

# Milk decreases urinary excretion but not plasma pharmacokinetics of cocoa flavan-3-ol metabolites in humans<sup>1–3</sup>

William Mullen, Gina Borges, Jennifer L Donovan, Christine A Edwards, Mauro Serafini, Michael EJ Lean, and Alan Crozier

## ABSTRACT

**Background:** Cocoa drinks containing flavan-3-ols are associated with many health benefits, and conflicting evidence exists as to whether milk adversely affects the bioavailability of flavan-3-ols.

**Objective:** The objective was to determine the effect of milk on the bioavailability of cocoa flavan-3-ol metabolites.

**Design:** Nine human volunteers followed a low-flavonoid diet for 2 d before drinking 250 mL of a cocoa beverage, made with water or milk, that contained 45  $\mu\text{mol}$  (–)-epicatechin and (–)-catechin. Plasma and urine samples were collected for 24 h, and flavan-3-ol metabolites were analyzed by HPLC with photodiode array and mass spectrometric detection.

**Results:** Milk affected neither gastric emptying nor the transit time through the small intestine. Two flavan-3-ol metabolites were detected in plasma and 4 in urine. Milk had only minor effects on the plasma pharmacokinetics of an (epi)catechin-*O*-sulfate and had no effect on an *O*-methyl-(epi)catechin-*O*-sulfate. However, milk significantly lowered the excretion of 4 urinary flavan-3-ol metabolites from 18.3% to 10.5% of the ingested dose ( $P = 0.016$ ). Studies that showed protective effects of cocoa and those that showed no effect of milk on bioavailability used products that have a much higher flavan-3-ol content than does the commercial cocoa used in the present study.

**Conclusions:** Most studies of the protective effects of cocoa have used drinks with a very high flavan-3-ol content. Whether similar protective effects are associated with the consumption of many commercial chocolate and cocoa products containing substantially lower amounts of flavan-3-ols, especially when absorption at lower doses is obstructed by milk, remains to be determined. *Am J Clin Nutr* 2009;89:1784–91.

## INTRODUCTION

Seeds of cocoa (*Theobroma cacao*) are a rich source of flavan-3-ols—a flavonoid subgroup that occurs as simple monomers, typically (–)-epicatechin and (+)-catechin, and straight-chain oligomeric and polymeric proanthocyanidins (1, 2). During processing, flavan-3-ols are lost and, as a consequence, are not necessarily major components in many commercial cocoas and dark chocolates (3, 4). Nonetheless, in recent years, a substantial body of evidence has accumulated indicating that consumption of high flavan-3-ol chocolates and cocoa-based beverages have protective effects on human health, particularly cardiovascular benefits (5–12). In addition, acute ingestion of the flavan-3-ol monomer (–)-epicatechin has also been shown to improve spatial memory retention in adult mice (13), whereas consumption of a flavan-3-ol-rich cocoa by

humans can increase cerebral blood flow to gray matter in the brain (14).

Many of these effects have been attributed to dark chocolate. In 2003, Serafini et al (15) reported that although the consumption of 100 g dark chocolate brought about an increase in plasma antioxidant capacity, this effect was substantially reduced when the chocolate was ingested with 200 mL milk, and no increase in antioxidant capacity was observed after the consumption of milk chocolate. They also showed that the absorption of (–)-epicatechin from chocolate was reduced when consumed with milk or as milk chocolate. They hypothesized that proteins in the milk bind to the flavan-3-ols and limit their absorption from the gastrointestinal tract (15). If this occurs, it further suggests that milk may negate the protective effects of cocoa flavan-3-ols. This report generated much controversy with another group, who carried out experiments with a flavan-3-ol-rich cocoa drink and published a reply disputing any effect of milk on plasma antioxidant capacity and (–)-epicatechin absorption (16–18). Two more recent studies have shown that drinking a cocoa beverage with milk does not affect plasma flavan-3-ol monomer concentrations (19) and does not influence the concentrations of flavan-3-ol metabolites in plasma or excreted in urine (20, 21). Interestingly, there has been a parallel debate and seemingly conflicting reports about the effect of milk on plasma flavan-3-ol and antioxidant concentrations associated with the consumption of black tea (22–26).

There is a need to clarify this controversy, and, to this end, 9 healthy volunteers ingested 10 g commercial cocoa dissolved in 250 mL either full-fat milk or water together with 1 g paracetamol and 5 g lactulose. Plasma and urine samples were collected over the ensuing 24-h period and flavan-3-ol metabolites analyzed by

<sup>1</sup> From the Division of Ecology and Evolutionary Biology, Faculty of Biomedical and Life Sciences (WM, GB, and AC); the Division of Developmental Medicine, University of Glasgow, Glasgow, United Kingdom (CAE and MEJL); the Medical School of South Carolina, Charleston, SC (JLD); and the National Institute for Food and Nutrition Research, Rome, Italy (MS).

<sup>2</sup> Supported by grant BB/C003802/1 from the UK Biotechnology and Biological Sciences Research Council to AC and CAE.

<sup>3</sup> Address correspondence to A Crozier, Plant Products and Human Nutrition Group, Graham Kerr Building, Division of Ecology and Evolutionary Biology, Faculty of Biomedical and Life Sciences, University of Glasgow, Glasgow, G12 8QQ, United Kingdom. E-mail: a.crozier@bio.gla.ac.uk.

Received December 10, 2008. Accepted for publication March 17, 2009. First published online April 29, 2009; doi: 10.3945/ajcn.2008.27339.

HPLC–tandem mass spectrometry (MS<sup>2</sup>). Plasma paracetamol and breath hydrogen were measured to determine the rate of gastric emptying and the mouth-to-cecum transit time of the head of the drink.

## SUBJECTS AND METHODS

### Chemicals and materials

HPLC-grade methanol and acetonitrile were obtained from Rathburn Chemicals (Walkerburn, Borders, United Kingdom). Formic acid was purchased from Riedel-DeHaen (Seeze, Germany), acetic acid was purchased from BDH (Poole, United Kingdom), and L-(+)-ascorbic acid, (+)-catechin, (–)-epicatechin, and procyanidin dimer B1 were purchased from Extrasynthese (Genay, France). Green and Black's Organic Cocoa (Green and Black's Ltd, London, United Kingdom) and full-fat milk (3.6 g fat/100 mL) were obtained from Sainsbury's Supermarket, Crow Road, Glasgow, United Kingdom.

### Study design

Because we did not have a priori estimates of the variability of cocoa flavan-3-ol metabolites to be analyzed in this study, a power calculation was based on data obtained in a bioavailability study we conducted with green tea in which (epi)catechin metabolites were monitored (27). Using the variability in these metabolites, we calculated that a sample size of 6 would be sufficiently powered to detect a 20% difference with  $P < 0.05$ . We decided to err on the side of caution and used 9 subjects for the current investigation.

The study protocol was approved by the Glasgow Royal Infirmary Local Research Ethics Committee. Six healthy non-smoking men and 3 women who were not taking any medication gave their written consent and participated in the study, which was conducted in February and March 2007. They were aged between 20 and 43 y and had a mean body mass index (in kg/m<sup>2</sup>) of  $24.7 \pm 2.7$  (range: 20.9–28.2). They followed a low flavonoid-diet, which excluded fruit and vegetables and beverages such as tea, coffee, fruit drinks, and wine for 2 d before the study. After an overnight fast, the subjects consumed 1 g paracetamol, 5 g lactulose, and 250 mL cocoa prepared by adding 250 mL hot milk to 10 g Green and Black's Organic Cocoa powder. Four weeks later under identical conditions, the same subjects again drank 250 mL cocoa but prepared with water rather than milk. Because there was a 4-wk interval between treatments and because flavan-3-ols are eliminated in <1 d (27), there would be little if any effect of treatment sequence.

Venous blood (12 mL) was collected into heparinized tubes from all subjects 0, 0.5, 1, 2, 3, 4, 6, 8, and 24 h after ingestion, and plasma obtained by centrifugation at 4000 g for 10 min at 4°C. Two 1-mL aliquots of plasma were acidified to pH 3 with 15  $\mu$ L of 50% formic acid, and 50  $\mu$ L of 10 mmol/L ascorbic acid was also added. The samples were then frozen in liquid nitrogen and stored at –80°C before extraction and analysis of flavan-3-ols by HPLC with photodiode array (PDA) and tandem mass spectrometric (MS<sup>2</sup>) detection. For paracetamol analysis, venous blood samples obtained 0, 15, 30, 45, 60, 75, 90, 105, and 120 min after ingestion were collected in EDTA-coated tubes, and the plasma obtained was stored as two 1-mL aliquots at –80°C before analysis.

Urine was collected before supplementation and over 4 time periods (0–2, 2–5, 5–8, and 8–24 h) after the ingestion of the cocoa drinks. The total volume for each period was recorded. Immediately after collection, the urine samples were acidified to pH 3 with formic acid, placed in an autosampler at 4°C, and analyzed by HPLC-PDA-MS<sup>2</sup>. Additional acidified aliquots were stored at –80°C.

End-expiratory breath hydrogen concentrations were monitored every 15 min for 8 h after ingestion of the cocoa drink supplement. Measurements were made by blowing into an EC60 Breath Hydrogen Monitor (Bedfont Scientific Ltd, Rochester, Kent, United Kingdom). The time of first sustained rise in breath hydrogen was taken as the mouth-to-cecum transit time. Volunteers ate ham or turkey with white bread rolls (low in non-digestible carbohydrate) 3 h after consuming the cocoa drinks and thereafter remained on a low-flavonoid diet until after the final 24-h samples were collected.

### Extraction of plasma

Plasma samples were extracted based on a modification of a method developed by Day et al (28). Triplicate 250- $\mu$ L volumes of plasma, to which had been added 50 ng (–)-epicatechin as an internal standard, 25  $\mu$ L of 10-mmol/L ascorbic acid containing 0.5 mmol/L EDTA, and 1.0 mL acetonitrile, were vortexed for 30 s every 2 min over a 10-min period before the mixture was centrifuged at 4000 g at 4°C for 10 min. The methanolic and acetonitrile extracts were combined and reduced to dryness under nitrogen at 38°C. The dried samples were then resuspended in 30  $\mu$ L methanol plus 220  $\mu$ L of 1% aqueous formic acid. Duplicate 100- $\mu$ L volumes were analyzed by HPLC-PDA-MS<sup>2</sup> on the day of extraction. Recoveries of the (–)-epicatechin internal standard were typically 55%.

### Processing of urine

Immediately after collection, 100- $\mu$ L samples of acidified urine were analyzed by HPLC-PDA-MS<sup>2</sup>.

### Analysis of plasma and urine by HPLC with PDA and MS<sup>2</sup> detection

Samples were analyzed on a Surveyor HPLC system composed of an HPLC pump, a diode array absorbance detector (scanning from 250 to 700 nm), and an autosampler cooled to 4°C (Thermo Electron Corporation, San Jose, CA). Separation was carried out by using a 250  $\times$  2 mm (internal diameter) 4- $\mu$ m Synergi RP-Max column (Phenomenex, Macclesfield, United Kingdom) eluted with a gradient over 30 min of 15–45% acetonitrile in 1% formic acid at a flow rate of 200  $\mu$ L/min; the column was maintained at 40°C. After passing through the flow cell of the diode array detector, the column eluate was directed to an LCQ Advantage ion-trap mass spectrometer fitted with an electrospray interface (Thermo Electron Corporation). Analyses used the negative ion mode, because this provided the best limits of detection for flavan-3-ols and were initially carried out by using full-scan, data-dependent MS scanning from  $m/z$  100 to 1000. The tuning of the mass spectrometer was optimized by infusing a standard of (–)-epicatechin into the source along with



the 15% acetonitrile in 1% aqueous formic acid, the initial HPLC mobile phase, at a flow rate of 0.3 mL/min. The capillary temperature was 250°C; the sheath gas and auxiliary gas were 60 and 20 units, respectively; and the source voltage was 4 kV.

Quantitative analysis used MS in the selected ion monitoring (SIM) mode at  $m/z$  369, 383, and 465. All quantitative estimates were based on SIM peak areas data expressed as (–)-epicatechin equivalents, with all peak identifications having been confirmed by full-scan consecutive reaction monitoring (MS<sup>3</sup>). The inter-assay variation was measured over the period of the analysis. The lowest and highest level standards used had CVs of 3% and 7%, respectively.

### Analysis of cocoa drink

Triplicate 500- $\mu$ L aliquots of the cocoa drink, made with boiled water as described above (10 g/250 mL), were used for quantitative analysis of the flavan-3-ol and procyanidin contents by the thiolysis degradation method of Alonso-Salces et al (29). Freeze-dried 500- $\mu$ L aliquots of the drink were reacted with 400  $\mu$ L benzylmercaptan (5% in methanol, vol:vol) and 200  $\mu$ L acidified methanol (3.3% HCl, vol:vol) at 40°C for 30 min, and vortex-mixed every 10 min. The reaction mix was then immediately cooled in an ice bath for 5 min. Samples were then filtered and stored at –80°C before analysis, together with samples that had not been subjected to thiolysis degradation, by HPLC-PDA-MS<sup>2</sup>, as described above but by using a gradient of 1% aqueous formic acid (A) in acetonitrile (B) programmed as follows: 0 min, 3% B; 5 min, 9% B; 15 min, 16% B; 50 min, 55% B; and 55 min, 55% B. The flow rate was 1 mL/min, and a fluorometric detector with excitation at 280 nm and emission at 310 nm was used.

To separate the (+) and (–) enantiomers of catechin and epicatechin, the cocoa drink was also analyzed by using a 250  $\times$  4.6 mm Cyclobond I-2000 RSP chiral column (Advances Separation Technologies, Whippany, NJ) containing derivatized  $\beta$ -cyclodextrin according to the procedures described by Donovan et al (30).

### Analysis of plasma paracetamol

Plasma paracetamol was measured with an acetaminophen assay kit (Cambridge Life Sciences, Cambridge, United Kingdom) with amounts of standard adapted to predicted paracetamol concentrations from initial measurements in this study (31). An index of the half-time for gastric emptying was obtained by applying curve-fitting analysis to data on plasma paracetamol concentrations (32).

### Pharmacokinetic analysis of flavan-3-ol metabolites in plasma

Maximum plasma concentrations of the metabolites from 0 to 8 h after dosing were defined as  $C_{max}$ . The time to maximum plasma concentration ( $t_{max}$ ) was defined as the time in hours at which  $C_{max}$  was reached. The elimination half-life ( $t_{1/2}$ ) for the metabolites was calculated as  $0.693/Ke$ , where  $Ke$  is the slope of the linear regression of the plasma metabolite concentrations. Areas under the curve (AUCs) were calculated by using the Kinetica software package (Thermo Electron Corporation).

### Statistical analysis

Data on flavan-3-ol concentrations are represented as means  $\pm$  SEs ( $n = 9$ ). When appropriate, data were subjected to statistical analysis by using analysis of variance and a paired  $t$  test with Minitab software, version 13 (Minitab Inc, Addison-Wesley Publishing, Reading, MA). Urinary flavan-3-ol catabolite profiles were submitted to 2-factor repeated-measures analysis of variance by using SPSS (version 16.0; SPSS Inc, Chicago, IL), in which treatment was the repeated measure and time was the independent factor.

## RESULTS

### Flavan-3-ol content of the cocoa drink

HPLC-PDA-MS<sup>2</sup> analysis showed that the 250 mL of the drink made from 10 g cocoa powder contained  $22.3 \pm 0.3 \mu\text{mol}$  catechin and  $23.0 \pm 0.4 \mu\text{mol}$  epicatechin, which corresponds to 6.5 and 6.7 mg, respectively. Chiral chromatography showed that the principal components were (–)-epicatechin and (–)-catechin, with only trace amounts of (+)-catechin. This is important because (+)-catechin is much more bioavailable than is (–)-catechin (30). Analysis before and after thiolysis degradation established that the procyanidin content of the drink was 70 mg/250 mL. The cocoa powder, therefore, contained 8.3 mg total flavan-3-ols/g. This is not atypical, being similar to or higher than the amounts found in 13 of the 21 commercial US cocoas analyzed by Miller et al (4).

### Qualitative analysis of flavan-3-ol metabolites in plasma and urine

Plasma extracts and urine samples, collected over a 24-h period after ingestion of 250 mL of the cocoa drink made with either water or with milk, and flavan-3-ol metabolites were analyzed by HPLC-PDA-MS<sup>3</sup>. Note that, without reference compounds, MS<sup>3</sup> is unable to distinguish between epicatechin and catechin metabolites. The identification of glucuronide, sulfate, and methyl metabolites of (epi)catechin, 2 of which were detected in plasma and 4 in urine (see the supplemental Figure under “Supplemental data” in the online issue), based on the following criteria, are summarized in **Table 1**.

Peak 1, which was detected in urine but not in plasma, had an HPLC retention time ( $R_t$ ) of 15.5 min and MS produced a negatively charged molecular ion  $[\text{M-H}]^-$  at  $m/z$  369, which when subjected to MS<sup>2</sup> fragmented to produce ions at  $m/z$  289, a loss of 80 amu, indicative of the cleavage of an  $\text{SO}_3^-$  unit. The  $m/z$  289 ion indicates the presence of (epi)catechin, which was confirmed when it yielded an MS<sup>3</sup> base ion at  $m/z$  245. Peak 1, therefore, is an (epi)catechin-*O*-sulfate.

Peak 2 ( $R_t = 17.4$  min), which was also present only in urine, had an  $[\text{M-H}]^-$  at  $m/z$  465 and fragmented with a 176-amu loss, characteristic of a glucuronide, to produce an MS<sup>2</sup> ion at  $m/z$  289, which, as for peak 1, yielded an MS<sup>3</sup> ion at  $m/z$  245. This fragmentation pattern is in keeping with the presence of an (epi)catechin-*O*-glucuronide. Peak 2 did not cochromatograph with (–)-epicatechin-7-*O*-glucuronide and is possibly (–)-epicatechin-3'-*O*-glucuronide, which has been identified in urine, collected after oral ingestion of (–)-epicatechin by humans (33).

**TABLE 1**HPLC-MS<sup>3</sup> identification of flavan-3-ol metabolites in plasma and urine collected after the ingestion of a cocoa drink by human volunteers<sup>1</sup>

Peak	$R_t$	[M-H] <sup>-</sup>	MS <sup>2</sup>	MS <sup>3</sup>	Identity	Location
	<i>min</i>	<i>m/z</i>	<i>m/z</i>	<i>m/z</i>		
1	15.5	369	289	245, 231, 205	(Epi)catechin- <i>O</i> -sulfate	Urine
2	17.4	465	289	245, 231, 205	(-)-Epicatechin- <i>O</i> -glucuronide	Urine
3	18.6	369	289	245, 231, 205	(Epi)catechin- <i>O</i> -sulfate	Plasma, urine
4	22.1	383	303	259, 245	<i>O</i> -Methyl-(epi)catechin- <i>O</i> -sulfate	Plasma, urine

<sup>1</sup>  $R_t$ , retention time; [M-H]<sup>-</sup>, negatively charged molecular ion; MS<sup>2</sup>, daughter ion produced by fragmentation of [M-H]<sup>-</sup>; MS<sup>3</sup>, daughter ions produced by fragmentation of MS<sup>2</sup> daughter ion.

Peak 3 ( $R_t = 18.6$  min) was detected in both plasma and urine and had a mass spectrum identical to that of peak 1. It too, thus, is an (epi)catechin-*O*-sulfate.

Peak 4 ( $R_t = 22.1$  min) was a plasma and urinary metabolite. It had a mass spectrum similar to that of the (epi)catechin-*O*-sulfates but with ions at values 14 amu higher, indicating that this metabolite is an *O*-methyl-(epi)catechin-*O*-sulfate.

These 4 metabolites were also the principal (epi)catechin metabolites detected in other bioavailability studies that we conducted with green tea-based products and in which epicatechin and catechin intakes were higher than in the cocoa study (27, 34). Additional (epi)catechin metabolites detected and quantified in these green tea studies were also detected after cocoa intake, but typically in trace, nonquantifiable amounts in samples from some but not all of the volunteers. HPLC-SIM analysis of plasma and urine samples was unable to find detectable quantities of procyanidin dimers and trimers.

### Quantitative analysis of flavan-3-ol metabolites in plasma

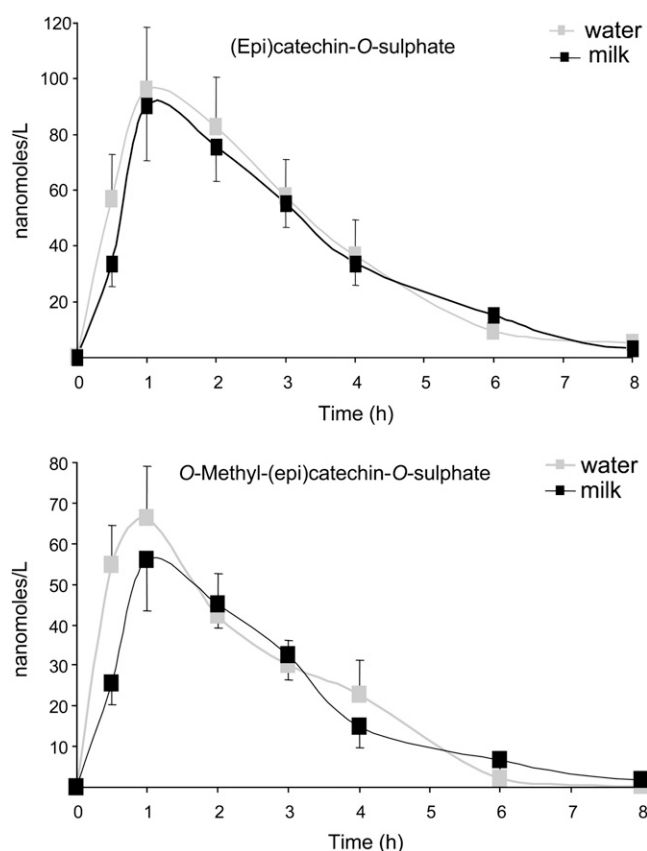
The pharmacokinetic data for the plasma metabolites (epi)catechin-*O*-sulfate (peak 3) and *O*-methyl-(epi)catechin-*O*-sulfate (peak 4) are presented in **Figure 1** and **Table 2**. The 0-h samples contained no detectable flavan-3-ols, and none of the volunteers had measurable concentrations of flavan-3-ol metabolites in plasma 24 h after ingestion of either of the cocoa drinks. However, metabolites were detected in plasma 30 min after ingestion of both drinks. The  $t_{max}$  for the main metabolite, (epi)catechin-*O*-sulfate (peak 3), was  $1.4 \pm 0.2$  h with the cocoa-water drink and  $1.3 \pm 0.2$  h with the cocoa-milk drink. The 2 values are not significantly different; likewise, milk did not have a significant effect on the  $C_{max}$  for (epi)catechin-*O*-sulfate. It did, however, significantly extend the  $t_{1/2}$  of (epi)catechin-*O*-sulfate from  $1.5 \pm 0.1$  to  $2.0 \pm 0.2$  h ( $P = 0.02$ ). Milk also significantly reduced the AUC for (epi)catechin-*O*-sulfate from  $173 \pm 27$  to  $135 \pm 16$  nmol · h/L, and this is reflected in the AUC/dose values, likewise, being significantly different ( $P = 0.001$  in both instances). The physiologic significance of these relatively minor changes remains to be determined. In contrast, milk did not have a significant effect on any of the pharmacokinetic parameters calculated for the second plasma metabolite, *O*-methyl-(epi)catechin-*O*-sulfate (Table 2).

### Quantitative analysis of flavan-3-ol metabolites in urine

Data on the excretion of the 4 flavan-3-ol metabolites in urine after the ingestion of the 2 cocoa drinks are presented in **Table 3**. The main excretory metabolite was the methyl-(epi)catechin-

*O*-sulfate (peak 4) followed by the (epi)catechin-*O*-sulfate (peak 3), both of which were detected in plasma. Also present in urine in smaller amounts were a further (epi)catechin-*O*-sulfate (peak 1) and the putative (-)-epicatechin-3'-*O*-glucuronide (peak 2), neither of which were present in plasma in detectable amounts.

The data in Table 3 are presented as the nmoles excreted for each metabolite over 24 h and for each of the 4 collection periods. In addition, the total metabolites excreted during each collection period are expressed as a percentage of the total 0–24-h excretion period for each drink. In both instances, ≈80% of the excretion occurred within 5 h of intake. The time-by-treatment interaction was highly significant for all of the variables ( $P < 0.001$ ).



**FIGURE 1.** Mean ( $\pm$ SE) concentrations of an (epi)catechin-*O*-sulfate and an *O*-methyl-(epi)catechin-*O*-sulfate in the plasma of human subjects 0–8 h after the ingestion of 250 mL cocoa containing 42  $\mu$ mol flavan-3-ol monomers, made with either water or milk.  $n = 9$ . Note that no flavan-3-ols or their metabolites were detected in plasma collected 24 h after ingestion of the cocoa.

**TABLE 2**

Pharmacokinetic parameters of an (epi)catechin sulfate and a methyl(epi)catechin sulfate in the plasma of 9 human subjects after consumption of a 250-mL cocoa drink, containing 42  $\mu\text{mol}$  flavan-3-ol monomers, made with either water or milk<sup>1</sup>

Metabolite and beverage	$C_{\text{max}}$	$t_{\text{max}}$	$t_{1/2}$	AUC	$C_{\text{max}}/\text{dose}$	AUC/dose
	nmol/L	h	h	nmol · h/L	nmol/L per $\mu\text{mol}$	nmol · h/L per $\mu\text{mol}$
(Epi)catechin- <i>O</i> -sulfate, peak 3						
Cocoa-water	83 $\pm$ 8	1.4 $\pm$ 0.2	1.5 $\pm$ 0.1 <sup>a</sup>	173 $\pm$ 27 <sup>a</sup>	2.0 $\pm$ 0.2	4.1 $\pm$ 1.9 <sup>a</sup>
Cocoa-milk	77 $\pm$ 14	1.3 $\pm$ 0.2	2.0 $\pm$ 0.2 <sup>b</sup>	135 $\pm$ 16 <sup>b</sup>	1.8 $\pm$ 0.3	3.2 $\pm$ 1.2 <sup>b</sup>
<i>O</i> -Methyl-(epi)catechin- <i>O</i> -sulfate, peak 4						
Cocoa-water	60 $\pm$ 8	1.0 $\pm$ 0.2	2.3 $\pm$ 0.3	123 $\pm$ 13	1.4 $\pm$ 0.2	2.9 $\pm$ 0.9
Cocoa-milk	50 $\pm$ 8	1.3 $\pm$ 0.2	1.9 $\pm$ 0.3	125 $\pm$ 17	1.2 $\pm$ 0.2	3.0 $\pm$ 1.2

<sup>1</sup> All values are means  $\pm$  SEMs;  $n = 9$ .  $C_{\text{max}}$ , maximum post-ingestion plasma concentration;  $t_{\text{max}}$ , time to reach  $C_{\text{max}}$ ;  $t_{1/2}$ , elimination half-life; AUC, area under the curve (0–8 h). Comparable values for the cocoa-milk and cocoa-water drinks with different superscript letters are significantly different,  $P < 0.02$  (paired  $t$  test). For metabolite identification and peak numbers, see Table 1.

Moreover, the combined urinary excretion of the metabolites for the 0–2-h and 2–5-h samples was significantly higher for the cocoa-water drink than for the cocoa-milk beverage. These values were 3938  $\pm$  538 nmol compared with 2115  $\pm$  323 nmol for the 0–2-h samples ( $P = 0.003$ ) and 2690  $\pm$  470 nmol compared with 1914  $\pm$  322 nmol for the 2–5-h samples ( $P = 0.04$ ). When calculated as a percentage of cocoa flavan-3-ols ingested, 0–24-h urinary metabolites were 18.3  $\pm$  1.9% of intake after ingestion of the cocoa-water drink compared with 10.5  $\pm$  1.1% of intake after ingestion of the cocoa-milk drink. These values were significantly different ( $P = 0.016$ ).

### Effect of milk on gastric emptying and mouth-to-cecum transit time

Consumption of the cocoa-water and cocoa-milk drinks resulted in peak plasma paracetamol times of 1.4  $\pm$  0.1 and 1.6  $\pm$

0.1 h, values that were not significantly different. Thus, milk did not affect the rate of gastric emptying. Likewise, the mouth-to-cecum transit time of the beverage, based on breath hydrogen measurements, was also not delayed significantly, with values of 2.2  $\pm$  0.4 h for the cocoa-water drink compared with 2.8  $\pm$  0.4 h for the cocoa-milk drink.

### DISCUSSION

This study showed that 2 metabolites, an (epi)catechin sulfate and an *O*-methyl-(epi)catechin sulfate, were detected in plasma after consumption of 250 mL of a commercial cocoa containing 42  $\mu\text{mol}$  flavan-3-ol monomers made with either water or milk (Table 1). Milk did not have a significant effect on the  $C_{\text{max}}$  of either metabolite nor did it affect the  $t_{\text{max}}$  values, which were indicative of absorption in the small intestine (Table 2).

**TABLE 3**

Quantities of flavan-3-ol metabolites in the urine of 9 human subjects 0–24 h after consumption of a 250-mL cocoa drink containing 42  $\mu\text{mol}$  flavan-3-ol monomers, made with water or milk<sup>1</sup>

Flavan-3-ol metabolites	0–2 h		2–5 h		5–8 h		8–24 h		Total	
	Cocoa-water	Cocoa-milk	Cocoa-water	Cocoa-milk	Cocoa-water	Cocoa-milk	Cocoa-water	Cocoa-milk	Cocoa-water	Cocoa-milk
(Epi)catechin- <i>O</i> -sulfate, peak 1 (nmol/d)	928 $\pm$ 110	476 $\pm$ 75	535 $\pm$ 55	377 $\pm$ 63	164 $\pm$ 18	87 $\pm$ 16	132 $\pm$ 58	111 $\pm$ 55	1760 $\pm$ 121	1010 $\pm$ 104
(–)-Epicatechin- <i>O</i> -glucuronide, peak 2 (nmol/d)	405 $\pm$ 44	123 $\pm$ 18	253 $\pm$ 43	136 $\pm$ 24	78 $\pm$ 16	35 $\pm$ 10	22 $\pm$ 9	12 $\pm$ 7	753 $\pm$ 70	297 $\pm$ 31
(Epi)catechin- <i>O</i> -sulfate, peak 3 (nmol/d)	1127 $\pm$ 196	694 $\pm$ 113	920 $\pm$ 190	737 $\pm$ 118	168 $\pm$ 42	156 $\pm$ 42	93 $\pm$ 50	73 $\pm$ 44	2433 $\pm$ 304	1614 $\pm$ 135
<i>O</i> -Methyl-(epi)catechin- <i>O</i> -sulfate, peak 4 (nmol/d)	1146 $\pm$ 231	823 $\pm$ 131	899 $\pm$ 171	660 $\pm$ 124	228 $\pm$ 41	153 $\pm$ 43	206 $\pm$ 146	66 $\pm$ 51	2761 $\pm$ 423	1624 $\pm$ 217
Total (nmol/d)	3938 $\pm$ 538 <sup>a</sup>	2115 $\pm$ 323 <sup>b</sup>	2690 $\pm$ 407 <sup>a</sup>	1914 $\pm$ 322 <sup>b</sup>	569 $\pm$ 111	410 $\pm$ 117	459 $\pm$ 172	262 $\pm$ 119	7706 $\pm$ 819 <sup>a</sup>	4397 $\pm$ 482 <sup>b</sup>
(% of total excreted/d)	51.1	48.1	34.9	24.8	7.4	5.3	6.0	3.4	100	100

<sup>1</sup> All values are means  $\pm$  SEMs;  $n = 9$ . Comparable values for the cocoa-milk and cocoa-water drinks with different superscript letters are significantly different,  $P < 0.04$  (paired  $t$  test). The time-by-treatment interaction was highly significant for all the variables ( $P < 0.001$ ). For metabolite identification and peak numbers, see Table 1.

However, in the case of the (epi)catechin-*O*-sulfate, milk extended the  $t_{1/2}$  and lowered the AUC and AUC/dose values that were obtained, although it had no effect on the pharmacokinetic parameters calculated for the *O*-methyl-(epi)catechin-*O*-sulfate (Table 2).

Analysis of urine excreted 0–24 h after cocoa consumption provided a much clearer view of the effect of milk on flavan-3-ol absorption (Table 3). An additional 2 metabolites were detected in urine: a second (epi)catechin-*O*-sulfate and an (epi)catechin-*O*-glucuronide. In contrast with the plasma data, milk had a major effect on the flavan-3-ol metabolite concentrations in urine, with significant reductions in the amounts excreted, especially in the initial 0–2-h and 2–5-h periods after ingestion of the cocoa drink. Overall, the 0–24 h excretion of metabolites corresponded to  $18.3 \pm 1.9\%$  of intake after ingestion of the cocoa-water beverage compared with  $10.5 \pm 1.1\%$  after ingestion of the cocoa-milk beverage. Whereas milk markedly reduced the concentration of cocoa flavan-3-ol metabolites appearing in the urine, it did not affect either gastric emptying ( $1.4 \pm 0.1$  h with water and  $1.6 \pm 0.1$  h with milk) or the time for the head of the meal to reach the colon ( $2.2 \pm 0.4$  h compared with  $2.8 \pm 0.4$  h). This indicates that the reduced excretion of the metabolites was not due to an effect on the rate of transport of the meal through the gastrointestinal tract, but was more likely to be a consequence of components in the milk that either 1) bound directly to flavan-3-ols or 2) interfered with the mechanism involved in their transport across the wall of the small intestine into the portal vein.

Although milk has a marked effect on the excretion, and hence the absorption, of cocoa flavan-3-ols, there was no parallel reduction in the concentration of flavan-3-ol metabolites in plasma. Whereas analysis of plasma provides valuable information on the identity,  $C_{max}$  and  $t_{max}$  values of circulating flavonoid metabolites, pharmacokinetic parameters do not necessarily provide an accurate quantitative assessment of uptake from the gastrointestinal tract because of the rapid turnover of these metabolites in the circulatory system (27). Urinary excretion provides a more realistic approach to compare bioavailability in different treatment groups, although the absolute numbers probably underestimate the total amount absorbed because urinary excretion values do not account for metabolites sequestered in body tissues. Urinary excretion values also do not take into consideration other routes of elimination, including possible excretion in bile. Nevertheless, it is interesting to note that, in humans, excretion of flavan-3-ol metabolites, as a percentage of intake, is substantially higher than that of many other flavonoids. In the present study, excretion after consumption of the cocoa-water beverage was equivalent to  $18.3 \pm 1.9\%$  of intake. Considering that  $\approx 50\%$  of the flavan-3-ol monomer content of the cocoa was (–)-catechin, which has low bioavailability (30), the observed  $18.3 \pm 1.9\%$  excretion is in keeping with the 25.3% reported after cocoa consumption (35) and a 28.5% excretion of (epi)catechin metabolites after ingestion of green tea (27). In the present study, urinary excretion of metabolites (nmol) divided by  $C_{max}$  (nmol/L) gave a value of 54 for the cocoa-water beverage and 35 for the cocoa-milk beverage. Comparable values obtained in our studies with metabolites of onion flavonols (36), orange juice flavanones (37), and strawberry anthocyanins (38) are 9.8, 13.6 and 6.2, respectively (27). This showed that, relative to these flavonoids, flavan-3-ol metabolites are excreted in

substantial amounts without a parallel increase in their concentrations in the circulatory system.

In contrast with our findings, other investigations have reported that milk does not affect the absorption of flavan-3-ols. These include a study by Roura et al (21), which monitored flavan-3-ol metabolites in urine after ingestion of cocoa containing 128  $\mu\text{mol}$  flavan-3-ol monomers, a 3-fold higher quantity than the 42  $\mu\text{mol}$  ingested in the present study. It is, however, interesting to note that although not significant, urinary excretion in the study of Roura et al (21) was 20% lower with the cocoa-milk beverage than with the cocoa-water beverage. Keogh et al (19), who analyzed plasma 0–8 h after consumption of a flavan-3-ol-rich cocoa drink, also reported that milk had no effect on the absorption of catechin and epicatechin. In this instance, the ingested dose of flavan-3-ol monomers was 2374  $\mu\text{mol}$ , which was 53-fold higher than in the present study. This high dose is reflected in a  $C_{max}$  of  $\approx 12$   $\mu\text{mol/L}$ , compared with  $\approx 150$   $\text{nmol/L}$  in the present study. Schroeter et al (17) reported that milk did not influence the AUC of plasma epicatechin after consumption of a cocoa beverage, which, in this instance, was consumed at a dose of 1314  $\mu\text{mol}$  flavan-3-ol monomers for a 70-kg human. It appears that, with high flavan-3-ol cocoas, the factors in milk that reduce absorption have a minimal overall effect. For drinks with a lower flavan-3-ol content, such as the one used in the present study—which is typical of many commercial cocoas that are on supermarket shelves (4) and available to the general public—milk does have the capacity to interfere with absorption.

Many elegant studies have shown potential protective effects of cocoa (5–14), with the exception of the study by Taubert et al (11), which showed a significant lowering of blood pressure, most involved the consumption of high amounts of flavan-3-ols (see reference 8 and the supplemental Table under “Supplemental data” in the online issue). For instance, a recent study on improved vascular function in diabetic patients involved feeding volunteers a cocoa drink containing 341 and 876  $\mu\text{mol}$  flavan-3-ol monomers (39). Whether similar protective effects are possible in the general public consuming chocolate and cocoa products containing substantially lower amounts of flavan-3-ols needs to be determined, especially because, at lower doses, absorption becomes subject to interference by milk and quite possibly to other components in the food matrix.

A final point to note is the varying flavan-3-ol metabolite profiles obtained in different studies. In the present investigation, an (epi)catechin-*O*-sulfate and an *O*-methyl-(epi)catechin-*O*-sulfate were detected in plasma and urine, whereas urine contained an additional (epi)catechin-*O*-sulfate and an (epi)catechin-*O*-glucuronide (Table 1). A pharmacokinetic study with cocoa by Baba et al (35), in which samples were subjected to glucuronidase and sulfatase treatment before analysis, indicated, albeit indirectly, the presence of sulfate and sulfoglucuronides of (epi)catechin and methyl-(epi)catechin as the main metabolites in both plasma and urine. Likewise, in a similar investigation with green tea, (epi)catechin sulfates were detected in plasma in higher concentrations than their glucuronide counterparts (40). A later cocoa study in which plasma and urine samples were analyzed by HPLC-MS<sup>2</sup>, an (epi)catechin glucuronide was the sole metabolite detected in plasma, whereas glucuronide and sulfate metabolites were detected in urine (41). Another study with cocoa using HPLC-MS<sup>2</sup> to analyze flavan-3-ol metabolites also

found an (epi)catechin-glucuronide in plasma, but, in addition, detected a methylated glucuronide and (epi)catechin and methyl-(epi)catechin sulfates. These metabolites were also detected in urine along with a methyl-(epi)catechin sulfoglucuronide (42). Without further investigation, the reason for these varying metabolite profiles, especially the absence of sulfated metabolites in plasma in some HPLC-MS<sup>2</sup>-based studies and not others, is difficult to ascertain because there are so many variables. These variables include 1) the method used to process the plasma before analysis; 2) the widely varying flavan-3-ol doses; 3) the amount of (+)-catechin and its less bioavailable (–)-isomer in cocoa, which has been determined only in the present study; 4) the time points at which plasma and urine samples are collected—the present study is the only HPLC-MS<sup>2</sup>-based study in which a full 0–24-h plasma pharmacokinetic profile was obtained; and 5) the ethnic origin and dietary habits of the human volunteers who participate in the feeding studies.

We thank the participants in the study and are grateful to Junji Terao and Yoshichika Kawai (University of Tokushima, Japan) for generously supplying a sample of (–)-epicatechin-7-*O*-glucuronide.

The authors' responsibilities were as follows—WM: helped design the study, conducted most of the experimental work, and contributed to the drafting of the manuscript; GB and JLD: contributed to the experimental work and helped revise the manuscript; AC and CAE: designed the study, supervised the experimental work, and contributed to the drafting of the manuscript and its revision; and MS and MEJL: contributed to the design of the study and helped revise the manuscript. None of the authors had a personal or financial conflict of interest.

## REFERENCES

- Crozier A, Jaganath IB, Marks S, Saltmarsh M, Clifford MN. Secondary metabolites as dietary components in plant-based foods and beverages. In: Crozier A, Clifford MN, Ashihara H, eds. *Plant secondary metabolites: occurrence, structure and role in the human diet*. Oxford, United Kingdom: Blackwell Publishing, 2006:208–302.
- Aron PM, Kennedy JA. Flavan-3-ols: nature, occurrence and biological activity. *Mol Nutr Food Res* 2008;52:79–104.
- Gu L, House SE, Wu X, Ou B, Prior RL. Procyanidin and catechin contents and antioxidant capacity of cocoa and chocolate products. *J Agric Food Chem* 2006;54:4057–61.
- Miller KB, Hurst WJ, Payne MJ, et al. Impact of alkalization on the antioxidant and flavanol content of commercial cocoa powders. *J Agric Food Chem* 2008;56:8527–33.
- Fisher ND, Hughes M, Gerhard-Herman M, Hollenberg NK. Flavanol-rich cocoa induces nitric-oxide-dependent vasodilation in healthy humans. *J Hypertens* 2003;21:2281–6.
- Engler MB, Engler MM, Chen CY, et al. Flavonoid-rich dark chocolate improves endothelial function and increases plasma epicatechin concentrations in healthy adults. *J Am Coll Nutr* 2004;23:197–204.
- Keen CL, Holt RR, Oteiza PI, Fraga CG, Schmitz HH. Cocoa antioxidants and cardiovascular health. *Am J Clin Nutr* 2005;81:S298–303.
- Hollenberg K, Norman MD. Vascular action of cocoa flavanols in humans: the roots of the story. *J Cardiovasc Pharmacol* 2006;47(suppl): S99–102.
- McCullough ML, Chevaux K, Jakson L, et al. Hypertension, the Kuna, and the epidemiology of flavanols. *J Cardiovasc Pharmacol* 2006;47: S103–9.
- Schroeter H, Heiss C, Balzer J, et al. (–)-Epicatechin mediates beneficial effects of flavanol-rich cocoa on vascular function in humans. *Proc Natl Acad Sci USA* 2006;103:1024–9.
- Taubert D, Roesen R, Lehmann C, Jung N, Schömig E. Effects of low habitual cocoa intake on blood pressure and bioactive nitric acid. A randomized control trial. *JAMA* 2007;298:49–60.
- Cooper KA, Donovan JL, Waterhouse AL, Williamson G. Cocoa and health: a decade of research. *Br J Nutr* 2008;99:1–11.
- van Praag H, Lucero MJ, Yeo GW, et al. Plant-derived flavanol (–)-epicatechin enhances angiogenesis and retention of spatial memory in mice. *J Neurosci* 2007;27:5869–788.
- Francis S, Head K, Morris PG, Macdonald IA. The effect of flavanol-rich cocoa on the fMRI response to cognitive task in healthy young people. *J Cardiovasc Pharmacol* 2006;47:S215–20.
- Serafini M, Bugianesi R, Maiaini G, et al. Plasma antioxidants from chocolate. *Nature* 2003;424:1013.
- Halliwell B. Plasma antioxidants (communication arising): health benefits of eating chocolate? *Nature* 2003;426:787.
- Schroeter H, Holt RR, Orozco TJ, Schmitz HH, Keen CL. Milk and the absorption of flavanols. *Nature* 2003;426:787–8.
- Serafini M, Crozier A. Milk and the absorption of flavanols—reply. *Nature* 2003;426:788.
- Keogh JB, McInerney J, Clifton PM. The effect of milk protein on the bioavailability of cocoa polyphenols. *J Food Sci* 2007;72:S230–3.
- Roura E, Andès-Lacueva C, Estruch R, et al. Milk does not affect the bioavailability of cocoa powder flavonoid in healthy human. *Ann Nutr Metab* 2007;51:493–8.
- Roura E, Andès-Lacueva C, Estruch R, et al. The effect of milk as a food matrix for polyphenols on the excretion profile of cocoa (–)-epicatechin metabolites in healthy human subjects. *Br J Nutr* 2008;100:846–51.
- Serafini M, Ghiselli A, Ferro-Luzzi A. *In vivo* antioxidant effect of green and black tea in man. *Eur J Clin Nutr* 1996;50:28–32.
- van het Hof KH, Kivits GA, Weststrate JA, Tijburg LB. Bioavailability of catechins from tea: the effect of milk. *Eur J Clin Nutr* 1998;52:356–9.
- Langley-Evans SC. Consumption of black tea elicits an increase in plasma antioxidant potential in humans. *Int J Food Sci Nutr* 2000;51:309–15.
- Leenen R, Roodenburg AJ, Tijburg LB, Wiseman SA. A single dose of tea with or without milk increases plasma antioxidant activity in humans. *Eur J Clin Nutr* 2000;54:87–92.
- Lorenz M, Jochmann N, von Krosigk A, et al. Addition of milk prevents vascular protective effects of tea. *Eur Heart J* 2007;28:219–23.
- Stalmach A, Troufflard S, Serafini M, Crozier A. Absorption, metabolism and excretion of Choldi green tea flavan-3-ols by humans. *Mol Nutr Food Res* (Epub ahead of print 31 October 2008). 10.1002/mnfr.200800169.
- Day AJ, Mellon F, Barron D, et al. Human metabolism of dietary flavonoids: identification of plasma metabolites of quercetin. *Free Radic Res* 2001;35:941–52.
- Alonso-Salces RM, Guyot S, Herrero C, et al. Chemometric characterisation of Basque and French ciders according to their polyphenolic profiles. *Anal Bioanal Chem* 2004;379:464–75.
- Donovan JL, Crespy V, Oliveira M, et al. (+)-Catechin is more bioavailable than (–)-catechin: relevance to the bioavailability of catechin from cocoa. *Free Radic Res* 2006;40:1029–34.
- Clements JA, Heading C, Nimmo WS, Prescott LF. Kinetics of acetaminophen absorption and gastric emptying in man. *Clin Pharmacol Ther* 1978;24:420–31.
- Maes BD, Mys G, Getpens BJ, et al. Gastric emptying flow curves separated from carbon-labeled octanoic acid breath test results. *Am J Physiol* 1998;275:G169–75.
- Natsume M, Osakabe N, Oyama M, et al. Structures of (–)-epicatechin glucuronide identified from plasma and urine after oral ingestion of (–)-epicatechin: differences between human and rat. *Free Radic Biol Med* 2003;34:840–9.
- Auger C, Hara Y, Crozier A. Bioavailability of polyphenon E flavan-3-ols in humans with an ileostomy. *J Nutr* 2008;138:1535S–42S.
- Baba S, Osakabe N, Yasuda A, et al. Bioavailability of (–)-epicatechin upon intake of chocolate and cocoa in human volunteers. *Free Radic Res* 2000;33:635–41.
- Mullen W, Edwards CA, Crozier A. Absorption, excretion and metabolic profiling of methyl-, glucuronyl-, glucosyl and sulpho-conjugates of quercetin in human plasma and urine after ingestion of onions. *Br J Nutr* 2006;96:107–16.
- Mullen W, Archeveque M-A, Edwards CA, Crozier A. Bioavailability and metabolism of orange juice flavanones in humans: impact of a full-fat yogurt. *J Agric Food Chem* 2008;56:1157–64.
- Mullen W, Edwards CA, Serafini M, Crozier A. Bioavailability of pelargonidin-3-glucoside and its metabolites in humans following the ingestion of strawberries with and without cream. *J Agric Food Chem* 2008;56:713–9.
- Balzer J, Rassaf T, Heiss C, et al. Sustained benefits in vascular function through flavanol-containing cocoa in medicated diabetic patients. *J Am Coll Cardiol* 2008;51:2141–9.



40. Lee M-J, Wang Z-Y, Li H, et al. Analysis of plasma and urinary tea polyphenols in human subjects. *Cancer Epidemiol Biomarkers Prev* 1995;4:393–9.
41. Roura E, Andrés-Lacueva C, Jáuregui O, et al. Rapid liquid chromatography tandem mass spectrometer assay to quantify plasma (–)-epicatechin metabolites after ingestion of a standard portion of cocoa beverage in humans. *J Agric Food Chem* 2005;53:6190–4.
42. Tomás-Barberán FA, Ceinfuegos-Jovellanos E, Marín A, et al. A new process to develop a cocoa power with higher flavonoid monomer content and enhanced bioavailability in healthy humans. *J Agric Food Chem* 2007;55:3926–35.

