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Bioavailability of [2-^{14}C]Quercetin-4′-glucoside in Rats

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[2-^{14}C]Quercetin-4′-glucoside (4 mg/kg body weight) was fed by gavage to rats housed in metabolic cages, and over an ensuing 72 h period, radiolabeled products in body tissues, plasma, feces, and urine were monitored by high-performance liquid chromatography with online radioactivity and MS^2 detection. One and 6 h after ingestion, while in the small intestine, the flavonol glucoside was converted to glucuronide and methylated and sulfated derivatives of quercetin, but only trace amounts of these metabolites were excreted in urine. On entering the cecum and the colon, the flavonol metabolites declined as they were converted to phenolic acids, principally 3-hydroxyphenylacetic acid and 3,4-dihydroxyphenylacetic acid, by the colonic microflora. Feces contained mainly 3-hydroxyphenylacetic acid. Urine collected 0−12 and 0−24 h after ingestion contained radiolabeled hippuric acid and 3-hydroxyphenylacetic acid. ^{14}C-Hippuric acid declined markedly in the 24−48 and 48−72 h urine samples, and there was a concomitant increase in labeled benzoic acid. There was minimal accumulation of radioactivity in plasma, despite a 69% recovery of label in urine over the 72 h period, and likewise, very little radioactivity was detected in body tissues out with the gastrointestinal tract. This is reflected in the fact that 72 h after ingestion 96% of the ingested radioactivity was recovered in feces, urine, and the cage washes, which comprise a mixture of urine and feces. The study reveals that as it passes through the gastrointestinal tract, almost all of the of [2-^{14}C]quercetin-4′-glucoside is converted to phenolic acids, compounds not monitored in previous flavonol bioavailability studies with model animal systems, some of which have used exceedingly high doses of the aglycone quercetin (500 mg/kg body weight), which is not a normal dietary component.

KEYWORDS: [2-^{14}C]Quercetin-4′-glucoside; metabolites; phenolic acids; rats; HPLC-tandem mass spectrometry

INTRODUCTION

Epidemiological studies suggest that the consumption of flavonol-rich diets decreases the risk of developing heart disease and certain cancers (*1, 2*). Recent studies with humans have detected quercetin metabolites in blood and urine following acute ingestion of quercetin glucoside-rich onions (*3, 4*). However, these studies accounted for only a small percentage of the ingested dose. In a survey of 97 bioavailability studies (*5*), the reported levels of bioavailability, based on urinary excretion, varied from as little as 0.02% for anthocyanins from elderberry juice (*6*) to 1−2% for anthocyanins from strawberries (*7, 8*). Reported flavonol glucoside bioavailability ranged from 1 to 6% of intake (*9, 10*). A figure of <0.1% has been reported for quercetin-3-*O*-rutinoside, which is absorbed in the large rather than the small intestine (*11*) with substantial person-to-person variation, reflecting the impact of the colonic microflora (*12*). Flavanone recoveries ranging from 1 to 30% have been

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reported (*5, 13*). Flavan-3-ols also show great variability, but this seems to relate to whether or not the feed is with a 3-*O*-gallated derivative. Nongallated flavan-3-ols such as (−)-epicatechin have been reported to be excreted in urine at levels of 25−30% of intake, whereas a feed with (−)-epigallocatechin gallate resulted in excretion of <0.02% (*14*−*16*).

While studies providing further data on bioavailability can be obtained with feeds using volunteers with an ileostomy, the information that can be obtained by analyzing human ileal fluid, plasma, and urine is not complete, as it does not assess to what degree the compounds of interest are sequestered in body tissues and organs. For obvious reasons, such data can only be obtained with animal models that are preferably fed a radiolabeled substrate. Previously, we have used [2-^{14}C]quercetin-4′-glucoside in a feeding study in which radiolabeled metabolites were monitored in plasma and body tissues for a 5 h period after ingestion using high-performance liquid chromatography (HPLC) with radioactivity and MS detection systems (*17, 18*). To investigate the bioavailability of [2-^{14}C]quercetin-4′-glucoside more thoroughly, the fate of the flavonol in rats has now been followed over a 72 h rather than 5 h period postingestion. In this study, unlike the earlier one, ethical permission was obtained to house the rats in metabolism cages for the duration of the experiment. This enabled all excreted components to be collected except for exhaled CO_2. Radiolabeled CO_2 has been reported to be the main product of [^{14}C]quercetin fed to humans by Walle et al. (*19*). However, as the quercetin used was labeled at the 4-position in the C ring, it was not possible to follow the fate of A or B ring metabolites remaining after loss of CO_2. With the [2-^{14}C]quercetin-4′-glucoside used in the current study, the ^{14}C-label is retained by B ring metabolites, enabling their production and subsequent fate to be determined.

MATERIALS AND METHODS

Synthesis of [2-^{14}C]Quercetin 4′-*O*-*β*-D-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) by the method previously reported for the synthesis of [2-^{13}C]quercetin 4′-*O*-*β*-D-glucoside (*20*), except that the intermediate ester was not purified by filtration through alumina. The compound was pure by ^{1}H spectroscopy, and prior to beginning the feeding study, only one radioactive peak that cochromatographed with quercetin-4′-glucoside was detected by HPLC-photodiode array (PDA)-radiocounting (RC).

Chemicals. HPLC grade methanol and acetonitrile were obtained from Rathburn Chemicals (Walkerburn, Peebleshire, United Kingdom). Formic acid was purchased from Riedel-DeHaen (Seeize, Germany), and acetic acid was from BDH (Poole, United Kingdom). L-(+)-Ascorbic acid and quercetin were supplied by Extrasynthese (Genay, France). Quercetin-3,4′-diglucoside and quercetin-4′-glucoside were from AASC Ltd. (Southampton, United Kingdom). Hippuric acid, 2,3-dihydroxyphenylacetic acid and 4-hydroxyphenylacetic acid, benzoic acid, 3,4-dihydroxyphenylacetic acid, 3,4-dihydroxybenzoic acid, 3-methoxy-4-hydroxyphenylacetic acid, 3-methoxy-4-hydroxybenzoic acid, and 3,5-dihydroxy-3-methoxybenzoic acid were purchased from Sigma (Poole, United Kingdom).

Animals and Sample Preparation. The experimental time points for analysis were 1, 6, 12, 24, 48, and 72 h. At each time point, three male Sprague−Dawley rats (Iffa Credo, L'Arbresle, France) weighing ca. 250 g were used. The animals were handled according to the guidelines of the Committee on Animal Care at the University of Montpellier and NIH guidelines (*21*) and were housed individually in stainless steel metabolic cages with free access to deionized water. They were kept under light from 0700 to 1900 h, at a temperature of 23 ± 1 °C, with constant humidity. The rats were deprived of food for 16 h before being force-fed by gavage with 0.5 mL of water/ethanol (92:8, v/v) containing of 18.4 × 10^6 dpm [2-^{14}C]quercetin-4′-glucoside. At the appropriate time points after feeding, three rats were anaesthetized with pentobarbital (60 g/L pentobarbital, 60 mg/kg body weight). Blood was drawn by cardiac puncture with heparin-moistened syringes, and plasma and erythrocytes were separated by centrifugation at 2300*g* for 10 min at room temperature and then stored at −80 °C until analysis. The whole body tissues were perfused in situ with chilled 0.15 M NaCl to remove residual blood, after which brain, heart, lungs, kidneys, liver, testis, muscle, and spleen were removed, rinsed in saline, blotted dry, frozen in liquid nitrogen, and stored at −80 °C until analysis. The gastrointestinal (GI) tract was removed intact along with its contents and then separated into stomach, duodenum, ileum and jejunum, cecum, and colon before being frozen. All tissues were weighed. Feces and urine were collected from each rat during the course of the study, and they too were stored at −80 °C prior to analysis.

Measurement of Radioactivity in Tissues and Body Fluids. Ten milligram aliquots of powdered freeze-dried tissue and feces and 100 μL of plasma and urine were treated with 0.5 mL of tissue solubilizer (National Diagnostics, Hull, United Kingdom) for 3 h at 50 °C in a shaking water bath. This solublization treatment produced clear solutions, and 150 μL aliquots were added to 5 mL of scintillation cocktail (Optiflow Safe One, Fisons, Loughborough, United Kingdom) before determination of radioactivity using a Wallac 1409 liquid scintillation counter (Pharmacia, Uppsala, Sweden).

Extraction of Tissues and Faeces. The size of aliquot taken for extraction was determined by the amount of radioactivity found in the sample. This ranged from 10 mg for stomach samples taken at the 1 h time point to 100 mg for the 6 h stomach samples. If only very low amounts of radioactivity were detected in a tissue, it was not extracted and further processed. Tissues were extracted by the addition of 50% aqueous methanol and continuous shaking. After 30 min, the mixture was centrifuged at 2000*g* for 20 min. The methanolic supernatant was decanted, and the pellet was re-extracted a second time. The two supernatants were combined, and the methanol was removed in vacuo, prior to freezing and lyophilization. The residue was resuspended in the initial HPLC mobile phase, and up to 200 μL was analyzed by HPLC-PDA-RC-MS.

HPLC with PDA, Radioactivity, and MS^2 Detection. Samples were analyzed on a Surveyor HPLC system comprised of an autosampler cooled to 4 °C, an HPLC pump, PDA detector, scanning from 250 to 700 nm (Thermo Finnigan, San Jose, CA), and an online radioactivity (RC) monitor (Reeve Analytical model 9701, LabLogic, Sheffield, United Kingdom). Separation of quercetin metabolites was carried out using a 250 mm × 4.6 mm i.d. 4 μm Synergi C_{18} RP-Max column (Phenomenex, Macclesfield, United Kingdom), maintained at 40 °C and eluted with a 60 min gradient of 5−40% acetonitrile in 1% formic acid, at a flow rate of 1 mL/min (HPLC system 1). After it passed through the flow cell of the absorbance monitor, the column eluate was directed to a radioactivity monitor fitted with a 500 μL heterogeneous flow cell packed with cerium-activated lithium glass scintillant (*22*), and it was then split and 0.3 mL/min directed to a LCQ DecaXP ion trap mass spectrometer. Analysis with an electrospray interface (ESI) in negative ion mode provided the best limits of detection for quercetin-based metabolites. This was carried out using full scan, data-dependent MS^2 scanning from *m*/*z* 100 to 1000. The capillary temperature was 350 °C, the sheath gas and auxiliary gas were 60 and 10 units, respectively, and the source voltage was 4 kV.

Separation of phenolic acids was also carried out using the same instrumentation but with HPLC using a 250 mm × 4.6 mm i.d. 4 μm Synergi C_8 Polar-RP column (Phenomenex) eluted with a 60 min gradient of 5−45% methanol in 0.1% acetic acid (HPLC system 2). To confirm the identification of the phenolic acid catabolites, a third HPLC system was used. This consisted of a 250 mm × 4.6 mm i.d. 4 μm Gemini Phenyl column (Phenomenex) eluted with the same solvents as the Polar column but with a 60 min, 12−20% gradient (HPLC system 3). With both HPLC systems, the mass spectrometer was used with an atmospheric chemical ionization interface (APCI) in negative ion mode, as this provided the best limits of detection for phenolic acids. This was carried out using full scan, data-dependent MS^2 scanning from 100 to 500 *m*/*z*. The APCI vaporizer temperature was 350 °C, the capillary temperature was 140 °C, the sheath gas and auxiliary gas were 80 and 40 units, respectively, and the source voltage was 5 kV.

Table 1. Distribution of Radioactivity in Tissues Outside the GI Tract 1−72 h after the Ingestion of 18.4 × 10^6 dpm of [2-^{14}C]Quercetin-4′-glucoside by Rats[a]

tissue	1 h	6 h	12 h	24 h	48 h	72 h
liver	80 ± 19 (0.4%)	95 ± 32 (0.5%)	87 ± 5 (0.5%)	95 ± 4 (0.5%)	94 ± 14 (0.5%)	81 ± 11 (0.4%)
kidney	54 ± 17 (0.3%)	98 ± 24 (0.5%)	116 ± 4 (0.6%)	41 ± 6 (0.2%)	51 ± 20 (0.3%)	27 ± 5 (0.2%)
brain	2 ± 2 (<0.1%)	2 ± 1 (<0.1%)	4 ± 3 (<0.1%)	4 ± 3 (<0.1%)	2 ± 0 (<0.1%)	2 ± 2 (<0.1%)
testes	2 ± 1 (<0.1%)	7 ± 1 (<0.1%)	4 ± 1 (<0.1%)	2 ± 0 (<0.1%)	1 ± 0 (<0.1%)	1 ± 0 (<0.1%)
lungs	3 ± 1 (<0.1%)	5 ± 3 (<0.1%)	1 ± 0 (<0.1%)	1 ± 0 (<0.1%)	1 ± 0 (<0.1%)	ND
heart	7 ± 4 (<0.1%)	8 ± 1 (<0.1%)	6 ± 2 (<0.1%)	3 ± 1 (<0.1%)	2 ± 1 (<0.1%)	1 ± 0 (<0.1%)
muscle	427 ± 249 (2.3%)	408 ± 101 (1.0%)	335 ± 105 (0.8%)	204 ± 64 (0.5%)	156 ± 40 (0.4%)	100 ± 40 (0.2%)
total	573 ± 293 (3.1%)	623 ± 162 (3.4%)	553 ± 120 (3.0%)	350 ± 79 (1.9%)	307 ± 75 (1.7%)	112 ± 58 (0.6%)

[a] Data on the recovery of radioactivity are presented as dpm × 10^3 ± standard error (n = 3) and as a percent of intake in parentheses. ND, not detected.

Table 2. Distribution of Radioactivity in the GI Tract, Plasma, Urine, Feces, and Cage Washings 1−72 h after the Ingestion of 18.4 × 10^6 dpm of [2-^{14}C]Quercetin-4′-glucoside by Rats[a]

	1 h	6 h	12 h	24 h	48 h	72 h
stomach	7458 ± 2202 (41%)	252 ± 285 (1.4%)	66 ± 71 (0.4%)	ND	ND	ND
duodenum	1628 ± 1035 (8.8%)	284 ± 159 (1.5%)	42 ± 28 (0.2%)	ND	ND	ND
jejunum/ileum	4597 ± 2116 (25%)	4713 ± 309 (26%)	419 ± 350 (2.3%)	48 ± 5 (0.2%)	25 ± 12 (0.1%)	18 ± 6 (0.1%)
cecum	ND	5363 ± 799 (29%)	2619 ± 1697 (14%)	230 ± 74 (1.4%)	36 ± 24 (0.2%)	19 ± 2 (0.1%)
colon	ND	2494 ± 1152 (14%)	316 ± 528 (1.7%)	342 ± 60 (1.9%)	ND	ND
total GI tract	13683 ± 767 (75%)	13106 ± 125 (71%)	3462 ± 1070 (19%)	620 ± 159 (3.4%)	61 ± 53 (0.3%)	51 ± 1 (0.3%)
plasma	83 ± 43 (0.5%)	74 ± 10 (0.4%)	40 ± 15 (0.2%)	19 ± 7 (0.1%)	ND	ND
urine	47 ± 20 (0.3%)	460 ± 144 (2.5%)	9398 ± 1290 (52%)	11687 ± 2236 (64%)	11430 ± 3126 (63%)	12641 ± 3181 (69%)
feces	111 ± 96 (0.9%)	ND	1715 ± 111 (9.4%)	2077 ± 825 (11%)	2906 ± 984 (16%)	3349 ± 249 (18%)
cage wash	69 ± 17 (0.4%)	776 ± 84 (4.3%)	2254 ± 1401 (12%)	1982 ± 1106 (11%)	2231 ± 877 (12%)	1684 ± 461 (9.2%)
other tissues	573 ± 293 (3.1%)	623 ± 162 (3.4%)	553 ± 120 (3.0%)	350 ± 79 (1.9%)	307 ± 75 (1.7%)	212 ± 58 (1.2%)
total	14566 ± 1029 (79 ± 6%)	15039 ± 2580 (82 ± 14%)	16869 ± 1977 (92 ± 11%)	16735 ± 1638 (91 ± 9%)	16935 ± 1095 (92 ± 6%)	17937 ± 2988 (97 ± 16%)

[a] Data on the recovery of radioactivity are presented as dpm × 10^3 ± standard error (n = 3) and as a percentage of intake in parentheses. Radioactivity in the GI tract and plasma indicates amounts present at each time point. Radioactivities in the urine, feces, and cage washings are the cumulative amounts that have left the body since ingestion and are thus for the 0−1, 0−6, 0−12, 0−24, 0−48, and 0−72 h periods. Samples containing trace levels of radioactivity, less than 0.1% of the ingested dose, are listed as not detected (ND). Data on radioactivity in "other tissues" are taken from **Table 1**.

RESULTS

Distribution of Radioactivity in Rat Tissues and Fluids. Each rat ingested 18.4 × 10^6 dpm of [2-^{14}C]quercetin-4′-glucoside, weighing 1.0 mg. Three rats were sacrificed at each time point 1, 6, 12, 24, 48, and 72 h after feeding the radiolabeled substrate, and the distribution of radioactivity in tissues, plasma, urine, feces, and the cage washings, which consisted of a mixture of urine and feces, was determined. The data obtained are presented in **Tables 1** and **2**. The amounts of radioactivity in the GI tract, tissues, organs, and plasma are the amounts present at each time point, while that in urine, feces, and cage washings are the cumulative amounts that have left the body after ingestion.

There was minimal accumulation of radioactivity in tissues outside the GI tract with typically <0.1% of intake being detected in the brain, testes, lungs, and heart (**Table 1**). Slightly more radioactivity appeared in the liver and kidney samples with higher amounts being detected in muscle tissue with the sample taken 1 h after ingestion of [2-^{14}C]quercetin-4′-glucoside containing 2.3% of intake. However, there is a substantial amount of muscle tissue; it accounts for ca. 25% of the body mass of the rats, so when expressed on a dpm/g basis, the concentration of radioactivity in the muscle samples is no greater than that in the other organs.

The amounts of radioactivity detected in the various sections of the GI tract, plasma, urine, feces, and cage washings are presented in **Table 2**. After 1 h, most of the radioactivity, 41% of intake, was still in the stomach, while 25% had moved to the jejunum/ileum via the duodenum. At this point, no radioactivity was present in either the cecum or the colon. After 6 h, the radioactivity had moved down the GI tract with ca. 26 and 29% of intake being detected in the jejunum/ileum and cecum, respectively, and 14% in the colon. In samples collected at later time points, the amount of radioactivity associated with the GI tract, including that in the cecum and colon, declined markedly (**Table 2**). This would appear to be mainly the result of absorption from the large intestine and excretion in urine coupled with the feces being expelled from the body. The radioactivity in urine increased substantially between 6 and 12 h, at which point more than 50% of the ingested dose had been excreted and thereafter increased only slowly. Despite the high level of urinary excretion, only trace amounts of radioactivity were detected in plasma. Radioactivity corresponding to 9.4% of intake appeared in feces in the 12 h samples, and after 72 h, a total of 18% of intake was associated with fecal material (**Table 2**).

Identification of Radiolabeled Compounds. HPLC-PDA-RC-MS^2 detected 32 radiolabeled compounds in the tissues and body fluids of rats following the ingestion of [2-^{14}C]quercetin-4′-glucoside (**Table 3**). In addition to the quercetin-4′-glucoside substrate and its aglycone, quercetin, 16 quercetin derivatives comprising a quercetin hydrate and a range of glucuronide, sulfated, and methylated metabolites were identified along with five phenolic acids. Nine of the radiolabeled compounds, all of which had nonflavonol absorbance spectra (i.e., a λ_{max} not at ca. 360 nm), could not be identified, as they did not ionize with either ESI or APCI to produce recognizable mass spectra.

Flavonol metabolite identifications were based on absorbance spectra and ESI mass spectrometric fragmentation data, as described by Mullen et al. (*17*), and are as follows:

Quercetin Monoglucuronides (Peaks 19 and 20). Two quercetin monoglucuronides were detected, each being characterized by a negatively charged molecular ion ($[M - H]^-$) at m/z 477,

Table 3. HPLC-MS Identification of Radiolabeled Flavonol Metabolites and Phenolic Acid Catabolites in Extracts of Tissues and Body Fluids of Rats after the Ingestion of 18 × 10^6 dpm of [2-^{14}C]Quercetin-4′-glucoside[a]

			flavonols		phenolic acids	
peak no.	R_t	compound	$[M - H]^-$ (*m/z*)	MS^2 daughter ions (*m/z*)	$[M - H]^-$ (*m/z*)	MS^2 daughter ions (*m/z*)
1	5.5	unknown				
2	6.7	unknown				
3	10.2	unknown				
4	10.5	3,4-dihydroxybenzoic acid[b]			153	109
5	11.6	3,4-dihydroxyphenylacetic acid[b]			167	123
6	18.3	hippuric acid[b]			178	134
7	18.8	quercetin diglucuronide	653	477, 301		
8	19.6	3-hydroxyphenylacetic acid[b]			151	121, 107, 93
9	21.6	unknown				
10	22.1	methyl quercetin diglucuronide	667	491, 315		
11	23.5	methyl quercetin diglucuronide	667	491, 315		
12	25.4	quercetin diglucuronide	653	477, 301		
13	26.5	unknown				
14	27.2	quercetin hydrate	349	317, 299		
15	27.8	methyl quercetin diglucuronide	667	491, 315		
16	28.0	quercetin diglucuronide	653	477, 301		
17	29.1	quercetin diglucuronide	653	477, 301		
18	29.8	quercetin diglucuronide	653	477, 301		
19	31.2	quercetin glucuronide	477	301		
20	32.2	quercetin glucuronide	477	301		
21	32.6	benzoic acid[b]			121	61
22	34.4	quercetin-4′-glucoside[a]	463	301		
23	36.6	methyl quercetin glucuronide	491	315		
24	37.5	methyl quercetin glucuronide	491	315		
25	38.3	methyl quercetin glucuronide	491	315		
26	39.4	methyl quercetin sulfate glucuronide	571	395, 315		
27	43.5	unknown				
28	44.3	quercetin	301	179, 151		
29	44.3	unknown				
30	45.2	unknown				
31	49.0	unknown				
32	50.0	quercetin sulfate	381	301		
33	53.3	unknown				

[a] All retention times were obtained with HPLC system 1. Flavonol mass spectra were obtained with HPLC system 1 and ESI-MS. Phenolic acid mass spectra were obtained with HPLC systems 2 and 3 and APCI-MS. [b] The identity was confirmed by cochromatography with a reference compound.

which on MS^2 fragmented with a 176 amu loss, indicative of cleavage of a glucuronide unit to produce a quercetin fragment at *m/z* 301.

Methyl Quercetin Monoglucuronides (Peaks 23−25). Three methyl quercetin glucuronides were detected. They were characterized by a mass spectrum with fragment ions at *m/z* values 14 amu higher than those obtained with quercetin glucuronides.

Quercetin Diglucuronides (Peaks 12, 16−18). Four quercetin diglucuronide were detected in the GI tract. Each had a $[M - H]^-$ at *m/z* 653, which yielded MS^2 fragments at *m/z* 477 (M − 176) and *m/z* 301 (M − 352, cleavage of two glucuronide units). This indicates that the two glucuronyl units are attached at different positions on the quercetin skeleton. If they had been disaccharides linked at one 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 sugar moiety (*23*).

Methylquercetin Diglucuronides (Peaks 10, 11, and 15). Three peaks were identified as methylquercetin diglucuronides on the basis of mass spectra with fragment ions at *m/z* values 14 amu higher than obtained with quercetin diglucuronides.

Quercetin Sulfate (Peak 32). This peak had a $[M - H]^-$ at *m/z* 381, and MS^2 yielded a major quercetin ion at *m/z* 301, which is in keeping with the an 80 amu cleavage of a sulfate unit.

Quercetin Hydrate (Peak14). This peak yielded a $[M - H]^-$ at *m/z* 349, which on MS^2 fragmented with an 18 amu loss to produce a base peak at *m/z* 331 and a smaller fragment at *m/z* 299, a loss of 32 amu. This fragmentation pattern can be explained by the presence of a hemiacetal hydrate derivative of quercetin that has reacted with methanol during extraction as described by Dangles et al. (*24*). A similar fragmentation pattern is observed when quercetin reacts with the peroxyl radical generator 2,2′-azobis-isobutyronitrile (*25*).

Phenolic acids were identified by cochromatography with authentic standards, absorbance spectra, and MS^2 fragmentation patterns obtained using the three HPLC systems. 3,4-Dihydroxybenzoic acid, 3,4-dihydroxyphenylacetic acid, 3-hydroxyphenylacetic acid, hippuric acid, and benzoic acid, all cochromatographed with authentic standards when analyzed using the Synergi C_{18} RP-Max column, eluted with a acetonitrile−water mobile phase containing 1.0% formic acid (HPLC system 1). However, because of ion suppression, no ESI mass spectrometric data were obtained, except for hippuric acid. Additional analysis using a Synergi Polar-RP column eluted with 0.1% aqueous acetic acid and methanol (HPLC system 2), in conjunction with APCI-MS^2, also produced cochromatographing peaks and provided confirmatory mass spectral data. Further verification of the identifications was obtained using a Gemini Phenyl HPLC column (HPLC system 3) in combination with APCI-MS^2. When used with a methanol-based mobile phase, this column provides HPLC elution profiles very different to the C_{12} and C_{18} columns because of aromatic interactions with the phenyl stationary phase.

3,4-Dihydroxybenzoic Acid (Peak 4). 3,4-Dihydroxybenzoic acid (aka protocatechuic acid) had a $[M - H]^-$ at *m/z* 153, which on MS^2 fragmented with a 44 amu loss, indicative of cleavage

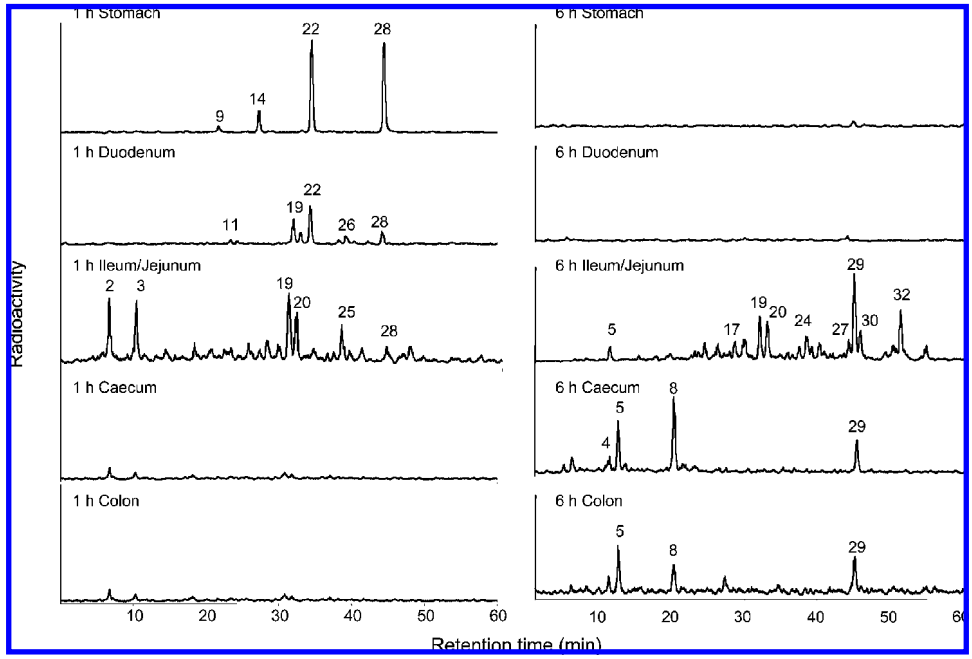

Figure 1. HPLC-RC analysis of radiolabeled compounds in the individual sections of the GI tract of individual rats 1 and 6 h after the ingestion of [2-^{14}C]quercetin-4′-glucoside. Extracts of the stomach, duodenum, jejunum/ileum, cecum, and colon were analyzed using a 250 mm × 4.6 mm i.d. 4 μm Synergi RP-Max HPLC column maintained at 40 °C and eluted with a 60 min gradient of 5−40% acetonitrile in water containing 1% formic acid at a flow rate of 1 mL min^{-1}. Detection was with an online radioactivity monitor operating in the heterogeneous mode. For identification of peaks and peak numbers, see **Table 3**.

of a carboxyl function, produced a single daughter ion at *m/z* 109. This spectrum matched that of a reference compound, which also cochromatographed with peak 4.

3,4-Dihydroxyphenylacetic Acid (Peak 5). 3,4-Dihydroxyphenylacetic acid (aka homoprotocatechuic acid) produced a [M − H]$^-$ at *m/z* 167, which also fragmented with a 44 amu loss, yielding a MS^2 ion at *m/z* 123. This mass fragmentation pattern was also seen in the standard compound.

Benzoylaminoacetic Acid (Peak 6). Benzoylaminoacetic acid (aka hippuric acid) ionized in all three systems to produce a [M − H]$^-$ at *m/z* 178, which with a 44 amu loss gave rise to a single MS^2 daughter ion at *m/z* 134. A hippuric acid standard cochromatographed with peak 6 and had the same MS^2 spectrum.

3-Hydroxyphenylacetic Acid (Peak 8). 3-Hydroxyphenylacetic acid had a [M − H]$^-$ at *m/z* 151, which fragmented with a 44 amu loss to produce a MS^2 base ion at *m/z* 107, as well as two additional ions at *m/z* 121 and 93. This mass spectral pattern is characteristic of 2-, 3-, and 4-hydroxyphenylacetic acids, which can be readily separated by HPLC. Cochromatography with a reference compound established that peak 8 was 3-hydroxyphenylacetic acid.

Benzoic Acid (Peak 21). Benzoic acid produced a [M − H]$^-$ at *m/z* 121. No MS^2 data were acquired because of masking by bleed ions from the HPLC column. However, the *m/z* 121 peak cochromatographed with a benzoic acid standard.

Radiolabeled Compounds in the GI Tract after 1 and 6 h. HPLC-RC profiles of radioactivity in the various sections of the GI tract 1 and 6 h after ingestion of [^{14}C]quercetin-4′-glucoside are illustrated in **Figure 1**. Quantitative data on the radioactivity associated with the individual metabolites, expressed as dpm × 10^3 and as a percentage of the total radioactivity in the individual sections of the GI tract, are presented in **Table 4**. As shown in **Table 2** at the 1 h time point, most of the ingested radioactivity remained in the stomach. HPLC-RC-MS^2 showed that this was mainly the ingested [^{14}C]quercetin-4′-glucoside (peak 22) and its aglycone, quercetin (peak 28), with smaller amounts of a putative quercetin hydrate (peak 14) and trace quantities of two unidentified nonflavonol compounds, peaks 3 and 9 (**Figure 1** and **Table 4**). Radioactivity that had moved to the jejunum/ileum had a different profile, and although quercetin-4′-glucoside and quercetin were still present, a number of other radiolabeled compounds were detected including two quercetin glucuronides (peaks 19 and 20), a methylquercetin glucuronide (peak 25), a quercetin diglucuronide (peak 7), and a further unknown metabolite (peak 3).

Six hours after feeding, most of the radioactivity in the GI tract was in the jejunum/ileum, cecum, and colon (**Table 2**). Quercetin-4′-glucoside and quercetin were no longer present. The complex HPLC-RC profile of the jejunum/ileum indicated that substantial metabolism had occurred (**Figure 1** and **Table 4**) with the main radiolabeled component being an unknown (peak 29) together with a quercetin sulfate (peak 32). Also present were the two quercetin glucuronides (peaks 19 and 20) detected in the 1 h sample, along with a number of minor peaks that included 3,4-dihydroxybenzoic acid (peak 4). The 6 h metabolite profiles further down the GI tract in the cecum and colon were different and less complex than that in the jejunum/ileum, with small amounts of peak 29, an unknown, remaining together with the phenolic acids 3,4-dihydroxybenzoic acid (peak 4), 3,4-dihydroxyphenylacetic acid (peak 5), and 3-hydroxyphenylacetic acid (peak 8).

Radiolabeled Compounds in the GI Tract after 12−72 h. Between 6 and 12 h after ingestion of [^{14}C]quercetin-4′-glucoside, the radioactivity in the GI tract fell from 71 to 19% of intake, with most of the radioactivity in the cecum, and after a further 12 h, only 3.4% remained (**Table 2**). Analysis of the 12 and 24 h jejunum/ileum samples showed that hippuric acid was the main radiolabeled compound present along with trace amounts of 3-hydroxyphenylacetic acid. The only radiolabeled compound in the 12 and 24 h cecum and colon extracts was

Table 4. Levels of ^{14}C-Labeled Compounds in the GI Tract of Rats 1 and 6 h after the Ingestion of 18 × 10^6 dpm of [2-^{14}C]Quercetin-4′-glucoside[a]

			1 h GI tract					6 h GI tract
peak no.	R_t (min)	compound	stomach	duodenum	jejunum/ ileum	jejunum/ ileum	cecum	colon
1	5.5	unknown					316 (6.0%)	
2	6.7	unknown			789 (17%)			
3	10.2	unknown	245 (3.3%)		572 (13%)			
4	10.5	3,4-dihydroxybenzoic acid				151 (3.3%)	255 (4.8%)	149 (6.0%)
5	11.6	3,4-dihydroxyphenylacetic acid					1348 (25%)	793 (32%)
6	18.3	hippuric acid						
7	18.8	quercetin diglucuronide			269 (5.9%)	39 (0.9%)		
8	19.6	3-hydroxyphenylacetic acid					1631 (31%)	796 (32%)
9	21.6	unknown	337 (4.5%)					
10	22.1	methylquercetin diglucuronide				73 (1.6%)		
11	23.5	methylquercetin diglucuronide				71 (1.5%)		
12	25.4	quercetin diglucuronide				29 (0.6%)		
13	26.5	unknown					424 (8.0%)	241 (9.7%)
14	27.2	quercetin hydrate	651 (8.7%)					
15	27.8	methylquercetin diglucuronide				303 (6.5%)		
16	28.0	quercetin diglucuronide		38 (2.4%)	144 (3.1%)			
17	29.1	quercetin diglucuronide				202 (4.4%)		
18	29.8	quercetin diglucuronide		10 (0.6%)	173 (3.8%)			
19	31.2	quercetin glucuronide		471 (29%)	1072 (23%)	398 (8.6%)		
20	32.2	quercetin glucuronide		195 (12%)	504 (11%)	350 (7.6%)		
21	32.6	benzoic acid						
22	34.4	quercetin-4′-glucoside	3024 (41%)	534 (33%)	325 (7.7%)			
23	36.6	methyquercetin glucuronide				47 (1.0%)		
24	37.5	methylquercetin glucuronide		31 (1.9%)	147 (3.2%)	311 (6.7%)		
25	38.3	methylquercetin glucuronide		188 (12%)	321 (7.0%)	159 (3.4%)		
26	39.4	methylquercetin glucuronide sulfate				307 (6.6%)		
27	43.5	unknown				252 (5.4%)		
28	44.3	quercetin	3201 (43%)	161 (9.9%)	255 (5.6%)			
29	44.3	unknown				756 (16%)	1330 (25%)	514 (21%)
30	45.2	unknown				67 (1.5%)		
31	49.0	unknown				220 (4.8%)		
32	50.0	quercetin sulfate				765 (16%)		
33	54.3	unknown				125 (2.7%)		

[a] Data are mean values ($n = 3$) expressed as dpm × 10^3 with figures in parentheses presenting data as a percentage of total radioactivity in the individual sections of the GI tract. For peak numbers and basis of identifications, see **Table 3** and **Figure 1**.

3-hydroxyphenylacetic acid (data not shown). The radiolabeled compounds in 48 and 72 h GI tract extracts contained less than 0.4% of the ingested dose, and this precluded identification.

Radiolabeled Compounds in Urine. As can be seen from the results in **Table 1**, most radioactivity was excreted in urine 6−12 h after [^{14}C]quercetin-4′-glucoside, and there was a slower increase over the ensuing 60 h period. Urine collected over the 0−12, 0−24, 24−48, and 48−72 h periods was analyzed by HPLC-PDA-RC-MS^2. With the exception of hippuric acid, it was not possible to identify flavonol and phenolic acids using the same HPLC conditions. Each sample was, therefore, analyzed using two systems. First, HPLC system 1 and ESI-MS^2 were used to identify flavonol metabolites, and second, HPLC system 2 and APCI-MS^2 were used for the analysis of potential phenolic acids.

Urinary Flavonol Metabolites. Analysis of the 0−12 h urine samples, using HPLC with MS in the full scan MS^2 mode, detected most of the 15 glucuronide, sulfated, and methylated quercetin conjugates listed in **Table 3**. However, they were present in only trace amounts, so it was not possible to quantify them by radioactivity or absorbance detection. All of the quantifiable radiolabeled HPLC peaks in urine at this and later time points were associated with either unknown compounds or phenolic acids. No flavonols were detected by HPLC-MS in urine collected 12 h after ingestion of the [^{14}C]quercetin4′-glucoside.

Urinary Phenolic Acids. HPLC-RC profiles of radiolabeled compounds in 0−12, 0−24, 24−48, and 48−72 h urine obtained from individual rats are illustrated in **Figure 2**, while mean quantitative data ($n = 3$) are presented in **Table 5**. Radioactivity collected 0−12 and 0−24 h after ingestion of [^{14}C]quercetin-4′-glucoside comprised mainly hippuric acid and 3-hydroxyphenylacetic acid with trace amounts of benzoic acid and three previously undetected unknowns A, B, and C. Hippuric acid declined substantially in the 24−48 and 48−72 h urine, while proportionally benzoic acid increased. The overall level of radioactivity excreted at these later time points was, however, much lower than that in the initial 0−12 h urine.

Radiolabeled Compounds in Plasma. Although radioactivity was rapidly absorbed into the plasma, the amount was low, 0.5% of intake in the 1 h samples. Assuming ca. 10 mL of plasma per rat, this corresponds to a concentration of around 1 μmol/L. Plasma samples collected after 1 h contained declining levels of radioactivity, and none was detected in the 48 and 72 h samples (**Table 2**). HPLC-PDA-RC-MS analysis showed that hippuric acid was present in all of the plasma extracts, but ^{14}C-labeled hippuric acid was detected only in the 6 and 12 h samples. Although a range of [^{14}C]quercetin conjugates were detected in 0.5 h plasma samples in a previous investigation (*17, 18*), they were present in only trace, unquantifiable amounts in the present study where the intake of [^{14}C]quercetin-4′-glucoside was 18.4 × 10^6 dpm per rat rather than the 58.5 × 10^6 dpm used earlier.

Radiolabeled Compounds in Feces. As shown in **Table 2**, most radioactivity, 9.4% of intake, initially appeared in feces 6−12 h after feeding [^{14}C]quercetin-4′-glucoside. The amounts gradually increased thereafter so that over the full 0−72 h, a total of 18% was associated with fecal material. Typical HPLC-

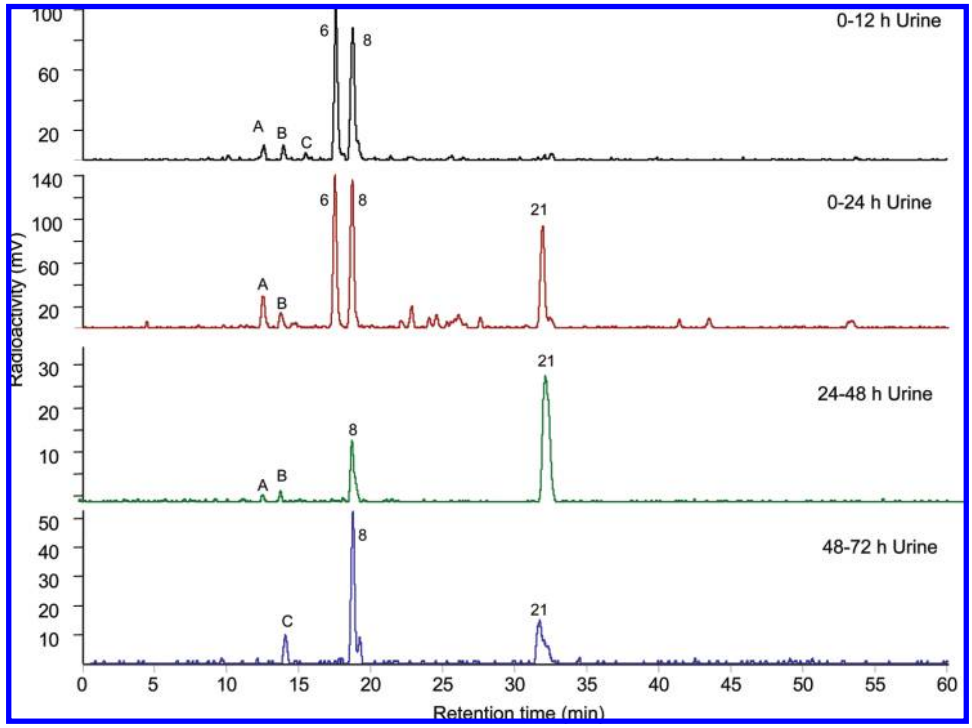

Figure 2. HPLC-RC analysis of radiolabeled compounds in urine of individual rats collected 0−12, 0−24, 24−48, and 48−72 h after the ingestion of [2-^{14}C]quercetin-4′-glucoside. Urine was analyzed using a 250 mm × 4.6 mm i.d. 4 μm Synergi RP-Max HPLC column maintained at 40 °C and eluted with a 60 min gradient of 5−40% acetonitrile in water containing 1% formic acid at a flow rate of 1 mL min^{-1}. Detection was with an online radioactivity monitor operating in the heterogeneous mode. Peaks A, B, and C are unknowns, peak 6 is hippuric acid, peak 8 is 3-hydroxyphenylacetic acid, and peak 21 is benzoic acid (see **Table 3**).

Table 5. Levels of ^{14}C-Labeled Compounds in the Urine and Feces of Rats Collected 0−72 h after the Ingestion of 18 × 10^6 dpm of [2-^{14}C]Quercetin-4′-glucoside[a]

		urine				feces			
HPLC peak	compound	0−12 h	0−24 h	24−48 h	48−72 h	0−12 h	0−24 h	24−48 h	48−72 h
A	unknown	505 (5%)	ND	80 (3%)	ND	ND	ND	ND	6 (2%)
B	unkown	267 (3%)	404 (5%)	110 (5%)	63 (4%)	ND	ND	ND	ND
C	unknown	74 (1%)	220 (2%)	163 (7%)	136 (9%)	ND	ND	ND	ND
6	hippuric acid	4857 (53%)	5840 (67%)	23 (1%)	ND	120 (7%)	ND	28 (4%)	24 (10%)
8	3-hydroxyphenylacetic acid	3155 (32%)	2029 (23%)	859 (37%)	1003 (63%)	1595 (93%)	1670 (100%)	676 (96%)	221 (88%)
21	benzoic acid	539 (6%)	213 (2%)	1110 (47%)	388 (24%)	ND	ND	ND	ND
	total radioactivity	9398 ± 759	8704 ± 1596	2346 ± 446	1591 ± 533	1715 ± 651	1670 ± 249	704 ± 296	251 ± 45

[a] Data are mean values (n = 3) expressed as dpm × 10^3 with figures in parentheses presenting data as a percentage of total radioactivity in the individual sections of the gastrointestinal tract. For HPLC peak numbers and basis of identifications, see **Table 3** and **Figures 4** and **5**.

RC traces of radiolabeled compounds in feces are illustrated in **Figure 3**, while quantitative data are presented in **Table 5**. The major component in all of the fecal samples was 3-hydroxyphenylacetic acid, along with small and varying amounts of hippuric acid. No flavonols were detected in the feces.

Radioactivity in Kidneys, Liver, and Other Tissues. Although low levels of radiolabeled quercetin conjugates were found in kidney and liver extracts in a previous study (*17*), they were not detected in the present investigation. The level of radioactivity in the kidneys stayed relatively constant at around 0.5% of intake over the 72 h period (**Table 1**). HPLC-PDA-RC-MS analysis revealed the presence of [^{14}C]hippuric acid in the 6 h kidney sample and labeled hippuric acid and 3-hydroxyphenylacetic acid in liver 12 h after [^{14}C]quercetin-4′-glucoside intake.

Like the kidneys, the level of radioactivity associated with the liver remained low, ranging from 0.2 to 0.6% of intake for the duration of the study (**Table 1**). The amount of radioactivity per gram was much lower than that in the kidneys so, with the exception of the 12 h sample, no metabolites could be identified; this was also the case with the other tissues and organs within the body.

DISCUSSION

This study has provided a comprehensive picture of the fate of flavonol glucoside, [2-^{14}C]quercetin-4′-glucoside, over a 72 period following its ingestion by rats. One hour after feeding the radiolabeled flavonol glucoside by gavage, the bulk of the radioactivity was present in upper part of the GI tract (**Table 2**). Six to 12 h after intake, the radioactivity had progressed down the GI, and this was associated with its appearance in feces together with sizable amounts being excreted in urine, seemingly after absorption principally in the large intestine. This occurred while the levels of radioactivity in the circulatory system remained extremely low, indicating that the compounds involved were being rapidly removed from the bloodstream by

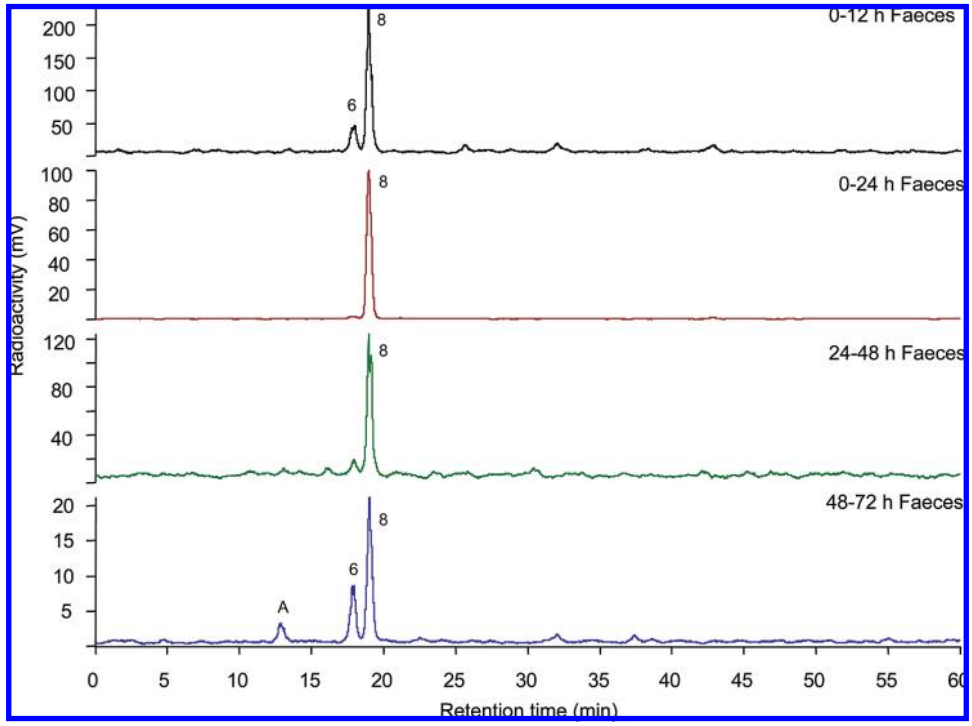

Figure 3. HPLC-RC analysis of radiolabeled compounds in feces of individual rats collected 0−12, 0−24, 24−48, and 48−72 h after the ingestion of [2-^{14}C]quercetin-4′-glucoside. Extracts were analyzed using a 250 mm × 4.6 mm i.d. 4 μm Synergi RP-Max HPLC column maintained at 40 °C and eluted with a 60 min gradient of 5−40% acetonitrile in water containing 1% formic acid at a flow rate of 1 mL min^{-1}. Detection was with an online radioactivity monitor operating in the heterogeneous mode. Peak A is an unknown, peak 6 is hippuric acid, and peak 8 is 3-hydroxyphenylacetic acid (see **Table 3**).

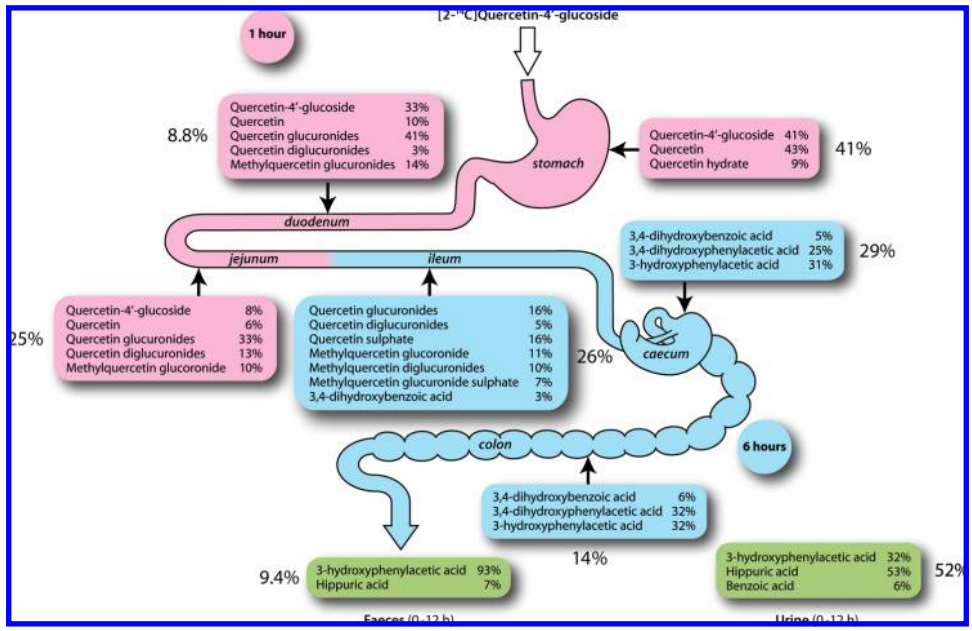

Figure 4. Schematic of the metabolism and catabolism of [2-^{14}C]quercetin-4′-glucoside in rats following its ingestion, movement down the GI tract, and its subsequent appearance in feces and excretion in urine. Figures for quercetin-4′-glucoside and metabolites inside the boxes represent their percentage of total radioactivity in the individual sections of the GI tract and/or feces and urine. Figures outside the boxes indicate radioactivity as a percentage of the amount ingested.

the kidneys. Outside the GI tract, only trace amounts of radioactivity were detected in the organs and tissues of the rat (**Table 1**).

Analysis of samples by HPLC-PDA-RC-MS^2 enabled detailed information to be obtained on the metabolism and catabolism of the quercetin-4′-glucoside as it passed through the body of the rat. The data obtained are summarized pictorially in **Figure 4**. One hour after being fed by gavage, 41% of the radioactivity remained in the stomach principally as the unmetabolized radiolabeled substrate. Also present was the aglycone, quercetin and a quercetin hydrate, both of which may be produced from quercetin-4′-glucoside by the action of microorganisms known to colonize the rat stomach. After 1 h, 25% of the ingested radioactivity had also moved from the stomach to the jejunum/ileum via the duodenum, which contained 8.8% of intake. Once in the GI tract, glucuronidation occurred with the duodenum

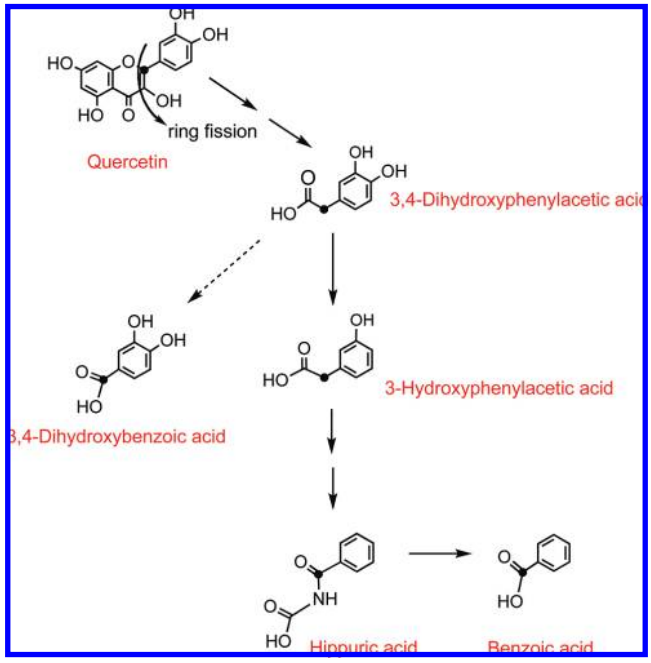

Figure 5. Proposed routes for the catabolism of quercetin. The ● indicates the position of ^{14}C, and the dashed arrow indicates a minor route.

containing quercetin glucuronides and diglucuronides as well as quercetin-4′-glucoside and quercetin. On reaching the jejunum/ileum, further metabolism had occurred with the levels of quercetin-4′-glucoside and quercetin being reduced and those of the quercetin glucuronides increasing along with a methylquercetin glucuronide. Presumably, this is a consequence of lactase phloridizin hydrolase activity and/or cytosolic β-glucosidase cleaving the glucoside and the released aglycone being subjected to the action of UDP-glucuronosyltransferases and methyltransferases in the wall of the small intestine (*26*). After 6 h, the jejunum/ileum retained 26% of the ingested radioactivity with the cecum and colon, which at 1 h had been devoid of radioactivity, now containing 29 and 14% of intake, respectively. There was also evidence of substantial metabolism occurring in the GI tract as quercetin-4′-glucoside and quercetin were no longer present in the jejunum/ileum, which now contained a quercetin sulfate as well as methylated and glucuronidated quercetin conjugates. A small amount of 3,4-dihydroxybenzoic acid was also present (**Figure 4**). No quercetin conjugates were detected in either the cecum or the colon, both of which contained substantial amounts of 3-hydroxyphenylacetic acid and 3,4-dihydroxyphenylacetic acid and smaller quantities of 3,4-dihydroxybenzoic acid (**Figure 4**). The accumulation of these phenolic acids is probably a consequence of the degradation of the flavonol conjugates by the colonic microflora.

Between 6 and 12 h after [^{14}C]quercetin-4′-glucoside intake, the radioactivity in the GI tract declined, and there was a concomitant increase in the amount of radioactivity appearing in feces and urine, 9.4 and 52% of intake, respectively. The urine contained mainly radiolabeled hippuric acid and 3-hydroxyphenylacetic acid, with a small amount of benzoic acid and only trace amounts quercetin metabolites. Radioactivity in the fecal material comprised mainly 3-hydroxyphenylacetic acid along with a smaller amount of hippuric acid (**Figure 4**).

Because of the radiolabel and the 72 h time course, this is first study in which an accurate picture has been obtained of the metabolism of quercetin in the small intestine, its degradation by the microflora in the colon, and the subsequent fate of the phenolic acids that are produced. The phenolic acids are difficult to follow in feeding studies with unlabeled substrates: first, because of subject-to-subject variations in the colonic microflora (*12*) and, second, because some of the phenolic acids are produced by routes independent of dietary flavonoid intake. Hippuric acid, for instance, can be derived from benzoic acid, quinic acid (*27*), tryptophan, tyrosine, and phenylalanine (*28*−*30*). The current study is, therefore, the first investigation in which it has been demonstrated unequivocally that hippuric acid is a degradation product of quercetin.

Proposed pathways for the catabolism of quercetin are presented in **Figure 5**. Upon reaching the large intestine, the various glucuronide conjugates will be hydrolyzed by the colonic microflora, releasing the aglycone, which is subjected to ring fission producing [^{14}C]3,4-dihydroxyphenylacetic acid, which first appears in substantial quantities along with labeled 3-hydroxyphenylacetic acid and traces of 3,4-dihydroxybenzoic acid, in the cecum and colon 6 h after [2-^{14}C]quercetin-4′-glucoside intake (**Table 4**). At the 12 and 24 h time points, only [^{14}C]3-hydroxyphenylacetic acid remained in the GI tract and feces had a similar profile except for the additional presence of trace quantities of [^{14}C]hippuric acid. It is plausible that 3,4-dihydroxyphenylacetic acid is subject primarily to dehydroxylation and conversion to 3-hydroxyphenylacetic acid, and as a minor route, it is also converted to 3,4-dihydroxybenzoic acid. Urine collected 0−12 and 0−24 h after intake also contained 3-hydroxyphenylacetic acid along with substantial amounts of [^{14}C]hippuric acid. 3-Hydroxyphenylacetic acid was also present in the 24−48 and 48−72 h urine samples, but labeled hippuric acid declined substantially, and there was a concomitant increase in [^{14}C]benzoic acid (**Table 5**). These observations imply that 3-hydroxyphenylacetic acid undergoes α-oxidation and dehydroxylation and is converted to hippuric acid (**Figure 5**). Where this conversion occurs is very much a matter of conjecture. Labeled hippuric acid was present in the 12 and 24 h jejunum/ileum samples, while trace, transient quantities were detected in kidney and liver extracts. The labeled benzoic acid that appears in urine is most probably derived from hippuric acid. Many microorganisms produce a hippuricase enzyme, and this and a range of mammalian carboxypeptidase enzymes are capable of hydrolyzing hippuric acid (*31, 32*). This conversion might be a feature of microorganisms in the urinary tract, kidney aminoacylase, or microorganisms in the urine prior to collection and freezing.

The overall recovery of radioactivity expressed as a percentage of intake was as follows: 1 h, 79 ± 6%; 6 h, 82 ± 14%; 12 h, 92 ± 11%; 24 h, 91 ± 9%; 48 h, 92 ± 6%; and 72 h, 97 ± 16% (**Table 2**). The fact that the 1 and 6 h recoveries, when most of the radioactivity was associated with the tissues of the GI tract, were lower than the later time points when the radioactivity was found principally in urine, feces, and the cage washings is probably a reflection of losses encountered during tissue extraction. The 90+% recoveries from 12 h onward indicate that losses through evolution of $^{14}CO_2$ are, at best, minimal. This contrasts with the study by Walle et al. (*19*), where [4-^{14}C]quercetin was fed orally to humans and 52% of the radioactivity was recovered as $^{14}CO_2$, which presumably was a product of colonic bacteria-mediated breakdown of quercetin. In the present investigation, the use of material labeled in the C-2 position resulted in retention of the ^{14}C-label by phenolic acids rather than the CO_2. This reflected in the 69% recovery of radioactivity in urine in the current study (**Table 2**) as compared with the 5% obtained by Walle et al. (*19*), which probably reflects the low level urinary excretion of glucuronide, sulfated, and methylated metabolites of [^{14}C]quercetin.

The current investigation demonstrates the value of not just the use of a radiolabeled substrate when monitoring the fate of flavonoids in animals test systems but also the detailed information that can be obtained through the use HPLC-MS. In two recent investigations into the bioavailability of quercetin in rats and pigs, metabolites in plasma and body tissues were analyzed indirectly, as aglycones released by treating samples with glucuronidase/sulfatase preparations (*33, 34*). HPLC-MS provides detailed information on postabsorption fate of quercetin-4′-glucoside that far exceeds anything produced by analysis of aglycones released by enzyme hydrolysis. As well as sacrificing major amounts of information on the metabolism and catabolism of quercetin, the enzyme hydrolysis approach also has potential quantitative limitations as the mollusc glucuronidase/sulfatase preparations contain a mixture of enzyme activities and there can be substantial batch-to-batch variation in their specificity (*35*). There are no reports of flavonol bioavailability studies using glucuronidase/sulfatase preparations where information on the identity, number, and quantity of the individual sulfate and glucuronide conjugates in the samples of interest has been obtained. Consequently, there are no direct data on the efficiency with which the enzymes hydrolyze the individual metabolites and release the aglycone. This introduces a varying, unmeasured error factor that impacts the accuracy of quantitative estimates. The fact that enzyme hydrolysis results in very reproducible data is an irrelevance as reproducibility is a measure of precision, although it is frequently mistaken for accuracy (*36*). These shortcomings of analyses based on enzyme hydrolysis apply not just to flavonols but to bioavailability studies with all dietary flavonoids, and in this context, it is interesting to note that the use of enzyme hydrolysis results in underestimates of isoflavone metabolites in rat tissues (*37*).

The rats used in the current study ingested quercetin-4′-glucoside at a dose of 4 mg/kg body weight, which corresponds to a 70 kg human consuming ca. 75 mg, which is the quercetin glucoside content of ca. 150 g of a flavonol-rich batch of onions (*38*). This is small as compared with the doses used in some investigations with rats and pigs. In these studies, some of which involved long-term supplementation, doses as high as 500 mg/kg/day were used—vastly in excess of that used in the current study and orders of magnitude in excess of a normal dietary intake. Also, it was the aglycone quercetin that was fed to the animals rather than a quercetin glycoside that would be the normal flavonol constituent of fruits and vegetables. In addition to their analytical shortcomings discussed earlier, the nutrition and physiological relevance of such studies, which claim "...for the first time to identify target tissues for quercetin, which may help to understand its mechanism of action in vivo" (*33*) are of highly questionable value.

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