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A New LC/MS/MS Rapid and Sensitive Method for the Determination of Green Tea Catechins and their Metabolites in Biological Samples

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A new rapid and sensitive method has been developed, using liquid chromatography in tandem mass spectrometry (LC-ESI-MS/MS) to identify green tea catechin metabolites in plasma and urine after oral intake of a green tea extract. (−)-Epigallocatechin-3-gallate (EGCG), (−)-epicatechin-3-gallate (ECG), (−)-epigallocatechin (EGC)-glucuronide, (−)-epicatechin (EC)-glucuronide, and EC-sulfate were identified in plasma, whereas in urine only the conjugated catechins were detected (EGC-glucuronide, EGC-sulfate, EC-glucuronide, and EC-sulfate). Standard calibration curves prepared in plasma were found to be linear in the range of 10.9–1379.3 nmol/L for EGCG, EGC, ECG, and EC. The accuracy and precision of this assay showed a coefficient of variation of <15%. The method allowed the detection and quantification limits (for 20 μL injection) from 1.1 to 2.6 nmol/L and 3.8–8.7 nmol/L, respectively, in plasma and 0.8–1.8 nmol/L and 2.6–6.0 nmol/L, respectively, in urine. This method can be applied for future clinical and epidemiological studies, allowing the identification of the active metabolites that will reach the target tissues.

KEYWORDS: Flavonoids; green tea; absorption; metabolites

INTRODUCTION

Tea is an ancient beverage steeped in history. Three billion kilograms of tea are produced each year. Green tea is produced by steaming fresh leaves (from the plant *Camellia sinensis*) for 1 min (to inactivate polyphenol oxidase), followed by drying (*1*). Because of the high rates of tea consumption in the global population, even small effects on humans could have large implications for public health (*2*). Green tea polyphenols have been extensively studied as cardiovascular disease (CVD) and cancer chemopreventive agents (*3–6*). To date, four studies (*7–10*) have examined the association between green tea consumption and mortality, but their sample sizes were small and the results were inconsistent. The Ohsaki study (*11*), with a very large sample size, concluded that tea consumption is associated with reduced mortality due to all causes, in addition to CVD, but not with reduced mortality due to cancer. They also reported that their study has its limitations and further clinical trials are ultimately necessary to confirm the protective effect of green tea on mortality.

(−)-Epigallocatechin-3-gallate (EGCG) is the major polyphenolic constituent found in green tea (dried fresh leaves of *C. sinensis*) (*12*). Several other polyphenolic compounds known as catechins are also found in lower abundance in this beverage. Others include (−)-epicatechin-3-gallate (ECG), (−)-epigallocatechin (EGC), and (−)-epicatechin (EC) (**Figure 1**).

Most molecular forms of flavonoids (the major class of polyphenols) that reach the peripheral circulation and the tissues are different from those present in foods (*13–19*) , and the biological effect may depend on their circulating metabolites (*20, 21*). Whereas glucuronides and sulphates are generally assumed to be rapidly excreted and pharmacologically inactive, it has been increasingly recognized that these conjugates may be more pharmacologically active than some of the parent compounds. Morphine-6β-glucuronide, the major metabolite of morphine, possesses more potent analgesic activity than the precursor itself (*22–24*). Very little is known about the biological activities of the conjugated metabolites of polyphenols. Glucuronides of isoflavones and epicatechin were shown to have much weaker estrogenic activity and provided no protection against oxidative stress in cells grown in vitro (*25, 26*); nevertheless, additional studies have shown that the 5-*O*-β-D-glucuronide of catechin and epicatechin excreted in rat urine does not interfere with their antioxidant properties as assessed

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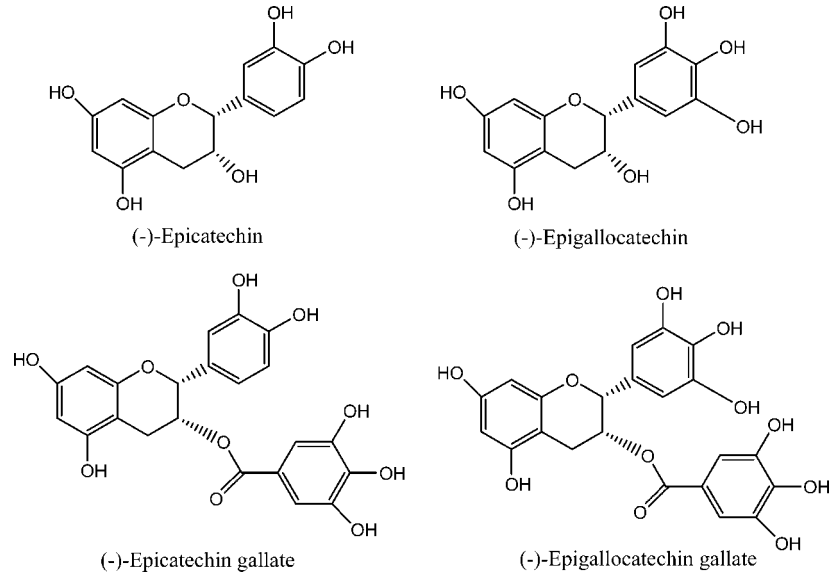

Figure 1. Chemical structures of green tea catechins.

by their ability to scavenge superoxide (*27, 28*), thus suggesting that in plasma they may still act as antioxidants.

Green tea catechin in vivo activity is limited by effective concentrations at target sites because it is assumed that catechin plasma concentration is dynamically equilibrated with its concentration in successfully reached tissues; changes observed in the plasma concentration are a reflection of what the metabolites undergo in those tissues. Therefore, there is a special interest to deeply study catechin behavior in the organism, that is, to evaluate the process of absorption, distribution, metabolism, and excretion through the metabolite's plasma and urine levels.

Several high-performance liquid chromatographic (HPLC) methods have been published regarding tea catechins analysis in blood plasma. Enzymatic hydrolysis is applied in the majority of these studies, and information about individual metabolites is lost, with these metabolites being the possible biologically active molecules that arrive at the target cells (*29–35*). Most of these methods involve the extraction of tea catechins from plasma or tissue homogenates with ethyl acetate (*33, 34, 36–39*), acetonitrile (*40*), or methanol (*41*), followed by an HPLC separation coupled with electrochemical (*29*) or MS detection (*42*). Liquid chromatography coupled with mass spectrometry (LC/MS/MS) has emerged as the preferred technology for quantitative determination of metabolites in different biomatrices, due to its sensitivity and selectivity through MS/MS experiments and the fact that it enables structural identification (*19, 43–45*). The present study aims to develop to our knowledge the most rapid method without enzymatic hydrolysis using solid phase extraction (SPE) and LC/MS/MS; both have proved to be excellent techniques for the identification of green tea catechin metabolites in biological samples. The method offers an extraction technique capable of simultaneously analyzing a high number of samples, an excellent method for future clinical and epidemiological studies.

EXPERIMENTAL SECTION

Standards and Reagents. EGCG (95%), EGC, and EC-gallate (98%) from green tea, EC, blank dog plasma and β-glucuronidase, type H-2, from helix pomatia were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ethylgallate (internal standard, IS) was purchased from Extrasynthese (Genay, France). Methanol, acetonitrile, and *n-n*-dimethylformamide (DMF) HPLC grade and formic acid were purchased from Scharlau Chemie S. A. (Barcelona Spain). *o*-Phosphoric acid 85% was purchased from Panreac Quimica SA (Barcelona Spain). Ultrapure water (Milli-Q) was obtained from a Millipore System (Bedford, MA England).

Subjects and Study Design. Two adult (15 and 13.40 kg) healthy female beagle-strain dogs were randomly chosen and were deprived of food overnight before conducting the experiment. The dogs were orally administered with two capsules containing a total of 200 mg of green tea extract; one additional dog was chosen as a control and was given an excipient. The blood was drawn before capsule administration and at 90 min after extract intake. The dogs were fed a polyphenol-free diet 2 h after the capsules were given. The blood samples (5 mL) were collected in vacutainier tubes containing ethylenediaminetetraacetic acid (EDTA) as an anticoagulant (Becton Dickinson, Franklin Lakes, NJ). Plasma was obtained after blood centrifugation at 1000 g for 10 min and stored in Eppendorf tubes at −80 °C until analysis was performed. Urine samples were taken before and 2 h after oral administration of green tea extract. Urine samples were acidified with 200 mM HCl and stored at −80 °C until analysis was carried out.

The study was done at the University of Zaragoza (Spain), in accordance with the *Guide for the Care and Use of Laboratory Animals* (*46*) and the Helsinki Declaration of 1975, as revised in 1996. The study protocol was approved by the Ethics Committee of the University of Zaragoza.

Sample Extraction Procedure for Green Tea Catechins and Their Metabolites. Green tea catechins in plasma and urine were extracted by SPE. Dog plasma samples were treated as follows: 280 μL of IS (2525 nmol/L) was added to 1 mL of plasma and was then mixed with 370 μL of antioxidant solution (containing 0.02 g/mL ascorbic acid and 1 mg/mL EDTA) and with 20 μL of *o*-phosphoric acid. After 2 min of vortex mixing, the samples were diluted with 3 mL of water. A solid phase extraction with Waters Oasis^{R} HLB 96-well plate 30 μm (30 mg) (Milford, MA, USA) was applied to the mixture. Cartridge activation was achieved by adding 1 mL of methanol, water, 70% (v/v) DMF containing 0.1% (v/v) formic acid and water, respectively. The cartridges were washed with 2 mL of water and 1 mL of 30% (v/v) methanol. Tea catechin metabolites were then eluted with 0.7 mL of 70% (v/v) DMF containing 0.1% (v/v) formic acid. After filtration of the sample with a 4 mm, 0.45 μm PTFE filter (Waters Corporation, USA), 20 μL of the resulting filtrate was injected into the LC/MS/MS system.

Standards preparation and sample treatments were performed in a darkened room with a red safety light to avoid the oxidation of the analytes.

Sample Hydrolysis. One milliliter of urine was mixed with 200 μL of 2 M sodium acetate pH 5 and 10 μL of a mixture of β-glucuronidase [(95000 units/mL) and sulfatase (773 units/mL)]. The reaction mixture

Table 1. Quantity (mg/g) of Green Tea Catechins Present in the Extract

green tea catechin	quantity (mg/g)
(−)-epicatechin (EC)	103.5
(−)-epicatechin-3-gallate (ECG)	93
(−)-epigallocatechin (EGC)	47
(−)-epigallocatechin-3-gallate (EGCG)	622

was incubated at 37 °C for 45 min (*47*), followed by an addition of 2 μL of 6 M HCl for acidification (pH 2). The resulting solution was subjected to the SPE procedure as described above.

Instrumentation. *LC/MS/MS.* Plasma and urine green tea catechin metabolites were identified and quantified by LC/MS/MS analysis. LC analysis was performed using a Perkin-Elmer series 200 (Norwalk, CT) equipped with a quaternary pump and a refrigerated autosampler. A Luna C18 column (50 × 2.0 mm i.d., 5 μm) from Phenomenex (Torrance, CA) was used at room temperature with an injected volume of 20 μL. Gradient elution was carried out with water (0.1% formic acid) and acetonitrile (0.1% formic acid) at a constant flow-rate of 600 μL/min. The gradient profile with the following proportions (v/v) of acetonitrile (0.1% formic acid) was applied (*t* (min), % acetonitrile): (0, 0.5), (3, 15), (6, 100), (9, 100); (10, 0.5), (15, 0.5) (6 min: time needed to reach initial conditions). A triple quadrupole mass spectrometer, API 3000 (Applied Biosystems, PE Sciex, Concord, Ontario, Canada), equipped with a TurboIonSpray source, was used to obtain the MS and MS/MS data. Prior to its use, the instrument was adjusted to meet the acceptance specifications defined by the manufacturer. TurboIonspray source settings were as follows: capillary voltage −3500 V, nebulizer gas (N_2) 10 (arbitrary units), curtain gas (N_2) 12 (arbitrary units), collision gas (N_2) 4 (arbitrary units), focusing potential −200 V, entrance potential −10 V, drying gas (N_2) heated to 400 °C and introduced at a flow-rate of 8000 cm^3/min. The declustering potential (DP) and the collision energy (CE) were optimized for each compound in infusion experiments: individual standard solutions (2.18–3.45 μmol/L) dissolved in 80:20 mobile phase were infused at a constant flow-rate of 5 μL/min into the mass spectrometer using a model syringe pump (Harvard Apparatus, Holliston, MA, USA). Full scan data were acquired by scanning from *m*/*z* 100 to 800 in profile mode, using a cycle time of 2 s with a step size of 0.1 u. For MS/MS, a product ion scan utilizing a cycle time of 2 s was used. MS/MS product ions were produced by collision-activated dissociation (CAD) of the selected precursor ions in the collision cell of the triple-quadrupole mass spectrometer and mass analysed using the second analyzer of the instrument. The multiple reaction monitoring (MRM), the method of choice due to the highest selectivity and sensitivity in quantitative LC/MS/MS, monitored several transitions for each analysis. Both quadrupoles (Q1 and Q3) were operated at unit resolution. The criteria for identification of green tea catechin metabolites such as retention time, MRM transition as mentioned above, and transitions 481 → 305, 385 → 305, 305 → 125, 465 → 289, 369 → 285, 289 → 245 (at a higher DP value) were chosen as confirmation of the MRM trace for each metabolite in collisionally induced dissociation (CID-MS/MS) experiments (*18*, *45*). Sample hydrolysis as explained above was also applied before extraction as a second confirmation analysis.

Safety Considerations. Dog plasma samples were considered as being potentially infectious. The general guidelines were respected regarding working with organic solvents and acids. Universal precautions regarding handling of chemicals and fluids were applied.

RESULTS AND DISCUSSION

Catechin Composition of Green Tea Extract. The catechin composition of the given green tea extract is shown in **Table 1**. A total amount of 173 mg of green tea flavan-3-ols was orally administered to the subjects.

Quality Parameters of the Method, for the Determination of Green Tea Catechin Metabolites in Dog Plasma. Selectivity, linearity, sensitivity, recovery, precision, and accuracy were the selected parameters for method evaluation.

LC/MS/MS Optimization. To establish the optimum MS and MS/MS conditions, infusion of individual EC, ECG, EGC, and EGCG standard solutions were performed. Declustering potential (DP) varied from −5 to −120 V and collision energy (CE) varied from −5 to −45 V. Optimal DP and CE selected were as follows: DP = −60 and CE = −25 for EC, ECG, and ECG; DP = −60, CE = −20 for EGCG and DP = −50, CE = −20 for ethyl gallate (EG) (IS). All MS and MS/MS data were collected in negative ion mode. Both quadrupoles were operated at unit resolution.

Selectivity. To discard any endogenous peaks at the same analyte retention time, blank dog plasma was analyzed with full scan and MRM analysis.

Linearity. The linearity of the method was investigated by spiking blank dog plasma and dog urine (obtained before capsule administration) with known concentrations of EC, ECG, EGC, EGCG, and EG (IS) at eight concentration levels ranging from 10.9 to 1379.3 nmol/L. Sample concentration was determined by weighted ($1/X^2$) linear regression of the standard line (*48*). The eight-point calibrator concentration showed linear and reproducible curves with the following correlation coefficients for EC, ECG, EGC, and EGCG: 0.9987, 0.9955, 0.9972, and 0.9973 for plasma and 0.9982, 0.9992, 0.991, and 0.9984 for urine. The residual analyses for this concentration range corresponding to each catechin were as follows: mean (SD); EC 102.3% (±10.3); ECG 92.3% (±6.9); EGC 90.9% (±12.1); EGCG 97.9% (±6.5).

Sensitivity. The sensitivity of the method was evaluated by determining the limit of detection (LOD) and the limit of quantification (LOQ). LOD was defined as the concentration

Table 2. Mean Values, Precision, Limits of Detection, and Quantification Data Obtained from the LC/MS/MS Analysis of EC, ECG, EGC, EGCG in Dog Plasma and Urine ($n = 3$)[a]

	EC				ECG				EGC				EGCG			
	plasma		urine		plasma		urine		plasma		urine		plasma		urine	
concn (ng/mL)	mean	RSD (%)	mean	RSD (%)	mean	RSD (%)	mean	RSD (%)	mean	RSD (%)	mean	RSD (%)	mean	RSD (%)	mean	RSD (%)
5	5.6	10.6	5.4	6.2	4.4	2.9	5.3	14.5	5.4	4.4	4.9	3.6	5.2	6.0	4.9	10.4
10	9.0	3.6	10.9	2.3	8.7	6.3	9.9	10.1	10.3	6.3	9.9	10.8	10.1	7.3	10.9	16.0
20	18.6	3.4	20.8	8.5	15.2	1.9	13.3	9.3	15.2	5.9	20.4	6.2	19.3	8.7	17.4	9.0
40	32.9	2.3	39.4	4.1	37.3	11.2	43.8	7.3	34.4	6.9	41.2	11.5	35.9	10.1	39.6	3.5
80	70.9	9.3	77.1	8.3	72.4	12.0	78.5	4.2	69.6	11.0	90.1	8.6	71.1	8.9	83.9	2.3
100	93.9	9.9	106.3	7.1	93.0	6.3	103.4	5.9	98.3	4.4	106.8	6.4	91.9	8.6	105.4	8.7
200	193.2	7.7	185.6	8.8	189.7	0.1	190.8	7.7	182.6	6.3	176.7	6.9	187.1	9.2	193.6	7.4
400	406.0	1.2	386.6	7.6	415.6	2.00	401.6	5.9	408.0	3.20	347.7	8.6	412	6.77	395.9	7.9
LOD nmol/L	1.2		0.8		2.4		1.4		2.6		1.8		1.1		1.1	
LOQ nmol/L	3.9		2.6		7.8		4.8		8.7		6.0		3.8		3.7	

[a] RSD, relative standard deviation; LOD, limit of detection for 20 μL; LOQ, limit of quantification for 20 μL.

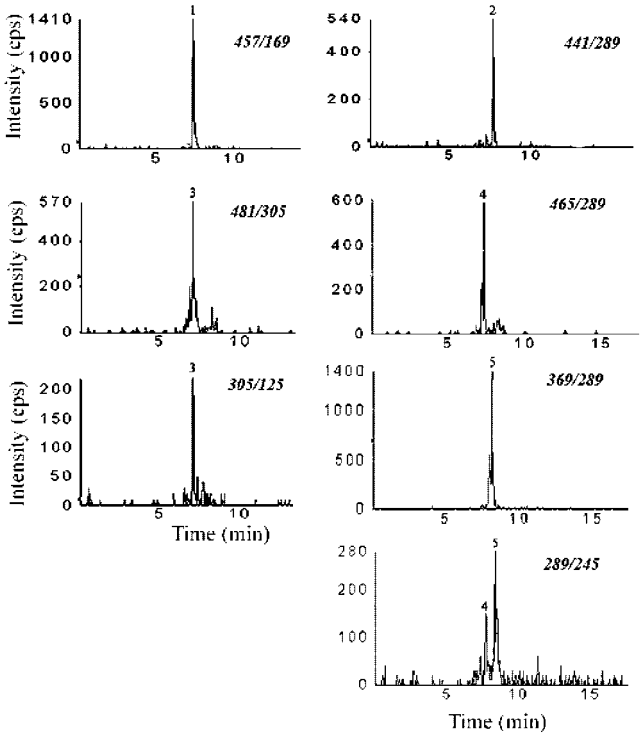

Figure 2. Multiple reaction monitoring chromatogram (MRM) of dog plasma after intake (90 min) of green tea extract. Peak 1, (−)-epigallocatechin-3-gallate (*m*/*z* 457/169); peak 2 (−)-epicatechin-gallate (*m*/*z* 441/289); peak 3 (−)-epigallocatechin-glucuronide (*m*/*z* 481/305); peak 4 (−)-epicatechin-glucuronide (*m*/*z* 465/289); peak 5 (−)-epicatechin-sulfate (*m*/*z* 369/289).

of EC, ECG, EGC, and EGCG with a signal-to-noise ratio of 3, whereas LOQ was the lowest standard with a signal-to-noise ratio of 10. LOD and LOQ for a 20 μL injection for each catechin in plasma and urine are shown in **Table 2**.

Recovery. Methanol and ethyl acetate as elution solvents have proven to be solvents that give better polyphenol recoveries. However, in this study, these solvents did not produce recoveries for green tea catechins with a galloyl moiety that have proved to generate specific affinities with HLB resins and are strongly retained. Unno et al. (*29*) resolved this problem with the correct choice of elution solvent, employing 70% (v/v) DMF containing 0.1% (v/v) phosphoric acid, which was used in this study as well but with 0.1% (v/v) of formic acid instead for better performance. Extraction efficiency (%) was investigated by spiking blank dog plasma and dog urine (obtained before capsule administration) with known quantities of catechins (EC, ECG, EGC, EGCG) at different concentration levels within the linear range of the calibration curve (5–400 ppb) and IS (EG) at a constant concentration. The recoveries of known amounts of catechins were as follows: EC (100.15 ± 19.60), ECG (95.53 ± 16.74), EGC (99.99 ± 18.88), and EGCG (99.99 ± 15.88) for plasma and EC (100.02 ± 8.03), ECG (100.07 ± 15.21), EGC (99.67 ± 8.70), and EGCG (100.05 ± 6.69) for urine.

Precision and Accuracy. A triplicate analysis of samples containing known amounts of catechins (EC, ECG, EGC, and EGCG) and EG (IS) prepared in blank dog plasma and dog urine (urine before capsule administration) was made. The analysis of standard calibration curves yielded precision and accuracy data found in **Table 2**. The criteria for acceptance precision and accuracy were accepted at all concentration levels (*49*).

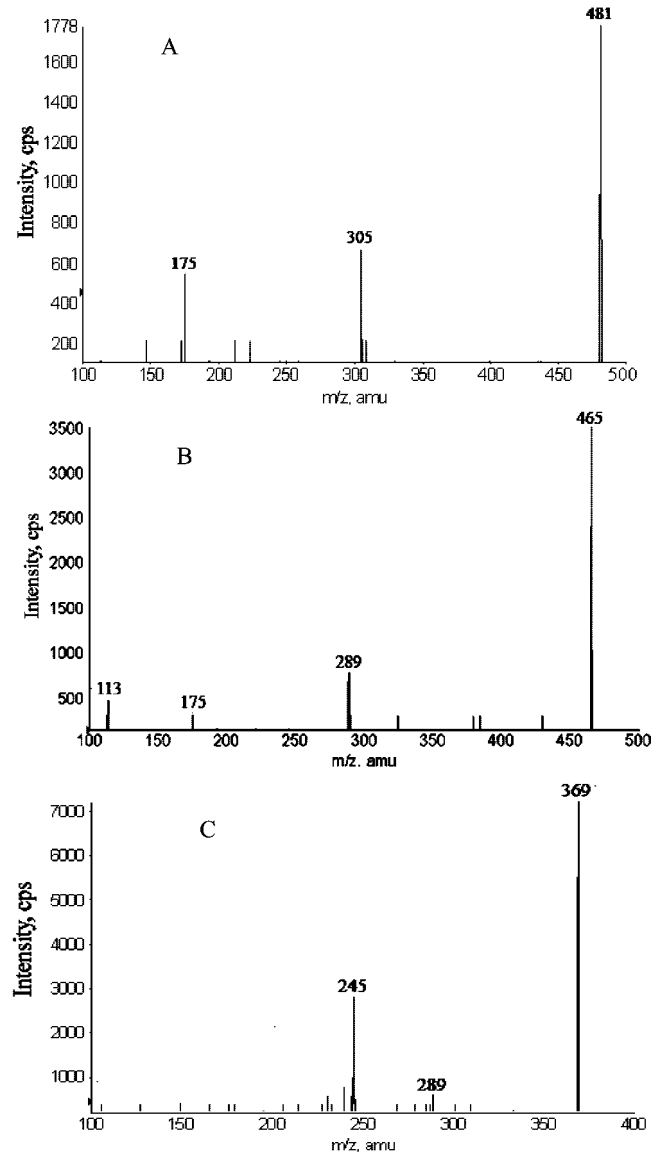

Figure 3. Product ion spectra of (**A**) (−)-epigallocatechin-glucuronide; (**B**) (−)-epicatechin-glucuronide; and (**C**) (−)-epicatechin-sulfate.

Repeatability and reproducibility for retention time was also calculated. Within-day precision (n = 10) was in the range of 1–7.4% for catechins and IS EG. Between-day precision (n = 30) was in the range of 6.3–11.6%.

Identification and Confirmation of Green Tea Catechins in Dog Plasma and Urine. HLB resins generated good recoveries during the extraction and clean-up of biological samples in phenolic compounds analyses ((*19*, *44*), and (*45*)). In this study, the application of HLB 96-well plates yielded a rapid sample preparation providing a less time-consuming extraction method capable of simultaneously analyzing a high number of biological samples.

The identification of green tea catechin metabolites was based on three parameters: comparison of the retention time with those available standards, MRM metabolite transition, transitions of the unconjugated catechins with higher DP in CID/MS/MS or product ion spectra and sample hydrolysis.

Figure 2 shows the chromatograms of a dog plasma sample in MRM acquisition mode. EGC-glucuronide could be confirmed by the presence of a peak in transition 305 → 125 at the same retention time but with lower intensity than in transition

Table 3. Description of Molecular Weight, Retention Time, Multiple Reaction Monitoring Transitions in Negative Mode, and Quantification in nmol/L of Green Tea Catechins and Their Metabolites in Dog Plasma and Urine

compound	MW	R_t (min)	MS/MS ions (*m*/*z*)	nmol/L (dog plasma)	nmol/L (dog urine)
(−)-epigallocatechin-3-gallate (EGCG)	458	7.3	457 → 169	312.3 ± 93.9	n.d.[a]
(−)-epicatechin-gallate (ECG)	442	7.6	441 → 289	94.8 ± 8.2	n.d.
(−)-epigallocatechin-glucuronide	482	6.9	481 → 305	174.1 ± 34.9[b]	28.9 ± 26.7[b]
(−)-epigallocatechin-sulfate	386	8.1	385 → 305	n.d.	224.9 ± 63.3[b]
(−)-epicatechin-glucuronide	466	7.1	465 → 289	107.9 ± 74.7[b]	254.9 ± 37.7[b]
(−)-epicatechin-sulfate	370	8.2	369 → 289	572.1 ± 13.1[b]	596.3 ± 142.0[b]

[a] n.d., not detected. [b] Quantified with their corresponding equivalent.

481 → 305 (**Figure 2**, peak 3); EC-glucuronide and EC-sulfate could be confirmed by the presence of a peak in transition 289 → 245 at the same retention time but with lower levels than in transitions 465 → 289 and 369 → 289 (**Figure 2** peaks 4 and 5). To ensure the identity of the conjugated metabolites, product ion scan mode as a second experiment was applied. **Figure 3** shows the product ion scan spectra for 481 (EGC-glucuronide) and 465 (EC-glucuronide), which produced an ion at *m*/*z* 305 and *m*/*z* 289, respectively, due to the loss of 176 units, which corresponded to a glucuronic acid; fragment ions 175 and 113, which are characteristic features of glucuronides, as Nobilis et al. reported in their study, were also present (*50*). Product ion scan spectra for *m*/*z* 385 (EGC-sulfate) and *m*/*z* 369 (EC-sulfate) produced an ion at *m*/*z* 305 and *m*/*z* 289, respectively, due to the loss of 80 units, which corresponded to a sulfate moiety. PIS for 369 also yielded an ion at 245 characteristic of EC fragmentation. The position of the glucuronic group could not be determined due to the lack of reference standards.

Urine samples were also incubated with β-glucuronidase and sulfatase as a second analysis before extraction to confirm that the conjugated catechins were glucuronides and sulfates. After extraction and analysis with LC/MS/MS, the only catechins detected in these hydrolysed urine samples were EGC and EC as their free forms, indicating that major conjugates were glucuronides and sulfates. Since hydrolysis was only applied for the confirmation of catechin conjugates, specifically glucuronides and sulphates, and due to the lacking of commercial conjugated standards, recoveries were determined for EC and EGC in urine, quantifying them after hydrolysis and comparing them with the values of EC conjugates (glucuronides and sufates) and EGC (glucuronides and sulfates) obtained with our method. Recoveries obtained were 83.38 and 97.87% for EC and ECG, respectively.

Green Tea Catechins Present in Plasma and Urine Samples. Green tea catechins and their metabolites were neither present in dog plasma at time 0, prior to consumption of the green tea extract, nor in the control subjects that had been given an excipient.

Catechins with the gallate moiety were present in plasma (EGCG and ECG), and three conjugated forms were also detected, whereas in urine mainly the conjugated forms of EC and EGC were present. Regarding conjugated catechins found in plasma, EGC was mainly in the glucuronide form, whereas EC was in both the glucuronide and the sulfate form. Urine samples were collected just before ingesting the green tea extract and at 2 h after ingestion. Tea catechins were mostly in the conjugated forms.

Green tea catechins and their metabolites quantified in dog plasma and urine in this study after oral administration of green tea extract are shown in **Table 3**, along with their retention times and concentration (nmol/L). Because of the lack of conjugated catechins reference standards, quantification was made with the corresponding equivalent, in the case of EGC-glucuronide, EGC-sulfate, EC-glucuronide, and EC-sulfate, which were quantified with its equivalents, EGC and EC, respectively.

It should be taken into consideration that although the compounds selected for analysis represent most of the abundant metabolites, the complete green tea catechin metabolites profile may have contained additional metabolites (*51*, *52*) that were not identified in this study.

We have developed a new LC/MS/MS procedure that determines green tea catechins and their metabolites present in biological samples with an extraction method capable of simultaneously analyzing a high number of samples. To our knowledge, this is the first ever method identified to detect and quantify green tea catechin and their metabolites in plasma and urine with the highest rapidity. Because of the lack of conjugated standards, the quantification of conjugated metabolites was made with their corresponding equivalents. This procedure offers excellent selectivity and sensitivity. The use of LC/MS/MS to obtain the fragmentation patterns of the deprotonated catechin and catechin metabolites led to the confident assignment of their structural classes. This method can be applied for future clinical and epidemiological studies, with a high number of subjects to identify the real active compounds of green tea and, consequently, to study their effects on mortality.

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LITERATURE CITED

(1) Kris-Etherton, P. M.; Keen, C. L. Evidence that the antioxidant flavonoids in tea and cocoa are beneficial for cardiovascular health. *Curr. Opin. Lipidol.* **2002**, *13*, 41–49.

(2) Rimm, E. B.; Stampfer, M. Diet, Lifestyle, and Longevity–The Next Steps. *JAMA* **2004**, *292*, 1490–1492.

(3) Zaveri, N. T. Green tea and its polyphenolic catechins: Medicinal uses in cancer and noncancer applications. *Life Sci.* **2006**, *78*, 2073–2080.

(4) Cooper, R.; Morre, D. J.; Morre, D. M. Medicinal benefits of green tea: Part I. Review of noncancer health benefits. *J. Altern. Complement. Med.* **2005**, *11*, 521–528.

(5) Frei, B.; Higdon, J. V. Antioxidant Activity of tea polyphenols in vivo: Evidence from animal studies. *J. Nutr.* **2003**, *133*, 3275S–3284S.

(6) Bettuzi, S.; Brausi, M.; Rizzi, F.; Castagnetti, G.; Peracchia, G.; Corti, A. Chemoprevention of human prostate cancer by oral administration of green tea catechins in volunteers with high-grade prostate intraepithelial neoplasia: A preliminary report from a one-year proof-of-principle study. *Cancer Res.* **2006**, *66*, 1234–1240.

(7) Yoshikazu, S.; Haruo, N.; Takao, W.; Shigeru, H.; Hiroyuki, S.; Susumu, F.; Yoshimi, I.; Yasuo, I.; Seiki, S.; Kunio, K.; Masayuki, I. Possible contribution of green tea drinking habits to the prevention of stroke. *Tohoku J. Exp. Med.* **1989**, *157*, 337–343.

(8) Nakachi, K.; Matsuyama, S.; Miyake, S.; Suganuma, M.; Imai, K. Preventive effects of drinking green tea on cancer and cardiovascular disease: Epidemiological evidence for multiple targeting prevention. *Biofactors* **2000**, *13*, 49–54.
(9) Iwai, N.; Ohshiro, H.; Kurozawa, Y.; Hosoda, T.; Morita, H.; Funakawa, K.; Okamoto, M.; Nose, T. Relationship between coffee and green tea consumption and all-cause mortality in a cohort of a rural Japanese population. *J. Epidemiol.* **2002**, *12*, 191–198.
(10) Nakachi, K.; Eguchi, H.; Imai, K. Can teatime increase one's lifetime. *Ageing Res. Rev.* **2003**, *2*, 1–10.
(11) Kuriyama, S.; Shimazu, T.; Ohmori, K.; Kikuchi, N.; Nakaya, N.; Nishino, Y.; Tsubono, Y.; Tsuji, I. Green tea consumption and mortality due to cardiovascular disease, cancer, and all causes in Japan: The Ohsaki Study. *JAMA* **2006**, *296*, 1255–65.
(12) Graham, H. N. Green tea composition, consumption, and polyphenol chemistry. *Prev. Med.* **1992**, *21*, 334–350.
(13) Day, A. J.; Mellon, F.; Barron, D.; Sarrazin, G.; Morgan, M. R. A.; Williamson, G. Human metabolism of dietary flavonoids: Identification of plasma metabolites of quercetin. *Free Radic. Res.* **2001**, *35*, 941–952.
(14) Day, A. J.; Williamson, G. Biomarkers of exposure to dietary flavonoids a review of the current evidence for the identification of quercetin glycosides in plasma. *Br. J.Nutr.* **2001**, *86*, S105–S110.
(15) Graefe, E. U.; Wittig, J.; Mueller, S.; Riethling, A. K.; Uehleke, B.; Drewelow, B.; Pforte, H.; Jacobasch, G.; Derendorf, H.; Veit, M. Pharmacokinetics and bioavailability of quercetin glycosides in humans. *J. Clin. Pharmacol.* **2001**, *41*, 492–499.
(16) Natsume, M.; Osakabe, N.; Oyama, M.; Sasaki, M.; Baba, S.; Nakamura, Y.; Osawa, T.; Terao, J. Structures of (−)-epicatechin glucuronide identified from plasma and urine after oral ingestion of (−)-epicatechin: Differences between human and rat. *Free Radic. Biol. Med.* **2003**, *34*, 840–849.
(17) Zhang, Y.; Hendrich, S.; Murphy, P. A. Glucuronides are the main isoflavone metabolites in women. *J. Nutr.* **2003**, *133*, 399–404.
(18) Kroon, P. A.; Clifford, M. N.; Crozier, A.; Day, A. J.; Donovan, J. L.; Manach, C.; Williamson, G. How should we assess the effects of exposure to dietary polyphenols in vitro? *Am. J. Clin. Nutr.* **2004**, *80*, 15–21.
(19) Roura, E.; Andres-Lacueva, C.; Jauregui, O.; Badia, E.; Estruch, R.; Izquierdo-Pulido, M.; Lamuela-Raventos, R. M. Rapid liquid chromatography tandem mass spectrometry assay to quantify plasma (−)-epicatechin metabolites after ingestion of a standard portion of cocoa beverage in humans. *J. Agric. Food Chem.* **2005**, *53*, 6190–6194.
(20) Harada, M.; Kan, Y.; Naoki, H.; Fukui, Y.; Kageyama, N.; Kiso, W. Identification of the major antioxidative metabolites in biological fluids of the rat with ingested (+)-catechin and (−)-epicatechin. *Biosci. Biotechnol. Biochem.* **1999**, *63*, 973–977.
(21) Spencer, J. P.; Schroeter, H.; Rice-Evans, C. Epicatechin and its in vivo metabolite, 3′-O-methyl epicatechin, protect human fibroblasts from oxidative-stress-induced cell death involving caspase-3 activation. *Biochemistry* **2001**, *354*, 493–500.
(22) Osborne, R.; Joel, S.; Trew, D.; Slevin, M. Analgesic activity of morphine-6-glucuronide. *Lancet* **1988**, *8589*, 828.
(23) Paul, D.; Standifer, K. M.; Inturrisi, C. E.; Pasternak, G. W. Pharmacological characterization of morphine-6 beta-glucuronide, a very potent morphine metabolite. *J. Pharmacol. Exp. Ther.* **1989**, *251*, 477–483.
(24) Osborne, R.; Thompson, P.; Joel, S.; Trew, D.; Patel, N.; Slevin, M. The analgesic activity of morphine-6-glucuronide. *Br. J. Clin. Pharmacol.* **1992**, *34*, 130–138.
(25) Zhang, Y.; Song, T. T.; Cunnick, J. E.; Murphy, P. A.; Hendrich, S. Daidzein and genistein glucuronides in vitro are weakly estrogenic and activate human natural killer cells at nutritionally relevant concentrations. *J. Nutr.* **1999**, *129*, 399–495.
(26) Spencer, P. E.; Schroeter, H.; Crossthwaithe, A. J.; Kuhnle, G.; Williams, R. J.; Rice-Evans, C. Contrasting influences of glucuronidation and O-methylation of epicatechin on hydrogen peroxide-induced cell death in neurons and fibroblasts. *Free Radic. Biol. Med.* **2001**, *31*, 1139–1146.
(27) Okushio, K.; Suzuki, M.; Matsumoto, N.; Nanjo, F.; Hara, Y. Identification of (−)-epicatechin metabolites and their metabolic fate in the rat. *Drug Metab. Dispos.* **1999**, *27*, 309–316.
(28) Harada, M.; Kan, Y.; Naoki, H.; Fukui, Y.; Kageyama, N.; Nakai, M.; Miki, W.; Kiso, Y. Identification of the major antioxidative metabolites in biological fluids of the rat with ingested (+)-catechin and (−)-epicatechin. *Biosci. Biotechnol. Biochem.* **1999**, *63*, 973–977.
(29) Unno, T.; Sagesaka, Y. M.; Kakuda, T. J. Analysis of tea catechins in human plasma by high-performance liquid chromatography with solid-phase extraction. *J. Agric. Food Chem.* **2005**, *53*, 9885–9889.
(30) Henning, S. M.; Niu, Y.; Liu, Y.; Lee, N. H.; Hara, Y.; Thames, G. D.; Minutti, R. R.; Carpenter, C. L.; Wang, H.; Heber, D. Bioavailability and antioxidant effect of epigallocatechin gallate administered in purified form versus as green tea extract in healthy individuals. *J. Nutr. Biochem.* **2005**, *16*, 610–616.
(31) Lee, M. J.; Maliakal, P.; Chen, L.; Meng, X.; Bondoc, F.; Prabhu, S.; Lambert, G.; Mohr, S.; Yang, C. Pharmacokinetics of tea catechins after ingestion of green tea and (−)-epigallocatechin-3-gallate by humans: formation of different metabolites and individual variability. *Cancer Epidemiol. Biomark. Prev.* **2002**, *11*, 1025–1032.
(32) Chow, H. H.; Cai, Y.; Alberts, D. S.; Hakim, I.; Dorr, R.; Shahi, F.; Crowell, J. A.; Yang, C. S.; Hara, Y. Phase I Pharmacokinetic Study of Tea Polyphenols following Single-dose Administration of Epigallocatechin Gallate and Polyphenon E. *Cancer Epidemiol. Biomark Prev.* **2001**, *10*, 53–58.
(33) Lee, M. J.; Wang, X. Y.; Li, H.; Chen, L.; Sun, Y.; Gobbo, S.; Balentine, D. A.; Yang, C. S. Analysis of Plasma and Urinary tea Polyphenols in Human Subjects. *Cancer Epidemiol., Biomarkers Prev.* **1995**, *4*, 393–399.
(34) van Amelsvoort, J. M.; van het Hof, K. H.; Mathot, J. N. J. J.; Mulder, T. P. J.; Wiersma, A.; Tijnerg, L. B. M. Plasma concentrations of individual tea catechins after a single oral dose in humans. *Xenobiotica* **2001**, *31*, 891–901.
(35) Yang, C. S.; Chen, L.; Lee, M. J.; Balentine, D.; Kuo, M. C.; Schatz, S. P. Blood and urine levels of tea catechins after ingestion of different amounts of green tea by human volunteers. *Cancer Epidemiol., Biomarkers Prev.* **1998**, *7*, 351–354.
(36) Maiani, G.; Serafini, M.; Salucci, M.; Azzini, E.; Ferreo-Luzzi, A. Application of a new high-performance liquid chromatographic method for measuring selected polyphenols in human plasma. *J. Chromatogr. B.* **1997**, *692*, 311–317.
(37) Pietta, P.; Simonetti, P.; Gardana, C.; Brusamolino, A.; Morazoni, P.; Bombardelli, E. Catechin metabolites after intake of green tea infusions. *Biochem. Mol. Biol. Int.* **1998**, *46*, 895–903.
(38) Lee, M. J.; Prabhu, S.; Meng, X.; Li, C.; Yang, C. S. Pharmacokinetics of tea catechins after ingestion of green tea and (−)-epigallocatechin-3-gallate by humans: formation of different metabolites and individual variability. *Anal. Biochem.* **2000**, *279*, 164–169.
(39) Kotani, A.; Miyashita, N.; Kusu, F. Determination of catechins in human plasma after commercial canned green tea ingestion by high-performance liquid chromatography with electrochemical detection using a microbore column. *J. Chromatogr. B.* **2003**, *788*, 269–275.
(40) Umegaki, K.; Sugisawa, A.; Yamada, K.; Higuchi, M. Analytical method of measuring tea catechins in human plasma by solid-phase extraction and HPLC with electrochemical detection. *J. Nutr. Sci. Vitaminol.* **2001**, *47*, 402–408.
(41) Nakagawa, K.; Miyazawa, T. Chemiluminescence-high-performance liquid chromatographic determination of tea catechin, (−)-epigallocatechin 3-gallate, at picomole levels in rat and human plasma. *Anal. Biochem.* **1997**, *248*, 41–49.
(42) Masukawa, Y.; Matsui, Y.; Shimizu, N.; Kondou, N.; Endou, H.; Kuzukawa, M.; Hase, T. Determination of green tea catechins in human plasma using liquid chromatography-electrospray ionization mass spectrometry. *J. Chromatogr. B.* **2006**, *834*, 26–34.

(43) Murphy, A. T.; Bonate, P. L.; Kasper, S. C.; Gillespie, T. A.; Delong, A. F. Determination of xanomeline in human plasma by ionspray tandem mass-spectrometry. *Mass Spectrom.* **1994**, *23*, 621–625.

(44) Mata, M. L.; Lacueva, C. A.; Roura, E.; Jáuregui, O.; Escribano, E.; Torre, C.; Lamuela-Raventós, R. M. Absorption and pharmacokinetics of grapefruit flavanones in beagles. *Br. J. Nutr.* **2007**, *98*, 86–92.

(45) Urpí-Sardà, M.; Jáuregui, O.; Lamuela-Raventós, R. M.; Jaeger, W.; Miksits, M.; Covas, M. I.; Andrés-Lacueva, C. Uptake of diet resveratrol into the human low-density lipoprotein. Identification and quantification of resveratrol metabolites by liquid chromatography coupled with tandem mass spectrometry. *Anal. Chem.* **2005**, *77*, 3149–3155.

(46) Committee on care and use of laboratory animals. *Guide for the Care and Use of Laboratory Animals*; Institute of Laboratory Animals Resources, National Research Council: Washington, DC, 1985; 83 pp.

(47) Gonthier, M. P.; Rios, L. Y.; Verny, M. A.; Remesy, C.; Scalbert, A. Novel liquid chromatography-electrospray ionization mass spectrometry method for the quantification in human urine of microbial aromatic acid metabolites derived from dietary polyphenols. *J. Chromatogr., B* **2003**, *789*, 243–255.

(48) Kiser, M.; Dolan, J. W. Selecting the Best Curve Fit. *LC-GC Eur.* **2004**, *17*, 138–143.

(49) U. S. Department of Health and Human Services, Food and Drug Administration. *Guidance for Industry. Bioanalytical Method Validation*; Washington, DC, 2001.

(50) Nobilis, M.; Holcapek, M.; Kolárová, L.; Kopecky, J.; Kunes, M.; Svoboda, Z.; Kvetina, J. Identification and determination of phase II nabumetone metabolites by high-performance liquid chromatography with photodiode array and mass spectrometric detection. *J. Chromatogr., A.* **2004**, *1031*, 229–236.

(51) Li, C.; Meng, X.; Winnik, B.; Lee, M.; Lu, H.; Sheng, S.; Buckley, B.; Yang, C. S. Analysis of urinary metabolites of tea catechins by liquid chromatography/electrospray ionization mass spectrometry. *Chem. Res. Toxicol.* **2001**, *14*, 702–707.

(52) Meng, X.; Sang, S.; Zhu, N.; Lu, H.; Sheng, S.; Lee, M.; Ho, C.; Yang, C. S. Identification and characterization of methylated and ring-fission metabolites of tea catechins formed in humans, mice, and rats. *Chem. Res. Toxicol.* **2002**, *15*, 1042–1050.

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