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Determination of Flavonol Metabolites in Plasma and Tissues of Rats by HPLC−Radiocounting and Tandem Mass Spectrometry Following Oral Ingestion of [2-^{14}C]Quercetin-4′-glucoside

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Epidemiological studies suggest that consumption of flavonol-rich diets decreases the risk of developing heart disease and certain cancers. Recent studies have detected flavonol conjugates in blood and urine following various dietary interventions. To assess to what extent flavonols also accumulate in tissues, where they might be expected to exert anti-carcinogenic and anti-atherogenic effects, [2-^{14}C]quercetin-4′-glucoside was synthesized and fed to rats. After 60 min, 93.6% of the ingested radioactivity was recovered from the intestine, incorporated into 18 metabolites that had undergone deglycosylation followed by varying degrees of glucuronidation, methylation, and/or sulfation. [^{14}C]Quercetin, the aglycon of the radiolabeled substrate, was present in the intestine and in trace amounts in the liver but was not detected in the plasma and kidneys. The original [2-^{14}C]-quercetin-4′-glucoside was detected exclusively in the intestine, where it accounted for only 26.2% of the radioactivity. The remainder of the recovered radioactivity was located mainly in the plasma, liver, and kidneys as ^{14}C-labeled metabolites. However, compared to the quantities in the gastrointestinal tract, the levels of metabolites in plasma and body tissues were very low, indicating only limited absorption into the blood stream. The data demonstrate that quercetin-4′-glucoside, which is a major flavonol in onions, undergoes rapid and extensive metabolism in the intestine, and this appears not to be associated to any extent with transport across the gut wall into the blood stream.

KEYWORDS: [2-^{14}C]Quercetin-4′-glucoside; rat tissues; tandem mass spectrometry; methylated metabolites; glucuronides; sulfated derivatives

INTRODUCTION

Several epidemiological studies indicate that consumption of a flavonol-rich diet is associated with a lower incidence of cardiovascular disease and cancer (*1*). This may reflect the diverse ability of flavonols such as quercetin to show potentially anti-atherogenic and anti-carcinogenic effects in model systems. For example, in relation to heart disease, some flavonols can prevent the oxidation in vitro of low-density lipoprotein to a potentially atherogenic form (*2*), inhibit platelet aggregation, and dilate blood vessels (*3*). Potential anticancer effects include the modulation of enzyme activities associated with carcinogen activation and detoxification (*4*), the prevention of oxidative DNA damage (*5*), and the modulation of gene expression, apoptosis, and malignant transformation (*6*). Early animal studies suggested that flavonols had limited nutritional relevance, as they appeared to be poorly absorbed and subjected to rapid excretion via the bile. However, an increasing number of studies (*7*−*11*) have detected flavonols and their metabolites in plasma and urine of human subjects following consumption of pure compounds and flavonol-rich foods. Although there is wide individual variation in absorption, possibly reflecting type of flavonol, degree of conjugation, and assay methodology, the presence of conjugates in body fluids provides necessary

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evidence for the potential to exert effects in vivo analogous to those observed in model systems.

There is little information on the presence of flavonols and associated metabolites in tissues where putative anti-carcinogenic and anti-atherogenic effects on cellular homeostasis may be expected to occur. Consequently, as a prerequisite to assessing the mechanisms by which flavonols modulate cellular metabolism and gene expression, the aim of this study was to identify tissue metabolites of quercetin-4′-glucoside, a major flavonol conjugate in onions (*12*), which are one of the main sources of flavonols in the Dutch diet (*13*). Rats were fed [2-^{14}C]-quercetin-4′-glucoside, and the radiolabeled components in plasma and body tissues were analyzed by high-performance liquid chromatography (HPLC) with detection using an on-line radioactivity monitor and a tandem mass spectrometer with an electrospray interface.

MATERIALS AND METHODS

Synthesis of [2-^{14}C]Quercetin-4′-*O*-*β*-D-glucoside. [2-^{14}C]Quercetin-4′-*O*-*β*-D-glucoside (specific activity 3.75 mCi/mmol) was synthesized in four steps from barium [^{14}C]carbonate by the method that we have previously reported for the synthesis of [2-^{13}C]quercetin-4′-*O*-*β*-D-glucoside (*14*), 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 species was detected by HPLC−radiocounting (RC).

Animals and Sample Preparation. After an overnight fast, three male rats of the Rowett Hooded Lister strain (mean weight 430 ± 4 g) were offered 1 g of stock rat feed (CRM, Special Diet Services, Witham, Essex, UK) containing 3.26 mg of [2-^{14}C]quercetin-4′-glucoside, to give a dose of 58.5 × 10^6 dpm. Rats consumed 99.3% of the ration within 2 min. After 60 min, animals were terminally anesthetized with isofluorane, and blood was obtained by cardiac puncture into heparinized evacuated tubes (Becton Dickinson, Oxford, UK). Plasma was obtained by centrifugation at 1500*g* for 10 min at 4 °C. The pelleted red blood cells were resuspended in phosphate-buffered saline (pH 7.4). Livers were perfused in situ with chilled 0.15 M KCl and then removed along with brain, heart, kidneys, lungs, muscle, spleen, and testes. The gastrointestinal tract was removed intact along with its contents. All samples were frozen in liquid nitrogen and stored at −80 °C prior to lyophilization, after which they were ground to a powder using a mortar and pestle and again stored at −80 °C. All tissues were weighed before and after lyophilization.

Measurement of Radioactivity. Ten-millogram aliquots of freeze-dried tissue, plasma, and red blood cells were treated with 0.5 mL of tissue solubilizer (National Diagnostics, Hull, UK) for 3 h at 50 °C in a shaking water bath. With the exception of red blood cells, which were bleached using 1.75 mL of a 25% solution of sodium hypochlorite, the solublization treatment produced clear solutions, and 150-*μ*L aliquots were taken and added to 5 mL of scintillation cocktail (Optiflow Safe One, Fisons, Loughborough, UK) before determination of radioactivity using a Wallac 1409 liquid scintillation counter (Pharmacia, Uppsala, Sweden).

Extraction of Samples. One-gram aliquots of freeze-dried tissues were extracted by continuous shaking with 15 mL of 50% methanol in 0.1 M phosphate buffer (pH 7.0) containing 20 mM sodium diethyldithiocarbamate. After 30 min, the mixture was centrifuged at 2000*g* for 20 min. The methanolic supernatant was decanted and the pellet re-extracted a further two times. The three methanolic supernatants were combined, and the methanol was removed in vacuo. The remaining aqueous phase was adjusted to pH 3.0 and partitioned three times with an equal volume of ethyl acetate. The ethyl acetate extracts were combined and reduced to dryness in vacuo prior to the measurement of radioactivity and analysis. A gentle stream of nitrogen was used to remove residual ethyl acetate from the aqueous phase before it was loaded onto a 2-g C_{18} Sep Pak cartridge (Waters, Milford, MA), which was washed with 15 mL of distilled water adjusted to pH 3.0 with H_2SO_4. The cartridge was then eluted with 30 mL of methanol to remove polar radiolabeled metabolites. The methanolic extract was dried in vacuo, and aliquots were taken for radioactivity measurements and analysis.

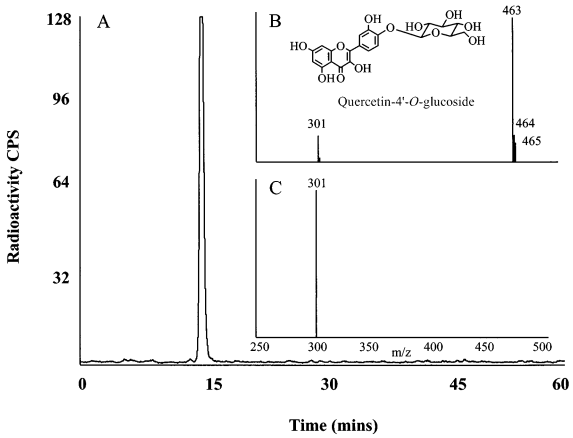

Figure 1. (A) Gradient HPLC–RC trace of 30 × 10^3 dpm aliquot of [2-^{14}C]-quercetin-4′-glucoside. Detection was with an on-line radioactivity monitor operating in the heterogeneous mode. (B) Full-scan negative-ion MS of the peak in panel A. (C) MS–MS of the molecular ion from the scan in panel B.

Proteins in plasma were precipitated by treating with 2.5 vol of acetone for 10 min. Samples were centrifuged, the supernatant was collected, and the pellet was re-extracted with acetone. The two acetone extracts were combined and reduced to the aqueous phase in vacuo, and ethyl acetate and aqueous extracts were obtained as described above.

HPLC with Diode Array, Radioactivity Detection, and Tandem Mass Spectrometry. Samples were analyzed on a P4000 liquid chromatograph fitted with an AS 3000 autosampler, with the initial detection by a UV6000 diode array absorbance monitor scanning from 250 to 700 nm (Thermo Finnigan, San Jose, CA). Separation was carried out using a 250 × 4.6 mm i.d., 4 *μ*m, Synergy RP-Max column (Phenomenex, Macclesfield, UK), eluted with a gradient over 60 min of either 0−100% or 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 absorbance monitor, the column eluate was directed to a radioactivity monitor (Reeve Analytical model 9701, Lab Logic, Sheffield, UK), fitted with a 500-*μ*L heterogeneous flow cell packed with cerium-activated glass scintillant, before being split, with 50% entering to a Finnigan LCQ Duo tandem mass spectrometer with an electrospray interface in negative-ion mode operating in full-scan mode from 100 to 2000 amu.

RESULTS

Distribution of Radioactivity in Rat Tissues and Extracts. Each rat consumed 1 g of stock rat feed containing 58.5 × 10^6 dpm [2-^{14}C]quercetin-4′-glucoside. An aliquot of the feed was extracted with methanol and analyzed by HPLC−RC−MS−MS, which demonstrated that the [2-^{14}C]quercetin-4′-glucoside was radiochemically pure (**Figure 1A**). Full-scan MS analysis was also carried out (**Figure 1B**), which shows a molecular ion (M^-) at *m*/*z* 463. The M + 2 ion at *m*/*z* 465, from the ^{14}C label, can also be seen. The calculated abundance of the M + 2 ion is 11.4%, from the specific activity of the label. MS−MS was carried out on the *m*/*z* 463 molecular ion (**Figure 1C**) rather than the smaller M + 2 ion. This yielded a secondary spectrum with the major ion at *m*/*z* 301, which corresponds to the aglycone quercetin.

After 60 min, the rats were sacrificed, and the distribution of radioactivity in the body was determined. The data obtained are presented in **Table 1** and show that by far the majority of the recovered radioactivity (93.6%) was associated with the

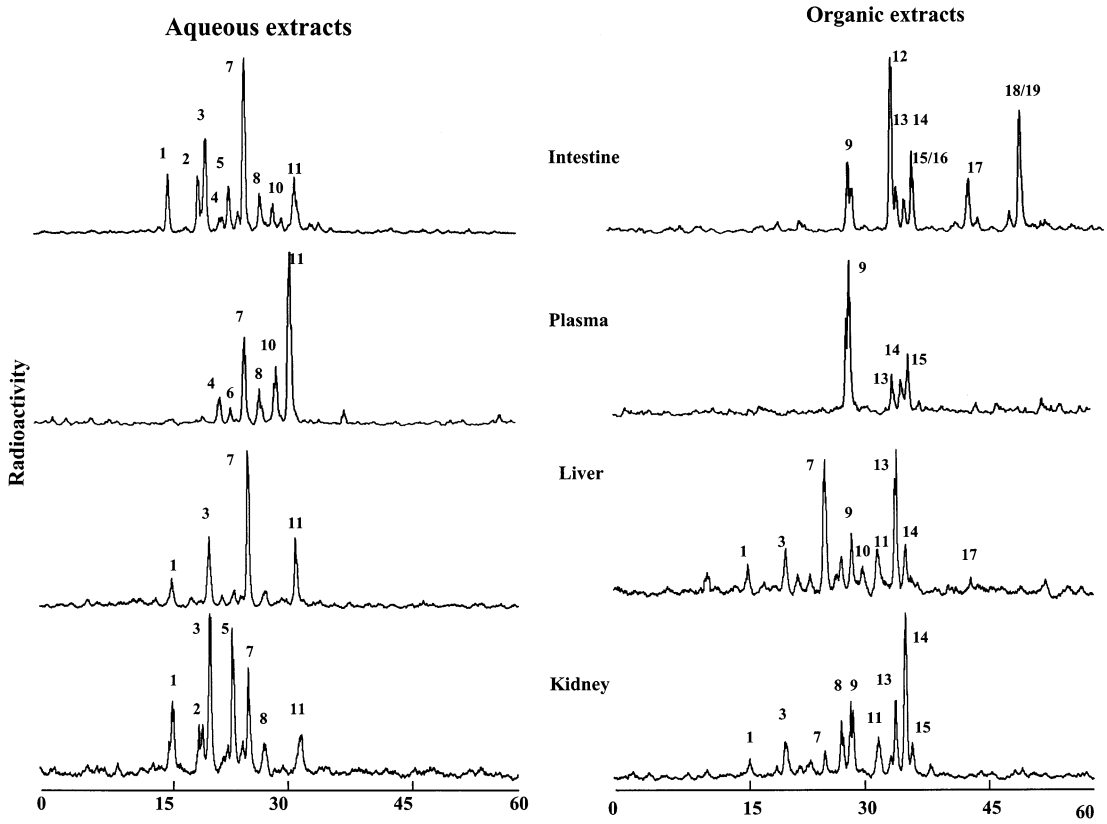

Figure 2. Gradient HPLC–RC traces of 30×10^3 dpm aliquots of ethyl acetate and aqueous extracts obtained from intestine, plasma, liver, and kidneys of rats 60 min after the ingestion of [2-^{14}C]quercetin-4′-glucoside. After passing through the flow cell of a diode array detector (data not shown), the column eluate was directed to a radioactivity monitor operating in heterogeneous mode, after which it was split and 50% of the eluate directed to a tandem mass spectrometer with an electrospray interface in negative-ion mode.

Table 1. Distribution of Radioactivity in Rats after Ingestion of [2-^{14}C]Quercetin-4′-glucoside[a]

tissue/fluid	total radioactivity	concentration of radioactivity
intestine and contents	54530 ± 2733 (93.6%)	2117 ± 106
plasma	1644 ± 39 (2.8%)	110 ± 3
red blood cells	4 ± 1 (–)	0 ± 0
liver	684 ± 61 (1.2%)	37 ± 3
kidneys	468 ± 76 (0.8%)	147 ± 31
spleen	6 ± 0 (–)	7 ± 0
brain	1 ± 0 (–)	0 ± 0
lungs	38 ± 3 (0.07%)	21 ± 1
heart	18 ± 1 (0.03%)	14 ± 1
muscle	839 ± 113 (1.4%)	4 ± 1
testes	25 ± 1 (0.04%)	8 ± 1

[a] Distribution of radioactivity in rats 60 min after ingestion of [2-^{14}C]quercetin-4′-glucoside. Radioactivity measured by liquid scintillation counting after dissolving tissues with a solubilizer. Radioactivity expressed as dpm $\times 10^3$ per tissue/fluid per rat ± standard error ($n = 3$) and in parentheses as a percentage of the recovered radioactivity. Concentration of radioactivity expressed as dpm $\times 10^3$ per g tissue/mL fresh weight ± standard error ($n = 3$).

intestine, which comprised the stomach and both small and large intestine and their contents. The plasma contained 1.64×10^6 dpm (2.8% of recovered radioactivity), indicating that some absorption had taken place, while 0.68×10^6 dpm (1.2%) was detected in the liver and 0.47×10^6 dpm (0.8%) in the kidneys. Muscle tissues contained 0.84×10^6 dpm, representing 1.4% of the recovered radioactivity. However, the concentration of radioactivity was very low (**Table 1**), and this precluded further analysis. The levels and/or concentrations of radioactivity located in other tissues were very low, and as a consequence only the radiolabeled components in the intestine, plasma, liver, and kidneys were processed further. The total radioactivity recovered was 58.3×10^6 dpm, which represents a 99.6% recovery of the 58.5×10^6 dpm consumed by each rat.

Identity of [2-^{14}C]Quercetin-4′-glucoside Metabolites. To reduce the sample weight to manageable proportions for HPLC, and obtain cleaner mass spectra, the extracts were processed as described in the Materials and Methods, and ethyl acetate and aqueous extracts were obtained which were analyzed separately. The samples from three individual rat tissues were combined, and aliquots of the ethyl acetate and aqueous fractions from the intestine, plasma, liver, and kidneys, containing between 10×10^3 and 30×10^3 dpm of radioactivity were analyzed by HPLC with tandem mass spectrometry in conjunction with a radioactivity monitor. The HPLC–RC profiles obtained show a complex pattern of metabolites, with 19 major peaks being detected (**Figure 2**). The results of negative-ion, electrospray mass spectrometric analyses of these peaks are presented in **Table 2**, and the MS–MS spectra of six of the metabolites are shown in **Figure 3**.

Peak 1 (retention time [t_R] = 16.7 min) had an M^- at *m*/*z* 653, which fragmented to produce ions at *m*/*z* 477 (M − 176, loss of a glucuronyl unit) and 301, which corresponds with the aglycone quercetin, and results from a further neutral loss of 176 amu, indicating that the metabolite is a quercetin diglucuronide with the glucuronyl moieties attached at different positions on the flavonol ring. If the two glucuronyl residues had been attached at the same position, it is unlikely that an M − 176 fragment would have been produced at *m*/*z* 477, as it

Table 2. Identification of Metabolites of [2-^{14}C]Quercetin-4′-glucoside Detected in Rats[a]

peak	t_R (min)	compound	M^- (*m*/*z*)	fragment ions (*m*/*z*)
1	16.7	quercetin diglucuronide	653	477 (M^- – GlcUA), 301[Q](M^- – GlcUA – GlcUA)
2	20.6	unknown		
3	21.6	methylquercetin diglucuronide	667	491 (M^- – GlcUA), 315 [MQ](M^- – GlcUA – GlcUA)
4	23.7	quercetin diglucuronide	653	477 (M^- – GlcUA), 301[Q](M^- – GlcUA – GlcUA)
5	24.3	methylquercetin diglucuronide	667	491 (M^- – GlcUA), 315 [MQ](M^- – GlcUA – GlcUA
6	25.1	quercetin diglucuronide	653	477 (M^- – GlcUA), 301[Q](M^- – GlcUA – GlcUA)
7	26.4	quercetin diglucuronide	653	477 (M^- – GlcUA), 301[Q](M^- – GlcUA – GlcUA)
8	28.3	quercetin diglucuronide	653	477 (M^- – GlcUA), 301[Q](M^- – GlcUA – GlcUA)
9	29.6	quercetin-3-glucuronide	477	301 [Q], (M^- – GlcUA)
10	30.3	quercetin glucuronide sulfate	557	477 (M^- – SO_3), 381 (M^- – GlcUA), 301 [Q](M^- – SO_3 – GlcUA)
11	32.6	methylquercetin glucuronide sulfate	571	491 (M^- – SO_3), 315 [MQ](M^- – SO_3 – GlcUA)
12	34.5	quercetin-4′-glucoside	463	301 [Q](M^- – Glc)
13	35.3	methylquercetin glucuronide	491	315 [MQ](M^- – GlcUA)
14	36.5	methylquercetin glucuronide	491	315 [MQ](M^- – GlcUA)
15	37.4	methylquercetin glucuronide	491	315 [MQ](M^- – GlcUA)
16	37.4	quercetin glucuronide	477	301 [Q](M^- – GlcUA)
17	44.6	quercetin	301	
18	51.1	methylquercetin sulfate	395	315 [MQ](M^- – SO_3)
19	51.2	quercetin sulfate	381	301 [Q]](M^- – SO_3)

[a] HPLC–RC retention times, negative-ion MS–MS fragmentation patterns, and identities of metabolites detected in rat tissues 60 min after oral ingestion of [2-^{14}C]quercetin-4′-glucoside. Peak number refers to peaks in **Figure 2**. Q, quercetin; MQ, methylquercetin; GlcUA, glucuronyl unit; Glc, glucosyl unit; M^-, molecular ion.

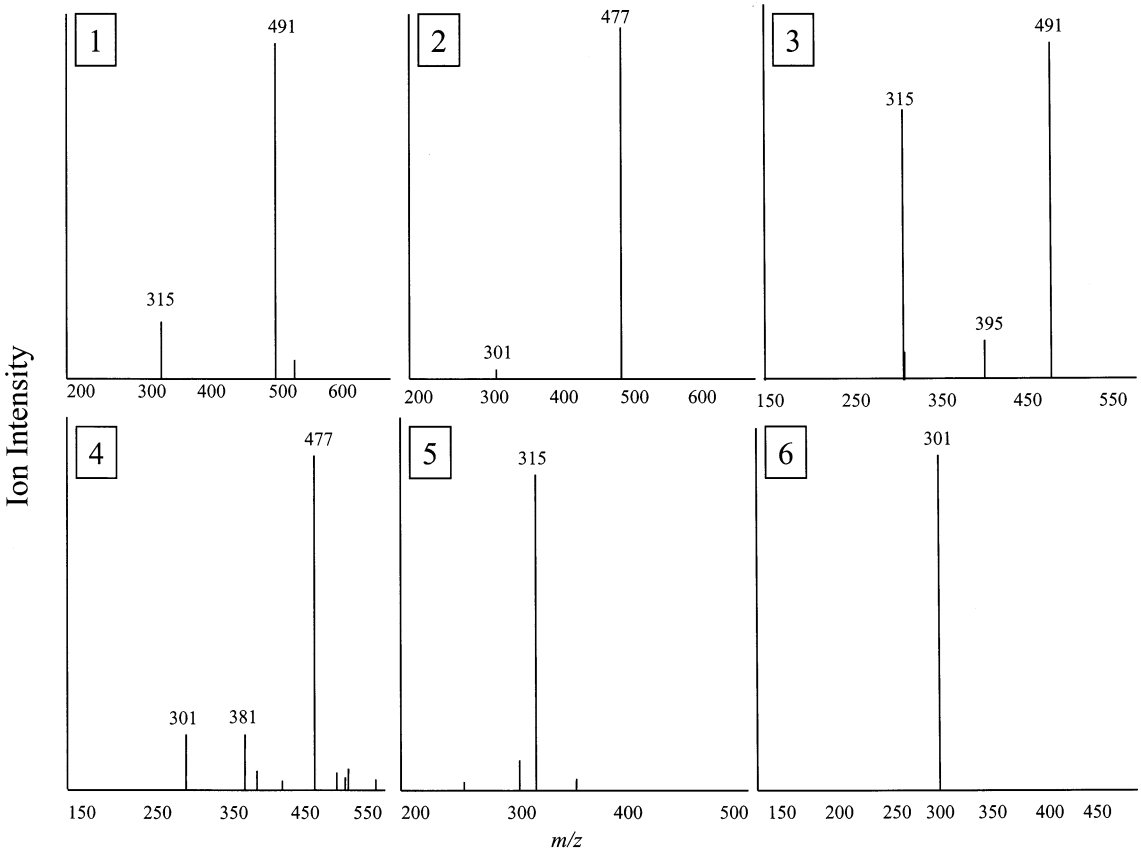

Figure 3. Tandem mass spectra of [2-^{14}C]quercetin-4′-glucoside metabolites. Spectrum 1: MS–MS of ion *m*/*z* 667, methylquercetin diglucuronide. Spectrum 2: MS–MS of ion *m*/*z* 653, quercetin diglucuronide. Spectrum 3: MS–MS of ion *m*/*z* 571, methylquercetin glucuronide sulfate. Spectrum 4: MS–MS of ion *m*/*z* 557, quercetin glucuronide sulfate. Spectrum 5: MS–MS of ion *m*/*z* 491, methylquercetin glucuronide. Spectrum 6: MS-MS of ion *m*/*z* 477, quercetin glucuronide.

has been shown that anthocyanin disaccharide conjugates fragment with loss of the intact disaccharide unit (*15*).

Peak 2 (t_R = 20.6 min) was not identified, as it did not yield recognizable mass spectral data.

Peak 3 (t_R = 21.6 min) had an M^- at *m*/*z* 667, 14 mass units higher than the quercetin diglucuronide in peak 1. It ionized to produce two M − 176 fragments at *m*/*z* 491 and 315, the latter being the M_r of a methylated analogue of quercetin. Peak 3 therefore contains a methylquercetin diglucuronide. The most likely methylated quercetin derivative is 3′-methylquercetin, isorhamnetin. However, as methylation at other positions cannot be ruled out, and there is an absence of reference compounds, the aglycone cannot be identified definitively.

Peak 4 (t_R = 23.7 min), like peak 1, is a quercetin diglucuronide, as it produced an MS−MS spectrum with an M^- at *m*/*z* 653, which fragmented to produce *m*/*z* 477 (M − 176) and 301 (quercetin).

Peak 5 (t_R = 24.3 min), like peak 3, is a methylquercetin diglucuronide, as it produced an MS−MS spectrum with an M^-

Table 3. Radioactivity Incorporated into Metabolites in Plasma and Tissues after Ingestion of [2-^{14}C]Quercetin-4′-glucoside by Rats[a]

peak	compound	intestine	plasma	liver	kidneys
1	quercetin diglucuronide	223 (0.8%)	n.d.	20 (6.4%)	13 (7.4%)
2	unknown	223 (0.8%)	n.d.	n.d.	8.2 (4.5%)
3	methylquercetin diglucuronide	364 (1.3%)	n.d.	41 (13.0%)	31 (17.0%)
4	quercetin diglucuronide	60 (0.2%)	54 (6.4%)	n.q.	n.d.
5	methylquercetin diglucuronide	16 (0.6%)	n.d.	n.q.	23 (12.5%)
6	quercetin diglucuronide	n.d.	32 (3.7%)	n.d.	n.d.
7	quercetin diglucuronide	685 (2.5%)	175 (20.5%)	105 (33.5%)	21 (11.7%)
8	quercetin diglucuronide	152 (0.6%)	70 (8.2%)	13 (4.0)	15 (8.4%)
9	quercetin-3-glucuronide	2877 (10.6%)	18 (2.1%)	21 (6.9%)	14 (7.9%)
10	quercetin glucuronide sulfate	1892 (6.9%)	118 (13.8%)	9.2 (3.0%)	n.d.
11	methylquercetin glucuronide sulfate	201 (0.7%)	372 (43.6%)	40 (12.8%)	5.6 (3.1%)
12	quercetin-4′-glucoside	7134 (26.2%)	n.d.	n.d.	n.d.
13	methylquercetin glucuronide	1783 (6.5%)	4.5 (0.5%)	42 (13.4%)	14 (7.7%)
14	methylquercetin glucuronide	1333 (4.9%)	3.7 (0.4%)	18 (5.6%)	31 (16.8%)
15/16	methylquercetin/quercetin glucuronides	3337 (12.2%)	6.6 (0.8%)	n.d.	5.7 (3.1%)
17	quercetin	1956 (7.2%)	n.d.	4.6 (1.5%)	n.d.
18/19	methylquercetin/quercetin sulfates	4890 (17.9%)	n.d.	n.d.	n.d.

[a] Radioactivity associated with quercetin-4′-glucoside and metabolites in intestine, plasma, liver, and kidneys of rats 60 min after oral ingestion of [2-^{14}C]quercetin-4′-glucoside. Data expressed as dpm $\times 10^3$ and in parentheses as a percentage of the overall level of recovered radioactivity per organ/plasma. n.q., identified, but below level of quantification; n.d., not detected.

at *m*/*z* 667, which fragmented to produce *m*/*z* 491 (M − 176) and 315 (methylquercetin).

Peaks 6−8 (t_R = 25.1, 26.4, and 28.3 min) were quercetin diglucuronides, as they all had an M^- at *m*/*z* 653, which yielded fragment ions at *m*/*z* 477 and 301.

Peak 9 (t_R = 29.6 min) had an M^- at *m*/*z* 477, which fragmented to produce an M − 176 ion at *m*/*z* 301, indicating the presence of a quercetin monoglucuronide, probably quercetin-3-glucuronide, as it co-chromatographed with a standard of this compound.

Peak 10 (t_R = 30.3 min) had an M^- at *m*/*z* 557, which ionized yielding fragment ions at *m*/*z* 477 (M − 80, loss of SO_3), 381 (M − 176, loss of glucuronyl unit), and 301 (quercetin). This metabolite is therefore a quercetin glucuronide sulfate conjugate.

Peak 11 (t_R = 32.6 min) is a methylquercetin glucuronide sulfate, as indicated by an M^- at *m*/*z* 571 that produced an M − 80 fragment at *m*/*z* 491, which with a further loss of *m*/*z* 176 yielded an *m*/*z* 315 methylquercetin ion.

Peak 12 (t_R = 34.5 min) is the parent compound, quercetin-4′-glucoside. Its mass spectrum had an M^- at *m*/*z* 463, which fragmented to produced *m*/*z* 301 (quercetin, M − 162, loss of a glucosyl unit).

Peaks 13−15 (t_R = 35.6, 36.5, and 37.4 min), all of which have an M^- at *m*/*z* 491 and fragment to produce an M − 176 ion at *m*/*z* 315, are methylquercetin glucuronide regioisomers.

Peak 16 (t_R = 37.4 min) is a quercetin glucuronide on the basis of an M^- at *m*/*z* 477, which fragmented, yielding *m*/*z* 301 (quercetin, M − 176, loss of glucuronyl unit).

Peak 17 (t_R = 44.6 min) is quercetin, with an M^- at *m*/*z* 301.

Peak 18 (t_R = 51.1 min), with an M^- at *m*/*z* 395 that produced an M − 80 fragment at *m*/*z* 315, is a methylquercetin sulfate conjugate.

Peak 19 (t_R = 51.2 min) yielded a mass spectrum with ions at *m*/*z* 381 (M^-) and 301 (M − 80) and is a quercetin sulfate derivative.

Amount of Radioactivity Incorporated into [2-^{14}C]Quercetin-4′-glucoside Metabolites. The data presented in **Figure 2** and **Table 2** are qualitative. The overall levels of radioactivity associated with [2-^{14}C]quercetin-4′-glucoside and its metabolites are presented in **Table 3**. These values were calculated from the peak areas in **Figure 2**, the amount of radioactivity in the ethyl acetate and aqueous extracts from the intestine, plasma, liver, and kidneys.

The information in **Table 3** shows clearly that the majority of the radioactivity was recovered from the intestine and that, within the 60 min period since ingestion, the [2-^{14}C]quercetin-4′-glucoside had undergone extensive metabolism, as it represented only 26.2% of the recovered radioactivity. The major metabolites in the intestine, which comprised the stomach and gastrointestinal tract along with their contents, were quercetin-3-glucuronide (peak 9, 10.6%), quercetin glucuronide sulfate (peak 10, 6.9%), methylquercetin glucuronide (peaks 13 and 14, 6.5 and 4.9%), and quercetin (peak 17, 7.2%). There were two significant peaks, both containing two unresolved compounds. Peaks 15 and 16 (12.2%) contained methylquercetin glucuronide and quercetin glucuronide, and peaks 18 and 19 (17.9%) contained methylquercetin sulfate and quercetin sulfate. Although these compounds could be identified by MS−MS within each peak, radiochemically they appeared as one peak and were therefore quantified as such.

The intestine contained other methylated derivatives, diglucuronides, and sulfate conjugates. Quercetin-4′-glucoside was not detected in extracts from plasma, liver, or kidneys (**Table 3**). The intestine contained, in varying amounts, all 18 metabolites of [2-^{14}C]quercetin-4′-glucoside, with the exception of quercetin diglucuronide (peak 6), which was detected only in plasma.

The main metabolites in plasma were peak 11 (methylquercetin glucuronide sulfate, 43.6% of radioactivity recovered in plasma), peak 7 (quercetin diglucuronide, 20.5%), and peak 10 (quercetin glucuronide sulfate, 13.8%). The quercetin diglucuronide in peak 7 (33.5%) was the main metabolite in the liver, while a range of low-level metabolites were detected in the kidneys, the largest being methylquercetin glucuronide (peak 14, 21.7%).

Figure 4 summarizes the types of metabolites that were detected in the rat samples. Diglucuronides were major components in all samples outwith the intestine. Plasma had the simplest metabolic profile, having only two major metabolite groups. The kidney and liver contained all the metabolite groups found in the intestine, with the exception of the parent compound.

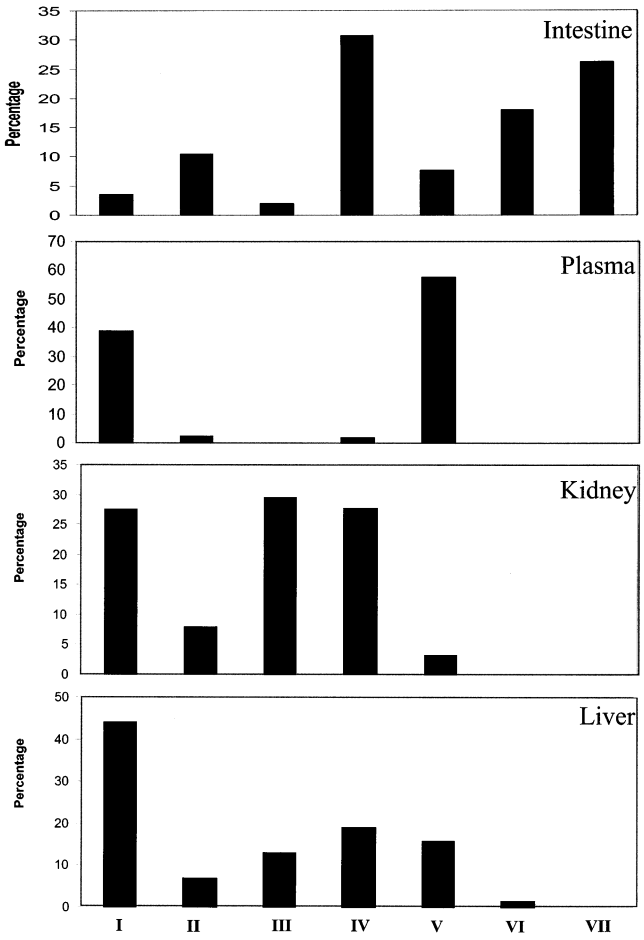

Figure 4. Metabolite profile of the plasma and tissue extracts of rats obtained 60 min after the ingestion of [2-^{14}C]quercetin-4′-glucoside. The intestine contained 95.5% of the recovered radioactivity, whereas the plasma, liver, and kidney comprised 2.5%, 1%, and 1%, respectively. **I**, quercetin diglucuronides; **II**, quercetin monoglucuronides; **III**, methylquercetin diglucuronides; **IV**, methylquercetin monoglucuronides; **V**, methylquercetin glucuronide sulfates and quercetin glucuronide sulfates; **VI**, methylquercetin sulfates and quercetin sulfates; **VII**, quercein-4′-glucoside.

DISCUSSION

Feeding of quercetin to rats results in detectable concentrations of the flavonol in blood and tissues (*16*−*20*). However, the nutritional relevance of such studies is unclear, as quercetin and other flavonols are found in foods almost exclusively as sugar conjugates (*21*). Consequently, we have fed custom-synthesized, high specific activity [2-^{14}C]quercetin-4′-glucoside to rats at an amount equivalent to a 70 kg human subject consuming 250 g fresh weight of onions (*12*, *22*). HPLC with an on-line radioactivity monitor and tandem MS with an electrospray interface was then used to provide detailed identification of radiolabeled metabolites in plasma and body tissues.

Sixty minutes after [2-^{14}C]quercetin-4′-glucoside was fed to rats, 93.2% of the ingested radioactivity was recovered from the intestine. However, this radioactivity comprised only 26.2% unmetabolized [2-^{14}C]quercetin-4′-glucoside, the remainder having undergone deglycosylation and varying degrees of glucuronidation, sulfation, and/or methylation in the process of being incorporated into 18 metabolites. The extent of this metabolism, in terms of both the number of metabolites and the exceptionally high levels of radioactivity incorporated into the metabolites, is on a scale far greater than might have been envisaged from previous feeding studies with unlabeled flavonols.

Quercetin glucosides from onion have been reported to be converted to glucuronide conjugates in human intestinal epithelial cells (*23*) after hydrolysis with β-glucosidase (*24*) and conjugation catalyzed by uridine-5-diphosphoglucuronyl transferase (UDP-GT) (*25*). The extensive formation of ^{14}C-labeled metabolites observed in rat intestine in the present study may occur by similar mechanisms, as rat intestinal mucosal preparations also have cytosolic β-glucosidase activity with broad specificity (*26*) and UDP-GT activity (*27*). Such transformations are likely to have occurred in the small intestine, as substantial quantities of glucuronide and sulfate conjugates of quercetin and methylquercetin accumulated within 60 min of ingestion of [2-^{14}C]quercetin-4′-glucoside. If the radiolabeled flavonol metabolites had reached the large intestine, the available evidence indicates that colonic bacteria may have induced ring fission, leading to the formation of compounds such as 4-hydroxyphenylacetic acid, 3,4-dihydrophenylacetic acid, and 4-hydroxy-3-methoxyphenylacetic acid (*28*−*30*), all of which would have retained the ^{14}C-label and thus have been readily detected.

The results obtained in the present study with rats suggest that absorption of quercetin-4′-glucoside from the gut is relatively poor, as 60 min after consumption only 6.4% of the recovered radioactivity was detected outside the gastrointestinal tract. Moreover, extensive deglycosylation, glucuronidation, sulfation, and methylation occurred, and no [2-^{14}C]quercetin-4′-glucoside was found in plasma, liver, and kidneys. Consequently, cell culture and in vitro studies investigating the effects of this flavonol on systemic cellular processes and gene expression should apply the metabolites rather than the parent compound. Moreover, as 73.8% of the [2-^{14}C]quercetin-4′-glucoside was metabolized within 60 min in the intestine, the data indicate that flavonol glucosides are not absorbed per se but undergo extensive metabolism, primarily involving deglycosylation and glucuronidation, prior to passage into the blood stream. It has been proposed that such metabolism occurs during passage across the gut wall (*31*−*34*). However, the extremely high levels of metabolites in the gut compared to those in plasma and body tissues suggest that most of the observed metabolism of [2-^{14}C]quercetin-4′-glucoside occurs independently of movement into the blood stream. The fact that different spectra of metabolites were detected in plasma, liver, and kidneys (**Figure 2**, **Table 3**) and the relatively high proportion of sulfated metabolites in plasma, compared to those in the intestine, liver, and kidneys (**Figure 4**), indicate that some postabsorption metabolism also occurred.

Human liver cells contain both β-glucosidase (*24*) and UDP-GT activity (*35*), so further hepatic modification of flavonol metabolites after entering the blood stream cannot be excluded, although in the present study this would be a relatively minor event compared to metabolism occurring in the gut. Some enterohepatic circulation of the metabolites could have occurred, though it would have to have happened very quickly. It is not possible to identify enterohepatic circulation in this system, as the rat does not have a gall bladder, and this makes the collection of bile difficult.

On the basis of in vitro studies with cell-free systems, it has been proposed that circulating quercetin glucuronides are subject to the action of β-glucuronidase in liver and other human tissues, resulting in the release of quercetin (*36*). The current in vivo study with rats provides no support for this hypothesis, as glucuronide conjugates and quercetin represented >98% and

1.5%, respectively, of the radioactivity recovered from the liver, and the aglycone was not detected in either plasma or kidneys (**Table 3**).

The biological significance of the extensive metabolism of [2-^{14}C]quercetin-4′-glucoside that occurred so rapidly in the intestine of rats, and the relatively small amounts of the quercetin glucuronide, sulfate, and methyl conjugates that pass into the blood stream and body tissue, is far from clear. Recently, it has been shown that quercetin-3-glucuronide, which was detected as a minor metabolite in the present study (**Table 3**), exerts a considerable inhibitory effect on lipid peroxidation in phospholipid membranes induced by reactive oxygen species and peroxidative enzymes (*37*). Although the effectiveness of the glucuronide conjugate was less than that of quercetin, it should be noted that the aglycone is not a genuine dietary component. It was also established that quercetin-3-glucuronide exhibited an affinity to phospholipid membranes that was significantly higher than that of ascorbic acid. On the basis of these observations, it was suggested that quercetin glucuronides and other metabolites, that accumulate in plasma after the consumption of food rich in quercetin glucosides contribute to the antioxidative defenses against membranous phospholipid peroxidation occurring in vivo (*37*). However, as around 93.6% of the radiolabeled flavonol consumed, and its metabolites, were found in the stomach and intestines after 1 h, the major site of potential protection against oxidative damage must be in the gastrointestinal tract itself.

Quercetin-3-glucuronide has been identified in rat plasma after oral administration of the aglycone (*38*). There are also two reports on the use of HPLC−MS to analyze human plasma after the consumption of onions (*11*, *39*), and neither of these studies was able to obtain full-scan mass spectral data on the putative metabolites that were detected. Wittig et al. used tandem mass spectrometry in the selected reaction monitoring mode to detect five quercetin glucuronide conjugates in a single sample of plasma (*11*). Day et al. analyzed four plasma samples and highlighted 11 putative quercetin metabolite peaks, which appeared on the $A_{370\,nm}$ HPLC traces. However, when the samples were combined and analyzed by HPLC−MS, only three of these peaks could be identified by MS operating in the selected-ion mode (*39*). The present study, in which 18 metabolites of quercetin-4′-glucoside were detected and 17 identified, provides a far more detailed picture of the metabolism and absorption of a flavonol glucoside than any previous reports that have appeared in the literature.

ABBREVIATIONS USED

Glc, glucosyl unit; GlcUA, glucuronyl unit; HPLC, high-performance liquid chromatography; RC, radiocounting; MS, mass spectrometry; M^-, molecular ion; MQ, methylquercetin; Q, quercetin; UDP-GT, uridine-5-diphosphoglucuronyl transferase.

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