Department of Pharmaceutical Sciences, St. John's University, College of Pharmacy and Allied Health Professions, Jamaica, New York, USA

Assessment of the effect of type of dairy product and of chocolate matrix on the oral absorption of monomeric chocolate flavanols in a small animal model

D. Gossai, C. A. Lau-Cam

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Dr. Cesar A. Lau-Cam, Department of Pharmaceutical Sciences, St. John's University, College of Pharmacy and Allied Health Professions, 8000 Utopia Parkway, Jamaica, New York, 11439 USA
claucamc@usa.net

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This study has examined the effects of type of dairy product (whole milk, skim milk, heavy cream) and chocolate matrix (baking, dark, dairy milk, white) on the oral absorption of the chocolate flavanols (+)-catechin and (−)-epicatechin in a small animal model. In the study, each flavanol compound, as a solution in water or a dairy product or as a chocolate dispersion in water, was administered intragastrically to male Sprague-Dawley rats in an amount equal to or equivalent to 350 mg/kg. In each instance, blood samples were collected over a 5 h period, and used to measure plasma total catechin concentrations by HPLC after enzymatic hydrolysis of flavanol conjugates. Pharmacokinetic data were evaluated using a one compartment approach. Whole milk and heavy cream, and to a much lesser extent skim milk, lowered the oral absorption of both (+)-catechin and (−)-epicatechin and altered the AUC, C_{max}, k_a, k_e and $t_{1/2}$ values in direct proportion to their fat, but not to their protein, content. In addition, the t_{max} for solutions of (−)-epicatechin in water and skim milk occurred 2 h earlier than from solutions in whole milk and heavy cream. Similarly, dispersions of baking chocolate in water and in whole milk yielded plasma levels of monomeric catechins that were, respectively, about equal to and much lower than those from aqueous solutions of authentic flavanols. A determining role for a chocolate matrix (dark, dairy milk or white chocolate) on the oral absorption of its constitutive monomeric flavanols was suggested by the apparent variability in plasma total catechins levels that existed among them both before and after their spiking with equal amounts of exogenous (+)-catechin and (−)-epicatechin Such a variability could reflect differences among different chocolates in terms of their physical properties, matrix components, and matrix characteristics imposed by the manufacturing process used for each type of chocolate. In all the experiments, (+)-catechin demonstrated a higher oral absorption than (−)-epicatechin.

1. Introduction

Chocolate contains several monomeric, oligomeric and polymeric flavonoids which, by virtue of their antioxidant properties (Da Silva et al. 1998; Rein et al. 2000; Richelle et al. 2001, Mursu et al. 2004), may be of value in the prevention of certain types of cancer (Weisburger 2001) and coronary heart disease (Arts et al. 2001; Weisburger 2001; Steinberg et al. 2003; Ding et al. 2006), and in the maintenance of cardiovascular health by modulating platelet activity (Rein et al. 2000; Murphy et al. 2003). Studies evaluating the oral absorption of chocolate flavonoids have indicated that the monomeric flavan-3-ols (+)-catechin (CAT) and (−)-epicatechin (ECAT) are the only ones to be absorbed from the gastrointestinal tract in quantities discernible by routine analytical methods (Donovan et al. 1999; Nelson and Sharpless 2003). However, in spite of reports describing the oral absorption, metabolism and plasma kinetics of CAT and ECAT from various types of chocolate and from cocoa powder in humans (Richelle et al. 1999; Baba et al. 2000a; Wang et al. 2000; Murphy et al. 2003; Schramm et al. 2003; Schroeter et al. 2003; Serafini et al. 2003) and laboratory animals (Baba et al. 2000b, 2001), controversy still exists on the effect that whole milk could have on the oral absorption of chocolate flavanols. Thus, while some laboratories have verified that whole milk reduces the oral absorption of these compounds (Serafini et al. 2003; Serafini and Crozier 2003), others have failed to confirm this effect (van het Hof et al. 1998; Leenen et al. 2000; Schroeter et al. 2003; Schramm et al. 2003). Furthermore, the degree of interference by whole milk on the oral absorption of chocolate flavanols is reported to vary depending on whether the milk is consumed concurrently with a chocolate or it is present as an ingredient of the chocolate matrix (Serafini et al. 2003).

To more clearly and conclusively define the contributing role of whole milk to the oral absorption and ensuing plasma pharmacokinetics of monomeric flavan-3-ols present in chocolate, the present study has investigated the extent of absorption of CAT and ECAT from the gastrointestinal tract following their individual oral administration to rats as equipotent solutions in both water and whole milk. In addition, experiments were carried out with solutions of CAT and ECAT in skim milk and heavy cream, with suspensions of commercial chocolates in whole milk and water, and with suspensions of various types of commercial chocolates in water, to be able to assess the contribution of variations in milk and in chocolate matrix composition to the oral absorption and ensuing plasma kinetics of monomeric chocolate catechins.

2. Investigations and results

2.1. Analysis of CAT and ECAT in plasma and chocolate samples

An isocratic HPLC method with fluorometric detection previously developed and validated in this laboratory (Gossai and Lau-Cam 2006) was used for the assay of CAT and ECAT in rat plasma and in commercial chocolates. When applied to plasma samples, this method demonstrated excellent selectivity (CAT 6.7 min, ECAT 13.8 min, acetylsalicylic acid, the internal standard, 21.3 min), satisfactory sensitivity (limit of detection values were 0.75 nM of CAT and 1.5 nM of ECAT), and excellent reproducibility (RSD for interday assays of triplicate samples of CAT and ECAT added to blank rat plasma at levels between 0.75–12 nM were in the range 1.3–4.1% and 0.5–2.0%, respectively). A linear relationship between concentrations of analyte and detector responses was observed over the concentration range 1.5–12 nM of CAT and ECAT. Plasma CAT and ECAT values following enzymatic hydrolysis of the plasma samples with a mixture of β-glucuronidase and aryl sulfatase are reported as total catechin levels (the sum of the parent molecule and its glucuronide, sulfate and sulfoglucuronide metabolites).

2.2. Plasma concentrations and kinetics of CAT and ECAT from solutions.

To determine the effect of the vehicle on the oral absorption of CAT and ECAT, rats were separately treated with solutions of each of these catechins (350 mg/kg) in distilled water and, later on, with equipotent solutions in a common dairy product (whole milk, skim milk or heavy cream), and their blood collected at predetermined intervals for the measurement of corresponding plasma total CAT and ECAT contents.

The temporal changes in plasma CAT and ECAT concentrations resulting from the oral administration of their various solutions to rats are shown in Figs. 1 and 2. The values of the pharmacokinetic parameters calculated from these plasma levels are presented in Table 1. When administered orally as aqueous solutions, both CAT and ECAT were absorbed rather readily from the gastrointestinal tract, with their plasma concentrations rising in a parallel manner and reaching a maximum (t_{max}) at 1 h post-administration. The values for C_{max} and AUC for CAT (∼32.1 nM and 79.7 nM · hr) were about the same and higher, respectively, than those for ECAT (∼32.4 nM and 59.2 nM · hr). In contrast, some marked differences were noted in the oral absorption of these flavanols when they were administered as solutions in a dairy product. For example, in the case of CAT, while the t_{max} for solutions in either skim milk, whole milk or heavy cream did not differ from that in water, the corresponding C_{max} and AUC values were significantly lowered both by whole milk (by 34% and 18%, respectively) and by heavy cream (by 62% and 55%, respectively) relative to an aqueous solution. Under identical conditions, the solution of CAT in skim milk showed the same AUC value and a lower C_{max} (by 19%) than its counterpart in water. In the case of ECAT, however, the same kinetic parameters varied according to the type of dairy product that served as a vehicle. Thus, in skim milk the t_{max} was reached in 1 h, but in whole milk and heavy cream it took 3 h. Furthermore, the C_{max} and AUC values for ECAT were lower than those of an equipotent aqueous solution when delivered as a solution in a dairy product, with the lowering effect declining in the order: heavy cream (by 68% and ∼48%) > whole milk (by 55% and 8%) > skim milk (by 15% and ∼16%).

In comparison to an aqueous solution, all the dairy products were found to delay the k_a, to slow the k_e, and to prolong the $t_{1/2}$ of both CAT and ECAT, although to different extents. Likewise all dairy products lowered the k_a (by 19–62%) and k_e (by 10–29%) and lengthened the $t_{1/2}$ (by 14–39%) of CAT relative to those values from an equipo-

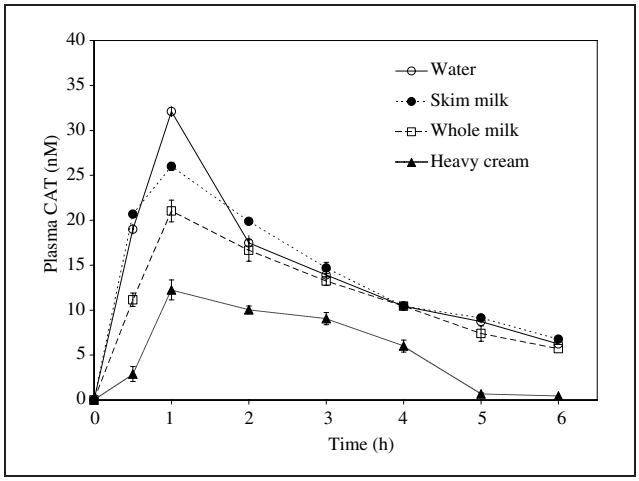

Fig. 1: Plasma concentration-time courses for solutions of CAT in water or a dairy product after oral administration to rats at the dose of 350 mg/kg. Each point represents the mean ± S.E.M. (n = 6)

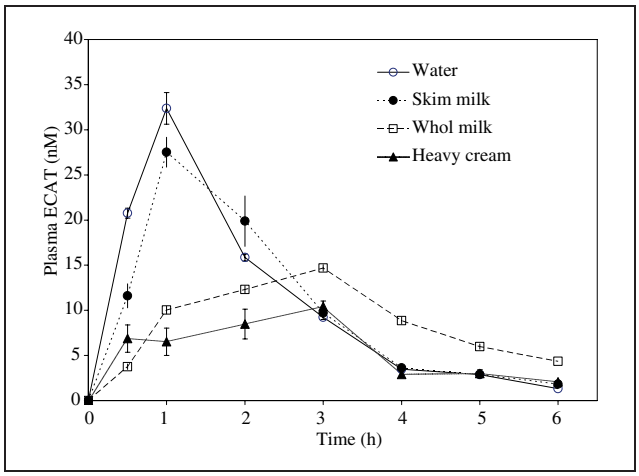

Fig. 2: Plasma concentration-time courses for solutions of ECAT in water or a dairy product after oral administration to rats at the dose of 350 mg/kg. Each point represents the mean ± S.E.M. (n = 6)

Table 1: Plasma pharmacokinetic data for CAT and ECAT after oral administration to rats as solutions in water or a dairy product[a,b]

Pharmacokinetic parameter	Water	Skimmed milk	Whole milk	Heavy cream
CAT				
$AUC_{0-\infty}$ (nM · h)	79.74 ± 0.29	79.41 ± 0.18*	65.36 ± 0.63***	35.73 ± 0.49***
C_{max} (nM)	32.09 ± 0.56	26.00 ± 0.45***	21.04 ± 1.21***	12.26 ± 1.10***
t_{max} (h)	1.00 ± 0.01	1.00 ± 0.01*	1.00 ± 0.01*	1.00 ± 0.01*
$t_{1/2}$ (h)	2.22 ± 0.11	2.53 ± 0.13***	2.71 ± 0.14***	3.09 ± 0.15***
k_a (h^{-1})	32.09 ± 0.31	26.00 ± 0.21***	21.04 ± 0.59***	12.26 ± 0.58***
k_e (h^{-1})	0.31 ± 0.02	0.28 ± 0.01**	0.26 ± 0.01***	0.22 ± 0.01***
ECAT				
$AUC_{0-\infty}$ (nM · h)	59.17 ± 0.35	49.67 ± 0.35***	54.29 ± 0.33***	30.78 ± 0.88***
C_{max} (nM)	25.08 ± 0.55	21.82 ± 0.34**	19.39 ± 0.64***	9.78 ± 0.60***
t_{max} (h)	1.00 ± 0.05	1.00 ± 0.03*	3.00 ± 0.15*	3.00 ± 0.15*
$t_{1/2}$ (h)	1.18 ± 0.06	1.28 ± 0.06**	2.13 ± 0.11***	1.42 ± 0.07***
k_a (h^{-1})	25.08 ± 0.36	21.82 ± 0.29**	6.29 ± 0.05***	2.97 ± 0.11***
k_e (h^{-1})	0.59 ± 0.03	0.54 ± 0.03**	0.33 ± 0.02***	0.49 ± 0.02***

[a] Values are reported as the mean ± SEM for n = 6 rats
[b] Statistical comparisons were versus water: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$

tent aqueous solution (i.e., 32.09 h^{-1}, 0.31 h^{-1} and 2.21 h, respectively), with the altering effect decreasing in the order heavy cream > whole milk > skim milk. Furthermore, by analogy to the results gathered with CAT, the k_a, k_e and $t_{1/2}$ values for an aqueous solution of ECAT (i.e., 25.08 h^{-1}, 0.59 h^{-1}, 1.18 h, respectively) were similarly affected by a dairy product, with the k_a and k_e becoming decreased by 13–88%, and ~8.5–17%, respectively, and the $t_{1/2}$ becoming lengthened by 8–103%. In general, the altering effects of a dairy product on the plasma kinetic of ECAT decreased in the order previously verified for CAT, namely heavy cream > whole milk > skim milk.

To ascertain the contributory roles that proteins and fats in each dairy product might play on the oral absorption of CAT and ECAT, the concentrations of these constituents (g/240 mL) in each dairy product (mean protein content found and declared fat content values were, respectively: skim milk 7.16 and 2.00; whole milk 7.05 and 8.00, heavy cream 0.29 and 0.00) was correlated with the corresponding plasma AUC values (Table 1). In this manner it was possible to determine that the AUC values of CAT and ECAT decreased proportionally to an increase in the fat (heavy cream > whole milk > skim milk), but not in the protein (whole milk = skim milk > heavy cream) content of the dairy product. A plot of AUC values for CAT an ECAT versus the fat content in a dairy yielded the graphs shown in Fig. 3. The variance, r^2, values of the lines for CAT and ECAT were 0.9413 and 0.9421, respectively. For comparative purposes, this graph also shows the correlations for aqueous solutions of CAT and ECAT which, owing to the absence of interfering substances, yielded the highest AUC values.

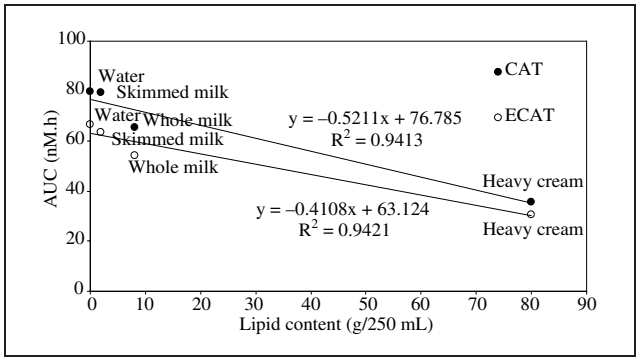

Fig. 3: Correlation plots of plasma $AUC_{0-\infty}$ for solutions of CAT and ECAT in a dairy product vs. concentration of fat in a dairy product. Each point is the mean value for n = 6. For comparative purposes, data derived from solutions of CAT and ECAT in water are shown alongside

2.3. Plasma concentrations and kinetics of CAT and ECAT from chocolate dispersions

The effect of a chocolate matrix and of exogenous milk added to this matrix on the oral absorption chocolate monomeric catechins was examined using four commercial types of chocolate, namely, baking, dairy milk, dark, and white. A preliminary assay of the CAT and ECAT content of these chocolates revealed that their contents in each of these phenolics and the ratio of one phenolic to the other varied from chocolate to chocolate. Thus, they were readily measurable in baking (~91 mg/g CAT and 103 mg/g ECAT) and dark chocolate (~63 mg/g CAT and 87 mg/g ECAT), of unequal occurrence in dairy milk chocolate (0 mg/g CAT, ~40 mg/g ECAT), and absent from white chocolate. On the basis of these results, *in vivo* experiments with baking chocolate, dairy milk chocolate and dark chocolate required two separate oral dosings of the chocolate, one to deliver 350 mg/kg of CAT and the other to deliver the same dose of ECAT. Furthermore, in the same experiments, dairy milk chocolate and white chocolate were supplemented with sufficient quantities of the missing catechin and capable of delivering 350 mg/kg of each catechin to rats.

The influence of milk on the oral absorption of monomeric flavan-3-ols in chocolate was inferred from the plasma levels (Figs. 4 and 5) gathered for samples of baking chocolate delivered by the oral gavage technique either as an aqueous solution or as a uniform dispersion in whole milk. The plasma C_{max}, t_{max} and AUC values derived from these experiments are presented in Table 1. Relative to results for equipotent solutions of CAT or ECAT in plain water, and taken as 100%, it is evident that in the presence of whole milk, the plasma C_{max} and AUC, but not the t_{max}, of both catechins from baking chocolate were affected, in this case negatively and to about the same extent, with the C_{max} showing a ~2-fold decrease and the AUC a ~1.8-fold decrease.

Two types of experiments were carried out to investigate the possible effect that a chocolate matrix could have on the absorption of its constitutive monomeric catechins from the GI tract. In the first set of experiments, aqueous

Table 2: Plasma pharmacokinetic data for CAT and ECAT, present in or added to, commercial chocolates after oral administration of the chocolate to rats as dispersions in water or whole milk[a,b,c]

Type of chocolate	CAT			ECAT		
	C_{max} (nM)	t_{max} (h)	AUC (nM · h)	C_{max} (nM)	t_{max} (h)	AUC (nM · h)
Baking in water	$32.32 \pm 1.23^{*}$	1.00	$77.19 \pm 2.03^{*}$	$25.49 \pm 2.17^{*}$	1.00	$60.02 \pm 4.12^{*}$
Baking in milk	$16.86 \pm 0.96^{***}$	1.00	$42.51 \pm 2.62^{***}$	$12.66 \pm 1.03^{***}$	1.00	$32.75 \pm 3.62^{***}$
Dark in water	$2.00 \pm 0.56^{***}$	1.00	$2.22 \pm 1.36^{***}$	$0.00 \pm 0.14^{***}$	1.00	$0.00 \pm 0.35^{***}$
Dark in water, spiked	$23.66 \pm 1.65^{**}$	1.00	$57.12 \pm 2.95^{***}$	$18.66 \pm 1.06^{**}$	1.00	$42.57 \pm 1.09^{**}$
Dairy milk in water	$0.01 \pm 0.12^{***}$	1.00	$0.01 \pm 0.11^{***}$	$0.01 \pm 0.18^{***}$	1.00	$0.01 \pm 0.22^{***}$
Dairy milk, spiked	$0.05 \pm 0.16^{***}$	1.00	$0.07 \pm 0.08^{***}$	$0.02 \pm 0.20^{***}$	1.00	$0.03 \pm 0.12^{***}$
White in water, spiked	$20.37 \pm 3.02^{**}$	1.00	$42.79 \pm 1.09^{***}$	$15.32 \pm 0.99^{***}$	1.00	$31.26 \pm 3.58^{***}$

[a] Values are reported as the mean ± SEM (n = 6)
[b] Dark chocolate, dairy milk and white chocolate were spiked with CAT and ECAT to deliver 300 mg/kg of each flavanol
[c] Statistical comparisons were versus water: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$

dispersions (∼2 mL) of either dark chocolate or dairy milk chocolate were separately given to rats in quantities that delivered 350 mg/kg of each monomeric catechin. In the other set of experiments, rats were dosed with aqueous dispersions of the same types of chocolate and of white chocolate after they had been spiked with sufficient monomeric catechin to represent an oral dose of 350 mg/kg of the relevant catechin. From the ensuing plasma levels shown in Figs. 4 and 5, the plasma kinetics for CAT and ECAT presented in Table 2 were calculated.

When the plasma pharmacokinetic values for aqueous dispersions of dark and dairy milk chocolate are compared with those of an equipotent dispersion of baking chocolate, it is readily apparent of the existence of marked differences not only from baking chocolate but also from each other. Thus, while the C_{max} and AUC values for CAT from dark chocolate were ∼16-fold and ∼35-fold lower, respectively, than those of baking chocolate, no corresponding plasma pharmacokinetic values could be calculated for ECAT from this type of chocolate dispersion since its plasma values remained below the limits of detection of the analytical method used.

Spiking dark chocolate with sufficient CAT to deliver this compound in a dose of 350 mg/kg, dramatically narrowed the differences in the plasma pharmacokinetic values between this chocolate (C_{max} ∼1.4 fold lower, AUC ∼1.35-fold lower) and baking chocolate but without making them equal. The same trend of results was obtained for the spiked samples of white chocolate (C_{max} ∼1.60-fold lower, AUC ∼1.80-fold lower for CAT; C_{max} ∼1.7-fold lower, AUC ∼1.9-fold lower for ECAT). To test the contribution of the chocolate matrix on the release of the constitutive monomeric catechins, the pharmacokinetics of CAT and ECAT from aqueous dispersions of dairy milk chocolate were studied before and after a spiking with exogenous amounts of these catechins. As shown in Table 2, even after spiking dairy milk chocolate, the C_{max} and AUC for both catechins from dairy milk chocolate remained below 0.1 nM and 0.1 nM · h, respectively. Overall, and regardless of the type of chocolate and vehicle tested, the t_{max} (1 h) remained unchanged throughout.

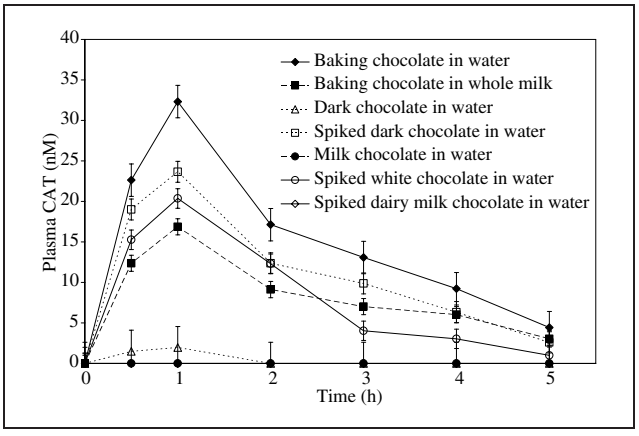

Fig. 4: Plasma concentration-time courses for CAT in dispersions of baking chocolate and milk chocolate in water after oral administration to rats in an amount delivering 350 mg/kg of CAT. Each point represents the mean ± S.E.M. (n = 6)

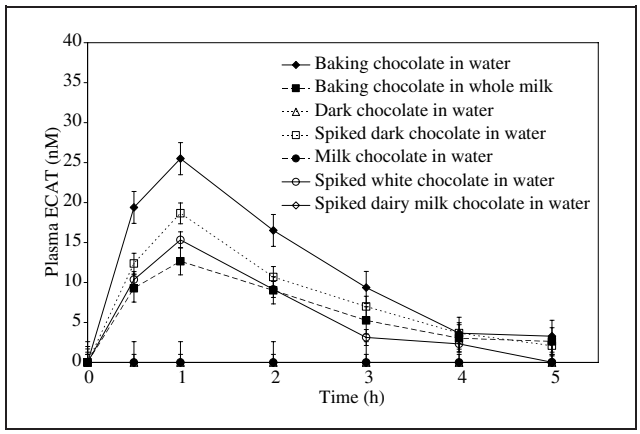

Fig. 5: Plasma concentration-time profiles for ECAT in dispersions of baking chocolate and milk chocolate in water after oral administration to rats in an amount delivering 350 mg/kg of ECAT Each point represents the mean ± S.E.M. (n = 6)

3. Discussion

The main purpose of this study was to determine whether the oral absorption of CAT and ECAT, the major monomeric catechin components of chocolate, is influenced by the concurrent ingestion of whole milk or by differences in matrix composition and physical characteristics of chocolates containing them. The study was accomplished using the rat, an animal model that is found to yield oral bioavailability data for CAT and ECAT that compares favorably with that gathered in human subjects (Baba et al. 2001; Piskula and Terao 1998; Rein et al. 2000; Richelle et al. 1999; Tsang et al. 2005; Wang et al. 2000).

In spite of a rapid absorption from the intestinal tract, the oral bioavailability of CAT and ECAT is quite low, with reported values ranging from less than 1% (Goldberg et al. 2003) to about 6.5% (Catterall et al. 2003; Goldberg et al. 2003; Xu et al. 2004; Zhu et al. 2000). Some of the rea-

sons underlying such a low bioavailability may be factors operating within the gastrointestinal tract and including, among others, a limited membrane permeability, transporter-mediate intestinal secretion, and gut wall metabolism, wide tissue distribution, and a high first-pass hepatic elimination (Cai et al. 2002; Zhu et al. 2000). To compensate for a poor oral bioavailability and, at the same time, to ensure plasma levels that will fall within the limits of detection of the HPLC method used for assaying CAT and ECAT in the circulation, in the present study these flavanols were administered intragastrically in a rather high (i.e., 350 mg/kg) dose, either as pure compounds or as equipotent amounts of a commercial chocolate. Furthermore, since both CAT are ECAT susceptible to extensive intestinal conversion to glucuronide, sulfate and sulfoglucuronide conjugates (Baba et al. 2000a, 2001; Donovan et al. 1999; 2001; Kuhnle et al. 2001; Tsang et al. 2005), the plasma samples containing them were subjected to enzymatic hydrolysis with β-glucuronidase and arylsulfatase prior to their assay for unconjugated catechin content (Piskula and Terao 1998; Baba et al. 2000a, b; Donovan et al. 2001). Hence, the plasma values of CAT and ECAT that are reported here represent total catechins, i.e., the sum of unmetabolized (free) catechin plus conjugated catechin.

Results on the effect of whole milk on the oral absorption of monomeric chocolate catechins are surrounded by controversy and subject to variability among different laboratories. For example, it has been documented that whole milk can reduce the oral absorption and plasma levels of ECAT as well as the total antioxidant ability (TPAA) to a greater extent in individuals that ingested either dark chocolate along with whole milk or as part of a milk chocolate than individuals who only ingested dark chocolate (Serafini et al. 2003). The lowering effect of whole milk on the oral absorption of ECAT has been related to an interaction between this compound and proteins in milk, to form a complex that absorbs poorly from the intestinal tract. While confirmatory evidence on this assumption was suggested by a previous study in which milk proteins were found to mask the antioxidant capacity of CAT from green and black tea (Arts et al. 20001b), evidence to the contrary has also become available. Thus, there is one report indicating that that the addition of milk to tea has no effect on the oral absorption of monomeric catechins from tea (van het Hoff et al. 1998); and another describing the lack of effect of adding whole milk to both black and green tea infusions on the extent of TPAA derived by healthy human from the infusions, but without providing information on the accompanying plasma catechin levels (Leenen et al. 2000). Other laboratories have shown that the addition of 10% milk to a black tea infusion or of 66% milk to a cocoa beverage will not alter the antioxidant activity derived from these beverages (Richelle et al. 2001); and that drinking a milk-cocoa beverage will result in plasma AUC and TPPA values for ECAT that are not significantly different from those of individuals who drink a water-cocoa beverage supplemented with the amounts of carbohydrate and fat found in milk (Schroeter et al. 2003). Furthermore, in a more recent study it was verified that the consumption of a chocolate drink mixed with skim milk powder was devoid of any altering effect on the oral bioavailability of CAT and ECAT, thus ruling out a role for milk proteins (Keogh et al. 2007). However, there is also evidence to suggest effects of milk on the oral absorption and pharmacokinetics of cocoa flavanols that are at variance with those encountered by most investigators. More specifically, the plasma AUC, C_{max} and $t_{1/2}$ values for CAT and ECAT were found slightly higher in human subjects that had ingested a dispersion of cocoa in whole milk than in those that had consumed the same amounts of cocoa but dispersed in plain water (Schramm et al. 2003).

To determine the influence of a dairy product and of components in a chocolate matrix on the oral absorption of CAT and ECAT, two types of experiments were conducted with these flavanols. In one, each catechin compound was administered as a solution in plain water (the control treatment) or in a dairy product (the test treatment). In the other, the catechins were administered as part of a chocolate product, which was dispersed either in water or in a relevant dairy product. Since the concentrations of CAT and ECAT in commercial chocolates are not the same, the chocolate samples were administered in two portions, each one containing a concentration of monomeric catechin equal to that used in experiments with the pure compounds. In the event that a catechin was either absent (for example CAT in dark chocolate, CAT and ECAT in white chocolate) or in a very low concentration, the chocolate was spiked with the missing or limiting catechin until it provided a dose of catechin equipotent to that administered as a solution of the pure catechin.

By comparing the pharmacokinetics values for solutions of a catechin compound in whole milk and in plain water, it was verified that milk exerted a significant reducing effect on the values of AUC, C_{max} and k_a for both CAT and ECAT relative to those recorded for aqueous solutions, but had little or no effect on the respective plasma t_{max}, $t^{l}_{/2}$, and k_e values. The possibility that fats and protein components of a dairy product might be playing a determining role on the oral absorption of CAT and ECAT was investigated by comparing the oral absorption of these flavanols from their individual solutions in whole milk and in other common dairy products (i.e., skim milk, heavy cream), and correlating the results with the corresponding contents in fats (declared values) and proteins (experimentally determined values). In this manner it was established that the AUC and C_{max} values of CAT and ECAT decreased in proportion to an increase in the fat content of the dairy product. Accordingly, skim milk, having the lowest declared fat content (0.83 g/dL), was found the least inhibitory; heavy cream, having the highest fat content (33.3 g/dL), the most inhibitory, and whole (full fat) milk (3.33 g/dL) exhibiting an intermediate effect. In addition, finding that the magnitude of the effects of skimmed and whole milk on the AUC and C_{max} values were different in spite of having equal protein contents (3.33 g/dL), and that heavy cream was the most inhibitory even though it was found to be practically devoid (~0.29 g/dL) of proteins, militates against the previously held concept that milk proteins are relevant to the absorption of monomeric chocolate catechins from the gastrointestinal tract (Arts et al. 2002).

Three additional experiments were carried out to verify the contributory role of whole milk in the oral absorption of CAT and ECAT from a chocolate matrix. In the first experiment, the plasma pharmacokinetic values of these catechins were examined using baking chocolate, a chocolate with simpler composition than either dark or dairy milk chocolate and yielding plasma levels that approximated those observed with solutions of the individual catechins in water. In addition, the ingestion of a dispersion of baking chocolate in whole milk yielded plasma AUC and C_{max} values for CAT and ECAT that were nearly one-

half lower than those attained with equipotent dispersions in plain water. The second experiment, aimed at comparing the plasma pharmacokinetics of CAT and ECAT from baking chocolate against those from dairy milk chocolate and dark chocolate following their intragastric administration as equipotent dispersions in distilled water, found the existence of marked differences among the various types of chocolate. Thus, while the AUC and C_{max} values for CAT and ECAT from baking chocolate approached the values recorded for aqueous solutions of these monomeric flavanols, those from dark chocolate and milk chocolate were very low and virtually nil, respectively, to permit their calculation. These extreme differences in oral absorption may reflect differences in matrix composition and in the physical characteristics of the product (Beckett 1999). For example, baking chocolate is a molded solid product, contains no sugar, is rather friable when compressed, and has little unctuousity as a result of a much lower content in cocoa butter than dark, milk or white chocolate. In contrast, cocoa powder as a result of an even lower content in cocoa butter than baking chocolate, behaves as a dry and free flowing product that is easily dispersible in water. On the basis of the existing physical differences among commercial chocolates, it is plausible that the CAT and ECAT present in cocoa powder will be in a better position to enter the intestinal mucosa, even in the presence of whole milk, than from chocolates with a higher content in cocoa butter since they can diffuse out of their matrix more readily than they would from chocolates with a higher content of cocoa butter. The third experiment was designed to investigate the influence of the type chocolate on the oral absorption of CAT and ECAT. For this purpose, the plasma pharmacokinetics of CAT and ECAT released from aqueous dispersions of dark and white chocolate, both spiked with equal quantities (175 mg/mL) of CAT and ECAT were compared with those from unspiked samples. While the unspiked samples of dark chocolate and white chocolate yielded very low and no levels of the two CAT and ECAT, respectively, both spiked chocolate samples yielded readily detectable plasma levels of these monomeric catechins. However, in comparison to AUC values for CAT and ECAT obtained from spiked aqueous dispersion of baking chocolate, those from spiked dispersions of dark chocolate and white chocolate were 1.4–1.8-fold and 1.4–1.9-fold lower, respectively. At least two factors could be invoked to account for these diverging results. One is the differences in relative proportions of cocoa butter, whole milk (in powdered or condensed form), lecithin, flavorings and sugar present in each of chocolate types used here (Beckett 1999; Fryer and Pinschower 2000). The other could be the extent of the physical interactions that may take place between matrix components during the manufacturing process of a chocolate. This assumption is tenable since cocoa butter and other fat sources in chocolate are known to undergo cycles of melting and solidification along with continuous mixing. Hence, fat components will ultimately coat solid components in a chocolate matrix, including monomeric catechins, with polymorphic crystal networks that melt just below body temperatures (Toro-Vazquez et al. 2004). This event might be responsible for the better absorption of monomeric catechins of exogenous origin from the intestinal tract than those found within a chocolate matrix, which are more susceptible to becoming trap within the chocolate matrix than their exogenous counterparts. On the other hand, earlier reports that cocoa powder had little effect on the bioavailability of monomeric catechins in rats (Baba et al. 2001) and humans (van het Hof et al. 1998) could simply reflect the presence of an interfering matrix than is less complex than that present in the chocolate evaluated here. On the other hand, the extremely low plasma C_{max} and AUC values for CAT and ECAT following their release from dairy milk chocolate, even after spiking this chocolate with both monomeric catechins could be due to the combined effect of a very complex matrix and the presence of both milk and fats, factors which, when co-occurring, may jointly contribute to impeding the release of monomeric catechins from a chocolate for eventual uptake into the intestinal mucosa.

The fat components in chocolates would appear to influence the release characteristics of CAT and ECAT from a chocolate matrix not simply by its physical presence but, rather, by modifying matrix characteristics by interacting with catechins and other matrix components during the manufacturing process of a chocolate. This conclusion is based on a comparison of the amount of petroleum ether solubles, found in the various chocolates evaluated here with the corresponding fat contents declared by the manufacturers. In this present situation, three out of the four commercial chocolates tested (i.e., dairy milk, dark, white) had declared quantities of total fats that were within about 2% of each other (dairy milk 30.0; dark 32.5; white 28.6) and petroleum ether solubles contents that differed by not more than about 5% of each other in the most extreme case (dairy 31.4; dark 32.0; white 27.3). A further indication that fats alone are not as important as are other features associated with commercial chocolates is to find that the monomeric catechins of baking chocolate exhibited a higher oral absorption than from the other chocolates even though this type of chocolate had the highest labeled content of total fats (50.0 g/100 g) and was found to contain the highest concentration of petroleum ether solubles (52.6 g/100 g). Additional, although less important, factors that may have contributed to the low plasma levels of CAT and ECAT that follow their oral consumption as part of milk and dark chocolates are an extensive first-pass hepatic effect and a competitive absorption in the gastrointestinal tract when in the presence of each other (Baba et al. 2001).

CAT and ECAT appear to differ in the extent of their absorption from the intestinal tract. For example, following the oral administration of these compounds to Sprague-Dawley rats, both individually and as a mixture, the sum of the urinary metabolites for CAT was found to be significantly lower than that for ECAT (Baba et al. 2001). In the same study, the existence of a competitive intestinal absorption between CAT and ECAT was inferred from the decrease in the sum of the urinary metabolites of ECAT when the two flavanols were administered together. The present work also finds the existence of difference in oral absorption between CAT and ECAT and, at the same time, verifies the absorption of CAT to be more affected by whole milk and heavy cream than is the absorption of ECAT. However, the trend of these results contradicts that reported elsewhere (Baba et al. 2001), namely that the absorption of CAT from the gastrointestinal tract is greater than that of ECAT. Although the exact reason for this discrepancy is not apparent at this time, especially since both laboratories used rats of the same strain and gender and of approximately the same body weight, one possibility could be the size of the oral dose used in each laboratory, being 7-fold higher (i.e., 350 mg/kg vs. 50 mg/kg) here than that used by Baba et al. (2001). On the other hand, the results reported here are more in line with those found

for CAT and ECAT both in rats and in isolated preparations of rat jejunum and ileum. Thus, the administration of a single dose of 3-^{3}H-labeled flavanol to male Wistar rats resulted in values of total dose excretion in urine and feces that were of about the same magnitude for both compounds even though the dose of CAT was only one-seventh that of ECAT (Catterall et al. 2003); and perfusing rat jejunum and ileum preparations for 90 min with the individual catechins led to cumulative absorptions that were in both instances higher for CAT than for ECAT (Kuhnle et al. 2000).

In summary, dairy products and commercial chocolates are found to negatively influence the oral absorption of the chocolate flavanols CAT and ECAT to differing extents. In the case of dairy products the influence appears to be directly related to the fat, but not to the protein, content of the dairy product. In the case of commercial chocolates, the release characteristics of CAT and ECAT from the chocolate matrix and, hence, the fraction at which they reach the general circulation, could be a function of the characteristics of the chocolate matrix as imposed by the interactions between catechins and chocolate matrix components, notably fats, during the manufacturing of a chocolate. Hence, the amounts of monomeric chocolate catechins appearing in the circulation after the consumption of a chocolate product-liquid dairy product beverage or of a commercial chocolate will be determined, respectively, by the type and volume of dairy product added or the type of chocolate consumed.

4. Experimental

4.1. Materials

Samples of CAT (hydrate) and ECAT, acetylsalicylic, aryl sulfatase (type H-1 from *Helix pomatia* with 15,300 U/g), bovine β-D-glucuronidase (type B-3 with 2.63×10^8 Fishman U/g), disodium EDTA and reagents for measuring proteins (Kit No.690-A) were purchased from Sigma Chemical Company (St. Louis, MO). Sodium acetate (anhydrous) was from Aldrich Chemical Company (Milwaukee, WI), perchloric acid (70%) and formic acid came from J. T. Baker (Phillipsburg, NJ), and petroleum ether (b.p. 35–60 °C) and solvents for HPLC were obtained from EM Science (Gibbstown, NJ). The samples of whole milk, skim milk and heavy cream (Oak Tree Farm Dairy, Inc., East Northport, NY), unsweetened baking chocolate (Baker's®, Kraft Foods North America, Inc., Rye Brook, NY), semisweet dark chocolate (Cadbury® Royal Dark, The Hershey Company, Hershey, PA), milk chocolate (Cadbury® Dairy Milk, The Hershey Company, Hershey, PA) and white chocolate (Nestlé USA, Inc., Solon, OH) were purchased from local stores.

4.2. Animals and treatments

This study was approved by the Institutional Animal Care and Use Committee of St. John's University. The animals received humane care according to guidelines set by the United States Department of Agriculture. The male Sprague-Dawley rats used in the study (276–300 g) were obtained from Taconic Farms (Germantown, NY). The animals were housed in groups of 3, in plastic cages with open-mesh wire lids, in a room maintained at a temperature controlled room (21.1 ± 3 °C) and an inverted 12 h light/dark cycle. The animals were allowed to acclimate to their new environment for a period of 5 days before an experiment, and to have free access to a commercial rodent diet (LabDiet® 5001, Purina Mills, St. Louis, MO) and tap water. The food was removed 14 h before an experiment.

Solutions of CAT and ECAT were prepared on the day of the experiment in water, skim milk, whole milk, or high fat (heavy) cream, to contain 175 mg/mL. The samples of chocolate were prepared by dispersing 1 g of sample in warm water with the aid of sonication until a uniformly fluid paste was obtained. When a chocolate sample lacked a catechin (e.g., CAT in dark chocolate; CAT and ECAT in white chocolate), it was spiked prior to oral dosing in an amount providing 350 mg/kg of each missing catechin.

Each experimental group consisted of 6 fasted rats. The treatment solutions were delivered as a single dose by the intragastric route via a 14 gauge oral needle attached to a 5 mL plastic syringe, in a volume not exceeding 2 mL. The dose of catechin compound (or an equipotent volume of chocolate suspension) was, in all instances, 350 mg/kg.

4.3. Samples for plasma catechin analysis

Blood samples (~0.5 mL), were collected from the tail vein of conscious rats every 0.5 h for the first 2 h, and at 1 h intervals for the next 4 h, into polyethylene microtubes containing a small amount of disodium EDTA. Each blood sample was vortex-mixed for about 30 s, and centrifuged at 1000 × g for 10 min to separate its plasma. The plasma samples were transferred to clean microtubes and stored at 4 °C pending their analysis.

A sample of frozen plasma was allowed to thaw in a warm water bath, and then shaken gently to ensure homogeneity. To 100 μL volume of plasma, 50 μL of enzyme preparation (a mixture of 2 mg of β-glucuronidase and 165 mg of aryl sulfatase in 5 mL of 0.1 M sodium acetate, adjusted to a pH 5.0 with glacial acetic acid) was added. The mixture was incubated at 37 °C for 4 h, allowed to cool to ambient temperature, and immediately treated with 50 μL of internal standard solution (prepared by mixing 3 mL of a solution of catechin compound, 22 μg/mL, or of acetylsalicylic acid, 100 μg/mL, in methanol, with 2 mL of perchloric acid). The mixture was vortex-mixed for 30 s, and next centrifuged at 1000 × g for 10 min. A 50 μL portion of the supernatant was injected into the liquid chromatograph. A standard preparation (containing 11 μg/mL of CAT, ECAT or of both compounds, in methanol) was treated in similar manner as the sample preparation and analyzed alongside.

4.4. Pharmacokinetic analysis

The following pharmacokinetic parameters were calculated from the plasma CAT and ECAT levels using standard equations (Harvey 1980), and assuming a one compartment model: C_{max} (the maximum plasma concentration), t_{max} (time for maximum plasma concentration), $t_{1/2}$ (the plasma half-life), k_a (the rate constant of the oral absorption phase), k_e (the rate constant of the elimination phase), and $AUC_{0-\infty}$ (the area under the plasma concentration-time curve).

4.5. Assay of CAT and ECAT in chocolate

A 1 g quantity of chocolate (baking, milk chocolate), was placed in a mortar, mixed with 6 mL of methanol, and thoroughly triturated with the aid of a pestle. To the mixture, 7 mL of water was added and the mixing continued until a fluid paste was obtained. The fluid paste was filtered through a 0.47 μm membrane filter, diluted with water in a ratio of 25 μL of filtrate to 1 ml of water, and the dilution analyzed for CAT and ECAT by the same HPLC used for the analysis of plasma samples but using acetylsalicylic acid as the internal standard.

4.6. Assay of proteins in dairy products

Total protein content was measured using a commercially available colorimetric assay kit (Kit 690-A, Sigma Chemical Co.). A 200 μL aliquot of dairy product (whole milk, skim milk, heavy cream) was placed in a clean glass test tube and mixed with 2.2 mL of Biuret reagent with the aid of a vortex mixer. After allowing the mixture to stand at room temperature for 10 min, it was mixed with 100 μL of Folin color reagent, and allowed to stand for an additional 30 min. The reaction mixture was centrifuged at 2500 × g for 10 min to remove insolubles, and its absorption was read on a spectrophotometer at 725 nm. The concentration of protein (in mg/dL) was calculated by reference to a calibration curve of bovine serum albumin prepared on the day of the analysis.

4.7. Nonpolar (petroleum ether) extractables from chocolates

A 1 g quantity of commercial chocolate (baking, dark, dairy or white) was placed in a 250 mL stoppered Erlenmeyer flask, mixed with about 100 mL of petroleum ether b.p. 35–60 °C, and mechanically stirred for 14 h. The suspension was filtered with the aid of vacuum through a preweighed disc of Whatman No. 1 filter paper, held on a Buchner funnel, into a suction flask. After thoroughly washing the filter paper and flask with fresh solvent, the filtrate and washings were pooled together and evaporated to dryness under a stream of dry air. This residue, representing the nonpolar petroleum ether extractables, and the filter paper with the insoluble material, were placed in a vacuum oven, dried further at 45 °C for 12 h, and their weights recorded on an analytical balance.

4.8. HPLC analysis of plasma total catechins

The analyses of monomeric plasma catechins were carried out in the isocratic mode on a Microsorb-MV C18™, 250 × 4.9 mm i.d., 100 Å particle size, column (Varian, Palo Alto, CA), using a mixture of water-methanol-formic acid (84 : 15 : 1, by volume) as the mobile phase and a flow rate of 1 mL/min. The effluent was monitored fluorometrically using an excitation wavelength of 280 nm and an emission wavelength of 310 nm. The internal standard was selected based on the type of sample tested. In experiments with a single catechin, one catechin served as the internal standard

for the other, but in experiments with a chocolate specimen acetylsalicylic acid was used instead. The concentration of the analyte of interest was calculated on the basis of the ratio of the peak area of the sample (A_{sp}) to the peak area of the internal standard (A_{IS}), and by reference to calibration curves CAT and ECAT, prepared on the day of the experiment, in catechin-free rat plasma, and covering the concentration range 1–60 nM.

4.9. Statistical analysis

The results are reported as the mean ± SEM for $n = 6$. Statistical differences from control values were determined by unpaired Student's t-test with the help of a commercial software program (GraphPad Prism 3.2®). Intergroup differences were considered to be significant when $p < 0.05$.

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