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Absorption, Metabolism, and Excretion of Cider Dihydrochalcones in Healthy Humans and Subjects with an Ileostomy

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The phloretin-*O*-glycosides, phloretin-2′-*O*-glucoside and phloretin-2′-*O*-(2″-*O*-xylosyl)glucoside, are thought to be unique to apples and apple products. To investigate the metabolism and bioavailability of these compounds, nine healthy and five ileostomy human subjects consumed 500 mL of Thatchers Redstreak apple cider containing 46 μmol of phloretin-*O*-glycosides. Over the ensuing 24 h period, plasma, urine, and ileal fluid were collected prior to analysis by high-performance liquid chromatography−mass spectrometry (HPLC−MS). The sole metabolite present in quantifiable amounts in plasma was phloretin-2′-*O*-glucuronide, which reached a peak concentration (C_{max}) of 73 nmol/L and 0.6 h after ingestion (T_{max}) with the healthy subjects, and statistically similar values were obtained with the ileostomy volunteers. Phloretin-2′-*O*-glucuronide was also detected in urine along with two additional phloretin-*O*-glucuronides and a phloretin-*O*-glucuronide-*O*-sulfate. The quantity of phloretin metabolites excreted in urine represented 5.0 ± 0.9% of intake in healthy volunteers and 5.5 ± 0.6% in ileostomy volunteers. The similarity in the excretion levels of the two groups and the rapid plasma T_{max} indicate absorption of the dihydrochalcones in the small intestine. Of the two major phloretin-*O*-glycosides in cider, only phloretin-2′-*O*-(2″-*O*-xylosyl)glucoside was recovered in ileal fluid in quantities corresponding to 22% of intake. The absence of phloretin-2′-*O*-glucoside in ileal fluid suggests that it is more readily absorbed than phloretin-2′-*O*-(2″-*O*-xylosyl)glucoside. Phloretin-2′-*O*-glucuronide, two other phloretin-*O*-glucuronides, one phloretin-*O*-glucuronide-*O*-sulfate, two phloretin-*O*-sulfates, and the aglycone phloretin were also detected in the ileal fluid. This implies that the wall of the small intestine contains β-glycosidase, sulfuryltransferase, and UDP-glucuronosyltransferase activities and that, as well as being absorbed, sizable amounts of the phloretin metabolites that are formed efflux back into the lumen of the gastrointestinal tract. The overall recovery of the dihydrochalcones and their metabolites in the ileal fluid was equivalent to 38.6% of intake.

KEYWORDS: Cider; dihydrochalcones; bioavailability; healthy humans; ileostomists

INTRODUCTION

Apples contain significant amounts of phenolic compounds in the form of hydroxycinnamates, flavan-3-ols, anthocyanins, flavonols, and dihydrochalcones (*1*, *2*). Cider apples have been shown to contain higher levels of these compounds than dessert apples (*3*, *4*). The phenolic compounds can survive the cider-making process, with some ciders containing relatively high concentrations (*5*−*7*). Dihydrochalcones have a distinctive open C-ring structure, and they occur in apples as phloretin-2′-*O*-(2″-*O*-xylosyl)glucoside, phloretin-2′-*O*-glucoside, and the aglycone phloretin (*3*, *7*−*9*) (**Figure 1**). Rooibos tea also contains dihydrochalcones, in the form of aspalathin and nothofagin, but these are *C*- rather than *O*-glycosides (*10*). Although trace levels of phloretin-2′-*O*-glucoside have been detected in strawberries (*11*), the presence of sizable amounts of phloretin-*O*-glycosides is thought to be unique to apples and apple products, such as cider, and their detection has been used to confirm the presence of apples in juices, jams, and purees (*12*, *13*). The detection of phloretin in urine, after enzyme hydrolysis, to convert putative glucuronide and sulfate metabolites to the aglycone, is a feature of the consumption of apple products (*14*), including cider (*15*).

Phloretin-2′-*O*-glucoside is an inhibitor of the sodium-dependent glucose transporter 1 (SGLT1) located in the intestinal lumen (*16*). By decreasing the absorption of glucose, phloretin-2′-*O*-glucoside may, therefore, reduce postprandial blood glucose levels, and it is thought this action may be beneficial to the treatment of diabetes mellitus. Attempts have been made to produce synthetic SGLT1 inhibitors that induce glucosuria as a potential treatment for diabetes (*17*).

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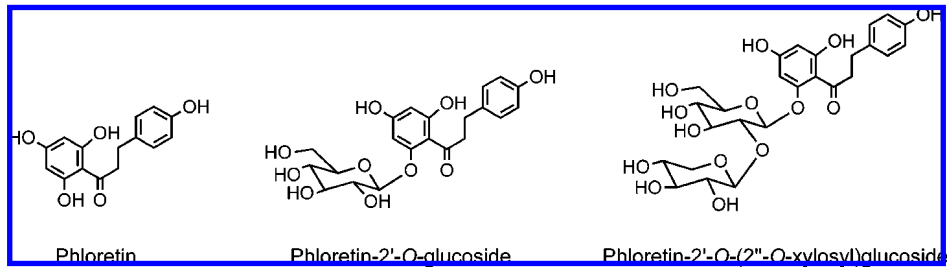

Figure 1. Structure of cider dihydrochalcones.

This paper describes an investigation of the metabolism, absorption, and excretion of cider dihydrochalcones in both healthy human volunteers and healthy human volunteers with an ileostomy. Plasma, ileal fluid, and urine were collected over a 24 h period after the consumption of 500 mL of cider. The samples were then analyzed, without prior enzyme hydrolysis, by high-performance liquid chromatography−photodiode array−mass spectrometry (HPLC−PDA−MS^n).

MATERIALS AND METHODS

Chemicals. HPLC-grade methanol and acetonitrile were purchased from Rathburn Chemicals (Walkerburn, Scotland), and formic acid was purchased from Riedel-DeHaen (Seeize, Germany). Phloretin-2′-*O*-glucoside, ethylenediaminetetraacetic acid (EDTA), L-(+)-ascorbic acid, and sodium diethyldithiocarbamate were obtained from Sigma-Aldrich (Poole, U.K.). Kaempferol-3-glucoside was purchased from Extrasynthese (Genay, France). Redstreak cider containing 5% alcohol, was supplied by the Thatchers Cider Company (Sandford, Somerset, U.K.). Phloretin-2′-*O*-β-glucuronide was prepared as described by Kahle et al. (*8*).

Study Design. The Glasgow Royal Infirmary Research Ethics Committee approved the study protocol. Nine healthy human subjects with an intact functioning colon and five subjects with an ileostomy who were nonsmokers and not on any medication participated in this study and gave their written consent. The healthy subjects were aged between 21 and 42 years and had a mean BMI of 26.1 ± 1.0 (standard error). The ileostomy subjects, who were otherwise healthy, were aged between 40 and 54 years and had a mean BMI of 26.0 ± 1.5. They followed a diet low in flavonoids and phenolic compounds, which excluded fruits and vegetables, high-fiber products, and beverages such as tea, coffee, fruit juices, cider, beer, and wine, for 2 days before the study. After an overnight fast, the volunteers consumed 500 mL of Thatchers Redstreak cider. Volunteers ate ham or turkey with white bread rolls 3 h after drinking the cider and thereafter remained on a low flavonoid diet for a further 21 h until the final blood and urine samples were collected.

Plasma and urine were collected for healthy volunteers before the intake of cider (time 0 h) and over a 24 h period after supplementation. Ileal fluid and urine was taken from all ileostomy subjects before the intake of cider (time 0 h) and over the following 24 h period. Plasma samples were also taken from three ileostomy volunteers.

A cannula was inserted into the arm of each volunteer, and venous blood was withdrawn at 0, 0.5, 1, 2, 3, 4, 6, 8, and 24 h post-ingestion of the cider. A total of 10 mL of blood was collected in heparinized tubes at each time point and immediately centrifuged at 4000*g* for 10 min at 4 °C. The plasma was removed from the red blood cells; 1 mL aliquots were acidified to pH 3 with 30 μL of 50% aqueous formic acid; and 100 μL of ascorbic acid (10 mmol/L) was added to prevent oxidation. The plasma samples were then stored at −80 °C prior to analysis. Urine and ileal fluid were collected prior to supplementation and over 0−2, 2−5, 5−8, and 8−24 h periods after the consumption of the cider. Upon collection, the volume/weight of each sample was recorded, after which aliquots were immediately stored at −80 °C.

Analysis of Cider. Triplicate 10 μL aliquots of cider were taken for direct quantitative analysis of their dihyrochalcone content by HPLC−PDA−MS^2.

Plasma Extraction. Plasma samples were extracted in triplicate according to the method developed by Day et al. (*18*). A total of 250 ng of phloretin-2′-*O*-glucoside was added as an internal standard to a 450 μL aliquot of plasma along with acetonitrile (1.2 mL) and 50 μL of 10% (m/v) ascorbic acid containing 0.5 mmol/L EDTA (*19*). Samples were vortexed for 30 s every 2 min over a 10 min period, before centrifuging at 3000*g* at 4 °C for 20 min. The supernatant was collected, and the pellet was re-extracted as described above but with methanol instead of acetonitrile. The supernatants were combined and dried under nitrogen. The extract was then resuspended in 25 μL of methanol and 225 μL of 0.1% formic acid in water and centrifuged at 16000*g* at 4 °C for 20 min, after which 200 μL was injected into HPLC−PDA−MS. Average recoveries of phloretin-2′-*O*-glucoside were 65%.

Ileal Fluid Extraction. Defrosted duplicate 2.5 g aliquots of ileal fluid were spiked with an internal standard, kaempferol-3-glucoside (100 μg), and then mixed with 10 mL of a 50% aqueous methanol solution containing 1% formic acid and 20 mmol/L sodium diethyldithiocarbamate. The mixture was homogenized for 1 min using an Ultra Turrax homogenizer (T-25, IKA-WERKE, Staufen, Germany). Samples were further extracted for 30 min using an orbital shaker (IKA-WERKE) before being centrifuged at 4900*g* for 20 min at 4 °C. The resulting supernatant was collected, and the pellet was re-extracted using methanol containing 1% formic acid and 20 mmol/L diethyldithiocarbamate following the same procedure as described above. The supernatants were combined and reduced to dryness in vacuo using a rotary evaporator at 35 °C. The samples were freeze-dried, and the residues were resuspended in 2 mL of 10% aqueous methanol containing 0.1% formic acid and centrifuged at 16100*g* for 20 min at 4 °C. The supernatant was analyzed by triplicate injections of 5 μL into the HPLC−PDA−MS system. Average recoveries of the kaempferol-3-glucoside internal standard were 70%.

Urine. Samples were defrosted and centrifuged at 16100*g* for 20 min at 4 °C. Aliquots (25 μL) were injected and analyzed in triplicate by HPLC−PDA−MS^n.

HPLC−MS Analysis. Samples were analyzed using a Surveyor HPLC system comprising a HPLC pump, a PDA detector, scanning from 250 to 700 nm, and an autosampler cooled to 6 °C (Thermo Electron, San Jose, CA). Separations were carried out using a Synergi Max-RP column, 250 × 4.6 mm inner diameter, 4 μm (Phenomenex, Macclesfield, U.K.). The column, maintained at 40 °C, was eluted at a flow rate of 1 mL min^{-1} with a 55 min gradient of 20−55% acetonitrile in 0.1% formic acid for biological samples and a 50 min gradient of 3−35% acetonitrile in 1% formic acid for cider. After the eluate passed through the flow cell of the PDA detector, it was split and 0.2 mL min^{-1} was directed to a LCQ DecaXP ion-trap mass spectrometer fitted with an electrospray interface. The capillary temperature was 350 °C; sheath and auxiliary gas were both 60 units; and the source voltage was 5 kV for negative ionization. Identification was carried out using consecutive reaction monitoring (CRM), and quantification was carried out using selected ion monitoring (SIM). The limit of detection using SIM was 0.1 ng injected onto the column. The metabolites were quantified as phloretin-2′-*O*-glucoside equivalents.

Pharmacokinetic Analysis of Plasma Metabolites. The maximum post-ingestion plasma concentration of phloretin metabolites was defined as C_{max}. The time to reach maximum plasma concentration (T_{max}) was defined as the time in hours at which C_{max} was reached. The elimination half-life for the metabolites in hours was computed using the following formula: $T_{1/2} = 0.693/K_e$, where K_e is the slope of the linear regression of the log of 0−24 h plasma metabolite concentrations. Area-under-the-curve (AUC) calculations were determined using a Kinetic software package (Thermo Electron).

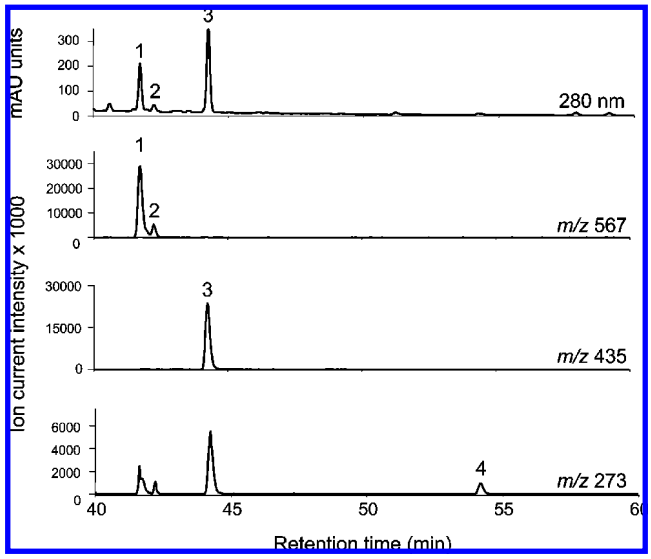

Figure 2. HPLC−PDA−MS analysis of Thatchers Redstreak cider. Chromatograms represent gradient, reversed-phase HPLC analysis with the detection of phloretin conjugates by SIM at *m*/*z* 567 for phloretin-*O*-(*O*-xylosyl)hexosides, *m*/*z* 435 for phloretin-2′-*O*-glucoside, *m*/*z* 273 for phloretin and with a PDA detector at 285 nm. Peak 1, phloretin-2′-*O*-(2″-*O*-xylosyl)glucoside; peak 2, phloretin-*O*-(*O*-xylosyl)glucoside; peak 3, phloretin-2′-*O*-glucoside; peak 4, phloretin.

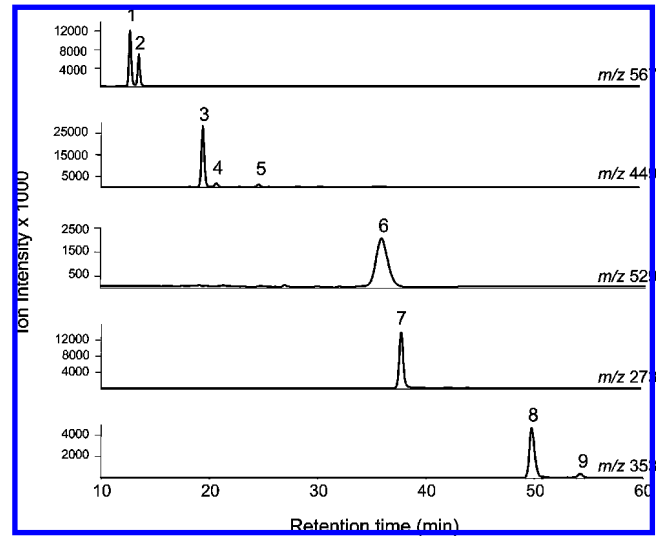

Figure 3. HPLC−SIM analysis of phloretin metabolites in ileal fluid collected after the ingestion of 500 mL of cider. Chromatograms represent gradient, reversed-phase HPLC analysis with the detection of phloretin conjugates and their metabolites by mass spectrometry using SIM at *m*/*z* 567 for phloretin-*O*-(*O*-xylosyl)hexosides, *m*/*z* 449 for phloretin-*O*-glucuronides, *m*/*z* 529 for phloretin-*O*-glucuronide-*O*-sulfates, *m*/*z* 273 for phloretin, and *m*/*z* 353 for phloretin-*O*-sulfates. For identifications of peaks see **Table 1**.

RESULTS

Dihydrochalcone Content of Cider. Gradient, reverse-phase HPLC with PDA detection and full-scan data-dependent MS^2, as described by Marks et al. (*7*), was used to identify and quantify the dihydrochalcone content of the cider. Two major and two minor dihydrochalcones were detected (**Figure 2**). Peak 1, with a retention time (R_t) of 41.7 min, and peak 2, a minor component with a R_t of 42.2 min, both yielded a mass spectrum with a negatively charge molecular ion ($[M - H]^-$) at *m*/*z* 567 and a MS^2 ion at *m*/*z* 273, indicative of a phloretin-xylosyl-hexose conjugate. Although no reference compounds were available, it is well-known that apples contain phloretin-2′-*O*-(2″-*O*-xylosyl)glucoside (*2, 4, 8, 13, 20*). We, therefore, assume that the main component, peak 1, is most probably phloretin-2′-*O*-(2″-*O*-xylosyl)glucoside. The minor constituent, peak 2, is a phloretin-*O*-(*O*-xylosyl)hexoside, with the position(s) of the substituent groups undetermined. There is one report of the presence of a phloretin-*O*-(*O*-xylosyl)galactoside in apples (*21*), but it is unclear as to whether or not peak 2 could be this conjugate, especially because, with flavonols at least, galactoside conjugates typically elute from reverse-phase HPLC systems before rather than after the corresponding glucoside (*7*).

Peak 3, the main component in the HPLC trace (**Figure 2**) (R_t = 44.2 min) had a $[M - H]^-$ at *m*/*z* 435, which produced a MS^2 fragment at *m*/*z* 273. The loss of 162 amu indicates the cleavage of a hexose unit, and co-chromatography with an authentic standard confirmed this peak to be phloretin-2′-*O*-glucoside. Peak 4, a minor constituent with a R_t of 54.2 min, produced a $[M - H]^-$ at *m*/*z* 273, which fragmented, yielding a MS^2 ion at *m*/*z* 167. This indicates the presence of the aglycone phloretin, and the identification was confirmed by co-chromatography with a reference compound.

It was estimated that 500 mL of cider contained 14 ± 0.2 μmol of phloretin-2′-*O*-(2″-*O*-xylosyl)glucoside and 31 ± 0.2 μmol of phloretin-2′-*O*-glucoside. The phloretin content was 78 ± 19 nmol, and that of phloretin-*O*-(*O*-xylosyl)hexoside was 848 ± 14 nmol, making a total dihydrochalcone content of 46 ± 0.3 μmol per 500 mL.

Identification of Phloretin Derivatives and Their Metabolites in Ileal Fluid, Plasma, and Urine. Plasma extracts, ileal fluid extracts, and urine samples were analyzed by HPLC−PDA−MS^n. **Figure 3** illustrates typical HPLC−SIM traces, which, together with CRM data, led to the tentative identification of the dihydrochalcones presented in **Table 1**.

Peak 1 (R_t = 12.6 min) and peak 2 (R_t = 13.6 min) both had a $[M - H]^-$ at *m*/*z* 567 and, with a loss of 294 amu, a MS^2 ion at *m*/*z* at 273. This fragmentation pattern is characteristic of a phloretin-*O*-(*O*-xylosyl)hexoside. Peak 1 is probably phloretin-2′-*O*-(2″-*O*-xylosyl)glucoside, and peak 2 is probably a phloretin-*O*-(*O*-xylosyl)hexoside (see the previous section).

Peaks 3−5 (R_t = 19.4, 20.7, and 24.6 min) all had a $[M - H]^-$at *m*/*z*449, which on MS^2 fragmented with a 176 amu loss, characteristic of the cleavage of a glucuronide unit, to produce an ion at *m*/*z* 273. Further fragmentation of the ion at *m*/*z* 273 produced a spectrum of ions matching that of an authentic standard of phloretin. All three peaks are, therefore, phloretin-*O*-glucuronides. However, co-chromatography with an authentic standard showed that peak 3 was phloretin-2′-*O*-glucuronide.

Peak 6 (R_t = 35.0 min) had a $[M - H]^-$ ion at *m*/*z* 529, which with a 176 amu loss yielded a MS^2 fragment ion at *m*/*z* 353. MS^3 of *m*/*z* 353 resulted in an 80 amu loss to produce a base ion at *m*/*z* 273, and MS^4 of this ion yielded a base ion at *m*/*z* 167. This is in keeping with the fragmentation pattern of a phloretin-*O*-glucuronide-*O*-sulfate. This peak is much broader than the other peaks in the chromatogram (**Figure 3**) and may comprise two unresolved phloretin-*O*-glucuronide-*O*-sulfates.

Peak 7 (R_t = 37.8 min) co-chromatographed with and had the same mass spectrum ($[M - H]^-$ at *m*/*z* 273 and MS^2 ion at *m*/*z* 167) as phloretin.

Table 1. Tentative HPLC−MS^n Identifications of Phloretin Metabolites in Plasma, Ileal Fluid, and Urine Collected after the Ingestion of Cider by Human Volunteers[a]

peak	R_t (min)	$[M - H]^-$ (*m*/*z*)	MS^2 (*m*/*z*)	MS^3 (*m*/*z*)	dihydrochalcones	location
1	12.8	567	273	167	phloretin-2′-*O*-(2″-*O*-xylosyl)glucoside	ileal fluid
2	13.6	567	273	167	phloretin-*O*-(*O*-xylosyl)hexoside	ileal fluid
3	19.4	449	273	167	phloretin-2′-*O*-glucuronide	plasma, ileal fluid, urine
4	20.7	449	273	167	phloretin-*O*-glucuronide	ileal fluid, urine
5	24.6	449	273	167	phloretin-*O*-glucuronide	ileal fluid, urine
6	35.0	529	353	273	phloretin-*O*-glucuronide-*O*-sulfate	ileal fluid, urine
7	37.8	273	167	167	phloretin	ileal fluid
8	49.4	353	273	167	phloretin-*O*-sulfate	ileal fluid
9	53.8	353	273	167	phloretin-*O*-sulfate	ileal fluid

[a] For peak numbers, see **Figure 3**. R_t, retention time; $[M - H]^-$, negatively charged molecular ion; MS^2, daughter ion produced by fragmentation of $[M - H]^-$; MS^3, daughter ion produced by fragmentation of the MS^2 ion.

Table 2. Pharmacokinetic Parameters of Phloretin-2′-*O*-glucuronide in the Plasma of Nine Healthy Human Subjects after the Consumption of 500 mL of Cider[a]

metabolite	C_{max} (nmol/L)	T_{max} (h)	$T_{1/2}$ (h)	AUC (nmol L^{-1} h^{-1})	C_{max}/dose (nmol L^{-1} μmol^{-1})	AUC/dose (nmol L^{-1} h^{-1} μmol^{-1})
phloretin-2′-*O*-glucuronide	73 ± 11	0.6 ± 0.1	0.7 ± 0.1	129 ± 19	1.5 ± 0.2	3.3 ± 0.4

[a] Data are expressed as mean values ± standard error ($n = 9$). C_{max}, maximum post-ingestion plasma concentration; T_{max}, time to reach C_{max}; $T_{1/2}$, the elimination half-life; AUC, area under the curve (0−8 h). Phloretin-2′-*O*-glucuronide is peak 3 in **Table 1** and **Figure 3**.

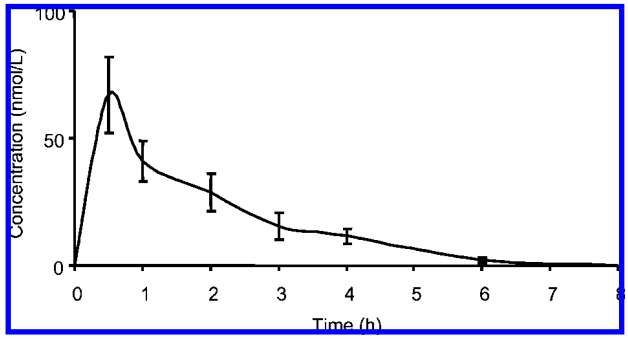

Figure 4. Concentration of phloretin-2′-*O*-glucuronide (peak 3, **Table 1**) in the plasma of human subjects 0−8 h after the ingestion of 500 mL of cider. Data are expressed as mean values with their standard errors ($n = 9$) depicted by vertical bars.

Peaks 8 and 9 ($R_t = 49.4$ and 53.8 min) both had a $[M - H]^-$ at *m*/*z* 353, which on MS^2fragmentation showed a loss of 80 amu to give a fragment ion at *m*/*z* 273. This indicates that peaks 9 and 10 are phloretin-*O*-sulfates.

Quantitative Analysis of Phloretin Metabolites in Plasma. Phloretin-2′-*O*-glucuronide (peak 3, **Table 1**) was present in quantifiable amounts in plasma. A smaller amount of another phloretin-*O*-glucuronide (peak 4) was also present but in trace levels that could not be quantified accurately. The pharmacokinetic analysis of phloretin-2′-*O*-glucuronide in plasma from healthy subjects is summarized in **Table 2** and **Figure 4**. The 0 and 24 h plasma samples contained no detectable phloretin metabolites. The phloretin-2′-*O*-glucuronide C_{max} of the post-ingestion plasma was 73 ± 11 nmol/L, which was reached after 0.6 ± 0.1 h. The $T_{1/2}$ was 0.7 ± 0.1 h, and the AUC was 129 ± 19 nmol L^{-1} h^{-1}. The C_{max}/dose was 1.5 ± 0.2 nmol L^{-1} μmol^{-1}, and the AUC/dose was 3.3 ± 0.4 nmol L^{-1} h^{-1} μmol^{-1}. The plasma from three ileostomy volunteers was also analyzed. The C_{max} of 93 ± 29 nmol/L was reached after 0.5 ± 0.0 h. The $T_{1/2}$was 0.9 ± 0.3 h, and the AUC was 140 ± 28.8 nmol L^{-1} h^{-1}. None of these values is statistically different from those obtained with the subjects with an intact colon, indicating that absorption of the dihydrochalcones occurs principally in the small intestine.

Quantitative Analysis of Phloretin Metabolites in Ileal Fluid. All of the dihydrochalcones detected in ileal fluid are listed in **Table 3**. Overall, 17 699 ± 1862 nmol of phloretin derivatives were identified and quantified in the 0−24 h samples. A total of 3154 ± 1051 nmol of phloretin-2′-*O*-(2″-*O*-xylosyl)glucoside was recovered in the ileal fluid sample, which is 22% of the original intake. Recovery of the phloretin-*O*-(*O*-xylosyl)hexoside was 713 ± 170 nmol, 88% of the amount ingested, while none of the 31 ± 0.2 μmol of the phloretin-2′-*O*-glucoside in the cider was excreted in the ileal fluid. The amount of phloretin detected in ileal fluid, 2411 ± 477 nmol, was greater than the 78 ± 19 nmol present in the ingested cider initial dose, demonstrating that hydrolysis of phloretin glycosides had occurred during their passage through the stomach and small intestine.

The majority of the phloretin metabolites detected in the ileal fluid were present as phloretin-*O*-glucuronides in amounts corresponding to 18.7% of intake. The phloretin-*O*-glucuronide-*O*-sulfate and phloretin-*O*-sulfate content of the ileal fluid was relatively small, being equivalent to 3.6 and 3.0% of the ingested dose, respectively. Overall, the non-absorbed parent compounds and metabolites excreted in ileal fluid over the 24 h period after drinking the cider represented 38.6% of the ingested dihydrochalcones.

Quantitative Analysis of Phloretin Metabolites in Urine. The total quantity of phloretin metabolites excreted in urine by the healthy subjects was 2300 ± 391 nmol compared to 2524 ± 203 nmol by the ileostomy volunteers. This equates to an excretion of 5.0 and 5.5% of the ingested dose, respectively (**Tables 4** and **5**). The values obtained with the two groups are not statistically different, providing further evidence that the dihydrochalcones are absorbed in the small rather than large intestine. The major urinary metabolite was phloretin-2′-*O*-glucuronide (peak 3, **Table 1**), which also predominated in plasma, and the amount excreted was equivalent to 4.2% of intake with the healthy volunteers (**Table 4**) and 4.4% with the ileostomy volunteers (**Table 5**). Lower levels of two other phloretin-*O*-glucuronides were detected, with their combined amounts being 216 ± 43 nmol/L in urine from healthy volunteers and 353 ± 49 nmol/L with the ileostomy subjects. Phloretin-*O*-glucuronide-*O*-sulfate was also detected in urine from both groups: 151 ± 35 nmol/L with the health subjects and 145 nmol/L with the ileostomists. Most excretion of phloretin metabolites occurred 0−2 h after ingestion of the cider.

Table 3. Quantities of Dihydrochalcones and Their Metabolites Recovered in Ileal Fluid after the Consumption of 500 mL of Cider[a]

phloretin derivatives (peak)	0−2 h	2−5 h	5−8 h	8−24 h	total (0−24 h)
phloretin-2′-(2″-*O*-xylosyl)glucoside (1)	811 ± 329	1834 ± 739	480 ± 369	29 ± 38	3154 ± 1051
phloretin-*O*-(*O*-xylosyl)hexoside (2)	144 ± 64	467 ± 153	85 ± 48	16 ± 21	713 ± 170
phloretin-2′-*O*-glucuronide (3)	501 ± 238	4467 ± 1337	1854 ± 1460	220 ± 181	7043 ± 1376
phloretin-*O*-glucuronide (4)	17 ± 12	114 ± 41	116 ± 92	12 ± 15	259 ± 113
phloretin-*O*-glucuronide (5)	116 ± 75	710 ± 550	431 ± 293	19 ± 17	1275 ± 539
phloretin-*O*-glucuronide-*O*-sulfate (6)	12 ± 16	745 ± 512	649 ± 838	nd	1406 ± 801
phloretin (7)	171 ± 145	1429 ± 842	545 ± 318	267 ± 212	2411 ± 477
phloretin-*O*-sulfate (8)	28 ± 15	575 ± 169	418 ± 460	331 ± 213	1352 ± 550
phloretin-*O*-sulfate (9)	2 ± 2	38 ± 16	32 ± 36	14 ± 12	86 ± 58
total	1802 ± 568 (3.9%)	10380 ± 3998 (22.6%)	4610 ± 2647 (10.0%)	907 ± 454 (2.0%)	17699 ± 1862 (38.6%)

[a] Data are expressed in nmoles as mean values ± standard error ($n = 5$). Values in parentheses represent recovery as a percentage of intake. nd = not detected. For phloretin derivatives and peak numbers, see **Table 1** and **Figure 3**.

Table 4. Phloretin Metabolites Excreted in Urine of Healthy Humans 0−24 h after the Ingestion of 500 mL of Cider[a]

phloretin metabolites (peak)	0−2 h	2−5 h	5−8 h	8−24 h	total (0−24 h)
phloretin-2′-*O*-glucuronide (3)	762 ± 95	601 ± 131	477 ± 367	93 ± 60	1933 ± 354
phloretin-*O*-glucuronide (4)	88 ± 17	56 ± 14	58 ± 26	nd	202 ± 44
phloretin-*O*-glucuronide (5)	2 ± 2	7 ± 4	5 ± 5	nd	14 ± 9
phloretin-*O*-glucuronide-*O*-sulfate (6)	47 ± 20	75 ± 23	30 ± 16	nd	151 ± 35
total	868 ± 103 (2.0%)	738 ± 147 (1.7%)	570 ± 406 (1.2%)	93 ± 60 (0.2%)	2300 ± 391 (5.0%)

[a] Data are expressed as nmoles ± standard error ($n = 9$). Values in parentheses represent excretion as a percentage of the intake. For phloretin metabolites and HPLC peak numbers refer to **Table 1** and **Figure 3**.

Table 5. Phloretin Metabolites Excreted in Urine of Human Subjects with an Ileostomy 0−24 h after the Ingestion of 500 mL of Cider[a]

phloretin metabolites (peak)	0−2 h	2−5 h	5−8 h	8−24 h	total (0−24 h)
phloretin-2′-*O*-glucuronide (3)	1362 ± 149	510 ± 129	89 ± 22	65 ± 18	2026 ± 186
phloretin-*O*-glucuronide (4)	174 ± 21	71 ± 15	38 ± 9	nd	283 ± 21
phloretin-*O*-glucuronide (5)	7 ± 7	23 ± 12	40 ± 21	nd	70 ± 36
phloretin-*O*-glucuronide-*O*-sulfate (6)	29 ± 18	76 ± 27	40 ± 28	nd	145 ± 63
total	1572 ± 192 (3.4%)	680 ± 199 (1.5%)	207 ± 92 (0.5%)	65 ± 18 (0.1%)	2524 ± 203 (5.5%)

[a] Data are expressed as nmoles ± standard error ($n = 5$). Values in parentheses represent excretion as a percentage of the intake. For phloretin metabolites and HPLC peak numbers refer to **Table 1** and **Figure 3**.

The overall quantity of metabolites in the 0−2 h urine samples was equivalent to 2.0% of dihydrochalcone intake with the healthy subjects and 3.4% in the case of the ileostomy volunteers.

DISCUSSION

This is first report on the use of HPLC−MS to analyze the levels of the phloretin metabolites in plasma and urine. Previous studies were based on HPLC analysis, with absorbance or electrochemical detection, of the amount of phloretin released by treating samples with mollusc enzymes containing sulfatase and β-glucosidase activity (*15*, *22*, *23*). With this approach, even with enzymes of established specificity, only indirect and far less detailed information is obtained on the metabolites and the nature of the conjugating moiety (*24*).

In the present study, healthy and ileostomy subjects consumed 500 mL of a cider containing 46 μmol of phloretin glycosides. After the ingestion of the cider, the main metabolite in all biological samples was phloretin-2′-*O*-glucuronide (peak 3, **Table 1**). In the plasma samples, this was the only dihydrochalcone to be detected and quantified. The mean C_{max}, 73 nmol/L, was accompanied by a T_{max} of 0.6 h (**Figure 4** and **Table 2**), and similar values were obtained with the ileostomists. This indicates that the dihydrochalcones are rapidly hydrolyzed and glucuronidated prior to absorption, principally in the small intestine.

Ileal fluid collected 0−24 h after cider consumption contained 38.6% of the dihyrochalcone intake, with the major compound being phloretin-2′-*O*-glucuronide, the same metabolite that appeared in plasma. The level of urinary excretion and the spectrum of phloretin metabolites were similar in healthy and ileostomy volunteers, providing further evidence of absorption occurring principally in the small intestine. Phloretin-*O*-glucuronide-*O*-sulfate (peak 6, **Table 1**) and phloretin-*O*-sulfate (peak 8, **Table 1**) were present in the ileal fluid in similar amounts (**Table 3**), but only the phloretin-*O*-glucuronide-*O*-sulfate was detected in urine (**Tables 4** and **5**). This suggests that sulfates probably undergo glucuronidation prior to urinary excretion. The main metabolite in urine was phloretin-2′-*O*-glucuronide, which also predominated in plasma and ileal fluid. This compound accounted for 84% of the metabolites found in the urine of healthy volunteers and 77% with the ileostomy volunteers.

The values obtained with the healthy subjects for C_{max}/dose and AUC/dose, 1.5 nmol L^{-1} μmol^{-1} and 3.3 nmol L^{-1} h^{-1} μmol^{-1}, respectively, for phloretin-2′-*O*-glucuronide (**Table 2**), are similar to those of 1.1 nmol L^{-1} μmol^{-1} and 3.9 nmol L^{-1} h^{-1} μmol^{-1} obtained with pelargonidin-*O*-glucuronide, following the ingestion of strawberries containing pelargonidin-3-*O*-glucoside (*25*). However, the AUC/dose of 16.5 nmol L^{-1} h^{-1} μmol^{-1} obtained with plasma hesperetin glucuronides after the ingestion of orange juice containing hesperetin-7-*O*-rutinoside (*24*) is much higher than the corresponding values obtained in the cider and strawberry studies. These differences are probably due to subtle variations in chemical structure rather than simply being a consequence of dihydrochalcones and the anthocyanin being absorbed in the small intestine, while the orange juice

flavanone is absorbed in the large intestine. This is because there are also major differences in the C_{max}/dose values obtained with structurally similar flavonoids that are absorbed in the large intestine. For instance, both quercetin-3-*O*-rutinoside and hesperetin-7-*O*-rutinoside are absorbed in the large intestine, and when ingested in similar amounts, the respective C_{max}/dose values for the flavonol and the flavanone were 0.09 and 16.5 nmol L^{-1} μmol^{-1} (*24*, *26*). It is evident that minor differences in the structure of either the aglycone or the conjugating sugar can have a major impact on the fate of dietary flavonoids and phenolic compounds within the body following ingestion. Further study is necessary to understand the mechanisms involved and their potential effects on the bioactivity of the compounds involved.

Kahle et al. (*8*) have reported the recovery of phloretin glycosides and metabolites in ileal fluid after the consumption of apple juice, containing 81.2 μmol of dihydrochalcones, by humans. The recovery of phloretin-2′-*O*-(2″-*O*-xylosyl)glucoside in ileal fluid was 20% of intake, which is similar to the 22% obtained after drinking cider. The apple juice study identified one phloretin glucuronide, phloretin-2′-*O*-glucuronide, in the ileal fluid, compared to three detected after cider consumption. It also failed to detect the presence of small quantities of phloretin-*O*-sulfates and the phloretin-*O*-glucuronide-*O*-sulfates in ileal fluid (**Table 3** and **Figure 3**). This may be due to the use of HPLC with PDA detection as the means of analysis. The use of HPLC−MS allows for the identification and quantification of minor metabolite peaks, which are either below the PDA detection limit or, in the absence of reference compounds and/or the structural information provided by MS, not recognizable as phloretin metabolites.

Despite being the main dihydrochalcone in the cider used in the current study, phloretin-2′-*O*-glucoside, unlike phloretin-2′-*O*-(2″-*O*-xylosyl)glucoside, was not detected in ileal fluid (**Table 3**). A similar observation was made by Kahle et al. (*8*) in their study with apple juice. This implies that the glucoside is absorbed better than the xylosyl-glucoside. Arguably, because it is more readily cleaved by lactase, phloridzin hydrolase in the brush border of the small intestine or cytosolic β-glucosidase in the epithelial cells, releasing phloretin, is glucuronidated prior to entry into the circulatory system. The presence of substantial quantities of phloretin glucuronide, sulfate, and glucuronide-sulfate metabolites in ileal fluid (**Table 3**) indicates that the wall of the small intestine also contains sulfuryltransferases and UDP-glucuronosyltransferases, and a sizable portion of the phloretin metabolites that are produced efflux back into the lumen of the gastrointestinal tract.

Dihydrochalcones are similar in structure to flavanones, with the difference being the open-ringed structure of the C ring. In planta, dihydrochalcones are converted to flavanones by the enzyme chalcone isomerase. Similarities can be seen in their human metabolites, with the domination of monoglucuronide metabolites (*24*). Structure seems key to the nature of metabolites, with flavan-3-ols forming methyl as well as glucuronide and sulfate derivatives (*27*), while metabolism of flavonols is more complex, producing multiple methyl, glucuronyl, sulfuryl, and glucosyl derivatives and conjugates that are mixtures of all three metabolite moieties (*28*).

While the dihydrochalcones are not the major compounds in cider, they are characteristic of apples and apple products. They are also known to have the ability to inhibit active SGLT1 (*16*). An apple juice study looked at the effect of different levels of phloretin-2′-*O*-glucoside on blood glucose levels; a cloudy apple and a clear juice were used (*29*). The clear apple juice gave volunteers a 27 μmol dose of phloretin-2′-*O*-glucoside, similar to the intake in the cider study. The cloudy apple juice contained 60 μmol of phloretin-2′-*O*-glucoside. In comparison to the control (equimolar for glucose), the clear apple juice significantly delayed glucose absorption and lowered plasma glucose concentration. Although the impact of other apple juice phenolics may have had an influence, it indicates that the dihydrochalcones in the cider used in our current study may produce similar responses. It would be interesting to repeat this study using phloretin-2′-*O*-glucoside in the absence of other phenolic compounds.

Future *in vitro* and *ex vivo* studies using cell lines investigating potential health benefits of consuming products containing dihydrochalcones should not use apple extracts or phloretin-*O*-glycosides. Instead, they should use low- and sub-μmol/L concentrations of phloretin-2′-*O*-glucuronide that is absorbed into the circulation. However, investigations on colonic health could employ phloretin-2′-*O*-(2″-*O*-xylosyl)glucoside and phloretin because these dihydrochalcones are found in modest amounts in ileal fluid and, in humans with an intact colon, will pass from the small to the large intestine.

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