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Chocolate Matrix Factors Modulate the Pharmacokinetic Behavior of Cocoa Flavan-3-ol Phase II Metabolites Following Oral Consumption by Sprague−Dawley Rats

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The impact of carbohydrates and milk on the bioavailability of catechin (C) and epicatechin (EC) from chocolate has been previously studied. However, little data exist regarding potential modulation of the phase II metabolism by these chocolate matrix factors. The objectives of this study were to assess the impact of matrix composition on qualitative and quantitative profiles of circulating catechins and their metabolites following administration of commercially relevant chocolate confections. Sprague−Dawley rats were administered 1.5 g of a confection (reference dark, high sucrose, or milk chocolate) by intragastric gavage, and plasma samples were collected over 8 h. High-performance liquid chromatography−mass spectrometry analysis was performed to quantify C, EC, and their metabolites. The predominant metabolites were *O*-glucuronides (two metabolites) and *O*-Me-*O*-glucuronides (three metabolites). Plasma concentrations of metabolites were generally the highest for high sucrose treatment and lowest for milk treatment, while the reference dark treatment generally resulted in intermediate concentrations. The *O*-Me-(±)-C/EC-*O*-β-glucuronide (peak 4) was significantly higher for the high sucrose treatment (2325 nM h) versus the milk treatment (1300 nM h). Additionally, C_{MAX} values for (±)-C/EC-*O*-β-glucuronide (peak 3) and two *O*-Me-(±)-C/EC-*O*-β-glucuronides (peaks 4 and 6) were significantly higher for the high sucrose treatment (4012, 518, and 2518 nM, respectively) versus the milk treatment (2590, 240, and 1670 nM, respectively). Milk and sucrose appear to modulate both metabolism and plasma pharmacokinetics and, to a lesser extent, the overall bioavailability of catechins from chocolate confections.

KEYWORDS: Bioavailability; chocolate; flavan-3-ols; epicatechin; phase II metabolism; Sprague−Dawley rat

INTRODUCTION

Chocolate from *Theobroma cacao* has been investigated as a functional food with potential health-protective and therapeutic activities against several chronic and degenerative diseases. Chocolate products are rich sources of a variety of monomeric, oligomeric, and polymeric flavan-3-ols (*1−5*). Although the profile of flavan-3-ol species present in chocolate can vary greatly, monomers are one of the major forms present (*4*, *6*). The predominant flavan-3-ol monomers present in chocolate include (−)-epicatechin (EC) and (+)-catechin and (−)-catechin (referred to as C, **Figure 1**) (*1*). Many of the proposed biological activities associated with chocolate have been linked to the flavan-3-ols, including C and EC. These activities include improved biomarkers of cardiovascular health and function (*7−17*), increased serum and/or plasma antioxidant capacity (*17*, *18*), and increased insulin sensitivity and decreased insulin resistance (*11−13*).

Continued interest in the potential benefits of cocoa flavan-3-ols has underscored the need to better understand the bioavailability and metabolism of flavan-3-ol monomers in vivo. The poor absorption of flavan-3-ols (*19−21*) highlights the potential for optimization of bioavailability as an important strategy for maximizing the effects of chocolate flavan-3-ols in vivo. To accomplish this, a more detailed understanding of the effect of product matrix composition on absorption and metabolism of flavan-3-ols from commercial chocolate products is required.

Previous investigations of the overall bioavailability of C and EC from chocolate in human subjects have reported conflicting results regarding the influence of food matrix factors such as milk protein and carbohydrate (sucrose, starch, etc.). Serafini et al. (*22*) reported that the EC area under the plasma pharmacokinetic curve (AUC) was lower for a milk chocolate confection as compared to a dark chocolate confection of equal EC content,

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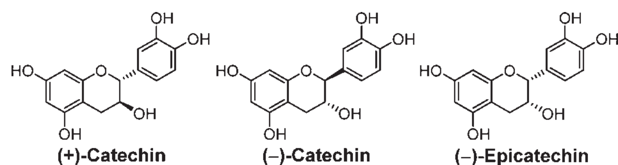

Figure 1. Structures of C and EC, the major monomeric flavan-3-ols present in chocolate.

while the consumption of liquid milk with the dark chocolate confection resulted in an intermediate AUC value. However, Schramm et al. (*23*) reported that the consumption of a cocoa beverage formulated with milk resulted in a slightly, but not significantly, higher AUC than the consumption of a cocoa beverage formulated with water. Additionally, four separate studies found no difference in the overall bioavailability of EC between cocoa beverages formulated with liquid milk versus water (*24*–*27*). Interestingly, all studies of beverage formulations have shown no difference in overall bioavailability, while the study by Serafini is the only study performed with confections and also the only study to show a pronounced negative effect of milk on overall bioavailability.

Schramm et al. (*23*) also investigated the effect of carbohydrate and found that the coconsumption of cocoa beverages with sucrose or bread resulted in increased plasma AUC and maximal plasma concentrations (C_{MAX}) of EC after the consumption of cocoa with water. However, limited data exist regarding whether carbohydrate levels similarly affect EC bioavailability when chocolate is consumed in confection form. Recently, we investigated the influence of matrix composition and physical form (beverage vs confection) of the bioavailability of EC from chocolate (*28*). We found that the overall bioavailability was generally similar between formulations but that the physical form greatly modulated the serum pharmacokinetic behavior (C_{MAX} and T_{MAX}) of EC.

While the impact of matrix formulation on the bioavailability of flavan-3-ol monomers from chocolate has been extensively studied, there is little data regarding the potential modulation of phase II metabolism and subsequent circulating profiles by milk and/or carbohydrate. Studies of the influence of matrix formulation on phase II metabolite profiles have predominantly examined urine, with little data on plasma, and therefore do not completely reflect alterations of circulating profiles. Roura et al. (*26*) reported that the consumption of a milk-based cocoa beverage by human subjects resulted in increased excretion of EC sulfates and decreased excretion of EC glucuronides in urine, with no effect on total bioavailability, as compared to the consumption of a water-based cocoa beverage. Mullen et al. reported that the consumption of cocoa beverages resulted in the presence of C/EC sulfates and *O*-Me sulfates in plasma and that the presence of milk increased the plasma elimination time and decreased the AUC of the sulfate metabolites relative to water-based beverages (*29*). Mullen et al. also reported that the total urinary excretion of C/EC metabolites (sulfates, *O*-Me sulfates, and *O*-glucuronides) was significantly lower from the milk-based beverages than from the water-based beverages from 0 to 2 and 2 to 5 h and for total (24 h) excretion. However, the studies by Roura et al. and Mullen et al. examined beverages but not solid confections. Other studies in rats and humans have shown that C and EC are extensively metabolized in vivo and are present in plasma as 3′-*O*-Me and 4′-*O*-Me derivatives, 5-*O*-β-glucuronides, 3′/4′-*O*-Me-5-*O*-β-glucuronides, and a variety of sulfated metabolites (*26*, *30*–*37*). Although the reported quantitative profiles of these metabolites vary greatly, the 5-*O*-β-glucuronides appear to predominate, while the sulfated metabolites are typically present in low concentrations in the plasma, as they are rapidly excreted into the urine.

Table 1. Formulations of the Three Chocolate Confection Treatments

	product		
	CDK	CHS	CMP
treatment	reference dark	high sucrose	milk protein
ingredient	solid components (mg/1.5 g serving)		
cocoa butter	750.00	450.00	525.00
cocoa powder	502.50	502.50	502.50
sucrose	245.55	545.55	245.55
vanilla flavor	1.20	1.20	1.20
soya lecithin	0.75	0.75	0.75
milk protein[a]	0.00	0.00	225.00
total	1500.00	1500.00	1500.00
flavan-3-ol	composition (mg/1.5 g serving)[b]		
C	0.49 ± 0.03	0.51 ± 0.01	0.53 ± 0.04
EC	1.09 ± 0.03	1.13 ± 0.03	1.14 ± 0.01
total	1.58 ± 0.06	1.64 ± 0.04	1.67 ± 0.05

[a] Milk protein concentrate (80%). [b] The flavan-3-ol composition was determined as described previously (*28*). Data represent means of $n = 3$ independent analyses of finished products, which were all formulated using equal amounts of the same cocoa powder. There were no significant differences between product compositions.

The present study was designed to use a relevant animal model to further investigate the effects of matrix composition on the bioavailability of flavan-3-ols from cocoa, which we previously observed in humans (*28*). The objectives of this study were to assess the impact of food matrix composition on the bioavailability and metabolism of C and EC monomers and to characterize the qualitative and quantitative profiles of circulating native compounds and their predominant phase II metabolites, following administration of commercially relevant chocolate confections. An understanding of how chocolate matrix factors potentially modulate systemic flavan-3-ol bioavailability and alter the profile of circulating species could potentially lead to the development of formulation and/or dietary strategies designed specifically to optimize the bioavailability and, by extension, the in vivo activities of monomeric cocoa flavan-3-ols.

MATERIALS AND METHODS

Reagents and Standards. (±)-C and (−)-EC standard material, formic acid, ascorbic acid, Na_2EDTA, and NaCL were obtained from Sigma-Aldrich (St. Louis, MO). MeOH (LC-MS grade) was obtained from Mallinckrodt Baker (Phillipsburg, NJ). ACN (Optima grade) was obtained from Fisher Scientific (Pittsburgh, PA). All H_2O was distilled/deionized using a Barnstead MegaPure MP-1 system (Dubuque, IA).

Chocolate Confections. Chocolate matrices were prepared by Kraft Foods, Inc. (Munich, Germany). Three solid confections [reference dark chocolate (CDK), high sucrose (CHS), and high milk protein (CMP)] providing commercially relevant levels of C and EC (approximately 1.37 mg C + EC/1.5 g serving for rats, equivalent to ~36.5 mg C + EC/40 g serving for humans, based on analysis of raw materials) from cocoa were formulated (**Table 1**). The flavan-3-ol content of the confections was determined as described previously (*28*). To balance serving sizes, the cocoa butter content was adjusted as needed. Therefore, the CDK treatment was higher in fat (0.75 g/1.5 g serving) than the CHS and CMP treatments, which had similar fat contents (0.45 and 0.52 g/1.5 g serving, respectively).

Animal Pharmacokinetic Experiment. Animal care and experimental protocols were approved by Purdue University's Animal Care and Use Committee (PACUC). Twenty-four male Sprague–Dawley rats (~250 g) were obtained from Harlan (Indianapolis, IN) and maintained on a 12 h light/dark cycle in a climate-controlled facility. Rats were initially fed a 14% protein rodent maintenance diet (Teklad 2014, Harlan), followed by

a nutritionally complete polyphenol-free purified maintenance diet (AIN-93M, Dyets, Inc., Bethlehem, PA) for 7 days prior to the study. Food and H_2O were available ad libitum. After the 7 day diet adjustment period, rats were anaesthetized with isoflurane and implanted with jugular catheters exteriorized dorsally. Catheters were kept patent by flushing every 12 h postsurgery with heparinized saline (100 U/mL). Following a 48 h recovery period, food was removed for 6 h, and baseline blood samples (400 μL) were collected via the catheter. Rats then received 1.5 g of melted CDK, CHS, or CMP by intragastric gavage (n = 8 per treatment, chocolate products were melted at 50 °C for 25 min prior to gavage). Following gavage, food was restored, and blood samples were collected at 0.5, 1, 2, 4, 6, and 8 h. Blood samples were placed in Li-heparin tubes to prevent clotting and then centrifuged (5000 rpm, 10 min, 4 °C). Following centrifugation, 100 μL of plasma was collected and stabilized by the addition of 25 μL of 1% aqueous ascorbic acid solution (w/v). Samples were then blanketed in N_2 and stored at −80 °C prior to analysis.

Solid-Phase Extraction (SPE). Plasma samples were thawed at room temperature, and 1 mL of acidified saline [0.9% NaCl (w/v) and 0.1% formic acid (v/v)] containing 20 mg/mL ascorbic acid and 1 mg/mL Na_2EDTA was added to each sample. Samples were ultrasonicated for 15 s and centrifuged at 18000 rcf for 2 min to break the resulting foam. SPE was performed on Waters Oasis HLB (30 mg, 1 cc) SPE cartridges (Milford, MA). SPE cartridges were preconditioned with 1 mL of methanol (MeOH) and 1 mL of H_2O, followed by loading of the plasma samples. Cartridges were washed with 2 mL of 1.5 M aqueous formic acid (v/v) and 2 mL of 5% aqueous MeOH (v/v). Compounds of interest were then eluted with 2 mL of acidified MeOH (0.1% formic acid, v/v). Extracts were dried under vacuum at 37 °C. Dried extracts were then solubilized in 150 μL of 0.1% aqueous formic acid (v/v), vortexed for 10 s, and immediately analyzed by high-performance liquid chromatography−mass spectrometry (HPLC-MS) (40 μL injection volume).

HPLC-MS Analysis. HPLC separations were performed on a Waters 2695 separations module equipped with a Waters XTerra RP-C_{18} column (2.1 mm × 100 mm, 3.5 μm particle size). The column temperature was 35 °C, and samples were maintained at 8 °C. The binary mobile phase system was comprised of 0.1% aqueous formic acid (v/v, phase A) and 0.1% formic acid in acetonitrile (v/v, phase B). The system flow rate was 0.3 mL/min. Elution was performed based on the following linear gradient: 90% A at 0 min, 30% A at 5.5 min and held until 7 min, 0% A at 7.01 and held until 7.5 min, 90% A at 7.51 min and held until 12 min. The postcolumn effluent was split 1:1 prior to (−)-electrospray ionization (ESI)-MS analysis on a Waters ZQ 2000 mass spectrometer. The ESI capillary voltage was −3.5 kV, and the source and desolvation temperatures were 150 and 350 °C, respectively. The desolvation gas and cone gas were N_2 at flow rates of 400 and 60 L/h, respectively. The cone, extractor, and RF lens voltages were 30, 3, and 0.5 V, respectively. Selected ion response (SIR) detection was performed simultaneously for m/z 289, 303, 465, and 479 to detect native catechins and their predominant phase II metabolites including O-Me catechins (m/z 303), glucuronides (m/z 465), and O-Me glucuronides (m/z 479). SIR m/z spans were ±0.75 for each mass. Dwell times for individual SIRs were 0.3 s, with an interchannel delay of 0.01 s and interscan delay of 0.05 s. SIR data were collected from 0 to 7 min.

Data Analysis. Native and metabolite species in plasma were quantified by MS peak area using a standard curve based on EC response. Statistical analyses were performed using SAS 9.1.3 software (SAS Institute Inc., Cary, NC). Plasma metabolite levels are reported as means ± standard errors of the mean (SEMs). AUCs were calculated using the linear trapezoidal rule. The maximum plasma concentrations (C_{MAX}) and the times at which the maximum plasma concentration (T_{MAX}) were determined directly from individual plasma concentration vs time curves and expressed as means ± SEMs. Differences in pharmacokinetic parameters of total as well as individual metabolites between treatments were determined by a paired Student's t test (α = 0.05).

RESULTS

A representative SIR chromatogram from the HPLC-MS analysis of pooled plasma samples (0.5−8 h) collected from a rat following consumption of CMP and extracted by SPE as described above is shown in **Figure 2**. This separation demonstrates that six species arising from C and EC were detected in the plasma of rats fed the chocolate confections (none of the labeled peaks were detected in the 0 h baseline samples for any rat, data not shown). Peaks 2, 4, and 6 were assigned as O-Me glucuronides of C/EC due to the $[M - H]^-$ = 479 and the m/z 479 → m/z 303 fragmentation (loss of the glucuronyl residue to form O-Me-C/EC). Peaks 1 and 3 were assigned as glucuronides of C/EC due to the $[M - H]^-$ = 465 and the m/z 465 → m/z 289 fragmentation (loss of the glucuronyl residue to form C/EC). Peak 5 was assigned as (±)-EC based on the $[M - H]^-$ = 289 and identical retention time (approximately 4 min) to authentic EC standard material. On the basis of the results from previous studies of the phase II metabolism of C and EC (*30−35*, *37*) and elution order of each peak in relation to C and

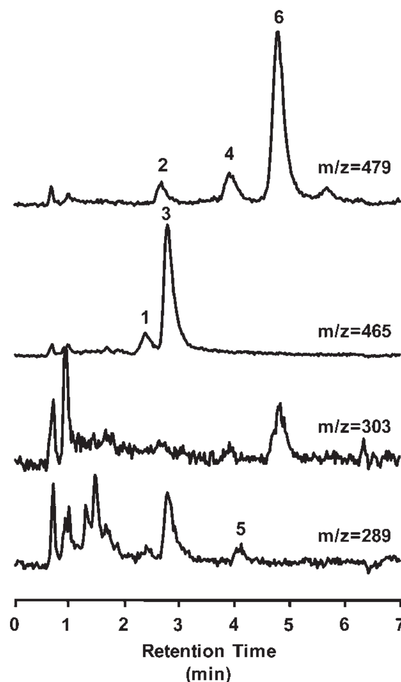

Figure 2. Representative reverse-phase HPLC-MS separation of an extract of pooled rat plasma (0.5−8 h) obtained following the consumption of 1.5 g of CMP chocolate matrix. SIR chromatograms are shown for deprotonated pseudomolecular ions $[M - H]^-$ representing native monomers (m/z 289) and predominate phase II metabolites: O-glucuronides (m/z 465) and O-methyl-O-glucuronides (479). Additionally, a SIR is shown for m/z 303, which is an MS fragment of the O-methyl-O-glucuronides.

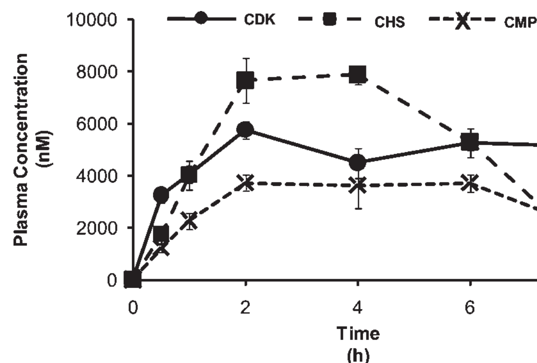

Figure 3. Plasma pharmacokinetic curves (0−8 h) of total monomeric flavan-3-ols (native and phase II metabolites) from rats fed each of the chocolate confections. Curve values represent the mean plasma concentration from n = 4 rats per time point for each treatment; error bars represent the SEM. Plasma concentrations are expressed as EC equivalents.

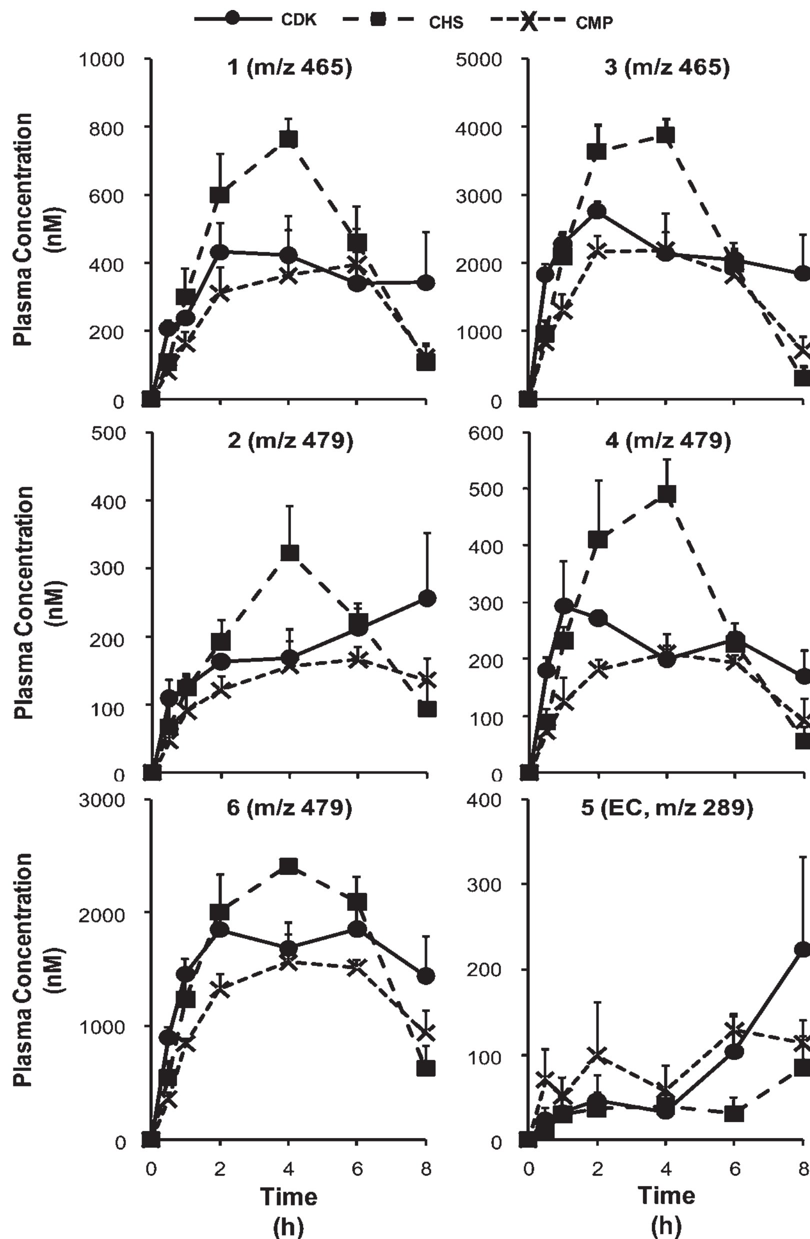

Figure 4. Plasma pharmacokinetic curves (0−8 h) of native flavan-3-ols and predominant phase II metabolites (glucuronides and *O*-Me glucuronides) from rats fed each of the chocolate confections. Peak 1, (±)-C/EC-*O*-glucuronide; peak 2, *O*-Me-(±)-C/EC-*O*-glucuronide; peak 3, (±)-C/EC-*O*-glucuronide; peak 4, *O*-Me-(±)-C/EC-*O*-glucuronide; peak 5, (±)-EC; and peak 6, *O*-Me-(±)-C/EC-*O*-glucuronide. Curve values represent the mean plasma concentration from $n = 4$ rats per time point for each treatment; error bars represent the SEM. Plasma concentrations are expressed as EC equivalents. Note the distinct scales for each set of curves.

EC, the tentative peak assignments were made as follows: peak 1, (±)-C/EC-5-*O*-*β*-glucuronide; peak 2, *O*-Me-(±)-C/EC-5-*O*-*β*-glucuronide; peak 3, (±)-C/EC-5-*O*-*β*-glucuronide; peak 4, *O*-Me-(±)-C/EC-5-*O*-*β*-glucuronide; peak 5, (±)-EC; and peak 6, *O*-Me-(±)-EC-5-*O*-*β*-glucuronide. HPLC-MS analysis conducted on these samples was not able to distinguish between 3′- and 4′-*O*-Me metabolites. As such, all methylated metabolites were identified as *O*-Me-C/EC derivatives. Additionally, the stereochemistry of these metabolites is unknown, as they may have arisen from (±)-C, which are both present in cocoa, or (±)-EC, due to isomerization postconsumption. Therefore, each is designated as being a metabolite of (±)-C/EC. No intact (±)-C, (±)-C/EC sulfated metabolites, or unconjugated *O*-Me-(±)-C/EC metabolites were detected in the plasma for any confection treatment. On the basis of these data, *O*-glucuronides and *O*-Me glucuronides appeared to be the major phase II metabolites present in plasma. Free EC was a minor component in blood, consistent with previous studies on catechins in vivo in rats (*32*, *33*).

Following identification of native EC and the phase II metabolites of C and EC in the plasma, pharmacokinetic curves of the mean plasma concentrations over 0−8 h were prepared for total metabolite sums (**Figure 3**) as well as for each compound (**Figure 4**). Examination of these curves shows that the plasma concentrations of the phase II metabolites (total as well as individual metabolite species) were generally the highest for the CHS treatment and the lowest for the CMP treatment, while the CDK treatment generally resulted in intermediate concentrations over the entire 8 h period. Additionally, plasma concentrations appeared to peak at approximately 4 h postgavage.

Examination of the calculated pharmacokinetic parameters (**Table 2**) shows that the AUC was significantly lowered by CMP as compared to CHS treatment for total circulating species as well

Table 2. Pharmacokinetic Parameters (0−8 h) of Native Flavan-3-ols and Predominant Phase II Metabolites in Plasma of Rats

			pharmacokinetic parameters[a]		
compound	peak	treatment	AUC (nM h)	C_{MAX} (nM)	T_{MAX} (h)
(±)-C/EC-*O*-glucuronide	1	CDK	2796 ± 625 a	504 ± 125 a	4.0 ± 1.4 a
		CHS	3733 ± 584 a	763 ± 61 a	4.0 ± 0.0 a
		CMP	2266 ± 516 a	458 ± 125 a	4.0 ± 0.8 a
(±)-C/EC-*O*-glucuronide	3	CDK	16952 ± 1574 ab	2849 ± 214 b	3.5 ± 1.5 a
		CHS	19503 ± 1890 a	4012 ± 306 a	3.5 ± 0.5 a
		CMP	13343 ± 1496 b	2590 ± 420 b	3.0 ± 0.6 a
O-Me-(±)-C/EC-*O*-glucuronide	2	CDK	1411 ± 188 a	292 ± 80 a	5.5 ± 1.5 a
		CHS	1598 ± 259 a	322 ± 69 a	4.0 ± 0.0 a
		CMP	1054 ± 230 a	190 ± 27 a	5.0 ± 0.6 a
O-Me-(±)-C/EC-*O*-glucuronide	4	CDK	1757 ± 122 a	341 ± 61 ab	3.8 ± 1.3 a
		CHS	2325 ± 322 a	518 ± 72 a	3.5 ± 0.5 a
		CMP	1300 ± 49 b	240 ± 18 b	5.0 ± 0.6 a
O-Me-(±)-C/EC-*O*-glucuronide	6	CDK	12843 ± 1325 ab	1988 ± 180 ab	5.0 ± 1.3 ab
		CHS	13847 ± 1221 a	2518 ± 139 a	3.5 ± 0.5 a
		CMP	9901 ± 830 b	1670 ± 203 b	5.5 ± 0.5 b
(±)-EC	5	CDK	601 ± 164 a	229 ± 107 a	6.5 ± 1.5 a
		CHS	308 ± 86 a	104 ± 25 a	5.5 ± 1.5 a
		CMP	708 ± 217 a	171 ± 38 a	3.6 ± 1.4 a
total	1−6	CDK	36360 ± 3856 ab	5916 ± 659 b	5.5 ± 1.5 a
		CHS	41314 ± 3876 a	8110 ± 530 a	3.5 ± 0.5 a
		CMP	28573 ± 2926 b	5112 ± 757 b	4.5 ± 1.0 a

[a] Values represent the means ± SEMs from n = 4 rats per formulation. Pharmacokinetic parameters with different letters are significantly different for that compound or metabolite ($P < 0.05$). The time at which the maximum serum concentration was observed (T_{MAX}), and the maximum serum concentrations observed (C_{MAX}).

as for the three EC metabolites detected, (±)-C/EC-*O*-glucuronide (peak 3), *O*-Me-(±)-C/EC-*O*-glucuronide (peak 4), and *O*-Me-(±)-C/EC-*O*-glucuronide (peak 6). Additionally, the AUC was significantly lowered by CMP as compared to CDK treatment for *O*-Me-(±)-C/EC-*O*-glucuronide (peak 4). No significant differences in AUC values were observed between treatments for (±)-EC (which was present in low concentrations as compared to the other metabolites), (±)-C/EC-*O*-glucuronide (peak 1), or *O*-Me-(±)-C/EC-*O*-glucuronide (peak 2).

The C_{MAX} values were significantly greater for CHS (CHS > CDK = CMP) for both total metabolites as well as (±)-C/EC-*O*-glucuronide (peak 3, the predominant metabolite observed). In addition, C_{MAX} values were significantly lowered by CMP vs CHS treatment for *O*-Me-(±)-C/EC-*O*-glucuronide (peak 4) and *O*-Me-(±)-C/EC-*O*-glucuronide (peak 6) (for this metabolite, the difference in C_{MAX} approached significance between CDK and CHS, $P = 0.058$). Similar to AUC values, no significant differences in C_{MAX} values were observed between treatments for (±)-EC, (±)-C/EC-*O*-glucuronide (peak 1), or *O*-Me-(±)-C/EC-*O*-glucuronide (peak 2).

The trends in the pharmacokinetic data consistently reflected AUC and C_{MAX} values ordered CHS > CDK > CMP, with the exception of EC. The calculated pharmacokinetic parameters reflect the general shapes of the pharmacokinetic curves, which suggest that the CHS treatment resulted in significantly higher plasma concentrations of the predominant phase II metabolites of C and EC than the CMP treatment, while CDK resulted in intermediate plasma concentrations.

T_{MAX} values ranged from 3.0 to 6.5 h. Statistically significant differences between T_{MAX} values were only observed for *O*-Me-(±)-C/EC-*O*-glucuronide (peak 6) between CHS (3.5 ± 0.5 h) and CMP (5.5 ± 0.5 h) treatments. No other significant differences were observed between T_{MAX} values for different treatments. Generally, the CHS treatment resulted in the lowest T_{MAX} values, with the exception of (±)-EC and (±)-C/EC-*O*-glucuronide (peak 3), for which the CMP treatment resulted in the lowest T_{MAX} values.

DISCUSSION

Both absorption and metabolism of cocoa-derived catechins appear to be influenced by chocolate matrix. Specific differences and trends observed for the AUC of C and EC metabolites [CHS had the highest AUC for all compounds except (±)-EC] generally agree with the results of Schraam et al. (*23*), who reported that consumption of carbohydrates along with cocoa beverages increased the AUC of EC relative to control. Additionally, the significant differences and the general trend observed for the C_{MAX} values [CHS had the highest C_{MAX} for all compounds except (±)-EC] in the present study also agree with Schraam et al. (*23*), who demonstrated that carbohydrates increased the C_{MAX} values of EC relative to control. The lower overall bioavailability of C relative to EC (*38*) may have minimized observed differences for any metabolites derived from (±)-C.

Several studies have been performed regarding the influence of milk protein on the bioavailability of EC from cocoa beverages and chocolate. Serafini et al. (*22*) reported that milk resulted in a reduced AUC for EC relative to control in chocolate confections, while Schroeter et al. (*27*), Schraam et al. (*23*), Roura et al. (*25*), and Keogh et al. (*24*) reported no statistical difference between the AUC of EC from cocoa beverages consumed with water or milk. The present results found a significantly lower AUC from the milk formulation (CHS > CMP) for total species as well as all three EC metabolites detected, as well as a trend where the AUCs from CMP were the lowest from the milk formulation for all compounds except (±)-EC. It is critical to note that we examined

confections, like Serafini et al. (*22*), while the studies demonstrating no difference between milk and control (*23*−*25*, *27*) were performed using cocoa beverages.

Recently, we performed a study in human subjects (*28*) using the same confections used in the present study, as well as beverages, and demonstrated that the AUC of EC from CMP was lower, although not significantly different, than CHS and CDK. Additionally, the C_{MAX} of EC was lower from CMP than both CHS (statistically different) and CDK (not statistically different). However, the highest AUC and C_{MAX} values in this study were observed from milk-containing beverage forms of these chocolate formulations. Taken together, these studies suggest that milk and sucrose appear to modulate the pharmacokinetics of EC and formation of predominant C/EC phase II metabolites from confections. The presence of milk protein also appears to exert mild suppressive effect on the bioavailability of these compounds from confections. However, in most cases, this effect is not large enough to be statistically significant. One explanation for this is that milk may drive the phase II generation of sulfated metabolites (*26*), which are rapidly excreted into the urine, thus reducing the formation of glucuronides and *O*-Me glucuronides. While sulfated metabolites were not detected in rodent plasma in the current study, the extent to which food matrix factors influence the formation and clearance of these metabolites merits further investigation.

Milk does not appear to exert these effects to the same extent in beverages matrices when compared to confections, as our previous study demonstrated that milk-containing beverages produced generally higher serum AUC and C_{MAX} values than confections formulated with or without milk (*28*). This may be due to the rapid emptying of beverages from the stomach, which facilitates more rapid appearance in the blood, as well as the relative ease of digestive release and solubilization from beverages as compared to confections. This process would serve to facilitate subsequent catechin absorption. It is important to note that this increase in the formation of sulfated metabolites from cocoa was previously observed following the consumption of milk beverages (*26*). A significant uncertainty therefore remains regarding the mechanisms by which matrix composition and physical form (beverage vs confections) of chocolate products interact to modulate bioavailability and metabolism.

In conclusion, our data combined with that of previous investigations (*28*, *22*) suggest that chocolate confections containing high levels of sucrose may enhance plasma levels of the predominant C and EC metabolites as compared to milk chocolate confections, while confections containing moderate levels of sucrose and no milk deliver intermediate plasma levels of these compounds. However, the physical state of the product may significantly modulate this effect, as our prior study comparing confections and beverages (*28*) demonstrated that milk-containing beverages produced generally higher serum AUC and C_{MAX} values than confections with or without milk, and numerous studies have shown no difference in the overall bioavailability of EC between cocoa beverages formulated with milk versus water (*24*−*27*). In the future, simultaneous investigation of the distribution of native species and phase II metabolites into both plasma and urine will be required to quantitatively assess the influence of chocolate matrix composition and physical form on the bioavailability, metabolism, and systemic distribution of C and EC from chocolate.

ABBREVIATIONS USED

EC, (−)-epicatechin; C, (+)-catechin and (−)-catechin; AUC, area under the plasma pharmacokinetic curve; ESI, electrospray ionization; CMP, high milk protein; CHS, high sucrose; HPLC-MS, high-performance liquid chromatography−mass spectrometry; C_{MAX}, maximal plasma concentrations; SEM, mean ± standard error of the mean; MeOH, methanol; *O*-Me, *O*-methyl; CDK, reference dark chocolate; SIR, selected ion response; SPE, solid-phase extraction; T_{MAX}, time at which the maximum plasma concentration was observed.

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LITERATURE CITED

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