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Absorption, Disposition, Metabolism, and Excretion of [3-^{14}C]Caffeic Acid in Rats

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ABSTRACT: Male Sprague−Dawley rats ingested 140 × 10^6 dpm of [3-^{14}C]*trans*-caffeic acid, and over the ensuing 72 h period, body tissues, plasma, urine, and feces were collected and the overall levels of radioactivity determined. Where sufficient radioactivity had accumulated, samples were analyzed by HPLC with online radioactivity and tandem mass spectrometric detection. Nine labeled compounds were identified, the substrate and its cis isomer, 3′-*O*- and 4′-*O*-sulfates and glucuronides of caffeic acid, 4′-*O*-sulfates and glucuronides of ferulic acid, and isoferulic acid-4′-*O*-sulfate. Four unidentified metabolites were also detected. After passing down the gastrointestinal tract, the majority of the radiolabeled metabolites were excreted in urine with minimal accumulation in plasma. Only relatively small amounts of an unidentified ^{14}C-labeled metabolite were expelled in feces. There was little or no accumulation of radioactivity in body tissues, including the brain. The overall recovery of radioactivity 72 h after ingestion of [3-^{14}C]caffeic acid was ∼80% of intake.

KEYWORDS: *[3-^{14}C]caffeic acid, absorption, disposition, metabolism, excretion, rats*

INTRODUCTION

Hydroxycinnamates, such as caffeic acid and ferulic acid, are of dietary significance, being widely distributed in fruits, vegetables, grains, and coffee.[1] They occur predominantly as esterified forms linked with sugars or organic acids such as quinic acid and tartaric acid (Figure 1).[1,2] Esterification with quinic acid produces a family of compounds collectively known as chlorogenic acids. The most widely distributed chlorogenic acid is 5-*O*-caffeoylquinic acid, which can occur along with its 3-O- and 4-O-isomers (Figure 1), and is especially abundant in coffee, apples, and maté.[3−5] These compounds can be major constituents in the human diet and are of interest, as they have antibacterial and antiinflammatory properties,[6,7] and have been linked to potential protective effects against cardiovascular disease, diabetes, Alzheimer's disease, and ischemia-reperfusion injury.[8−10] The potential biopharmacological properties of hydroxycinnamates are dependent on their absorption in the gastrointestinal tract and the associated metabolism. The bioavailability of caffeoylquinic acids and their primary metabolite, caffeic acid, have been investigated using a diversity of ex vivo test systems, and human and animal models[11−14] with especially detailed studies being recently reported after the ingestion of coffee by healthy subjects and volunteers with an ileostomy.[15,16] Nonetheless, there is still a gap in acquiring the information on the extent to which hydroxycinnamates are sequestered in the body tissues and organs, a topic on which detailed information can only be obtained with animal-based investigations.

Hydroxycinnamate disposition and metabolism are most readily monitored using an animal model and feeding radiolabeled substrates as demonstrated in a flavonol bioavailability study in which [2-^{14}C]quercetin-4′-*O*-glucoside was fed to rats.[17] To this end, the current study focused on the absorption, disposition, metabolism, and excretion of [3-^{14}C]*trans*-caffeic acid (Figure 1) over a 72 h period following ingestion of the labeled hydroxycinnamate by male Sprague−Dawley rats.

MATERIALS AND METHODS

Synthesis of [3-^{14}C]*trans*-Caffeic Acid. [3-^{14}C]*trans*-Caffeic acid (specific activity 2.2 mCi/mmol) was synthesized from [^{14}C]carbon dioxide prepared from [^{14}C]barium carbonate by the route tested on the synthesis of nonradioactive [3-^{13}C]*trans*-caffeic acid (Figure 2). ^{1}H NMR spectroscopy indicated that the [3-^{14}C]*trans*-caffeic acid was pure, and only one peak was detected by HPLC with photodiode array (PDA) and online radioactivity detection (RC). In the test route, 3,4-diallyloxy[carboxy-^{13}C]benzoic acid **2** was prepared from aryl bromide **1** using [^{13}C]barium carbonate as a source of [^{13}C]carbon dioxide.[18]

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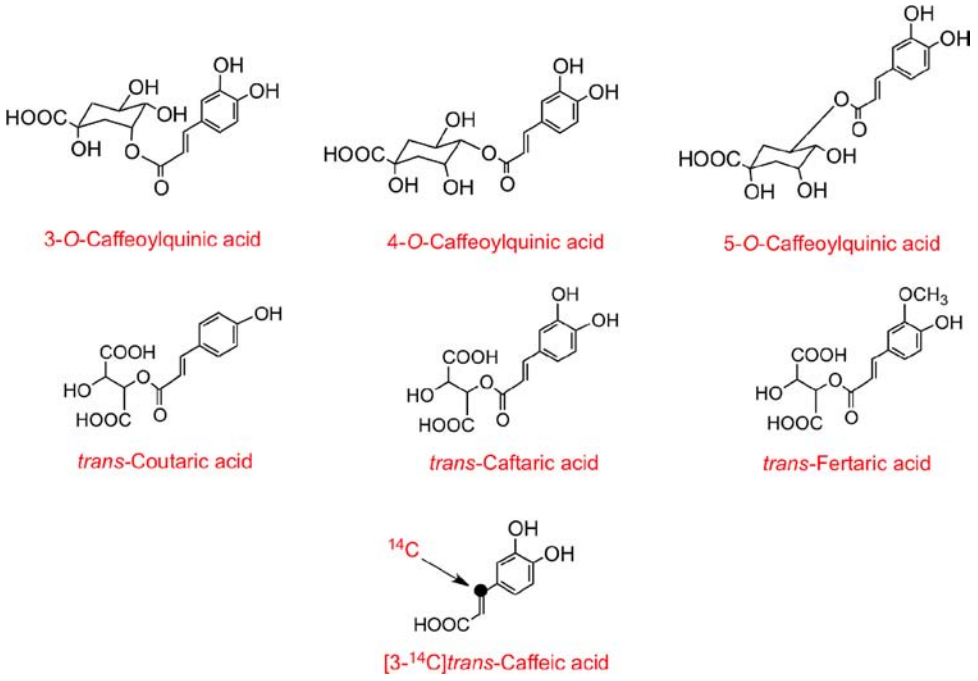

Figure 1. Structures of common dietary hydroxycinnamates.

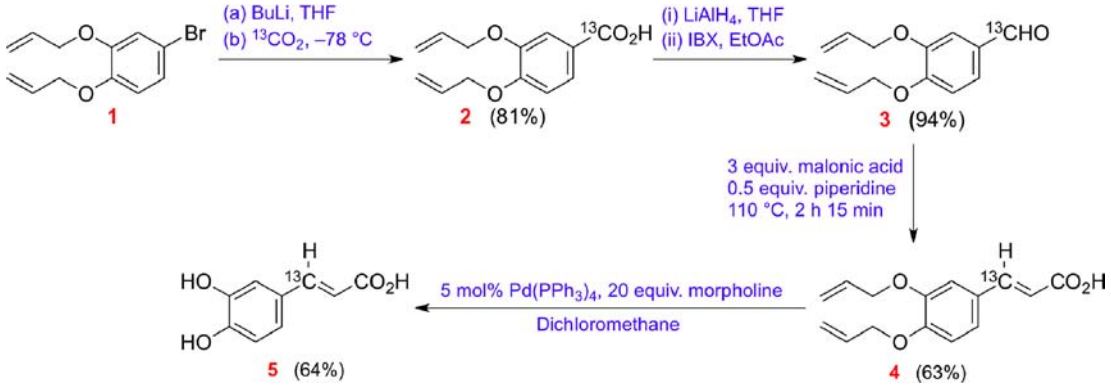

Figure 2. Synthesis of [3-^{13}C]*trans*-caffeic acid.

Reduction to the alcohol followed by oxidation gave aldehyde **3** in excellent yield. Knoevenagel condensation with malonic acid then gave protected *trans*-caffeic acid **4**, and deprotection gave [3-^{13}C]*trans*-caffeic acid as outlined below.

Synthetic Chemistry. 3,4-Diallyloxy[carboxy-^{13}C]benzoic acid **2** was prepared as described by Caldwell et al.,[18] and other chemicals were purchased from commercial suppliers. ^{1}H and ^{13}C NMR spectra were obtained on a Bruker DPX/400 spectrometer operating at 400 and 100 MHz, respectively. DEPT was used to assign the signals in the ^{13}C NMR spectra as C, CH, CH_2, or CH_3. Each compound was also synthesized without a label (data not presented), and the ^{13}C NMR spectra of unlabeled material were used to identify coupling in the ^{13}C NMR spectra of labeled material. Mass spectra (MS) were recorded on a Jeol JMS700 (MStation) spectrometer. Infrared (IR) spectra were obtained on a Perkin-Elmer 983 spectrophotometer. A Golden Gate attachment that uses a type IIa diamond as a single reflection element was used in some cases so that the IR spectrum of the compound (solid or liquid) could be directly detected without any sample preparation. Tetrahydrofuran was dried over sodium and benzophenone, and dichloromethane was dried over calcium hydride.

Preparation of 3,4-Diallyloxy[carbaldehyde-^{13}C]benzaldehyde ***3****.* $LiAlH_4$ (319 mg, 8.40 mmol, 2.2 equiv) was added portionwise to a solution of 3,4-diallyloxy[carboxy-^{13}C]benzoic acid **3** (898 mg, 3.82 mmol) in dry tetrahydrofuran (20 mL) cooled to 0 °C and under an atmosphere of argon. The solution was then stirred at room temperature for 3 h. The reaction mixture was carefully quenched with H_2O and then 2 M HCl added until the precipitate had dissolved. The reaction mixture was extracted into ethyl acetate (EtOAc) (100 mL) and the aqueous layer reextracted with EtOAc (50 mL). The combined organics were washed with H_2O (200 mL) and brine (200 mL). The EtOAc layer was dried over magnesium sulfate and concentrated under vacuum to give the alcohol as a brown oil (836 mg). 2-Iodoxybenzoic acid (IBX) (2.42 g, 8.65 mmol, 3.0 equiv) was added to a solution of the alcohol (637 mg, 2.88 mmol) in EtOAc (10 mL) under an atmosphere of argon. The resulting suspension was heated to 80 °C for 3 h. After cooling, the precipitate was filtered off and then washed with EtOAc (30 mL). The filtrate was washed with sodium thiosulfate solution (100 mL), dried over magnesium sulfate, and concentrated under vacuum to give the aldehyde **3** as a brown oil (602 mg, 94% over two steps). R_f[silica, ether] 0.57. δ_H (400 MHz: $CDCl_3$): 4.65 (2H, td, *J* 1.6, 5.2 Hz, OCH_2), 4.69 (2H, td, *J* 1.6, 5.2 Hz, OCH_2), 5.29–5.34 (2H, m, 2 × CH_AH_B=CH), 5.42–5.47 (2H, m, 2 × CH_AH_B=CH), 6.03–6.13 (2H, m, CH_AH_B=C*H*), 6.96 (1H, d, *J* 7.9 Hz, H-5), 7.36–7.43 (2H, m, H-2 and 6), 9.83 (1H, d, *J* 87.6 Hz, ^{13}CHO). δ_C (100 MHz: $CDCl_3$): 69.52 (CH_2), 69.54 (CH_2), 111.33 (d, *J* 3.1 Hz, CH), 112.23 (CH, d, *J* 5.2 Hz), 117.95 (CH_2), 118.13 (CH_2), 126.40 (CH, d, *J* 5.5 Hz, CH), 129.94 (C, d, *J* 55.0 Hz), 132.23 (CH), 132.55 (CH), 148.66 (C, d, *J* 4.9 Hz), 153.75 (C),

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Table 1. Distribution of Radioactivity (dpm × 10^3) in the Tissues, GI Tract, Plasma, Red Blood Cells, Cage Washings, Urine, and Feces 1−72 h after Ingestion of 140 × 10^6 dpm of [3-^{14}C]*trans*-Caffeic Acid by Rats[a]

tissue	1 h	3 h	6 h	12 h	24 h	48 h	72 h
stomach	87349 ± 14248	9940 ± 8426	260 ± 89	519 ± 275	105 ± 25	313 ± 263	108 ± 31
duodenum	11134 ± 2672	11325 ± 9052	262 ± 70	408 ± 243	84 ± 6	78 ± 5	15 ± 3
jejunum/ileum	12701 ± 1460	22343 ± 4141	1508 ± 308	1891 ± 766	1472 ± 851	960 ± 119	60 ± 6
cecum	18 ± 4	8446 ± 5215	27484 ± 19727	5441 ± 1493	1896 ± 320	440 ± 107	205 ± 96
colon	34 ± 19	10303 ± 7683	9273 ± 2124	5503 ± 2839	2593 ± 545	411 ± 194	57 ± 31
subtotal GI tract (A)	**111236 ± 16507**	**62356 ± 12761**	**38787 ± 18749**	**13769 ± 2760**	**6150 ± 1234**	**2202 ± 521**	**444 ± 114**
kidney	4496 ± 1257	1678 ± 384	4493 ± 2956	195 ± 25	96 ± 24	49 ± 15	13 ± 1
brain	9 ± 3	3 ± 0	4 ± 0	3 ± 2	1 ± 0	1 ± 0	1 ± 0
testes	86 ± 36	20 ± 2	18 ± 3	8 ± 4	7 ± 4	1 ± 0	1 ± 0
lungs	119 ± 18	54 ± 10	28 ± 5	6 ± 2	12 ± 6	3 ± 1	1 ± 0
heart	81 ± 11	20 ± 5	18 ± 2	3 ± 9	5 ± 3	2 ± 1	60 ± 1
muscle	129 ± 42	69 ± 42	48 ± 19	9 ± 3	10 ± 3	4 ± 1	3 ± 1
liver	474 ± 40	142 ± 35	190 ± 44	50 ± 6	46 ± 17	13 ± 2	6 ± 0
spleen	10 ± 2	2 ± 0	3 ± 1	0.4 ± 0	0.6 ± 0	0.8 ± 0	0.2 ± 0
red blood cell	4 ± 2	34 ± 9	12 ± 4	17 ± 12	6 ± 6	0	0
subtotal tissues (B)	**5407 ± 1340**	**2021 ± 390**	**4815 ± 2953**	**293 ± 17**	**184 ± 41**	**75 ± 14**	**26 ± 2**
total (A + B)	**116643 ± 17596**	**64377 ± 13128**	**43601 ± 21692**	**14055 ± 2762**	**6333 ± 1275**	**2276 ± 531**	**470 ± 113**

sample	0−1 h	0−3 h	0−6 h	0−12 h	0−24 h	0−48 h	0−72 h
plasma	3388 ± 3299	41 ± 11	37 ± 5	5 ± 1	9 ± 8	2 ± 1	nd[b]
urine	5435 ± 1868	11157 ± 2759	23089 ± 1651	24178 ± 3719[c]	87363 ± 11381	93708 ± 33689	95597 ± 34018
feces	nd	nd	nd	1519 ± 1463	1928 ± 739	5701 ± 1265	6419 ± 936
cage washings	3950 ± 3667	2841 ± 759	4818 ± 1693	12531 ± 2194	5940 ± 1883	7153 ± 1941	8928 ± 2143
subtotal (C)	**12773 ± 5001**	**14039 ± 3298**	**27944 ± 1269**	**38233 ± 2539**	**95240 ± 34986**	**106564 ± 35498**	**110944 ± 35240**
total (A + B + C)	**129417 ± 17268**	**78416 ± 10685**	**71546 ± 22939**	**52308 ± 4866**	**101573 ± 35408**	**108840 ± 34968**	**111414 ± 35231**

[a]Radioactivity in the cage washings, urine, and feces are the cumulative amounts that have left the body since ingestion at 0 h. [b]nd = not detected. [c]This value is low because of spillage of an undetermined volume of urine during collection. 0−24, 0−48, and 0−72 h estimates are cumulative based on a combination of data obtained with 0−12, 12−24, 24−48, and 48−72 h samples.

190.66 (^{13}CH). *m/z* (EI): 219.1 (M^+, 55%), 178.1 (20), 150.0 (15), 41.1 (100). HRMS: 219.0977. $C_{12}{}^{13}CH_{14}O_3$ requires (M^+), 219.0976.

Preparation of 3-(3′,4′-Diallyloxyphenyl)[3-^{13}C]trans-propenoic Acid **4**. Piperidine (52 μL, 0.53 mmol, 0.5 equiv) was added to a solution of aldehyde **3** (233 mg, 1.06 mmol) and malonic acid (331 mg, 3.19 mmol, 3.0 equiv) in pyridine (3 mL). The solution was then heated to 110 °C for 2 h. After cooling, the solution was acidified to pH 1 and extracted into EtOAc (25 mL) and the aqueous layer reextracted with EtOAc (10 mL). The combined organic layers were washed with 1 M HCl (2 × 50 mL) and brine (50 mL). The EtOAc layers were dried over magnesium sulfate and concentrated under vacuum to give the acid (232 mg, 84%). Note: prolonged standing in solution causes a small degree of double bond isomerization to occur, this is most noticeable in $CDCl_3$. R_f[silica, ether] 0.38. mp 155−158 °C. δ_H (400 MHz: acetone-d_6): 4.65−4.75 (4H, m, 2 × OCH_2), 5.25−5.29 (2H, m, 2 × CH_AH$_B$=CH), 5.44−5.48 (2H, m, 2 × CH$_A$H$_B$=CH), 6.06−6.18 (2H, m, CH$_A$H$_B$=C*H*), 6.42 (1H, dd, *J* 0.8, 15.9 Hz, H-2), 7.03 (1H, d, *J* 8.3 Hz, H-5′), 7.22 (1H, ddd, *J* 2.0, 3.2, 8.3 Hz, H-6′), 7.36 (1H, dd, *J* 2.0, 2.4 Hz, H-2′), 7.62 (1H, dd, *J* 15.9, 155.2 Hz, H-3). δ_C (100 MHz: acetone-d_6): 70.47 (CH_2), 70.64 (CH_2), 113.91 (CH, d, *J* 3.3 Hz), 114.87 (CH, d *J* 5.1 Hz), 117.22 (CH, d, *J* 70.1 Hz), 117.79 (CH_2), 117.90 (CH_2), 124.06 (CH, d, *J* 2.7 Hz), 128.99 (C, d, *J* 56.1 Hz), 134.90 (CH), 135.16 (CH), 145.98 (^{13}CH), 150.12 (C, d, *J* 5.5 Hz), 152.06 (C), 168.59 (C). υ_{max} (nujol)/cm^{-1}: 1662 (COOH), 1594 (Ar), 1510 (Ar), 968 (CH=CH). *m/z* (EI): 261.1 (M^+, 90%), 220.2 (35), 190.1 (30), 180.1 (15), 83.0 (70), 41.1 (100). HRMS: 261.1083. $C_{14}{}^{13}CH_{16}O_4$ requires (M^+), 261.1082.

Preparation of [3-^{13}C]trans-Caffeic Acid **5**. Acid **4** (100 mg, 0.38 mmol) and morpholine (0.67 mL, 7.66 mmol, 20 equiv) in dry dichloromethane (1.5 mL) was degassed and tetrakis-(triphenylphosphine)palladium (22 mg, 19 μmol, 20 mol %) added. The resulting green solution was stirred under an atmosphere of argon at room temperature for 2.5 h. The reaction mixture was extracted into EtOAc (20 mL) and washed with 1 M HCl (2 × 100 mL) and then brine (100 mL). The EtOAc was washed with $NaHCO_3$ solution (2 × 100 mL) and the aqueous layer acidified to pH 1 and extracted into EtOAc (2 × 50 mL). The combined organics were dried over magnesium sulfate and concentrated under vacuum to give the acid **5** as an off-white solid (44 mg, 64%). δ_H (400 MHz: acetone-d_6): 6.28 (1H, dd, *J* 1.0, 15.9 Hz, H-2), 6.89 (1H, d, *J* 8.0 Hz, H-5′), 7.06 (1H, ddd, *J* 2.0, 5.0, 8.2 Hz, H-6′), 7.18 (1H, dd, *J* 2.2, 4.6 Hz, H-2̀), 7.56 (1H, dd, *J* 15.9, 154.4 Hz, H-3), 8.17 (1H, broad s, OH), 8.41 (1H, broad s, OH). δ_C (100 MHz: acetone-d_6): 115.56 (CH, d, *J* 2.5 Hz), 116.12 (CH, d, *J* 70.0 Hz), 116.75 (CH, d, 5.2 Hz), 122.89 (CH, d, 2.4 Hz), 128.07 (C, d, *J* 56.1 Hz), 146.35 (^{13}CH), 146.68 (C), 149.02 (C), 168.62 (C). *m/z* (EI): 181.1 (M^+, 95%), 164.1 (30), 135.1 (45), 90.1 (30). HRMS: 181.0454. $C_{12}{}^{13}CH_8O_4$ requires (M^+), 181.0456.

Chemicals. *trans*-Caffeic acid was obtained from AASC Ltd. (Southampton, UK), and *cis*-caffeic acid was prepared by exposing a methanolic solution of *trans*-caffeic acid to UV light.[19] HPLC grade methanol and acetonitrile were purchased from Rathburn Chemicals (Walkerburn, UK). Formic acid and scintillation cocktail (Optiflow Safe One) were supplied by Fisher Scientific (Leicestershire, UK). Tissue solubilizer was obtained from National Diagnostic (Hull, UK).

Rats and Feeding Procedures. Male Sprague−Dawley rats (Iffa Credo, L'Arbresle, France) weighing ∼250 g, handled according to the guidelines of the Committee on Animal Care at the University of Montpellier and NIH guidelines, were housed individually in stainless steel metabolic cages with free access to deionized water. They were kept under a 0700 to 1900 h light regime, at a temperature of 23 ± 1 °C, with constant humidity.

The rats were deprived of food for 16 h before being fed by gavage with 0.5 mL of water/ethanol (92:8, v/v) containing of 1.52 mg (140 × 10^6 dpm) [3-^{14}C]*trans*-caffeic acid, a dose equivalent to ∼6.08 mg/

kg body weight. At the end of each of the following time points: 1, 3, 6, 12, 24, 48, and 72 h, three rats were anaesthetized with pentobarbital (60 g/L pentobarbital, 60 mg/kg body weight). Blood was drawn by cardiac puncture with a heparin-moistened syringe and plasma and erythrocytes were separated by centrifugation at 2300*g* for 10 min at room temperature, before being frozen in liquid nitrogen and stored at −80 °C. 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, testes, muscle, and spleen were removed, rinsed in saline, blotted dry, frozen, and stored at −80 °C. The gastrointestinal (GI) tract was removed intact along with its contents and then separated into stomach, duodenum, ileum/jejunum, cecum, and colon. All tissues were weighed before being frozen. Feces and urine from each rat were collected from metabolic cages during the course of the study, and they too were frozen and stored at −80 °C. Samples were shipped to the University of Glasgow on dry ice. Upon arrival tissues and feces were freeze-dried, weighed, ground to a powder, and stored at −80 °C, along with plasma and urine, prior to analysis.

Measurement of Radioactivity in Tissues and Body Fluids. Ten milligram aliquots of powdered freeze-dried samples and 100 μL volumes of plasma and urine were treated overnight with 1 mL of tissue solubilizer at 50 °C in a shaking water bath. This produced clear solutions to which was added 10 mL of scintillation cocktail before the determination of radioactivity using a LS 6500 multipurpose liquid scintillation counter (Beckman).

Sample Preparation. One hundred milligram aliquots of powdered tissues and 100 μL of plasma were extracted with 1.5 mL of methanol:water (1:1, v/v) containing 0.1% formic acid. If only very low amounts of radioactivity had been detected in a sample, it was not extracted and further processed. After 30 min, the extract was centrifuged at 1000*g* for 10 min, the methanolic supernatant decanted, and the pellet reextracted. The two supernatants were combined and concentrated to one-third of volume in vacuo after which 10 μL aliquots were taken to measure radioactivity and ascertain the amount of sample required for analysis by HPLC with RC and tandem mass spectrometry (MS^2) detection. Urine was analyzed directly without further processing.

HPLC Analysis with PDA, Radioactivity, and MS^2 Detection. HPLC was used with RC and MS^2 detection for identification and quantification of radiolabeled compounds as described previously.[17] Samples were analyzed on a Surveyor HPLC system comprising an autosampler cooled to 4 °C, an HPLC pump, and a PDA detector, scanning from 250 to 700 nm (Thermo Finnigan, CA). HPLC was carried out using a 250 mm × 4.6 mm i.d. 4 μm Synergi C_{12} RP-Max column (Phenomenex, Macclesfield, UK) maintained at 40 °C and eluted at flow rate 1 mL/min with a 60 min gradient of 5−40% acetonitrile in 0.1% formic acid. After passing through the flow cell of the absorbance monitor, the column eluate was split and 0.3 mL directed to a Finnigan LCQ Duo tandem mass spectrometer with an electrospray interface in negative-ionization mode operating in full scan mode from *m/z* 150 to 600. The remaining 0.7 mL of eluate was mixed at a "T" with scintillation cocktail pumped at 3.0 mL/min and directed to a radioactivity monitor (Reeve Analytical Model 9701, LabLogic, Sheffield, UK) fitted with a 1.0 mL homogeneous flow cell.

The mass spectrometer was operated in negative ionization mode. Tuning was optimized by infusing a standard of ferulic acid dissolved in the initial HPLC mobile phase into the source at a flow rate of 0.3 mL/min. Analyses were carried out using full scan, data-dependent MS^2 scanning from *m/z* 100 to 600. Capillary temperature was 300 °C, sheath and auxiliary gases were 80 and 60 units/min, respectively, and collision energy was set at 35%. Data was processed by Xcalibur software program version 2.1.

RESULTS

Distribution of Radioactivity in Rat GI Tract. Three rats were sacrificed 1, 3, 6, 12, 24, 48, and 72 h after each ingested 140 × 10^6 dpm of [3-^{14}C]caffeic acid, and the distribution of radioactivity in tissues, plasma, urine, and feces, as well as the cage washings that consisted of a mixture of urine and feces, was determined. The data obtained are presented in Tables 1 and 2. In Table 1, data are presented as the amounts of radioactivity recovered in the various tissues and fluids while in Table 2, it is presented as radioactivity recovered as a percentage of the ingested 140 × 10^6 dpm of [3-^{14}C]caffeic acid. The 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.

Table 2. Distribution of Radioactivity Expressed as a Percentage Intake in Tissues, GI Tract, Plasma, Red Blood Cells, Cage Washings, Urine, and Feces 1−72 h after Ingestion of 140 × 10^6 dpm of [3-^{14}C]*trans*-Caffeic Acid by Rats[a]

tissue	1 h	3 h	6 h	12 h	24 h	48 h	72 h
stomach	62	7.1	0.2	0.4	0.1	0.2	0.1
duodenum	7.9	8.1	0.2	0.3	0.1	0.1	<0.1
jejunum/ileum	9.1	16	1.1	1.4	1.1	0.7	<0.1
cecum	<0.1	6.0	19.6	3.9	1.4	0.3	0.1
colon	<0.1	7.4	6.6	3.9	1.9	0.3	<0.1
subtotal GI tract (A)	**80**	**45**	**28**	**9.8**	**4.4**	**1.6**	**0.3**
kidney	3.2	1.2	3.2	0.1	0.1	<0.1	<0.1
brain	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
testes	0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
lungs	0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
heart	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
muscle	0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
liver	0.3	0.1	0.1	<0.1	<0.1	<0.1	<0.1
spleen	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
red blood cell	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	nd
subtotal tissues (B)	**3.9**	**1.4**	**3.4**	**0.2**	**0.1**	**0.1**	**0**
total (A + B)	**83**	**46**	**31**	**10**	**4.5**	**1.6**	**0.3**
sample	0−1 h	0−3 h	0−6 h	0−12 h	0−24 h	0−48 h	0−72 h
plasma	2.4	<0.1	<0.1	<0.1	0.1	<0.1	nd[b]
urine	3.9	8.0	16	17[c]	62	67	68
feces	nd	nd	nd	1.1	1.4	4.1	4.6
cage washings	2.8	2.0	3.4	9	4.2	5.1	6.4
total (C)	**9.1**	**10**	**20**	**27**	**68**	**76**	**79**
total (A + B + C)	**92**	**56**	**51**	**37**	**73**	**78**	**80**

[a]Radioactivity in the cage washings, urine, and feces are the cumulative amounts that have left the body since ingestion at 0 h. [b]This value is low because of spillage of an undetermined volume of urine during collection. 0−24, 0−48, and 0−72 h estimates based on a combination of data obtained with 0−12, 12−24, 24−48, and 48−72 h samples. [c]nd = not detected.

One hour after ingestion, 62% of the ingested radioactivity remained in the stomach with 7.9% having moved to the duodenum and 9.1% reaching the jejunum/ileum. Overall, 80% of the radioactivity was still present in the GI tract, but after 3 h, this dropped to 45% of intake. After 3 h, the level in the stomach declined to 7.1% as radioactivity appeared in increased amounts in the jejunum/ileum, cecum, and colon. After 6 h, radioactivity in the GI tract had fallen to 28% of the quantity ingested and was present mainly in the cecum and colon. At later time points, the amount of radioactivity declined further and what remained was present in the distal rather than the proximal GI tract (Tables 1 and 2).

Table 3. HPLC-MS^2 Identification of *trans*-Caffeic Acid Metabolites in Extracts of Tissues and Body Fluids after Ingestion of 140 × 10^6 dpm of [3-^{14}C]*trans*-Caffeic Acid by Rats

peak no.	HPLC t_R (min)	compound	$[M - H]^-$ (m/z)	MS^2 (m/z)	MS^3 (m/z)	location
1	15.6	unknown A	–			cecum
2	16.4	unknown B	–			feces
3	16.8	caffeic acid-4′-*O*-glucuronide	355	179	135	urine
4	18.1	*trans*-caffeic acid[a]	179	135		GI tract, urine
5	19.2	*cis*-caffeic acid[a]	179	135		GI tract
6	18.3	caffeic acid-3′-*O*-glucuronide[a]	355	179	135	urine
7	20.2	ferulic acid-4′-*O*-glucuronide[a]	369	193, 113, 175		urine
8	22.8	caffeic acid-4′-*O*-sulfate[a]	259	179	135	urine
9	25.4	caffeic acid-3′-*O*-sulfate[a]	259	179	135	jejunum/ileum, cecum, urine
10	26.1	ferulic acid-4′-*O*-sulfate[a]	273	193	178, 149, 134	urine
11	29.7	isoferulic acid-3′-*O*-sulfate[a]	273	193	178	cecum, urine
12	30.4	unknown C	–			urine
13	43.0	unknown D	–			cecum, colon

[a]Indicates cochromatography with a reference compound.

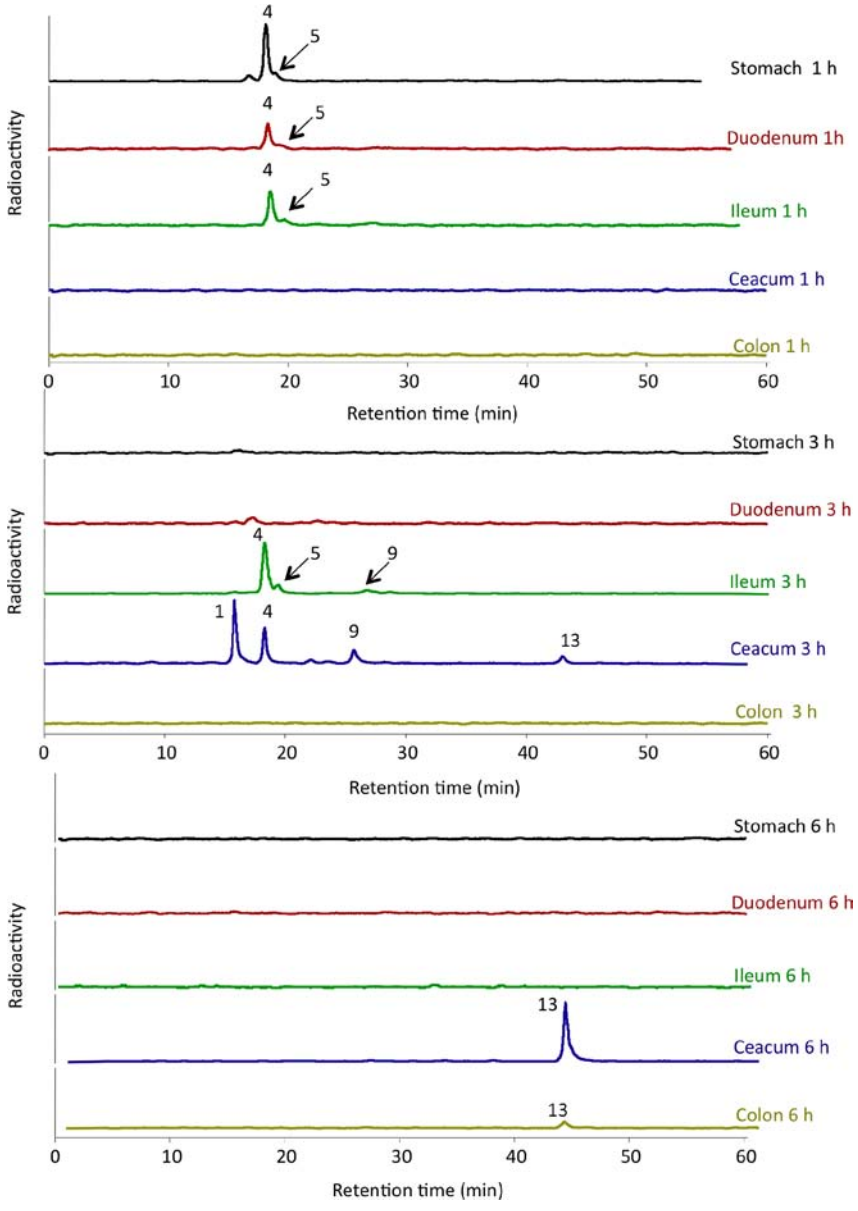

Figure 3. HPLC-RC analysis of radiolabeled compounds in the individual sections of the GI tract rats 1, 3, and 6 h after the ingestion of [^{14}C]caffeic acid. For the identification of the numbered peak, see Table 3.

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Table 4. Levels of ^{14}C-Labeled Compounds in the GI Tract 1, 3, 6, and 12 h after Ingestion of 140 × 10^6 dpm of [3-^{14}C]*trans*-Caffeic Acid by Rats[a]

compound (peak no.)	1 h stomach	1 h duodenum	1 h ileum	3 h ileum	3 h cecum	6 h cecum	6 h colon	12 h cecum	12 h colon
unknown A (1)	nd[b]	nd	nd	nd	3460 (41%)	nd	nd	nd	nd
trans-caffeic acid (4)	73681 (87%)	9060 (81%)	9712 (76%)	16128 (72%)	2538 (30%)	nd	nd	nd	nd
cis-caffeic acid (5)	11739 (13%)	2075 (19%)	2988 (24%)	3567 (16%)	nd	nd	nd	nd	nd
caffeic acid-4′-*O*-sulfate (8)	nd	nd	nd	nd	nd	nd	nd	nd	nd
caffeic acid-3′-*O*-sulfate (9)	nd	nd	nd	2647 (12%)	1536 (18%)	nd	nd	nd	nd
unknown D (13)	nd	nd	nd	nd	912 (11%)	27484 (100%)	9272 (100%)	5441 (100%)	5502 (100%)

[a]Data expressed as dpm × 10^3 and in parentheses as a percentage of the radioactivity in each sample at individual time points. [b]nd = not detected.

Distribution of Radioactivity in Rat Plasma, Urine, Feces, and Cage Washings. After 1 h, 2.4% of the ingested radioactivity was detected in plasma. However, there was rapid decline, and at subsequent time points, radioactivity in the circulatory system (plasma and red blood cells) had fallen to <0.1% of intake (Tables 1 and 2). Over the 3−72 h period, radioactivity in the GI tract fell from 45% to 0.3% of intake while there was a 68% excretion in urine. This indicates that the [^{14}C]caffeic acid-derived compounds, despite passing through the circulatory system in quantity, do not accumulate to any extent, as they are removed extremely rapidly by urinary excretion from the kidneys. The majority of the radioactivity in urine was excreted within 24 h of the ingestion of [3-^{14}C]caffeic acid.

The total radioactivity passing out of the body in feces over the 72 h period after ingestion was 4.6% of intake, and even if the cage washings were composed mainly of fecal material, this is still much lower than the 68% excreted in urine.

Distribution of Radioactivity in Rat Tissues. The amount of radioactivity detected in the various body tissues outside the GI tract are also presented in Tables 1 and 2. The quantities detected in brain, testes, lungs, heart, muscle, and spleen were minimal, <0.1% of intake. However, 1.2−3.2% of intake was present in the kidneys with ∼10-fold lower levels in the liver after 1 and 3 h. Despite the 140 × 10^6 dpm intake of [3-^{14}C]caffeic acid, the maximum amount of radioactivity transferred to the brain of the rats was a mere 9 × 10^3 dpm 1 h after ingestion (Table 1).

Identification of Radiolabeled Compounds. Aliquots of methanolic extracts of urine, feces, and sections of the GI tract containing >20 × 10^3 dpm of radioactivity were analyzed with HPLC-PDA-RC-MS^2 and MS^3 in the consecutive reaction monitoring (CRM). The data obtained for MS^2 and MS^3 daughter ions were then compared with the available standards for *trans*- and *cis*-caffeic acid and in the case of other metabolites were referred to published MS^n fragmentation patterns.[15,20,21] A total of 13 radiolabeled compounds were detected, and 9 were identified on the basis of the properties summarized in Table 3. These are described below.

Peaks 3 (t_R, 16.8 min) and 6(t_R, 18.3 min) had a negatively charged molecular ion ([M − H]$^-$) at *m*/*z* 355 which yielded an M − 176 daughter ion at *m*/*z* 179 which equates with the loss of a glucuronide moiety, and a further MS^2 fragment at *m*/*z* 135 corresponding to caffeic acid. These metabolites which were detected in urine and, in keeping with the data of Farrell et al.,[20] were tentatively identified as a caffeic acid-4′-*O*-glucuronide (peak 3) and caffeic acid-3′-*O*-glucuronide (peak 6).

Peaks 4 (t_R, 18.1 min) and 5 (t_R, 19.2 min), both having a [M − H]$^-$ at *m*/*z* 179, which upon MS^2 produced an ion at *m*/*z* 135, were caffeic acid constituents. Based on cochromatography with reference compounds, the earlier eluting peak 4 was identified as *trans*-caffeic acid, the radiolabeled substrate ingested by the rats, while the later eluting minor component, peak 5, was *cis*-caffeic acid. Both compounds were detected in the GI tract at the 1 and 3 h time points.

Peaks 7 (t_R, 20.2 min), which was detected in urine, had a [M − H]$^-$ at *m*/*z* 369 that produced an MS^2 ion *m*/*z* 193, an 176 amu loss indicative of cleavage of a glucuronide moiety. The fragmentation pattern and chromatographic properties suggest that this peak is ferulic acid-4′-*O*-glucuronide, and this was confirmed by cochromatography with a chlorogenic acid metabolite previously identified as ferulic acid-4′-*O*-glucuronide by Stalmach et al.[15]

Peaks 8 (t_R, 22.8 min) and 9 (t_R, 25.4 min) both had a [M − H]$^-$ at *m*/*z* 259, which upon MS^2 produced an ion at *m*/*z* 179, with an 80 amu loss of a sulfate unit. Peak 8 was identified as caffeic acid-4′-*O*-sulfate and peak 9 as caffeic acid-3′-*O*-sulfate on the basis of cochromatography with previously identified chlorogenic acid metabolites.[15]

Peak 10 (t_R, 26.1 min) and peak 11 (t_R, 29.7 min) both had a [M − H]$^-$ at *m*/*z* 273, which produced a daughter ion at *m*/*z* 193, an 80 amu loss of sulfate. The identity of these metabolites were confirmed as ferulic acid-4′-*O*-sulfate (peak 10) and isoferulic acid-3′-*O*-sulfate (peak 11) on the basis of cochromatography with previously identified metabolites of chlorogenic acids.[15]

It was not possible to identify peaks 1, 2, 12, and 13, named respectively as unknowns A, B, C, and D, because they did not ionize and produce recognizable mass spectra. Unknowns A and D were detected in cecum and colon, unknown B in the feces, and unknown C in urine (Table 3).

Radiolabeled Compounds in the GI Tract. HPLC-RC profiles of radioactivity in the various segments of the GI tract 1, 3, and 6 h after [^{14}C]*trans*-caffeic intake are illustrated in Figure 3. The amounts of the various radiolabeled compounds detected are expressed both as dpm × 10^3 and as a percentage of the total radioactivity of each segment of the GI tract in Table 4.

One hour after ingestion of [^{14}C]*trans*-caffeic acid most radioactivity remained in the GI tract. The main components in the stomach, duodenum, and ileum were the unmetabolized trans isomer (76−87%) along with smaller amounts of *cis*-

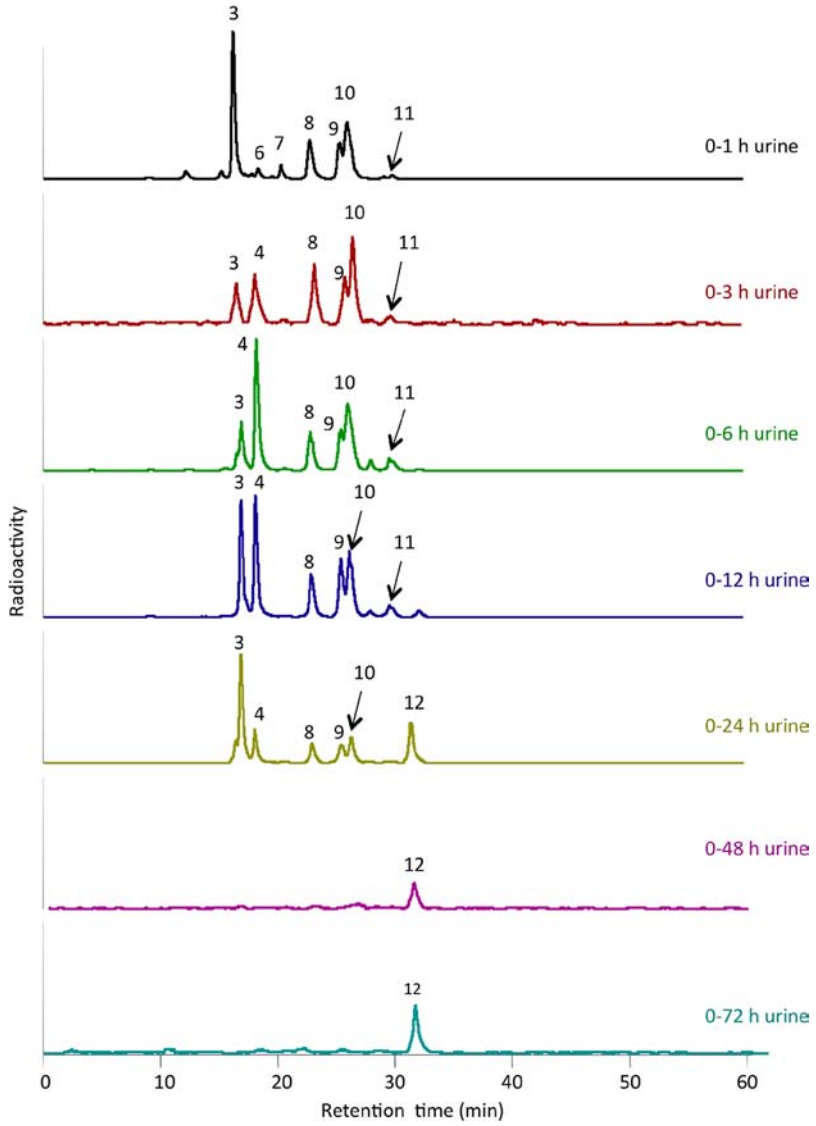

Figure 4. HPLC-RC analysis of radiolabeled compounds in the urine 0−72 h after the ingestion of [^{14}C]caffeic acid. For the identification of numbered peaks, see Table 3.

caffeic acid (13−24%). Three hours after intake the radioactivity had progressed down to the distal GI tract with *trans*-caffeic acid remaining the main radiolabeled component in the ileum (72%) and cecum (30%). The ileum also contained *cis*-caffeic acid (16%). The cecum contained unknown A (41%), unknown D (11%), and caffeic acid-3′-*O*-sulfate (18%). No radiolabeled compound was present in the colon in detectable amounts at this stage.

Six hours after [^{14}C]*trans*-caffeic acid intake most of the radioactivity remaining in the GI tract was in the cecum and colon in the form of the unknown metabolite D, peak 13 (Figure 3, Table 4). At the later 12, 24 , 48, and 72 h time points, only traces of radioactivity remained in the GI tract with no metabolites present in sufficient quantities to facilitate identification.

Radiolabeled Compounds in Urine and Feces. Radioactivity in urine and feces (Tables 1 and 2) are the cumulative amounts that had been excreted from the body of the rats after ingestion of [^{14}C]*trans*-caffeic acid. HPLC-RC profiles of radiolabeled metabolites detected in urine are illustrated in Figure 4, with mean quantitative data presented in Table 5. In the first hour after intake, seven radiolabeled metabolites were excreted in urine. These were caffeic acid-4′-*O*-glucuronide (38%), caffeic acid-3′-*O*-glucuronide (2.1%), ferulic acid-4′-*O*-glucuronide (3.2%), caffeic acid-4′-*O*-sulfate (16%), caffeic acid-3′-*O*-sulfate (11%), ferulic acid-4′-*O*-sulfate (28%), and isoferulic acid-3′-*O*-sulfate (1.4%).

Analysis of the urine samples detected the presence of unmetabolized *trans*-caffeic acid in the 0−3 h, 0−6 h, 0−12 h, and 12−24 h samples. Caffeic acid-3′-*O*-glucuronide and ferulic acid-4′-*O*-glucuronide were detected only in 0−1 h urine. Caffeic acid-4′-*O*-sulfate, caffeic acid-3′-*O*-sulfate, ferulic acid-4′-*O*-sulfate, and the smaller quantities of isoferulic acid-3′-*O*-sulfate were all excreted in the 0−1 h urine, and the amounts remained relatively constant in the 0−3 h, 0−6 h, and 0−12 h samples but declined in the 0−24 urine. Caffeic acid-4′-*O*-glucuronide followed a similar profile, but its excretion was more prolonged with relatively high amounts in the 12−24 h urine. None of these metabolites were excreted after 24 h, and the 24−48 h and 48−72 h samples contained only low levels of the unknown metabolite C in quantifiable amounts (Figure 4, Table 5).

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Table 5. Levels of ^{14}C-Labeled Metabolites in Urine 0−72 h and Feces 12 and 24 h after Ingestion of 140 × 10 ^{6} dpm of [3-^{14}C] *trans*-Caffeic Acid by Rats[a]

	urine							feces	
compound (peak no.)	0−1 h	0−3 h	0−6 h	0−12 h	0−24 h	24−48 h	48−72 h	0−12 h	0−24 h
caffeic acid-4′-*O*-glucuronide (3)	2084 (38%)	1536 (14%)	3308 (14%)	5671 (24%)	35489 (42%)	nd[b]	nd	nd	nd
unknown B (2)	nd	nd	nd	nd	nd	nd	nd	1519 (100%)	1928 (100%)
*trans*caffeic acid (4)	nd	2437 (22%)	7466 (32%)	6185 (26%)	8358 (9.9%)	nd	nd	nd	nd
caffeic acid-3′-*O*-glucuronide (6)	112 (2.1%)	nd	nd	nd	nd	nd	nd	nd	nd
ferulic acid-4′-*O*-glucuronide (7)	171 (3.2%)	nd	nd	nd	nd	nd	nd	nd	nd
caffeic acid-4′-*O*-sulfate (8)	875 (16%)	2242 (20%)	2995 (13%)	3073 (13%)	6813 (8.1%)	nd	nd	nd	nd
caffeic acid-3′-*O*- sulfate(9)	569 (11%)	1364 (12%)	2563 (11%)	3506 (15%)	7383 (8.8%)	nd	nd	nd	nd
ferulic acid-4′-*O*- sulfate (10)	1545 (28%)	3169 (28%)	5642 (24%)	4691 (19%)	9693 (12%)	nd	nd	nd	nd
isoferulic acid-3′-*O*-sulfate (11)	684 (1.4%)	409 (4.8%)	1114 (5%)	1052 (4.4%)	nd	nd	nd	nd	nd
unknown C (12)	nd	nd	nd	nd	16627(20%)	6345 (100%)	1889 (100%)	nd	nd

[a]Data expressed as dpm × 10^{3} and in parentheses as a percentage of the radioactivity in each sample at individual time point. [b]nd = not detected.

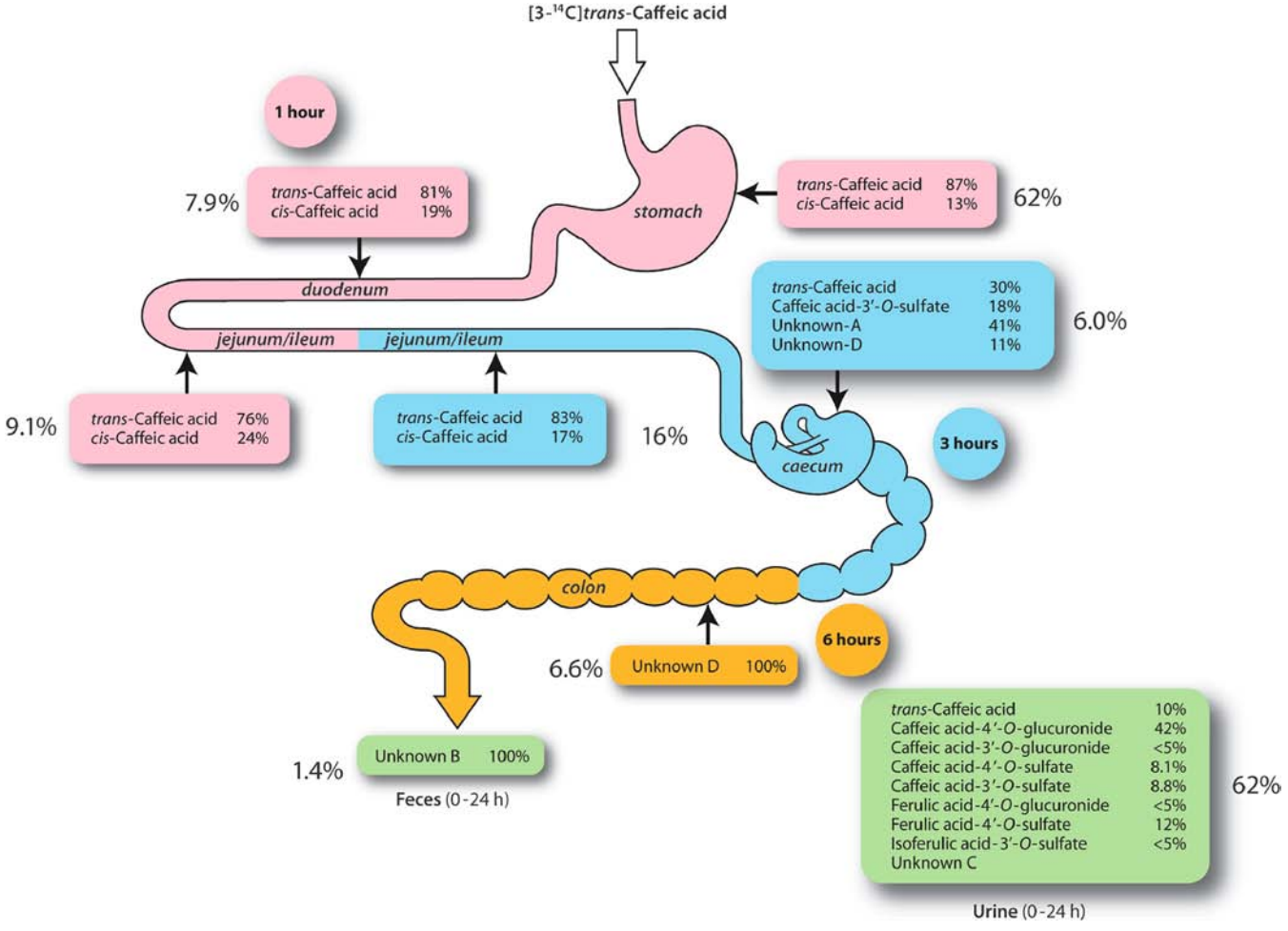

Figure 5. Metabolism of [^{14}C]caffeic acid in rats following ingestion, movement down the GI tract, and subsequent appearance of radiolabeled metabolites in feces and urine. Values for caffeic acid and metabolites inside the boxes represent their percentage of total radioactivity in the individual sections of the GI tract and/or feces and urine. Values outside the boxes indicate radioactivity as a percentage of the amount ingested.

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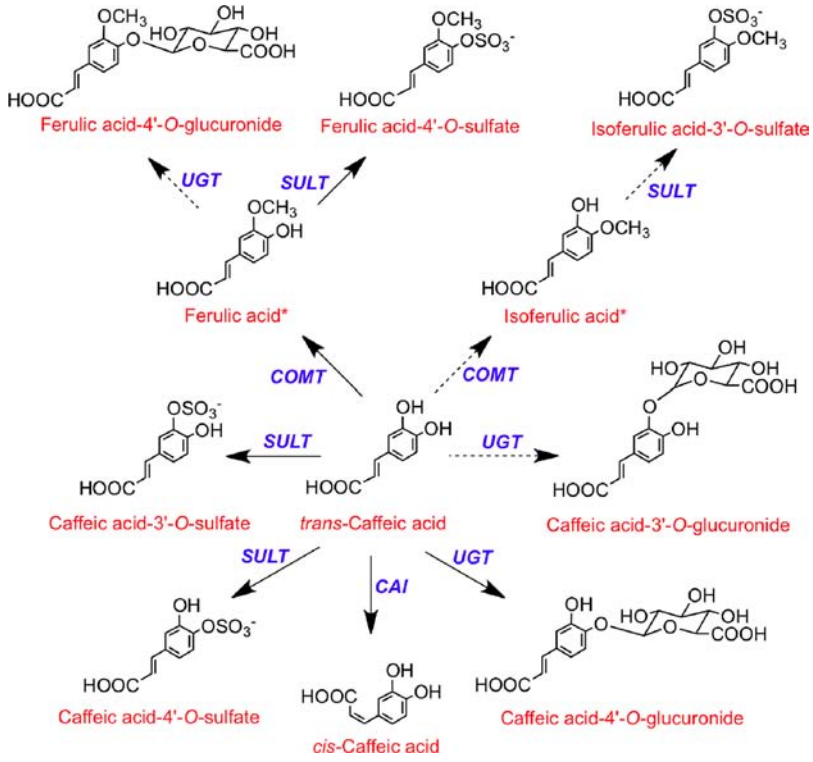

Figure 6. Proposed routes of metabolism of *trans*-caffeic acid following ingestion by rats. CAI, caffeic acid isomerase; COMT, catechol-*O*-methyltransferase; SULT, sulfotransferase; UGT, uridine-5′-diphosphate-glucuronosyltransferase.

The 0−12 and 0−24 h feces contained the unknown metabolite B (Table 5), and although increased amounts of radioactivity were present in the 24−48 and 48−72 h fecal material, no metabolites were present in sufficient amounts to facilitate identification and quantification.

Radiolabeled Compounds in Kidney, Liver, and Other Tissues. The amount of the radioactivity to accumulate in tissues outside the GI tract was minimal throughout the study with most being detected in the kidneys 1, 3, and 6 h after the intake (1.2−3.2%). Despite the high amount of the radioactivity excreted in the urine, only trace amounts of radioactivity were detected in plasma. Due to the low level of radioactivity in all these samples, no metabolites were identified by HPLC-RC-PDA-MS^2.

DISCUSSION

The study was designed to obtain information on the absorption, metabolism, and overall bioavailability of [^{14}C] *trans*-caffeic acid following its ingestion by rats. Based on the data obtained from HPLC-PDA-RC-MS^2 analyses, information on the metabolism and catabolism of the radiolabeled substrate as it passed through the GI tract and was excreted in urine and, to a lesser degree, feces are presented in Tables 4 and 5 and summarized in Figure 5.

After 1 h, most of the radioactivity remained in the stomach in the form of *trans*-caffeic acid and its cis isomer. Smaller amounts of these compounds were also present in the duodenum and the ileum/jejunum. At this point, the plasma contained 2.4% of the ingested radioactivity, but no metabolite was present in identifiable quantities. Urine collected in the first hour contained caffeic acid metabolites, indicative of absorption in the proximal GI tract. The metabolites were caffeic acid-4′-*O*-glucuronide, caffeic acid-3′-*O*-glucuronide, ferulic acid-4′-*O*-glucuronide, caffeic acid-3′-*O*-sulfate, caffeic acid-4′-*O*-sulfate, ferulic acid-4′-*O*-sulfate, and smaller amounts of isoferulic acid-3′-*O*-sulfate which reflect phase II metabolism of caffeic acid upon its removal from the lumen of the GI tract, possibly in the intestinal epithelium or the liver.

In the jejunum/ileum, *trans*- and *cis*-caffeic acid were the main compounds detected after 1 and 3 h of ingestion of ^{14}C-caffeic acid. At this juncture, the main radioactive compound in the cecum was *trans*-caffeic acid along with smaller amounts of caffeic acid-3′-*O*-sulfate and the unidentified compounds, metabolites A and D. Radioactivity in the cecum and colon at 6 and 12 h was exclusively in the form of the unknown metabolite D while the unknown B was the sole radiolabeled metabolite to be detected in feces. Urine collected 0−6 h after [^{14}C]*trans*-caffeic acid intake contained radiolabeled caffeic acid-4′-*O*-glucuronide, caffeic acid-3′-*O*-sulfate and caffeic acid-4′-*O*-sulfate, ferulic acid-4′-*O*-sulfate, isoferulic acid-4′-*O*-sulfate, and caffeic acid. The increased amounts of radiolabeled caffeic acid-4′-*O*-glucuronide in the 0−12 h and 0−24 h and isoferulic acid-4′-*O*-sulfate in the 0−12 h urine samples suggest that the formation of these derivatives may occur in the lower GI tract. Sulfates were the main hydroxycinnamate metabolites, but it is not possible to determine the extent to which sulfation occurs in the wall of the small intestine and the colon or is hepatic in origin.

Over the 72 h postingestion period, a total of 68% of the radioactivity was excreted in urine, indicating that, despite the low levels in plasma, substantial amounts of radioactivity were passing through the circulatory system en route to the kidneys for excretion in urine. The urinary excretion and the low levels of radioactivity in fecal samples also demonstrate efficient absorption and high bioavailability of caffeic acid. This is in keeping with the high urinary excretion of hydroxycinnamate

metabolites after the consumption of caffeoylquinic acid-rich coffee by human volunteers.[15]

The proposed pathways for the metabolism of caffeic acid as it passes through the body of the rats is illustrated in Figure 6. *trans*-Caffeic acid underwent some degree of conversion to its cis isomer, as well as methylation, sulfation, and glucuronidation, events that were readily monitored through the use of a ^{14}C-labeled substrate. The routes are similar, but not identical, to those operating in humans after the ingestion of 5-*O*-caffeoylquinic acid.[15,16] In humans, methylation appears more extensive as evidenced by the occurrence of a more diverse spectrum of ferulic acid, isoferulic acid, and dihydroferulic acid metabolites appearing in plasma and urine as major components. Also in humans, in contrast to rats, dehydrogenation of the hydroxycinnamate side chain occurs as indicated by formation of dihydrocaffeic acid and dihydroferulic acid metabolites. Arguably the absence of these metabolites in rats reflects differences in the composition of their colonic microflora. Humans, unlike rats, also produce feruloylglycine as a caffeic acid metabolite, and this is probably a reflection of differences in hepatic enzymes.

The formation of *cis*-caffeic acid in the stomach within 1 h is of interest, as it was not a contaminant of the [^{14}C]*trans*-isomer that was ingested by the rats. Possibly there is a mammalian or gut bacterial enzyme able to produce this transformation, but reduction to dihydrocaffeic acid and a nonspecific oxidation to yield both *cis*- and *trans*-caffeic acid is another possibility. However, the absence of dihydrocaffeic acid does not support this option. The relative proportions change little after the bolus has left the stomach (Table 4), suggesting that the gut microflora are not involved. Although Poquet et al.[21] have observed a putative ferulic acid glucuronide, no radiolabeled metabolites of *cis*-caffeic acid were detected in the present study.

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