4′″-Sulfation Is the Major Metabolic Pathway of Epigallocatechin-3-gallate in Humans: Characterization of Metabolites, Enzymatic Analysis, and Pharmacokinetic Profiling

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ABSTRACT: Epigallocatechin-3-gallate (EGCG), a major green tea polyphenol, has beneficial effects on human health. This study aimed to elucidate the detailed EGCG sulfation process to better understand its phase II metabolism, a process required to maximize its health benefits. Results show that kinetic activity of sulfation in the human liver and intestinal cytosol is 2-fold and 60- to 300-fold higher than that of methylation and glucuronidation, respectively, suggesting sulfation as the key metabolic pathway. Moreover, SULT1A1 and SULT1A3 are responsible for sulfation in the liver and intestine, respectively. Additionally, our human ingestion study revealed that the concentration of EGCG-4″-sulfate in human plasma (C\text{max}: 177.9 nmol-L\textsuperscript{-1}, AUC: 715.2 nmol-h-L\textsuperscript{-1}) is equivalent to free EGCG (C\text{max}: 233.5 nmol-L\textsuperscript{-1}, AUC: 664.1 nmol-h-L\textsuperscript{-1}), suggesting that EGCG-4″-sulfate is the key metabolite. These findings indicate that sulfation is a crucial factor for improving EGCG bioavailability, while also advancing the understanding of the bioactivity and toxicity of EGCG.

KEYWORDS: metabolism, pharmacokinetics, bioavailability, epigallocatechin-3-gallate, sulfation

INTRODUCTION

Green tea consumption has beneficial effects on human health. These benefits are attributed to tea catechins: epigallocatechin-3-gallate (EGCG), epigallocatechin (EGC), epicatechin-3-gallate (ECG), and epicatechin (EC). Among these catechin analogs, EGCG (Figure 1), which comprises more than 50% of the total catechins, has the highest antioxidant activity, while also serving to alleviate metabolic syndrome\textsuperscript{2,3} and protect against cancer.\textsuperscript{4,5} Hence, EGCG may have potential applications in functional foods and pharmaceuticals. However, although numerous in vivo and in vitro studies have been conducted to clarify the effects and molecular mechanisms of EGCG,\textsuperscript{6–8} its extensive metabolism and poor bioavailability make it difficult to translate the findings to humans.\textsuperscript{9} Thus, understanding the metabolism of EGCG in humans is required to adapt EGCG for medicine and functional foods efficiently.

Previous human and animal studies have indicated that following ingestion, EGCG undergoes phase II metabolism in enterocytes and hepatocytes.\textsuperscript{10} Given that multiple phase II conjugation reactions participate in EGCG metabolism, it is important to determine the metabolic pathway(s) and isozymes that exert the greatest impact on EGCG bioavailability and bioefficacy.\textsuperscript{11} Among the various types of conjugation, previous studies have provided a detailed understanding of methylation and glucuronidation. Regarding methylation, several in vitro studies have reported that catechol-0-methyltransferase (COMT) catalyzes the formation of 4″-methyl-EGCG and 4″,4‴-dimethyl-EGCG in the liver.\textsuperscript{12,13} Meanwhile, pharmacokinetic studies have detected these methylated forms of EGCG in rat plasma and urine, mouse urine, and human plasma.\textsuperscript{14–17} While the kinetic activity of human COMT is reportedly higher than that of mouse and rat COMT, the differences between them are not remarkable (1.16- to 1.17-fold).\textsuperscript{14} Moreover, EGCG glucuronidation is catalyzed by UDP-glucuronosyltransferase (UGT)-1A1, 1A8, and 1A9 in humans and occurs at the 4″-, 3‴-, or 3′-hydroxy moiety in humans, whereas the 7′-hydroxy moiety is also glucuronidated in rats.\textsuperscript{18} Considering that the in vitro catalytic activity level of 4″- and 3′-glucuronidation in the liver decreases in the following order: mouse > human > rat, it is believed that species differences also exist in vivo. Indeed, EGCG-glucuronide is detectable within

Figure 1. Chemical structure of EGCG.
mouse and rat plasma;\textsuperscript{19,20} however, it is not directly detected in human plasma.

Moreover, since sulfated conjugates are detected in rat bile and urine, as well as in human ial fluids, plasma,\textsuperscript{15,21} and cellular metabolites,\textsuperscript{22} sulfation catalyzed by sulfotransferase (SULT) is considered another important EGCG metabolic pathway. Sulfation is not only a critical factor for improving EGCG bioavailability and bioefficacy but also for understanding potential species differences between humans and experimental animals.\textsuperscript{19,21} However, the details associated with EGCG sulfation have not yet been elucidated despite its importance. This is partially due to the lack of an authentic standard for EGCG-sulfate, which impedes the accurate analysis of sulfate conjugations. Although previous data for EGCG-sulfate formation have been calculated based on analysis of β-glucuronidase- and sulfatase-treated samples,\textsuperscript{15,25} a previous report suggested that certain sulfated polyphenols are highly resistant to sulfatase hydrolysis, indicating that quantification based on hydrolysis may not be accurate.\textsuperscript{26} Consequently, the kinetic activity of sulfation, accurate plasma concentration of EGCG-sulfate, and sulfotransferase isozymes responsible for EGCG sulfation have not been identified, thus preventing a comprehensive understanding of EGCG metabolism.

Accordingly, the primary aim of this study was to elucidate the details of EGCG sulfation to better understand the phase II metabolism. In particular, we used a recently developed and chemically synthesized EGCG-sulfate standard to provide accurate quantification of EGCG-sulfates in vitro and human samples.\textsuperscript{27} To verify the contribution of sulfation to the first pass effects and EGCG clearance, we quantified the kinetic activity of EGCG sulfation using human liver or intestinal cytosol and compared it to other metabolic pathways. In addition, to facilitate a discussion on species differences and bioavailability at the molecular level, we determined the specific isozymes responsible for EGCG sulfation in humans by using recombinant SULTs. These in vitro studies were further confirmed by qualitative analysis of human plasma metabolites and quantification of EGCG-sulfate.

\section*{MATERIALS AND METHODS}

\textbf{Chemicals, Reagents, and Enzyme Source.} EGCG and EGCG-4′-methyl standard was obtained from Nagara-Science (Gifu, Japan). EGCG-4′-sulfate, EGCG-3′-sulfate, EGCG-3′-glucuronide, and EGCG-4′-glucuronide standards (purity >94\%) were synthesized as previously reported.\textsuperscript{19,23} Pooled human liver and intestinal cytosols and microsomes from 15–50 donors of either sex were obtained from Xenotech (Kansas City, KS, USA). Recombinant human sulfotransferases (SULTs; SULT1A1, SULT1A3, SULT1B1, SULT1E1, and SULT2A1) were obtained from R&D Systems, Inc. (Minneapolis, MN, USA). EGCG-sulfate extracted from metabolites of human SULT expressed in yeast cytosol was purchased from TOPU-BIO Research Co., Ltd. (Toyama, Japan).

\textbf{EGCG Sulfation by Human Cytosolic Fractions or Recombinant Human SULTs.} The reaction mixture consisted of cytosolic or recombinant protein, 100 mM potassium phosphate buffer (pH 7.4), 10 mM magnesium chloride, 0.15 mM L(+)-ascorbic acid, and various concentrations of EGCG in a final volume of 50 μL. The cytosolic proteins and recombinant proteins (SULT1A1, SULT1A3, SULT1B1, SULT1E1, and SULT2A1) were used at 0.1 mg/mL, 0.5, 2, 8, and 2 μg/mL, respectively. These protein concentrations were determined by preliminary experiments to check the linearity of metabolism. The reaction was initiated by adding 10X concentration of 3′-phosphoadenosine-5′-phosphosulfate (PAPS, final concentration: 0.15 mM), and the samples were incubated at 37 °C for 10 min. The reaction was terminated by adding 50 μL of ice-cold acetone containing 0.15 mM L(+)-ascorbic acid. Samples were centrifuged at 15,000×g for 10 min at 4 °C, and 50 μL of the supernatant was used for analysis; 0.065–250 μM EGCG gradient was used for human liver cytosol (HLC), 0.0262–100 μM for human intestinal cytosol, 0.025–6.4 μM for SULT1A1, and 0.164–40 μM for SULT1A3, SULT1B1, SULT1E1, and SULT2A1. The maximum and minimum concentrations of EGCG were determined using the following criteria: a minimum of two points for concentrations lower than K<sub>e</sub> and two points for concentrations in a plateau. Moreover, with respect to SULT1A1 and the liver cytosolic fraction, a minimum of two points was examined at the concentration at which substrate inhibition occurred.

\textbf{Methylation of EGCG by Human Cytosolic Fractions.} The reaction mixture consisted of 0.1 mg/mL cytosolic proteins, different concentrations of EGCG (0.065–16 μM), 1 mg/mL S-adenosyl methionine (SAM), 0.15 mM ascorbic acid, 10 mM magnesium chloride, and 100 mM KPI buffer (pH 7.4) in a final volume of 50 μL. The reaction mixtures were incubated at 37 °C for 10 min, and the reaction was stopped by adding 50 μL of acetonitrile containing 0.15 mM ascorbic acid. Samples were centrifuged at 15,000×g for 10 min, and 50 μL of the supernatant was used for analysis.

\textbf{Glucuronidation of EGCG by Human Microsomal Fractions.} The reaction mixture consisted of 0.5 mg/mL microsomal proteins, different concentrations of EGCG (0.409–250 μM), 1 mM uridine 5′-diphosphoglucuronic acid (UDPGA), 0.15 mM ascorbic acid, 10 mM magnesium chloride, and 100 mM KPI buffer (pH 6.8) in a final volume of 50 μL. The reaction mixtures were incubated at 37 °C for 10 min, and the reaction was stopped by 50 μL of acetonitrile containing 0.15 mM ascorbic acid. Samples were centrifuged at 15,000×g for 10 min, and 50 μL of the supernatant was used for analysis.

\textbf{Human Testing.} This study was conducted in accordance with the ethical guidelines for clinical research and ethical principles based on the Declaration of Helsinki. The study protocol was approved (UMIN-CTR ID No. UMIN00033192) by the local ethics committee (Human Ethics Committee at Kao, registration number T139-180531), and informed consent was obtained from all volunteers. Ten healthy male volunteers participated in this study (mean ± SEM age: 33.7 ± 2.7, body height: 171.1 ± 2.3 cm, and body weight: 60.1 ± 2.1 kg). Volunteers were instructed to refrain from ingesting catechin analogs and any food or drink, except water, from 1 day or evening before the study day, respectively. On the first study day, volunteers ingested 350 mL of a beverage containing 615 mg of green tea catechins (135 mg of EGCG, 127 mg of EGC, 45 mg of ECG, 38 mg of GC, 97 mg of GCG, 120 mg of GC, 22 mg of CG, and 33 mg of C). Venous blood samples were collected 0.5, 1, 1.5, 2, 2.5, and 6 h after ingestion, and the plasma was separated by centrifugation at 2130×g for 10 min. Samples collected the blood samples and checked the physical condition of the participants. Phosphate buffer (0.4 M, pH 3.6) with 20% (w/v) L(+)-ascorbic acid and 0.1% (w/v) EDTA disodium (EDTA 2Na) was added to the plasma with a 10% plasma volume prior to storage at −80 °C.

\textbf{Plasma Extraction.} A solid-phase extraction procedure was performed using an Oasis HLB column (1 cc, 10 mg, Oasis, Milford, MA, USA) to isolate EGCG and its metabolites. Plasma samples (200 μL) were placed in 2 mL microtubes and diluted to 1000 μL using 0.2 M acetic acid containing 0.015 M phosphate buffer. For quantification, 100 μL of 40 mg/mL ethyl gallate was added as an internal standard. The HLB column was activated with 1 mL of DMF containing 0.1% acetic acid and 1 mL of water (1 min, vacuum suction). After loading the sample onto the column, 1 mL of water containing 30% methanol was used to wash the column. EGCG and its metabolites were eluted with 100 μL of DMF containing 0.1% acetic acid and analyzed by liquid chromatography–tandem mass spectrometry (LC–MS/MS). The rate of recovery of this method was 90.6, 87.3, and 88.4% for EGCG, EGCG-4′-sulfate, and EGCG-4′-glucuronide, respectively.

\textbf{1H NMR Analysis.} 1H NMR spectra were obtained on Advance III 600 (Bruker, Ettlingen, Germany) with a cryoprobe. EGCG and EGCG-sulfate extracted from metabolites of human SULT were dissolved in acetonitrile-d₄ (99.8% atom% D contains 0.03% (v/v) TMS, Sigma-Aldrich, St. Louis, MO, USA).
Quantification in In Vitro Samples by Liquid Chromatography–Mass Spectrometry (LC–MS). EGCG metabolites were analyzed using an LC–MS2020 (Shimadzu, Kyoto, Japan) operating in the selected ion monitoring (SIM) mode. Separations were performed using Poroshell 120 EC-C18 (2.7 μm, 4.6 mm × 50 mm, Agilent) coupled with a Poroshell 120 EC-C18 guard column (2.7 μm, 4.6 mm × 5 mm, Agilent), maintained at 40 °C in a column oven. Mobile phases (0.4 mL/min) comprised solution A (0.1% formic acid) and solution B (acetonitrile containing 0.1% formic acid). Injections were carried out at 2 μL using an autosampler maintained at 4 °C. The gradient program was: 0.0–0.5 min: 10–12% B; 0.5–2.5 min: 12% B; 2.5–4.5 min: 12–14% B; 4.5–7.5 min: 14% B; 7.5–10.5 min: 14–15% B; 10.5–11.5 min: 15–50% B; 11.5–13.0 min: 50% B; 13.0–13.5 min: 50–10% B; and 13.5–15.0 min: 10% B. Each metabolite was quantified by peak area measurements in comparison with a standard curve of the authentic standard. The range of the calibration curve was 0.065–6.4 μM.

Metabolite Identification in In Vitro and Plasma Samples and Quantification of Plasma EGCG and Its Metabolites. EGCG and its metabolites in human plasma were detected using a Vanquish UPLC with a Q-Exactive Focus Orbitrap mass spectrometer (Thermo Fisher Scientific, Walhama, MA, USA) in full-scan MS mode (150–1000 m/z). Resolution and automated gain control (AGC) were set at 70,000 and 1 × 10⁶, respectively. The sheath gas flow rate, Aux gas flow rate, spray voltage, and S-lens RF level were set to 40, 10, 2.0 kV, and 50.0, respectively. HPLC programs were the same as mentioned in the previous section. Concurrently, we quantified EGCG, EGCG-4'-sulfate, and EGCG-4'-glucuronide in plasma samples by peak area measurements in comparison with a standard curve of the authentic standard using TraceFinder software ver. 4.0 (Thermo Fisher Scientific). The range of the calibration curve was 1–500 ng/mL. 

Data Analysis. Kinetic analyses were carried out using Prism 7 (GraphPad) to calculate the Vₘₐₓ and Kₘ of each metabolic pathway and recombinant enzyme. The intrinsic clearance (Vₘₐₓ/Kₘ), viz., the specific activity of each cytosol and recombinant protein, was compensated by the amount of protein used in the experiment. The plasma concentrations of each compound versus the time profile were subjected to a noncompartmental analysis using Phoenix WinNonlin 7.0 (Pharsight). T₁/₂ of each compound of the same group was analyzed by Wilcoxon’s rank sum test. P-values of less than 0.05 were considered statistically significant.

## RESULTS

Identification of EGCG-Sulfate Generated by Human SULT or Human Cytosolic Fractions. Table 1 and Figure S1 summarize the ¹H NMR data for EGCG-sulfate extracted from a metabolite of human SULT, as well as the ¹H NMR data for EGCG. A comparison of these NMR spectra revealed that EGCG-sulfate had a slight chemical shift change at H2' and H6'', indicating that sulfation occurred at the D-ring of EGCG. The equivalent signals for H2'' and H6'' protons demonstrated molecular structure symmetry at the D-ring, suggesting that the H4'' position undergoes sulfation. Note that H2 and H3 of EGCG and EGCG-sulfate did not split and were detected as singlets, suggesting low coupling constants, and inferring that they are epi type catechins.

Figure 2 shows a portion of the chromatogram generated via LC–MS/MS analysis of EGCG-sulfate metabolized by human liver or small intestinal cytosol. Additionally, several standards with sulfation in the D-ring were analyzed. In both metabolized samples, the retention time of the main EGCG-sulfate peak was the same as the EGCG-4'-sulfate standard; however, it differed compared to the EGCG-3'-sulfate. Moreover, the spike test showed that EGCG-sulfate generated by HLC is identical to EGCG-4'-sulfate (Figure S2). Note that the separation method in this study can differentiate EGCG and EGCG-sulfate from gallocatechin-3-gallate (GCG) and GCG-sulfate, which are non-epi type EGCG catechins (Figure S3). These results demonstrate that EGCG sulfation in humans generates EGCG-4'-sulfate.

Table 1. ¹H NMR Data of EGCG and EGCG-Sulfate Generated by Human SULTs

| position | ¹H (CD3CN) | ¹H (CD3CN), extract by human SULT | Δδ |
|----------|------------|----------------------------------|----|
| 2        | 4.99(s)    | 5.00(s)                          | 0.01|
| 3        | 5.47(s)    | 5.48(s)                          | 0.01|
| 4        | 2.80(dd, J = 2.2, 17.6 Hz) | 2.80(dd, J = 2.2, 17.6 Hz) | 0.00|
| 6        | 2.95(dd, J = 4.6, 17.4 Hz) | 2.96(dd, J = 4.5, 17.4 Hz) | 0.01|
| 8        | 6.00(d, J = 2.3 Hz) | 6.00(d, J = 2.3 Hz) | 0.00|
| 2'       | 6.50(s)    | 6.50(s)                          | 0.00|
| 6'       | 6.50(s)    | 6.50(s)                          | 0.00|
| 2''      | 6.71(s)    | 6.87(s)                          | -0.04|
| 6''      | 6.91(s)    | 6.87(s)                          | -0.04|

*Each number and notation denote chemical shift (ppm), peak splitting, and coupling constants (J) in turn. Δδ represents the differences in chemical shift values in EGCG-sulfate compared with EGCG. s, singlet; d, doublet; and dd, doublet of doublets.
Kinetic Activity of Sulfation and Other Metabolic Pathways Using Human Liver and Small Intestinal Fractions. The formation of EGCG-4″-sulfate by HLC exhibited a Michaelis–Menten kinetic profile at 0–16 μM (Figures 3A and S4A). In contrast, substrate inhibition occurred at EGCG concentrations >40 μM (Figure S4G). As reported previously, methylation and glucuronidation by human liver fractions also exhibited a Michaelis–Menten kinetic profile (Figures 3C,E and S4C,E). The maximum velocity (V_{max}) and affinity for EGCG (K_{m}) of sulfation were determined by fitting the Michaelis–Menten equation with a concentration-dependent curve of EGCG conjugation. Values represent the mean ± SD of three independent experiments. The kinetic curve at lower or higher concentrations is shown in Figure S4.

Table 2. V_{max}, K_{m}, and Intrinsic Clearance (V_{max}/K_{m}) of EGCG Sulfation and Comparison with Other Conjugations

| conjugation          | V_{max} (nmol·mg-cytosol or microsome⁻¹·min⁻¹) | K_{m} (μM) | V_{max}/K_{m} (μL·mg-cytosol or microsome⁻¹·min⁻¹) |
|----------------------|-----------------------------------------------|------------|--------------------------------------------------|
| human liver          |                                               |            |                                                  |
| 4″sulfation           | 0.58 ± 0.44                                   | 0.24 ± 0.08| 2417                                             |
| 4″glucuronidation     | 1.43 ± 0.027                                  | 49.5 ± 2.7 | 28.8                                             |
| 3″glucuronidation     | 0.41 ± 0.007                                  | 51.1 ± 2.4 | 8.0                                              |
| 4″methylation         | 0.17 ± 0.007                                  | 0.17 ± 0.04| 1000                                             |
| human intestine       |                                               |            |                                                  |
| 4″sulfation           | 1.03 ± 0.04                                  | 0.82 ± 0.17| 1256                                             |
| 4″glucuronidation     | 0.18 ± 0.005                                  | 8.80 ± 0.85| 20                                               |
| 3″glucuronidation     | 0.18 ± 0.005                                  | 8.09 ± 0.79| 20                                               |
| methylation           | N.D.                                          | N.D.       | N.D.                                             |

"V_{max} and K_{m} values were determined by fitting the Michaelis–Menten equation with a concentration-dependent curve of EGCG conjugation. Values represent the mean ± SD of three independent experiments. V_{max} and K_{m} values were used to calculate the intrinsic clearance (V_{max}/K_{m}). The V_{max} and intrinsic clearances were compensated by the amount of protein used in experiments."
concentration-dependent curve at 0–16 μM and compared with those of other conjugates (Table 2). The affinity of sulfation (K_m: 0.24 μM) was approximately 200-fold higher than that of glucuronidation (49.5 and 51.1 μM), and the sulfation V_max (0.58 nmol·mg⁻¹·min⁻¹) was slightly lower than that of glucuronidation (1.43 and 0.41 nmol·mg⁻¹·min⁻¹). Moreover, although the affinity of sulfation was slightly lower than that of methylation (0.17 μM), the V_max for sulfation was 3-fold higher than that for methylation (0.17 nmol·mg⁻¹·min⁻¹). Thus, based on intrinsic clearance (V_max/K_m), sulfation in the liver cytosol had the highest metabolic activity (2417 μL·mg-cytosol⁻¹·min⁻¹) among the three metabolic pathways.

Next, we quantified the sulfation kinetic activity in human small intestinal cytosol and compared it to that of other conjugates (Figure 3B,D,F, Table 2, and Figure S4). Of note, methylation did not occur in the cytosol of the human intestine. Sulfation and glucuronidation exhibited a Michaelis–Menten kinetic profile in the small intestinal fraction. Moreover, substrate inhibition did not occur in the sulfation of the small intestine fraction. The intrinsic clearance (1256 μL·mg-cytosol⁻¹·min⁻¹) of sulfation by the small intestine was 63-fold higher than that of glucuronidation (20 μL·mg-microsome⁻¹·min⁻¹).

Concentration-Dependent Sulfation by Human Recombinant SULTs. The kinetic activities of EGCG sulfation by individual human SULTs (SULT1A1, SULT1B1, SULT1E1, SULT1A3, and SULT2A1) were determined (Figures 4, S5, and Table 3). SULT1A1, SULT1E1, and SULT1A3 catalyzed EGCG to EGCG-4'-sulfate, whereas SULT1B1 and SULT2A1 did not induce sulfation under the conditions employed. Moreover, at 0–0.4 μM, SULT1A1 exhibited higher affinity (K_m: 0.38 μM) and intrinsic clearance (V_max/K_m: 94.03 mL·mg-enzyme⁻¹·min⁻¹) than other SULTs, indicating its central role in EGCG sulfation. Moreover, at >0.8 μM, SULT1A1 demonstrated substrate inhibition, and SULT1A3 exhibited the highest rate of sulfation among the SULTs, suggesting its key role in EGCG sulfation at higher concentrations.

Qualitative Analysis of EGCG Metabolites in Human Plasma. Plasma samples were analyzed following ingestion of 350 mL of a catechin-rich beverage containing 135 mg of EGCG using high-resolution accurate mass spectrometry. Notably, mass spectrometry cannot distinguish the metabolites of EGCG and GCG without reference compounds. Representative chromatograms are shown in Figure 5A, while the detailed information such as accurate mass and MS/MS fragments of these metabolites is shown in Table 4. Three metabolites were detected as EGCG or GCG metabolites. Moreover, the EGCG-sulfate in plasma samples was identified as EGCG-4'-sulfate, based on the EGCG-4'-sulfate standard. Peak 1 (retention time: 4.64) showed significant [M-H]⁻ signals at m/z 633.110. Although MS/MS fragments of this peak were not acquired, the accurate mass and retention time were equivalent to those of EGCG-4'-glucuronide, indicating that...
Peak 1 represents EGCG-4″-glucuronide. Peak 2 (retention time: 12.92 min) showed significant [M-H]⁻ signals at m/z 565 for (E)GCG-methyl, m/z 551 for (E)GCG-methyl-sulfate, m/z 633 for (E)GCG-glucuronide, and m/z 565 for (E)GCG-dimethyl-sulfate. The presence of a product ion at m/z 183 indicated that Peak 1 represented a methylated galloyl moiety, while the m/z 319 represented another methylated moiety on the B-ring. Thus, this peak likely represents (E)GCG-dimethyl-sulfate, which is methylated in the B- and D-rings.

**Quantitative Analysis of EGCG, EGCG-4″-Sulfate, and EGCG-4″-Glucuronide in Human Plasma.** Following ingestion of a catechin-rich beverage by human volunteers, the plasma concentrations of EGCG, EGCG-4″-sulfate, and EGCG-4″-glucuronide were quantified by LC−MS/MS over 0−6 h. The time-concentration curve and pharmacokinetic profiles of these compounds are shown in Figure 6 and Table 5. EGCG-4″-sulfate had a C_max of 177.9 nmol·L⁻¹ and AUC of 715.2 nmol·h·L⁻¹, which is equivalent to that of EGCG (C_max = 233.5 nmol·L⁻¹, AUC 664.1 nmol·h·L⁻¹). The plasma concentration of EGCG-4″-sulfate was higher than that of EGCG-4″-glucuronide (C_max = 75.3 nmol·L⁻¹, AUC 198.9 nmol·h·L⁻¹), partly reflecting the results of the in vitro kinetic test using the cytosolic and microsomal fractions. EGCG-4″-sulfate had a longer T_{1/2} (3.9 h) than that of EGCG (2.1 h) and EGCG-4″-glucuronide (1.5 h), indicating that it was removed more slowly.

**DISCUSSION**

To our knowledge, this is the first report to elucidate the details of EGCG sulfation. These findings advance the current

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**Figure 5.** EGCG metabolite profile in human plasma after oral ingestion of catechin-rich tea. (A) Representative extracted ion chromatogram of EGCG metabolites from human plasma collected 2 h after ingestion of 615 mg of extracted catechin (135 mg of EGCG). Each chromatogram represents the detection of EGCG or its metabolites: m/z 457 for free (E)GCG, m/z 537 for (E)GCG-sulfate, m/z 471 for (E)GCG-methyl, m/z 551 for (E)GCG-methyl-sulfate, m/z 633 for (E)GCG-glucuronide, and m/z 565 for (E)GCG-dimethyl-sulfate. (B) MS/MS spectrum of EGCG-sulfate. (C) MS/MS spectrum of (E)GCG-diMe-sulfate.

**Table 4.** EGCG and Its Metabolites Detected in Human Plasma after Ingestion of Catechin-Rich Tea

| metabolites       | theoretical m/z | actual m/z     | the number of peaks | MS/MS m/z | retention time (min) | relative intensity (EGCG = 1) |
|-------------------|-----------------|----------------|---------------------|-----------|----------------------|-----------------------------|
| EGCG              | 457.07763       | 457.0780       | 1                   | 125, 169, 305 | 5.88                 | 1.00                        |
| EGCG-4″-sulfate   | 537.03445       | 537.0347       | 1                   | 125, 169, 305 | 7.21                 | 0.76                        |
| EGCG-4″-glucuronide | 633.10972     | 633.1100       | 1                   | N.D.       | 4.64                 | 0.39                        |
| (E)GCG-diMe-sulfate | 565.06575     | 565.0659       | 1                   | 168, 183, 224, 280, 319, 485 | 12.92               | 0.54                        |

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understanding of EGCG phase II metabolism, which will maximize the potential benefits of EGCG in humans. Specifically, we revealed that following ingestion of EGCG by humans, EGCG-4'-sulfate is present within plasma at concentrations comparable to those of EGCG. Furthermore, we determined that SULT1A1- and SULT1A3-mediated sulfation make significant contributions to the first pass effects and EGCG clearance. Collectively, these findings provide fundamental insights regarding the bioavailability, species differences, bioactivity, and toxicity of EGCG at the molecular level.

Our in vitro kinetic study using recombinant SULTs revealed that SULT1A1 and SULT1A3 are primarily responsible for EGCG sulfation. SULT1A1 is expressed in the liver and small intestine, whereas SULT1A3 is expressed in the small intestine and other organs, including the kidneys and lungs, but it is absent within the liver. In the current study, the kinetic curve of sulfation in the recombinant SULT1A1 exhibited substrate inhibition at >0.8 μM. Considering that the inhibitory concentration was higher than the observed plasma EGCG concentration (C_{max} < 0.3 μM), the substrate inhibitory effect of SULT1A1 in the liver was deemed negligible following EGCG intake. In contrast, substrate inhibition of SULT1A1 is presumed to occur in the small intestine as the enterocytic concentration of EGCG may exceed 0.8 μM. Meanwhile, with an in vitro metabolism study with purified SULT1A1 protein, Wang et al. reported that EGCG is not a SULT1A1 substrate. These differences in study results may be due to the high EGCG concentration (250 μM) used in the previous study, which likely caused SULT1A1 to exhibit substrate inhibition. These results indicate that SULT1A1 is the predominant isozyme responsible for EGCG sulfation in the liver, while SULT1A3 serves as the primary contributor to EGCG sulfation in the small intestine.

Based on our findings, SULT1A1- and SULT1A3-mediated sulfation appear to be key factors capable of improving the poor bioavailability of EGCG, which agrees with the results of previous studies. For example, quercetin, an inhibitor of SULT1A1 and SULT1A3, reportedly has the potential to increase EGCG bioavailability in rats and humans. The mechanism discussed in these studies includes inhibition of multidrug resistance-associated protein 2 (MRP2)—the efflux transporter of EGCG—and COMT. However, the inhibition of SULT1A1 (IC_{50}: 0.41–13 μM) and SULT1A3 (IC_{50}: 7 μM) by quercetin is equivalent to that of COMT (IC_{50}: 0.9–8.5 μM) and MRP2 (IC_{50}: 7.3–22.1 μM), suggesting that the combined effects of SULT1A1 and SULT1A3 inhibition may also serve to increase bioavailability. By contrast, a population pharmacokinetic study in humans demonstrated that SNP rs750155—polymorphisms in SULT1A1 genes—exerted no significant effect on the oral clearance (CL/F) of EGCG, a pharmacokinetic parameter that partially reflects bioavailability. However, the effect of SNP rs750155 on the sulfation activity of xenobiotics, including EGCG, has not been demonstrated. Furthermore, previous results for a single SNP are not sufficient to discuss the effect of SULT1A1 on the bioavailability of EGCG. Thus, further in vitro and clinical studies are required to confirm the importance of sulfation by SULT1A1 and SULT1A3 on EGCG bioavailability.

In functional foods and drugs, animal models are often used to assess the toxicity and bioactivity of compounds. However, when extrapolating data from animals to humans, differences among species must be considered. This study suggests that SULT1A1 and SULT1A3 could be effectively applied if differences among species are considered in EGCG bioactivity and toxicity. Since SULT1A1 is conserved in other species, including rodents, sulfation of EGCG could also occur in animals. However, a previous study on other substrates reported that activity of SULT1A1 varies among species at a dozen-fold scale, suggesting that the metabolic activity of EGCG in the liver significantly differs among species. Furthermore, SULT1A3 is found only in primates, indicating that the enteroctylic sulfation of EGCG in other species is much lower than that in humans. Therefore, previous results on the
pharmacokinetics and metabolites obtained in animals should be treated with care in terms of differences in species. SULTs have been reported to metabolize flavonoid structures without galloyl groups. However, our results showed that only the 4'-position is sulfated in EGCG, which has a galloyl moiety. One reason for this may be that the incorporation of a galloyl moiety induces steric hindrance in the flavonoid structure. In fact, the small active pocket in SULT1A3 does not prefer steric hindrance. Moreover, in addition to sulfate conjugation, methyl and glucuronide conjugations also show regioselectivity at the 4'-hydroxyl moiety, suggesting a high reactivity and low steric hindrance at this site. To further verify these possibilities, the interaction between the active site of SULT1A1 and SULT1A3 and EGCG should be examined via docking simulations.

Finally, our human ingestion study revealed that EGCG-4'-sulfate is one of the main forms circulating in the plasma, comparable to free EGCG and that sulfation is more prevalent than the other metabolic pathway, i.e., glucuronidation. In contrast, a previous study on green tea did not detect EGCG-sulfate despite detecting other sulfated catechins, such as EGC-sulfate. The previous study employed nitrogen-drying methods during plasma extraction, which might explain the low stability of EGCG due to the dimerization between the B-ring and galloyl moiety, which occurs during the concentration process. The EGCG recovery rate using the plasma extraction method employed in the previous study was only 41%, whereas that of EGCG, which does not have a galloyl moiety, was 74%, suggesting that EGCG is specifically unstable when extracted using this method. In contrast, in our study, we used the solid-phase extraction method in which the recovery rates of EGCG and EGCG-4'-sulfate were 90 and 87%, respectively. Thus, EGCG-sulfate might have been missed upon using the plasma extraction procedure employed in the previous study.

It is unclear whether EGCG-4'-sulfate itself exerts beneficial effects in humans. In general, the antioxidant activities of catechol contribute to the beneficial effects of EGCG. On the one hand, as the catechol structure in the galloyl moiety is blocked in EGCG-4'-sulfate, it might be less bioactive than free EGCG if the mechanism of efficacy depends on antioxidant activity. On the other hand, various activities such as the antihypertensive effect are maintained or enhanced by methylated conjugates of EGCG, even though a portion of the catechol structure is lost. The maintenance and enhancement of methylation efficacy are attributed to protein interactions, including enzyme inhibition and gene expression regulation. Therefore, it is possible that sulfate conjugates themselves also exert biological effects via functional protein interactions.

Recent studies have suggested that the sulfate conjugates of polyphenols act as stable forms of their parent compound for delivery to the target organ. For example, it has been proposed that resveratrol sulfates gradually regenerate their active parent compounds in the target organ, contributing to prolonged resveratrol exposure in vivo. Quercetin sulfates are also reported to serve as storage forms for quercetin in the plasma, liver, and kidneys. Thus, if other polyphenols share the same mechanism, EGCG-4'-sulfate might act as a free-form precursor. In our study, a slower half-life of EGCG-4'-sulfate represents a stable form in humans, capable of providing a prolonged supply of active EGCG. In contrast, a previous study revealed that within tissues, EGCG is present in its free form, whereas EGCG is largely conjugated in the plasma of mice. It further concluded that conjugation limits the EGCG distribution to target organs. To resolve this discrepancy, it is necessary to assess the transport activity of EGCG-4'-sulfate and sulfatase activity in target organs.

In conclusion, we revealed that SULT1A1- and SULT1A3-mediated sulfation are crucial for EGCG metabolism, highlighting their importance in enhancing EGCG bioavailability in humans. However, our study is limited in that it focused exclusively on metabolism in the first pass effects of EGCG; other factors, such as absorption and hepatic uptake, should also be considered when discussing EGCG bioavailability. Nevertheless, we also revealed that in humans, EGCG-4'-sulfate represents the main circulating EGCG derivative, suggesting its significance in the health benefits elicited by EGCG. Thus, future studies focusing on the impact of EGCG-4'-sulfate on the bioefficacy and toxicity of catechin intake may improve EGCG utilization in pharmaceutical and functional food applications.
Comparison of catechin profiles in human plasma and urine after single dose of catechins and inhibition of catechol-o-methyltransferase by (−)-epigallocatechin-3-gallate; PAPS, 3′-phosphoadenosine-5′-phosphosulfate; SULT, sulfotransferase; SAM, S-adenosyl methionine; UDPGA, uridine 5′-diphosphoglucuronic acid; UGT, UDP-glucuronosyltransferase

Notes
The authors declare no competing financial interest.

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ABBREVIATIONS
COMT, catechol-o-methyltransferase; EGCG, epigallocatechin-3-gallate; PAPS, 3′-phosphoadenosine-5′-phosphosulfate; SULT, sulfotransferase; SAM, S-adenosyl methionine; UDPGA, uridine 5′-diphosphoglucuronic acid; UGT, UDP-glucuronosyltransferase

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