-C_3-C_6 compounds which occur widely in plants and plant-derived foods (Saltmarch *et al.*, 2003) and beverages (Duthie and Crozier, 2003). They have several potential nutritional and health promoting roles in the human body but there is still much unknown 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 coronary heart disease 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 mass spectrometry (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

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of quercetin-3,4'-diglucoside (I in *Figure 1*), quercetin-4'-glucoside (II), and smaller amounts of other conjugates including isorhamnetin-4'-glucoside (III) (Tsushida and 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 and Williamson, 2002). 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.*, 1996a, b, 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 the selected reaction monitoring mode detected five quercetin glucuronides in human plasma collected 1 hr after ingestion of an 800 g onion supplement (Wittig *et al.*, 2001). A further study in which plasma, collected 1.5 hr post-ingestion of 200 g of fried onions, was analysed by HPLC identified in total 12 putative quercetin metabolite peaks. 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), isorhamnetin-3-glucuronide (V) and quercetin-3'-sulphate (VI) (Day *et al.*, 2001). In the study reported here, HPLC with photodiode array (PDA) and MS^2 detection was used to analyse human plasma and urine collected from six volunteers at a series of time points over a 24 hr period after the ingestion of red onions which contained high levels of a range of anthocyanins and flavonols.

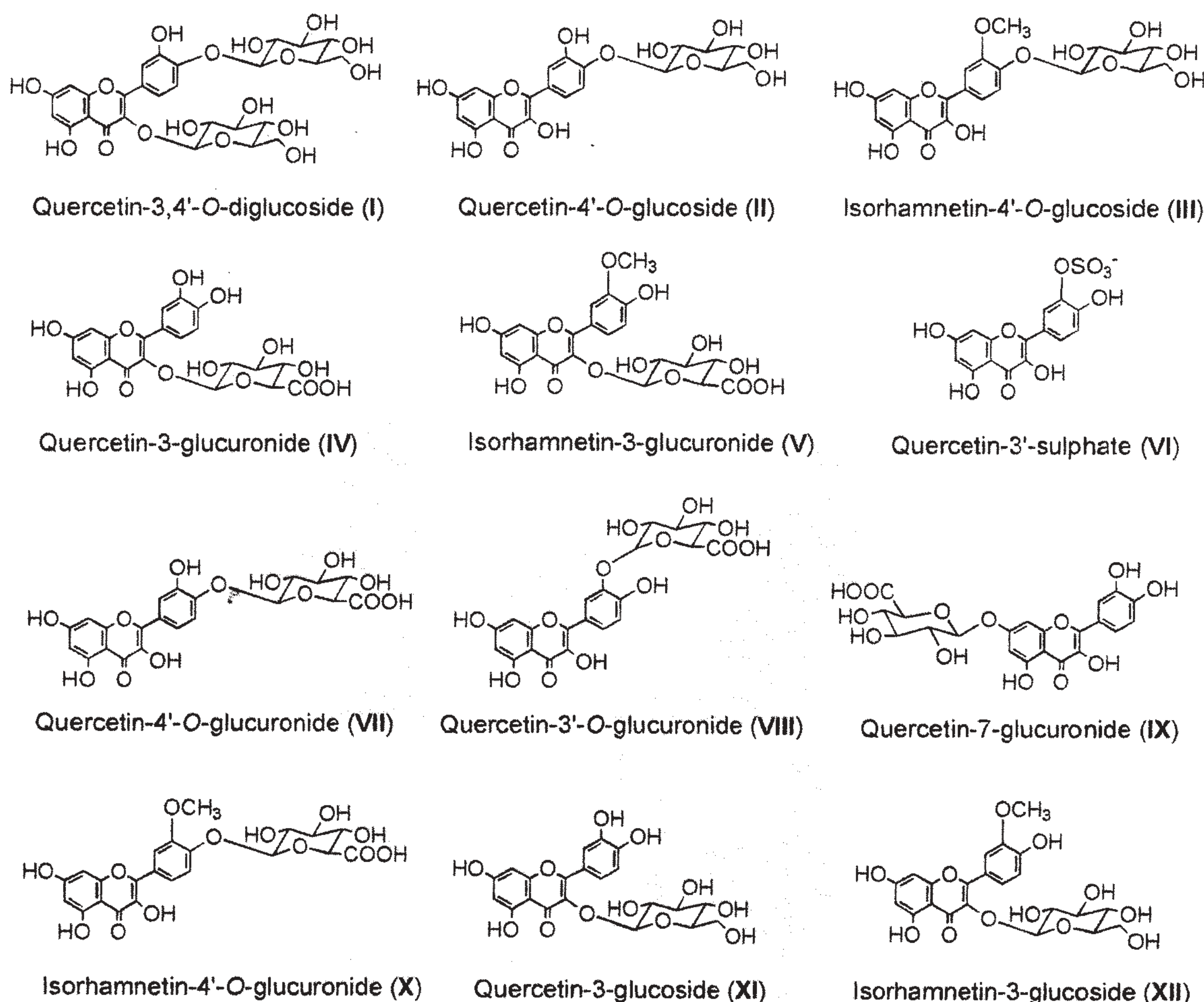

Figure 1. Structures of flavonol glucosides and their metabolites.

EXPERIMENTAL

Study Design

Six volunteers (four males and two females), who were healthy, non-smokers and not on any medication, participated in this study and gave their written consent. They were aged between 23 and 45 years and had a mean body mass index of 23.7 ± 1.2 (range 20.9-27.6). Subjects were required to follow a low flavonoid diet for two days and to fast overnight prior to supplementation. This diet excluded most fruits, vegetables and beverages such as 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.

All subjects consumed 270 g of fried red onions. Venous blood samples were taken before (0 hr) and 0.5, 1, 2, 3, 6 and 24 hr post-ingestion. Blood (12 ml) was collected in heparinized tubes at each time point and immediately centrifuged at 4000 *g* for 10 min at 4°C. The plasma was separated from the red blood cells and 500 µl aliquots were acidified to pH 3 with 15 µl of 50% aqueous formic acid and 50 µl of 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 hr 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 plc, 80, Crow Road, Glasgow G11, United Kingdom). 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, United Kingdom). L-(+)-ascorbic acid, quercetin and isorhamnetin-3-glucoside were purchased from Extrasynthese (Genay, France). AASC Ltd (Southampton, England) supplied quercetin-3,4'-diglucoside, quercetin-4'-glucoside, quercetin-3-glucoside, isorhamnetin-4'-glucoside, malvidin-3-glucoside and cyanidin-3-glucoside.

[2-^{14}C]Quercetin-4'-*O*-β-D-glucoside was synthesized in four steps from barium [^{14}C]carbonate (specific activity 3.75 mCi mmol^{-1}) 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-7-glucuronide, quercetin-3'-sulphate and isorhamnetin-3- glucuronide were donated by Dr Paul Needs and Dr Paul Kroon (Institute of Food Research, Norwich, United Kingdom).

Extraction of Onions and Plasma

Aliquots of fried onions were extracted with 70% methanol and processed as described by Mullen *et al.* (2004). Triplicate samples of plasma were treated according to the method of Day *et al.* (2001). Quantitative analysis of the flavonol and anthocyanin content of extracts was carried out with HPLC-PDA-MS^2. Prior to extraction [^{14}C]quercetin-4'-glucoside was added to plasma samples as an internal standard.

Urine

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

HPLC with Photodiode 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 auto sampler cooled to 4°C (Thermo Finnigan, San Jose, USA). Separation was carried out using a 250 x 4.6 mm I.D. 4 µm Synergi Max-RP column (Phenomenex, Macclesfield, United Kingdom) eluted with a 60 min gradient of 5%-40% acetonitrile in 1% formic acid at a flow rate of 1 ml min^{-1} 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^{-1} was directed to a LCQ DecaXP ion trap mass spectrometer fitted with an electrospray interface (Thermo Finnigan). Analyses utilized the negative ion mode for flavonols and positive ionization for anthocyanins as this provided the best limits of detection. Analysis was carried out using full scan, data dependant 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 ionization and 1 kV for positive ionization.

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. Anthocyanins in red onion extracts were quantified by reference to a 515 nm cyanidin-3-glucoside calibration curve.

Additional analyses were carried out specifically for the separation of quercetin-3-glucuronide from quercetin-7-glucuronide. These used the same MS^2 conditions and negative ionization, with a 250 x 4.6 mm I.D. 4 µm Synergi Polar-RP column (Phenomenex, Macclesfield, United Kingdom), maintained at 40°C, eluted with a 60 min gradient of 10%-18% acetonitrile in 1% formic acid at a flow rate of 1 ml min^{-1}.

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 $T_{1/2} = 0.693/K_e$ where K_e is the slope of the linear regression of the plasma metabolite concentrations.

RESULTS

Analysis of Fried Red Onions

Gradient reverse phase HPLC with absorbance detection and full scan data dependent MS^2 was used to identify and quantify the flavonol and anthocyanin content of the fried red onion meals. Absorbance at 365 nm and negative ionization MS^2 were used for flavonol analysis. The total amount of flavonols in the 270 g onion meal was 275 ± 8.8 µmoles. In keeping with the data of Tsushida and Suzuki (1995), the major components were quercetin-3,4'-diglucoside (I) (107 ± 1.4 µmol), quercetin-4'-glucoside (II) (143 ± 12 µmol) and isorhamnetin-4'-glucoside (III) (11 ± 1.4 µmoles) which accounted for 95% of the 275 ± 8.8 µmoles flavonol intake.

The anthocyanins were monitored at 515 nm and positive ion full scan MS^2 data were obtained. In accordance with previously published work, seven anthocyanins were detected (Donner *et al.*, 1997) and main components in the 270 g meal were cyanidin-3-glucoside (9.3 ± 0.3 µmoles), cyanidin-3-(6''-malonylglucoside) (48 ± 1.7 µmoles) and cyanidin-3-(6''-malonyllaminaribioside) (14 ± 0.5 µmoles). The total anthocyanin content of the onion meal was estimated to be 75 ± 2 µmoles.

Qualitative Analysis of Plasma and Urine

Plasma and urine samples, collected as previously described, were analysed by HPLC with PDA and MS^2 detection. No peaks were apparent in the A_{515} nm traces obtained with either urine or plasma. With the sample sizes analysed and the limit of detection at A_{515} nm, anthocyanins at levels $\geq 0.1\%$ of the amounts ingested would have been detected. In contrast to the anthocyanins, flavonol metabolites were present in plasma and urine, corresponding to ca. 4% of the intake, with a total of 23 quercetin-based compounds being detected. Typical HPLC traces obtained at A_{365} nm are illustrated in *Figures 2a* and *b* and the identifications based on MS^2 spectra and t_R data are summarized in *Table 1*. The different classes of metabolites that were detected can be summarized as follows:

Quercetin monoglucuronides. Three quercetin monoglucuronides were detected, each being characterized by a negatively charged molecular ion ($[M-H]^-$) at *m/z* 477 which on MS^2 fragments with a loss of 176 *u*, corresponding to the cleavage of a glucuronide unit, to produce an ion at *m/z* 301 from the quercetin fragment. Co-chromatography with reference compounds on the Synergi Max-RP HPLC column facilitated the identification of quercetin-4'-glucuronide (VII) (*peak 18*) and quercetin-3'-glucuronide (VIII) (*peak 19*). However, peak 10, a significant component in both plasma and urine, co-chromatographed with both quercetin-3-glucuronide (IV) and quercetin-7-glucuronide (IX) standards. A second HPLC protocol using a Synergi Polar-RP column was therefore used to resolve these components. Quercetin-7-glucuronide had a t_R of 34.6 min and quercetin-3-glucuronide eluted later with a t_R of 36.2 min. When urine was analysed using the Polar-RP column, peak 10 co-chromatographed with quercetin-3-glucuronide and there was no evidence of the presence of quercetin-7-glucuronide (*Figure 3*). Similar data were obtained with plasma samples. This finding contradicts claims by Spencer *et al.*, (2003) that quercetin-7-glucuronide is one of the major *in vivo* metabolites of quercetin in humans.

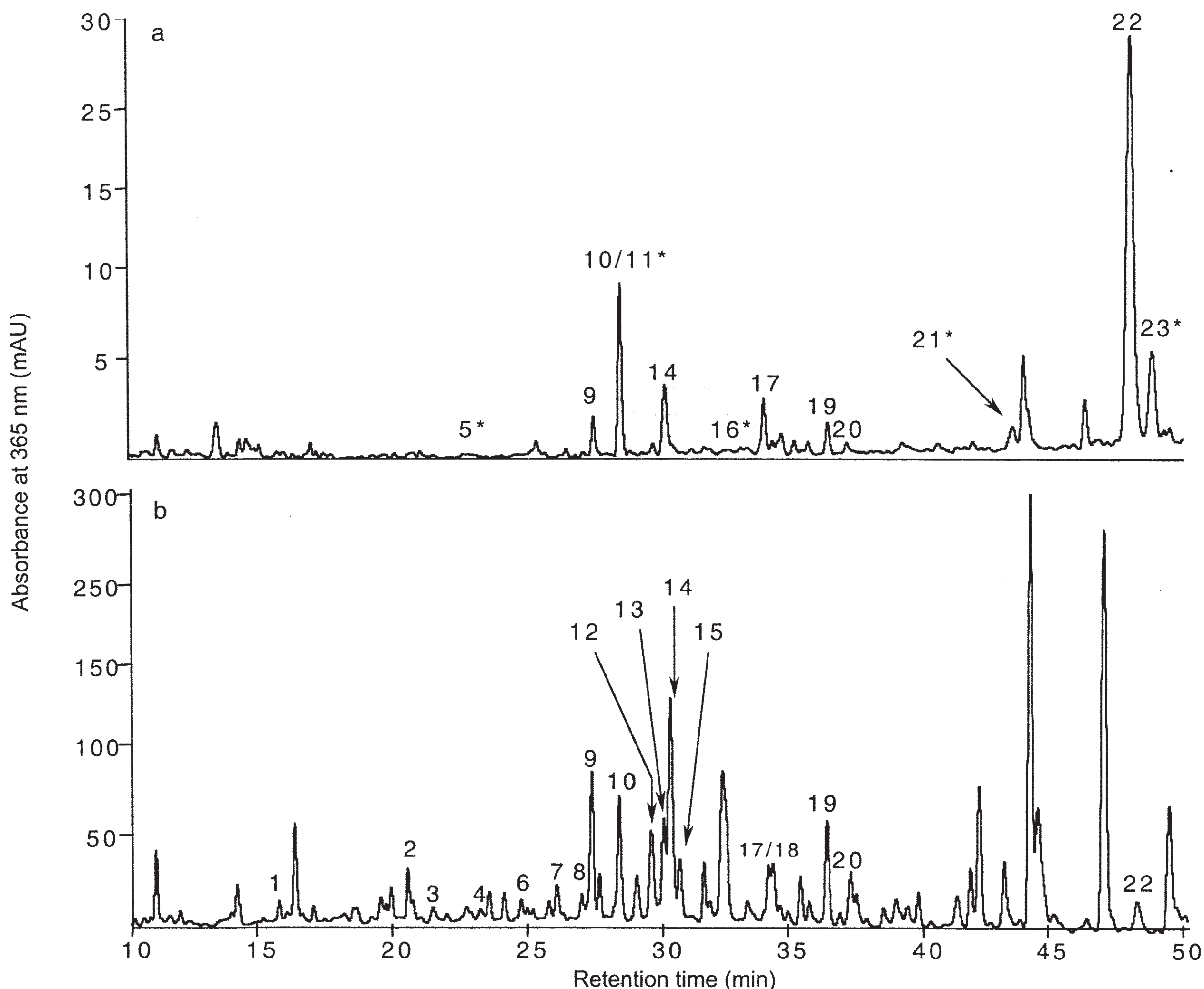

Figure 2. Gradient reversed-phase HPLC with detection at 365 nm of quercetin metabolites in (a) a plasma extract and (b) urine obtained from a human volunteer after the consumption of fried red onions. Samples analysed on a 250 x 4.6 mm I.D., 4 µm Synergi Max-RP column at 40°C and eluted at a flow rate of 1 ml min^{-1} 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 MS^2 with an electrospray interface with negative ionization – for identity of peaks 1–23, (Table 1). ** Indicates peaks detected in samples from only one of six volunteers.*

TABLE 1. HPLC-MS^2 IDENTIFICATION OF QUERCETIN METABOLITES DETECTED IN PLASMA AND URINE AFTER THE CONSUMPTION OF 270 g OF FRIED ONIONS BY SIX HUMAN VOLUNTEERS

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

Notes: Peak numbers and HPLC retention times refer to HPLC trace in *Figures 2a* and *b*. t_R - retention time; $[M-H]^-$ - negatively charged molecular ion; Glc - glucosyl unit; GlcUA – glucuronyl unit; *indicates compounds detected only in the plasma of one of the six volunteers.

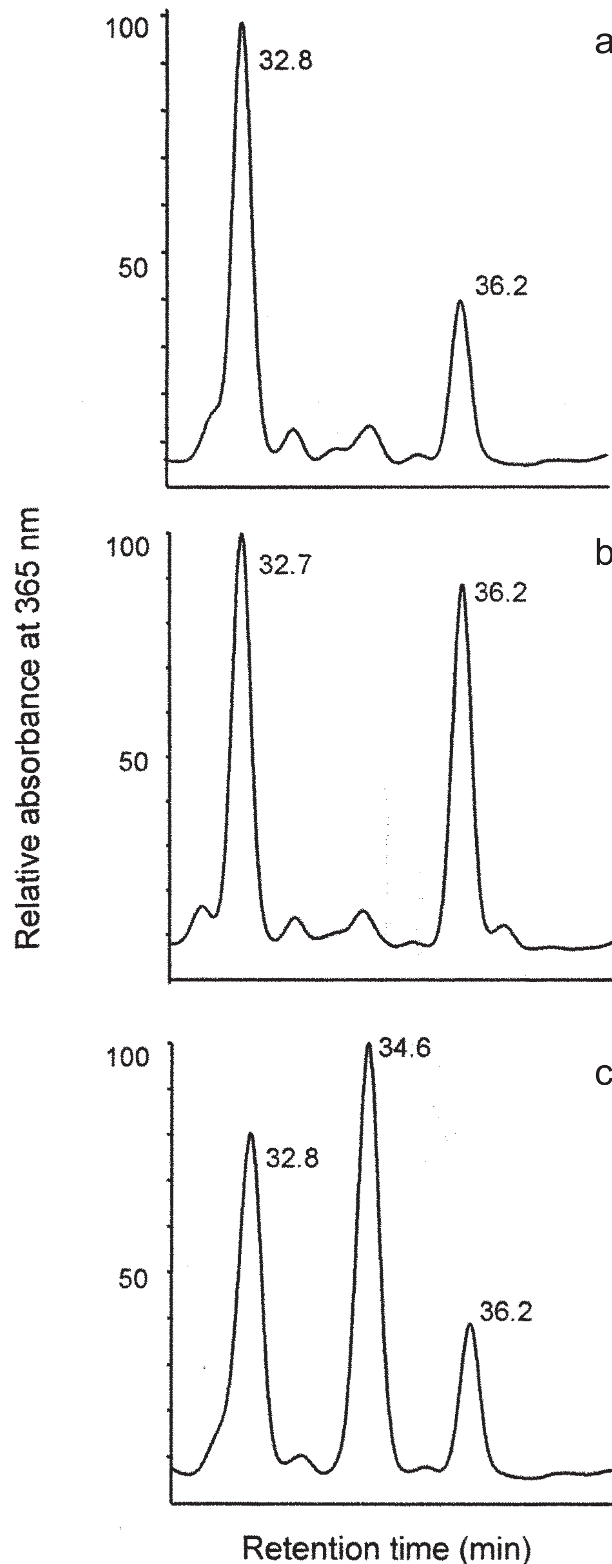

Figure 3. Gradient HPLC co-chromatography of urine, obtained from a human volunteer after the consumption of 270 g of fried red onions, with quercetin-3-glucuronide and quercetin-7-glucuronide. Samples, (a) urine, (b) urine and quercetin-3-glucuronide and (c) urine and quercetin-7-glucuronide, analysed on a 250 x 4.6 mm I.D. 4μm Synergi Polar-RP column at 40°C at a flow rate of 1 ml min^{-1} with a 60 min gradient of 5%-25% acetonitrile in water containing 1% formic acid. Detection was with a diode array detector operating at 365 nm. Numbers associated with individual peaks indicate retention times in minutes.

***Quercetin diglucuronides**.* Low levels of three quercetin diglucuronides, peaks 1, 6 and 9, were present in urine. Each had a $[M-H]^-$ at *m/z* 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). This indicates that the two glucuronyl units are attached at different positions on the quercetin skeleton. If they have been linked at the same position, it is unlikely that a M-176 fragment would have been produced at *m/z* 477 as it has been shown that anthocyanin disaccharide conjugates fragment with loss of the intact disaccharide moiety (Giusti *et al.*, 1999).

***Methylquercetin monoglucuronides**.* Two methyl quercetin glucuronides were detected, peaks 17 and 20, which were characterized by a mass spectrum with fragment ions at *m/z* values 14 *u* higher than obtained with quercetin glucuronides. Co-chromatography with a reference compound established that peak 17 was 3'-methylquercetin-3-glucuronide (isorhamnetin-3-glucuronide, V). Peak 20 was tentatively identified as 3'-methylquercetin-4'-glucuronide (isorhamnetin-4'-glucuronide, X) on the basis of previous work (Day *et al.*, 2001).

***Methylquercetin diglucuronides**.* Peaks 2 and 4 were identified as methylquercetin diglucuronides on the basis of mass spectra with fragment ions at *m/z* values 14 *u* higher than obtained with quercetin diglucuronides.

***Quercetin**.* The plasma of one of the six volunteers contained traces of the aglycone, quercetin, which had a $[M-H]^-$ at *m/z* 301 and MS^2 fragment ions at *m/z* 179 and 151.

***Quercetin sulphates**.* Peaks 22 and 23 had a $[M-H]^-$ at *m/z* 381 and MS^2 yielded a major ion at *m/z* 301 ($[M-H]^-$-80) which is in-keeping with the fragmentation of a quercetin sulphate. On the basis of co-chromatography with a reference compound, peak 22 was identified as quercetin-3'-sulphate (VI), which was the major quercetin metabolite in plasma (*Figure 2a*). The position of the sulphate group on the quercetin sulphate in peak 23 remains undetermined. However, the close t_R with that of quercetin-3'-sulphate indicates that it might be quercetin-4'-sulphate.

***Quercetin glucuronide sulphates**.* Peaks 13 and 14 both produced a $[M-H]^-$ at *m/z* 557 and MS^2 yielded ions at *m/z* 477 ($[M-H]^-$-80, loss of SO_3), *m/z* 381 ($[M-H]^-$-176, loss of a glucuronide unit) and *m/z* 301 (quercetin) indicating that both compounds are quercetin glucuronide sulphates. Peak 14 was the main quercetin metabolite in the urine of all volunteers.

***Quercetin glucosides**.* The plasma of one volunteer contained traces of quercetin-3,4'-diglucoside as well as small amounts of quercetin-3-glucoside (XI) and its methylated derivative isorhamnetin-3-glucoside (XII). The mass spectra of these compounds are summarized in *Table 1* and in all three instances identity was established by co-chromatography with the appropriate standard.

***Quercetin glucoside glucuronides**.* Peaks 3, 7 and 8 which were detected in 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 glucuronide unit) and *m/z* 301 indicating that both compound are quercetin glucoside glucuronides.

***Quercetin glucoside sulphates**.* Peaks 12 and 15 were characterized by a $[M-H]^-$ at *m/z* 543 which when fragmented produced MS^2 ions at *m/z* 463 ($[M-H]^-$-80, loss of SO_3), *m/z* 381 (M-162, loss of a glucoside unit) and *m/z* 301 demonstrating the presence of quercetin glucoside sulphates.

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, isorhamnetin-3-glucuronide and quercetin-3'-sulphate. Quercetin-3'-glucuronide and isorhamnetin-4'-glucuronide were present in the plasma of all volunteers in low, non-quantifiable, amounts while other flavonol derivatives, quercetin-3,4'-diglucoside, quercetin-3-glucoside, isorhamnetin-3-glucoside and the aglycone quercetin, were detected, albeit in very small quantities, only in the plasma of volunteer 6 (*Table 1*).

The 0-24 hr pharmacokinetic profiles of the five major plasma flavonol metabolites are illustrated in *Figure 4* and the pharmacokinetic analysis summarized 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 ± 82 nM and 351 ± 27 nM, respectively. In both instances T_{max} was less than 1 hr after the ingestion of the onion supplement (*Table 2*). A quercetin diglucuronide (*peak 9*) had a similar T_{max} (0.80 ± 0.12 hr) but a lower C_{max} (62 ± 12 nM) than the two main metabolites. The levels of all three metabolites declined after reaching C_{max} (*Figure 4*) and they had a similar $T_{1/2}$ with values of 1.71-2.33 hr (*Table 3*). The pharmacokinetic profiles of isorhamnetin-3-glucuronide and quercetin

glucuronide sulphate (*peak 14*) were different to those of the other metabolites illustrated in *Figure 4*. Isorhamnetin-3-glucuronide had a C_{max} of 112 ± 18 nM and a T_{max} of 0.60 hr and there was a slow rate of decline after C_{max} which is reflected in a $T_{1/2}$ of 5.34 hr (*Table 3*). The C_{max} of the quercetin glucuronide sulphate (*peak 14*) was 123 ± 26 nM while its T_{max} at 2.5 ± 0.22 hr was delayed compared to that of the other metabolites and the $T_{1/2}$ (4.54 hr) was much slower than that observed with quercetin-3'-sulphate, quercetin-3-glucuronide and the quercetin diglucuronide (*Table 3*).

The cumulative C_{max} of the five major plasma metabolites was 1.1 μmoles l^{-1}. Assuming 3 litres of plasma per person, this is equivalent to a total of 3.3 μmoles, which is 1.2% of the quercetin glucosides in the ingested onion supplement. This broadly similar to the figures of 0.43% and 0.9% of intake obtained in earlier studies with onions (Hollman *et al.*, 1996a; Aziz *et al.*, 1998). It is, however, much lower than the 6.4% obtained by Graefe *et al.* (2001) who reported a C_{max} of 7.1 μmoles l^{-1}. after the ingestion of an onion supplement containing 331 μmoles of quercetin glucosides.

TABLE 2. PHARMACOKINETIC PARAMETERS OF QUERCETIN METABOLITES IN THE PLASMA OF HUMAN VOLUNTEERS AFTER THE CONSUMPTION OF ONIONS

Metabolite (*peak number*)	C_{max}	T_{max}	$T_{1/2}$
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

Notes: C_{max} – maximum concentration in plasma expressed in nM. T_{max} – time to reach C_{max} expressed in hr. $T_{1/2}$ – the elimination half-life of metabolites in hr. Data presented as mean values ± standard error (n = 6). Peak numbers as in *Figure 2* and *Table 1*.

TABLE 3. QUANTITIES OF QUERCETIN METABOLITES IN URINE OF HUMAN SUBJECTS 0-24 hr AFTER THE CONSUMPTION OF AN ONION SUPPLEMENT

Metabolites (*peak number*)	0 - 4 hr	4 -8 hr	8 -24 hr	Total
Quercetin-3-glucuronide *(10)*	512 ± 101	400 ± 113	n.d.	912 ± 149
Quercetin-3'-glucuronide *(19)*	979 ± 220	804 ± 194	62 ± 30	1 845 ± 193
Quercetin diglucuronide *(9)*	1 007 ± 253	942 ± 273	274 ± 98	2 223 ± 417
Quercetin glucuronide glucoside *(3)*	99 ± 21	64 ± 16	n.d.	163 ± 23
Quercetin glucuronide sulphate *(13)*	608 ± 124	566 ± 143	210 ± 73	1 384 ± 163
Quercetin glucuronide sulphate *(14)*	743 ± 170	418 ± 98	68 ± 50	1 229 ± 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	1 789 ± 239
Isorhamnetin-4'-glucuronide *(20)*	451 ± 11	249 ± 2	n.d.	700 ± 114
Methylquercetin diglucuronide *(2)*	439 ± 132	475 ± 67	89 ± 69	1 003 ± 156
Methylquercetin diglucuronide *(4)*	189 ± 49	163 ± 41	74 ± 36	426 ± 99
Total	6 558 ± 1 323	5 329 ± 1 018	999 ± 267	12 886 ± 1 038

Notes: Mean values expressed as nmoles ± standard error (n = 6). Peak numbers as in *Figure 2* and *Table 1*. n.d. – not detected.

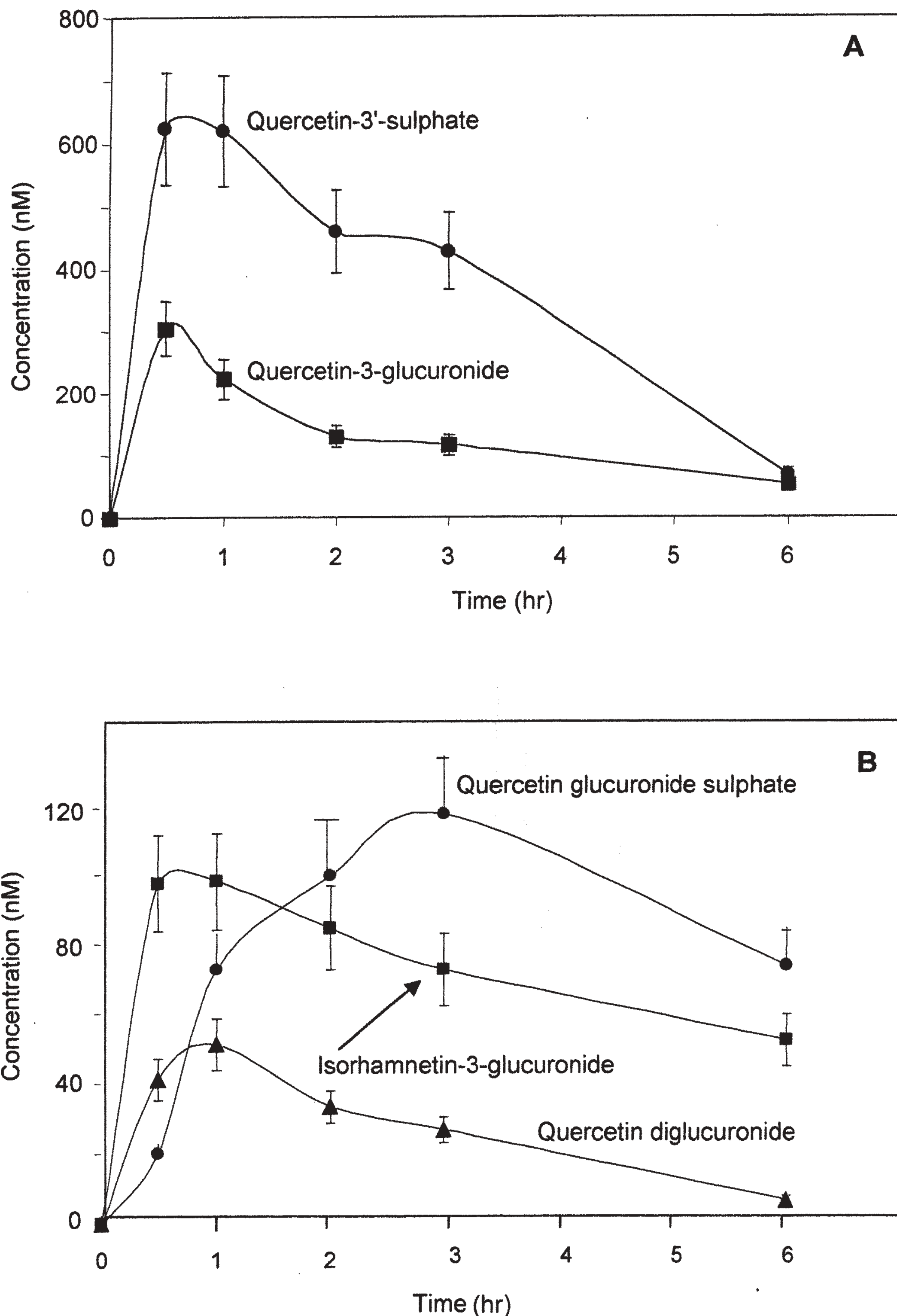

Figure 4. Concentration of (a) quercetin-3′-sulphate, quercetin-3-glucuronide (b) a quercetin glucuronide sulphate, isorhamnetin-3-glucuronide and a quercetin diglucuronide in plasma from six human volunteers collected 0-6 hr after the ingestion of red onions. Data expressed as mean values in nmoles/litre ± standard error (n = 6).

Quantitative Analysis of Flavonol Metabolite Excretion in Urine

Eighteen flavonol metabolites were detected in urine samples collected 0-4, 4-8 and 8-24 hr 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 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 ± 417 nmoles being excreted over the 24 hr period following ingestion of the onion supplement. Substantial amounts of quercetin-3'-glucuronide (1845 ± 193 nmoles), isorhamnetin-3-glucuronde (1789 ± 27 nmoles) and two quercetin glucuronide sulphates (*peak 13*, 1384 ± 163 nmoles; *peak 14*, 1229 ± 190 nmoles) were also detected.

DISCUSSION

The results of this 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 volunteers after ingestion of onions. The pharmokinetics 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 hr and $T_{1/2}$ values of 1.71 and 2.33 hr respectively. (*Figure 4*, *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

TABLE 4. QUERCETIN METABOLITES DETECTED IN PLASMA AND URINE AFTER THE CONSUMPTION OF 270 g OF FRIED ONIONS BY SIX HUMAN VOLUNTEERS. ESTIMATES OF LEVELS IN PLASMA EXPRESSED AS nM ± S.E. (N = 6). AMOUNTS IN URINE EXPRESSED AS AMOUNT EXCRETED IN nM ± S.E. OVER A 24 hr PERIOD POST-INGESTION OF ONIONS

Metabolite	Plasma	Urine
quercetin diglucuronide	n.d.	trace
methylquercetin diglucuronide	n.d.	1003 ± 156
quercetin glucoside glucuronide	n.d.	163 ± 23
methylquercetin diglucuronide	n.d.	426 ± 99
quercetin diglucuronide	n.d.	trace
quercetin glucoside glucuronide	n.d.	trace
quercetin glucoside glucuronide	n.d.	trace
quercetin diglucuronide	51 ± 13	2 223 ± 417
quercetin-3-glucuronide	306 ± 42	912 ± 149
quercetin glucoside sulphate	n.d.	393 ± 60
quercetin glucuronide sulphate	n.d.	1 384 ± 163
quercetin glucuronide sulphate	117 ± 12	1 229 ± 190
quercetin glucoside sulphate	n.d.	821 ± 156
isorhamnetin-3-glucuronide	98 ± 17	1 789 ± 27
quercetin-4'-glucuronide	n.d.	trace
quercetin-3'-glucuronide	trace	1 845 ± 193
isorhamnetin-4'-glucuronide	trace	700 ± 11
quercetin-3'-sulphate	539 ± 46	trace

Notes: Peak numbers refer to HPLC traces in *Figures 2a* and *b*. n.d. - not detected. 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 (*Table 1*) are not presented.

(*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 hr. This $T_{1/2}$ is much shorter than similar absorption studies carried out previously (Hollman *et al.*, 1996a; 1997; Aziz *et al.*, 1998; Moon *et al.*, 2000; Graefe *et al.*, 2001).

More than 90% of the urinary flavonol metabolites were excreted within the first 8 hr after ingestion of onions (*Table 4*). Total 0-24 hr flavonol metabolite excretion in urine for the individual volunteers were 13.9, 13.7, 10.1, 16.4, 9.6 and 14.0 µmoles and the mean value of 12.9 ± 1.1 µmoles corresponds to 4.7% of intake. This is in agreement with the level excretion of flavonols in urine after onion consumption by humans 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 enteroctye 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 and 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 *Figures 5* and *6*. 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 synthesized *in vivo*. A single step sulphation of the aglycone in the enterocyte, as illustrated in *Figure 5*, would appear to be a more direct, but not necessarily exclusive, route.

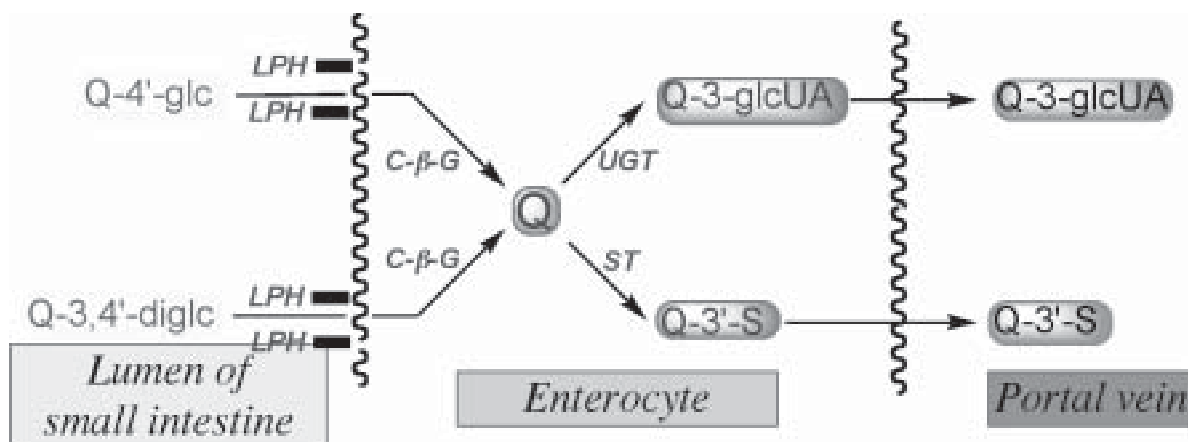

Figure 5. Schematic of the possible metabolic fate of quercetin-4'-glucoside and quercetin-3,4'-diglucoside as they pass from the lumen of the small intestine into the hepatic portal vein. Q – quercetin; glc – glucoside; diglc - diglucoside; glcUA - glucuronide; S - sulphate; LPH – lactase phlorizen hydrolase; C-β-G – cytosolic β-glucosidase; UGT – glucuronyltransferase; ST – sulphofotransferase.

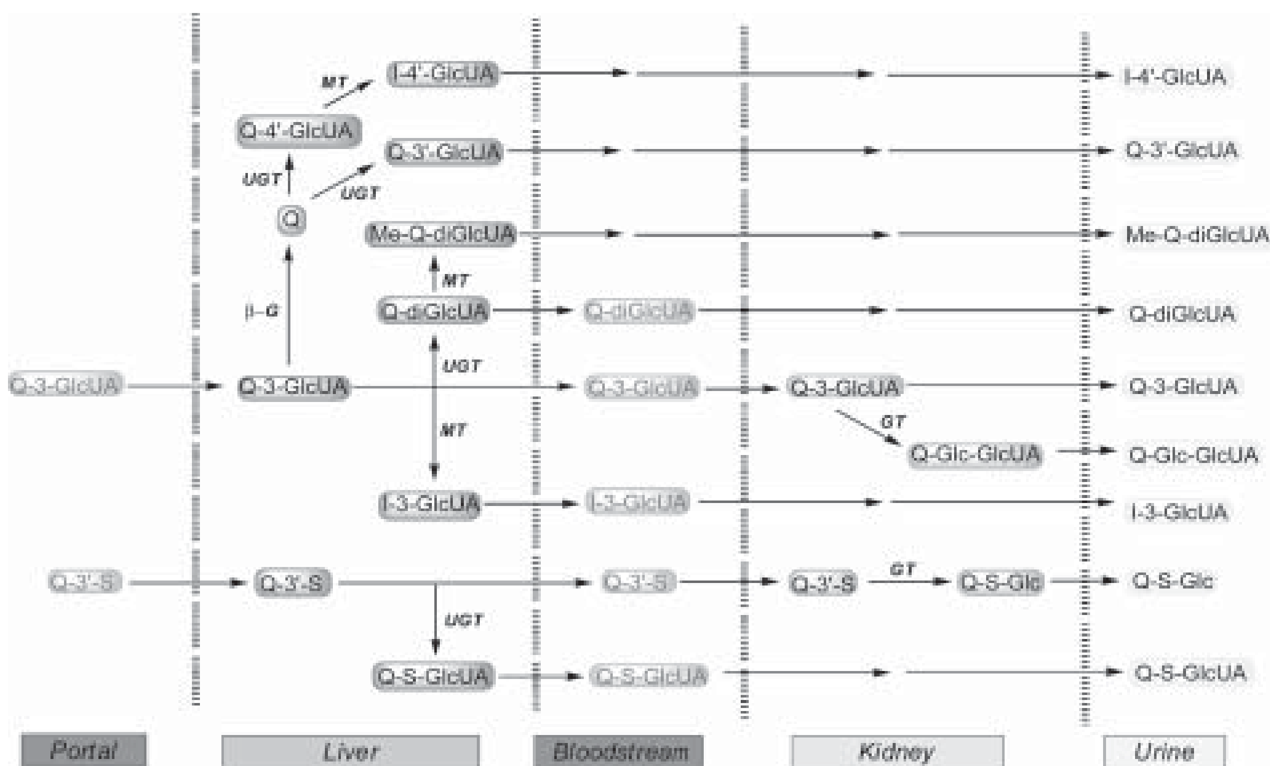

Figure 6. Schematic of the possible metabolic fate of quercetin-3-glucuronide and quercetin-3′-sulphate as they are transported from the small intestine to the liver where they are further metabolized before returning to the bloodstream and being excreted in urine via the kidneys. Q – quercetin; I – isorhamnetin; glc – glucoside; glcUA - glucuronide; diglcUA - diglucuronide S – sulphate; β-G –β-glucosidase; UGT - glucuronyltransferase; MT – methyltransferase; GT – glucosyltransferase.

The C_{max} values of plasma metabolites and 24 hr urinary excretion of the flavonol metabolites (*Table 4*) detected after consumption of onions and present 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, querctin-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 via excretion by 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 *Figure 6*, may be the formation of the glucoside conjugates in the kidneys, which are known to possess β-glucosyltransferase activity (Matern and Matern, 1987; Shipkova *et al.*, 2001).

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 derivatization procedure that produced fluorescent flavonol derivatives (Hollman *et al.*, 1996b). It has been suggested that the putative flavonol glucoside peaks were flavonol glucuronides which have very similar retention properties (Day and 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 and 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 dosed unaccounted for. The most likely fate of these 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 ionize readily when subjected to MS with an electrospray interface.

The data obtained in this 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 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 online radioactivity monitor (Mullen *et al.,* 2002; 2003; Graf *et al.,* 2005).

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 *et al.* (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 humans 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 mass spectrometry using ion trap MS^n has made it much easier to identify metabolite peaks observed in the improved absorbance traces.

Anthocyanin Analysis

No anthocyanins were detected in either plasma or urine after the ingestion of onions containing a total of 75 μmoles of anthocyanins, principally cyanidin-3-(6''-malonylglucoside). However, this is in keeping with other reports on the fate of dietary anthocyanins following absorption. The picture that has emerged is that a variety of anthocyanins appear in urine after supplementation with very high doses of berries or berry extracts but, at best, in extremely low concentrations, typically 0.1% or less of the ingested dose (McGhie *et al.,* 2003; Cooney *et al.,* 2004).

CONCLUSION

The present study with human volunteers, 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 23 metabolites were either identified or partially identified with five being quantified in plasma and 12 in urine. If these samples had been subjected to hydrolysis only quercetin and isorhamnetin would have been detected and quantified. This data is 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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