Absorption and pharmacokinetics of green tea catechins in beagles

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The present study evaluates for the first time in dogs, the kinetics of green tea catechins and their metabolic forms in plasma and urine. Ten beagles were administered 173 mg (12·35 mg/kg body weight) of catechins as a green tea extract, in capsules. Blood samples were collected during 24 h after intake and urine samples were collected during the following periods of time: 0–2, 2–6, 6–8 and 8–24 h. Two catechins with a galloyl moiety and three conjugated metabolites were detected in plasma. Most of the detected forms in plasma reached their maximum plasma concentration (C_{max}) at around 1 h. Median C_{max} for (−)-epigallocatechin-3-gallate (EGCG), (−)-epicatechin-3-gallate (ECG), (−)-epigallocatechin glucuronide (EGC-glucuronide), (−)-epicatechin glucuronide (EC-glucuronide), (−)-epicatechin sulphate (EC-sulphate) were 0·3 (range 0·1–1·9), 0·1 (range 0–0·4), 0·8 (range 0·2–3·9), 0·2 (range 0·1–1·7) and 1 (range 0·3–3·4) μmol/l, respectively. The areas under the plasma concentration *v.* time curves ($AUC_{0\rightarrow24}$) were 427 (range 102–1185) μmol/l × min for EGC-glucuronide, 112 (range 53–919) μmol/l × min for EC-sulphate, 71 (range 26–306) μmol/l × min for EGCG, 40 (range 12–258) μmol/l × min for EC-glucuronide and 14 (range 0·1–124) μmol/l × min for ECG. The values of mean residence time ($MRT_{0\rightarrow24}$) were 5 (range 2–16), 2 (range 1–11), 10 (range 2–13), 3 (range 2–16) and 2·4 (range 1–18) h for EGCG, ECG, EGC-glucuronide, EC-glucuronide and EC-sulphate, respectively. In urine, catechins were present as conjugated forms, suggesting bile excretion of EGCG and ECG. Green tea catechins are absorbed following an oral administration and EGC-glucuronide is the metabolic form that remains in the organism for a longer period of time, suggesting that this compound could suffer an enterohepatic cycle.

Flavanols: Metabolites: Pharmacokinetic: Extract: Plasma: Dogs: Urine

Green tea is the most consumed beverage in the world aside from water, and has recently attracted significant attention, both in the scientific and in consumer communities for its health benefits for a variety of disorders, protection against cancer and promotion of weight loss. The beneficial effects of green tea are attributed to the polyphenolic compounds present in this beverage. Green tea polyphenols have been extensively studied as CVD and cancer chemopreventive agents[1–4]. The natural product (−)-epigallocatechin-3-gallate (EGCG) is the major polyphenolic constituent found in green tea (dried fresh leaves of the plant *Camellia sinensis*)[5]. Several other flavanols are also found in lower abundance in this beverage, including (−)-epicatechin-3-gallate (ECG), (−)-epigallocatechin (EGC), (−)-epicatechin (EC) and (+)-catechin (Fig. 1). More than 50 % of the mass of this catechin combination is composed of EGCG which has proven beneficial effects in studies of Parkinson's disease[6], Alzheimer's disease[7,8],

stroke[9,10], obesity[11], diabetes[12,13], chemoprevention[14–16] and high antioxidant activity[17]. It has also been demonstrated to be a potential anti-inflammatory molecule[18,19].

The metabolic fate of polyphenols in the organism after their ingestion as food or beverages should be thoroughly studied, since these are the active forms that may generate the biological effect of these compounds. Green tea catechin *in vivo* activity is limited by effective concentrations at target sites, since it is assumed that plasma concentration of catechins and their metabolites are dynamically equilibrated with their concentration at successfully reached tissues. The changes observed in plasma concentrations reflect transformations the metabolites undergo in the tissues. It is, therefore, of special interest to study in depth the behaviour of catechins in the organism, that is, to evaluate the process of absorption, distribution, metabolism and excretion through plasma and urine levels of their metabolites. The use of enzymatic
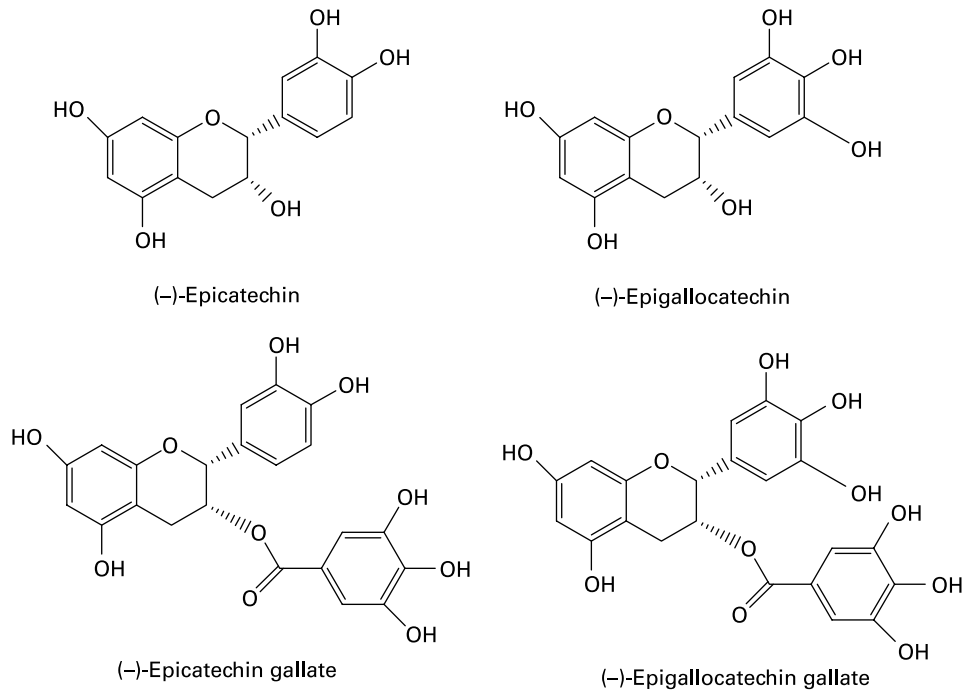

Fig. 1. Chemical structures of green tea catechins.

hydrolysis for the determination of tea catechins in plasma has been extensively applied in previous studies[20–26]. However, information about individual metabolites is not available in these studies, nor have they carried out a pharmacokinetic evaluation of the specific active molecules that could reach target cells.

The use of dogs as a model has been shown to be useful in evaluating the absorption of polyphenols from different food sources, allowing identification of specific forms present in the systemic circulation. In a previous study we evaluated in plasma, the kinetics of flavanones and their metabolites in beagle dogs after oral ingestion of a grapefruit fruit extract[27]. Our earlier results demonstrated the absorption of grapefruit flavanone via the presence of its metabolites in plasma. Swezey et al.[28] studied in beagles the absorption, tissue distribution and elimination of 4-[³H]epigallocatechin-3-gallate after intravenous and oral administration of radiolabelled EGCG. Their results indicate that, after oral administration, EGCG is rapidly and widely distributed to tissues where it can exert its biological effects.

Therefore, the purpose of the current study is to assess, for the first time in beagles, the major flavanol forms in plasma and urine after oral administration of a green tea extract. We also evaluate the pharmacokinetics of these metabolic forms in both biological matrices by considering their biotransformation, thus providing a general model that can be used for studies on flavonoid bioavailability.

Materials and methods

Standards and reagents

EGCG (95 %), EGC and ECG (98 %) from green tea, (−)-epi-catechin and blank dog plasma were purchased from Sigma-Aldrich (St Louis, MO, USA). Ethylgallate (internal standard) was purchased from Extrasynthese (Genay, France). Methanol, acetonitrile and n-n-dimethylformamide (HPLC grade) and formic acid were purchased from Scharlau Chemie SA (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, USA). Green tea capsules contained 124 mg EGCG, 9 mg EGC, 21 mg EC, 19 mg ECG, 4·38 mg (+)-catequin and talc as an excipient. The capsules were stored at room temperature and protected from extreme environmental conditions.

Subjects and study design

Twelve healthy adult (14 (SD 3) kg) female beagle dogs were randomly chosen and were deprived of food overnight before the experiment. The dogs were orally administered with two capsules containing 200 mg green tea extract; two dogs were chosen as a control and were given an excipient containing talc. Blood samples (5 ml) were drawn before capsule administration and at the following times: 30, 45, 60, 75, 90 min, 2, 4, 8 and 24 h and were collected in vacutainer tubes containing EDTA as anticoagulant (Becton Dickinson, Franklin Lakes, NJ, USA). Plasma was obtained after blood centrifugation at 1000 g for 10 min and stored in Eppendorf tubes at −80°C until analysis. Urine samples were taken before and at the following intervals: 0–2, 2–6, 6–8 and 8–24 h, after oral administration of green tea extract. Urine was acidified with 200 mM-HCl and stored at −80°C until analysis.

The study was carried out at the University of Zaragoza (Spain) in accordance with the Guide for the Care and Use of Laboratory Animals[29]. 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 obtained by solid-phase extraction. Dog plasma samples were treated as follows: 280 μl internal standard (2525 nmol/l) were added to 1 ml plasma and then mixed with 370 μl antioxidant solution (containing 0·2 g/ml ascorbic acid and 1 mg/ml EDTA) and with 20 μl *o*-phosphoric acid. After 2 min of vortex mixing, samples were diluted with 3 ml water. A solid-phase extraction (30 mg) with a Waters Oasis® HLB 30 μm ninety-six-well plate (Milford, MA, USA) was applied to the mixture. Cartridge activation was achieved by adding 1 ml: methanol, water, 70 % (v/v) *n-n*-dimethylformamide containing 0·1 % (v/v) formic acid and water, respectively. The cartridges were washed with 2 ml water and 1 ml 30 % (v/v) methanol. Tea catechin metabolites were then eluted with 0·7 ml 70 % (v/v) *n-n*-dimethylformamide containing 0·1 % (v/v) formic acid. After filtration with a 4 mm, 0·45 μm polytetrafluoroethane filter (Waters Corporation, USA), 20 μl of the resulting filtrate was injected into the LC–MS/MS system.

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

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, USA) equipped with a quaternary pump and a refrigerated auto-sampler. A Luna C18 column (50 × 2·0 mm internal diameter, 5 μm) from Phenomenex (Torrance, CA, USA) was used at room temperature, and the injected volume was 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. A gradient profile with the following proportions (v/v) of acetonitrile (0·1 % formic acid) was applied: 0 min, 0·5 %; 3 min, 15 %; 6 min, 100 %; 9 min, 100 %. A triple quadrupole mass spectrometer (API 3000; Applied Biosystems, PE Sciex, Concord, Ontario, Canada), equipped with a Turbo IonSpray source was used to obtain the MS and MS/MS data. Prior to its use, the instrument was checked to meet the acceptance specifications defined by the manufacturer. The triple quadrupole mass spectrometer was calibrated with the Turbo IonSpray using a test mixture solution of poly (propyleneglycol) obtained from Applied Biosystems. The mass spectrometer was calibrated so that mass accuracy specifications and sensitivity were achieved over the entire mass range. Turbo Ionspray 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 and the collision energy were optimized for each compound in infusion experiments: individual standard solutions (21·8–34·5 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 unit. 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 of selected precursor ions in the collision cell of the triple–quadrupole mass spectrometer and mass analysed using the second analyser of the instrument. The multiple reaction monitoring, the method of choice owing to its highest selectivity and sensitivity in quantitative LC–MS/MS, has 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, multiple reaction monitoring transition as mentioned earlier and transitions $481 \rightarrow 305$ ((−)-epigallocatechin-glucuronide), $385 \rightarrow 305$ ((−)-epigallocatechin-sulphate), $305 \rightarrow 125$ ((−)-epigallocatechin), $465 \rightarrow 289$ ((−)-epicatechin-glucuronide), $369 \rightarrow 289$ ((−)-epicatechin-sulphate (*m/z* 369/289), $289 \rightarrow 245$ ((−)-epicatechin) (at a higher declustering potential value) were chosen as confirmation of the multiple reaction monitoring trace for each metabolite in collisionally induced dissociation MS/MS experiments[30,31].

Safety considerations

Dog plasma samples were considered as potentially infectious. We have respected general guidelines regarding working with biological fluids, organic solvents and acids. Universal precautions regarding handling of chemicals and fluids were applied.

Pharmacokinetic analysis

Pharmacokinetic parameters were determined by means of a non-compartmental analysis using the WinNonlin® Professional software version 3.3 (Pharsight Corporation, USA). The linear trapezoidal method was used to calculate the area under the plasma concentration curve (AUC_{0-t}) from time 0 until the last detectable concentration. The maximum plasma concentration (C_{max}) and the time needed to reach C_{max} (T_{max}) were determined by visual inspection of the experimental data. MRT was estimated by means of the AUMC/AUC ratio, where AUMC is from the first measurement of the plasma concentration *v.* time curve. The maximum accumulated amount of catechins excreted in urine was also determined.

Statistical analysis

The pharmacokinetic parameters of green tea catechins and its metabolites were compared by one-way ANOVA on ranks followed by a Scheffé's multiple comparison test. $P < 0.05$ was considered significant. The statistical analysis was performed using SPSS software version 11.5 for Windows (SPSS Inc., Chicago, IL, USA).

Results

Green tea catechins present in plasma and urine samples

Two catechins with a galloyl moiety were present in plasma (EGCG and ECG) and three conjugated forms were also detected, however, only the conjugated forms of EC and EGC were present in urine. Regarding conjugated catechins found

in plasma, EGC was mainly present in the glucuronide form while EC was both in the glucuronide and sulphate form. Figs. 2 and 3 show plasma concentration *v.* time curves corresponding to the catechins and catechin metabolites detected in plasma. In urine tea catechins EGC and EC were mostly present in the conjugated forms, as glucuronides and sulphates.

Pharmacokinetics of green tea catechins after oral intake of green tea extract

The following pharmacokinetic parameters corresponding to each of the catechins and catechin metabolites (C_{max}, $AUC_{0\to24}$, $MRT_{0\to24}$, T_{max}) are summarized in Table 1. Values are expressed as median and range, together with the results of the statistical analysis carried out. No significant statistical differences were found between most of the pharmacokinetic parameters corresponding to both catechins and catechin conjugates. This was not the case, however, with $AUC_{0\to24}$, whose values showed significant differences between (−)-epigallocatechin glucuronide (EGC-glucuronide) and (−)-epicatechin glucuronide (EC-glucuronide), and EGC-glucuronide and ECG.

Fig. 4 represents the curves of accumulated excretion in urine for each of the conjugated catechins. Values are expressed as means and standard deviations for ten dogs.

Discussion

During the past 10 years, there have been several pharmacokinetics studies[22–26,32–36] with green tea catechins where only unchanged catechins were evaluated, suggesting that the calculated bioavailability in these studies were probably overestimated. Thus, additional studies, where catechin metabolites are determined, are necessary. Once the polyphenol has been ingested it may suffer a number of changes during absorption and when it reaches the systemic circulation it may be present in its native or in its metabolic form. Thus, in order to understand the mechanism by which polyphenols exert their beneficial effects, extensive data from metabolic and distribution studies are required. Previous studies with green tea
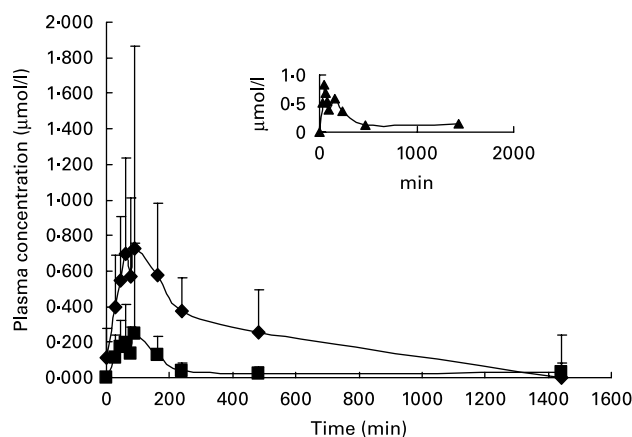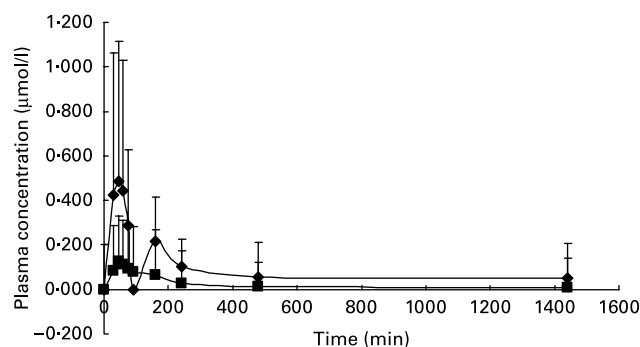
Fig. 3. Time *v.* plasma concentration curves for the following catechins: (−)-epigallocatechin-3-gallate (◆) and (−)-epicatechin-3-gallate (■) for ten beagles receiving 173 mg green tea catechins. Values are means with their standard deviations depicted by vertical bars.

catechins have applied different forms of administration, including green tea as a beverage, green tea extracts, pure catechins (EGCG) or polyphenolic preparations (Polyphenon E), demonstrating that the form of administration has a pronounced effect on the bioavailability of these compounds.

In the present study, we have administered green tea catechins as a green tea extract contained in capsules. The presence of conjugated EGCG forms in dog plasma was not detected, only the free forms of EGCG and ECG were present. The present results were in accordance with Chow *et al.* [23], who indicated that after ingestion of EGCG and Polyphenon E (a tea polyphenol preparation) by human volunteers, plasma EGCG was mainly present in the free (unconjugated) form and may be eliminated in faeces by biliar excretion. The presence of unconjugated forms of EGCG and ECG in dog plasma could be explained by the fact that EGCG and ECG have a galloyl group on the C ring and EGCG has a hydroxyl group in the $5'$ position (Fig. 1). Possibly this group could have decreased access to the active site of uridine diphosphate glucuronosyl transferase, since EGCG (galloyl group and hydroxyl in $5'$ position) has shown an even lower glucuronidation rate than that of ECG[37]. Another explanation, as has been reported before, may be the fact that green tea catechins can be strong inhibitors of phase II enzymes (caffeic *o*-methyltransferases, uridine diphosphate glucuronosyl transferases and sulfotransferases). It has also been found that methylation of EGC can be strongly inhibited by ECG and EGCG[38], justifying the absence of methylated conjugate.

As can be observed in Table 1, EGC-glucuronide showed the highest $AUC_{0\to24}$ values and also the highest $MRT_{0\to24}$, indicating that this specific metabolite remains within the organism for approximately 10 h. Considering that in the orally administered green tea extract, EGC is in the lowest proportion (4·7 %) compared to the other catechins, these high AUC and MRT values may be due to an enterohepatic cycle. The glucuronide formation could take place during EGC absorption and/or in the liver by the action of uridine diphosphate glucuronosyl transferase. The resulting conjugated product (EGC-glucuronide) could be again excreted to the intestinal lumen through the bile. This conjugate could be hydrolysed by action of the intestinal enzyme β-glucuronidase generating EGC that could once again be absorbed, generating the enterohepatic cycle, and remaining in the organism

Fig. 2. Time *v.* plasma concentration curves for the following catechin metabolites: (−)-epigallocatechin glucuronide (◆), (−)-epicatechin glucuronide (■) and (−)-epicatechin sulphate (▲) for ten beagles receiving 173 mg green tea catechins. Values are means with their standard deviations depicted by vertical bars.

Table 1. Pharmacokinetic parameters of green catechins and their metabolites in beagles, after oral intake of green tea extract

(Median values and ranges for ten beagles)

Parameter	EGCG		EGC-glucuronide		EC-glucuronide		EC-sulphate		ECG	
	Median	Range	Median	Range	Median	Range	Median	Range	Median	Range
C_{max} (μmol/l)	0·3	0·1–2	0·8	0·2–4	0·2	0·1–2	1	0·3–3	0·1	0–0·4
T_{max} (h)	1·1	0·5–2·7	1·3	0·8–24	1	0·8–2·7	1·1	0·5–4	0·9	0·5–8
$AUC_{0\rightarrow24}$ (μmol/l × min)	71	26–306	427	102–1185	40*	12–258	112	53–919	14*	0·1–124
$MRT_{0\rightarrow24}$ (h)	5	2–16	10	2–13	3	2–16	2·4	1–18	2	1–11

AUC, area under the plasma concentration *v.* time curve; C_{max}, maximum plasma concentration; ECG, (−)-epicatechin-3-gallate; EC-glucuronide, (−)-epicatechin glucuronide; EC-sulphate, (−)-epicatechin sulphate; EGCG, (−)-epigallocatechin-3-gallate; EGC-glucuronide, (−)-epigallocatechin glucuronide; MRT, mean residence time; T_{max}, time needed to reach C_{max}.
Median values were significantly different from those of the EGC-glucuronide group: *$P < 0.05$.

for a longer period of time. As has been reported before, the health benefits of flavonoids can be due to different properties of their major phase II metabolites, such as antioxidants, protein inhibitors of human Multidrug Resistance Proteins 1 and 2, and monocyte–endothelial cell interaction[39–41]. The high MRT value is very interesting because EGC-glucuronide could be a potentially active molecule that remains in the organism long enough to exert its beneficial actions.

T_{max} and AUC values for EGCG compared well with data generated in previous studies with human subjects[21,24,34,42]. However, C_{max} values differ, suggesting that the bioavailability of green tea catechins is variable and could be due to differences in species, quantity and form of catechin administration. It should be considered that the presence or absence and the time-dependent appearance and disappearance of specific catechin metabolites in plasma can vary significantly among subjects and species, suggesting that interindividual variation is an important factor that must always be taken into consideration when performing dietary assessment studies.

It is assumed that plasma concentrations of catechins and their metabolites are equilibrated with their concentrations at successfully reached tissues, Swezey *et al.*[28] demonstrated that radioactive EGCG was distributed to a variety of epithelial tissues in beagles; the highest concentrations being observed in the liver and gastrointestinal tract

tissues. However, additional investigation is needed on tissue distribution, specifically with the metabolites present in plasma after oral intake, since these compounds, such as EGC-glucuronide, are the ones that remain in the organism for a longer period of time.

The maximum amount of metabolites excreted in urine is directly related to the quantity of the absorbed compound[43]. In Fig. 4, the accumulative amount excreted per sampling interval is referred to the mid-point of the collection period, for example, for the 0–2 h period, the mid-point would be 1 h; for the interval 2–6 h, the mid-point would be 4 h. The values of the 7 h mid-point correspond to the interval 6–8 h. Unfortunately not all the subjects could urinate during these hours, obtaining less sample and consequently, lower values. This tendency is only observed in the graphics with mean values; individual graphics do not show this tendency. As presented in Fig. 4, EC conjugates are the metabolites with the highest excreted amounts, sulphates being the major ones; (−)-epicatechin sulphate is the metabolite with the highest maximum amount excreted. The cumulated urine excretion during 24 h after ingestion of the green tea extract accounted for 1·12 and 0·58 % for EC and EGC, respectively, suggesting that a considerable amount of these metabolites could have bile excretion as an elimination pathway as well. On the other hand, the absence of unconjugated EGCG and ECG in urine may possibly be due to most of the absorbed unconjugated EGCG being excreted through the bile[28,44].

To our knowledge, we have evaluated, for the first time in beagles, the pharmacokinetics of green tea catechins and their metabolites in plasma and urine after oral administration of a green tea extract. We have demonstrated that catechins are absorbed from the intestine as their intact form or as conjugates following oral administration, and that the conjugated catechins remain in the organism for a longer period of time probably due to an enterohepatic cycle. The present finding is very interesting because if the conjugated metabolites of green tea catechins are indeed active molecules, their MRT values are high enough to exert potentially beneficial effects. These data also provide a general model for future pharmacokinetics studies.
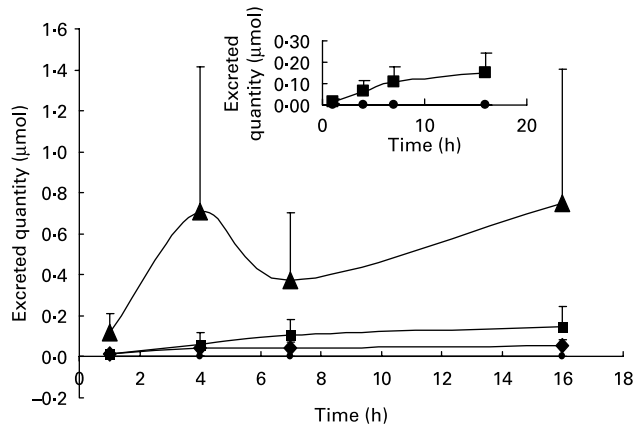

Fig. 4. Accumulated excreted quantities (μmol) of catechin conjugates in urine during 24 h, after capsule administration for ten beagles. Values are means with their standard deviations depicted by vertical bars. ◆, (−)-Epicatechin glucuronide; ▲, (−)-epicatechin sulphate; ●, (−)-epigallocatechin glucuronide; ■, (−)-epigallocatechin sulphate.

Acknowledgements

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