Consumption of both black tea and green tea results in an increase in the excretion of hippuric acid into urine\textsuperscript{1–4}

Theo P Mulder, Anton G Rietveld, and Johan M van Amelsvoort

ABSTRACT

Background: A major portion of the catechins in green tea is not absorbed in the small intestine. Bacteria in the colon convert non-absorbed catechins into simpler phenolic compounds, which may also be absorbed. During the production of black tea, most catechins are polymerized to complex molecules called thearubigins. Little is known about the microbial degradation of these complex polyphenols, but hippuric acid has been identified as a major excretion product associated with black tea consumption.

Objective: To investigate whether green tea and black tea have the same metabolic fate in humans.

Design: Seventeen healthy male volunteers were studied with a randomized, full-crossover design. Each intervention period lasted 4 d, ie, a 2-d run-in period with a low-polyphenol diet followed by a 2-d treatment period. Volunteers consumed a daily dose of 6 g green tea solids, 6 g black tea solids, or 360 mg caffeine. Intervention periods were separated by a 10-d washout period. Twenty-four-hour urine samples were collected during the second day of each treatment period. Hippuric acid was analyzed with HPLC-tandem mass spectrometry.

Results: The mean excretion of urinary hippuric acid during black tea and green tea consumption was 3.75 ± 0.28 mmol/24 h and 4.22 ± 0.28 mmol/24 h, respectively (95% CI for the difference: −0.37 to +1.30 mmol/24 h). The hippuric acid excretion during the control treatment was much lower (1.89 ± 0.28 mmol/24 h; \( P < 0.0001 \), compared with both black tea and green tea).

Conclusion: The ingestion of either green tea or black tea results in a major increase in the excretion of hippuric acid into urine.


KEY WORDS

Tea, flavonoid, catechin, theaflavin, thearubigin, metabolism, benzoic acid, hippuric acid

INTRODUCTION

Flavonoids are very common in the human diet, and high concentrations can be found in, for example, red wine, beer, chocolate, and tea. Green and black teas originate from the leaves of \textit{Camellia sinensis}. The leaves of tea plants contain large amounts (10–25% dry weight) of monomeric flavonoids called catechins. Green tea is made by inactivating the enzymes in the freshly picked leaves. Black tea is produced from fresh green leaves through a process called fermentation. In an enzymatic oxidation, catechins are condensed into theaflavins (dimers) and thearubigins (polymers). Approximately 10% of the flavonoids in black tea are catechins, 10% are theaflavins, and 70% are thearubigins (1, 2). The molecular structure of the thearubigins has not been completely resolved (1, 3).

It has been shown that catechins and theaflavins are absorbed from the intestine (4–7). However, only a small amount of the oral dose can be recovered from urine. The fate of the bulk of the catechins and theaflavins is unknown (8). The bioavailability of the thearubigins has not been studied because of the lack of suitable analytic methods.

Flavonoids that are not absorbed in the small intestine are metabolized by the bacterial flora in the colon (9–11). Experiments with cultured intestinal bacteria have demonstrated that fission of the central C3 ring of catechins is mediated by colonic microorganisms (Figure 1). This type of fission is decisive for the basic structure of the resulting metabolites, ie, hydroxyphenyl-\(\gamma\)-valerolactones and phenolic acids (10). These metabolites are absorbed from the colon, and their urinary concentrations exceed that of the intact flavonoid (12–15).

The microbial degradation of theaflavins and thearubigins has been studied much less, but it is possible that these yield very similar metabolites. Clifford et al (16) used \(^1\)H nuclear magnetic resonance spectroscopy to analyze the urine of healthy volunteers before and after consumption of black tea. They reported black tea consumption to be associated with a 3-fold increase in the urinary excretion of hippuric acid. Hippuric acid excretion after green tea consumption has not been studied, but hippuric acid may be a major metabolite produced from green tea catechins. The aim of this study was to compare the urinary excretion of hippuric acid after consumption of the same amounts of green tea or black tea.

SUBJECTS AND METHODS

Subjects

The protocol for the study and the information brochure for the healthy volunteers were approved by the Medical Ethical Committee of the Nederlandse Unilever Bedrijven BV. Male non-smoking volunteers were recruited through written invitations sent to male participants of previous studies. The men had been recruited initially through advertisements in the local newspapers. Inclusion criteria were as follows: age of 18–70 y, body

\textsuperscript{1} From the Unilever Health Institute, Unilever Research and Development, Vlaardingen, The Netherlands.

\textsuperscript{2} Presented at the 1st International Conference on Polyphenols and Health, held in Vichy, France, November 18–21, 2004.

\textsuperscript{3} Supported by Unilever Health Institute.

\textsuperscript{4} Reprints not available. Address correspondence to TP Mulder, Unilever Health Institute, Unilever Research and Development, PO Box 114, 3130 AC Vlaardingen, The Netherlands. E-mail: theo.mulder@unilever.com.
mass index of 18–32 kg/m², consumption of alcoholic beverages of < 28 glasses/wk, habitual coffee or tea consumption, < 10 h/wk of intense sporting activities, not on a specific diet, not taking flavonoid-containing supplements, no prescribed medication, and had not participated in a biomedical trial for 3 mo before the start of the study. Of 48 eligible men, 39 apparently healthy volunteers were selected on the basis of these inclusion criteria, a medical history questionnaire, and several routine clinical laboratory values measured in blood, serum, and urine. Finally, 18 men were assigned by lot to participate in the study, and 3 served as backup subjects during the run-in period. The protocol was fully explained to these 21 men, and all gave their written informed consent before the start of the study.

One of the volunteers withdrew from the study during the first run-in period, for personal reasons. He was replaced with the first backup subject. Another participant withdrew from the study during the first intervention, because he judged the taste of the study beverage to be unacceptable. He was not replaced. One subject reported the use of the cholesterol-lowering drug pravastatin (3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitor) in a routine questionnaire assessment after completion of the first intervention period. He had been taking the drug at a stable dose for several years but had “forgotten” to mention it during the screening visit. No effect of pravastatin on the measures studied was found in the medical literature; therefore, it was decided that the subject would not be excluded from the study. Seventeen participants completed the study. The characteristics of these 17 male participants were as follows (means ± SD): age, 59.2 ± 11.5 y; height, 1.80 ± 0.09 m; body weight, 80.4 ± 10.4 kg; body mass index, 24.8 ± 2.9 kg/m².

**Experimental design**

The subjects were studied with a randomized, full-crossover design with 3 treatments, ie, green tea solids, black tea solids, and caffeine (placebo). Each intervention period lasted 4 d, with a 2-d run-in period with a low-polyphenol diet followed by a 2-d tea treatment period. An overview of the study design is presented in Table 1. Intervention periods were separated by a washout period of almost 10 d, during which the volunteers consumed their habitual diets without any restrictions or additions.

Tea solids were prepared by extracting black or green tea (Lipton Research Blends, Lipton Englewood Cliffs, NJ) with boiling water and spray-drying the resulting extracts. The black and green tea solids were provided as prepacked 1-g portions. Volunteers were instructed to dissolve one portion of tea solids in hot water, to add sugar and milk according to their liking, and to drink the beverage while it was still warm. Volunteers were asked to consume 6 of these 1-g portions per day, at 2–3-h intervals (equivalent to ~12 cups of tea per day).

During the control intervention, a daily dose of 360 mg caffeine (USP quality; Sigma-Aldrich, Zwijndrecht, The Netherlands) was supplemented in 6 gelatin capsules, each containing

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**TABLE 1**

<table>
<thead>
<tr>
<th>Study design</th>
<th>Sunday</th>
<th>Monday</th>
<th>Tuesday</th>
<th>Wednesday</th>
<th>Thursday</th>
<th>Friday</th>
<th>Saturday</th>
</tr>
</thead>
<tbody>
<tr>
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<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Stop, 07:00</td>
<td>No</td>
</tr>
<tr>
<td>Main hot meal supplied</td>
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<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Supplement</td>
<td>No</td>
<td>Caffeine</td>
<td>Caffeine</td>
<td>Test article</td>
<td>Test article</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Urine collection</td>
<td>No</td>
<td>No</td>
<td>Start, 07:00</td>
<td>Stop, 07:00</td>
<td>Start, 07:00</td>
<td>Stop, 07:00</td>
<td>No</td>
</tr>
<tr>
<td>Blood collection</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>~16:00</td>
<td>No</td>
<td>No</td>
</tr>
</tbody>
</table>

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*The scheme was repeated three times, with 1 wk free of dietary restrictions between the intervention weeks.*
60 mg caffeine. This amount of caffeine was equivalent to that in the tea solids. Volunteers were instructed to ingest the capsules at 2–3-h intervals during the day.

For the low-polyphenol diet, volunteers were instructed to refrain from drinking coffee, tea, fruit juices, beer, or wine and from eating onions, kale, broccoli, applesauce, or chocolate during the treatment periods. To compensate for the low caffeine content of the low-polyphenol diet, a daily dose of 360 mg caffeine was supplemented during the run-in days. The main hot meals were provided as commercial frozen meals (IgloMora Group, 's-Hertogenbosch, The Netherlands) with low polyphenol contents. Dietary records were maintained during all 3 intervention periods. Volunteers were asked to replicate the diet that they consumed on the 2 run-in days during the subsequent 2 treatment days. They were also encouraged to repeat their diet from the first intervention during subsequent interventions. A printed copy of their first dietary record was given to them as a reminder.

Urine collection

Twenty-four-hour urine samples were collected both during the second day of the run-in period and during the second day of the treatment period (last day of the intervention). Urine was collected into 500-mL polystyrene flasks containing 3.5 mL of a 50% (by vol) solution of m-phosphoric acid. The pH of 24-h urine samples was adjusted to a value between 2.0 and 3.0 with 50% (by vol) m-phosphoric acid solution. Samples of the acidified urine were stored at −20 °C until analysis, within 2 mo. Creatinine concentrations in urine were analyzed with an Hitachi 912 clinical chemical analyzer (Roche, Almere, The Netherlands).

Analysis of hippuric acid in urine

Because preliminary experiments indicated that the concentrations in some of the samples were below the detection limit of the routine HPLC-ultraviolet method, hippuric acid concentrations in urine were analyzed with HPLC-tandem mass spectrometry. HPLC–electrospray-ionization tandem mass spectrometry was performed by injecting 20 μL of urine that had been diluted 20-fold in mobile phase onto an Xterra C18 column (150 mm, 5 μm; Waters, Milford, MA). The isocratic mobile phase consisted of 20 mmol/L ammonium acetate:acetonitrile (92:8, by vol), pH 2.8, at a flow rate of 0.3 mL/min. 4-Aminohippuric acid was used as an internal standard. Detection was performed with a Scieix API 3 Plus triple quadrupole (Sciex/Perkin Elmer/ Applied Biosystems, Gouda, The Netherlands) with turbo-ion spray in positive mode. Tandem mass spectrometry parameters were as follows: source temperature, 500 °C; ion spray, 5500 V; orifice, 37 V; nebulizing gas and curtain gas (both nitrogen) flow rates, 50 and 1.2 L/h, respectively. The mass spectrometry data were collected with multiple-reaction monitoring, with a dwell time of 200 ms, a pause time of 49 ms, and m/z ratios for the parent and daughter ions of 179.9 and 105.1 for hippuric acid and 195.1 and 119.9 for 4-aminohippuric acid (internal standard), respectively.

Calibration curves were obtained with the standard-addition method, because preliminary experiments indicated variable recovery when external calibration was used. Samples were spiked with 0, 0.5, 2.0, 3.5, or 5.0 μmol/L hippuric acid and 50 μmol/L 4-aminohippuric acid. The lower limit of quantification was not assessed, but the sample with the smallest amount produced a signal > 10 times the background noise. Within- and between-assay CVs were 13% and 18%, respectively.

Analysis of catechins in plasma

To check for compliance, blood was collected in EDTA-containing tubes on the second day of each tea treatment, at ~16:00. The concentrations of catechin, epicatechin, epigallocatechin, epicatechin gallate, and epigallocatechin gallate in plasma were measured essentially as described previously (7).

Analysis of tea samples

Catechins, theaflavins, gallic acid, and caffeine were analyzed with HPLC with diode-array detection. Flavonol-glycosides were converted to the corresponding free aglycones (quercetin, kaempferol, and myricetin) with acid hydrolysis before analysis. Total polyphenol content was analyzed with the Folin-Ciocalteu method (17), with gallic acid as a standard.

Statistical analyses

Statistical analyses were performed with analysis of variance, with treatment as a factor, urinary hippuric acid excretion at the end of the run-in period as a covariable, and subjects and period as blocks. Differences between treatments and 95% CIs were established with Tukey’s test. The SAS version 8.2 software package (SAS Institute, Cary, NC) was used to perform the calculations. Two-sided P values were considered significant at P < 0.05. Urinary hippuric acid excretion and plasma catechin concentrations are reported as least-squares mean ± SEM.

RESULTS

Tea solids

Green tea solids contained 57 mg/g dry weight caffeine, and black tea solids contained 55 mg/g. The total amounts of chemically well characterized polyphenols (ie, catechins, theaflavins, flavonols, and free gallic acid) in the green tea solids and the black tea solids were 0.90 and 0.28 mmol/g, respectively (Table 2). Concentrations of total polyphenols (including thearubigins)
were estimated with the Folin-Ciocalteu assay. Results were 2.21 and 1.51 mmol/g of gallic acid equivalents for the green tea solids and the black tea solids, respectively.

**Hippuric acid in urine**

Mean excretions of urinary hippuric acid were not significantly different during consumption of black tea (3.75 ± 0.28 mmol/24 h) and green tea (4.22 ± 0.28 mmol/24 h; 95% CI of the difference between green tea and black tea: −0.37 to +1.30 mmol/24 h) (Figure 2). Hippuric acid excretion during the control treatment was less than one-half of those values (1.89 ± 0.28 mmol/24 h; *P* < 0.0001, compared with black tea and compared with green tea). Individual increases in hippuric acid excretion attributable to the consumption of green or black tea (tea control) also were not significantly different (Figure 3). Amounts of hippuric acid excreted during the second day of the run-in period with the low-polyphenol diet (1.87 ± 0.15 mmol/24 h; 51 samples) were not significantly different from the quantities excreted during the control treatment with caffeine, indicating that urinary hippuric acid concentrations had reached low stable values after 35 h with the low-polyphenol diet.

Results were also calculated as millimoles of hippuric acid per mole of creatinine, to correct for small mistakes made during urine collection (2 urine samples missed, 2 samples collected in the wrong container, and 8 mistakes made in the timing of collection were reported). Results were 311 ± 26, 335 ± 26, and 150 ± 26 mmol hippuric acid/mol creatinine during the black tea, green tea, and control supplementation, respectively (*P* < 0.0001, control compared with black tea and compared with green tea).

The excretion of hippuric acid during the control period varied among individual volunteers by a factor of almost 10 (Figure 2). The responses to the tea treatments also varied extensively, but the consumption of both kinds of tea solids resulted in increases in the urinary excretion of hippuric acid for most volunteers. Hardly any effect of either type of tea on urinary hippuric acid excretion was noticed for 2 volunteers, however, and green tea had a much larger effect than did black tea for 2 other volunteers.

**Catechins in plasma**

Mean plasma catechin concentrations (compliance check) during the control, black tea, and green tea interventions were 20 ± 1, 112 ± 12, and 765 ± 122 mmol/L, respectively. For all volunteers, the lowest plasma catechin concentration was found in the sample collected during the control period and the highest concentration was detected after the green tea intervention, consistent with the much greater amounts of catechins in green tea.

**DISCUSSION**

In this study, the mean urinary excretion of hippuric acid by 17 healthy human volunteers increased by 1.87 and 2.34 mmol/24 h after the consumption of black or green tea solids (6 g/d), respectively. Clifford et al (16) were the first to note the massive increase in urinary hippuric acid excretion after black tea consumption among humans. Among the 9 volunteers they studied, urinary hippurate excretion increased by 1.50 mmol/24 h after the consumption of 8 mugs of black tea per day. The urinary excretion of hippuric acid by the 20 volunteers studied by Olthof et al (14) increased by 1.90 mmol/24 h, on average, after consumption of 4 g of black tea solids per day. The urinary excretion of some phenolic acids [3-hydroxybenzoic acid, 3,4,5-trihydroxybenzoic acid, 3-hydroxyphenylacetic acid, 3,4-dihydroxyphenylacetic acid, 3,4-dihydroxyphenylpropionic acid, and 3-(3,4-dihydroxyphenyl)propionic acid] also increased (14); for most of those phenolic acids, however, the increase attributable to the tea supplementation was only on the order of 0.005–0.050 mmol/24 h. The hydroxyphenyl-γ-valerolactones may account for 6–39% of the dose of catechins ingested in green tea.
of simple polyphenols in the daily dose of 6 g of black tea was calculated and arrived at a similar conclusion; the total amount consumed both green tea and black tea, in a crossover design. Consumption of the 2 types of tea solids resulted in comparable increases in urinary hippuric acid excretion, indicating similar extents of fermentative degradation.

Clifford et al (16) calculated the total amounts of catechins, theaflavins, gallic acid, and flavonols consumed by their volunteers and concluded that these simple polyphenols could not fully account for the increased urinary excretion of hippuric acid. Consequently, they suggested that the complex thearubigins were also converted to hippuric acid. We performed the same calculation and arrived at a similar conclusion; the total amount of simple polyphenols in the daily dose of 6 g of black tea was 1.65 mmol/24 h, compared with a mean increase in hippuric acid excretion during the black tea intervention of 1.87 mmol/24 h. Our results obtained with green tea indicated that only ~45% of the catechins and flavonols were converted to hippuric acid, ie, 5.43 mmol/24 h catechins and flavonols consumed, resulting in additional excretion of 2.34 mmol/24 h urinary hippuric acid. Thearubigins cannot be analyzed because of their complex nature (3). However, the Folin-Ciocalteu assay can be used to measure the total content of polyphenols, including thearubigins, in tea samples relative to a standard such as gallic acid. The daily dose of polyphenols in the black tea solids was 9.08 mmol gallic acid equivalents, and the corresponding increase in hippurate excretion in urine was 1.86 mmol/24 h (20%). For green tea, the corresponding values were 13.3 mmol gallic acid equivalents consumed per day, resulting in an increase in urinary hippurate excretion of 2.33 mmol/24 h (18%). Therefore, black tea and green tea consumption had comparable effects on urinary hippuric acid excretion. These results indicate that the lactones and phenolic acids produced from black tea and green tea polyphenols in the human colon are probably quantitatively similar, irrespective of the flavonoid source from which they are derived.

The health benefits of dietary flavonoids have often been attributed to their antioxidant activities, but the microbial metabolites of dietary flavonoids have lower antioxidant activities than do their parent compounds (18). The lower antioxidant activity, however, may be offset by greater bioavailability for these smaller molecules. In addition, dietary flavonoids may have significant effects on the colonic flora (19) and thus confer a type of prebiotic effect. In this respect, it has been noted that not only tea drinking but also wine (15), cider (20), and coffee (14) consumption can result in increases in urinary hippuric acid excretion, indicating that polyphenols from different dietary sources may have similar effects on the colonic flora.

In conclusion, green tea consumption and black tea consumption result in similar amounts of microbial degradation products that are absorbed by the body. These microbial metabolites, and not the native tea flavonoids, may be responsible for at least some of the health effects attributed to tea consumption.

REFERENCES