Synthesis of (−)-[4-^3H]Epigallocatechin Gallate and Its Metabolic Fate in Rats after Intravenous Administration

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Because a great deal of attention has been focused on the metabolism of (−)-epigallocatechin gallate (EGCg), quantitative analysis of this compound is required. For this purpose we developed a method of chemical synthesis of [4-^3H]EGCg. Synthesized [4-^3H]EGCg showed 99.5% radiochemical purity and a specific activity of 13 Ci/mmol. To clarify the excretion route of EGCg, the radioactivity levels of bile and urine were quantified after intravenous administration of [4-^3H]EGCg to bile-duct-cannulated rats. Results showed that the radioactivity of the bile sample excreted within 48 h accounted for 77.0% of the dose, whereas only 2.0% of the dose was recovered in the urine. The excretion ratio of bile to urine was calculated to be about 97:3. These results clearly showed that bile was the major excretion route of EGCg. Time-course analysis of the radioactivity in blood was also performed to estimate the pharmacokinetic parameters following intravenous administration of [4-^3H]EGCg. In addition, EGCg metabolites excreted in the bile within 4 h after the intravenous dose of [4-^3H]EGCg were analyzed by HPLC. The results showed that 4′,4″-di-*O*-methyl-EGCg was present in the conjugated form and made up about 14.7% of the administered radioactivity.

Keywords: *(−)-Epigallocatechin gallate; (−)-[4-^{3}H]epigallocatechin gallate; catechin; tea; biliary excretion*

INTRODUCTION

Green tea derived from the leaves of *Camellia sinensis* contains a large amount of catechins. Green tea catechins have been reported to have a wide range of physiological functions, including antibacterial (*1*), antiviral (*2*), antioxidative (*3*), hypocholesterolemic (*4*, *5*), hypoglycemic (*6*), and anticarcinogenic activities (*7*, *8*). In particular, because (−)-epigallocatechin gallate (EGCg) is the most abundant component of green tea catechins and has stronger physiological activities than the other catechin compounds, there are expectations that it will exert beneficial effects on human health. Accordingly, there has also been a growing interest in determining the metabolic profiles of EGCg in order to clarify the mechanisms of the physiological activities in vivo. It has been demonstrated that ingested EGCg was absorbed, at least in part, into the portal vein in rats (*9*, *10*), and that a part of ingested EGCg entered into the systemic circulation in rats (*11*−*13*) and humans (*12*, *14*−*16*). In addition, Chen et al. (*13*) have reported that the bioavailability of ingested EGCg was less than 2% of the dose in rats. With regard to excretion, EGCg and its conjugates were not detected in human urine (*14*, *16*), although ingested EGCg was detected mostly in conjugated forms in the human plasma. These findings suggested that bile could be the main excretion route of EGCg. Recently, Suganuma et al. (*17*) reported on the distribution of radioactivity in various organs and blood, and on the excretion of radioactivity in the urine and feces after oral administration of [^3H]EGCg to mice. They showed that maximum levels of radioactivity in most of the organs and blood were observed at 24 h postdose, and about 6% of radioactivity of dose was excreted in the urine within 24 h. However, no information was available with respect to biliary excretion of [^3H]EGCg. In addition, although Suganuma et al. showed the exchange rate of [^3H]EGCg with water in vitro was less than 5% for 24-h incubation, it still remains that the labeled EGCg may be susceptible to hydrogen−tritium exchange in vivo due to the presence of tritium atoms in the aromatic B ring and/or the galloyl moiety of EGCg. In this study, we established chemical synthesis of [4-^3H]EGCg, which is resistant to hydrogen−tritium exchange. With this labeled EGCg, the excretion route of EGCg was traced after intravenous administration of [4-^3H]EGCg to rats. Further, apparent pharmacokinetic parameters of EGCg were calculated on the basis of radioactivity in the blood after intravenous dose, and some biliary metabolites were also identified by HPLC analysis.

MATERIALS AND METHODS

General. EGCg was prepared from green tea by the method previously described (*18*). Sodium borodeuteride (NaB^2H_4) was obtained from Aldrich Chemical Co. (15681-89-7) and sodium

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boro[^3H]hydride (NaB^3H_4) was obtained from Amersham-Pharmacia (TRK838), and all other reagents were of analytical grade. For structural analyses of EGCg peracetate, 4-bromo-EGCg peracetate, and [4-^2H]EGCg, mass spectra were recorded on a JEOL DX-303 mass spectrometer and NMR spectra were obtained on a JEOL Lambda-500 system. The NMR chemical shifts were given in ppm (δ) with tetramethylsilane (TMS) as an internal standard. High-resolution fast atom bombardment mass spectrometry (HR−FABMS) was measured on a Finnigan Mat 95 instrument at the Mass Spectrometry Service, University of Minnesota. Elemental analysis was performed at Microanalytica Narita (Japan). Mass and ^{3}H NMR spectra of [4-^3H]EGCg were measured at Amersham Pharmacia Biotec (UK).

Preparation of EGCg Peracetate (2). EGCg (1 g, 2.18 mmol) (1) was added to a mixture of pyridine (14 mL) and acetic anhydride (16 mL, 157 mmol) with stirring, and the mixture was kept for 20 h at 45 °C in an oil bath. The reaction mixture was then poured into ice-cold water (500 mL) with vigorous stirring and was left to stand for 1 h to form a white precipitate. The resulting precipitate was collected by filtration with a sintered glass funnel (4 G), washed with 500 mL of chilled water, and dried under reduced pressure. The resulting product was chromatographed on a column of silica gel 60 (100−210 μm, 25 mm i.d. × 500 mm), with *n*-hexane/ethyl acetate (1:3) (solvent A) as the eluent. According to TLC analysis with solvent A, the fractions containing the compound showing R_f value of 0.57 were pooled and evaporated to give the compound **2** (1.7 g, 98.3% yield). The purity of the product was found to be 99% by analytical HPLC. The analytical HPLC was performed in a JASCO liquid chromatograph apparatus with a JASCO 870 UV detector. Following are the conditions: column, CAPCELL PAK C18 UG120 (Shiseido Co., Ltd., 4.6 mm i.d. × 250 mm); mobile phase, 50% aqueous acetonitrile containing 0.01% trifluoroacetic acid (TFA); flow rate, 1 mL/min; temperature, 35 °C; detection, UV 280 nm. Full assignments of ^{1}H and ^{13}C NMR spectra were done by heteronuclear multiple bond correlation (HMBC) and heteronuclear multiple quantum coherence (HMQC) experiments.

EI-MS *m/z*: 794 (M^+). ^{1}H NMR (500 MHz, $CDCl_3$): δ 2.21 (CH_3−Ac × 2), 2.23 (CH_3−Ac), 2.25 (CH_3−Ac), 2.26 (CH_3−Ac × 3), 2.27 (CH_3−Ac), 2.98 (dd, 1H, H-4b (provisionally determined), J = 2.3, 17.9), 3.04 (dd, 1H, H-4a (provisionally determined), J = 4.6, 17.9), 5.16 (br s, 1H, H-2), 5.62 (m, 1H, H-3), 6.59 (d, 1H, H-8, J = 2.2 Hz), 6.71 (d, 1H, H-6, J = 2.2 Hz), 7.24 (s, 2H, H-2′, 6′), 7.60 (s, 2H, H-2″, 6″). ^{13}C NMR (125 MHz, $CDCl_3$): δ 20.1−21.1 (CH_3−Ac × 8), 25.9 (C-4), 68.0 (C-3), 76.5 (C-2), 108.1 (C-8), 109.0 (C-6), 109.4 (C-4a), 118.8 (C-2″, 6″), 122.4 (C-2′, 6′), 127.5 (C-1″), 134.4 (C-4′), 135.1 (C-1′), 139.0 (C-4″), 143.3 (C-3″, 5″), 143.5 (C-3′, 5′), 149.7 (C-7), 149.8 (C-5), 154.8 (C-8a), 163.5 (C═O), 166.2 (C═O−Ac), 166.7 (C═O−Ac), 167.4 (C═O−Ac × 2), 167.5 (C═O−Ac × 2), 168.4 (C═O−Ac), 168.8 (C═O−Ac).

Preparation of 4-Bromo-EGCg Peracetate (3). The compound **2** (1.2 g, 1.51 mmol) was dissolved in carbon tetrachloride (150 mL) by heating at 60 °C. *N*-Bromosuccinimide (290 mg, 1.63 mmol) and α,α′-azobisisobutyronitrile (3 mg) were added to the solution. The mixture was refluxed for 1 h. The reaction mixture was then cooled to room temperature and evaporated to dryness under reduced pressure. The resulting product was chromatographed on a column of silica gel 60 (100−210 μm, 25 mm i.d. × 500 mm) with solvent A as the eluent. The aliquot of each fraction was checked by the same TLC analysis described in the method for the preparation of **2**. The fractions (R_f value of 0.63) were pooled and evaporated to dryness under reduced pressure. For further purification, the residue (1.23 g) was dissolved in acetonitrile (3 mL) and the aliquots (3 × 1 mL) of the solution were subjected to preparative HPLC. The preparative HPLC was performed with a CAPCELL PAK C18 UG120 (Shiseido Co., Ltd., 20 mm i.d. × 250 mm) column in the same HPLC systems described above. Following are the HPLC conditions: mobile phase, 50% aqueous acetonitrile containing 0.01% TFA; flow rate, 9.9 mL/min; temperature, room temperature; detection, UV 280 nm. The peak with a retention time of 61 min was collected and concentrated to remove acetonitrile. The product deposited in the concentrated solution was collected with a membrane filter (Advantec) and then washed with 500 mL of distilled water. The resulting product was dried under reduced pressure to give the compound **3** (600 mg, 46% yield). The purity of the product was found to be 99% by analytical HPLC under the same conditions as described in the preparation of **2**. Full assignments of ^{1}H and ^{13}C NMR spectra were done by HMBC and HMQC experiments.

HR−FABMS (positive) *m/z*: 873.0935 $[M+H]^+$, 875.0915 $[M+H]^+$ (Calcd. for $C_{38}H_{33}O_{19}BrH$, 873.0878; 875.0858). ^{1}H NMR (500 MHz, $CDCl_3$): δ 2.24 (s, 6H, CH_3−Ac × 2), 2.26 (s, 3H, CH_3−Ac), 2.27 (s, 3H, CH_3−Ac), 2.28 (s, 6H, CH_3−Ac × 2), 2.29 (s, 3H, CH_3−Ac), 2.37 (s, 3H, CH_3−Ac), 5.38 (d, 1H, H-4, J = 2.0 Hz), 5.64 (dd, 1H, H-3, J = 0.9, 2.0 Hz), 5.87 (br s, 1H, H-2), 6.74 (d, 1H, H-8, J = 2.5 Hz), 6.77 (d, 1H, H-6, J = 2.5 Hz), 7.30 (s, 2H, H-2′, 6′), 7.59 (s, 2H, H-2″, 6″). ^{13}C NMR (125 MHz, $CDCl_3$): δ 20.2 (CH_3−Ac × 2), 20.6 (CH_3−Ac × 4), 21.1 (CH_3−Ac × 2), 37.2 (C-4), 72.4 (C-3), 72.7 (C-2), 108.1 (C-8), 109.9 (C-6), 110.4 (C-4a), 118.8 (C-2″, 6″), 122.5 (C-2′, 6′), 126.6 (C-1″), 134.1 (C-4′), 134.7 (C-1′), 139.3 (C-4″), 143.4 (C-3″, 5″), 143.6 (C-3′, 5′), 150.1 (C-7), 151.9 (C-5), 154.3 (C-8a), 163.2 (C═O), 166.1 (C═O−Ac), 166.7 (C═O−Ac), 167.4 (C═O−Ac × 2), 167.6 (C═O−Ac × 2), 167.9 (C═O−Ac), 168.5 (C═O−Ac).

Preparation of [4-^2H]EGCg (4). The compound **3** (200 mg, 229 μmol) was dissolved in 15 mL of anhydrous methanol, and then 360 mg of NaB^2H_4 was added to the solution. After the solution was gently stirred for 6 h at room temperature, 5% phosphoric acid (80 mL) was added to the reaction mixture to terminate the reaction. The resulting mixture was concentrated to remove methanol and then was applied to a DIAION HP-20 (Mitsubishi Chemica Co.) column (20 mm i.d. × 150 mm) that had been equilibrated with distilled water. After washing with 200 mL of distilled water, the column was eluted with 100 mL of acetonitrile and the eluent was evaporated to dryness. The resulting residue was further purified by preparative HPLC. The preparative HPLC conditions were the same as those for the purification of **3** except for using acetonitrile/ethyl acetate/0.05% phosphoric acid (12:0.6:90, by volume) as a mobile phase. The peak with a retention time of 31 min was collected and concentrated to remove organic solvents. Desalting of the concentrated solution was performed with the DIAION HP-20 column under the same conditions as described above. The eluent was evaporated to dryness under reduced pressure to obtain the compound **4** (55 mg, 52% yield). The purity of the product was found to be more than 99% by HPLC under the same analytical conditions used for **3** except for using acetonitrile/ethyl acetate/0.05% phosphoric acid (12:0.6:90, by volume) as a mobile phase. Full assignments of ^{1}H and ^{13}C NMR were done by HMBC and HMQC experiments.

Elemental analysis calcd. for $C_{22}H_{17}D_1O_{11}\cdot 2H_2O$: C, 53.32%; H(+D), 4.68%. Found: C, 53.42%; H(+D), 4.63%. FABMS (positive) *m/z*: 460 $[M+H]^+$. FABMS (negative) *m/z*: 458 $[M-H]^-$. ^{1}H NMR (500 MHz, CD_3OD): δ 2.82 (d, 1H, H-4, J = 2.5 Hz), 4.96 (br s, 1H, H-2), 5.52 (dd, 1H, H-3, J = 1.5, 2.5 Hz), 5.95 (s, 2H, H-6, 8), 6.49 (s, 2H, H-2′, 6′), 6.94 (s, 2H, H-2″, 6″). ^{13}C NMR (125 MHz, CD_3OD): δ 28.1 (C-4), 69.9 (C-3), 78.7 (C-2), 95.9 (C-8), 96.6 (C-6), 99.4 (C-4a), 106.9 (C-2″, 6″), 110.3 (C-2′, 6′), 121.5 (C-1″), 130.9 (C-4′), 133.8 (C-1′), 139.9 (C-4″), 146.4 (C-3″, 5″), 146.7 (C-3′, 5′), 157.3 (C-8a), 157.9 (C-7), 158.0 (C-5), 167.7 (C═O).

Preparation of [4-^3H]EGCg (5). The compound **3** (10 mg, 11.5 μmol) was dissolved in 0.7 mL of anhydrous methanol, and then 17 Ci of NaB^3H_4 (11 mg, 283 μmol) was added. The reaction mixture was left to stand overnight at room temperature. Subsequently, the reaction mixture was left to stand for 3 h at room temperature after the addition of $NaBH_4$ (8 mg, 211 μmol). Then the reaction was quenched by the addition of 5% phosphoric acid (4 mL). The resulting mixture was concentrated to about half of its original volume in vacuo. The concentrated solution was extracted four times with ethyl acetate (3 mL). The combined extracts were washed three times with distilled water (3 mL) and evaporated to dryness.

The residue was purified by preparative HPLC. The HPLC was carried out on a Prodigy ODS-2 in a liquid chromatograph apparatus equipped with a UV detector (280 nm) and a β-radioactivity detector. The elution was done with methanol/water/acetic acid (20:80:0.1, by volume) at a flow rate of 3 mL/min at room temperature. The main radioactive peak was collected, and the solvent was evaporated to dryness (4.2 mg, 19% yield). The residue was dissolved in ethanol solution containing L-ascorbic acid at 8 mg/mL (30 mL). The ethanol solution showed 30 mCi of radioactivity. The radiochemical purity of the product was found to be 99.5% by HPLC. The HPLC was carried out on a CAPCELL PAK C18 UG120 (Shiseido Co., Ltd., 4.6 mm i.d. × 250 mm) column in a liquid chromatograph apparatus equipped with a UV detector (280 nm) and a β-radioactivity detector. The elution was done with acetonitrile/ethyl acetate/0.05% phosphoric acid (12:0.6:90, by volume) at a flow rate of 1 mL/min at 40°. The specific activity was 13 Ci/mmol calculated by MS analysis. This procedure was carried out at Amersham Pharmacia Biotec (UK). Following are the analytical data: FABMS (positive) *m/z*: 459 $[M+H]^+$; *m/z*: 461 $[M+H]^+$. ^3H NMR (500 MHz, CD_3OD): δ 2.98.

Animals and Diets. Male Wistar rats (6 weeks of age, 180−210 g) purchased from Charles River Japan, Inc., were given a polyphenol-free diet (*10*) for a week. Water was provided ad libitum throughout this experiment (for bile-duct-cannulated rats, 0.9% NaCl solution was supplied as drinking water). The rats were fasted overnight before dosing labeled EGCg and the diet was resumed 4 h after dosing.

Surgical Procedure for Bile-Duct Cannulation. After the rats were anesthetized with pentobarbital sodium, the skin of their head was incised and the abdomen was opened. A micropolyethylene tube (outside diameter 0.61 mm) was fed from the incision of the head skin to the abdomen through the back subcutaneously and then the end of the tube was inserted into the bile duct. The other end of the tube was put into a vial containing 0.1 M sodium acetate buffer (pH 5.0) to collect the bile. The skin and abdomen wall were sutured and bandaged. The rats were left overnight to recover from anesthetization prior to dosing.

Dose. The [4-^3H]EGCg preparation (13 Ci/mmol) was diluted with unlabeled EGCg to a specific activity of 22.9 mCi/mmol. Thus, 200 μCi of the [4-^3H]EGCg preparation (13 Ci/mmol) was added to 2 mL of physiological saline containing cold EGCg (4 mg). The solution was intravenously administered to the rats ($n = 6$) at a dose of 2 mL per kg.

Sample Collection. After dosing, the bile-duct-cannulated rats were placed in stainless steel metabolism cages. Urine was collected in chilled vessels with dry ice. Bile was collected as described in surgical procedure. The time intervals of the collection were 0−4, 4−8, 8−12, 12−24, and 24−48 h after dosing. Serial blood samples (50 μL) were collected from the caudal vein at 1, 2, 4, 8, and 24 h. All rats were sacrificed at 48 h and blood samples (200 μL) were collected from inferior vena cava. These samples were stored at −20 °C until use.

Determination of Total Radioactivity in Samples. Each urine and bile sample was weighed, and aliquots (200 mg) of both samples were placed in scintillation vials. Each blood sample (50 or 200 μL) was also put into a scintillation vial. To the above vials containing urine, bile, and blood samples was added 1 mL of Solvable (Packard Bio Science B. V.). The scintillation vials were incubated for several hours at 45 °C, then 0.5 mL of propanol and 200−300 μL of 30% H_2O_2 were added, and the vials were incubated for 1 h at 50 °C to decolorize the samples. After the vials were cooled, 10 mL of Hionic Flour (Packard Bio Science B. V.) was added and the resulting mixtures were left to stand in a darkroom for 1 h. The radioactivities of the mixtures were determined by liquid scintillation spectroscopy (Aloka, LSC-3100).

HPLC Analysis of Metabolites in Bile. An aliquot (1 mL) of bile sample collected from six rats during the first 4 h was evaporated to dryness, and the residue was dissolved in 1 mL of 0.1M sodium acetate buffer (pH 5.0) containing 1% ascorbic acid and 0.15 mM ethylenediaminetetraacetic acid (EDTA). The solution was mixed with 20 μL of 0.1M sodium acetate buffer (pH 5.0) containing β-glucuronidase type H-1 (748 units of β-glucuronidase and 39.2 units of sulfatase). The mixture was incubated at 37 °C for 2 h with gentle shaking, then it was extracted three times with the same volume of ethyl acetate. The organic phase was concentrated to dryness and the residue was dissolved in 1 mL of acetonitrile/ethyl acetate/0.05% phosphoric acid (12:0.6:90, by volume) then subjected to analytical HPLC. The HPLC was carried out on a CAPCELL PAK C18 UG120 (Shiseido Co., Ltd., 4.6 mm i.d. × 250 mm) in a JASCO liquid chromatograph apparatus equipped with a JASCO UV-870 detector (280 nm) and a β-radioactivity detector (Beckman). The radioactivity detector was operated in homogeneous liquid scintillation counting mode with addition of 0.8 mL/min of Ultima-Flou M (Packard Bio Science B. V.) to the eluent passed through the UV detector. The elution was done with acetonitrile/ethyl acetate/0.05% phosphoric acid (12:0.6:90, by volume) at a flow rate of 0.8 mL/min at 40 °C.

RESULTS

Synthetic Considerations. We have developed chemical synthesis of [4-^2H]EGCg and [4-^3H]EGCg. The 4 position of EGCg was selected for the labeling position of deuterium or tritium to avoid hydrogen exchange. The synthetic routes to [4-^2H]EGCg and [4-^3H]EGCg are outlined in Figure 1. Thus, EGCg (compound **1**) was first peracetylated with acetic anhydride in pyridine and EGCg peracetate (compound **2**) was obtained in a good yield (98%). Compound **2** was brominated with *N*-bromosuccinimide and α, α′-azobisisobutyronitrile in carbon tetrachloride for 1 h at reflux. The molecular formula of the product was established as $C_{38}H_{33}O_{19}Br$ by HR−FABMS analysis. The ^1H NMR chemical shifts of the brominated product were found to be similar to those of **2**. However, in the brominated product one of two double-doublet signals (δ 3.04, 2.98) derived from H-4a and H-4b protons of **2** disappeared and another one (δ 5.38) emerged as a doublet with the downfield shift. The ^{13}C NMR chemical shifts of the brominated product were analogous to those of **2**, except for the downfield shifts of C-4 (about 11 ppm) and C-3 (about 4 ppm), and the upper-field shift of C-2 (about 4 ppm). These observations indicated that a hydrogen atom at the 4 position of the compound **2** was replaced by a bromine atom in the product. As a result, the product was identified as 4-bromo-EGCg peracetate (the compound **3**). Compound **3** was treated with NaB^2H_4 in anhydrous methanol at room temperature. The positive and negative FABMS data of the product showed $[M+H]^+$ ion peak at *m/z* 460 and $[M-H]^-$ ion peak at *m/z* 458, respectively. The MS analysis suggested that the product is monodeuterated EGCg. The ^1H NMR chemical shifts of the deuterated product were found to be superimposable on those of **1** (data not shown) except for the disappearance of the signal (δ 2.98) derived from the H-4b proton of **1**. In addition, the ^{13}C NMR data of the product were almost the same as those of **1**. These results demonstrated that substitution of a bromine at C-4 for a deuterium and complete deacetylation of **3** occurred simultaneously. Consequently, the product was identified as [4-^2H]EGCg (the compound **4**).

Synthesis of [4-^3H]EGCg (the compound **5**) from **3** was conducted by modification of the procedure for preparation of **4**, as described in Materials and Methods. Thus, compound **3** was first treated overnight with NaB^3H_4, followed by additional $NaBH_4$ for 3 h. This process contributed to reducing the usage of NaB^3H_4 markedly. The HPLC analysis revealed that the retention time of the product coincided with that of compound **1**. The

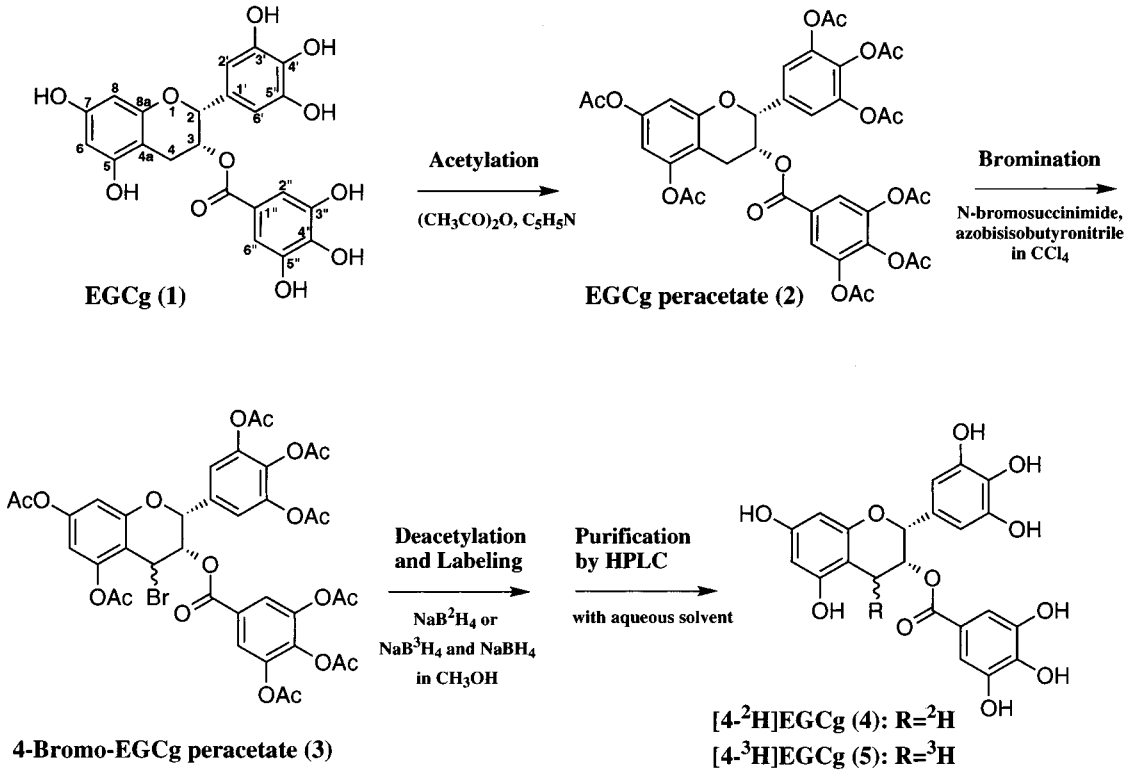

Figure 1. Synthetic route to [4-^2H]EGCg and [4-^3H]EGCg.

positive FABMS data of the product showed $[M+H]^+$ ion peak at *m/z* 459 and $[M+H]^+$ ion peak at *m/z* 461, suggesting that the product is a mixture of compound **1** and monotritiated EGCg. The ^{3}H NMR analysis of the product showed a signal only at δ 2.98. The chemical shift was superimposable on that of the signal (δ 2.98) derived from H-4b proton of compound **1**, which disappeared in the case of **4**. From these data, [4-^3H]EGCg (the compound **5**) was confirmed to be produced from **3**. The radiochemical purity of **5** was found to be 99.5% by HPLC analysis, and the specific activity was calculated to be 13 Ci/mmol by MS analysis. This is the first report on labeled EGCg in which deuterium or tritium is located at the 4 position.

Excretion of Radioactivity in Bile and Urine. [4-^3H]EGCg was administered intravenously to bile-duct-cannulated rats to clarify the excretion route of EGCg. The cumulative excretion of radioactivity in bile and urine, expressed as percentage of the dose, is illustrated in Figure 2. Only 2.0% of the dose was recovered in the urine over a period of 48 h. On the other hand, cumulative biliary excretion of radioactivity accounted for 77.0% of the dose in 48 h and rapid biliary excretion (56.9% of the dose) was observed within the first 4 h after intravenous dosing. These results showed that bile was the major excretion route for EGCg and the excretion ratio of bile to urine was about 97:3.

Pharmacokinetic Parameters of Radioactivity Derived from [4-^3H]EGCg in Blood. Figure 3 shows the time-course of the radioactivity in the blood after intravenous administration of [4-^3H]EGCg to bile-duct-cannulated rats. These time-course data were analyzed by the MULTI program (*19*). The best fit was achieved with a two-compartment mode, and apparent pharmacokinetic parameters for EGCg were then calculated. Distribution half-life ($T_{1/2\alpha}$), elimination half-life ($T_{1/2\beta}$), and distribution volume (V_d) were 1.04 h ± 0.6, 9.34 h ± 2.8. and 14.45 ± 9.2 dl/kg, respectively. The area

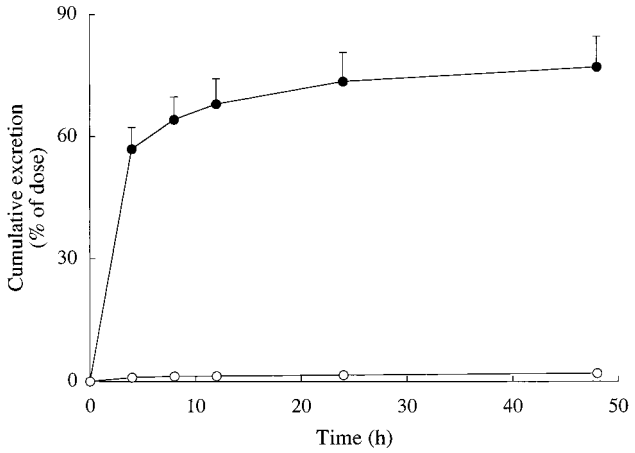

Figure 2. Cumulative excretion of radioactivity in bile (●) and urine (○) after intravenous administration of [4-^3H]EGCg to bile-duct-cannulated rats. The amount of excreted radioactivity was expressed as cumulative percentage of administered dose. Each point represents the mean ± SD of 6 rats.

under the curve ($AUC_{0-\infty}$) was estimated to be 2242.8 ± 1222 μg-equivalent·min/mL.

HPLC Analysis of Metabolites in Bile. In this study, to deconjugate the biliary metabolites, bile samples collected during the first 4 h after intravenous administration of [4-^3H]EGCg to rats were treated with glucuronidase/sulfatase mixture as described in Materials and Methods. Then, the metabolites formed were extracted with ethyl acetate and analyzed by HPLC equipped with radioisotope and UV detectors. Identification of the metabolites was performed by comparison with the retention time of six deconjugated biliary metabolites (EGCg, 4″-*O*-methyl-EGCg, 3″-*O*-methyl-EGCg, 3′-*O*-methyl-EGCg, 4′-*O*-methyl-EGCg, and 4′,4″-di-*O*-methyl-EGCg) identified in the rat bile collected after oral dose of EGCg in our previous study (*20*).

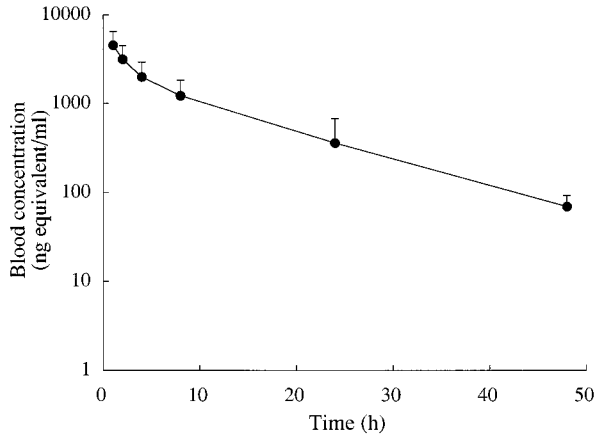

Figure 3. Blood concentration−time profiles of radioactivity after intravenous administration of [4-^3H]EGCg to bile-duct-cannulated rats. Nanogram equivalent of EGCg was calculated from the amount of radioactivity in blood. Each point represents the mean ± SD of 6 rats.

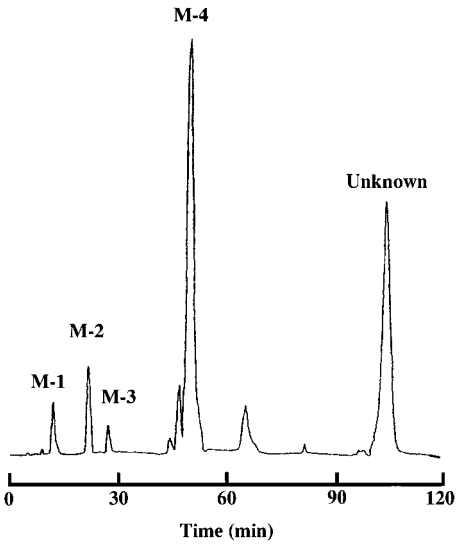

Figure 4. Representative HPLC profile of deconjugated forms of radioactive metabolites in rat bile sample. Prior to HPLC analysis bile samples were treated with β-glucuronidase/sulfatase mixture and the metabolites formed were extracted with ethyl acetate as described in Materials and Methods.

Figure 4 shows a representative HPLC profile of the deconjugated forms of radioactive metabolites in the bile. Four radioactive peaks of M-1, M-2, M-3, and M-4 were detected with a retention time of 13.2 , 22.5, 27.7, and 48.5 min, respectively. The peaks of M-1, M-2, M-3, and M-4 were identified as EGCg, 4″-*O*-methyl-EGCg, 4′-*O*-methyl-EGCg, and 4′,4″-di-*O*-methyl-EGCg, respectively; whereas 3′-*O*-methyl-EGCg and 3″-*O*-methyl-EGCg were not detected. The radioactivity in EGCg, 4″-*O*-methyl-EGCg, 4′-*O*-methyl-EGCg, and 4′,4″-di-*O*-methyl-EGCg accounted for 0.8, 1.5, 0.6, and 14.7% of administered radioactivity, respectively. Furthermore, a major unknown peak with the retention time of 100 min was detected, and the radioactivity accounted for 8% of the dose.

DISCUSSION

Radiolabeling technique is an invaluable tool for investigating metabolic fates of compounds in in vivo studies. Suganuma et al. (*17*) have reported that [^3H]-EGCg labeled with tritium gas was synthesized, and the positions labeled were estimated to be in the B ring and/or galloyl moiety. In general, it is likely that tritium labeled in aromatic rings will be susceptible to undergoing hydrogen exchange. With this concern in mind, in this study we developed chemical synthesis of [4-^3H]-EGCg with high radiochemical purity (99.5%) and specific activity (13 Ci/mmol). The radiochemical purity of [4-^3H]EGCg stored at −20 °C in ethanol solution was still 98.6% after three months. Further, after an oral dose of [4-^3H]EGCg to rats the urine sample excreted within 48 h was analyzed by HPLC, but no tritiated H_2O peak was observed (data not shown). Judging from these results, it is thought that the tritium at the 4 position of EGCg is not replaced by hydrogen from water in the body and there is no concern over the stability of the labeled EGCg.

In some previous studies on the metabolism of EGCg, it has been reported that part of the EGCg orally administered to rats or humans was detected in the plasma as EGCg or the glucuronide/sulfate conjugated forms thereof (*11*−*16*). On the other hand, neither EGCg nor its conjugates were detected in the urine (*13*, *14*). In light of these results it is supposed that EGCg absorbed into the body is excreted mainly in the bile. However, studies up until now have still been lacking in a clear explanation of the excretion route of EGCg. We attempted to define this point using [4-^3H]EGCg intravenously administered to bile-duct-cannulated rats. Results showed that most of the radioactivity derived from [4-^3H]EGCg was excreted in the bile, and the excretion ratio of bile to urine was 97:3. Millburn et al. (*21*) proposed that an important factor in facilitating the excretion of aromatic compounds into the bile was a molecular weight of more than 325 ± 50. Millburn et al. (*21*) and Bülles et al. (*22*) reported that facilitation of excretion of a compound into the bile was influenced not only by the original molecular weight of a compound, but also by an increase in the molecular weight and the polarity due to conjugation of a compound with glucuronic acid and sulfate. In the case of EGCg, not only does it have a molecular weight of 458, but it also can be found mostly in the forms of glucuronide and/or sulfate conjugates in the plasma (*14*) and bile (*20*). The present study confirmed that EGCg is mostly excreted in the bile, and hence EGCg is a representative compound which follows the biliary excretion rules proposed by Millburn et al. (*21*) and Bülles et al. (*22*).

In the present study we calculated the apparent pharmacokinetic parameters of EGCg on the basis of radioactivity (total amount of EGCg and its metabolites) in the blood after intravenous administration of [4-^3H]-EGCg. Previously, Chen et al. (*13*) had calculated the pharmacokinetic parameters of EGCg based on the amount of EGCg and its conjugates in the plasma. Results of the present study gave values of $T_{1/2\alpha}$ (1.04 h) and $T_{1/2\beta}$ (9.34 h) which were much larger than those ($T_{1/2\alpha}$ (11.8 min) and $T_{1/2\beta}$ (135.1 min)) reported by Chen et al. Moreover, although the dosage in this study was lower than that used by Chen et al., the value of *AUC* (2242.8 μg-equivalent·min/mL) was much larger than that (*AUC* (143.2 μg-equivalent·min/mL)) reported by Chen et al. A possible explanation for these differences may be that the parameters in the present study were calculated on the basis of the total amount of all metabolites, whereas Chen et al. calculated the parameters on the basis of only EGCg and its conjugates. Accordingly if metabolites with a slower elimination rate

are produced from EGCg and its conjugates in the blood, it could be expected that the values of half-life and *AUC* would increase. Piskula and Terao (*23*) reported that methylated (−)-epicatechin (EC) conjugates were metabolites with a slower elimination rate than EC and its conjugates. Thus, it seems likely that methylated EGCg conjugates were metabolites with a slower elimination rate and this may possibly explain the reason for the increase in values of half-life and *AUC*.

In our previous study (*20*) we found that after oral administration of EGCg, most EGCg metabolites in the bile were in glucuronide and/or sulfate forms. In addition, following treatment of the bile with β-glucuronidase/sulfatase, the deconjugated forms of the biliary metabolites were found to be EGCg, 3′-*O*-methyl-EGCg, 3″-*O*-methyl-EGCg, 4′-*O*-methyl-EGCg, 4″-*O*-methyl-EGCg, and 4′,4″-di-*O*-methyl-EGCg. In this study, using the above six EGCg metabolites as standards, we identified the biliary metabolites after intravenous administration of [4-^3H]EGCg to rats. Following are the biliary metabolites that we detected: 4′,4″-di-*O*-methyl-EGCg (14.7% of dose), 4″-*O*-methyl-EGCg (1.5% of dose), EGCg (0.8% of dose), and 4′-*O*-methyl-EGCg (0.6% of dose). In addition, a major unknown peak with a longer retention time (100 min) than that of 4′,4″-di-*O*-methyl-EGCg (48.5 min) was detected. The amount of the peak was estimated to be 8% of the dose. No information is available on this peak, but if it is a methyl-derivative of EGCg, judging from the retention time obtained from HPLC analysis it could be assumed to be trimethylated or further methylated EGCg. Furthermore, 3′-*O*-methyl-EGCg and 3″-*O*-methyl-EGCg were not observed in this study. The main biliary metabolite (in the deconjugated form) was EGCg in the previous study but was 4′,4″-di-*O*-methyl-EGCg in the present study. Thus, there are some differences in the metabolites between oral and intravenous administration. After intravenous administration, EGCg enters the liver directly. On the other hand, in the case of oral administration, since there is absorption process from the intestine, a large part of EGCg absorbed may first undergo glucuronidation (*23*), after which the EGCg conjugate enters the liver. Subsequently, intact EGCg and its conjugate may undergo further modification, in particular methylation in the liver. Although intact EGCg seems to be susceptible to methylation, the conjugated EGCg may be more resistant to methylation than intact EGCg, and this could be surmised to be the reason for the differences in the biliary metabolites according to the administration route.

In this study we developed the method of chemical synthesis of [4-^3H]EGCg which is resistant to hydrogen−tritium exchange. With this labeled EGCg we confirmed that the main excretion route of EGCg is by way of the bile. By comparison of apparent pharmacokinetic parameters of EGCg calculated in the present study with those calculated by Chen et al., it can be postulated that metabolites with a slower elimination rate than that of EGCg and its conjugates, were present in blood after intravenous administration of EGCg to rats. In addition, HPLC analysis of the bile showed that the main biliary metabolite of EGCg dosed intravenously was the conjugated form of 4′,4″-di-*O*-methyl-EGCg and it is suggested that the metabolites produced varied according to different administration routes (oral and intravenous).

ABBREVIATIONS USED

EGCg, (−)-epigallocatechin gallate; EDTA, ethylenediaminetetraacetic acid; TMS, tetramethylsilane; HR−FABMS, high-resolution fast atom bombardment mass spectrometry; $T_{1/2\alpha}$, distribution half-life; $T_{1/2\beta}$, elimination half-life; V_d, distribution volume; *AUC*, area under the blood concentration vs time curve.

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