Comparison of (−)-Epigallocatechin-3-O-gallate (EGCG) and O-Methyl EGCG Bioavailability in Rats

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Tea (Camellia sinensis L.) is consumed in many countries, and green tea consumption is particularly common in Asian countries. (−)-Epigallocatechin-3-O-gallate (EGCG), the major catechin present in green tea, has shown various bioactivities, such as anti-hypertensive,1,2 anti-allergenic,3 and anti-cancer effects.4 O-Methyl EGCGs, (−)-epigallocatechin-3-O-(3-O-methyl)gallate (EGCG3′Me) and (−)-epigallocatechin-3-O-(4-O-methyl)gallate (EGCG4′Me) (Fig. 1) were identified in some cultivars, such as Benifuuki, Benihomare, and Tung ting oolong tea.5,6 These tea extracts and purified O-methyl EGCGs prevented allergies in vivo and in vitro.5,7,8 Furthermore, our group found that Benifuuki tea extract had a stronger anti-obesity effect in mice receiving a high-fat diet than Yabukita tea extract did, which does not contain O-methyl EGCGs.9,10 A clinical trial revealed that Benifuuki tea also had stronger anti-hypertensive effects than those of Yabukita tea.11 Although EGCG3′Me and the teas containing it have strong bioactivities in vivo, the bioactivities of EGCG3′Me and Benifuuki tea extract in vitro were not stronger than those of EGCG or Yabukita tea extract.1,10 These findings suggested that the absorption of O-methyl EGCGs and Benifuuki tea extract may be related to their strong bioactivities in vivo. However, the pharmacokinetics of purified O-methyl EGCGs have not yet been examined. In this study, we elucidated the bioavailability and apparent distribution volumes of O-methyl EGCGs in rats, and compared the pharmacokinetics of EGCG and O-methyl EGCGs.

MATERIALS AND METHODS

Chemical Compounds and Reagents EGCG (TEAVIGO®; purity, ≥97%) was obtained from DSM Nu.
tritional Products (Basel, Switzerland). EGCG3’Me (purity, ≥98%) and EGCG4’Me (purity, ≥95%) were prepared by the method described by Yano et al.6 Briefly, a methanolic solution of EGCG was prepared and mixed with a diazomethane-ether solution. The mixture was maintained at −20°C for 0.5–4 h. After neutralization by addition of acetic acid, the methanolic solvent was removed in vacuo. Methyl catechins were separated using a JASCO LC 800 series HPLC system equipped with a TSKgel ODS-80TS column (Tosoh Biosciences, Tokyo, Japan). O-Methyl EGCGs were eluted with an isocratic gradient of 15% aqueous acetonitrile at a flow rate of 45 mL/min. The structures of methyl catechins were identified by 1H- and 13C-NMR (Bruker Avance 500 MHz NMR spectrometer, Bruker, Tokyo, Japan). Chemical shifts are given in δ (ppm), with tetramethylenesilane as an internal standard (s=singlet, d=doublet, dd=double doublet, and m=multiplet). Chemical shifts were presented as follows: EGCG3’Me, 1H-NMR (500 MHz, CD3OD) δ: 2.87 (1H, dd, J=17.3, 2.5), 2.99 (1H, dd, J=17.3, 4.4), 3.80 (3H, s), 4.99 (1H, brs), 5.49 (1H, m), 7.06 (1H, br, J=2.2), 5.97 (1H, brd, J=2.2), 6.51 (2H, brs), 7.01 (1H, brd, J=1.9), 7.06 (1H, br, J=1.9). 13C-NMR (125 MHz, CD3OD) δ: 26.8, 60.7, 70.3, 121.5, 130.9, 133.7, 140.6, 145.0, 146.8, 147.9, 157.1, 157.8, 157.9, 167.6. EGCG4’Me, 1H-NMR (500 MHz, CD3OD) δ: 2.84 (1H, dd, J=17.4, 2.5), 2.98 (1H, dd, J=17.4, 4.4), 3.81 (3H, s), 4.97 (1H, brs), 5.53 (1H, m), 5.95 (2H, brs), 6.49 (2H, brs), 6.91 (2H, brs). 13C-NMR (125 MHz, CD3OD) δ: 26.8, 60.7, 70.3, 78.5, 95.9, 96.5, 99.3, 106.8, 106.7, 111.9, 121.5, 130.9, 133.7, 140.6, 146.0, 147.9, 157.1, 157.8, 157.9, 167.1. Chemical shifts of O-methyl EGCGs corresponded to those described in a previous study by Aihara et al.2β-D-Glucuronidase from Escherichia coli (G-7896, EC 3.2.1.31) and sulfatase from abalone entrails (S-9754, EC 3.1.6.1) were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Other reagents and solvents were of the highest grade of commercially available materials.

**Experimental Designs** All animal experiments were approved by the Animal Experimental Committee of Morinaga & Company, Ltd., and were performed in accordance with the Guidelines for Proper Conduct of Animal Experiments of the Science Council of Japan. All efforts were made to minimize the number of animals used and their suffering. Male Sprague-Dawley rats (7 weeks old) were obtained from Japan SLC, Inc. The rats had free access to food (AIN 93G: Oriental Yeast, Tokyo, Japan) and water. The rats were maintained at a temperature of 23±2°C under a 12-h light/dark cycle. Food was withheld from the rats 16 h prior to their use in the experiment. The right jugular veins of the rats were cannulated for blood sampling and intravenous administration under anesthesia. The cannulated rats were maintained for an additional 1 week during recovery.

The dose of each catechin in the current study was based on previous investigations of EGCG.13,14 Briefly, in the oral administration group, EGCG, EGCG3’Me, and EGCG4’Me were dissolved in 1 mL HCl/1 mL ascorbic acid/10% polyethylene glycol prior to oral administration at a dose of 100 mg/kg. Blood samples were withdrawn through the canula at 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 6, 8, and 24 h after administration. In the intravenous administration group, each catechin was dissolved in 1 mL ascorbic acid/10% polyethylene glycol in saline prior to intravenous administration at a dose of 10 mg/kg.

Blood samples were withdrawn through the canula at 2 min, and 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 6, 8, and 24 h after administration. The samples were collected in heparinized tubes and centrifuged (5000 rpm, 10 min, 4°C). Plasma samples were transferred to new tubes, and 1/10 volume of preservative solution (4% ascorbic acid/0.02% ethylenediaminetetraacetic acid (EDTA) /80 mM phosphate buffer, pH 3.6) was added to them. The plasma samples were stored at −30°C until use.

**Plasma Preparation** The plasma samples were hydrolyzed by β-D-glucuronidase and sulfatase according to the method described by Maeda-Yamamoto et al., with slight modification.15 Briefly, 50 μL of each plasma sample was mixed with 20 μL of 1% ascorbic acid/0.005% EDTA/0.2 mM phosphate buffer (pH 6.5), 5 μL of β-D-glucuronidase (15.6 Units), 5 μL of sulfatase (1.25 Units), and 20 μL of water, and subsequently incubated at 37°C for 45 min.

Catechins were extracted by solid-phase extraction from the plasma samples before and after hydrolysis by β-D-glucuronidase and sulfatase. Plasma samples were applied to 30 mg Strata-X™ 33u polymeric reversed phase cartridges (Phenomenex Inc., California, U.S.A.) after conditioning with 1 mL of methanol and 1 mL of water. The cartridges were washed with 1 mL of water and 1 mL of 5% methanol. One milliliter of ethyl acetate was added to each cartridge to remove the lipophilic compounds. The catechins were eluted with 5 mL of methanol–acetonitrile (50:50, v/v) at 35°C. After the addition of 10 μL of 0.01% ascorbic acid/0.05% EDTA, the eluate was dried in vacuo. The residues were dissolved in the mobile phase. For determination of recovery from plasma, the control plasma was spiked with each catechin. The catechins were extracted by solid-phase extraction as described above. The recovery of each catechin was 88.9% (EGCG), 94.0% (EGCG3’Me), and 102.6% (EGCG4’Me). The plasma samples before and after hydrolysis were used for quantification of the intact and the total catechin concentrations, respectively. The intact catechin was defined as the unmetabolized catechin in plasma, and the total catechin concentration represented the sum of the intact and the conjugated catechin concentration. The concentration of conjugated catechin, which was the sum of glucuronidated and sulfated catechins, was calculated by subtraction of the intact catechin concentration from the total catechin concentration.

**HPLC Analysis** The plasma concentrations of catechins were determined using the method described by Sano et al.16 Briefly, an HPLC system with an electrochemical detector (ECD) (NANOSPACE SI-2: Shiseido, Tokyo, Japan) was equipped with a Capcell pak C18 MGII (5 μm, 2 mm i.d.×150 mm; Shiseido, Tokyo, Japan). Aliquots of samples (20 μL) were applied to the HPLC–ECD system, and eluted with an isocratic mobile phase of acetonitrile–0.1% phosphoric acid (12:88, v/v) containing 0.1 mM EDTA, at the flow rate of 0.3 mL/min. The column temperature was maintained at 40°C. The eluent was monitored by the ECD at the applied potential of 600 mV.

**Pharmacokinetic Analysis and Statistical Analysis** The area under the concentration–time curve (AUC) was calculated using the linear trapezoidal rule. As described in previous studies on the absorption of EGCG,13,14 the pharmacokinetic parameters were calculated using a two-compartment model for intravenous administration of EGCG and O-methyl EGCG by the method of residuals. Each pharmacokinetic parameter...
was calculated as follows:

$$C = A \cdot e^{-\alpha t} + B \cdot e^{-\beta t} \tag{1}$$

where $K_{12} = \frac{A}{(A+B)(A+\alpha+B)}$, $K_{21} = \frac{A}{(A+B)}$, $K_{10} = \frac{\alpha}{(A+\beta+B)}$, $V_d = \frac{D}{(A+B)}$, $C$, concentration; $t$, time; $\alpha$, rate constant associated with the distribution phase of the concentration–time curve; $\beta$, rate constant associated with the elimination phase of the concentration–time curve; $K_{12}$, rate constant for distribution from the central compartment to the peripheral compartment; $K_{21}$, rate constant for distribution from the peripheral compartment to the central compartment; $K_{10}$, rate constant for elimination from the central compartment; $V_d$, apparent distribution volume of central compartment; $D_{po}$, dose of intravenous administration; and $C_{po}$, speculated concentration at 0h.

The rate constant associated with the distribution phase ($\alpha$) and the distribution phase ($\beta$) were calculated from the first 3 time points or the last 3 time points. Bioavailability was calculated as follows:

$$\text{Bioavailability } (%) = \left(\frac{AUC_{po}}{D_{po}} / \frac{AUC_{iv}}{D_{iv}}\right) \times 100 \tag{2}$$

where $AUC_{po}$, AUC after oral administration; $AUC_{iv}$, AUC after intravenous administration; and $D_{po}$, dose of oral administration.

Statistical analyses were performed using SPSS (Statistical Package for the Social Sciences) 13.0J for Windows (SPSS Inc., Tokyo, Japan). The differences between the pharmacokinetic parameters of each group were assessed using Tukey–Kramer’s multiple comparison tests.

RESULTS

**Plasma Chromatograms after Administration of O-Methyl EGCGs**

Figure 2 shows the HPLC chromatograms of pure EGCG and O-methyl EGCGs (A), the plasma before oral administration (B), and the plasma after oral administration of O-methyl EGCGs (C, D). Each catechin was individually detected at 7.6 min (EGCG), 13.9 min (EGCG4 Me), and 15.0 min (EGCG3 Me) with the HPLC–ECD system (Fig. 2A). No noticeable peak was detected in the plasma before administration (Fig. 2B). Following oral administration of O-methyl EGCGs, each intact catechin was detected as a distinct peak and several hydrophilic peaks were detected earlier than those of O-methyl EGCGs (Figs. 2C, D). These hydrophilic peaks disappeared after plasma treatment with $\beta$-glucuronidase and sulfatase, accompanied by increase in the peaks for each O-methyl EGCG. The chromatograms of the plasma after intravenous administration of each catechin were similar to those after oral administration (data not shown).

**Plasma Concentration Profiles of EGCG and O-Methyl EGCGs after Oral Administration**

Figure 3 shows the plasma concentration profiles of each catechin after oral administration. EGCG3 Me reached the maximal concentration ($C_{max}$) at 0.5h, and then decreased in a time-dependent manner (Fig. 3A). EGCG3 Me maintained a higher concentration than those of EGCG and EGCG4 Me up to 8h. All catechins were undetectable at 24h. As shown in Table 1, the $AUC_{po}$ of EGCG3 Me was significantly higher than those of EGCG ($p<0.01$) and EGCG4 Me ($p<0.01$).

The concentration of conjugated EGCG3 Me was higher than those of conjugated EGCG and EGCG4 Me (Fig. 3B) as well as in the intact form. The $AUC_{po}$ of conjugated EGCG3 Me was also higher than those of EGCG and EGCG4 Me (Table 1). Following oral administration of EGCG, a small amount of conjugated EGCG4 Me was detected in plasma, and conjugated EGCG was also detected in trace amounts after oral administration of EGCG4 Me. However, metabolism from EGCG3 Me to the other catechins was undetectable in plasma at the dose of 100 mg/kg.

**Plasma Concentration Profiles after Intravenous Administration and Bioavailabilities of EGCG and O-Methyl EGCGs**

Figure 4 shows the plasma concentration profiles of each catechin after intravenous administration, and Table 2 summarizes the pharmacokinetic parameters after intravenous administration calculated by using Eq. 1. The $C_{po}$ of EGCG3 Me was significantly higher than those of EGCG and EGCG4 Me. Similar to EGCG and EGCG4 Me, EGCG3 Me was rapidly eliminated from plasma, and all catechins were undetectable at 24h. The half-lives of catechins ($T_{1/2,α}$, $T_{1/2,β}$ and $T_{1/2,iv}$) were not significantly different from each other (Table 2). The $V_d$ of EGCG3 Me was calculated to be 0.26 L/kg, and it was considerably lower than those of EGCG ($p<0.01$) and EGCG4 Me ($p<0.01$). Furthermore, the $AUC_{po}$ of EGCG3 Me was significantly greater than those of EGCG ($p<0.01$) and EGCG4 Me ($p<0.01$), whereas the $AUC_{po}$ of conjugated EGCG3 Me was not different from those of conjugated EGCG and EGCG4 Me (Table 2).

The bioavailability of each catechin was calculated to be 0.14% (EGCG), 0.38% (EGCG3 Me), and 0.21% (EGCG4 Me) by using Eq. 2 (Table 2). The bioavailability of EGCG3 Me was 2.7-fold and 1.8-fold higher than that of EGCG and EGCG4 Me, respectively.

**DISCUSSION**

In the current study, we determined the bioavailabilities of O-methyl EGCGs and compared the pharmacokinetic parameters of EGCG and O-methyl EGCGs in rats (Table 2). The bioavailability of EGCG was similar to that previously reported values, and the bioavailability, $AUC_{po}$ and $AUC_{iv}$ of EGCG4 Me were similar to the corresponding values for EGCG (Tables 1, 2). However, the bioavailability, $AUC_{po}$ and $AUC_{iv}$ of EGCG3 Me were higher than the corresponding values for EGCG. In addition, our preliminary study on the dose dependency of $AUC$s for each catechin after oral administration (30, 100, and 300 mg/kg) and intravenous administration (3, 10, and 30 mg/kg) also supported the higher $AUC_{po}$ and $AUC_{iv}$ of EGCG3 Me at these doses (data not shown). Maeda-Yamamoto suggested that EGCG3 Me showed higher absorption than EGCG after oral administration of Benifuuki tea extract in rats and humans. In previous studies, overestimation of the $AUC_{po}$ of EGCG3 Me and other catechins after administration of tea extract could have occurred, because of metabolism from EGCG to O-methyl EGCGs, in rats. In addition, the elimination of EGCG from plasma was reported to be affected by other tea extract catechins. However, in the current study, we clearly demonstrated that EGCG3 Me was more effectively absorbed into the circulating blood than either EGCG or EGCG4 Me.

In the intravenous administration study, the $AUC_{iv}$ of intact EGCG3 Me was about 3-fold higher than those of EGCG and EGCG4 Me (Table 2), although the $T_{1/2}$ of each phase was...
not significantly different among the three catechins. These results might suggest that the elimination phase makes a minor contribution to the higher $AUC_{iv}$ of EGCG3\textsuperscript{Me}. On the other hand, the $V_d$ of EGCG3\textsuperscript{Me} was about one-third of those of EGCG and EGCG4\textsuperscript{Me}. The lower $V_d$ of EGCG3\textsuperscript{Me} suggested that more EGCG3\textsuperscript{Me} was retained in circulating blood than EGCG and EGCG4\textsuperscript{Me}. This characteristic of EGCG3\textsuperscript{Me} may contribute to its higher $AUC_{po}$ and $AUC_{iv}$.

In flavonoids, the methylation of a hydroxyl group generally increases hydrophobicity, and leads to improved absorption.\textsuperscript{20) However, in this study, EGCG3\textsuperscript{Me} had the highest $AUC_{po}$ and bioavailability. In addition, the $V_d$ of EGCG3\textsuperscript{Me} was significantly different from those of the other catechins. These findings suggested that methylation of the 3\textsuperscript{rd} position of the hydroxyl group, but not of the 4\textsuperscript{th} position, was important for modification of bioavailability and distribution (Fig. 1). There are several possible mechanisms related to this pharmacokinetic difference between the three catechins, including their binding affinities to organs or plasma and the pathways involved in their metabolism. Yano showed that the specific binding capacity of EGCG3\textsuperscript{Me} with cellular surfaces was not as strong as that of EGCG, but was similar to that of EGCG4\textsuperscript{Me}.\textsuperscript{11) In another study, Ishii indicated that the affinity of EGCG3\textsuperscript{Me} for human albumin, which is a major plasma protein related to the stability and the distribution of various compounds, was also weaker than that of EGCG, and the same as that of EGCG4\textsuperscript{Me}.\textsuperscript{21) These previous studies could not explain the pharmacokinetic difference between EGCG3\textsuperscript{Me} and EGCG4\textsuperscript{Me} sufficiently. Thus, the underlying reasons of the difference are not yet elucidated, and further investigations are necessary to clarify these reasons.

After oral administration, the conjugation ratios of EGCG, EGCG3\textsuperscript{Me}, and EGCG4\textsuperscript{Me} ($AUC_{po,\text{conjugated}}/AUC_{po,\text{intact}}$) were calculated to be 7.10, 2.17, and 2.65, respectively. These ratios indicated that most of the plasma EGCG and 0-methyl EGCGs were glucuronidated or sulfated (Table 1). In contrast, after intravenous administration, the conjugation ratios of EGCG, EGCG3\textsuperscript{Me}, and EGCG4\textsuperscript{Me} ($AUC_{iv,\text{conjugated}}/AUC_{iv,\text{intact}}$) were 0.18, 0.10, and 0.20, respectively, indicating that most of the EGCG and 0-methyl EGCGs were intact (Table 2). The
Fig. 3. Plasma Concentration Profiles of Catechins after Oral Administration (100 mg/kg) to Rats

The values are the means ± S.E.M. (n = 5–6). Intact catechin (A) and conjugated catechin (B); EGCG, open circles; EGCG3Me, closed circles; and EGCG4Me, open triangles.

Fig. 4. Plasma Concentration Profiles of Catechins after Intravenous Administration (10 mg/kg) to Rats

The values are the means ± S.E.M. (n = 5–6). Intact catechin (A) and conjugated catechin (B); EGCG, open circles; EGCG3Me, closed circles; and EGCG4Me, open triangles.

Table 1. Pharmacokinetic Parameters after Oral Administration

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Unit</th>
<th>EGCG</th>
<th>EGCG3Me</th>
<th>EGCG4Me</th>
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<tr>
<td>Dose</td>
<td>mg/kg</td>
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<td>100</td>
<td>100</td>
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<tr>
<td>AUCpo</td>
<td>Intact</td>
<td>µg·h/L</td>
<td>39.6±14.2*</td>
<td>317.2±43.7†</td>
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<td></td>
<td>Conjugated</td>
<td>µg·h/L</td>
<td>281.3±76.7*</td>
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<td>Cmax</td>
<td>Intact</td>
<td>µg/L</td>
<td>11.0±5.9*</td>
<td>86.7±14.9†</td>
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<tr>
<td></td>
<td>Conjugated</td>
<td>µg/L</td>
<td>47.5±8.8*</td>
<td>149.4±29.9†</td>
</tr>
</tbody>
</table>

a) The values are the means ± S.E.M. (n = 5–6). *p < 0.01 vs. EGCG3Me; †p < 0.01 vs. EGCG4Me, Tukey–Kramer’s multiple comparison test.

Table 2. Pharmacokinetic parameters after intravenous administration

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Unit</th>
<th>EGCG</th>
<th>EGCG3Me</th>
<th>EGCG4Me</th>
</tr>
</thead>
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<tr>
<td>Dose</td>
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<td>10</td>
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<tr>
<td>AUCiv</td>
<td>Intact</td>
<td>µg·h/L</td>
<td>2772.2±479.9*</td>
<td>8209.4±549.2†</td>
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<tr>
<td></td>
<td>Conjugated</td>
<td>µg·h/L</td>
<td>505.0±139.1</td>
<td>785.2±260.0</td>
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<tr>
<td>C0</td>
<td>µg/L</td>
<td>12269.5±2131.8*</td>
<td>39603.4±4403.5†</td>
<td>11257.1±1285.6</td>
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<td>T1/2 α</td>
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<td>0.12±0.00</td>
<td>0.11±0.01</td>
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<tr>
<td>T1/2 β</td>
<td>h</td>
<td>2.68±0.40</td>
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<tr>
<td>T1/2 β</td>
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<tr>
<td>Vd</td>
<td>L/kg</td>
<td>0.94±0.16*</td>
<td>0.26±0.02†</td>
<td>0.93±0.14</td>
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<td>Bioavailability</td>
<td>%</td>
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<td>0.38</td>
<td>0.21</td>
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</table>

a) The values are the means ± S.E.M. (n = 5–6). *p < 0.01 vs. EGCG3Me; †p < 0.01 vs. EGCG4Me, Tukey–Kramer’s multiple comparison test.
higher conjugation ratios in the oral administration study suggested that the conjugated EGCG and O-methyl EGCGs in plasma predominantly came from the intestinal mucosa.

According to previous reports about the conjugation site, EGCG was glucuronidated at the 7-, 3′-, 3″-, and 4′-positions in rat liver microsomes, and at the 4′-position in mice intestinal microsomes. Furthermore, EGCG4′Me was poorly glucuronidated by several types of human UDP-glucuronosyltransferase expressed in the intestinal mucosa, because the glucuronidation site was occupied by a methyl group. In the current study, the conjugation ratios of EGCG3′-Me (2.17) and EGCG4′Me (2.65) after oral administration were not as high as that of EGCG (7.10). These results suggested that O-methyl EGCGs may avoid conjugation because of hydroxyl group inactivation and steric hindrance by methylation.

In summary, we clarified that the AUC of EGCG3′Me was higher than the corresponding values for EGCG and EGCG4′Me, indicating that methylation at the 3′-position in the D-ring greatly modified the pharmacokinetics of catechins. Furthermore, the higher bioavailability and the lower Vd led to a higher AUC of EGCG3′Me. These findings explained the difference between bioactivities observed in vivo and in vitro. The higher AUC of EGCG3′Me may have contributed to the bioactivity of EGCG3′Me and teas containing it in vivo.

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