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RESEARCH ARTICLE

# Bioavailability of multiple components following acute ingestion of a polyphenol-rich juice drink

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A healthy diet involves eating fruit and vegetables on a daily basis, the benefits of which are in part linked to the ingestion of bioactive compounds including polyphenols. As a convenient means of delivering additional polyphenols to the diet, a polyphenol-rich (P-R) juice drink was prepared and the bioavailability of its diverse spectrum of constituents investigated. Ten human volunteers followed a low-flavonoid diet for 2 days before drinking 350 mL of the P-R beverage. Plasma and urine were collected for 24 h and analyzed by HPLC-PDA-MS. The plasma pharmacokinetics and recoveries of urinary metabolites of flavan-3-ols, flavanones, dihydrochalcones and 5-*O*-caffeoylquinic acid, both in terms of their identity and quantity, were, in most instances, not markedly different to those reported in other feeding studies with green tea, orange juice, apple cider and coffee. This indicates that the combination of polyphenolic compounds in the P-R beverage are absorbed and excreted to a similar extent whether fed individually or together in a single beverage. It is concluded that the P-R beverage can deliver the intended blend of bioavailable polyphenols, which would normally require consumption of several different plant-derived foods.

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## 1 Introduction

Diets rich in fruits and vegetables have positive effects on health, reducing mortality rates [1, 2] and protecting against degenerative conditions, including cardiovascular disease [3, 4] as well as lowering the incidence of Alzheimer's disease [5] and possibly delaying general mental decline in the elderly [6, 7]. These health benefits are attributable, in part, to the ingestion of bioactive compounds that include flavonoids and phenolics found in vegetables and, to a greater extent, fruits [8]. Although it is thought that these compounds act as antioxidants, there is a growing realization that they may also function in other ways [8, 9], for instance, in reducing inflammation [10], platelet aggregation and LDL oxidation [11, 12], improving endothelial function [13–15] and reducing blood pressure [16, 17].

There is evidence that beverages, such as cocoa [18–20], red wine [21–23], tea [24, 25] and fruit juices [26], containing a diversity of polyphenolic compounds [27], can have a favorable impact on human health. One practical way to deliver some of the potentially beneficial components of the diet in a convenient manner, which does not involve major changes in eating habits, would be to develop beverages containing a wide spectrum of polyphenolic compounds originating from different plant sources.

The aim of the present study was to investigate the bioavailability of a diversity of phenolic compounds, principally flavonoids, following acute ingestion by human

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**Abbreviations: P-R,** polyphenol-rich

subjects of 350 mL of a low-calorie, polyphenol-rich (P-R) juice drink. The P-R beverage seeks to provide a blend of polyphenols in a single serving of the drink that cannot be obtained from a portion of fruit. However, in view of the flavonoid complexity of the beverage matrix, it is important to establish whether or not bioavailability is limited by potential competitive interactions for absorption, transport and enzymatic sites of metabolism in the human gastrointestinal tract, and elsewhere in the body.

## 2 Materials and methods

### 2.1 Reagents

EDTA, ethyl gallate, 5-*O*-caffeoylquinic acid, procyanidin B2, (−)-gallocatechin, (−)-epicatechin, (+)-catechin and (−)-gallocatechin-3-*O*-gallate were purchased from Sigma-Aldrich (Poole, UK) and (−)-epigallocatechin, (−)-epigallocatechin-3-*O*-gallate and (−)-epicatechin-3-*O*-gallate were obtained from Apin Chemicals (Abingdon, UK). Quercetin-3-*O*-rutinoside, quercetin-3-*O*-glucoside, phloretin-2′-*O*-glucoside, hesperetin-7-*O*-rutinoside, naringenin-7-*O*-rutinoside, ferulic acid, caffeic acid, sinapic acid and *p*-coumaric acid were obtained from AASC (Southampton, UK). Cyanidin-3-*O*-glucoside, peonidin-3-*O*-glucoside, malvidin-3-*O*-glucoside and 3-*O*-methyl-gallic acid were purchased from Extrasynthese (Genay, France) and methanol and ACN were obtained from Rathburn Chemicals (Walkerburn, Scotland). Formic and acetic acid were obtained from Fisher Scientific (Loughborough, UK). Benzyl mercaptan was purchased from Lancaster Synthesis (Morecombe, UK). Dihydroferulic acid was obtained from Alfa Aesar (Heysham, Lancashire, UK). Feruloylglycine was a gift from Professor Takao Yokota (Teikyo University, Utsunomiya, Japan) and hesperetin-7-*O*-glucuronide was a gift from Dr Hikaru Matsumoto (National Institute of Fruit Tree Science, Shizuoka, Japan). Phloretin-2′-*O*-glucuronide was prepared as described by Kahle *et al.* [28]. Professor Junji Terao and Dr. Yoshichika Kawai, (University of Tokushima, Japan) supplied a sample of (−)-epicatechin-7-*O*-glucuronide and Dr. Yukihiko Hara (Mitsui Norin, Tokyo, Japan) donated standards of 3′- and 4′-*O*-methyl-(−)-epicatechin. A low-calorie P-R juice drink was supplied by the Coca Cola Company (Atlanta, USA).

### 2.2 Drink design

The beverage was designed to include polyphenolic compounds from various sources with known or potential health benefits. In the first instance, this involved performing a supplier survey to identify potential sources of high polyphenol-containing extracts, followed by testing potential ingredients to determine the quantity and type of phenolic compounds actually present. The quality of these products in terms of polyphenol content varied greatly but based on HPLC-MS analyses and an *ex-vivo* screening for impact on endothelial function [29], sources of ingredients were selected that enabled a low-calorie, 28% juice P-R beverage to be prepared containing green tea flavan-3-ols, grape seed and pomace procyanidins, apple dihydrochalcones, procyanidins and chlorogenic acid, citrus flavanones and grape anthocyanins. The drink also contained 168 mg of vitamin C, 13 g of carbohydrate and 51 calories *per* 350 mL.

### 2.3 Study design

The Glasgow University Faculty of Medicine Ethical Committee approved the study protocol (REC reference number: 46779/1). Six male and four female volunteers (19–51 years of age; mean body mass index 24.3, range 17.4–34.7) who were healthy, non-smokers and not on any medication gave their written consent and participated in the study. They followed a diet low in flavonoids, which excluded fruits and vegetables, high fiber products and beverages such as tea, coffee, fruit juices and wine for 2 days before the study. After an overnight fast, volunteers consumed 350 mL of the P-R beverage. Volunteers ate ham or turkey with white bread rolls 4 h after drinking the juice and thereafter remained on a low flavonoid diet for a further 20 h until the final blood and urine samples were collected. Twelve milliliter of venous blood were collected in heparinized tubes from all volunteers at 0, 0.5, 1, 2, 3, 4, 5, 6, 8 and 24 h post-ingestion and plasma separated by centrifugation at 4000 *g* for 10 min at 4°C. Two 1-mL aliquots of plasma were acidified to pH 3 with 30 μL of 50% aqueous formic acid and 100 μL of 10 mmol/L ascorbic acid, frozen in liquid nitrogen and stored at –80°C prior to analysis by HPLC-PDA-$MS^2$. Urine was collected prior to supplementation and over four time periods, 0–2, 2–5, 5–8 and 8–24 h, after the ingestion of the drink. The total volume for each period was recorded. After collection, urine samples were divided into aliquots and stored at –80°C prior to analysis by HPLC-PDA-$MS^2$.

### 2.4 Extraction of plasma

Plasma samples were extracted using a method developed by Day *et al.* [30]. A 450-μL aliquot of plasma, acidified with 13.5 μL of 50% aqueous formic acid, was added dropwise to 1125 μL of ACN to which was added 20 μL of 10% ascorbic acid containing 0.5 mmol/L EDTA, and 1 μg of ethyl gallate as an internal standard. Samples were vortexed for 30 s every 2 min over a 10-min period before centrifuging at 1500 *g* for 20 min at 4°C. The supernatant was decanted and the pellet re-extracted with 1125 μL of methanol and after centrifugation the two supernatants were combined and reduced to dryness under a stream of nitrogen at 35°C.

 

### 2.5 Processing of urine

Urine samples were defrosted and centrifuged for 5 min at 16 100 *g* at 4°C prior the analysis by HPLC-PDA-MS$^2$.

### 2.6 Analysis of plasma and urine by HPLC with PDA and MS detection

The P-R juice drink, plasma extracts and urine from this study were analyzed by HPLC-PDA-MS$^2$. Full details of the methodology are provided in Mullen *et al.* [31]. Quantification of the metabolites was carried out using HPLC-MS in both selected ion monitoring and selected reaction monitoring modes. The quantitative analysis of flavanone metabolites is described by Mullen *et al.* [32], hydroxychalcone metabolite quantification by Marks *et al.* [33], hydroxycinnamate metabolite quantification by Stalmach *et al.* [34] and flavan-3-ol metabolite quantification by Stalmach *et al.* [35]. Gallic acid metabolites were quantified using a 3-*O*-methyl-gallic acid calibration curve.

It should be noted that analysis of flavan-3-ols and their metabolites is more subtle than is generally appreciated. For instance, without reference compounds, which can be separated by reversed-phase HPLC, MS is unable to distinguish between (−)-epicatechin and (+)-catechin metabolites or (−)-epigallocatechin and (+)-gallocatechin metabolites. There is also evidence of both green tea processing and post-consumption, converting (+)-flavan-3-ols to their (−)-stereoisomers, which together with their associated metabolites, cannot be discriminated by reversed-phase HPLC-MS. We, therefore, refer to flavan-3-ol metabolites as (epi)catechins or (epi)gallocatechins.

### 2.7 Pharmacokinetic analysis of metabolites in plasma

Data on polyphenol metabolite levels are presented as mean values ± standard error ($n = 10$). Maximum plasma concentration of metabolites from 0 to 24 h post-dose was defined as $C_{max}$, with $t_{max}$ being the time at which $C_{max}$ was reached. The elimination half-life ($t_{1/2}$) for the metabolites was computed by using the following formula $t_{1/2} = 0.693/Ke$ where $Ke$ is the slope of the linear regression of the plasma metabolite concentration. Area under the curve was calculated between 0 and 8 h using a Kinetica software package (Thermo Electron Corporation).

## 3 Results

### 3.1 Identification of flavonoids and phenolic compounds in the P-R juice drink and their plasma and urinary metabolites

The basis of HPLC-PDA-MS$^2$ identifications of 26 flavonoids and related compounds in the P-R juice drink along with information on the identification of 13 metabolites in plasma and 33 in urine after consumption of the beverage by human volunteers are described in detail in a separate publication [31].

### 3.2 Quantitative analysis of flavonoids and phenolic compounds in the P-R juice drink

The 350 mL of the beverage consumed by the volunteers contained 101 mg of grape seed, grape pomace and apple procyanidins, with an average degree of polymerization of 3.9, as well as the compounds listed in Table 1. The predominant ingredients in the drink were green tea flavan-3-ols, with the principal component being 187 μmol of (−)-epigallocatechin.

**Table 1.** Flavonoid and phenolic composition of the P-R juice drink. Identification and quantification were based on HPLC with PDA and fluorescence detection and full scan data-dependent tandem MS[a)]

| Compounds | μmol/350 mL |
|---|---|
| Gallic acid | 52 |
| **Total phenolic acids** | 52 |
| (−)-Epicatechin | 77 |
| (+)-Catechin | 50 |
| (−)-Epigallocatechin | 187 |
| (+)-Gallocatechin | 48 |
| (−)-Epicatechin-3-*O*-gallate | 6.2 |
| (−)-Epigallocatechin-3-*O*-gallate | 65 |
| Procyanidin B1 dimer | 15 |
| Procyanidin B2 dimer | 39 |
| **Total flavan-3-ols** | 487 |
| Peonidin-3,5-*O*-diglucoside | 1.8 |
| Malvidin-3,5-*O*-diglucoside | 3.4 |
| Peonidin-3-*O*-glucoside | 0.6 |
| Malvidin-3-*O*-glucoside | 0.7 |
| Malvidin-3-*O*-(6″-*p*-coumaroyl)-5-*O*-diglucoside | 0.6 |
| Malvidin-3-*O*-*p*-(6″-*p*-coumaroyl)glucoside | 0.7 |
| **Total anthocyanins** | 7.8 |
| 5-*O*-Caffeoylquinic acid | 46 |
| **Total chlorogenic acids** | 46 |
| Hesperetin-7′-*O*-rutinoside | 45 |
| Naringenin-7-*O*-neohesperidoside | 5.9 |
| **Total flavanones** | 51 |
| Phloretin-2′-*O*-glucoside | 68 |
| Phloretin-2′-*O*-(2″-xylosyl)glucoside | 15 |
| **Total dihydrochalcones** | 83 |
| Quercetin-3-*O*-rutinoside | 1.3 |
| Quercetin-3-*O*-galactoside | 1.6 |
| Quercetin-3-*O*-glucoside | 1.3 |
| Quercetin-*O*-glucuronide | 2.3 |
| Myricetin-*O*-hexoside | 2.7 |
| Quercetin-*O*-rutinosylglucoside | 1.0 |
| **Total flavonols** | 10 |
| **Total flavonoids and phenolic compounds** | **737[b)]** |

a) Data expressed as mean values ± standard error ($n = 3$).
b) The P-R juice drink also contained 101 mg/350 mL of procyanidins.

 

Also present were 5-*O*-caffeoylquinic acid and dihydrochalcones from apples, citrus flavanones, gallic acid, which was also a green tea-derived compounds and trace amounts of a diversity of flavonols and the grape anthocyanins. The beverage, because of the selected ingredients, has a much more diverse composition of polyphenolic compounds than that found in any single component fruit juice or drink [36].

### 3.3 Quantitative analysis of plasma metabolites

No metabolites were detected in either the 0 h or 24 h plasma samples. The quantities present 0.5–8 h after ingestion of the P-R juice drink are depicted in Figure 1 with the pharmacokinetic parameters presented in Table 2. The main phenolic metabolites to accumulate in plasma were (epi)catechin-*O*-sulfates and *O*-methyl-(epi)catechin-*O*-sulfates which had $C_{max}$ values in excess of 500 nmol/L and, as a consequence, high area under the curve values. The $t_{max}$ values of most of the (epi)catechin and (epi)gallocatechin metabolites ranged from 0.6 to 1.1 h, indicative of absorption in the small intestine. The only flavan-3-ol metabolite outside this bracket was an *O*-methyl-(epi)gallocatechin-*O*-sulfate with a $t_{max}$ of $2.0 \pm 0.1$ h, which may be a consequence of post-absorption phase II metabolism. The dihydrochalcone metabolite, phloretin-2′-*O*-glucuronide, which had a $C_{max}$ of $204 \pm 26$ nmol/L, had a short $t_{max}$, $0.6 \pm 0.1$ h with a small secondary peak at 4.0 h (Fig. 1) as observed in our previous study with apple cider [33]. In contrast to the other metabolites, the two citrus-derived hesperetin-*O*-glucuronides had a delayed $t_{max}$ of $3.7 \pm 0.2$ h (Table 2), which indicates absorption in the large rather than the small intestine.

Note that no metabolites were detected in plasma collected immediately prior to ingestion of the beverage and Wilcoxon signed rank tests indicated that the quantities of metabolites detected 30 min after ingestion were significantly higher than to the zero level baseline values ($p = 0.002$).

### 3.4 Quantitative analysis of metabolites in urine

Data on the excretion of flavonoids and phenolic metabolites in urine 0–2, 2–5, 5–8 and 8–24 h after the ingestion of the

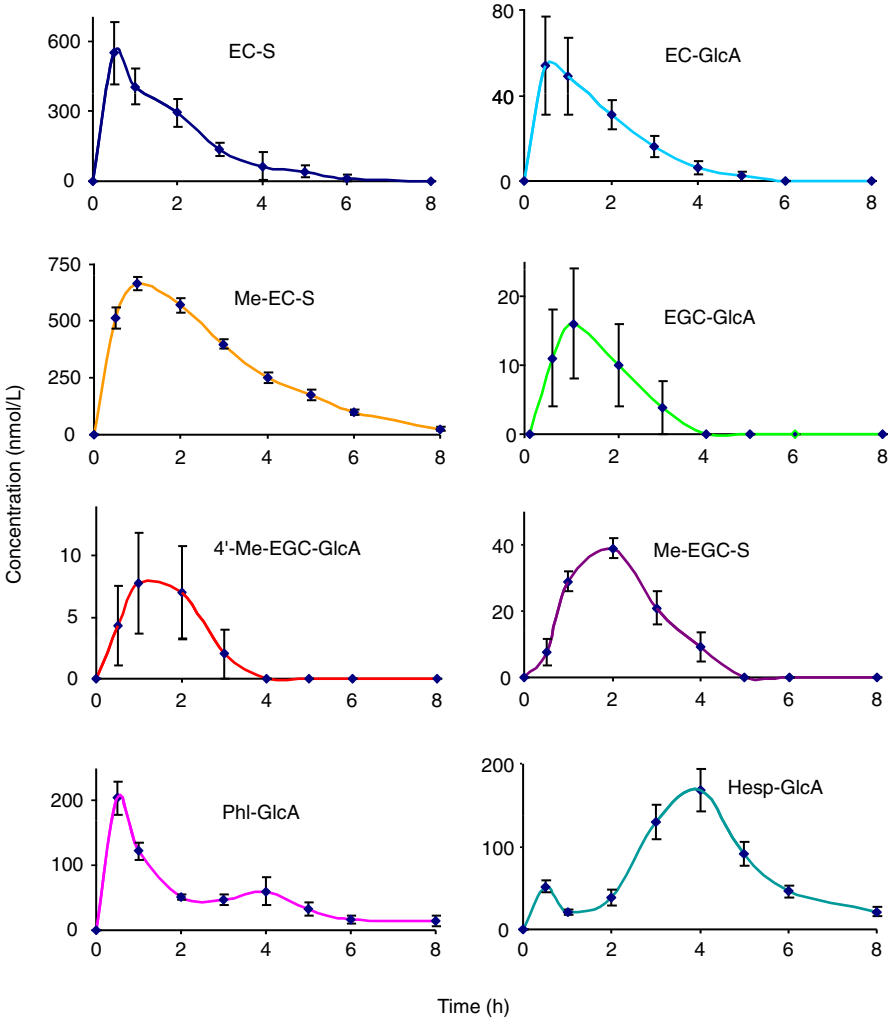


**Figure 1.** Concentrations of (epi)catechin-*O*-sulfates (EC-S), an (epi)catechin-*O*-glucuronide (EC-GlcA), *O*-methyl-(epi)catechin-*O*-sulfates (Me-EC-S), an (epi)gallocatechin-*O*-glucuronide (EGC-GlcA), a 4′-*O*-methyl-(epi)gallocatechin-*O*-glucuronide (4′-Me-EGC-GlcA), an *O*-methyl-(epi)gallocatechin-*O*-sulfate (Me-EGC-S), phloretin-2′-*O*-glucuronide (Phl-GlcA) and hesperetin-*O*-glucuronides (Hesp-GlcA) in the plasma of human subjects 0–8 h after the ingestion of 350 mL of a P-R juice drink. Data expressed in nmol/L as mean values with the standard errors ($n = 10$) depicted by vertical bars.

 

beverage are presented in Tables 3–5. The 0–24 h excretion of (epi)catechin metabolites was 31.9 ± 2.1 μmol, an extremely high amount which corresponds to 25.2% of intake. The excretion of (epi)gallocatechin metabolites was 14.4 ± 1.2 μmol equivalent to 6.2% of intake (Table 3).

Phloretin-2′-*O*-glucuronide, the main dihydrochalcone metabolite in plasma, also predominated in urine which contained small amounts of an additional phloretin-*O*-glucuronide and three phloretin-*O*-glucuronide-*O*-sulfates, metabolites not detected in plasma. The overall excretion of the dihydrochalcone metabolites corresponded to 4.9% of intake, and occurred mainly within the first 5 h after ingestion, suggesting absorption occurred principally in the small intestine (Table 4).

Small quantities of hesperetin-7-*O*-glucuronide, the main plasma flavanone metabolite, appeared in urine along with large amounts of two additional unassigned hesperetin-*O*-glucuronide isomers and a hesperetin-*O*-glucuronide-*O*-sulfate (Table 4). In total the hesperetin urinary metabolites accounted for 12.0% of the 45 μmol of ingested hesperetin-7-*O*-rutinoside. Most excretion of the hesperetin derivatives took place 2–8 h after ingestion (Table 4), which along with the plasma pharmacokinetic profile (Fig. 1) indicates that absorption may be occurring in both the small and the large intestine. The P-R drink contained 5.9 μmol of an additional flavanone, naringenin-7-*O*-neohesperidoside (Table 1) but, presumably because of this relatively low dose, no metabolites were detected in either plasma or urine, despite naringenin being potentially more readily bioavailable than hesperetin [32].

In addition, urine also contained two *O*-methyl-gallic acid-*O*-sulfates, which were excreted over the 24-h collection period in quantities correspond to 10.1% of gallic acid intake, presumably derived from 52 μmol of gallic acid itself and 71 μmol of 3-*O*-galloylated flavan-3-ols (Table 5). Also present in urine was an array of hydroxycinnamate metabolites that originated from 46 μmol of 5-*O*-caffeoylquinic acid (Table 5). The main components were dihydrocaffeic acid-3-*O*-sulfate, dihydroferulic acid, ferulic acid-4-*O*-sulfate and feruloylglycine. The combined 0–24 h excretion of the

**Table 2.** Pharmacokinetic analysis of metabolites detected in plasma of healthy volunteers following the ingestion of 350 mL of P-R juice drink[a)]

| Metabolites (*number of isomers*) | $C_{max}$ (nmol/L) | $t_{max}$ (h) | $t_{1/2}$ (h) | AUC (nmol/h) |
|---|---|---|---|---|
| (Epi)catechin-*O*-sulfates *(2)* | 545 ± 189 | 0.9 ± 0.1 | 1.1 ± 0.5 | 1130 ± 369 |
| (−)-(Epi)catechin-*O*-glucuronide | 54 ± 23 | 1.1 ± 0.2 | 1.0 ± 0.4 | 119 ± 43 |
| 3′ and 4′-Methyl-(epi)catechin-*O*-sulfates *(4)* | 663 ± 46 | 1.0 ± 0.0 | 1.4 ± 1.0 | 2317 ± 269 |
| (Epi)gallocatechin-*O*-glucuronide | 16 ± 8 | 0.6 ± 0.2 | 1.0 ± 0.5 | 31 ± 19 |
| 4′-*O*-Methyl-(epi)gallocatechin-*O*-glucuronide | 7.8 ± 4.1 | 0.6 ± 0.2 | 1.0 ± 0.5 | 17 ± 10 |
| *O*-Methyl-(epi)gallocatechin-*O*-sulfate | 39 ± 3 | 2.0 ± 0.1 | 1.0 ± 0.7 | 94 ± 16 |
| Phloretin-2′-*O*-glucuronide | 204 ± 26 | 0.6 ± 0.1 | 2.1 ± 1 | 425 ± 86 |
| Hesperetin-*O*-glucuronide *(2)* | 168 ± 45 | 3.7 ± 0.2 | 1.3 ± 0.5 | 559 ± 163 |

$C_{max}$, 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).
a) Data expressed as mean values ± standard error ($n = 10$).

**Table 3.** Quantities of metabolites of (epi)catechin and (epi)gallocatechin in the urine of 10 human subjects 0–24 h after the consumption of 350 mL of a P-R juice drink[a)]

| Metabolite *(number of isomers)* | 0–2 h | 2–5 h | 5–8 h | 8–24 h | Total |
|---|---|---|---|---|---|
| (Epi)catechin-*O*-sulfate *(3)* | 6.7 ± 0.8 | 5.3 ± 0.4 | 1.8 ± 0.3 | 0.6 ± 0.1 | 14.4 ± 1.0 |
| 3′- and 4′-*O*-Methyl-(epi)catechin-*O*-sulfate (*5*) | 3.9 ± 0.5 | 3.8 ± 0.3 | 1.9 ± 0.2 | 1.0 ± 0.2 | 10.6 ± 0.6 |
| (−)-Epicatechin-3′-*O*-glucuronide | 2.0 ± 0.4 | 1.6 ± 0.2 | 0.9 ± 0.1 | 0.5 ± 0.1 | 5.0 ± 0.5 |
| (−)-(Epi)catechin-*O*-glucuronide sulfate *(2)* | 0.5 ± 0.1 | 0.8 ± 0.1 | 0.5 ± 0.1 | 0.1 ± 0.0 | 1.9 ± 0.2 |
| **Total (epi)catechin metabolites** | **13.1 ± 3.9** | **11.5 ± 0.8** | **5.1 ± 0.5** | **2.2 ± 0.4** | **31.9 ± 2.1** |
| ***% Recovery*** | ***10.3 ± 3.1*** | ***9.1 ± 0.6*** | ***4.0 ± 0.4*** | ***1.8 ± 0.2*** | ***25.2 ± 2.9*** |
| (Epi)gallocatechin-*O*-glucuronide | 4.5 ± 0.6 | 3.6 ± 0.5 | 1.1 ± 0.2 | 0.3 ± 0.1 | 9.5 ± 0.9 |
| 4′-*O*-Methyl-(epi)gallocatechin-*O*-glucuronide | 0.3 ± 0.0 | 0.3 ± 0.0 | 0.2 ± 0.0 | 0.1 ± 0.0 | 0.9 ± 0.1 |
| 4′-*O*-Methyl-(epi)gallocatechin-*O*-sulfate *(2)* | 1.3 ± 0.1 | 1.6 ± 0.1 | 0.8 ± 0.1 | 0.3 ± 0.1 | 4.0 ± 0.4 |
| **Total (epi)gallocatechin metabolites** | **6.1 ± 1.8** | **5.5 ± 0.5** | **2.1 ± 0.4** | **0.7 ± 0.1** | **14.4 ± 1.2** |
| ***% Recovery*** | ***2.6 ± 0.8*** | ***2.4 ± 0.2*** | ***0.9 ± 0.2*** | ***0.3 ± 0.1*** | ***6.2 ± 0.5*** |

Figures in bold represent total metabolites excreted in μmol and in italics as a percentage of the total ingested. Intake of (+)-catechin and (−)-epicatechin = 127 μmol; (+)-gallocatechin and (−)-epigallocatechin intake = 235 μmol.
a) Data expressed as μmol ± standard error ($n = 10$).

 

**Table 4.** Quantities of metabolites of phloretin and hesperetin in the urine of 10 human subjects 0–24 h after the consumption of 350 mL of P-R juice drink[a)]

| Metabolite *(number of isomers)* | 0–2 h | 2–5 h | 5–8 h | 8–24 h | Total |
|---|---|---|---|---|---|
| Phloretin-2′-*O*-glucuronide | 1.6±0.2 | 1.0±0.2 | 0.5±0.1 | 0.2±0.1 | 3.3±0.3 |
| Phloretin-*O*-glucuronide | 0.1±0.0 | 0.04±0.01 | 0.03±0.02 | 0.01±0.0 | 0.2±0.0 |
| Phloretin-*O*-glucuronide-*O*-sulfate *(3)* | 0.2±0.0 | 0.1±0.0 | 0.1±0.0 | 0.1±0.0 | 0.5±0.0 |
| **Total phloretin metabolites** | **1.9±0.6** | **1.2±0.2** | **0.6±0.1** | **0.3±0.1** | **4.0±0.4** |
| ***% Recovery*** | ***2.3±0.7*** | ***1.4±0.2*** | ***0.8±0.2*** | ***0.4±0.2*** | ***4.9±0.5*** |
| Hesperetin-7-*O*-glucuronide | 0.03±0.0 | 0.2±0.1 | 0.2±0.0 | 0.03±0.0 | 0.4±0.1 |
| Hesperetin-*O*-glucuronide *(2)* | 0.2±0.0 | 0.9±0.1 | 0.8±0.1 | 0.2±0.1 | 2.1±0.2 |
| Hesperetin-*O*-glucuronide-*O*-sulfate | 0.1±0.0 | 1.2±0.4 | 1.2±0.2 | 0.4±0.1 | 2.9±0.5 |
| **Total hesperetin metabolites** | **0.3±95** | **2.3±0.6** | **2.2±0.3** | **0.6±0.2** | **5.4±0.7** |
| ***% Recovery*** | ***0.7±0.2*** | ***5.1±1.3*** | ***4.8±0.7*** | ***1.4±0.4*** | ***12.0±1.6*** |

Figures in bold represent total metabolites excreted in μmol and in italics as a percentage of the total ingested. Intake of phloretin-2′-*O*-(2″-*O*-xylosyl)glucoside and phloretin-2′-*O*-glucoside = 83 μmol; hesperetin-7-*O*-rutinoside intake = 45 μmol.
a) Data expressed as μmol ± standard error ($n = 10$).

**Table 5.** Quantities of metabolites of gallic acid and 5-*O*-caffeoylquinic acid in the urine of 10 human subjects 0–24 h after the consumption of 350 mL of a P-R juice drink[a)]

| Metabolites *(number of isomers)* | 0–2 h | 2–5 h | 5–8 h | 8–24 h | Total |
|---|---|---|---|---|---|
| *O*-Methyl-gallic acid-*O*-sulfate *(2)* | 2.4±0.5 | 4.5±1.0 | 2.9±0.6 | 2.6±0.4 | 12.4±1.8 |
| **Total gallic acid metabolites** | **2.4±0.5** | **4.5±1.0** | **2.9±0.6** | **2.6±0.4** | **12.4±1.8** |
| ***% Recovery*** | ***1.9±0.4*** | ***3.5±0.8*** | ***2.4±0.5*** | ***2.1±0.3*** | ***10.1±1.5*** |
| Caffeic acid-3-*O*-sulfate | 0.3±0.1 | 0.1±0.0 | 0.3±0.0 | 0.8±0.1 | 1.5±0.3 |
| Dihydrocaffeic acid-3-*O*-sulfate | 0.3±0.1 | 0.3±0.1 | 1.8±0.7 | 3.5±0.7 | 5.9±1.2 |
| Ferulic acid-4-*O*-sulfate | 0.3±0.0 | 0.2±0.0 | 0.9±0.1 | 2.2±0.4 | 3.6±0.5 |
| Dihydroferulic acid | 0.2±0.1 | 0.3±0.1 | 0.5±0.2 | 1.0±0.2 | 2.0±0.4 |
| Dihydroferulic acid-4-*O*-sulfate | 0.08±0.03 | 0.1±0.0 | 0.2±0.0 | 0.3±0.1 | 0.7±0.1 |
| Dihydro(iso)ferulic acid | 0.1±0.1 | 0.1±0.5 | 0.2±0.1 | 0.4±0.2 | 0.8±0.3 |
| Feruloylglycine | 0.6±0.1 | 0.5±0.1 | 1.1±0.2 | 4.1±0.4 | 6.3±0.8 |
| **Total ferulic acid metabolites** | **1.9±0.2** | **1.6±0.2** | **5.0±0.7** | **12.3±0.8** | **20.8±1.4** |
| ***% Recovery*** | ***4.2±0.5*** | ***3.8±0.4*** | ***10.8±1.5*** | ***26.7±1.8*** | ***45.6±3.2*** |

Figures in bold represent total metabolites excreted in μmol and in italics as a percentage of the total ingested. Gallic acid intake = 52 μmol and intake of 3-*O*-galloylated flavan-3-ols = 71 μmol; 5-*O*-caffeoylquinic acid intake = 46 μmol, which is converted to hydroxycinnamate metabolites.
a) Data expressed as μmol ± standard error ($n = 10$).

hydroxycinnamate metabolites corresponded to 45.6% of 5-*O*-caffeoylquinic acid intake. The timing of excretion implies that most absorption occurred in the large intestine. None of the urinary gallic acid or hydroxycinnamate metabolites were present in plasma in detectable quantities.

Note that no metabolites were detected in urine collected prior to ingestion of the beverage and Wilcoxon signed rank tests indicated that the quantities of metabolites detected 0–2 h, 2–5 h, 5–8 h and 8–24 h after ingestion were significantly higher than to the zero level baseline values ($p = 0.002$).

In the current study, no anthocyanins were detected in either plasma or urine. This is not surprising as the drink, despite its red color, contained low levels of peonidin-3,5-*O*-diglucoside, malvidin-3,5-*O*-diglucoside and four other anthocyanins (Table 1). With the exception of pelargonidin-3-*O*-glucoside, anthocyanins are poorly absorbed, being rarely detected in plasma and, typically, are excreted in quantities corresponding to < 0.05% of intake [8]. Although the drink also contained a substantial quantity of procyanidins, no procyanidin dimers, trimers or pentamers were detected in any of the plasma or urine samples. The drink also contained a number of flavonol glycosides but they were minor components (Table 1) and neither quercetin nor myricetin metabolites accumulated in either plasma or urine in detectable quantities.

## 4 Discussion

It is of interest to compare the data obtained in this acute bioavailability study using a multicomponent P-R beverage

 

with those obtained in our previous studies with orange juice [32], apple cider [33], coffee [34] and green tea products [35, 37, 38], which used identical feeding procedures and analytical techniques and in some, but not all, instances similar intakes of the individual flavonoids and related compounds.

The flavan-3-ols were the main components in the P-R juice drink (Table 1) and although similar (epi)catechin and (epi)gallocatechin metabolites were detected in plasma compared to an earlier study with green tea [35], there were substantial differences in the pharmacokinetic profiles of the different types of metabolites possibly due to the differences in ingested amounts and/or the differing matrices of the two beverages. The (epi)catechin-*O*-sulfates and *O*-methyl-(epi)catechin-*O*-sulfates had substantially higher $C_{max}$ values than were obtained after the ingestion of green tea which contained *ca.* 50% more (epi)catechins (Table 6). Conversely, there was a substantial lower plasma levels of (epi)gallocatechin metabolites with the P-R drink, where the combined $C_{max}$ values of the sulfate, glucuronide and methyl metabolites was 63 nmol/L (Table 2) compared with 251 nmol/L observed after ingestion of green tea containing similar amounts of (epi)gallocatechins (Table 6). These differences in plasma concentration were, however, not reflected in parallel changes in urinary excretion of the (epi)catechin and (epi)gallocatechin metabolites. Why this should be is unclear at this juncture and merits further investigation.

Most of the plasma $t_{max}$ values of the flavan-3-ol metabolites were $<1$ h (Table 2) indicating absorption in the small intestine. This is *ca.* 1 h more rapid than those obtained in an investigation in which 500 mL of a bottled green tea was fed to human volunteers [35]. This could be a consequence of the tea containing 36.5 g of carbohydrate, which might slow the rate of gastric emptying [39], compared with 13 g in the P-R drink. Supporting this possibility is our report that when a sugar-free green tea infusion was fed to ileostomists, the plasma $t_{max}$ values of the flavan-3-ol metabolites were similar to those presented in Table 2 [37].

The plasma pharmacokinetic profile of phloretin-2′-*O*-glucuronide (Fig. 1) resembled that obtained after feeding apple cider [33] although a $C_{max}$ of 204 nmol/L was obtained (Table 2) compared to 73 nmol/L with cider. This could be due to the more substantial intake of dihydrochalcones with the P-R beverage, 83 μmol, compared with the 46 μmol in the apple cider study. Likewise, the hesperetin-*O*-glucuronide plasma profile (Fig. 1) was similar to that obtained after drinking orange juice [32] although again different $C_{max}$ values were apparent. With the P-R drink, a $C_{max}$ of 168 nmol/L was obtained after a 45-μmol intake of hesperetin-7-*O*-rutinoside. This compares with 922 nmol/L attained following consumption of orange juice containing 168 μmol of the flavanone rutinoside. Arguably, the different $C_{max}$ values could reflect a dose effect.

While 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, due to the rapid turnover of the metabolites in the circulatory system. Urinary excretion provides a more realistic parameter for assessing the bioavailability of dietary phenolics although the absolute numbers probably under-estimate the total amount absorbed as they do not account for metabolites sequestered in body tissues. Nor do urinary excretion values take into consideration other routes of elimination, including possible excretion in bile [8]. Nevertheless, it is interesting to note that excretion of flavan-3-ol metabolites by humans, as a percentage of intake, is substantially higher than that of many other flavonoids. In the current study, after consumption of the P-R juice drink, excretion of (epi)catechin metabolites was $25.2 \pm 2.9$% of intake while that of (epi)gallocatechin was only 6.2% (Table 3). This in keeping with observations originally made after the ingestion of Polyphenon E [38] and subsequently confirmed in studies with green tea [35, 37]. The high excretion of (epi)catechin metabolites has also been noted after the ingestion of cocoa-based beverages with 17.1% of intake being reported by Mullen *et al.* [40] and 28.5% by Baba *et al.* [41] (Table 7).

The P-R juice drink contained 5-*O*-caffeoylquinic acid which yielded seven urinary metabolites in quantities equivalent to 45.6% of hydroxycinnamate intake. The main metabolites were feruloylglycine and dihydrocaffeic acid-3-*O*-sulfate (Table 5). There are small differences in the urinary hydroxycinnamate metabolite profiles obtained with the P-R beverage and coffee [34] but much of this may well be a consequence of the approximately tenfold higher chlorogenic acid intake in the study with coffee.

The data in Table 7 compare quantitative metabolite excretion in urine as a percentage of intake obtained in the

**Table 6.** Plasma $C_{max}$ values for (epi)catechin and (epi)gallocatechin metabolites detected after the consumption of P-R juice drink and green tea. The respective intakes of (epi)catechins were 127 and 87 μmol and for (epi)gallocatechins 235 and 240 μmol[a)]

| Metabolites | P-R drink | Green tea[b)] |
|---|---|---|
| (Epi)catechin-*O*-sulfates | $545 \pm 189$ | $89 \pm 15$ |
| *O*-Methyl-(epi)catechin-*O*-sulfates | $663 \pm 46$ | $90 \pm 15$ |
| (Epi)catechin-*O*-glucuronide | $54 \pm 23$ | $29 \pm 5$ |
| (Epi)gallocatechin-*O*-glucuronide | $16 \pm 8$ | $126 \pm 19$ |
| 4′-O-Methyl-(epi)gallocatechin-*O*-glucuronide | $8 \pm 4$ | $46 \pm 6$ |
| *O*-Methyl-(epi)gallocatechin-*O*-sulfate | $39 \pm 3$ | $79 \pm 12$ |

a) Data expressed in nmol/L as mean values ± standard error ($n = 10$).

b) Data on green tea from Stalmach *et al.* [35].

 

**Table 7.** Summary of quantitative data on excretion of flavonoid and phenolic metabolites in acute human feeding studies

| Phenolic components | Origin | Intake | Metabolite excretion | | Study |
|---|---|---|---|---|---|
| | | (μmol) | (μmol) | (% of intake) | |
| (Epi)catechin | P-R drink | 127 | 31.9 | 25.1 | Current study |
| | Green tea | 67 | 19.1 | 28.5 | Stalmach *et al.* [35] |
| | Cocoa | 45 | 7.7 | 17.1 | Mullen *et al.* [40] |
| | Cocoa | 974 | 246 | 25.3 | Baba *et al.* [41] |
| (Epi)gallocatechin | P-R drink | 235 | 14.4 | 6.2 | Current study |
| | Green tea | 293 | 33.3 | 11.4 | Stalmach *et al.* [35] |
| (Epi)gallocatechin-3-*O*-gallate | P-R drink | 65 | n.d. | 0 | Current study |
| | Green tea | 238 | n.d. | 0 | Stalmach *et al.* [35] |
| (Epi)catechin-3-*O*-gallate | P-R drink | 6.2 | n.d. | 0 | Current study |
| | Green tea | 49 | n.d. | 0 | Stalmach *et al.* [35] |
| Hesperetin-7-*O*-rutinoside | P-R drink | 45 | 5.4 | 12.0 | Current study |
| | Orange juice | 168 | 10.9 | 6.3 | Mullen *et al.* [32] |
| Phloretin-*O*-glycosides | P-R drink | 83 | 4.0 | 4.9 | Current study |
| | Apple cider | 45 | 2.3 | 5.0 | Marks *et al.* [33] |
| 5-*O*-Caffeoylquinic acid[a)] | P-R drink | 46 | 20.8 | 45.6 | Current study |
| | Coffee[b)] | 412 | 120 | 29.1 | Stalmach *et al.* [34] |
| Gallic acid[c)] | P-R drink | 123[d)] | 12.4 | 10.1 | Current study |
| Anthocyanins[c)] | P-R drink | 5.2 | n.d. | 0 | Current study |

n.d., not detected.
a) Excreted as hydroxycinnamate metabolites.
b) This study involved the ingestion of a number of chlorogenic acids of which 5-*O*-caffeoylquinic acid was the main component.
c) There are no reports in the literature on comparable feeding studies involving either gallic acid or the P-R juice drink anthocyanins.
d) Gallic acid intake includes gallic acid and 3-*O*-galloylated flavan-3-ols.

present study with excretion levels in feeds involving products containing fewer individual flavonoids. This shows that excretion of hesperetin metabolites was 12.0% with the P-R juice drink compared to 6.3% with orange juice. This could be a reflection of hesperetin-7-*O*-rutinoside intake with the orange juice being 3.7-fold higher than with the P-R juice drink. Recovery of phloretin metabolites with the P-R beverage was 4.9% of intake and, with a *ca*. 50% reduced intake, 5.0% with apple cider. Excretion of 5-*O*-caffeoylquinic acid metabolites after consumption of the P-R drink was 45.8% of intake while, with an approximately tenfold higher intake of chlorogenic acids, a 29.1% excretion occurred with coffee (Table 7).

The plasma pharmacokinetics (Fig. 1) and recoveries of urinary metabolites (Table 7), both in terms of their identity and quantity, are, with a few exceptions, in line with the findings of other feeding studies with orange juice [32], apple cider [33], coffee [34] and green tea products [35, 37, 38]. This is of special interest as it indicates that the various flavonoids and phenolic compounds are absorbed and excreted to a broadly similar extent whether fed individually or in combination in a single beverage. This further suggests that there is no substantial competition for transport across the gastrointestinal mucosa into the circulatory system for the diverse array of polyphenolic components in the P-R drink. More specifically, the spectrum of metabolites in plasma and urine obtained after ingestion of the P-R beverage, compared to those obtained in single component studies, was not markedly different for flavanones, dihydrochalcones and chlorogenic acids. Qualitative differences were, however, observed with the metabolites of (epi)catechins and (epi)gallocatechins suggesting that although their absorption was not influenced to any degree by the other phenolic compounds in the juice drink, there may have been some competition for access to sulfotransferases, uridine-5′-diphosphate glucuronyltranferases and catechol-*O*-methyltransferases that resulted in differences in the plasma and urinary flavan-3-ol metabolite profiles obtained with the P-R beverage compared to green tea.

The fact that metabolites of some of the phenolic compounds in the P-R drink were absorbed in the small intestine and others in the large intestine suggests that metabolites may be circulating in the body for a more sustained period of time than would occur after ingestion of a beverage containing a more restricted spectrum of polyphenolic components (see [8]). The data obtained on plasma and urinary metabolites are consistent across several classes of flavonoids and phenolic compounds with none showing evidence of major interactions that would limit their bioavailability. The results are, therefore, transferable to other specific combinations in beverages, allowing us to conclude that P-R drinks may, in principle, be explored as a convenient vehicle to deliver a blend of potentially protective polyphenols and flavonoids into the diet without major changes in eating habits.

 

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