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NMR-Based Metabonomic Studies on the Biochemical Effects of Epicatechin in the Rat

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Flavonoid consumption via tea drinking has been attributed a number of potential health benefits including cancer prevention, anti-inflammatory action, and cardioprotectant activity. Although the predominant flavonoids in fresh leaf and green tea are known to be flavan-3-ols and flavan-3-*O*-gallates ("the catechins"), the biochemical effects of tea polyphenol consumption on living systems are generally poorly understood. Metabonomic methods utilizing ^{1}H NMR spectroscopy of biofluids and principal component analysis (PCA) have been applied to investigate the bioavailability and metabolic responses of rats to a single dose of 22 mg of epicatechin (EC) dissolved in water. Urine samples were collected twice daily (0−8 and 8−24 h) from male Sprague−Dawley rats ($n = 10$) prior to dosing and for 2 days after dosing. A series of subtle urinary biochemical effects were evident from the ^{1}H NMR spectra showing that EC was both bioavailable and biochemically active. The identifiable biochemical effects associated with EC dosing included decreased urinary concentrations of taurine, citrate, dimethylamine, and 2-oxoglutarate. These effects were predominately seen within the first 8 h after dosing. EC metabolites were also observed in the urine during this time period. PCA of later time points after dosing (24−32 and 32−48 h) showed that the effects of EC were reversible. This is the first in vivo study demonstrating the overall endogenous metabolic effects of EC consumption and shows the bioavailability of EC via metabolic effects and excretion of EC metabolites.

KEYWORDS: Epicatechin; NMR; pattern recognition; bioavailability; polyphenol; tea; flavonoid

INTRODUCTION

^{1}H NMR spectroscopy of biofluids provides a useful approach with which to probe poorly understood endogenous and xenobiotic metabolic processes (*1*−*4*). This technique has proved to be both powerful and efficient in investigating the response of organisms to drugs/toxins based on perturbation of the composition of biofluids, because it generates comprehensive metabolic profiles that allow characterization of the physiological or pathophysiological status of an organism. Furthermore, these metabolic profiles can be obtained without preselection of measurement parameters or the selective derivatization procedures required for most other analytical methods (*1*).

Complex NMR spectral profiles of biofluids can be more efficiently interpreted with the use of automated data reduction and pattern recognition (PR) techniques (*3*, *4*). Computer-based multivariate approaches allow rapid characterization of the biofluid spectral profiles of samples based on the degree of inherent biochemical similarities between samples. The combination of NMR and PR allows better visualization of the changing endogenous biological profile resulting from a physiological challenge or stimulus such as a disease process, administration of a xenobiotic, genetic modification, or even a change in nutrition (*3*, *4*). We have termed this approach "metabonomics", which describes the multiparametric response of intact biological systems to external stimuli in time (*4*).

In the current work, a metabonomic approach has been applied to the study of the biochemical effect in the rat of consumption of epicatechin (EC), a flavonoid that is one of the major constituents of fresh tea leaf (**Figure 1**).

Tea (*Camellia sinensis*) is one of the most popular beverages in both Western and Eastern cultures (*5*) and is known to contain a range of different flavonoid compounds including epicatechin. Flavonoid compounds have been shown to have chemical and biological effects in both animal models and man, providing scientific support for anecdotal evidence suggesting that there

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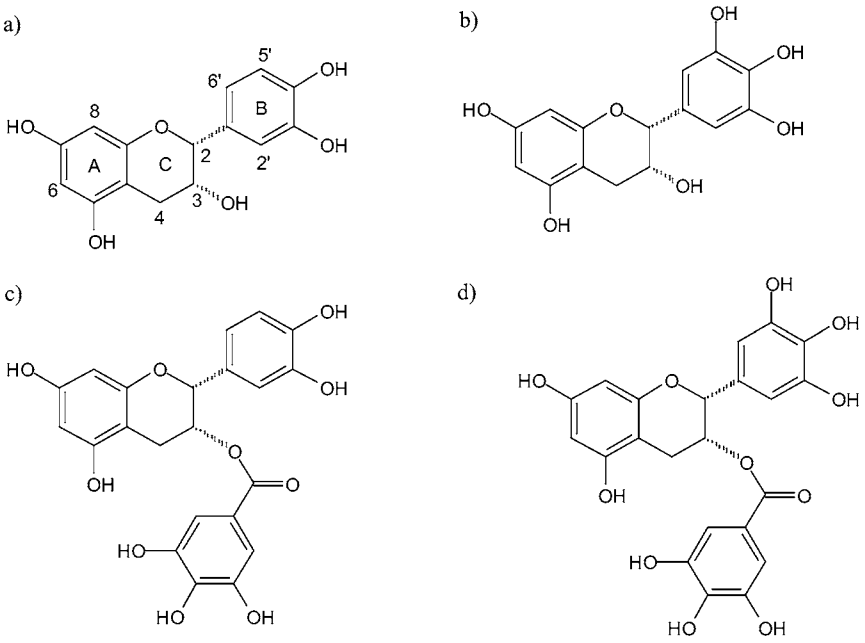

Figure 1. Molecular structures of selected tea flavonoids: (a) epicatechin (EC); (b) epigallocatechin (EGC); (c) epicatechin-3-*O*-gallate (ECG); (d) epigallocatechin-3-*O*-gallate (EGCG).

are health benefits associated with tea drinking. For example, in vitro and epidemiological studies have demonstrated indirect inhibition of cholesterol biogenesis (*6*) and lipid peroxidation (*7*), reduction of the risk of heart disease (*8*), and chemopreventive activity against various forms of tumors, including those of the breast, colon, pancreas, uterus, and rectum (*9*−*11*). Studies have also established that consumption of tea modulates the expression of genetic factors such as K-ras and capase-3, a factor involved in cell cycle regulation and apotosis (*12*, *13*).

Although previous studies have identified specific metabolic properties of catechins, they have not offered insight into their overall bioactivity or presented a holistic view of the bioactivity of EC in particular. These issues are addressed in this study, where the bioavailability and endogenous biochemical effects of EC have been investigated using ^{1}H NMR spectroscopy.

MATERIALS AND METHODS

Dosing and Sample Collection. Male Sprague−Dawley (SD) rats weighing 220−270 g (n = 10) were adapted to a polyphenol-free semisynthetic diet, thus ensuring any later observed metabolic effects were due to the administered polyphenol rather than any other dietary origin.

After an initial acclimatization period of 2 weeks in cages housing 10 rats per cage, the animals were transferred to individual metabolism cages and allowed to acclimatize for a further 24 h. Following this period, a 1 mL gavage of water was administered to the rats (sham treatment) and cooled urine (253 K) samples were obtained at 0−8 h and 8−24 h after administration of water. Free access to food and drinking water was available throughout the study period. The light cycle consisted of 12 h of light and 12 h of dark, the temperature was maintained at 295−297 K, and the humidity was maintained between 45 and 65% throughout the study. Following this 24 h predose collection, a 1 mL gavage containing 18 mg of epicatechin (minimal 98% pure, Sigma-Aldrich, Dorset, U.K.) dissolved in Milli-Q water was administered to the animals. The dose given was based on the equivalent of 10 cups of tea consumed by a human. Assuming that one cup of tea contains 50 mg of catechins and that the metabolic rate of rats is ∼10 times the metabolic rate of humans and that the average human weight is 70 kg (and rats 250 g), a single dose is thus (0.25 kg/70 kg × 10 cups × 10 × 50 mg) =18 mg of epicatechin.

Cooled urine samples (253 K) were collected as described above at the following times after EC administration: 0−8, 8−24, 24−32, and 32−48 h. All urine samples were stored at 203 K pending NMR spectroscopic analysis. Throughout the study, clinical observations were performed twice weekly during the run-in period and every day during the urine collection periods. All animals appeared well throughout the study, Body weights were determined once a week. After the final collection time point all animals were killed by decapitation after halothane anesthesia and subjected to autopsy.

Acquisition of ^{1}H NMR Spectra of Urine. Urine samples were thawed, and 400 μL aliquots were added to 200 μL of 0.2 M sodium phosphate buffer, pH 7.3, containing 0.5% (w/v) NaN_3, in a 1.5 mL polypropylene microcentrifuge tube. The samples were then left to stand for 10 min prior to centrifugation at 1700*g* for 10 min. An aliquot of 500 μL of the supernatant was added to 50 μL of 1 mM 3-(trimethylsilyl)propionic-(2,2,3,3-d_4) acid sodium salt (TSP) in D_2O. ^{1}H NMR spectra were acquired on a Bruker DRX 600 spectrometer operating at 600.13 MHz at 303 K using a standard pulse sequence for water peak suppression, that is, RD−90°−t_1−90°−t_m−90°−acquire free induction decay (FID) (*14*), where 90° represents a nonselective 90° radio frequency pulse, RD is a relaxation delay of 2 s during which the water resonance was selectively irradiated, and t_1 corresponds to a fixed interval of 3 μs. The water resonance was irradiated for a second time during the mixing time t_m (150 ms). A total of 64 transients were acquired into 64K data points using a spectral width of 7204 Hz and an acquisition time of 4.59 s. Prior to Fourier transformation (FT), an exponential line broadening function of 0.3 Hz was applied to the FID.

Data Processing and Pattern Recognition of NMR Spectroscopic Data. Each ^{1}H NMR spectrum was corrected for phase and baseline distortions, and^{1}H NMR chemical shifts in the urine were referenced to TSP at δ 0.0 using XWINNMR (version 2.1, Bruker GmbH). Each NMR spectrum was reduced to 256 regions of equal width (0.04 ppm), and the integral of each region was determined using AMIX (version 2.5, Bruker). The resulting table of the spectral intensity information was then exported to Microsoft Excel version 97 SR-2. The chemical shift regions between δ 4.5 and 6.0 (corresponding to the water and urea resonances) and between δ 0.0 and 0.22 (corresponding to TSP resonance) were removed prior to any statistical analysis in order to eliminate any spurious effects of variability in the suppression of the water resonance and the effects of variation in the urea signal caused by partial cross-solvent saturation via solvent-exchanging protons. Following the removal of these redundant regions, each spectral intensity data set was normalized to the total sum of the spectral integrals, thereby partially accounting for concentration differences due to the volume of urine excreted.

Principal component analysis (PCA) of the ^{1}H NMR spectra was performed using Pirouette (version 2.03, Infometrix, Inc., Woodinville,

WA). PCA can be used to display intersample and intervariable relationships in simple graphical terms. This is achieved by reduction of the dimensionality of the data set via two- or three-dimensional mapping procedures, facilitating the simultaneous comparison of a large number of complex objects (in this instance, NMR spectra). PCA creates linear combinations (principal components, PCs) of the original variables with appropriate weighting coefficients. The PCs are calculated such that each PC is orthogonal (uncorrelated) with all other PCs. The first PC (PC1) describes the largest amount of the variance of the data set, with subsequent PCs containing correspondingly smaller amounts of variance not accounted for by previous PCs.

^{1}H NMR data were analyzed using mean-centering (MC). With MC adjustment, the mean value of each variable is calculated and subtracted from the data, and therefore MC tends to highlight changes in high-concentration metabolites: it is typically used when the measurement units for variables are the same.

Information resulting from PCA is displayed as "scores plots", which represent the distribution of samples in multivariate space, and "loadings plots", which describe the relationship among the variables (in this case the spectral regions). The loadings provide information on how the old variables are linearly combined to form the new variables, that is, PC scores. Additionally, the loadings unravel the magnitude (large or small correlation) and manner (positive or negative correlation) in which the measured variables contribute to the formation of the scores (*15*).

PCA was initially used to identify anomalous samples, that is, samples that mapped separately from the main data set grouping on the bais of their biochemical profiles. The importance of identifying such samples is that they have a strong power to distort the PC models. Outlier samples were then removed from subsequent analysis after examination of the relevant NMR spectra to ensure the elimination was valid (typically due to poor spectral phasing or baseline artifacts).

Quantification of EC by ^{1}H NMR Spectroscopy. Quantification of the levels of EC excreted was achieved using ^{1}H NMR peak integration. ^{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.

Reporter resonances from EC metabolites suitable for quantification were identified (the 2′ protons from EC moieties; see **Figure 1** for labeling scheme). These signals, along with those from the TSP internal standard (which has a single resonance arising from nine protons), were integrated, and the total amount of EC metabolites in the sample was then calculated, using the formula

$$W_a = W_s(A_a/A_s)(\mathrm{MW}_a/\mathrm{MW}_s)(N_s/N_a)$$

where subscripts a and **s** denote metabolite and standard, respectively, W is the weight of the substance in the sample, A is the area under the integral of the NMR resonance, MW is the molecular weight, and N is the number of ^{1}H atoms giving rise to the signal.

Analysis of all the 0−8 and 8−24 h postdose (pd) ^{1}H spectra was carried out. The total amount of EC eliminated was calculated on the basis of the total urine volumes, allowing the bioavailability of EC to be determined.

RESULTS AND DISCUSSION

^{1}H NMR Spectroscopy of Urine. Comparison of the aliphatic regions of the ^{1}H NMR spectra from control and EC-treated rats (**Figure 2A**,**B**) indicated that endogenous metabolite levels changed as a consequence of EC dosing. These responses were typically manifested as changes in the concentration of creatinine, taurine, citrate, and 2-oxoglutarate (2-OG) and the presence of EC metabolite signals. Resonances assignable to EC metabolites could also be observed in the aromatic region of the spectra (**Figure 2C**,**D**).

PC Analysis of ^{1}H NMR Spectra. Preliminary PC analysis identified three outlier urine samples. These outlying spectra were examined for abnormalities; the major problems were artifacts arising from abnormal baselines as a result of poor water suppression, and one outlier differed due to the presence of a contaminant. These samples were excluded from subsequent analysis.

Metabonomic Analysis of ^{1}H NMR Spectra Using Mean-Centered Descriptors. PCA of spectral data derived from urine collected before dosing with EC revealed the inherent clustering behavior of the samples. The greatest separation was observed in the scores plot of PC1 versus PC3 (**Figure 3a**). The PCA scores plot indicated that the samples were predominantly divided into two groups corresponding to the samples obtained from daytime collection (0−8 and 24−32 h) and samples obtained from overnight collections (8−24 and 32−48 h). This clustering pattern suggested diurnal variation was a strong contributor to the biochemical profile and was the cause of primary differentiation within the sample population. The loadings plot (**Figure 3b**) allowed the determination of the spectral components that contributed to the observed diurnal variations. The basis of the separation between the daytime and overnight sampling time points was changes in metabolite resonances arising from citrate, taurine, dimethylglycine, and creatinine, supporting previous observations (*16*).

Rats are nocturnal and show greater activity at night. The observed diurnal variation in biochemical concentrations may therefore be explained by variation in activities, including feeding and locomotor. To interrogate the more subtle information on changes related to epicatechin dosing, the elimination of diurnal variation was essential. This was achieved by subdividing the data set into two subsets corresponding to samples collected during daytime and overnight time points.

Identification of Biochemical Effects of EC. Separate PC models based on the ^{1}H NMR spectra of daytime (0−8 and 24−32 h, control, and pd) and overnight (8−24 and 32−48 h, control, and pd) urine samples were constructed. These analyses showed that dosing with epicatechin resulted in significant variations in the metabolic profile of the rat urine in both data sets, with each scores plot showing three clusters based on collection times pre- and post-EC dosing (see **Figures 4** and **5**, daytime and overnight data, respectively). The results indicate that the alteration in rat urinary biochemistry was greatest in the first 24 h after EC administration. Furthermore, the degree of separation of samples according to sampling time point achieved in the daytime samples (**Figure 4**) was greater than that achieved in the overnight period (**Figure 5**). This suggests that the greatest biochemical effect occurred in the first 8 h after exposure, with the effects already reduced before the later overnight time points were sampled. Indeed, it can be seen that the PCA plot shows a metabolic trajectory, with 8 h pd points moving away from the control region of biochemical hyperspace during the period of greatest biochemical changes and then returning toward the control region of space by 24 h pd. Also of note was the fact that the temporal separation in the daytime data (**Figure 4**) was achieved using PC1 and PC2, whereas the separation in the overnight data (**Figure 5**) required PC2 versus PC3 for visualization. This indicated that the biochemical differences in the latter data set were more subtle because they were in PCs describing lower levels of variance in the data. These observations are consistent with previous studies in which it was determined that EC and its metabolites are eliminated in the urine within 9 h of consumption (*17*, *18*). Although (+)-catechin and (−)-epicatechin and their respective glucuronides have been identified in the plasma of rats following catechin ingestion (*19*, *20*), these metabolites were not identified in the urine samples obtained in the current study and therefore did

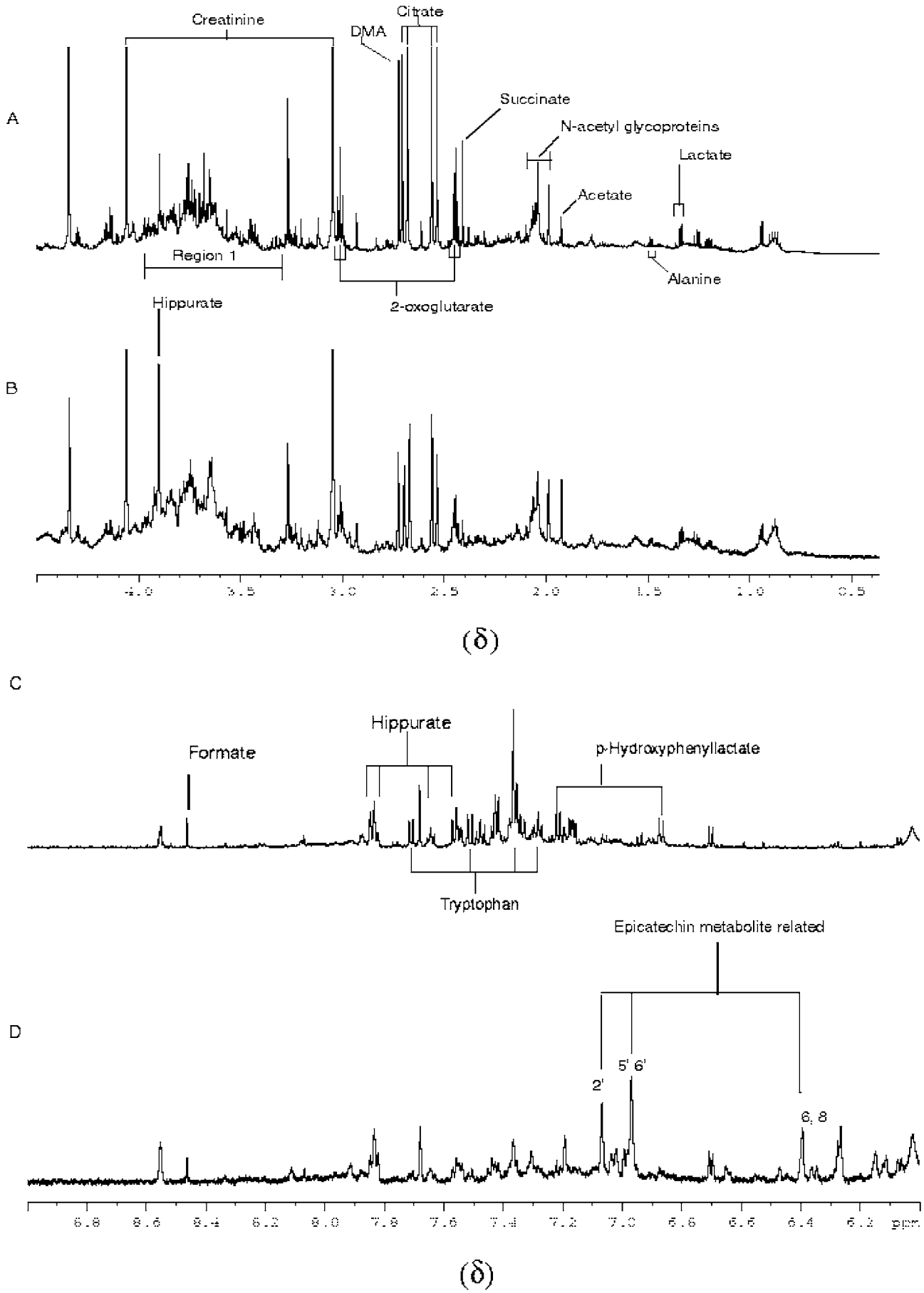

Figure 2. 600 MHz ^{1}H NMR spectra of rat urine (A) predose and (B) after epicatechin dosing (upfield region δ 0–4); (C) predose and (D) after epicatechin dosing (downfield region δ 6–8).

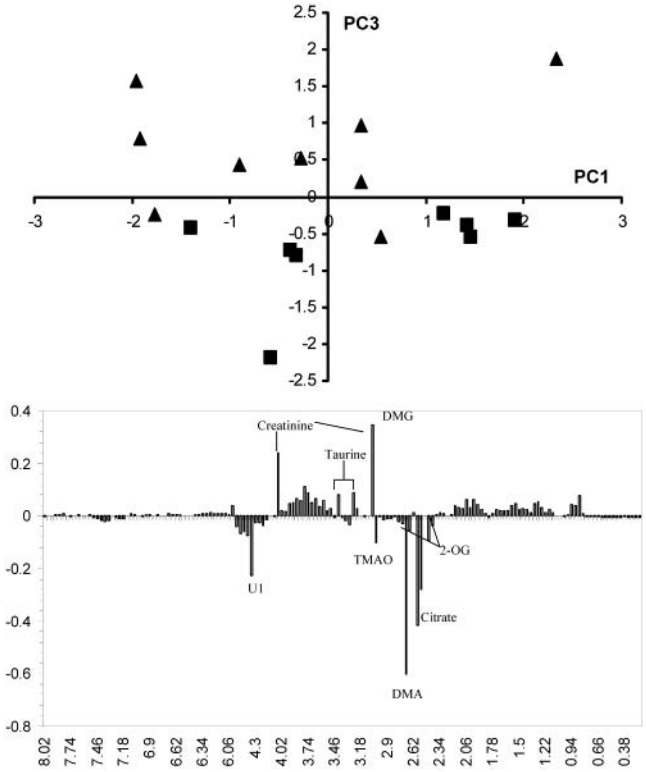

Figure 3. Scores plot (a, top) derived from the ^{1}H NMR urine spectra using MC scaling of the descriptors. The plot shows the clear separation between (▲) daytime (0–8 and 24–32 h) and (■) overnight (8–24 and 32–48 h) time points. This demonstrates inherent clustering of samples based on diurnal variation. Loadings plot (b, bottom) derived from the ^{1}H NMR urine spectra using MC scaling of the descriptors showing the contribution of the descriptors to the separation (see panel a) of daytime (0–8 and 24–32 h) and overnight (8–24 and 32–48 h) time points.

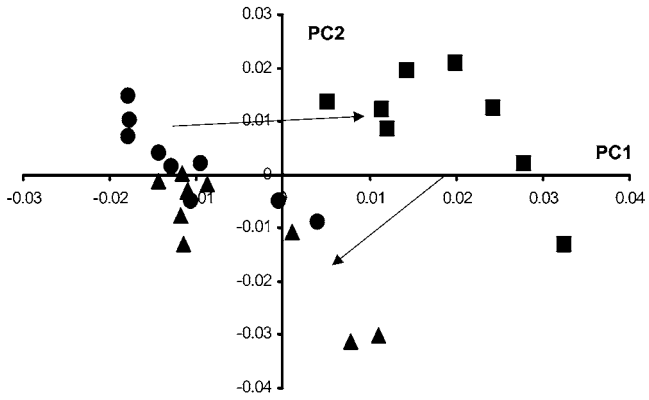

Figure 4. Scores plot derived from the ^{1}H NMR urine spectra (MC scaling) demonstrating the separation of pre- and postdose time points (daytime samples): (●) 0–8 h control; (■) 0–8 h pd; (▲) 24–32 h pd. PC1 described 32.32% and PC2 22.83% of variance. The arrows give an indication of the metabolic trajectory in time.

not influence the analysis of the effect of epicatechin on the endogenous profile.

Although the data suggest that biochemical recovery occurs within a few hours after dosing, the extent of the endogenous biochemical metabolite changes requires further analysis. The degree to which the distinctive separation of the 0−8 h control and after-dose urine collections is attributable to elimination of EC metabolites during the 0−8 h pd period (or not) is of particular interest, and this is discussed below.

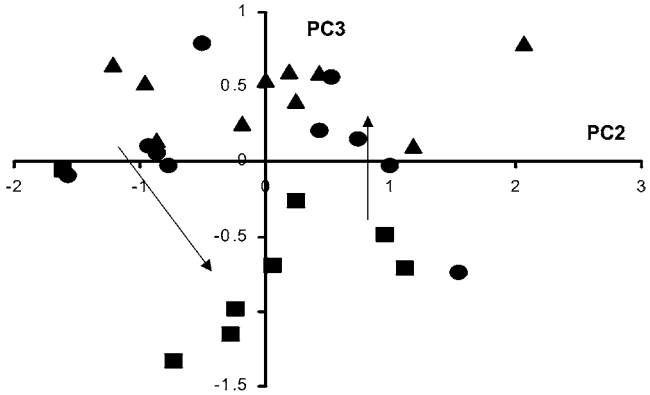

Figure 5. Scores plot derived from the ^{1}H NMR urine spectra (MC scaling) demonstrating the separation of pre- and postdose time points (overnight samples): (●) 8–24 h control; (■) 8–24 h pd; (▲) 32–48 h pd. The arrows give an indication of the metabolic trajectory in time.

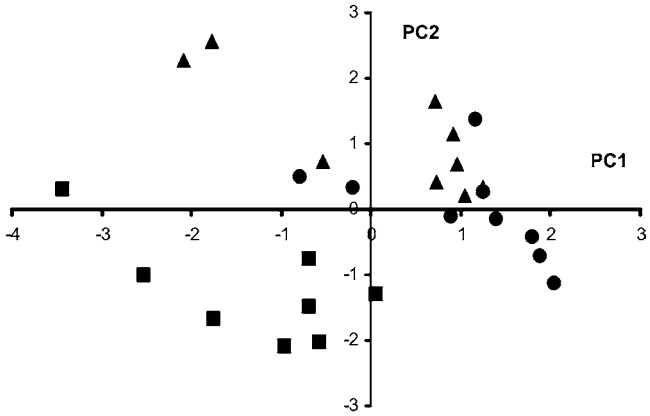

Figure 6. Scores plot derived from the aliphatic chemical shift region of the ^{1}H NMR urine spectra (MC scaling) demonstrating the separation of pre- and postdose time points (daytime samples): (●) 0–8 h control; (■) 0–8 h pd; (▲) 24–32 h pd. The plot describes 55.14% of the total variance.

PCA of Specific Chemical Shift Regions. To determine those factors important in obtaining the class separation observed in **Figure 4**, the data (0−8 h control and pd, 24−32 h pd) were examined in more detail by separate analysis of the downfield region (from δ 10.0 to 6.0) of the NMR spectra for EC metabolites, and the aliphatic region (from δ 4.5 to 0.22), where peaks primarily from endogenous metabolites are found.

PCA of the data from the upfield region (from δ 4.5 to 0.22) of the ^{1}H NMR spectra of rat urine following EC dosing is shown in **Figure 6**. The loadings plot (not shown) indicated decreases in the signals corresponding to specific endogenous metabolites following EC dosing. Elimination of descriptors attributable to EC metabolites within the aliphatic region did not alter the observed separation. There appeared to be no significant biochemical difference between the two PC plots (**Figures 4** and **6**). Consequently, it was concluded that endogenous metabolites must contribute to the separation of the pre- and postdose urine samples, indicating that metabolic variation in response to EC dosing was identifiable by NMR-based chemometric analysis.

Examination of the PC loadings and subsequent inspection of the corresponding urinary ^{1}H NMR spectra enabled identification of the endogenous metabolites for which levels were perturbed by exposure to EC and thus allowed identification of the biochemical markers related to the endogenous effects of EC dosing. These results are presented in **Table 1**. The spectral

Table 1. Changes in the Relative Levels of Endogenous and Exogenous Metabolites in Rat Urine before and after Epicatechin (EC) Dosing: ↓ Indicates a Decrease in Metabolite Levels and ↑ an Increase

NMR chemical shift or *loading region(s)[a]	metabolite significant for separation of 0–8 h time points	change in 0–8 h urine profile pd[b]	change in 8–24 h urine profile pd[c]	comparison of daytime urine profile pd[d]
*0.86			↑	
0.94 (t), 3.68 (d), 1.98 (m)	isoleucine	↓	↑	
1.33, 4.11	lactate		↑	
1.94 (s)	acetate	↓		
2.02	*N*-acetyl glycoproteins	↓		
2.06	*N*-acetyl glycoproteins	↓	↑	↑
2.42 (s)	succinate	↓	↑	
2.46 (t), 3.02 (s)	2-oxoglutarate	↓	↑	
2.56 (d), 2.72 (d)	citrate	↓	↑	↑
2.70 (s)	dimethylamine	↓		↑
2.94 (s)	trimethylamine-*N*-oxide	↓		↑
2.98 (s)	dimethylglycine		↑	
3.06 (s), 4.06 (s)	creatinine	↓	↑	↑
3.41 (t)	U2[e]	↓	↑	↑
3.26 (t), 3.42 (t)	taurine	↓	↑	↑
*3.30, 3.78, 3.86	carbohydrate/amino acid			↑
*3.58, 3.74, 3.78, 4.30, 4.38, 4.46, 6.06	carbohydrate/amino acid		↑	
3.82 (dd), 3.94 (dd)	serine		↑	↓
4.34 (s)	U1[e]	↓	↓	↑
2.98, 3.62, 3.66, 3.90, 4.38, 2.98, 6.26, 6.38, 6.94, 6.98, 7.06	EC metabolites	↑	↑	↓

[a] Letters in parentheses indicate the peak multiplicities: s, singlet; d, doublet; dd, doublet of doublets; t, triplet; m, multiplet. [b] Comparison of pre- and postdose 0–8 h urine profiles. [c] Comparison of pre- and postdose 8–24 h urine profiles. [d] Changes occurring between 0 and 8 h and 24–32 h urine profiles after dosing with EC. [e] Unidentified metabolite signals.

descriptors most affected by EC dosing were found to correspond to a decrease in the urinary concentrations of 2-oxoglutarate (δ 2.46 and 3.02), citrate (δ 2.58), dimethylamine (δ 2.70), creatinine (δ 3.06), and taurine (δ 3.26 and 3.42). These changes may be indicative of a decrease in the metabolic activity/carbohydrate metabolism of the rats and/or changes in liver and kidney functions (*16*), as the chemical shifts identified were indicative of components involved in cellular metabolism. Both dimethylamine and taurine have a function as renal osmolytes and have been shown to alter during episodes of renal toxicity or disease (*21*). However, whereas the current study indicated that epicatechin induced a decrease in the urinary excretion of dimethylamine, a recent study reported increased urinary excretion of dimethylamine and trimethylamine after the ingestion of green tea (*22*). These metabolite changes may be related to the proposed effects of dietary polyphenols on kidney function and could reflect a shift in energy metabolism from carbohydrate metabolism to lipid/amino acid metabolism.

PC analysis of the partial ^{1}H NMR spectra corresponding only to the δ 10.0−6.0 region of the spectra is shown in **Figure 7** illustrating that a number of aromatic signals contribute significantly to the separation of the 0−8 h pre- and postdose urine spectra. **Table 1** shows that an increase in signal intensities occurred between δ 7.5 and 6.0, a region associated with resonances from EC and its metabolites rather than endogenous species. The derived spectral loadings correspond well with literature values for ^{1}H NMR chemical shifts for metabolites of EC and its various gallates/conjugates (*18*).

Observations of the Time Course of Metabolic Effects of EC Dosing. To determine to what extent EC had been eliminated, PCA of the 8−24 h time points was carried out.

Analysis of the loadings plot involved in the separation of 8−24 h time points (results summarized in **Table 1**) indicated that the majority of the biochemical compounds that contributed to the discrimination were endogenous in origin. Many of these species coincided with those deemed to be significant in the analysis of the 0−8 h time points (**Table 1**). Whereas the levels

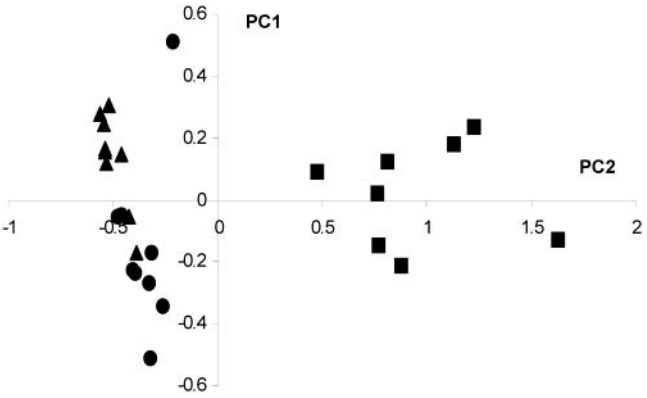

Figure 7. Scores plot derived from the aromatic chemical shift region of the ^{1}H NMR urine spectra (MC scaling) demonstrating the separation of the (●) 0–8 h control, (■) 0–8 h pd, and (▲) 24–32 h pd time points (daytime samples). The PC plot describes 59.8% of the total variance.

of biomarkers related to EC administration decreased during the 0−8 h time points, it was seen that they generally increased during the 8−24 h time period when compared with control. This was possibly due to a homeostatic compensatory/recovery process in response to EC administration. Furthermore, the values at 0−8 h pd for the descriptors corresponding to EC metabolites (from the aromatic region) were seen to decrease over the subsequent 8−32 h pd period: this indicated that EC had largely been eliminated 0−8 h after dosing.

Previous feeding studies of catechins have suggested that the major part of an oral dose is absorbed and metabolized by enzymes in the gut and liver and that the key metabolites formed are mixed sulfate (*18*), glucuronide, and *O*-methyl conjugates (*23*). Up to 90% of absorbed (+)-catechin is excreted into the urine as these mixed conjugates, although, more generally, the absorption ratio is in the range of 4−58% depending on the type of flavonoid (*18*). Preliminary HPLC-MS−NMR spectroscopic analysis of urine samples in the current work confirmed

that the ingested EC had been metabolized and excreted in the 0−8 h time period (data not shown). The total bioavailability of EC over 24 h was calculated by integration of appropriate peaks in the NMR spectra: it was found to be in the range of 30−54% of that administered. On average, 57% of the EC excreted was eliminated in the urine 0−8 h pd; the remainder was eliminated in the 8−24 h pd period. In view of this, it is concluded that the biochemical effects observed are directly related to the presence of EC in the system and that these effects of EC lasted for <24 h after dosing.

In summary, there were significant metabolic effects resulting from EC consumption in the rat that could be discerned from normal diurnal variations in urinary metabolite excretion. It has been shown here that EC is bioavailable and is subject to metabolism and subsequent urinary excretion. Metabonomic analysis indicated that exposure to EC induced changes in the endogenous urinary profile, confirming the bioactivity of this tea component. This metabonomic analysis, on its own, could not provide information as to whether the effects of epicatechin are beneficial to health but will provide a useful comparison of altered metabolism for ongoing longer term studies investigating tea consumption in human populations. Nevertheless, the analysis highlights the potential of NMR-based metabonomic analysis as a tool for in vivo investigations and the possibility that integrated metabonomic studies of other biofluids (i.e., plasma) and tissues could provide a clearer metabolic picture.

ABBREVIATIONS USED

δ, chemical shift; DMA, dimethylamine; DMG, dimethylglycine; EC, epicatechin. EGC, epigallocatechin; ECG, epicatechin-3-*O*-gallate; EGCG, epigallocatechin-3-*O*-gallate; FID, free induction decay; FT, Fourier transform; 2-OG, 2-oxoglutarate; ^{1}H NMR, proton nuclear magnetic resonance; pd, postdose; PCA, principal components analysis; TCA, tricarboxylic acid cycle; TMAO, trimethylamine-*N*-oxide; TSP, 3-(trimethylsilyl)propionic-(2,2,3,3-d_4) acid sodium salt.

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LITERATURE CITED

(1) Nicholson, J. K.; Wilson, I. D. High-resolution proton magnetic resonance spectroscopy of biological fluids. *Prog. NMR Spectrosc.* **1989**, *21*, 449−501.

(2) Lindon, J. C.; Nicholson, J. K.; Holmes, E. Metabonomics: Metabolic processes studied by NMR spectroscopy of biofluids. *Concepts Magn. Reson.* **2000**, *12*, 289−320.

(3) Holmes E.; Nicholls, A. W.; Lindon, J. C.; Ramos, S.; Spraul, M.; Neidig, P.; Connor, S. C.; Connelly, J.; Damment, S. J.; Haselden, J.; Nicholson, J. K. Development of a model for classification of toxin-induced lesions using 1H NMR spectroscopy of urine combined with pattern recognition. *NMR Biomed.* **1998**, *11* (4−5), 235−244.

(4) Nicholson, J. K.; Lindon, J. C.; Holmes, C. E. 'Metabonomics': understanding the metabolic response of living systems to pathophysiological stimuli via multivariate statistical analysis of biological NMR spectroscopic data. *Xenobiotica* **1999**, *29*, 1181−1189.

(5) Balentine, D. A.; Wiseman, S. A.; Bouwens, L. C. M. The chemistry of tea flavonoids. *Crit. Rev. Food Sci. Nutr.* **1997**, *37*, 693−704.

(6) Ikuro, A.; Takahiro, S.; Kaoru, U.; Toshio, M. Green tea polyphenols: Novel and potent inhibitors of squalene epoxidase. *Biochem. Biophys. Res. Commun.* **2000**, *268*, 767−771.

(7) Cherubini, A.; Beal, M. F.; Frei,B. Black tea increases the resistance of human plasma to lipid peroxidation in vitro, but not ex vivo. *Free Radical Biol. Med.* **1999**, *27*, 381−387.

(8) Cook, N. C.; Samman, S. Flavonoids: chemistry, metabolism, cardioprotective effects, and dietary sources. *J. Nutr. Biochem.* **1996**, *7*, 66−76.

(9) Toshihiko, O. Protective role of dietary polyphenols in oxidative stress. *Mechanisms Aging Dev.* **1999**, *111*, 133−139.

(10) Lyn-Cook, B. D.; Rogers, T.; Tan, Y.; Blann, E. B.; Kadlubar, F. F.; Hammons, G. J. Chemopreventive effects of tea extracts and various components on human pancreatic and prostate tumor cells *in vitro*. *Nutr. Cancer* **1999**, *35*, 80−86.

(11) Yukiaki, K.; Yukihiko, H. Antimutagenic and anticarcinogenic activity of tea polyphenols. *Mutat. Res./Rev. Mutat. Res.* **1999**, *43*, 69−97.

(12) Ahmad, N.; Gupta, S.; Mukhtar, H. Green tea polyphenol epigallocatechin-3-gallate differentially modulated nuclear factor kappa B in cancer cells versus normal cells. *Arch. Biochem. Biophys.* **2000**, *376*, 338−346.

(13) Islam, S.; Islam, N.; Kermode, T.; Johnstone, B.; Mukhtar, H.; Moskowitz, R. W.; Goldberg, V. M.; Chales Malemud, J.; Haqqi, T. M. Involvement of caspase-3 in epigallocatechin-3-gallate mediated apoptosis of human chondrosarcoma cells. *Biochem. Biophys. Res. Commun.* **2000**, *270*, 793−797.

(14) Nicholson, J. K.; Foxall, P. J. D.; Spraul, M.; Farrant, R. D.; Lindon, J. C. 750 MHz ^{1}H and ^{1}H-^{13}C NMR spectroscopy of human blood plasma. *Anal. Chem.* **1995**, *34*, 793−811.

(15) Eriksson, L.; Johansson, E.; Kettaneh, N.; Wold, S. Introduction to multi and megavariate data analysis using projection methods (PCA & PLS). *Umetrics* **1999**.

(16) Bollard, M. E.; Holmes, E.; Lindon, J. C.; Mitchell, S. C.; Branstetter, D.; Zhang, W.; Nicholson, J. K. Investigations into biochemical changes due to diurnal variation and estuarial cycle in female rats using high resolution ^{1}H NMR spectroscopy of urine and pattern recognition. *Anal. Biochem.* **2001**, *15*, 295 (2), 194−202.

(17) Lee, M. J.; Wang, Z. Y.; Li, H.; Chen, L.; Sun, Y.; Gobbo, S. Absorption, distribution and elimination of tea polyphenols in rats. *Drug Metab. Dispos.* **1997**, *25*, 1045−1050.

(18) Kazuo, O.; Masayuki, S.; Natsuki, M.; Fumio, N.; Yukihiko, H. Identification of (−)-epicatechin metabolites and their metabolic fate in the rat. *Drug Metab. Dispos.* **1999**, *27*, 309−316.

(19) Harada, M. Identification of the major antioxidative metabolites in biological fluids of the rat with ingested (+)-catechin and (−)-epicatechin. *Biosci., Biotechnol., Biochem.* **1999**, *63*, 973−977.

(20) Baba, S.; Osakabe, N.; Natsume, M.; Muto, Y.; Takizawa, T.; Terao, J. In vivo comparison of the bioavailability of (+)-catechin and (−)-epicatechin and their mixture in orally administered rats. *J. Nutr.* **2001**, *131*, 2885−2891.

(21) Holmes, E.; Bonner, F. W.; Sweatmann, B. C.; Lindon, J. C.; Beddell, C. R.; Rahr, E.; Nicholson, J. K. Nuclear megnetic resonance spectroscopy and pattern recognition analysis of the biochemical processes associated with the progression of and recovery from nephrotoxic lesions in the rat induced by mercury-(II) chloride and 2-bromoethanamine. *Mol. Pharmacol.* **1992**, *42*, 922−930.

(22) Choi, S. Y.; Chung, M. J.; Sung, N. J. Volatile N-nitrosamine inhibition after intake Korean green tea and Maesil (*Prunus mume* SIEB. Et ZACC.) extracts with an amine rich diet in subjects ingesting nitrate. *Food Chem. Toxicol.* **2002**, *40*, 949−957.

(23) Dullos, A. G.; Duret, C.; Rorer, D.; Girardier, L.; Mensi, N.; Fathi, M.; Chantre, P.; Vandermander, J. Efficacy of green tea extract rich in catechin polyphenols and caffeine increase 24-h energy expenditure and fat oxidation in humans. *Am. J. Clin. Nutr.* **1999**, *70*, 1040−1045.

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