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(+)-Catechin is more bioavailable than (−)-catechin: Relevance to the bioavailability of catechin from cocoa

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Abstract

Catechin is a flavonoid present in fruits, wine and cocoa products. Most foods contain the (+)-enantiomer of catechin but chocolate mainly contains (−)-catechin, in addition to its major flavanol, (−)-epicatechin. Previous studies have shown poor bioavailability of catechin when consumed in chocolate. We compared the absorption of (−) and (+)-catechin after *in situ* perfusion of 10, 30 or 50 μmol/l of each catechin enantiomer in the jejunum and ileum in the rat. We also assayed 23 samples of chocolate for (+) and (−)-catechin. Samples were analyzed using HPLC with a Cyclobond I-2000 RSP chiral column. At all concentrations studied, the intestinal absorption of (−)-catechin was lower than the intestinal absorption of (+)-catechin ($p < 0.01$). Plasma concentrations of (−)-catechin were significantly reduced compared to (+)-catechin ($p < 0.05$). The mean concentration of (−)-catechin in chocolate was 218 ± 126 mg/kg compared to 25 ± 15 mg/kg (+)-catechin. Our findings provide an explanation for the poor bioavailability of catechin when consumed in chocolate or other cocoa containing products.

Keywords: *Catechin, chocolate, enantiomer, chiral, bioavailability, rat*

Introduction

Flavonoids may be beneficial in the prevention of cancer, cardiovascular and neurodegenerative diseases. The pharmacokinetics of flavonoids when consumed in foods has been an active area of research as the beneficial effects of flavonoids will be dependent upon their uptake and distribution to target tissues and cells [1]. Catechin and epicatechin are abundant, naturally occurring flavonoids from the class known as flavanols. Cocoa products, berries, apples, tea and red wine are among the richest dietary sources [2–3].

In most plants only the (+) enantiomer of catechin and the (−)-enantiomer of epicatechin are produced [4]. Until recently, cocoa products were thought to contain (+)-catechin along with (−)-epicatechin [5,6]. It has been recently shown that catechin in chocolate is predominantly the (−)-catechin isomer, rather than the (+)-catechin isomer that is present in most other foods [7]. The (−)-catechin is likely formed from epimerization at the 2 position of (−)-epicatechin during processing [7].

Catechin, when consumed in cocoa products, appears to be less bioavailable than when consumed in other foods [8,9]. Catechin also appears to be far less bioavailable than epicatechin when both are consumed together in a cocoa beverage [9]. Previous animal and clinical studies performed on other foods

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and purified flavanols have suggested that catechin is less bioavailable than epicatechin but not the magnitude of difference observed after cocoa product consumption [8,10–13].

The specific chiral forms of the flavanols in foods and in plasma have not been documented in previous clinical studies because enantiomers display identical behavior when run on traditional C-18 reversed phase chromatography, as used in these studies. We hypothesized the reason for the lower plasma concentrations of catechin from cocoa products is that the (−)-catechin enantiomer is less bioavailable than the (+)-catechin enantiomer. To test this hypothesis we developed a chiral high performance liquid chromatography (HPLC) method to analyze plasma concentrations of (+) and (−)-catechin enantiomers along with their methylated metabolites. We then investigated the bioavailability of (+) and (−)-catechin after *in situ* perfusion in the jejunum and ileum in the rat. Finally, in order to confirm that catechin in most available chocolate samples is the (−) enantiomer, we assayed 23 samples of commercially available chocolate bars for their content of both enantiomers of catechin and epicatechin.

Materials and methods

Synthesis of 3′- and 4′-O-methylated metabolites of (+) and (−) catechin

Purified (+)-catechin and (−)-catechin were purchased from Sigma (St Louis, MO). The 3′- and 4′-*O*-methylated conjugates of each of the catechin enantiomers were synthesized using a mixture of 250 mg of (+/−)-catechin, 500 mg K_2CO_3 and 1 ml methyl iodide in 20 ml acetone which was irradiated in an ultrasonic bath for 2.5 h as previously described [14]. The conditions were optimized to increase the yield of the 3′ and 4′ *O*-methylated metabolites, and decrease the amounts of unwanted products. Optimal conditions were obtained by maintaining the reaction temperature between 35 and 40°C during the reaction and performing the reaction for 2.5 h. Under these conditions, there was ~80% conversion of catechin to products with ~35% converted to the 3′-*O*-methylated metabolite and ~45% converted to the 4′-*O*-methylated metabolite. The positions of the methyl groups for (+)-catechin were previously determined by one-dimensional difference nuclear overhauser effect spectroscopy [14]. The (−) forms of each of the methylated enantiomers were identified as those that co-eluted with the (+) forms when separated using reversed phase chromatography as previously described [14]. The methylation reactions were performed separately using 1 mg of the purified (+) and (−) forms of catechin for subsequent determination of the elution order of the enantiomers using the chiral HPLC method described below.

Chiral HPLC separation

Chiral HPLC analysis was performed using a Waters 2690 HPLC (Waters, Milford, MA) with a Cyclobond I-2000 RSP 250 × 4.6 mm column (Advanced Separation Technologies, Whippany, NJ) containing derivatized β-cyclodextrin. Mobile phase A consisted of 50 mM NaH_2PO_4 at pH 3.0 and mobile phase B consisted of 80% acetonitrile in 30 mM NaH_2PO_4 at pH 3.0. The flow rate was 1 ml/min and a linear gradient from 10.0 to 13.5% B over 45 min and then increasing to 45% B at 70 min. Detection was performed by fluorescence monitoring (λex 280 nm; λem 310 nm). A chromatogram of a mixture of the (+) and (−) enantiomers of catechin and epicatechin along with their respective methylated metabolites is shown in Figure 1.

Animals and sample collection

Fifteen male Wistar rats weighing 382 ± 29 g were housed in temperature controlled rooms (22°C), with a dark period of 8 to 20 h and access to food for 8 to 16 h. The rats were fed a standard semi-purified diet as previously described [15]. All experimental protocols were in agreement with the Nestlé ethical committee and approved by the ethical committee of Switzerland's veterinary office.

The rats were fasted for 18 h, anesthetized with sodium pentobarbital (60 mg/kg body weight, intraperitoneal injection) and xylazine (2 mg/kg body weight, intramuscular injection) and kept alive throughout the entire perfusion period. After tracheotomy, an incision of the peritoneal cavity was performed, and the bile duct was cannulated. A segment of small intestine, starting below the ligament of Treitz and finishing 10 cm above the valvula ileocoecalis, was selected. The segment was internally attached with inflow and outflow cannulas. The cannulated segment was continuously perfused *in situ* for 1 h at 0.5 ml/min with a buffer containing 5 mM KH_2PO_4, 2.5 mM K_2HPO_4, 5 mM $NaHCO_3$, 50 mM

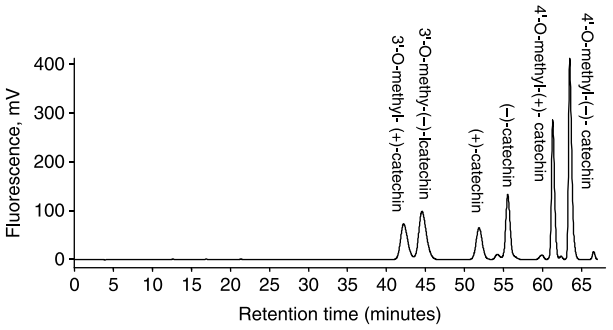

Figure 1. HPLC chromatogram of a mixture of the (+) and (−) enantiomers of catechin and epicatechin along with their respective methylated metabolites using a chiral analytical column containing derivatized β-cyclodextrin as described in materials and methods.

NaCl, 40 mM KCl, 10 mM tri-potassium citrate, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 8 mM glucose, 2 g/l PEG 4000, 5μCi/l ^{14}C- PEG 4000 and 1 mM taurocholic acid at pH 6.7. The buffer was supplemented with 10, 30 or 50 μmol/l of each of the catechin enantiomers. Aliquots were directly collected at the exit of the ileum after 1 h of perfusion. Water absorption was measured using ^{14}C labelled PEG 4000, and a correction factor was applied to the concentration of catechin measured in the effluent. At the end of the 1 h period, blood was drawn first from the mesenteric vein (0.5 ml) and then from the abdominal aorta (6 ml) into sodium citrate 0.1 M tubes. Plasma and perfusate samples were acidified with 10 μl/ml of 1 M acetic acid and stored at −20°C.

Analysis of (+) and (−) catechin in plasma and perfusion samples

Plasma samples (400 μl) were thawed and acidified with 24 μL of 0.58 M acetic acid. Samples were spiked with 4 μM (−)-epicatechin, the internal standard, which was not present in samples prior to analysis. Samples were incubated for 15 min at 37°C after the addition of 1200 units of β-glucuronidase (Sigma; St Louis, MO) and 25 units of arylsulfatase (Sigma) dissolved in 100 μl water. Samples were extracted by vigorous vortexing with 3 ml ethyl acetate and then centrifuged for 4 min at 14,000*g*. The resulting supernatant was evaporated with nitrogen at <30°C and redissolved in 50% acetonitrile. Calibration curves were prepared in blank plasma to correct for losses during the extraction procedure. Chiral HPLC was performed using the methodology described above except using an ESA (Chelmsford, MA) CoulArray Model 582/542 HPLC system coupled to a CoulArray 5600 coulometric detector. The detection potentials were set at 25, 100, 285 and 320 mV. Concentrations of (+) and (−)-catechin were determined using the responses on the first two potentials and concentrations of (+) and (−) methylated forms were determined using the 3rd due to a small interference sometimes present on the 4th potential. Perfusion samples were analyzed using the same procedure as plasma but with the Waters HPLC system with fluorescence detection described below.

Analysis of catechin and epicatechin enantiomers in chocolate samples

To determine the concentrations of both enantiomers of catechin and epicatechin in chocolate samples, we analyzed 23 chocolate bars sampled from those available in the United States, the European Union, and Africa (19 dark, 4 milk). Chocolate samples were ground to a fine homogeneous powder using a standard food processor. The chocolate powder (0.5 g) was defatted by extraction twice with 5 ml hexane, after which the residue was dried under a stream of nitrogen. The residue was then extracted three times using 2.5 ml of a mixture of acetone water and acetic acid (70:28:2 v:v:v). The supernatants were combined and evaporated to a volume of approximately 1 ml with nitrogen gas. Samples were then reconstituted to a volume of 5.0 ml with 10% acetonitrile prior to HPLC analysis. Concentrations were determined by comparison of values obtained from a standard curve prepared in commercial white chocolate which did not contain detectable levels of flavanols. Chiral HPLC analysis was performed using a Waters HPLC system with fluorescence detection as described above.

Data analysis

All numerical values are expressed as the arithmetic mean ± the standard deviation and significant differences were determined by conducting a two-tailed paired *t*-test using the Instat statistical program (San Diego, CA). Values of $p < 0.05$ were considered significantly different.

Results

Intestinal absorption of (+) and (−) catechin after in situ *perfusion of the rat small intestine*

In all animals and at all concentrations studied, the intestinal absorption of (−)-catechin was lower than the intestinal absorption of (+)-catechin. At 10 μmol/l concentration of each enantiomer perfused, 17 ± 10% of the dose of (+)-catechin was absorbed whereas 8 ± 10% of the dose of (−)-catechin was absorbed ($p < 0.01$). At 30 μmol/l perfusion concentration, 24 ± 15% of the dose of (+)-catechin was absorbed whereas 16 ± 17% of the dose of (−)-catechin was absorbed ($p < 0.001$). At 50 μmol/l perfusion concentration, 26 ± 28% of the dose of (+)-catechin was absorbed whereas 20 ± 30% of the dose of (−)-catechin was absorbed ($p < 0.01$).

Plasma concentrations of (+) and (−) catechin after in situ *perfusion of the rat small intestine*

Table I presents the plasma concentrations of (+) and (−) catechin after 1 h of perfusion with a solution containing 10, 30 or 50 μmol/l of each enantiomer of catechin. All concentration values represent those after hydrolysis by glucuronidase or sulfatase as neither (+) or (−)-catechin were detectable in aortic or mesenteric plasma prior to hydrolysis. In plasma obtained from the abdominal aorta, concentrations of the (−)-catechin enantiomer were 2–8-fold lower than the (+)-catechin enantiomer (all *p*-values <0.05). A chromatogram demonstrating the contrast in the plasma concentrations of (+) and (−) catechin

Table I. Plasma concentrations of (+) and (−)-catechin and their 3′ and 4′- *O*-methylated metabolites after *in situ* perfusion of an equal mixture of three concentrations of (+) and (−)-catechin enantiomers. All values are presented as means ± the standard deviations. *p*-values are comparisons between the (+) and (−) enantiomers from each of their respective columns. Total catechin is the sum of the catechin and its methylated metabolite; ns is not significant.

Perfusion concentration of each enantiomer (μmol/l)	(+)-catechin	3′-*O*-methyl-(+)-catechin	Total (+)-catechin	(−)-catechin	3′-*O*-methyl-(−)-catechin	Total (−)-catechin
			Concentration in plasma from abdominal aorta (μmol/l)			
10	0.09 ± 0.01	0.12 ± 0.07	0.21 ± 0.07	0.02 ± 0.00 ($p \leq 0.001$)	0.01 ± 0.02 ($p \leq 0.05$)	0.04 ± 0.01 ($p \leq 0.005$)
30	0.27 ± 0.04	0.43 ± 0.15	0.70 ± 0.17	0.03 ± 0.02 ($p \leq 0.001$)	0.14 ± 0.05 ($p \leq 0.005$)	0.18 ± 0.05 ($p \leq 0.001$)
50	0.45 ± 0.35	0.60 ± 0.63	1.06 ± 0.97	0.06 ± 0.06 ($p \leq 0.05$)	0.32 ± 0.39 ($p \leq 0.05$)	0.39 ± 0.45 ($p \leq 0.05$)
			Concentration in plasma from mesenteric vein (μmol/l)			
10	0.18 ± 0.12	0.15 ± 0.11	0.33 ± 0.23	0.05 ± 0.04 ($p \leq 0.05$)	0.04 ± 0.08 ($p \leq 0.05$)	0.09 ± 0.11 ($p \leq 0.05$)
30	0.70 ± 0.53	0.46 ± 0.51	1.16 ± 1.02	0.18 ± 0.16 ($p \leq 0.05$)	0.14 ± 0.22 (ns)	0.32 ± 0.34 ($p \leq 0.05$)
50	0.92 ± 0.47	0.51 ± 0.31	1.43 ± 0.76	0.23 ± 0.12 ($p \leq 0.01$)	0.46 ± 0.37 (ns)	0.69 ± 0.43 (ns)

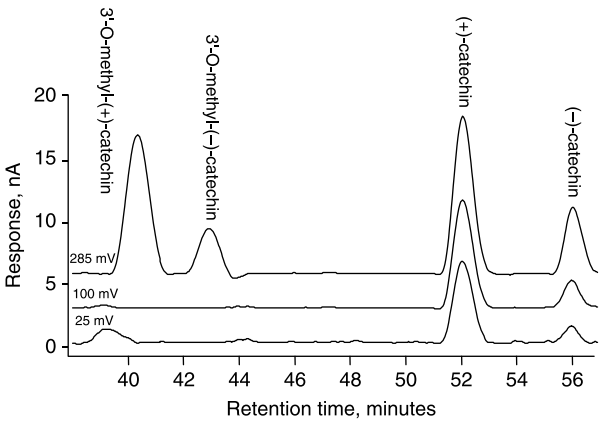

Figure 2. High performance liquid chromatogram with multi-electrode Coularray detection of plasma after *in situ* perfusion in the rat small intestine. Plasma was prepared from blood drawn from the abdominal aorta at the end of the perfusion experiment. Prior to extraction the plasma was hydrolyzed by β-glucuronidase and sulfatase and analyzed as described in materials and methods.

is shown in Figure 2. Both (+) and (−)-catechin were methylated at the 3′ position and concentrations of 3′-*O*-methyl-(−)-catechin were significantly lower than its antipode (all *p*-values ≤ 0.05). Approximately 50–60% of (+)-catechin was methylated and 30–80% of (−)-catechin was methylated. There was a trend towards increased methylation of the (−)-catechin enantiomer and this increase was significant after perfusion with 30 and 50 μmol/l of (+) and (−) catechin ($p < 0.05$). The enantiomers of 4′-*O*-methylated metabolites were very low or not detectable in most plasma samples.

Concentrations of (+) and (−)-catechin in plasma obtained from the mesenteric vein were generally higher than those obtained from aortic plasma. Concentrations of (−)-catechin in mesenteric plasma were several fold lower than (+)-catechin at all perfusion concentrations studied (all *p*-values < 0.05). The concentration of 3′-*O*-methyl-(−)-catechin was also lower than its (+) enantiomer but these differences were only significant after perfusion with 10 μmol/l catechin ($p < 0.05$).

Concentrations of (+) and (−)-catechin and (+) and (−)-epicatechin in chocolate samples

The extraction procedure and chiral HPLC methodology described here allowed separation of both enantiomers of catechin and epicatechin from chocolate samples. A representative HPLC chromatogram is shown in Figure 3. The chocolate samples contained 954 ± 537 mg/kg of total monomeric flavanols (both enantiomers of catechin and epicatechin). Epicatechin in chocolate was predominantly in the (−) form which accounted for 95 ± 1% of epicatechin present in the chocolate samples. The

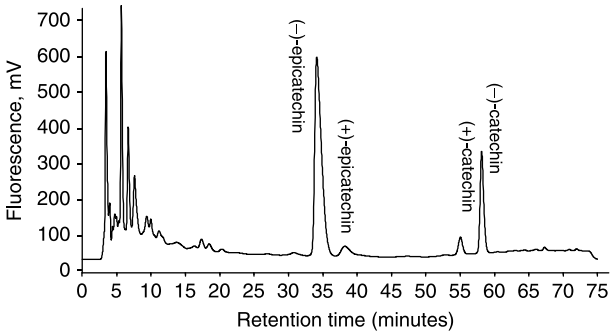

Figure 3. High performance liquid chromatogram of a representative chocolate sample. Chocolate was extracted and analyzed using a chiral analytical column containing derivatized β-cyclodextrin as described in materials and methods.

concentration of (−)-epicatechin in chocolate samples was 679 ± 413 mg/kg, whereas (+)-epicatechin was 33 ± 22 mg/kg. Catechin in chocolate was also predominantly in the (−) form which accounted for 89 ± 3% of catechin present in the chocolate samples. The mean concentration of (−)-catechin was 218 ± 126 mg/kg compared to a mean concentration of 25 ± 15 mg/kg (+)-catechin.

Discussion

In this study we demonstrated that plasma concentrations of (+) and (−)-catechin in the rat were markedly and consistently different when studied using an *in situ* model of small intestinal absorption. Plasma concentrations of the (−)-catechin enantiomer were several fold lower than plasma concentrations of the naturally occurring (+)-catechin enantiomer. We also documented that commercial chocolate samples available around the world contained a predominance of the less bioavailable (−)-catechin enantiomer, rather than (+)-catechin which is present in most other foods. Thus, our findings provide an explanation for the apparent low bioavailability of catechin when consumed in chocolate or other cocoa-containing products.

The decreased bioavailability of (−)-catechin in comparison to its antipode appears to be at least partially due to reduced absorption in the small intestine. In all of the animals studied, the intestinal absorption of (−)-catechin was lower than the intestinal absorption of (+)-catechin. Plasma concentrations of (−)-catechin obtained from the mesenteric vein were also several fold lower than (+)-catechin, evidence also supporting a significant difference in intestinal absorption. The mechanism by which catechin is absorbed by the small intestine is not known but has been suggested to be passive diffusion across the enterocyte [15]. The present study provides evidence that a more specific mechanism, such as directional transport, is likely involved in the intestinal absorption of catechin as both catechin enantiomers possess identical physical properties which govern passive diffusion.

The magnitude of the difference in intestinal absorption between the enantiomers was not as great as the differences between the plasma concentrations of the enantiomers. The absorption of (−)-catechin was approximately two thirds of the absorption of (+)-catechin, but plasma concentrations of the enantiomers were several fold different. Other potential contributing factors for the reduced bioavailability of (−)-catechin are differences in affinity for glucuronosyl transferase of sulfotransferase enzymes or transport proteins. It also cannot be excluded that some isomerization from (−)-catechin to (+)-catechin occurred in intestinal enterocytes or in the perfusion buffer. Preliminary *in vitro* stability studies (data not shown) investigating the catechin enantiomers at 37°C in the perfusion buffer did not suggest inter conversion of the enantiomers.

In this study both catechin enantiomers were administered simultaneously to each animal. There was a fairly large individual difference among animals as indicated by the standard deviations but analysis of paired data revealed a significant difference in intestinal absorption and plasma concentrations of the catechin enantiomers.

Although simultaneous administration of the enantiomers reduced the number of animals needed to observe differences, this study design did not reveal if the presence of possible competition during transport or metabolism.

Very little is known about the biological activity of (−)-catechin. In one study, at high concentrations, (+) and (−)-catechin showed opposite effects on glycogen metabolism in isolated rat hepatocytes [16]. Enantiomers of conventional drugs often have different pharmacokinetic properties as well as markedly different biological activities. It is not known whether (−)-catechin contributes to the biological effects observed after consumption of cocoa-containing products. Antioxidant effects of (−)-catechin due to free radical scavenging are unlikely to be important at the concentrations of (−)-catechin obtained in plasma after consumption of the doses of present in most cocoa containing foods.

Future studies will need to clarify if (−)-catechin has a specific activity that may contribute to the health effects of cocoa products. A recent study suggested that the effects on the vascular system are largely mediated by (−)-epicatechin [17]. As our understanding of the components responsible for biological effects of cocoa consumption increases, optimizing techniques used in processing to yield the highest levels of beneficial components could enhance the health benefits of cocoa product consumption.

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