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Nuclear Magnetic Resonance Spectroscopic Based Studies of the Metabolism of Black Tea Polyphenols in Humans

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Epidemiological studies indicate that a high intake of flavonoids is associated with an improved health status. Tea is one of the most abundant sources of flavonoids in the human diet. The bioavailability and biotransformation of tea flavonoids are, however, not clearly understood. The aim of the present study was to investigate the metabolism of black tea via a nonspecific screening method. ^{1}H nuclear magnetic resonance (NMR) spectroscopy was used to obtain nonselective profiles of urine samples collected from three human volunteers before and after a single dose of black tea. The complex spectroscopic profiles were interpreted with the use of pattern recognition techniques. Hippuric acid was confirmed as the major urinary black tea metabolite. One previously unknown metabolite was detected and identified as 1,3-dihydroxyphenyl-2-*O*-sulfate (sulfate conjugate of pyrogallol) using HPLC directly coupled to mass spectrometry and ^{1}H NMR spectroscopy. This study shows that NMR-pattern recognition studies can be used for the discovery of unknown flavonoid metabolites in humans.

KEYWORDS: Metabonomics; NMR spectroscopy; urine; black tea; polyphenols; phenolic acids; principal components analysis; metabolism; bioavailability

INTRODUCTION

Tea is a fragrant brew prepared from the leaves of two varieties of *Camellia sinensis*: *assamica* and *sinensis*. It is one of the most highly consumed beverages in the world. Fresh tealeaf is rich in water-soluble flavonoids, in particular, catechins (flavanols) (**Figure 1**) and flavonol glycosides (*1*). Many studies have indicated that dietary polyphenolic compounds provide beneficial antioxidant effects (*1*−*3*). Unlike green tea, black tea is the result of a controlled fermentation process in which mechanical maceration of tea shoots triggers enzyme catalyzed oxidation and partial polymerization of the tea catechins. The resulting condensed flavonoids known as theaflavins and thearubigins are responsible for the characteristic taste and color of black tea (*1*). Studies with green tea (*4*) and pure catechins (*5*) have shown that conjugates of the parent compounds can be detected in plasma. Very low levels of theaflavins in plasma and urine were also reported (*6*). The bioavailability of the thearubigins has not been studied because this class of compounds is still largely uncharacterized (*1*).

Only a few percent of an oral dose of catechins can be recovered from urine (*5*, *7*). The bacterial flora in the colon metabolizes flavonoids that were not absorbed in the small intestine (*8*−*10*). Fission of the central ring of catechins results in metabolites such as hydroxyphenyl-γ-valerolactones and phenolic acids (*9*). Some of these metabolites are absorbed and excreted into the urine (*11*−*13*). Clifford et al. (*14*) used proton nuclear magnetic resonance (^{1}H NMR) spectroscopy to monitor the urinary metabolite profiles of healthy volunteers on a low polyphenol diet before and during consumption of black tea. They reported that tea consumption resulted in a 3-fold increase in the urinary excretion of hippuric acid.

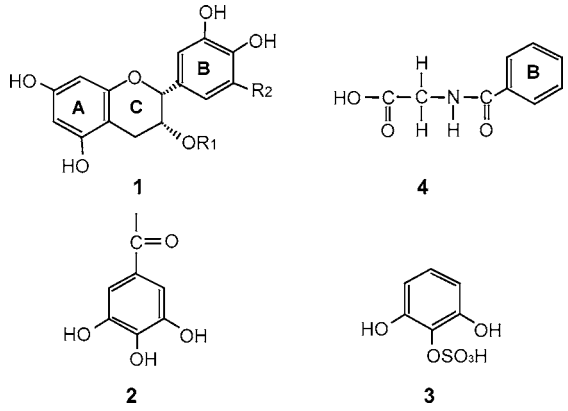

Figure 1. Basic structure of the flavonoid (–)-epicatechin (**1**; epicatechin R1 and R2 = H; epicatechin gallate R1 = gallate and R2 = H; epigallocatechin R1 = H and R2 = OH; and epigallocatechin gallate R1 = gallate and R2 = OH), gallate (**2**), 1,3-dihydroxyphenyl-2-*O*-sulfate (**3**), and hippuric acid (**4**).

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NMR spectroscopy can be used to obtain nonselective, information-rich metabolite profiles of biological samples with minimal sample preparation (*15*−*18*). A limiting factor in understanding the information from NMR spectra of biological samples is their complexity. Because of the large number of metabolites, signal overlap and crowding arises in the ^{1}H NMR spectra. The complex spectroscopic profile can be interpreted more efficiently by using automated data reduction and pattern recognition (NMR-PR) techniques (*18*−*21*). Automatic data reduction techniques convert the NMR spectra into a set of spectroscopic integrals, which are used as descriptors for principal components analysis (PCA). This approach eliminates the necessity of making a priori assumptions as to the relative importance of urinary metabolites in characterizing the metabolic effect(s) of interest. So far, the combination of NMR spectroscopy and pattern recognition has been applied successfully in the understanding of disease processes (*22*−*24*), toxicological processes (*25*−*28*), and the phenotypic outcome of gene expression (*29*−*31*). Most of these studies were applied to animal and cell culture models. The inherent metabolic variation in healthy people is typically large, and the effects of food ingredients on their metabolic profile are usually subtle in comparison to the effects of drugs or disease. In the present study, we address this challenge and investigate the metabolism and bioavailability of black tea components in healthy human volunteers via a nonspecific screening method.

MATERIALS AND METHODS

Samples. Healthy volunteers (one male and two females; mean age 38.3 years, range 33−44 years; mean body mass index 23.46, range 22.7−23.8; regular tea and coffee drinkers) were requested to minimize their consumption of polyphenol-rich foods (e.g., fruits, vegetables, herbs, spices, soy products, tea, coffee, beer, and wine) in their diet for five consecutive days. Midnight (0:00) at the start of first day of the low polyphenol diet was taken as the start of the study. Dietary records were kept during these five days and used to check compliance. At 09:00 h, after breakfast on day 4, each volunteer consumed 3 g of decaffeinated black tea extract (Lipton, Englewood Cliffs, NJ). This amount is equivalent to about 6 cups of black tea. All spontaneously voided urine was collected into separate plastic containers containing 5 mL of 0.2 N HCl to retard bacterial growth and ensure metabolite stability. The urine volume was recorded, and samples were stored at 4 °C until ^{1}H NMR spectroscopic analysis, which was carried out within 24 h of sample collection.

^{1}H NMR Spectroscopy. Samples were prepared for ^{1}H NMR spectroscopy by the pH of the urine sample being adjusted to 2.5 by addition of HCl (2 N). Acidified urine (0.5 mL) was then taken and placed in a 5 mm o.d. NMR tube. To each sample, 50 μL of deuterium oxide containing 1.9 g/L trimethylsilyl [2,2,3,3-$^2H_4$] propionate (TSP) was added. Spectra were obtained using a Bruker DMX 600 spectrometer operating at a 600.13 MHz ^{1}H-resonance frequency and equipped with a triple resonance (^{1}H, ^{13}C, ^{15}N) probe with *z*-gradients. Spectra were measured at 303 K using a standard water presaturation pulse sequence (i.e., RD-90°-t_1-90°-t_1-90°-acquire free induction decay, where 90° represents a nonselective 90° RF pulse, RD is a relaxation delay of 10 s during which the water resonance was selectively irradiated, and t_1 corresponds to a fixed interval of 3 μs). A total of 128 transients was collected into 32 000 data points with a spectroscopic width of 7000 Hz. Prior to Fourier transformation, the free induction decay was multiplied by an exponential weighting function corresponding to 0.3 Hz line broadening and zero-filled by a factor of 2. Spectra were referenced to the primary internal chemical shift reference, TSP at δ 0.0, and manually baseline corrected using XWINNMR (version 2.1, Bruker GmbH, Germany).

NMR Spectroscopic Data Reduction. To detect changes in urine due to black tea consumption, the aromatic region (δ 9.0−6.0) of the ^{1}H NMR spectra was studied. Data were reduced to ASCII format using AMIX (Analysis of MIXtures, version 2.8, Bruker GmbH, Germany). Each NMR spectrum was reduced to 75 discrete regions of equal width (0.04 ppm), and the integral of each region was determined (*27*). The resulting table of intensity information was then exported to Microsoft Excel version 97. Each region was normalized to the total area to remove the effects of concentration differences between the samples.

Principal Components Analysis (PCA). The Excel spreadsheet of the data-reduced NMR spectra was imported into Simca-P (version 7.01, Umetrics, Umeå, Sweden) for PCA. PCA is a data visualization method that is useful for overviewing relationships or groupings within multivariate data (*32*). Any prior knowledge relating to class membership is not used in the PCA analysis. The data are represented in *K*-dimensional space (*K* is the number of variables) and are subsequently reduced into a few principal components (PC). Successive PCs are orthogonal and describe successively less of the variance. The PC loadings or eigenvectors are the coefficients of the linear combinations of the original variables found by PCA.

^{1}H NMR spectral profiles of human urine samples are more diverse than, for example, rat samples (*33*). By mean-centering (subtracting of the average from each variable so that the mean for each variable is 0) the data individually for each volunteer, the effects of interperson variability are reduced. Therefore, PCA was performed on data that were mean-centered for each individual volunteer.

PCA analysis was repeated using data that had been mean-centered and scaled to unit variance. This method allows subtler detection but is also more sensitive to artifacts. In this case, scaling to unit variance showed no additional information from that gained by analysis using mean-centered data alone. Therefore, all data presented here are based on mean-centered data with no further scaling.

Quantification of Urine Metabolites. Quantification of altered exogenous excreted metabolites was achieved by integration of ^{1}H NMR peaks. ^{1}H NMR can be used to analyze a solution for the relative concentrations of the different components because the area under each resonance is directly proportional to the number of nuclei giving rise to that resonance. Resonances from these metabolites were integrated along with those from the internal standard, TSP. The amount of each metabolite was then calculated in mmol/L using the equation $C_m = (I_m/I_s)(N_s/N_m)C_s$, where m and s represent metabolite and standard, respectively, I is the peak area integral, N is the number of protons, and C is the concentration in mmol/L. The calculated concentration of each metabolite was then multiplied by the total volume of the appropriate urine sample to provide the absolute number of mmol of each metabolite per sample.

Quantification of Polyphenols in the Tea Extract. ^{1}H NMR spectroscopic analysis of the tea extract used in the study also allowed quantification of the tea polyphenols. The following polyphenols were quantified: catechins, theaflavins, gallic acid, caffeine, and theobromine. Since thearubigins are as yet uncharacterized components, their concentration was quantified by the difference between the total phenol content and the concentrations of those components characterized. A nominal molecular weight of 600 Da was assumed for thearubigins to calculate the moles of polyphenols taken in via black tea.

Isolation and HPLC NMR−MS Identification of Metabolite M1. The urine sample that contained the highest level of the unidentified black tea metabolite, hereafter referred to as metabolite 1 (M1) was taken and used for all further analysis. The presence and percentage of recovery of M1 after each stage of the cleanup procedure was determined by ^{1}H NMR.

Acidified urine (1 mL) was taken and precipitated with acetone (4:1 v/v, analytical grade, Merck) to remove inorganic salts and small proteins. The resultant suspension was centrifuged (20 min, 1000*g*), and the supernatant was retained, dried under nitrogen, and reconstituted in deionized H_2O (10 mL). This solution was separated into 10 aliquots, which were loaded onto Sep-Pak C18, 500 mg, solid-phase extraction cartridges (Waters, Milfort, MA) that had been activated with methanol and equilibrated with H_2O. The columns were eluted with 100% H_2O (1 mL), 90:10 (v/v) H_2O/methanol (1 mL), 50:50 (v/v) H_2O/methanol (1 mL), and 100% methanol (1 mL). M1 was found to consistently elute in the 90:10 (v/v) H_2O/methanol fraction with approximately 80% recovery. This fraction from each cartridge was combined, freeze-dried, and reconstituted in H_2O (1 mL). This sample was subjected to two additional HPLC purification steps, using a Hewlett-Packard series 1100

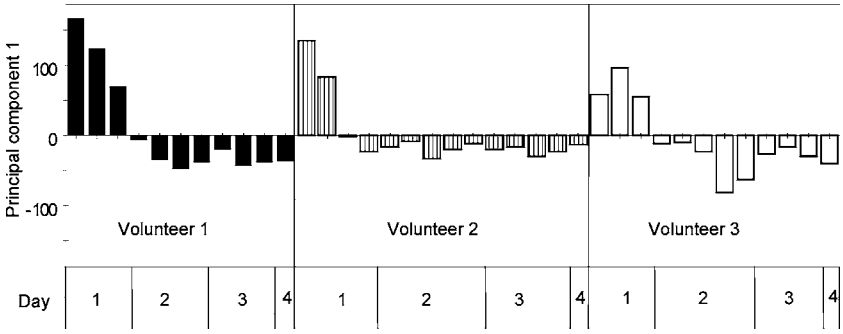

Figure 2. Principal component 1 (PC1) scores plot of all spontaneously voided urine samples collected between the start of the low polyphenol (midnight of day 1) diet and the tea consumption (9:00 AM at day 4). The first two to three urine samples collected after the start of the low polyphenol diet differ from the other samples collected during the dietary intervention.

instrument with a diode-array detector set to 254 nm (Agilent, Amstelveen, The Netherlands). In the first step, 100 μL of the 1 mL sample was injected onto a 250 × 4.6 mm i.d., 3 μm, Prontosil C30 reversed phase column (MAC MOD, Analytical Inc., Chadds Ford, PA) and eluted using a linear deuterium oxide/acetonitrile gradient ranging from 95:5 to 5:95 v/v using deuterated formic acid (0.4%; v/v) as a modifier. The retention time of M1 under these separation conditions was established by HPLC−NMR, operated in loop-storage mode with a Bruker peak sampling unit equipped with 200 μL loops (Bruker GmbH, Karlsruhe, Germany) using a Bruker DRX 600 NMR spectrometer with a 4 mm, LC−NMR probe (120 μL flow cell). The WET pulse sequence was used for suppression of the solvent peaks. For each fraction, 2000 transients were collected into 16 000 data points with a width of 6000 Hz. After establishing the retention time, one fraction of 4 min was collected (2 min before and 2 min after the measured retention time). This procedure was repeated 10 times, and the subsequently collected fractions were combined, freeze-dried, and reconstituted in 100 μL of deuterium oxide. This sample was then analyzed using HPLC−NMR−MS. One hundred μL was injected onto a 250 × 4.6 mm i.d., 5 μm, Chromspher PI C18 reversed phase column (Varian, Middelburg, The Netherlands) and eluted isocratically using 96:4 v/v deuterium oxide/acetonitrile with 0.4% formic acid (v/v) as the mobile phase and a detection wavelength of 254 nm. An Acurate model ICP-04-20 splitter (LC Packings, Amsterdam, The Netherlands) directed 95% of the flow to NMR and the remaining 5% to a Hewlett-Packard series 1100 MSD with electrospray interface (Agilent, Amstelveen, The Netherlands). The NMR was operated in loop-storage mode, as described previously. Electrospray ionization mass spectrometry spectra were acquired in negative ionization mode in a mass range of *m*/*z* 50−800 and a fragmentor voltage of 100 V. Nitrogen was used as drying gas at a flow-rate of 7.0 L/min at 300 °C. The nebulizer pressure was set to 35 psi, and the capillary voltage was optimized to 3500 V.

RESULTS AND DISCUSSION

Effects of Dietary Restrictions on Urinary ^{1}H NMR Spectra. PCA on the aromatic region of all the pre-tea NMR spectroscopic data indicated that the first two to three samples collected during the low-polyphenol diet run-in were significantly different from all other pre-tea samples. PC1 described 94.4% of the total variance and indicated a sharp decrease in urinary hippurate concentration after the start of the low-polyphenol diet (**Figure 2**). Therefore, the first three samples from all the volunteers were removed from all subsequent analyses. The remaining samples were then studied for metabolic alterations due to diurnal variation (*34*). Taking 07:00−19:00 h as being a daytime period and 19:00−07:00 h as overnight, subtle alterations were apparent in the scores plot of PC3 versus PC4, accounting for 11.5 and 8.8% of the total variance in the data, respectively. The 12 samples collected during the overnight periods were therefore also removed from the multivariate analysis.

The remaining daytime samples collected more than 36 h after the start of the polyphenol diet but before the tea dose were taken as controls. Spectroscopic data of these pre-tea control period urine samples were then compared with those from post-tea urine samples (daytime samples only). Only results arising from the analysis of the aromatic region are presented here since we were interested in metabolites with an intact aromatic ring.

Effects of a Single Dose of Black Tea on Urinary ^{1}H NMR Spectra. PCA was used as a visualization tool to detect tea-related patterns, which were consistent for all three volunteers. The first six PCs were inspected, and these PCs described 96.5% of the total variance. Distinct tea-related metabolic changes were evident in the scores plots of PC1 and PC5 (**Figure 3**), which described 71.0 and 1.6% of the total variance in the data, respectively. PC2, PC3, and PC4 showed no consistent effect for all three volunteers.

Differences in PC1 related primarily to an increase in the intensity of spectroscopic regions from hippuric acid (double doublet δ7.82, triplet δ7.66, and triple triplet δ7.54) (**Figure 4**). This increase in hippurate levels, together with the decrease at the start of the low-flavonoid diet, confirms a previous report by Clifford et al. (*14*) that hippurate is the main metabolite of black tea in humans. Hippurate is thought to be produced by colonic microbes cleaving two of the three rings of the flavonoid structure (the A-ring and the heterocyclic C ring) (**Figure 1**), while the B-ring appears to remain intact resulting in metabolites such as hydroxyphenyl-γ-valerolactones, phenylpropionic acids, phenylacetic acid, and benzoic acid (*11*, *12*, *14*). Benzoic acid is absorbed and conjugated in the liver with glycine to give hippuric acid (*N*-benzoylglycine) (**Figure 1**). The increase in urinary hippuric acid excretion was not detected in volunteers without a colon (*12*), indicating that hippuric acid is formed as a result of metabolism by gut micro-flora.

PC1 also indicated further smaller increases in a number of other spectroscopic regions. These included δ6.58 (doublet, M1), δ7.02 (visualized as a multiplet in the 1-D ^{1}H NMR spectra, M1), δ7.18 (singlet, tentatively assigned as gallic acid by comparison with an authentic reference standard), δ7.2 (doublet, metabolite 2), and δ7.26 (doublet, metabolite 2, a para-substituted aromatic compound). The corresponding loadings plot for PC5 showed a clear increase in M1 (**Figure 4**). These observed changes are highlighted in **Figure 5**.

The maximum sample change observed in PC1 occurred in samples collected at 24 ± 2.5 h post-tea dose, but those observed in PC5 occurred in samples collected at 8.5 ± 1.5 h. This finding suggests that the effect of the tea consumption on the urinary profile occurs in two main cycles: a low concentration fast effect and a high concentration slow effect.

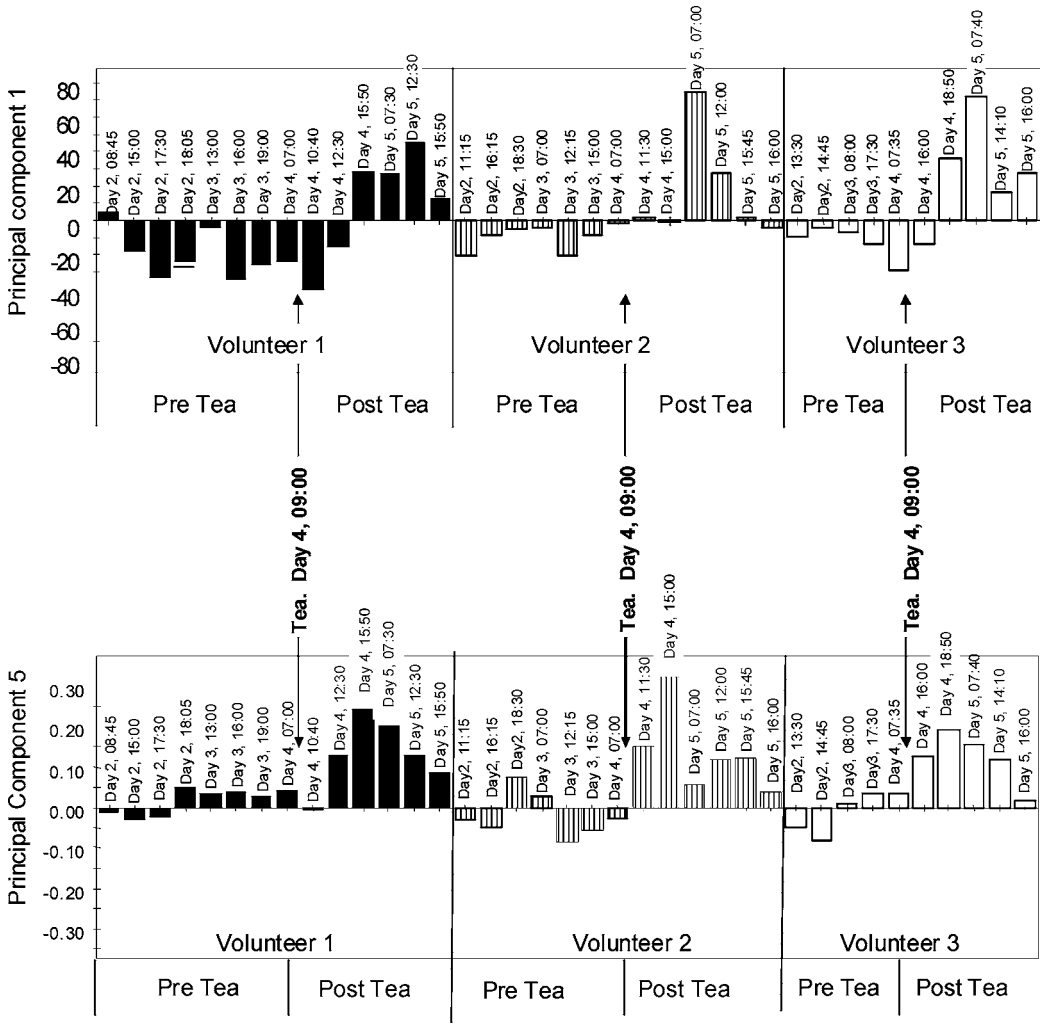

Figure 3. Scores plot of principal component 1 **(**PC1, top) and PC5 (bottom) highlighting the similarity of response from all three volunteers.

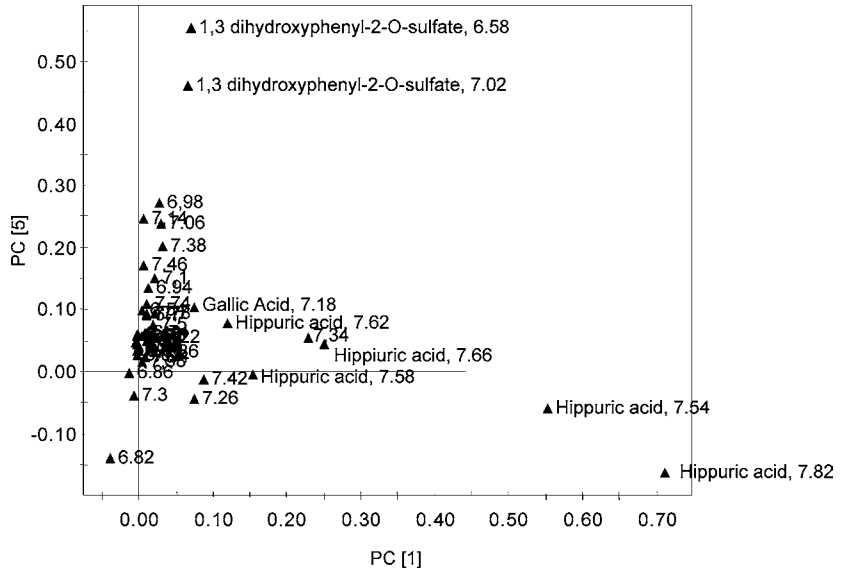

Figure 4. Loadings plot of principal component 1 (PC1) vs principal component 5 (PC5) illustrating that hippuric acid and 1,3-dihydroxyphenyl-*O*-2-sulfate were the compounds with the highest contribution in PC1 and PC5, respectively.

Identification of Metabolite M1 by Directly Coupled HPLC−NMR−MS. The loadings plot from PCA indicated a positive correlation between the doublet attributable to M1 at δ6.58 and another resonance at δ7.02. The coupling of these two resonances was confirmed by the application of ^{1}H−^{1}H total correlation spectroscopy (TOCSY) of the whole urine sample. The information relating to the multiplicity of the latter resonance, however, could not be determined from 1-D ^{1}H NMR data, due to extensive overlap and signal crowding. Furthermore, coupling to any other peaks in the ^{1}H NMR urine spectrum

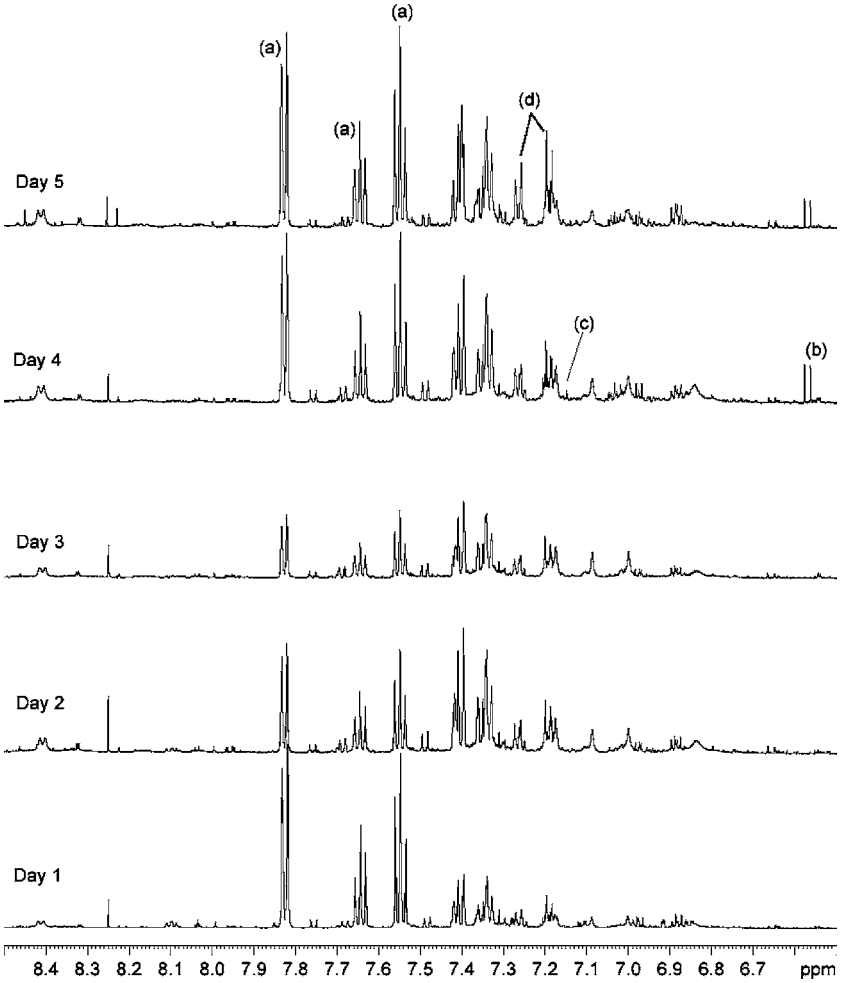

Figure 5. Aromatic region of ^{1}H NMR spectra δ(8.5–6.5) of 24 h urine samples from one of the volunteers, covering each of the five study days (a) indicates resonances from hippurate, (b) indicates resonances from 1,3-dihydroxyphenyl-*O*-2-sulfate, (c) indicates singlet resonance of gallic acid, and (d) unidentified metabolite Y (a para-substituted aromatic compound). Intensity (height) of all spectra was adjusted to the same scale using the trimethylsilyl [2,2,3,3-^{2}H$_4$] propionate signal.

was not established. Therefore, a selected urine sample was initially analyzed by HPLC−NMR with a view of establishing the spectroscopic identity of M1.

Initial directly coupled HPLC−^{1}H NMR data showed that M1 contained only two NMR detectable groups, a doublet at δ6.58 and a symmetrical triplet at δ7.02, indicating that M1 was a 1,2,3-trisubsituted symmetrical aromatic compound. An initial tentative assignment of this metabolite was pyrogallol (1,2,3-trihydroxybenzene) that has been reported in sheep as a metabolite of gallic acid (*35*). However, this was later disproved by standard addition of an authentic reference standard. Because of the lack of aliphatic NMR spectroscopic resonances attributable to M1, complete structural elucidation by HPLC−NMR alone was not possible. Furthermore, when HPLC−MS was utilized separately from HPLC−NMR, slight shifts in the chromatographic retention time of M1 prohibited a correlation of the NMR with the MS data. Therefore, directly coupled HPLC−NMR−MS was employed. The UV-detected peak of M1, with a retention time of 12.6 min was loop-stored and showed a doublet at δ6.60 and a triplet at δ7.06 in a purified 600 MHz ^{1}H NMR spectrum. The corresponding MS spectrum of M1 was extracted at the peak maximum of the total ion chromatogram (retention time 12.8 min). The MS spectrum indicated a deuterated molecular mass M* of *m*/*z* 209. A molecular mass of 206 Da was detected after solvent exchange, indicating three H/D-exchangeable protons in the molecule. Finally, MS fragments showed a loss of 80 m.u. corresponding to an SO_3^- group. Combining the results of the multiple detection system of on-line HPLC−NMR−MS, the structure of M1 is proposed as 1,3-dihydroxyphenyl-2-*O*-sulfate (**Figure 1**).

Most polyphenols excreted into urine are conjugated to glucuronide, sulfate, and methyl groups (*4*). Enzymes in the intestinal mucosa and in the liver perform the conjugation reactions (*36*). Consequently, 1,3-dihydroxyphenyl-2-*O*-sulfate is most probably produced by enzymatic conjugation of pyrogallol with sulfate. Meselhi et al. (*8*) showed that pyrogallol is a metabolite generated from both epicatechin gallate and gallic acid by human intestinal bacteria. Kohri et al. (*11*) reported that pyrogallol was the major metabolite detected in both plasma and urine of rats fed epigallocatechin gallate, but these authors analyzed samples that had been deconjugated by enzymatic treatment during which the sulfate-conjugated form was probably converted to the free, unconjugated pyrogallol. Our results show that the sulfated form of pyrogallol is also a major metabolite in humans. Hodgson et al. (*37*) indicated that 4-*O*-methylgallic acid can be used as a marker for black tea intake, and the levels they detect are also a few micromoles per day after drinking five cups of black tea per day (*37*, *38*). Our data, obtained in a few volunteers, indicated that 1,3-dihydroxyphenyl-2-*O*-sulfate is probably also a marker for black tea intake in humans, but the pharmacokinetic results demonstrate that the

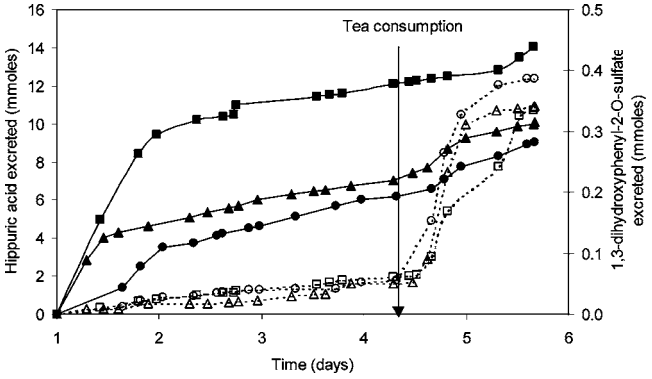

Figure 6. Cumulative urinary excretion of hippuric acid (solid line) and 1,3-dihydroxy-2-*O*-sulfate (dashed line) during the study from volunteer 1 (squares), volunteer 2 (triangles), and volunteer 3 (circles).

increase in urinary excretion of 1,3-dihydroxyphenyl-2-*O*-sulfate after tea consumption is only transient. Therefore, the metabolite can only be used to monitor consumption during 4−12 h after tea consumption.

Kinetics of Hippuric Acid and 1,3-Dihydroxyphenyl-2-*O*-sulfate. Following the observation of several dietary-related alterations within the ^{1}H NMR spectra, quantification of hippurate and 1,3 dihydroxyphenyl-2-sulfate was carried out, and traditional pharmacokinetic kinetics curves were constructed based on all samples collected (**Figure 6**), not just those used for PCA. There is a significant decrease of hippurate levels during the first 36 h on the low-flavonoid diet, after which, prior to tea intake, it was present only in low concentrations, as observed by PCA of the NMR spectra. This is illustrative of the high background level of hippurate in healthy humans on a normal diet that may be derived from polyphenols present in, for example, coffee (mainly chlorogenic acid) (*12*) and tea (*12*, *14*) but also from other plant-derived food items.

After intake of black tea, hippurate did not start to appear in the urine until 10.5 ± 2.9 h post-tea dose. On the other hand, 1,3-dihydroxyphenyl-2-*O*-sulfate was visible in samples collected 4.6 ± 2.4 h post-tea. This different kinetic behavior supports the observations made via PCA of the urine samples. The precursor of 1,3-dihydroxyphenyl-2-*O*-sulfate is probably gallic acid (*11*), and black tea contains approximately 1% of free gallic acid (*39*). In contrast, hippuric acid was probably formed via a slower, more complex route of degradation in the large intestine (*9*).

Total hippurate excreted after tea consumption until the end of the study, with an adjustment to allow for the baseline hippurate levels of each person, was 850 ± 320 μmol, while the total amount of 1,3-dihydroxyphenyl-2-*O*-sulfate excreted was 80 ± 48 μmol. The total amount of tea polyphenols consumed by each volunteer was estimated to be 1850 μmol (assuming a molecular mass of 600 Da for thearubigin).

ABBREVIATIONS USED

PC, principal component; PCA, principal components analysis; PR, pattern recognition; TOCSY, ^{1}H−^{1}H total correlation spectroscopy; TSP, trimethylsilyl [2,2,3,3-$^2H_4$] propionate.

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