Absorption and Metabolization of Cytoprotective Epicatechin Thio-Conjugates in Rats

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**List of Abbreviations:**

COMT, catechol-\(O\)-methyl transferase; Cya, cysteamine; Cya-EC, \(4\beta\)-(2-aminooethylthio)epicatechin or \(4\beta\)-(S-cysteaminyl)-epicatechin; Cys-EC, \(4\beta\)-(S-cysteiny1)epicatechin; EC, (−)-epicatechin; Gluc, glucuronidyl; HPLC–MS/MS, high performance liquid chromatography coupled to tandem mass spectrometry; Me, methyl; MRM, multiple reaction monitoring MS, mass spectrometry; PBS, phosphate buffer saline; SAM, S-adenosyl-L-methionine; TFA, trifluoroacetic acid; UDPGA, uridine diphosphate glucuronic acid.
Abstract

The epicatechin thio-derivatives 4β-(S-cysteinyl)-epicatechin (Cys-EC) and 4β-(S-cysteaminyl)-epicatechin (Cya-EC) are compounds that may provide protection from oxidation via mechanisms involving either the flavonoid moiety or the non-phenolic cysteine or cysteamine part of the molecule. Because the metabolically modified molecules may be the actual active species, we estimated the absorption/metabolization of the thio-derivatives through the small intestine in vitro and studied the body distribution of the compounds and their metabolites in rats. The analysis of the samples generated was done by high-performance liquid chromatography coupled to a UV detector and a tandem mass spectrometer. We show that Cya-EC follows the same phase II metabolization pattern as EC, while Cys-EC is transported with the intact catechol moiety through the small intestine and effectively metabolized systemically. We also find that Cya-EC generates Cys-EC in vivo, which provides evidence for a Cya-EC-mediated cytoprotective effect through cysteamine/cystine exchange with subsequent cysteine transport, ubiquitously throughout the organism.
Introduction

Polyphenols, particularly flavanols (flavonoids also known as catechins) have come under scientific scrutiny because they appear to have many desirable chemopreventive activities. However, there is no conclusive evidence that these activities have a net beneficial effect on animal health, particularly in humans. It is evident that polyphenols are effective radical scavengers in solution and under many experimental set-ups in vitro. Because free radicals trigger cell and tissue damage that can lead to disease and death, as described by Harman in his famous paper (Harman, 1956), it has long been assumed that free-radical scavengers such as polyphenols would prevent such damage and would eventually prolong life. Alas, things appear to be somewhat more complicated and polyphenols seem to fail in this apparently simple task. The reason for this is that radicals are not necessarily harmful and play key physiological roles in the organism as second messengers and part of controlled cytotoxic systems in crucial processes such as apoptosis. Thus the organism carefully regulates its redox homeostasis, which is not easily modified by exogenous agents (Halliwell, 2006). The extensive metabolization and effective excretion of polyphenols after ingestion is one of these control systems. A very small presence of intact catechol or pyrogallol moieties is detected in plasma and urine at any time in the post-prandial period; most of the catechins are rapidly converted into glucuronidates, methyl esters and sulphates in the small intestine and the liver, and also cleaved into smaller units by the gut microbiota (Kuhnle et al., 2000; Gonthier et al., 2003). If any biological activity is to be attributed to polyphenols in vivo, it seems probable that it would be triggered by the metabolites rather than the intact species.
We have proposed a series of thio-conjugates of catechins as novel putative antioxidants (Torres and Bobet, 2001; Torres et al., 2002). Apart from their classic role as free-radical scavengers, these thio-conjugates protect neurons in vitro from oxidative glutamate toxicity by a mechanism involving the maintenance of intracellular levels of glutathione rather than by scavenging free radicals (Torres et al., 2005). Because the mode of action of thio-catechins most probably involves the non-flavonoid part of the molecule (i.e., cysteine or cysteamine) we wanted to estimate the influence of thio-conjugation upon the absorption of antioxidants, and the fate of the conjugates after oral administration to rats. We first examined the absorption of 4β-(S-cysteinyl)-epicatechin (Cys-EC), 4β-(2-aminoethylthio)-epicatechin or 4β-(S-cysteaminyl)-epicatechin (Cya-EC) and non-conjugated (+)-epicatechin (EC) in the small intestine by the everted sacs technique; and then the metabolization and body distribution of the same molecules, in rats. The results should provide clues as to whether the activity observed in vitro has any significance in vivo.

Materials and Methods

Chemicals and Reagents. Water and solvents used were: milli-Q water, HPLC-grade acetonitrile (CH3CN, Merck, Darmstadt, Germany) for analytical RP–HPLC, and HPLC-grade acetonitrile (SDS, Peypin, France) for HPLC–MS. Acetic acid was from Merck. Biotech-grade trifluoroacetic acid (TFA; Fluorochem, Derbyshire, UK) was distilled in-house. Standards of EC (≥97%) and (+)-catechin (≥98%) were obtained from Sigma Chemical (Saint Louis, MO, USA). Thio-conjugates of EC, Cys-EC (98%) and Cya-EC (98%) were prepared in-house as described in the literature (Torres et al., 2002; Selga and Torres, 2005). Other enzymes and chemicals used were: β-
glucuronidase (type L-II from limpets, EC 3.2.2.3.1), catechol-\textit{O}\textendash\methyl transferase (COMT, from porcine liver, EC 2.1.1.6) and S-adenosyl-L-methionine (SAM) all from Sigma-Aldrich, (Steinheim, Germany).

**Animals and Diets.** Male Sprague-Dawley rats were provided by Harlan Interfauna Ibérica S.L. (Barcelona, Spain) and weighed 200 ± 15 g. The rats used for the everted sacs experiment (5 for each compound) were deprived of food for \(~\)18 h, anesthetized by a single dose of 90 mg of ketamine chlorohydrate/kg of body weight and 10 mg xilacine chlorohydrate/kg of body weight, administered intraperitoneally and kept alive during the extraction of the small intestine (jejunum and ileum) and then killed by an overdose of the anesthetic. The rats used to obtain fresh livers were killed by decapitation. The rats (3 for each compound) used for metabolism and body distribution studies were fed 400 mg of the test compounds/kg of body weight, and then killed 4 h later by decapitation to prevent interferences with liver metabolism.

The experimental protocols were approved by the Experimental Animal Ethical Research Committee of the University of Barcelona in accordance with current regulations for the use and handling of experimental animals (Decree 21497, *Generalitat de Catalunya*).

**HPLC–UV analysis.** Samples were analyzed by reversed-phase high performance liquid chromatography (RP–HPLC) on a LaChrom Elite HPLC system (Merck, Darmstadt, Germany) equipped with a quaternary pump, temperature control unit and photo-diode array UV detector (DAD) fitted with a Kromasil C18 column (25 cm x 0.4
Elution: [A] 0.10% (v/v) aqueous TFA, [B] 0.08% (v/v) TFA in water/CH$_3$CN (2:3) with a gradient of 8% to 28% [B] over 60 min. The flow rate was 1 mL/min. The UV detection wavelengths were 214 nm and 280 nm. Quantitative determination of the major components was performed from calibration curves (280 nm) of the corresponding peak areas, made using pure standards prepared as described elsewhere (Torres and Lozano, 2001). This system was also used for the micro-purification of metabolites for subsequent analysis by HPLC–MS and HPLC–MS/MS analysis.

**HPLC–MS and HPLC–MS/MS analysis.** An API 3000 triple quadrupole mass spectrometer (ABSciex, Concord, Ontario, Canada) with a Turbo Ion spray source was used in negative ion detection mode to obtain primary molecular ions and fragments (MS) and secondary fragments (MS/MS). Liquid chromatography separations were performed on an Agilent 1100 series liquid chromatography system (Agilent, Waldbronn, Germany) equipped with a Supelco Discovery HSC 18, 15 x 0.21 cm, 5 µm; 0.4 mL/min column. Gradient elution was performed using a binary system consisting of [A] 0.05% aqueous acetic acid and [B] CH$_3$CN, with a gradient of 0 to 18% of [B] over 20 min, followed by a cleaning step at 100% [B] (10 min) and a re-equilibration step (fast gradient to 0% [B] and then 10 min at these initial conditions). The MS settings were: capillary voltage -3500 V (negative mode), nebulizer gas (N$_2$) 10 arbitrary units (a.u.), curtain gas (N$_2$) 12 a.u., collision gas (N$_2$) 10 a.u., declustering potential (DP) between -30 and -60 V, focusing potential -200 V, entrance potential 10 V, and collision energy (CE) -30 V. The drying gas (N$_2$) was heated to 400°C and introduced at a flow rate of 8000 cm$^3$/min. Full-scan data acquisition was performed over values of $m/z$ ranging from 100 to 800 using a cycle time of 2 s with a step size of
0.1 units. Analyst 1.4.2 software from PE Sciex was used for data acquisition and processing. The samples were first analyzed by MS in full-scan mode (FS) and the results were compared with those from blank samples. FS data acquisition was performed by scanning from m/z 100 to 800 in profile mode and using a cycle time of 2 s with a step size of 0.1 u. and a pause of 2 ms between each scan. In product ion scan experiments, MS/MS product ions were produced by collision-activated dissociation (CAD) of selected precursor ions in the collision cell of the triple quadrupole mass spectrometer and mass analyzed using the second analyzer on the instrument. Multiple reaction monitoring (MRM) was performed for each transition with a dwell time of 500 ms. The identity of the putative metabolites found in FS was corroborated by MRM transitions, which give the best selectivity and sensitivity in HPLC–MS/MS (Liu and Hop, 2005) and confirmed by product ion scan MS/MS experiments on the molecular ion and/or fragment ions.

**Stability studies.** Stability under stomach conditions was tested at a concentration of 0.1 mM in simulated gastric juice (0.24% hydrochloric acid-0.2% sodium chloride solution, pH 2) as described elsewhere (Zhu et al., 2002b) by incubation with continuous shaking at 37°C for 1 h. Stability under intestinal conditions was tested at pH 7.4 and 6.5. Stability at pH 7.4 was assessed at a concentration of 0.1 mM with a Krebs-Henseleit bicarbonate buffer, with the following composition: 118 mM NaCl, 4.74 mM KCl, 1.18 mM MgSO₄·7H₂O, and 1.27 mM CaCl₂. The medium was gassed with carbogen (95% O₂, 5% CO₂) until pH 7.4 was obtained. At pH 6.5, stability was assessed at a concentration of 0.1 mM in the same buffer used before with 2-(4-morpholino)ethanesulfonic acid (MES) instead of carbonate, and the solution adjusted to the desired pH with Tris. Simulated intestinal juice solutions (10 mL) were incubated
for 30 min at 37ºC and continuously gassed with carbogen. The pH of all the solutions was kept constant during incubation. Samples were analyzed by HPLC.

**Metabolite standards.** Glucuronides of EC, Cys-EC and Cya-EC were obtained using liver microsomes essentially as described in the literature (Morand et al., 1998). Fresh rat livers were excised from decapitated rats then washed and homogenized in an ice-cold buffer (50 mM Tris pH 7.2, 100 mM Sucrose, 10 mM EDTA, 2 mM dithiothreitol (DTT), 1 µM Leupeptin) to obtain 50% (w/v) tissue homogenates. Samples were centrifuged at 11,000 g for 20 min at 4ºC and the supernatant was filtered and centrifuged at 105,000 g for 60 min. The final microsomal pellet was re-suspended to 50% (w/v) in HEPES buffer (100 mM HEPES, 100 mM sucrose) and kept frozen at –80ºC until use. The final preparation of microsomes had a protein concentration of 7 mg/mL, measured by BCA (Pierce, Rockford, IL, USA)/UV–Vis spectroscopy at 595 nm. The glucuronides were obtained by incubation of the polyphenols with the microsomal preparation. The incubation medium (750 µL) consisted of buffer (75 mM HEPES, 10 mM MgCl₂, pH 7, 540 µL), UDPGA (7 mM final concentration, 50 µL) and microsomal suspension (1/2) (~7 mg/mL, 100 µL) which was activated *in situ* by the addition of Triton X-100 (0.2% solution, 60 µL). The reaction was started by the addition of 2 µL aglycone solution to a final concentration of 0.3 mM and the mixture kept at 37ºC for 3 h. After the addition of acetonitrile (5% final concentration) and centrifugation, the samples were analyzed by HPLC–UV. To identify the peaks corresponding to glucuronide conjugates, aliquots of the incubation mixture were treated with β-glucuronidase or vehicle, and analyzed.

Methyl esters of EC, Cys-EC and Cya-EC were obtained with the microsomal preparation described before. The reaction mixture (1 mL) containing 50 µL of the liver
microsomes preparation in 0.1 M Tris-HCl buffer, pH 7.9, 10 mM MgCl₂, 1 mM DTT, 5 mM SAM and the corresponding substrate (20 µM) was incubated at 37°C in a shaking water bath for 6 h (Piskula and Terao, 1998). Samples were taken after 30 min and 6 h and analyzed by HPLC–UV. Methylation was also performed using COMT and the methyl group donor SAM (Ball et al., 1972; Piskula and Terao, 1998; Spencer et al., 2001). Briefly, COMT (500 units/mL) was pre-incubated in 10 mL of pH 7.5 buffer containing 20 mM cysteine, 2 mM MgCl₂, 10 mM NaH₂PO₄ and 0.5 mM DTT, at 37°C for 10 min. After incubation, nitrogen was bubbled through the mixture and SAM (5 mM, final concentration) and substrate (0.15 mM final concentrations of EC, Cys-Ec or Cya-EC) were added. The mixture was stirred at 37°C for 8 h. Additional SAM (same amount as before) was added after 2 h. Samples were taken at 0 h, 2 h, 4 h, 6 h and 8 h and analyzed by HPLC–UV.

These procedures generated mixtures from which the main metabolites were purified by HPLC and analyzed by HPLC–MS/MS. The samples were first analyzed in the FS mode and compared with blank samples. The identity of the putative metabolites found in the FS mode was corroborated by MRM transitions and product ion scan MS/MS analysis.

**Transport across the small intestine by the everted sacs technique.** Rats were deprived of food for 18 h, then anesthetized and kept alive during intestinal extraction. After abdominal laparotomy, a portion of both the jejunum and the ileum were removed, and immediately flushed with cold saline solution (4°C). The everted sacs were prepared following a described procedure (Wilson and Wiseman, 1954). The intestinal segments were turned inside-out (mucosa out – serosa in) and cut into 4-5
portions, ~2.5-3 cm long. Each portion was tied at the ends to form a sac, filled with the serosal medium (simulated intestinal juice at pH 7.4 without substrate) and incubated for 30 min at 37°C in mucosal medium (simulated intestinal juice at pH 6.5, 10 mL) in the presence of the test product (100 µM or 10 mM) with a continuous flow of carbogen. At the end of the incubation period, the sacs were weighed, carefully drained and the dried and weighed again. The decrease in weight was taken as the amount of fluid remaining after incubation. The serosal (i.e., inner) content was centrifuged (10,600 g, 5 min), and the supernatant was stored at ~80°C. The thawed samples were analyzed for the presence of the different test compounds and metabolites using HPLC. To detect glucuronides, aliquots of serosal samples were incubated with 0.5 M β-glucuronidase (final concentration of 1000 U/mL) for 120 min at 37°C in a 0.1 M phosphate buffer, pH 3.8 (Kuhnle et al., 2000). After centrifugation at 14,400 g for 5 min, the samples were analyzed by HPLC. Then the main metabolites were identified by comparison with the standards obtained by enzymatic glucuronidation and methylation. The metabolites were purified from serosal samples to corroborate their identity by HPLC-MS/MS. The quantification of EC and its thio-derivatives was carried out using HPLC–UV by comparison with standards. We quantified the total amount of glucuronides by comparing the amounts of aglycons before and after treatment of the serosal samples with β-glucuronidase. Because a standard for Me-EC was not available, we used the response curve for EC with a correction factor as described elsewhere (Piskula and Terao, 1998). To obtain the correction factor (1.1) a known amount of EC was methylated using the enzyme COMT in the presence of SAM as the methyl group donor, and the HPLC peak areas for EC and Me-EC were compared.
Metabolization in vivo and Body Distribution. The rats (3 for each compound) used for metabolization and distribution studies were fed with 400 mg of the corresponding compound (EC, Cys-EC and Cya-EC)/kg of body weight and killed after 4 h. Prior to administration, the rats were deprived of food for 12 h and given free access to water. Urine samples were collected and acidified with HCl (∼5 μL), and blood extracted and treated with heparin 1% to obtain plasma. Organs (liver, kidney, jejunum, ileum and colon) were removed and stored at −80°C.

Phenolic metabolites from plasma were precipitated and extracted as described in the literature (Mullen et al., 2002). Briefly, 1 mL of plasma was acidified with 5% HCl, then acetonitrile (2.5 mL) was added and the sample was vortexed for 30 s every 2 min over 10 min. The sample was then centrifuged at 3,000 g, 4°C for 10 min; the supernatant removed and the pellet extracted with MeOH (3 x 1 volume). The two supernatants were then combined, reduced to dryness and redissolved in 5% aqueous MeOH. The final solutions were filtered (0.45 μm, Millex-HV (PVDF) Millipore, Bedford, MA, USA) before analysis by HPLC–MS/MS. Urine samples were acidified with 5% HCl and extracted 3 times with ethyl acetate, the organic layer was vortexed for 30 s every 2 min over 10 min and then centrifuged at 2,400 g, 4°C for 10 min. The organic fraction was reduced to dryness and redissolved in 5% aqueous MeOH. The final solutions were filtered (0.45 μm, Millex-HV) before analysis. Tissue samples were defrosted and homogenized in MeOH/PBS (1:1, v/v). Then MeOH was evaporated and the aqueous fractions were extracted 3 times with ethyl acetate; the pooled organic layers were then vortexed for 30 s every 2 min over 10 min, and then centrifuged at 2,400 g, 4°C for 10 min. The supernatant was reduced to dryness and redissolved in 10% aqueous MeOH. The final solutions were filtered (0.45 μm, Millex-HV) before analysis.
Results

The epicatechin thio-conjugates Cys-EC and Cya-EC (Fig. 1) protect nerve cells (cell line HT22 and primary cultures) from oxidative glutamate toxicity by maintaining glutathione (GSH) levels through a mechanism that probably involves the non-phenolic part of the molecule (Maher et al., 2008). We describe here the absorption and metabolization of epicatechin thio-derivatives in rats, both in vitro (small intestine everted sacs technique) and in vivo.

Stability studies. We found that EC, Cys-EC and Cya-EC at a concentration of 100 μM were stable in simulated gastric juice (pH 2.0) and suffered 30-40% degradation in simulated intestinal juice (pH 7.4). This is in agreement with published results from other authors for EC and (+)-catechin (Zhu et al., 2002) who recorded a 50% degradation of EC together with some epimerization to catechin in simulated intestinal juice at pH 8.5 over 2 h. We opted to run the absorption tests at pH 6.5; at which the polyphenol derivatives were more stable (11% loss after 30 min).

Transport across the small intestine. We studied the transport of Cys-EC and Cya-EC across the jejunum and ileum in vitro in the rat intestinal everted sacs model, and the extent of their metabolization by conjugation (glucuronides, methyl groups). First, to identify the different metabolites, a non-physiological high concentration (10 mM) was used. Then the absorption/metabolization studies were performed at a concentration of
100 μM, which is the highest physiological concentration assayed by other authors (Mahé et al., 1992; Kuhnle et al., 2000; Donovan et al., 2001).

We first studied the metabolization of EC in our experimental set-up and compared our results with those in the literature. The HPLC chromatogram and the identification of the main EC metabolites by HPLC–MS/MS are included as supplemental data. Most of the EC identified in serosal samples was intact EC and glucuronidated EC (Gluc-EC). We also identified methylated EC (Me-EC) and Gluc-Me-EC. The HPLC profiles of the absorbed/metabolized products from Cys-EC and Cya-EC can be seen in Fig. 2. All of the Cys-EC identified in serosal samples incubated with 100 μM Cys-EC was the intact species (Fig. 2A, compound 1). In product ion scan mode, Cys-EC generated the corresponding molecular ion at \( m/z \) 408 ([M − H]−) which released a main MS/MS fragment at \( m/z \) 287 ([M − (HO₂CCH(NH₂)CH₂S) − H]−) and a characteristic fragment of the epicatechin moiety at \( m/z \) 243. The identity of the compound was corroborated by co-injection with a standard. At a high concentration of Cys-EC (10 mM) the metabolites Gluc-Cys-EC and Gluc-Me-Cys-EC were also detected. After incorporation of Cya-EC (100 μM) into the mucosal medium, the serosal compartment included intact Cya-EC (Fig. 2B, peak 2) identified by comparison with a standard. In product ion scan negative mode, Cya-EC generated the corresponding molecular ion at \( m/z \) 364 ([M − H]−) which released a main MS/MS fragment at \( m/z \) 287 ([M − (CH₂(NH₂)CH₂S) − H]−). In positive mode, Cya-EC gave more intense signals and generated the corresponding molecular ion at \( m/z \) 366 ([M + H]+) which released a main MS/MS fragment at \( m/z \) 289 ([M − (CH₂(NH₂)CH₂S) + H]+) and a fragment characteristic of the epicatechin moiety at \( m/z \) 245. Compound 3 in Fig. 2B was identified as Gluc-Cya-EC by comparison with the standard. Upon treatment with glucuronidase, peak 3 decreased while peak 2, corresponding to Cya-EC, became more pronounced (Fig. 2C). HPLC–
MS on compound 3 gave the molecular ion at m/z 540 ([M − H]⁻) which released a main MS/MS fragment at m/z 463 ([M − (CH₂(NH₂)CH₂S) − H]⁻ and the fragment of the epicatechin aglycon at m/z 287. MRM transitions were 540 → 463 and 463 → 287 (release of cysteamine followed by the loss of the glucuronyl moiety) and, less intense, 540 → 364 (release of glucuronyl moiety giving Cya-EC aglycone). Surprisingly, one of the compounds resulting from Cya-EC was Cys-EC (Fig. 2B, C; peak 1). Apart from showing the adequate retention time, the molecular ion was at m/z 408 ([M − H]⁻) and released an MS/MS fragment at m/z 287 (EC after loss of cysteine) and a fragment at m/z 243. At a concentration of 10 mM, some other metabolites were detected. A second Gluc-Cys-EC from both Cys-EC and Cya-EC gave a molecular ion at m/z 584 ([M − H]⁻) and released a main MS/MS fragment at 463 ([M − (HO₂CH(NH₂)CH₂S) − H]⁻ and the fragment of epicatechin aglycon at m/z 287. From Cys-EC we could also identify a second Gluc-Me-Cys-EC which yielded the molecular ion m/z 597 ([M−H]⁻) and released a main MS/MS fragment at 477 ([M − (HO₂CH(NH₂)CH₂S) − H]⁻, loss of cystein), and the fragment of Me-EC at m/z 301 (loss of glucuronate).

The quantitative results on the absorption of EC and its thio-conjugates across the small intestine are summarized in Fig. 3. Our values are lower than those obtained by other authors (Kuhnle et al., 2000) who used an experimental set up that is more faithful to the actual conditions in vivo. Since our purpose was to compare the absorption of EC with that of its thio-conjugates, we used a simpler yet informative approach and the quantitative results must not be taken as an estimation of the total absorption in the small intestine in vivo. It should be noted that, after the incubation time, absorption was still in process as indicated by the presence of metabolites in the intestinal tissue. Our data show that the thio-conjugates were less effectively absorbed than the parent EC. Moreover, two significant results were obtained. First, in contrast with EC and Cya-EC,
no glucuronidated Cys-EC was detected, meaning that, under our experimental conditions, Cys-EC is not metabolized in the intestine and consequently the free radical scavenging catechol group is available to the gut tissue during its transit. We also identified intact Cys-EC in tissue homogenates after 30 min of digestion. Second, a considerable portion of Cya-EC was converted into Cys-EC, probably by exchange with endogenous cystine (Maher et al., 2008).

**Body distribution and metabolization in vivo.** We examined the distribution of EC, Cys-EC and Cya-EC and their phase II metabolites in rats after the intake of a single dose of 400 mg/kg of body weight of each compound. It has previously been described that EC metabolites are still clearly detectable in plasma 4 h after administration with a maximum after around 2 h (Piskula and Terao, 1998). A sampling time of 4 h seemed adequate to evaluate the distribution of metabolites in organs, blood and urine.

Table 1 summarizes the occurrence of the metabolites. The MS data (MW, MRM transitions) corresponding to each compound are included as supplemental material. We detected glucuronides, methyl esters and conjugates with both moieties for the three compounds tested. Most of them were found both in urine and plasma. The phase II metabolism of the thio-conjugates essentially followed the same pattern. EC metabolites were more widely distributed than those from the thio-derivatives. Epimerization of epicatechin into catechin was only detected for EC in the ileum. The results in vivo confirm the observation in vitro that Cya-EC is converted into Cys-EC. Gluc-Cys-EC and Gluc-Me-Cys-EC, phase II metabolites of the cysteine conjugate, were detected in urine after administration of Cya-EC. Our results show that the conversion of the cysteamine thio-conjugate into the cysteine derivative is a process...
which clearly occurs in vivo. Non-metabolized species bearing the intact catechol group were detected after administration of EC and Cya-EC but not from Cys-EC. Fig. 4 illustrates the metabolization and thiol exchange of Cya-EC.

**Discussion**

We have reported that conjugates of catechins with thiol containing molecules such as cysteine and cysteamine share with other polyphenols their putatively beneficial antioxidant properties that are mainly attributed to the catechol or pyrogallol groups (Torres and Bobet, 2001; Lozano et al., 2006). We have also shown that the non-phenolic part of the molecule contributes significantly to the overall antioxidant effect. Thus, EC, Cys-EC and Cya-EC differ in their capacity to interact with biological membrane models (Lázaro et al., 2007) and to penetrate skin layers (Alonso et al., 2004). The free radical scavenging activity originates in the hydrogen atoms and/or electrons transferred from the catechol group on ring B (Figs. 1 and 4) and the non-phenolic part may influence the oxidative potentials or merely modify the capacity of the molecule to interact with surfaces. Our results show that the thio-conjugates are less efficiently transported through the small intestine than EC is. This confirmed the role of the non-flavonoid part of the molecule as a modulator of membrane interactions. The order of transport efficiency was: EC > Cya-EC ≈ Cys-EC in the small intestine, whereas in the stratum corneum (outer skin) the order was: Cya-EC > EC > Cys-EC (Alonso et al., 2004). Because EC is clearly the most lipophilic compound of those tested, the results from the everted sacs suggest that passive diffusion through membranes is the main transport route across the rat intestine. Apart from overall transport, it is of interest to estimate the extent of the phase II metabolization of the phenolics, because any conjugation with the catecholic hydroxyls (ring B, Fig. 4)
destroys the free radical scavenging capacity. The small intestine is the organ where most catechin glucuronidation occurs and it also plays a role in catechin methylation, while the liver is the most important site of sulphation as well as additional methylation (Donovan et al., 2001). Under our experimental conditions, Cya-EC followed the metabolization pattern described for EC and catechin (Piskula and Terao, 1998; Donovan et al., 2001) while Cys-EC behaved differently. We identified glucuronides, methyl esters and conjugates with both moieties from the three compounds tested but did not unequivocally identify any sulphate metabolite. All the metabolites from Cya-EC and Cys-EC detected in vitro were also seen in plasma, urine and organs after oral administration. Moreover, some other conjugates were identified in vivo, which may have resulted from post-absorption phase II metabolization. In the case of Cys-EC, the intact compound was the only species detected in vitro and surprisingly was not present in any of the samples from the in vivo experiment. It appears that Cys-EC is stable while transported through the small intestine and rapidly metabolized in the liver once it is internalized. This means that the intact free radical scavenging catechol group is conserved during its transport across the intestine and rapidly cleared systemically while EC and Cya-EC appear to be more extensively metabolized previously in the small intestine. This may have some significance for the putative antioxidant activity of the thio-conjugates. Cys-EC may be acting as a free-radical scavenger in the intestine for longer than Cya-EC and EC. We used rats because the thio-epicatechins cannot be tested in humans yet. We believe that our overall conclusions will prove to be valid for both species because phase II metabolites of EC have been reported to be the same in rats and humans, the main difference being that glucuronidation may occur preferentially at positions 3’ and 4’ (catecholic ring B) in humans (Natsume et al., 2003).
We have also reported that the thio-derivatives, particularly Cya-EC, protect nerve cells (cell line HT22 and primary cultures) from oxidative glutamate toxicity by maintaining intracellular GSH levels via a mechanism that is independent of the scavenging capacity of the catechol moiety (Torres et al., 2005). Here the non-flavonoid part of the molecule is a determining feature. We found that Cya-EC maintains GSH levels in vitro by enhancing the uptake of cysteine into the cell through a transporter that is different from the Na-independent system $x_c^-$, which is inhibited by extracellular glutamate (Maher et al., 2008). This is biologically and pharmacologically significant because it means that the incorporation of cysteine may occur even at toxic glutamate concentrations. We have also shown that pre-incubation of Cya-EC with cystine (Cys-Cys) is a necessary condition for this effect and we observed that when this happens, Cys-EC is generated in solution (Maher et al., 2008). There is a thiol exchange which may result in free cysteine and a mixed Cya-Cys disulphide that can be transported into the cell by a variety of transporters which are not inhibited by glutamate. We now present evidence that this disulphide exchange already occurs when Cya-EC is transported through the small intestine and may also occur virtually everywhere in the live organism. We detected Cys-EC in the serosal compartment of the everted sacs incubated with Cya-EC, and in ileum, jejunum, plasma and urine of rats administered Cya-EC orally. We even detected phase II metabolites that contained cysteine in the urine or plasma of rats given Cya-EC (Table 1 and Fig. 4). Our results prove that the protective effect of Cya-EC may take place in the organism wherever cystine is available for disulphide exchange, that is to say, virtually everywhere and for all the species, including humans. We also show that the active compounds are bioavailable for several hours after oral ingestion.
In conclusion, we have shown that Cya-Ec follows the same phase II metabolization pattern as EC, while Cys-EC is transported with the intact catechol moiety through the small intestine and effectively metabolized systemically. We have also seen that Cya-EC generates Cys-EC in vivo, which provides evidence that the antioxidant glutathione enhancing Cya-EC mediated cysteamine/cystine exchange may take place ubiquitously in the organism.
Acknowledgement. Language revision by Christopher Evans is appreciated.
References


Liu DQ and Hop CECA (2005) Strategies for characterization of drug metabolites using liquid chromatography tandem mass spectrometry in conjunction with chemical


Footnotes

This work was financed by the Spanish Ministry of Science and Technology [under awards PPQ2000-0688-C05-03 and PTR1995-0611-OP].
Legends for Figures

Fig. 1: Chemical structures of EC, Cys-EC and Cya-EC

Fig. 2: RP-HPLC profile of samples taken from the serosal compartment of the everted sac incubated with Cys-EC (A) and Cya-EC (B, C). C, serosal sample from Cya-EC incubation treated with β-glucuronidase. Conditions described in Materials and Methods
Peak 1, Cys-EC; peak 2, Cya-EC; peak 3, Gluc-Cya-EC

Fig. 3: Absorption and metabolization of EC, Cys-EC and Cya-EC in the small intestine by the everted sacs technique.

Fig. 4: Metabolites and thiol-exchange products from Cya-EC and possible pathways leading to them. Only the putatively major phase II metabolites in rats are depicted. Other isomeric species with different conjugation positions (regioisomers) were detected (see text).
### TABLE 1.

Body distribution of EC, Cys-EC and Cya-EC, their phase II metabolites and thiol-exchange products.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Fluid or Organ&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>From EC</td>
<td></td>
</tr>
<tr>
<td>EC</td>
<td>I, L, U</td>
</tr>
<tr>
<td>(+)-catechin</td>
<td>I</td>
</tr>
<tr>
<td>Gluc-EC</td>
<td>I, L, P, K, U</td>
</tr>
<tr>
<td>Gluc-Me-EC</td>
<td>I, L, P, K, U</td>
</tr>
<tr>
<td>Me-EC</td>
<td>U</td>
</tr>
<tr>
<td>From Cys-EC</td>
<td></td>
</tr>
<tr>
<td>Gluc-Cys-EC</td>
<td>U</td>
</tr>
<tr>
<td>Me-Cys-EC</td>
<td>P, U</td>
</tr>
<tr>
<td>Gluc-Me-Cys-EC</td>
<td>P, U</td>
</tr>
<tr>
<td>From Cya-EC</td>
<td></td>
</tr>
<tr>
<td>Cya-EC</td>
<td>I, U</td>
</tr>
<tr>
<td>Cys-EC</td>
<td>I, P, U</td>
</tr>
<tr>
<td>Gluc-Cya-EC</td>
<td>P, U</td>
</tr>
<tr>
<td>Gluc-Cys-EC</td>
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<td>Gluc-Me-Cys-Ec</td>
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<tr>
<td>Gluc-Me-Cya-EC</td>
<td>P</td>
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</tbody>
</table>

<sup>a</sup>I, Ileum; K, kidney; L, liver; P, plasma; U, Urine.
Figure 1

(-)-epicatechin (EC)

4β-(S-cysteiny1)epicatechin (Cys-EC)

4β-(S-cysteaminy1)-epicatechin (Cya-EC)
Figure 2
Figure 3
Figure 4
Absorption and Metabolization of Cytoprotective Epicatechin Thio-Conjugates in Rats

Ariadna Selga, María Pilar Vinardell, Raquel Martín-Venegas, Olga Jáuregui, Josefina Casas and Josep Lluís Torres

Drug Metabolism and Disposition

Supplemental Fig. 1 shows the chromatogram profile of the EC serosal sample. The identity of EC (peak 4) and Gluc-EC (peak 5) was corroborated in FS, product ion scan and MRM transition modes of HPLC–MS/MS. In the product ion scan mode, Gluc-EC generated the corresponding molecular ion at $m/z$ 465 ([M – H]$^-$) which released a main MS/MS fragment at $m/z$ 289 (loss of the glucuronide moiety) and the characteristic fragments of the glucuronide moiety at $m/z$ 175 and 113 (Levensen et al., 2005). From the results of other authors also working with rats (Natsume et al., 2003) we hypothesize that glucuronidation occurs at position 7. Me-EC (peak 6) was identified by comparison with the product of in vitro methylation by COMT in the presence of SAM (HPLC peak after approximately 47 min). We assumed that methylation occurred at position 3', in accordance with the literature (Piskula and Terao, 1998; Donovan et al., 2001). The most likely candidates for Gluc-EC and Gluc-Me-EC (peak 7) are (−)-epicatechin-7-O-glucuronide and 3'-O-methyl-(−)-epicatechin-7-O-glucuronide, which have been identified in rat plasma and urine after ingestion of (−)-epicatechin (Natsume et al., 2003).

We detected intact EC and its epimer (+)-catechin in tissue homogenates prepared after 30 minutes of digestion. Some epimerization of EC to catechin has previously been described (Zhu et al., 2002).
Supplemental Fig. 1: Structures of EC and its main metabolites in rats and RP-HPLC profile of a sample taken from the serosal compartment of the everted sac incubated with EC. Conditions described in Materials and Methods. Peak 4, EC; peak 5, Gluc-EC; peak 6, Me-EC; peak 7, Gluc-Me-EC.
SUPPLEMENTAL TABLE 1

MS/MS data that permitted the identification of intact compounds, their metabolites and thiol-exchange products with the help of synthetic and purified standards.

<table>
<thead>
<tr>
<th>Compound</th>
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<tbody>
<tr>
<td>EC</td>
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<td>289→245</td>
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<tr>
<td>Cat</td>
<td>289</td>
<td>289→245</td>
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<tr>
<td>Gluc-EC</td>
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<tr>
<td>Gluc-Me-EC</td>
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<td>479→303</td>
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<tr>
<td>Me-EC</td>
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From of Cys-EC

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<tbody>
<tr>
<td>Gluc-Cys-EC</td>
<td>584</td>
<td>584→463; 463→287</td>
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<tr>
<td>Me-Cys-EC</td>
<td>422</td>
<td>422→301</td>
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<tr>
<td>Gluc-Me-Cys-EC</td>
<td>598</td>
<td>598→477; 477→301</td>
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From Cya-EC

<table>
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<th>MRM</th>
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<tbody>
<tr>
<td>Cya-EC</td>
<td>364</td>
<td>364→287</td>
</tr>
<tr>
<td>Cys-EC</td>
<td>408</td>
<td>408→287</td>
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<tr>
<td>Gluc-Cya-EC</td>
<td>540</td>
<td>540→364</td>
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<tr>
<td>Gluc-Cys-EC</td>
<td>584</td>
<td>584→463; 463→287</td>
</tr>
<tr>
<td>Me-Cya-EC</td>
<td>378</td>
<td>378→301</td>
</tr>
<tr>
<td>Gluc-Me-Cya-EC</td>
<td>554</td>
<td>554→378</td>
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<tr>
<td>Gluc-Me-Cys-EC</td>
<td>598</td>
<td>598→477; 477→301</td>
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</table>
SUPPLEMENTAL DATA REFERENCES


