Intestinal absorption, metabolism, and excretion of (-)-epicatechin in healthy humans assessed by using an intestinal perfusion technique

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ABSTRACT

Background: (-)-Epicatechin is a dietary flavonoid present in many foods that affects vascular function, but its action is limited by incomplete absorption, conjugation, and metabolism. Factors that influence this activity may be attributed to instability in the gastrointestinal lumen, low permeability across the intestinal wall, or active efflux from enterocytes and extensive conjugation.

Objective: With the use of a multilumen perfusion catheter, we investigated the jejunal absorption, systemic availability, metabolism, and intestinal, biliary, and urinary excretion of (-)-epicatechin in humans.

Design: In a single-center, randomized, open, controlled study in 8 healthy volunteers, 50 mg purified (-)-epicatechin was perfused into an isolated jejunal segment together with antipyrine as a marker for absorption. (-)-Epicatechin and conjugates were measured in intestinal perfusates, bile, plasma, and urine.

Results: Forty-six percent of the dose was recovered in the perfusate either as unchanged (-)-epicatechin (22 mg) or conjugates (0.8 mg); with stability taken into account, this result indicates that ~46% of the dose had apparently been absorbed. The conjugates were predominantly sulfates, which indicated conjugation by sulfotransferases followed by efflux from the enterocytes. In contrast, epicatechin glucuronides were dominant in plasma, bile, and urine.

Conclusions: Almost one-half of the (-)-epicatechin is apparently absorbed in the jejunum but with substantial interindividual differences in the extent of absorption. The data suggest that the nature and substitution position of (-)-epicatechin conjugation are major determinants of the metabolic fate in the body, influencing whether the compound is effluxed into the lumen or absorbed into the blood and subsequently excreted. Am J Clin Nutr 2013;98:924–33.

INTRODUCTION

Dietary flavanols such as (-)-epicatechin are present at high concentration in many foods including cocoa, tea, and red wine. The consumption of foods rich in flavanols decreases risk of certain chronic diseases (1–4), although the exact mechanisms and metabolites responsible for these effects in vivo are unclear. Analogous to drugs taken orally, the extent and rate of absorption into the systemic circulation (the so-called bioavailability) of these compounds is crucial for their efficacy. For flavanols, conventional methods in healthy volunteers and ileostomists have indicated that <30% of the dose consumed is absorbed (5, 6).

To increase the systemic exposure of these compounds, high doses of polyphenols have been added to the diet and foods and tested in human-intervention studies. For example, (-)-epicatechin has been increased in certain test drinks or extracts (5, 7, 8) and significantly improved, both acutely and chronically, certain physiologically relevant biomarkers of cardiovascular risk (7, 9). Consequently, low flavanol bioavailability could explain the lack of strong in vivo biological effects, despite substantial effects in vitro, and partially explain the inconsistency of results shown in clinical intervention studies.

Many chemicals, including therapeutic drugs, have a low and variable bioavailability (10). Low oral bioavailability can occur through several mechanisms including poor permeability, extensive first-pass metabolism, saturation of uptake transporters, instability in gastrointestinal fluids, or, once absorbed, transport back into the lumen of the gastrointestinal tract by efflux transporters. Many in vitro models have been used to study how these different mechanisms can affect polyphenol bioavailability (11–15). However, the validity and biological relevance of these models are open for debate, such as the decreased expression of some enzymes and the overexpression of efflux transporters (16, 17).

The perfusion of a gut intestinal segment by catheters has been previously applied to study drug permeability, metabolism, transport, and the excretion of drugs (18, 19). The use of multilumen perfusion catheters in combination with stable-isotope-labeled control compounds in intestinal closed segments is one of the most advantageous intestinal perfusion techniques to study absorption and metabolism in vivo (19, 20). This method is a compelling way to elucidate the rate and extent of absorption, metabolism, and excretion of compounds. In addition, the difference between the solute concentration entering and leaving the
intestinal perfused segment provides the best way to measure the human intestinal permeability (P_{ed}). A similar methodology has been previously successfully applied for the study of intestinal metabolism of quercetin (21).

Consequently, we aimed to elucidate the steps that affect the intestinal absorption and metabolism of (−)-epicatechin and determine how conjugation affects absorption and excretion in humans. A better understanding about the rate-limiting steps of (−)-epicatechin absorption could eventually be relevant to improve the bioavailability of (−)-epicatechin and, therefore, its bioefficacy in humans.

SUBJECTS AND METHODS

Chemicals

Acetonitrile ultragradient HPLC grade was purchased from JT Baker Europe. HPLC-grade water was prepared by using a Milipore Milli-Q purification system (Millipore). All other solvents were HPLC grade and reagents were purchased from Merck. (−)-Epicatechin was purchased from Extrasynthese. Umbelliferone sulfate was purchased from Toronto Research Chemicals Inc.

Compounds for intestinal perfusion

Food-grade (−)-epicatechin was obtained from Atkin Chemicals Inc. For the tested batch, the supplier specifications indicated that the purity was 98.47%, with loss on drying at 0.74% and ashes at 0.44%. The reported concentration of heavy metals was ≤10 mg/kg, the microbiological contaminants total plate count was <100/g, the yeast and mold count was <10/g, and both Escherichia coli and Salmonella were negative. No residual solvent and pesticides could be detected. The main impurity was identified as (+)-catechin.

Antipyrine was used as a probe drug to assess the extent of absorption of an optimally bioavailable aqueous drug solution from isolated intestinal segments and compare it to the absorption and bioavailability of test compounds. This approach allowed for the elucidation of whether the low bioavailability of test compounds might have been a result of poor individual absorption or extensive first-pass metabolism. Antipyrine is well suited for this method of Goromaru et al (24).

The use of antipyrine labeled with 3 deuterium atoms ([2H3]antipyrine) in addition to the unlabeled drug allowed the simultaneous administration of 2 test compounds into 2 adjacent intestinal segments and ensured that the absorption process and bioavailability from the 2 segments were comparable. Moreover, this technique was used to see if any leakage had occurred from the proximal into the distal segment and vice versa. The presence of high amounts of antipyrine analog in the perfusate of the segment opposite to the segment into which it had been perfused would have indicated incomplete occlusion of the segment by the balloons. [2H3] antipyrine and the internal standard d6 ([2H6]antipyrine) were obtained by the methylation of 3-methyl-1-phenyl-3-pyrazolin-5-one with [6H6]-dimethylsulfate and followed by hydrogen-deuterium exchange. The isotope distribution of [2H3]antipyrine was as follows: 0.13% [2H1]antipyrine, 6.17% [2H2]antipyrine, 92.41% [2H3]antipyrine, and 1.9% [2H4]antipyrine. The isotope distribution of [2H4]antipyrine was 0.11% [2H1]antipyrine, 6.63% [2H2]antipyrine, and 92.49% [2H3]antipyrine.

Study protocol

The study protocol (Eudra-CT no. 2006–006008-12) was approved by the local ethics committee (Medical Faculty of the University of Tübingen), and all volunteers gave written informed consent. During the single-blind crossover study, 4 different polyphenols [(−)-epicatechin, procyanidin B2, hesperidin, and hesperetin-7-O-glucoside] were individually perfused in the intestinal tract by the multilumen perfusion catheter. Block randomization was applied for treatment allocation. In this article, we report only data from the (−)-epicatechin perfusion. Adjacent segments were perfused either with hesperidin or hesperetin-7-O-glucoside but not with procyanidin B2. Nine healthy volunteers, including 6 men (mean ± SEM age: 37.4 ± 6.5 y) and 3 women (mean age: 32.7 ± 11 y) enrolled in the study. Intakes of drugs, alcohol, coffee, grapefruit juice, or tea and smoking were not permitted from the evening before the study day (2000 h) throughout the study. In addition, a diet low in polyphenols and phenolic compounds, which excluded fruit and vegetables, high-fiber products, and beverages such as tea, coffee, fruit juices, and wine, was followed for 2 d before the study. A 350-cm-long and 4.6-mm-diameter silicone rubber catheter was used for perfusion of isolated jejunal segments. Details of this technique have been described elsewhere, including the intubation procedure, catheter design, composition of buffer solutions, and localization of the catheter via the transmucosal potential difference (18, 19, 25).

Briefly, 2 adjacent, 20-cm-long isolated segments were generated in the proximal jejunum by the inflation of 3 polypropylene balloons. These segments were independently perfused by separate infusion channels, and separate aspiration channels allowed the recovery of compound-containing perfusates from each segment.

After an overnight fast, the multilumen perfusion catheter was introduced orally and placed into the small intestine under endoscopic control. The transmucosal potential difference was used to determine the correct position of the device. The proximal balloon was positioned ≥20 cm distal from the pylorus and, thus, distal from the papilla of Vater. Subsequently, all 3 balloons were carefully inflated with air (Figure 1). Balloon pressures were kept constant (at ~25 mm Hg) throughout the study. Isolated segments were rinsed with a physiologic isotonic sodium chloride perfusion solution at 2.5 mL/min at 37°C for 30 min by use of a motor-driven syringe. In 8 volunteers, the catheter was successfully placed without any major adverse effect. One volunteer reported discomfort because of balloon distension and decided to discontinue the study.

Thereafter, 50 mg purified food-grade (−)-epicatechin that was dissolved in 30 mL perfusion buffer (10 mmol glucose/L, 5.4 mmol KC/l/L, 120 mmol NaCl/L, 2 mmol Na2HPO4/L, and 35 mmol mannitol/L at pH 7.4) was administered either into the proximal or distal isolated segment over 30 min at 1 mL/min, which contained either 2 mg antipyrine or [2H3]antipyrine. For the next 2 h, intestinal segments were continuously perfused with the perfusion buffer at 2 mL/min.

From each isolated intestinal segment, intestinal perfusates were continuously collected over 2.5 h at 15-min intervals.
Immediately after collection, perfusate samples were centrifuged and separated from shed enterocytes. Supernatant fluids were fractionated in 500-μL portions, spiked with 20 μL stabilization buffer (0.4 mol sodium dihydrogen phosphate/L, 20% ascorbic acid, and 0.1% EDTA at pH 3.6) and stored at −80°C until analysis.

Bile fluids were aspirated off above the proximal occluded segment, and their volume was measured. Bile samples were fractionated in 500-μL portions, spiked with 20 μL stabilization buffer, and stored at −80°C until analysis.

Venous blood samples obtained before and at 0.25, 0.5, 1, 2, 3, 4, 6, 8, and 24 h after the commencement of perfusion were immediately placed on ice and centrifuged for 10 min at 3000 × g at 4°C. Plasma samples were fractionated in 500-μL portions, spiked with 20 μL stabilization buffer, and kept at −80°C. Urine was collected predose and for 24 h after administration of (−)-epicatechin by using acid-washed urine-collection flasks that contained 1 mL 1 mol hydrochloric acid/L. During collection intervals, flasks were kept at 5°C. The total volume was measured, and 2 aliquots were stored at −80°C until analysis.

Quantification of antipyrine and [2H₃]antipyrine

Antipyrine and [2H₃]antipyrine were determined by liquid chromatography–tandem mass spectrometry (LC-MS/MS) analysis by using [2H₆]antipyrine as an internal standard as described previously (18). Briefly, 20 μL perfusate was mixed with 5 μL H₂O, 6 μL internal standard [2H₆]antipyrine (10 ng/μL in water) and 60 μL acetonitrile. After centrifugation, 50 μL supernatant fluid was diluted with 100 μL H₂O, and 10 μL were used for LC-MS/MS analysis. Plasma samples (100 μL) were spiked with 6 μL internal standard [2H₆]antipyrine (0.5 ng/μL in water). Protein precipitation was performed by the addition of 200 μL 1% (vol:vol) acetic acid in acetonitrile. After centrifugation, 100 μL supernatant fluid was diluted with 200 μL H₂O. After a second centrifugation step, 40 μL supernatant fluid was used for LC-MS/MS analysis. See “Supplemental data” in the online issue for the method performance for antipyrine and [2H₆]antipyrine in perfusate and plasma samples.

Quantification of (−)-epicatechin metabolites

Perfusate samples were centrifuged, diluted, and directly injected to ultra performance liquid chromatography coupled with tandem mass spectrometry for quantification of (−)-epicatechin metabolites. Plasma (200 μL), bile (100 μL), or urine (20 μL) samples were quantified by using a previously reported methodology (26). Briefly, samples were directly loaded onto a protein precipitation and phospholipids removal plate (Ostro; Waters Corp). Plasma protein precipitation was ensured by adding 600 μL acetonitrile and mixing thoroughly with a pipette. Samples were filtered by using a vacuum manifold for 5 min and recuperated in a 1-mL collection plate. The extraction procedure was enhanced by a washing step with 200 μL methanol. Afterward, eluates were reduced to dryness at room temperature under a flow of nitrogen. Finally, the residue was dissolved in 100 μL 8% acetonitrile in acidic water. Once resuspended, (−)-epicatechin metabolites were separated by reversed-phase ultraperformance liquid chromatography by using an Acquity HSS C₁₈ 2.1 × 100 mm, 1.8-μm column (Waters) equipped with an HSS C₁₈ VanGuard precolumn (Waters) as described elsewhere. The method repeatability, intermediate reproducibility, limit of detection, and limit of quantification for plasma and urine has been previously reported (26). Standardization and validation of the methodology in bile was performed by analyzing calibration samples prepared in bile (concentration range: 0.025–3 μmol/L). See Supplemental Table 1 under “Supplemental data” in the online issue for the method performance for bile samples.

Cell culture experiments

Caco-2 human epithelial colorectal adenocarcinoma cells were used to investigate the transport and efflux of (−)-epicatechin in vitro. Cells were grown in high-glucose DMEM (Gibco, Life Technologies) supplemented with 20% heat-inactivated fetal bovine serum, nonessential amino acids, t-glutamine (2 mmol/L), amphotericin B (1 μg/mL), penicillin (100 U/mL), and streptomycin (100 μg/mL) and maintained at 37°C and 5% CO₂. Cell passage numbers 38–50 were used in this study. The medium was replaced every 2 d, and cells were reseeded every 7 d. Cells were seeded in 12-well transwells inserts at a density of 20,000 cells/cm², and the medium on both sides was replaced every 2 d. After 21 d, cells were already differentiated. On the experiment day, medium was removed and replaced by Hank’s balanced salt solution supplemented with 25 mmol glucose/L, 10 mmol HEPES/L, and 1.8 mmol CaCl₂/L, Catalase (189 U/mL) and ascorbic acid (0.5 mmol/L) were added to prevent oxidation of the test compounds. (−)-Epicatechin (100 μmol/L) or umbelliferone (100 μmol/L) were placed in the apical side of cell monolayers and incubated for 2 h. Aglycone and conjugated compounds were
detected in the cell culture media by using the analytic conditions described for intestinal perfusates. Transepithelial electrical resistance values were measured before and after the transport study and indicated that the intact monolayer was not affected by the transport experiment.

Data analysis

Intestinal permeability \((P_{\text{eff}})\) was calculated according to a well-mix tank model as described by Lennernäs et al (27) as follows:

\[
P_{\text{eff}} = \left[\left(\frac{C_{\text{in}} - C_{\text{out}}}{Q_{\text{in}}}\right) + \left(\frac{2\pi rL}{Q_{\text{in}}}\right)\right] \times \left(\frac{Q_{\text{in}}}{2\pi rL}\right) (1)
\]

where \(C_{\text{in}}\) and \(C_{\text{out}}\) are the concentrations of the analyte entering and leaving the intestinal segment, and \(Q_{\text{in}}\) is the flow rate of the perfusion solution (2 mL/min). The surface of the cylinder of the jejunal segment was calculated by using the intestinal radius (1.75 cm) (21) and the length of the segment (20 cm).

All samples were analyzed at least in duplicate. The AUC was calculated by using the trapezoidal rule from 0 to 24 h after administration with Kinetica software version 5 (Thermo Fisher Scientific Inc). Actual blood sampling times were used.

Statistical analyses were conducted with GraphPad Prism and GraphPad Instat version 3.05 software (GraphPad Software). \(P < 0.05\) was considered statistically significant. Data are presented as means ± SEMs.

RESULTS

Human intestinal perfusion of (−)-epicatechin

Only (−)-epicatechin data are described in the current report; data on perfusion of procyanidin B2, hesperidin, and hesperetin-7-O-glucoside will be presented separately elsewhere. All perfusate samples collected from the isolated segment were visually colorless showing the absence of bile or blood.

Antipyrine and \([^{2}\text{H}_3]\)antipyrine were used as quality markers of the performance of the perfusion technique. The low amounts of antipyrine or \([^{2}\text{H}_3]\)antipyrine shown in the perfusate of the intestinal segment opposite to the segment in which the respective antipyrine analogs had been perfused showed the proper occlusion of the segments, and no substantial leakage had occurred in 6 volunteers (Table 1). In volunteers 6 and 8, a d3-antipyrine: antipyrine ratio >25% was shown in the distal segment, when d3-antipyrine was only perfused in the proximal segment (see Supplemental Table 2 under “Supplemental data” in the online issue). These findings suggested an incomplete occlusion of the segment, and thus, it was decided to exclude these volunteers from the analysis.

The low amount of antipyrine recovered in the perfusates showed good marker absorption (85% of the dose) (Table 1), with no significant differences between the proximal and distal segment \((P = 0.74)\). The average recovery of the perfusion buffer in volunteers was 26% lower than the perfused volume of 270 mL, which indicated the net absorption of water from the intestinal segments (see Supplemental Table 2 under “Supplemental data” in the online issue). No effect on the absorption of antipyrine was seen in relation to the net water absorption or secretion \((R^2 = 0.013, P = 0.786)\) (see Supplemental Figure 1A under “Supplemental data” in the online issue). In addition, the absorption of antipyrine from the intestinal segments were mirrored by the plasma AUC \((P = 0.03)\) (see Supplemental Figure 1B under “Supplemental data” in the online issue). The mean \(P_{\text{eff}}\) value for antipyrine \((8.1 \pm 0.8 \times 10^{-4} \text{ cm/s})\) was similar to the values previously reported by Lennernäs et al (28) \((5.6 \pm 1.6 \times 10^{-4} \text{ cm/s})\) (Table 1).

(−)-Epicatechin stability in the perfusion buffer was tested by in vitro incubation at 37°C. After 150 min incubation, 91.4% of the compound remained under experimental conditions (see Supplemental Figure 2 under “Supplemental data” in the online issue).

Human intestinal absorption of (−)-epicatechin

On average, ∼46% (−)-epicatechin was apparently absorbed because 22 ± 4 mg aglycone (−)-epicatechin were recovered after 2.5 h in the perfusion fluid, and ∼8% was lost because of stability (Table 2). There were pronounced interindividual differences that ranged from 31% to 90% because the recovery was between 4.7 and 32.1 mg. The mean calculated \(P_{\text{eff}}\) value for (−)-epicatechin was 1.80 ± 1.40 × 10⁻⁴ cm/s (Table 2). The time course of (−)-epicatechin and conjugates in the perfusate

| TABLE 1 | Individual data of d0- and d3-antipyrine recovered, percentage of antipyrine not recovered, and \(P_{\text{eff}}\) after administration of 2 mg antipyrine into the distal segment and \([^{2}\text{H}_3]\)antipyrine into the proximal jejunal segment of 8 healthy volunteers\(^1\) |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| | Distal | | | Proximal | | | | | | |
| Volunteer | d0- Antipyrine recovered | d3- Antipyrine recovered | Antipyrine not recovered | \(P_{\text{eff}}\) | d0- Antipyrine recovered | d3- Antipyrine recovered | Antipyrine not recovered | \(P_{\text{eff}}\) |
| ng/mL | ng/mL | µg | % | cm/s \((\times 10^{-4})\) | ng/mL | ng/mL | µg | % | cm/s \((\times 10^{-4})\) |
| 1 | 630 | 120 | 54.8 | 97.3 | 20.4 | 34.5 | 11.5 | 795 | 190 | 91.4 | 15.8 |
| 2 | 625 | 17.5 | 166.9 | 91.6 | 20.6 | 11.5 | 2810 | 730.9 | 81.4 | 3.1 |
| 3 | 2240 | 8.5 | 199.4 | 90.0 | 4.4 | 11.5 | 1870 | 591.9 | 70.4 | 5.6 |
| 4 | 3940 | 44.0 | 721.0 | 64.0 | 1.7 | 15.5 | 2540 | 657.9 | 67.1 | 3.6 |
| 5 | 1655 | 5.5 | 470.0 | 76.5 | 6.6 | 11.5 | 1625 | 432.3 | 78.4 | 6.7 |
| 6 | 15 | 277.5 | 4.7 | 99.8 | 933.8 | 17.0 | 1.0 | 2.2 | 99.9 | 1240.1 |
| 7 | 2450 | 22.0 | 668.9 | 66.5 | 3.8 | 18.5 | 2160 | 397.4 | 80.1 | 4.6 |
| 8 | 225 | 66.5 | 16.7 | 99.2 | 60.3 | 31.5 | 445 | 15.1 | 99.2 | 29.7 |

\(^1\) \(P_{\text{eff}}\): intestinal permeability.
showed a peak concentration of (−)-epicatechin at 30 min and, for conjugates, at 45 min (Figure 2A). There was no correlation between the amount of (−)-epicatechin recovered and the amount of antipyrene recovered in perfusates (see Supplemental Figure 3 under “Supplemental data” in the online issue).

(−)-Epicatechin and epicatechin metabolites were also shown in the adjacent segment [not perfused with (−)-epicatechin] in volunteers who received the compound in the distal segment (volunteers 1, 2, and 4) (Table 2, Figure 3A). We discounted above the absence of a leak in the perfused segments as assessed by the d0-antipyrene:d3-antipyrene ratio. In addition, there was a direct relation between the compound absorbed and amount of (−)-epicatechin measured in the adjacent segment, which suggested an absorption and a posteriori efflux of compounds in other region of the gut (Figure 3B). Moreover, higher amounts of epicatechin metabolites than aglycone (Figure 3A) plus a different epicatechin metabolite profile than that in the perfused segment supported this hypothesis (Figure 2B compared with 3C).

**Human intestinal metabolism of (−)-epicatechin**

Epicatechin metabolites [0.8 ± 0.2 mg as (−)-epicatechin equivalents] were quantified in the perfusates. Similar to that shown with (−)-epicatechin, relevant interindividual differences were observed in conjugates that ranged between 0.08 and 1.94 mg [as (−)-epicatechin equivalents]. The conjugated 3′-O-sulfate-epicatechin form was the most predominant metabolite shown in the intestinal fluid, which accounted for 48 ± 8% of total metabolites (Figure 2B).

In vitro experiments with Caco-2 cells were performed to compare (−)-epicatechin absorption and metabolism with results obtained in human intestinal perfusates. After the incubation of (−)-epicatechin for 2 h in the apical compartment, 3′-O-sulfate-epicatechin, 3′-O-methyl-4′-O-sulfate-epicatechin, 3′-O-methyl-5′-O-sulfate-epicatechin, and 3′-O-methyl-epicatechin were detected in both apical and basal compartments (Figure 4A). In (−)-epicatechin conjugates formed by cells, none of the compounds were glucuronides. Note that Caco-2 cells were able to produce glucoronide conjugates when umbelliferone was placed in the apical compartment (Figure 4B). In addition, significant concentrations of aglycone (−)-epicatechin were shown in the basal compartment.

**TABLE 2**

Individual data of (−)-epicatechin and (−)-epicatechin metabolites recovered, segment perfused with (−)-epicatechin, percentage of (−)-epicatechin recovered, percentage of (−)-epicatechin metabolites recovered, percentage of (−)-epicatechin not recovered, and $P_{eff}$ after administration of 50 mg (−)-epicatechin into the proximal jejunal segment of 8 healthy volunteers

<table>
<thead>
<tr>
<th>Volunteer</th>
<th>Segment perfused with epicatechin</th>
<th>Epicatechin recovered</th>
<th>Epicatechin metabolites recovered</th>
<th>Epicatechin recovered</th>
<th>Epicatechin metabolites recovered</th>
<th>Epicatechin not recovered</th>
<th>$P_{eff}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Distal</td>
<td>4.7 mg</td>
<td>0.13 mg</td>
<td>9.5%</td>
<td>0.2%</td>
<td>90.4%</td>
<td>4.60</td>
</tr>
<tr>
<td>2</td>
<td>Distal</td>
<td>29.3 mg</td>
<td>0.003 mg</td>
<td>58.5%</td>
<td>3.9%</td>
<td>37.6%</td>
<td>1.30</td>
</tr>
<tr>
<td>3</td>
<td>Distal</td>
<td>—</td>
<td>10 mg</td>
<td>61.7%</td>
<td>2.0%</td>
<td>36.3%</td>
<td>1.70</td>
</tr>
<tr>
<td>4</td>
<td>Distal</td>
<td>23.9 mg</td>
<td>0.02 mg</td>
<td>47.8%</td>
<td>0.8%</td>
<td>51.4%</td>
<td>0.79</td>
</tr>
<tr>
<td>5</td>
<td>Proximal</td>
<td>—</td>
<td>7.9 mg</td>
<td>64.2%</td>
<td>1.6%</td>
<td>34.2%</td>
<td>1.01</td>
</tr>
<tr>
<td>6</td>
<td>Proximal</td>
<td>—</td>
<td>19.6 mg</td>
<td>39.2%</td>
<td>1.7%</td>
<td>59.1%</td>
<td>4.60</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>—</td>
<td>—</td>
<td>46.8 ± 20.6</td>
<td>1.7 ± 1.3</td>
<td>51.5 ± 21.4</td>
<td>1.80 ± 1.40</td>
<td></td>
</tr>
</tbody>
</table>

$P_{eff}$, intestinal permeability.

**Human biliary excretion of (−)-epicatechin**

In a similar way to the assessment performed in intestinal fluid, the biliary excretion of (−)-epicatechin was evaluated by collecting samples every 15 min. The biliary elimination of (−)-epicatechin metabolites over the next 2.5 h after perfusion accounted for 2.6–
18.4 μg [as (–)-epicatechin aglycone equivalents] (Figure 5A). A substantial interindividual variability in the biliary elimination of (–)-epicatechin metabolites was observed. One volunteer excreted 5 times higher amounts of (–)-epicatechin metabolites in bile than those of other volunteers (P, 0.0001). The total amount of (–)-epicatechin metabolites excreted peaked at 90 min after the beginning of the perfusion (Figure 5B). In contrast to the conjugate pattern observed in the intestinal fluid, 3′-O-β-D-glucuronide-epicatechin, 3′-O-sulfate-epicatechin, and 3′-O-methyl-5-O-sulfate-epicatechin conjugates were significantly higher than the rest of the metabolites (31 ± 22%, 24 ± 11%, and 24 ± 11% of the total, respectively) (P < 0.0001) (Figure 5C).

Plasma and urine concentrations of (–)-epicatechin metabolites

The maximum concentration of (–)-epicatechin metabolites in plasma was observed 1 h after initiation of the perfusion (Figure 6A). 3′-O-β-D-glucuronide-epicatechin was the metabolite with the highest plasma concentration followed by 3′-O-sulfate-epicatechin and 3′-O-methyl-5-O-sulfate-epicatechin. Between 1.2 and 5.9 mg of the dose of (–)-epicatechin was excreted in the urine within 24 h as conjugated metabolites (Figure 6B). The total urinary excretion of (–)-epicatechin metabolites was significantly correlated (R² = 0.716; P = 0.016) with the amount of (–)-epicatechin absorbed (see Supplemental Figure 4 under “Supplemental data” in the online issue). As shown in plasma, conjugates 3′-O-β-D-glucuronide, 3′-O-sulfate, and 3′-O-methyl-5-O-sulfate epicatechin were the most prominent conjugates in urine (36 ± 15%, 12 ± 5%, and 12 ± 9% of total conjugates excreted, respectively) (Figure 7).
DISCUSSION

An understanding of the absorption, metabolism, distribution, and excretion of (–)-epicatechin in humans is instrumental to understand the factors that control the bioavailability of (–)-epicatechin. With the use of an intestinal perfusion technique, we were able to show the intestinal absorption, permeability, and metabolic pathways, including the role of biliary excretion of purified (–)-epicatechin, in healthy human volunteers.

The quantification of aglycone and (–)-epicatechin metabolites in intestinal perfusates allowed for an estimation of the contribution of the gut wall for the bioavailability of polyphenols. After 50 mg purified (–)-epicatechin was perfused for 2.5 h into the jejunum, 44 ± 8% of the compound was neither absorbed nor metabolized (Figure 7). Therefore, a significant amount of parent (–)-epicatechin can reach the large intestine and undergo metabolism by the gut microbiota, leading to compounds that can then be absorbed in the large intestine (29–31). In addition, similar to that recently shown with procyanidins (32), (–)-epicatechin could potentially modulate the growth of certain gut microbiota with potential prebiotic effects in humans. Consequently, additional investigations of the interaction of (–)-epicatechin with the gut microbiota and, especially, the bioavailability and bioefficacy of the breakdown products produced by these interactions are warranted.

The intestinal $P_{\text{eff}}$ represents a direct measurement of the local absorption rate and reflects the transport velocity across the epithelial barrier (28). In our experimental conditions, the mean calculated $P_{\text{eff}}$ of (–)-epicatechin was 1.80 ± 1.40 × 10^{-3} cm/s. Previously, perfusion experiments with quercetin-3,4′-diglucoside in humans calculated a $P_{\text{eff}}$ value of 8.9 ± 7.1 (21). Despite the prerequired action of lactase phlorizin hydrolase to cleave glucosidic moieties, the $P_{\text{eff}}$ suggests a better permeability of the quercetin-3,4′-diglucoside (after conversion to quercetin) than (–)-epicatechin.

The intestinal metabolic pathways of (–)-epicatechin in humans were elucidated. (–)-Epicatechin-3′-O-sulfate was the most predominant conjugate shown in intestinal fluids. Conjugates that contain a sulfate group (sulfate or methyl sulfate) accounted for 80 ± 8% of total metabolites analyzed (Figure 2B). The high expression and activity of sulfotransferases in enterocytes has been described (33). In addition, the expression of the main sulfotransferase isoform involved in the conjugation of phenolic compounds (sulfotransferase 1A) is higher in the...

FIGURE 5. A: Total amount of aglycone and metabolite forms in bile; B: Mean (±SEM) of aglycone and metabolite forms in bile at different time points. n = 6. C: Individual metabolite profile of conjugated forms in bile during the initial 2.5 h after administration of 50 mg (–)-epicatechin into an isolated 20-cm jejunal segment. 3′-G, 3′-S, and 3Me-SS conjugates were significantly higher than the rest of the metabolites (31 ± 22%, 24 ± 11%, and 24 ± 11% of the total, respectively) (P < 0.0001; 1-factor ANOVA). V, volunteer; 3′-G, 3′-O-β-D-glucuronide-epicatechin; 3′-Me-4′S, 3′-O-methyl-4′-O-sulfate-epicatechin; 3Me-SS, 3′-O-methyl-5′-O-sulfate-epicatechin; 3′-Me-7S, 3′-O-methyl-7′-O-sulfate-epicatechin; 3′-S, 3′-O-sulfate-epicatechin; 4′-G, 4′-O-β-D-glucuronide-epicatechin; 4′-Me-SS, 4′-O-methyl-5′-O-sulfate-epicatechin; 4′-Me-7S, 4′-O-methyl-7′-O-sulfate; 4′-S, 4′-O-sulfate-epicatechin; 5-SS, 5′-O-sulfate-epicatechin; 7-G, 7′-O-β-D-glucuronide-epicatechin; 7-S, 7′-O-sulfate-epicatechin.

FIGURE 6. A: Mean ± SEM total concentrations of conjugate forms in plasma. n = 6. B: Total amount of conjugate forms (as aglycone equivalents) in urine during the initial 24 h after administration of 50 mg (–)-EC into an isolated 20-cm jejunal segment of 6 healthy volunteers. EC, epicatechin; V, volunteer.
intestine than liver (33). These data support the concept that the conjugation with sulfate groups by the gut wall is a major metabolic barrier to the absorption of (–)-epicatechin.

Plasma, biliary, and urinary (–)-epicatechin conjugate profiles differed considerably from the forms quantified in intestinal fluid (Figure 7). The proportion of (–)-epicatechin glucuronide conjugates ranged from 20\%\% to 73\%\% in plasma. During metabolism in the intestine, the conjugation position and chemical group attached have been proposed to define whether the resulting molecule is absorbed or excreted back into the lumen (34). This clear difference in plasma and bile, compared with intestinal fluid, supports this hypothesis because the conjugates in the intestinal lumen have been effluxed from enterocytes via the apical surface, and the conjugates in the blood and bile have been absorbed from the enterocytes on the basolateral side. In contrast, in vitro experiments with Caco-2 cells showed a similar profile of (–)-epicatechin conjugates in the apical (efflux) and the basal (absorption) compartment after treatment with (–)-epicatechin. This result was probably attributable to the quantitative differences in gene expression in carrier transporters, low expression, or even the absence of certain isoforms of uridine-5'-diphosphate glucuronosyl-transferases (35) and lactase phlorizin hydrolase (36) between human enterocytes and Caco-2 cells. Thus, this clone of Caco-2 cells is not a good model to study the conjugation profile of (–)-epicatechin.

A noteworthy differential absorption of (–)-epicatechin was observed in 6 volunteers. The intestinal perfusate analysis showed that the amount of (–)-epicatechin recovered was between 4.7 and 32 mg. Similarly, the amount of (–)-epicatechin metabolites detected in bile and urine ranged from 2.6 to 18 µg and 1.2 to 5.9 mg, respectively (Figures 5A and 6B). The different rate of metabolism could be one of the most important variables for determining the overall absorption of polyphenols. A detailed analysis of the metabolite profile revealed a similar percentage of each form produced when the 6 volunteers were compared. For example, despite the different amount of (–)-epicatechin recovered from the intestinal perfusates, the percentage of total metabolites ranged only from 1.6\% to 6.6\% of the total aglycone form. An additional investigation of the precise molecular mechanisms that drove the production of the different types of conjugated forms will be relevant to understand the differences and similarities in the absorption of (–)-epicatechin. These investigations could potentially be useful for increasing the bioefficacy of certain polyphenols if one particular metabolite is especially bioactive.

The elimination of (–)-epicatechin metabolites by the intestine, bile, and urine accounted for 10.5 \(\pm\) 2.6\% of the total amount not shown in the perfusate. The fate of the missing (–)-epicatechin remains to be determined but could possibly have arisen from additional metabolism and excretion of absorbed compound and metabolites from the blood back into the gut lumen. Consequently, (–)-epicatechin and (–)-epicatechin metabolites were shown in adjacent segments in volunteers where the perfusion was placed in the distal and not proximal segment. This hypothesis was also supported by the higher amounts of (–)-epicatechin metabolites compared with aglycone shown in the adjacent segment and the different (–)-epicatechin metabolite profile between segments. On the reasonable assumption that this metabolism and efflux in the adjacent 20-cm segment could also have been occurring in the remainder of the intestinal wall, we inferred that some of the unaccounted (–)-epicatechin was lost by this mechanism.
Recently, (–)-epicatechin plasma metabolite profiles after oral consumption of chocolate (26) and cocoa drinks (37) have been described. 3′-O-β-D-Glucuronide-epicatechin accounted for ~32% and ~45% of total metabolites detected during the first 24 h after oral consumption of chocolate that contained 79 mg (–)-epicatechin (26) or 2 h after the oral ingestion of a cocoa drink that contained 1.8 mg (–)-epicatechin/kg body weight (37), respectively. Higher relative amounts of 3′-O-β-D-glucuronide-epicatechin (69%) were quantified in plasma samples after the intestinal perfusion of 50 mg purified (–)-epicatechin. The obvious difference in the metabolite pattern in plasma could have been a result of the route of administration (orally or intestinal perfusion) or treatment (food, beverage, or isolated compound). Properly designed experimental studies that evaluated the effect of forms of administration and food matrix by using the same dose and population group will help clarify how important these factors are for modifying the metabolite profile of (–)-epicatechin.

In conclusion, for the first time to our knowledge, the intestinal absorption and metabolism together with biliary and urinary excretion of pure (–)-epicatechin in healthy human volunteers have been studied. The results showed that approximately one-half of the amount of (–)-epicatechin that reaches the intestinal cells are absorbed with a percentage of metabolites (especially sulfate conjugates) eliminated by efflux into the intestinal lumen, and we showed a relatively modest elimination of (–)-epicatechin by bile. A potential absorption of (–)-epicatechin and elimination by efflux in another segment of the gut lumen was also observed in the current study. In addition, the data show a large inter-individual variability in the extent of absorption but much smaller differences in the variation of the overall metabolite profile.

We thank Sagar Thakkar, Frederic Destaillats, Francesca Giuffrida, and Fabiola Dionisi for their contribution to the current work.

The authors’ responsibilities were as follows—GW, MR, MS, and ME: designed the research; LA-G, AL, AT, CS, and UH: conducted the research; LA-G, AL, and HL: analyzed data; LA-G, MS, ME, and GW: wrote the manuscript; LA-G and GW: had primary responsibility for the final content of the manuscript; and all authors: read and approved the final manuscript. Some of the authors are (LA-G, AL, and MR) or were (HL and GW) employees of Nestle Ltd, which is a subsidiary of Nestle Ltd, and provides professional assistance, research, and consulting services for food, dietary, and pharmaceutical products of interest to Nestle Ltd. AT, CS, UH, MS, and ME had no conflicts of interest.

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