Identification and Pharmacokinetics of Novel Alkylresorcinol Metabolites in Human Urine, New Candidate Biomarkers for Whole-Grain Wheat and Rye Intake\(^1\)\(^-\)\(^3\)

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\(^3\)Supplemental Tables 1–4, Supplemental Figures 1–6, and Supplemental Methods are available from the “Online Supporting Material” link in the online posting of the article and from the same link in the online table of contents at http://jn.nutrition.org.

\(^4\)Abbreviations used: AA, acetic acid; ER\(_{\text{max}}\), maximum excretion rate; ESI, electrospray ionization; LC, liquid chromatography; LOD, limit of detection; M, molecular weight; RG, refined grain; \(t_{1/2}\), apparent half-life; \(t_{\text{max}}\), time to reach maximum excretion rate; WG, whole grain; 3,5-DHBA, 3,5-dihydroxybenzoic acid; 3,5-DHBA glycine, 2-(3,5-dihydroxybenzamido)acetic acid; 3,5-DHPPA, 3-(3,5-dihydroxyphenyl)propanoic acid; 3,5-DHPPTA, 3-(3,5-dihydroxyphenyl)pentanoic acid.

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Abstract

Biomarkers of dietary intake are prominent tools in nutritional research. Alkylresorcinol metabolites, 3,5-dihydroxybenzoic acid (3,5-DHBA) and 3-(3,5-dihydroxyphenyl)propanoic acid (3,5-DHPPA), have been proposed as exposure biomarkers of whole-grain (WG) wheat and rye intake. However, the profile of alkylresorcinol metabolites is not fully understood. The aim of this study was to investigate the metabolism of alkylresorcinols in mice and in humans, while further determining urinary pharmacokinetics of the novel alkylresorcinol metabolites to explore their potential as biomarkers of WG wheat intake. Utilization of the liquid chromatography–mass spectrometry approach resulted in 10 alkylresorcinol metabolites identified in mice and in humans, including 3 phenolic acids and 7 of their phase II conjugates. Among them, 2 novel metabolites were discovered: 5-(3,5-dihydroxyphenyl)pentanoic acid (3,5-DHPPTA) and 2-(3,5-dihydroxybenzamido)acetic acid (3,5-DHBA glycine). The structures of these 2 metabolites were confirmed by comparing with authentic standards synthesized in-house.

In the pharmacokinetic study, a group of 12 volunteers consumed a polyphenolic-restricted diet for 4 d before ingesting WG wheat bread containing 61 mg of alkylresorcinols. Urine samples were collected for 32 h, and alkylresorcinol metabolites were quantified with HPLC-coulometric electrode array detection. The mean urinary excretion rates and mean apparent half-life of 3,5-DHPPTA, 3,5-DHBA glycine, 3,5-DHBA, and 3,5-DHPPA at each time point were determined. Our results suggest that 3,5-DHPPTA and 3,5-DHBA glycine may be used in combination with 3,5-DHBA and 3,5-DHPPA as potential biomarkers to increase the accuracy of recording WG wheat and rye intake in epidemiologic studies. Further validation of 3,5-DHPPTA and 3,5-DHBA glycine as potential biomarkers is warranted. J. Nutr. doi: 10.3945/jn.113.184663.

Introduction

An impressive body of evidence from epidemiologic studies supports the notion that increasing consumption of whole grains (WGs)\(^7\) and/or cereal bran is associated with lower risk of chronic diseases, such as cancer, obesity, diabetes, and heart diseases (1–5). The main problem in epidemiologic studies is the lack of tools to precisely estimate the intake of various food components. There are inherent weaknesses in FFQs. To better understand the beneficial health effects of WGs, biomarkers for their exposure and effects are needed. One of the major groups of phenolic compounds in cereals is the \(n\)-alkylresorcinols. Alkylresorcinols are 1,3-dihydroxybenzene derivatives with an odd-numbered alkyl chain at position 5 of the benzene ring, a structure that gives them amphiphilic properties. Among commonly consumed foods, alkylresorcinols are only present in high amounts in wheat and rye, in which the length of the saturated alkyl tail varies between 15 and 27 carbons (C15:0–C27:0), with C19:0 and C21:0 being the most abundant alkylresorcinols in both wheat and rye (6–8). Therefore, alkylresorcinols have been suggested as biomarkers for the intake of WG wheat and rye. Under stable and frequent WG wheat and rye intake, alkylresorcinols can be considered as short-term biomarkers: estimated apparent half-lives and absorption half-lives in plasma are \(\sim 5\) h and \(\sim 6–8\) h, respectively (9). Recently, alkylresorcinols’ long-term reproducibility has been investigated in Sweden and was shown to reflect long-term exposure of WG wheat and rye.
It has been suggested that the metabolic pathway of alkylresorcinols is similar to that of tocopherols and is initiated by a cytochrome P450-mediated \( \omega \)-oxidation of the alkyl side chain, forming hydroxylated alkylresorcinols (11). They are further oxidized to produce carboxylated alkylresorcinols; subsequently, the side chain of alkylresorcinols is shortened by \( \beta \)-oxidation, yielding hydrophilic metabolites (11). A recent study was able to detect trifluoroacetic acid derivatives of carboxylated alkylresorcinol C19:0 and di-trifluoroacetic acid derivatives of hydroxylated alkylresorcinol C19:0 after incubation of alkylresorcinol C19:0 with human liver S9 fraction for 4 h, suggesting the degradation of alkylresorcinols to phenolic acid via cytochrome P450-mediated \( \omega \)-oxidation and subsequent \( \beta \)-oxidation (12). Alkylresorcinol metabolism in humans still remains largely unknown. At this time, there are only 2 phenolic acids, 3,5-dihydroxybenzoic acid (3,5-DHBA) and 3-(3,5-dihydroxyphenyl)propanoic acid (3,5-DHPPA), that have been identified as the major metabolites of alkylresorcinols in humans (13). Many studies have suggested that both plasma and urinary alkylresorcinol metabolites may serve as the exposure biomarkers of WG wheat and rye intake (13–19). Both 3,5-DHBA and 3,5-DHPPA showed longer apparent half-lives (10–12 h) when compared with their parent alkylresorcinols, suggesting that they may better reflect longer-term WG wheat and rye intake (18–20).

Cereal grains are understood to be the major sources of alkylresorcinol metabolites 3,5-DHBA and 3,5-DHPPA; however, these compounds have been characterized from alternative food sources. Low concentrations of 3,5-DHBA have been identified as the major metabolites of alkylresorcinols in humans (13). Many studies have suggested that both plasma and urinary alkylresorcinol metabolites may serve as the exposure biomarkers of WG wheat and rye intake (13–19). Both 3,5-DHBA and 3,5-DHPPA showed longer apparent half-lives (10–12 h) when compared with their parent alkylresorcinols, suggesting that they may better reflect longer-term WG wheat and rye intake (18–20).

| TABLE 1 | Nutrient contents and amounts of alkylresorcinols in the WG wheat bread and in the RG wheat bread used in this study† |
|------------------|------------------|------------------|------------------|------------------|
| Chemical composition | Per 100 g (dry weight) | Per test dose (dry weight) | |
| Carbohydrate, g | 79 | 66 | 104 | 91 |
| Fat, g | 9.2 | 5.1 | 12 | 7.0 |
| Protein, g | 24 | 10 | 32 | 14 |
| Dietary fiber, g | 12 | 5.1 | 16 | 7.0 |
| Energy, kJ | 2300 | 1573 | 3014 | 2171 |
| WG, g | 85 | — | 112 | — |
| Total alkylresorcinols ingested, mg | 47 | 0.97 | 61 (100) | 1.34 (100) |
| C17:0 | 1.8 | 0.070 | 2.4 (3.3) | 0.096 (7.1) |
| C19:0 | 16 | 0.99 | 21 (34.0) | 0.40 (30.0) |
| C21:0 | 22 | 0.41 | 28 (46.1) | 0.57 (42.4) |
| C23:0 | 5.5 | 0.11 | 7.2 (11.7) | 0.15 (11.3) |
| C25:0 | 2.1 | 0.087 | 2.7 (4.3) | 0.12 (9.2) |

† All analytical data except for alkylresorcinols in WG wheat bread and RG wheat bread are from Flowers Food, Inc. (Nature’s Own 100% Whole Wheat and Sunbeam Old Fashioned bread). RG, refined-grain; WG, whole-grain; —, not indicated in ingredients of RG wheat bread.

1 Calculated based on the amount of carbohydrates, fats, and proteins in the bread.

2 Relative compositions (wt%) of alkylresorcinol homologs are shown in parentheses.
urine samples were collected in plastic specimen containers, refrigerated during collection, returned to the laboratory every morning, and stored at 4°C. Hydrochloric acid (20%) was added to urine samples (0.4% of total urine volume). Samples were then portioned into aliquots and stored at −80°C until analysis.

**Treatment of mice and sample collection.** Experiments with mice were carried out according to protocols approved by the institutional review board for the Animal Care and Facilities Committee at North Carolina Central University. In brief, female C37BL/6J mice were purchased from Jackson Laboratory and allowed to acclimate for at least 1 wk before the start of the experiment. The mice were housed 5 per cage and maintained in air-conditioned quarters with a room temperature of 20 ± 2°C, relative humidity of 50 ± 10%, and an alternating 12-h light/dark cycle. Mice were fed Rodent Chow 5001 (LabDiet; Supplemental Table 1) and water and were allowed to eat and drink ad libitum. Alkyresorcinol C19:0 or C21:0 in DMSO were administered to mice by oral gavage (200 mg/kg). Urine and fecal samples were collected in metabolism cages (5 mice/cage) over 24 h after administration of vehicle (control group, n=5), alkylresorcinol C19:0 (treated group, n=5), or alkylresorcinol C21:0 (treated group, n=5). The samples were stored at −80°C until analysis.

**Urine and fecal sample preparation.** For acquisition of the metabolic profile, the urine samples [100 μL from each time point (human samples) or each group (mouse samples)] were added to 100 μL of methanol to precipitate proteins. After centrifugation at 17,000 × g for 5 min, the supernatant was transferred into vials for liquid chromatography (LC–MS) analysis. For quantifying the concentrations of the metabolites of alkylresorcinols in human urine, the samples were prepared by using the method of Koskela et al. (30), protocol A, with slight modification. Briefly, 198 ng of syringic acid was used as an internal standard. Urine and fecal samples (100 μL) were treated with 300 μL PBS (50 mmol/L, pH 6.8) containing β-glucuronidase (250 U) and sulfatase (3 U) for 24 h at 37°C. After incubation, the medium was diluted 5 times by adding methanol containing 0.2% acetic acid (AA). The resulting solution was centrifuged, and 10 μL of supernatant was analyzed by HPLC–coulometric electrode array detection. The mouse fecal samples were prepared as previously described (31).

**Chemical synthesis.** To determine the identities of 5-(3,5-dimethoxyphenyl)pentanoic acid (3,5-DHPPTA) and 2-(3,5-dihydroxybenzamido)acetic acid (3,5-DHBA, and 3,5-DHPPA), authentic standards were synthesized as described in the Supplemental Methods. In brief, a Wittig reaction of 3,5-dimethoxybenzaldehyde with (4-ethoxy-4-oxobutyl) triphenylphosphonium bromide followed by reduction by H2 produced methyl 5-(3,5-dimethoxyphenyl)pentanoate, which was demethylated and subsequently subjected to base hydrolysis to yield 3,5-DHPPTA (Supplemental Fig. 1A). 3,5-DHBA glycine was synthesized by amide coupling of 3,5-DHBA with 2-methoxy-2-oxoethanamine chloride followed by base hydrolysis (Supplemental Fig. 1B).

**LC-MS analysis.** LC-MS analysis was carried out with a Thermo Finnigan Spectra System, which consisted of an Accela high-speed MS pump, an Accela refrigerated autosampler, and an LCQ Fleet ion trap mass detector (Thermo Electron) incorporated with an electrospray ionization (ESI) interface or an atmospheric pressure chemical ionization interface. A Gemini-NX C18 column (150 × 3.0 mm i.d., 5 μm; Phenomenex) was used to analyze human urine, mouse urine, and mouse fecal samples with a flow rate of 0.3 mL/min. The binary mobile phase system consisted of water with 0.2% AA as phase A and methanol with 0.2% AA as phase B. The column was eluted by a gradient progress (0% B from 0 to 5 min; 0% to 40% B from 5 to 20 min; 40–80% B from 20 to 30 min; 80–100% B from 30 to 40 min; 100% B from 40 to 55 min, and 0% B from 55 to 65 min). The injection volume was 10 μL for each sample. The collision-induced dissociation was conducted with an isolation width of 2 Da and normalized collision energy of 35 for MS/MS analysis. The mass range was measured from 50 to 1000 m/z. Data acquisition was performed with Xcalibur version 2.0 (Thermo Electron). HPLC-ESI/MS/MS data for alkyresorcinol metabolites (1-10) in mice and in humans are listed in Supplemental Table 2.

**HPLC analysis.** An HPLC-coulometric electrode array detection system (ESA Laboratories) consisting of an ESA model 584 HPLC pump, an ESA model 542 autosampler, an ESA organizer, and an ESA coularray detector coupled with 2 ESA model 6210 4-sensor cells was used in the current study. Alkyresorcinol contents in WG wheat bread, RG wheat bread, or rodent diet were analyzed as previously described (32) with a modified gradient program: 90% B from 0 to 5 min, 90–100% B from 5 to 18 min, 100% B from 18 to 28 min, and 90% B from 28.1 to 33 min. A Gemini C18 column (150 × 4.6 mm, 5 μm; Phenomenex) was used to analyze at a flow rate of 1.0 mL/min. The concentrations of alkylresorcinol metabolites in human urine were measured as previously described (18) but with a modified gradient program: 5% B from 0 to 10 min at a flow rate of 0.5 mL/min, 5–20% B from 10 to 15 min with an increasing flow rate ranging from 0.5 to 0.8 mL/min, 20–100% B from 15 to 48 min at a flow rate of 0.8 mL/min, 100% B from 48 to 53 min, and 5% B from 53.2 to 60 min. The analytical column was a Gemini C18 column (150 × 4.6 mm, 5 μm; Phenomenex). The injection volume of the sample was 10 μL. 3,5-DHBA and 3,5-DHPHA were quantitated at 600 nm, 3,5-DHPTPA and 3,5-DHPA glycine at 670 nm, and syringic acid at 380 nm (Supplemental Fig. 2). The limits of detection (LODs) were 6, 10, 25, and 2 pg per injection for 3,5-DHPTPA, 3,5-DHBA glycine, 3,5-DHBA, and 3,5-DHPHA, respectively; and the limits of quantification for these 4 metabolites were 18, 31, 77, and 7 pg per injection, respectively. The linearity for 3,5-DHPTPA, 3,5-DHBA glycine, 3,5-DHBA, and 3,5-DHPHA ranged from the LOD to 65,000 pg per injection (0.5–6500 μg/L; the upper limit was not tested). The correlation coefficients were 0.999 for the 4 metabolites, respectively. Eight-point calibration curves (2–220 μg/L for 3,5-DHPTPA and 3,5-DHBA glycine and 15–2000 μg/L for 3,5-DHBA and 3,5-DHPHA) were used. The precisions for the 4 metabolites are presented in Supplemental Table 3. The recovery of all added concentrations for the 4 metabolites is shown in Supplemental Table 4.

**Pharmacokinetics.** The highest excretion rates of 3,5-DHPTPA, 3,5-DHBA glycine, 3,5-DHBA, and 3,5-DHPHA were quantitated from baseline (0 h) to 32 h after WG wheat bread intake were defined as maximum excretion rate (ERmax, μmol/h) and time to reach ERmax (tmax). The apparent half-life (t1/2) was defined as the time at which the metabolite urinary excretion rate decreased to half of the ERmax.

**Statistical analysis.** All data are given as means ± SEMs unless otherwise stated. The pharmacokinetic parameters ERmax, tmax, and AUC for 3,5-DHPTPA, 3,5-DHBA glycine, 3,5-DHBA, and 3,5-DHPHA were conducted by using GraphPad Prism software (version 5), and an unpaired t test was used to determine potential differences between 2 groups. One-factor ANOVA followed by the Brown-Forsythe test was used to test for differences between groups. For correlation analysis, Spearman rank correlation coefficients and partial correlations were calculated. P < 0.05 was considered to indicate significance.

**Results**

**Identification of alkyresorcinol metabolites.** By using LC-MS, both phase I and phase II metabolites of alkyresorcinols were searched from 24-h urine and fecal samples collected from mice treated with purified alkyresorcinol C19:0, alkyresorcinol C21:0, or vehicle and urine samples collected from human participants after either a RG wheat bread or a WG wheat bread breakfast. As a result, 10 metabolites (1–10) were identified, including 3 phenolic acids (1, 5, and 8) and 7 phase II conjugates (2-4, 6, 7, 9, and 10) (Supplemental Table 2, Supplemental Fig. 3). Among the 3 phenolic acids, metabolites 1 and 5 were identified as 3,5-DHBA and 3,5-DHPA by comparing their retention times and tandem mass spectra with those of authentic 3,5-DHBA and 3,5-DHPA (Fig. 1). Metabolite 8 had a...
molecular ion at m/z 209 [Molecular weight (M)-H]− under negative ESI/MS mode, which was 28 mass units higher than that of 3,5-DHPPA, indicating that 2 more –CH₂ groups were present in the structure of metabolite 8 than in 3,5-DHPPA. In addition, similar to 3,5-DHBA and 3,5-DHPPA, metabolite 8 had the fragment ion that lost 1 –COOH group at m/z 165 (M-COOH-H)² (Fig. 1). All of these spectra features suggested that metabolite 8 is 3,5-DHPPTA, an analog of phenolic acids 3,5-DHBA and 3,5-DHPPA. To further confirm the structure of metabolite 8, we synthesized 3,5-DHPPTA (Supplemental Fig. 1A) and confirmed its structure by analyzing the ¹H and ¹³C NMR spectra (Supplemental Methods). Metabolite 8 had the same retention time as well as the same molecular mass and fragment ion mass spectra as those of the synthetic 3,5-DHPPTA (Fig. 1). Therefore, metabolite 8 was identified as 3,5-DHPPTA (Supplemental Fig. 3). 3,5-DHPPTA was identified as a major metabolite in C19:0-treated mouse urine and a minor metabolite in C21:0-treated mouse urine but was not detectable in vehicle-treated control mice after enzymatic deconjugation (Supplemental Table 2), indicating that 3,5-DHPPTA is a metabolite of alkylresorcinols. Detection of 3,5-DHPPTA only in WG-treated human urine but not in RG-treated human urine coincided with the finding from the mouse study, unambiguously supporting 3,5-DHPPTA as a new metabolite of alkylresorcinols. To our knowledge, this is the first report of 3,5-DHPPTA as a metabolite of alkylresorcinols. On the other hand, 3,5-DHPPA was found in abundance in both C19:0- and C21:0-treated mice, and 3,5-DHBA was detectable in large quantities only in C19:0-treated mice (Supplemental Table 2). However, both 3,5-DHBA and 3,5-DHPPA were detectable in vehicle-treated control mouse urine probably due to the unpurified diets used in this study containing wheat (Supplemental Table 1), and despite having deprived the mice of food overnight.

The current study further investigated the phase II metabolites of 3,5-DHBA, 3,5-DHPPA, and 3,5-DHPPTA in both human and mouse urine. Selected-ion monitoring mode was used to search the potential mono-glucuronidated (M + 176), di-glucuronidated (M + 176 × 2), mono-sulfated (M + 80), di-sulfated (M + 80 × 2), and mono-glucuronidated and mono-sulfated (M + 176 + 80) phase II metabolites of these 3 phenolic acids. Only mono-glucuronidated or mono-sulfated 3,5-DHBA, 3,5-DHPPA, and 3,5-DHPPTA (2, 3, 6, 7, 9, and 10) were detectable in human or mouse urine (Supplemental Table 2). Their structures were confirmed on the basis of analysis of their tandem mass spectra. LC-MS data are summarized in Supplemental Table 2. In addition to glucuronidated and sulfated

**FIGURE 1** LC chromatograms and MS² (MS/MS) spectra of 3,5-DHPPTA (A), 3,5-DHBA glycine (B), 3,5-DHBA (C), and 3,5-DHPPA (D) in WG wheat bread–treated human urine and alkylresorcinol C19:0-treated mouse urine after enzyme deconjugation as well as corresponding authentic standards obtained by negative ESI/MS interface. ESI, electrospray ionization; LC, liquid chromatography; WG, whole-grain; 3,5-DHBA, 3,5-dihydroxybenzoic acid; 3,5-DHBA glycine, 2-(3,5-dihydroxybenzamido)acetic acid; 3,5-DHPPA, 3-(3,5-dihydroxyphenyl)propanoic acid; 3,5-DHPPTA, 5-(3,5-dihydroxyphenyl)pentanoic acid.
conjugates, metabolites generated from glycine conjugation (M + 57) were also searched in this study. 3,5-DHBA glycine (metabolite 4) was the only glycine conjugate detected in human urine but not in mouse urine under the LOD of our LC-MS (Fig. 1B). The peak at m/z 210 (M-H) in mass spectrum of metabolite 4 was 57 mass units higher than that of 3,5-DHBA, corresponding to a glycine with 1 H2O loss, suggesting that metabolite 4 is the mono-glycine-conjugated 3,5-DHBA. Similar to 3,5-DHBA, metabolite 4 had a major daughter ion at m/z 166 (M-COOH-H) (Supplemental Table 2, Fig. 1B), indicating that metabolite 4 also possessed a carboxylic acid group. To further confirm the structure of metabolite 4, 3,5-DHBA glycine was synthesized in-house, and its structure was confirmed by analyzing its 1H and 13C NMR data (Supplemental Methods). Metabolite 4 had an almost identical retention time as well as the same molecular mass and fragment ion mass spectra as those of the synthetic 3,5-DHBA glycine (Fig. 1B). Adding the authentic standard into WG-treated human urine further confirmed that metabolite 4 was identical to synthetic 3,5-DHBA glycine (Supplemental Fig. 4). Therefore, metabolite 4 was confirmed to be 3,5-DHBA glycine, which is a novel metabolite of alkylresorcinols. There was no mono-glucuronidated or mono-sulfated 3,5-DHBA glycine detected in samples collected from this study under our LC-MS conditions.

Both alkylresorcinol C19:0 and C21:0 were present in mouse feces in their intact form. Their structures were confirmed by comparison with the authentic standards that we purified from wheat bran (Supplemental Fig. 5). However, no alkylresorcinol metabolites were found in mouse feces under the LC-MS conditions used in this study. In addition, no intact or conjugated alkylresorcinol C19:0 or C21:0 was detected in urine samples collected from mice treated with purified alkylresorcinol C19:0 or C21:0. Only a small amount of alkylresorcinol C19:0 was detected and confirmed in human urine samples collected at 12–24 h after consumption of WG wheat bread (data not shown).

Pharmacokinetics of alkylresorcinol metabolites 3,5-DHPPTA, 3,5-DHBA glycine, 3,5-DHBA, and 3,5-DHPPA in human urine. Total alkylresorcinol contents in WG wheat bread (131 g dry weight) and RG wheat bread (138 g dry weight) were 61 and 1.34 mg, respectively. The mean urinary excretion rates of the 4 metabolites 3,5-DHPPTA, 3,5-DHBA glycine, 3,5-DHBA, and 3,5-DHPPA at each time point for the whole group who consumed WG wheat bread (n = 12) are presented in Fig. 2, and the pharmacokinetic data are shown in Table 2. The excretion rates of these 4 metabolites were calculated on the basis of the quantitative urine collected and expressed as μmol/h. The mean baseline (0 h) urinary excretion rate for the new metabolite 3,5-DHPPTA was zero, and the rates for the rest of metabolites (3,5-DHBA glycine, 3,5-DHBA, and 3,5-DHPPA) were also low (0.0076 ± 0.004, 0.0089 ± 0.003, and 0.0092 ± 0.002 μmol/h, respectively), indicating that all participants had avoided cereal bran–related products and foods containing phenolic compounds before the study. Two hours after ingestion of WG wheat bread, urinary excretion rates increased rapidly, and every participant reached ERmax fairly simultaneously for the 4 metabolites (Supplemental Fig. 6) based on the mean tmax of 6.6 ± 0.5 h for 3,5-DHPPTA, 9.5 ± 0.5 h for 3,5-DHBA glycine, 8.3 ± 0.6 h for 3,5-DHBA, and 7.4 ± 0.6 h for 3,5-DHPPA (P = 0.78) (Table 2). Eleven of the 12 participants reached ERmax for DHPPA between 6 and 9 h, whereas 1 participant reached ERmax at 4 h; 9 of the 12 participants reached ERmax for 3,5-DHBA glycine between 6 and 9 h, whereas 3 reached ERmax at 12 h (Supplemental Fig. 6). Most of the participants reached ERmax between 6 and 9 h for 3,5-DHBA and 3,5-DHPPA (Supplemental Fig. 6). Excretion rates decreased gradually after ERmax, and the mean t1/2 for 3,5-DHBA glycine and 3,5-DHPPA was determined at 11.1, 16.6, 15.9, and 14.8 h, respectively (P = 0.96). The final (32 h) mean urinary excretion rates for the 4 metabolites [3,5-DHPPTA (0.0041 ± 0.002 μmol/h), 3,5-DHBA glycine (0.037 ± 0.008 μmol/h), 3,5-DHBA (0.18 ± 0.05 μmol/h), and 3,5-DHPPA (0.28 ± 0.03 μmol/h)] were significantly different from baseline (P < 0.01 for all). No significant differences were observed between women and men in any of the pharmacokinetic parameters for 3,5-DHBA glycine, 3,5-DHBA, and 3,5-DHPPA (P > 0.10 for all) (Table 2). Significant differences between sexes in ERmax (P = 0.03) and AUC (P = 0.037) of 3,5-DHPPTA, respectively, however, were observed in this current study (Table 2). None of the pharmacokinetic parameters were significantly associated with age (P > 0.40 for all) or BMI (P > 0.08 for all). The mean total urinary recovery of the 4 metabolites at 32 h was 35.0% calculated from the ingested alkylresorcinols. The relative composition of the 4

![FIGURE 2 Urinary pharmacokinetics of 3,5-DHPPTA (A), 3,5-DHBA glycine (B), 3,5-DHBA (C), and 3,5-DHPPA (D) between baseline (0 h) and 32 h after a single intake of WG wheat bread containing 61 mg of alkylresorcinols (n = 12). Values are means ± SEMs. RG, refined-grain; WG, whole-grain; 3,5-DHBA, 3,5-dihydroxybenzoic acid; 3,5-DHBA glycine, 2-(3,5-dihydroxybenzamido)acetic acid; 3,5-DHPPA, 3-(3,5-dihydroxyphenyl)propanoic acid; 3,5-DHPPTA, 5-(3,5-dihydroxyphenyl)pentanoic acid.](https://example.com/figure2.png)
The concentrations of these 4 metabolites in the urine samples collected from the same group of participants (n = 12) who consumed RG wheat bread were also analyzed. Their mean urinary excretion rates at each time point are also presented in Fig. 2. Our results clearly showed that the excretion rates of these 4 metabolites dramatically increased after WG wheat bread consumption, suggesting that all 4 compounds are the metabolites of alkylresorcinols.

**Discussion**

The aim of this study was to further investigate alkylresorcinol metabolism and to determine urine pharmacokinetics of novel alkylresorcinol metabolites to explore their potential as biomarkers of WG wheat intake. Studies have suggested that the metabolic pathway of alkylresorcinols is similar to that of tocopherols. Several intermediates generated from the \( \omega \)-oxidation and \( \beta \)-oxidation of tocopherols have been reported as the metabolites of tocopherols (31). Unlike tocopherols, only 2 small phenolic acids, 3,5-DHBA and 3,5-DHPPA, have been proposed as the metabolites of alkylresorcinols that follow the same metabolic route. Recently, 3,5-dihydroxyxynamic acid and 3,5-dihydroxyphenylethanol have been tentatively proposed as the metabolites of 3,5-DHPPA (33). At the time of this study, direct evidence for the degradation of alkylresorcinols to 3,5-DHBA and 3,5-DHPPA, however, was not sufficient. Ross et al. (13) first proposed 3,5-DHBA and 3,5-DHPPA as the major alkylresorcinol metabolites in humans in 2004. Several studies have found that the concentrations of 3,5-DHBA and 3,5-DHPPA significantly increased after WG consumption (16,34). A recent in vitro study reported that hepatic biotransformation of alkylresorcinol C19:0 formed the hydroxylated C19:0 (OH-C19) and carboxylated C19:0 (COOH-C19) metabolites after incubation with human cytochrome P450 4F2 or human liver subcellular fraction S9 (12). This result seemed to clearly support the proposed metabolic pathway by which alkylresorcinols are degraded to phenolic acids via \( \omega \)-oxidation and subsequent \( \beta \)-oxidation, referred to as \( \omega \)-tocopherol metabolism (31), even though the 2 new oxidized metabolites, OH-C19 and COOH-C19, were tentatively identified by GC-MS due to the lack of authentic standards. To further study alkylresorcinol metabolism, a targeted LC-MS approach was used in this study. Ten alkylresorcinol metabolites were identified, including 2 novel metabolites, 3,5-DHPPTA and 3,5-DHPPA, in humans and in mice. 3,5-DHPPA was identified as a novel metabolite of alkylresorcinols from urine samples collected from mice treated with either pure alkylresorcinol C19:0 or C21:0 as well as from human urine samples collected from participants after consumption of WG wheat bread. 3,5-DHBA glycine was detected only from human urine samples but was beyond the limits of detection in mouse urine. 3,5-DHPPTA was identified as a novel metabolite of alkylresorcinols from urine samples collected from mice treated with either pure alkylresorcinol C19:0 or C21:0 as well as from human urine samples collected from participants after consumption of WG wheat bread. 3,5-DHBA glycine was detected only from human urine samples but was beyond the limits of detection in mouse urine. 3,5-DHPPTA was also confirmed by using authentic standards synthesized in-house. To further study alkylresorcinol metabolism, a targeted LC-MS approach was used in this study. Ten alkylresorcinol metabolites were identified, including 2 novel metabolites, 3,5-DHPPTA and 3,5-DHPPA, in humans and in mice. 3,5-DHPPA was identified as a novel metabolite of alkylresorcinols from urine samples collected from mice treated with either pure alkylresorcinol C19:0 or C21:0 as well as from human urine samples collected from participants after consumption of WG wheat bread. 3,5-DHBA glycine was detected only from human urine samples but was beyond the limits of detection in mouse urine. 3,5-DHBA glycine was detected only from human urine samples but was beyond the limits of detection in mouse urine.

**TABLE 2** Pharmacokinetic parameters of the 4 alkylresorcinol metabolites (3,5-DHPPTA, 3,5-DHBA glycine, 3,5-DHBA, and 3,5-DHPPA) in human urine samples after a single intake of WG wheat bread containing 61 mg of alkylresorcinols

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<thead>
<tr>
<th>Metabolite</th>
<th>( \text{ER}_{\text{max}} ) (( \mu \text{mol/h} ))</th>
<th>( t_{\text{max}} ) (h)</th>
<th>AUC (( \mu \text{mol/32 h} ))</th>
<th>( t_{1/2} ) (h)</th>
<th>Recovery of ingested alkylresorcinols</th>
<th>Relative composition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3,5-DHPPA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All (n = 12)</td>
<td>0.20 ± 0.04</td>
<td>6.6 ± 0.5</td>
<td>1.8 ± 0.3</td>
<td>11.1 ± 0.8</td>
<td>1.2 ± 0.2</td>
<td>3.8 ± 0.6</td>
</tr>
<tr>
<td>Women (n = 6)</td>
<td>0.12 ± 0.03</td>
<td>6.7 ± 0.8</td>
<td>1.3 ± 0.2</td>
<td>11.2 ± 1.5</td>
<td>0.78 ± 0.2</td>
<td>2.8 ± 0.5</td>
</tr>
<tr>
<td>Men (n = 6)</td>
<td>0.28 ± 0.06(^2)</td>
<td>6.5 ± 0.5</td>
<td>2.4 ± 0.4(^2)</td>
<td>10.9 ± 0.7</td>
<td>1.6 ± 0.2</td>
<td>4.9 ± 0.8</td>
</tr>
<tr>
<td>3,5-DHBA glycine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All (n = 12)</td>
<td>0.24 ± 0.05</td>
<td>9.5 ± 0.5</td>
<td>3.1 ± 0.5</td>
<td>16.6 ± 1.0</td>
<td>2.0 ± 0.3</td>
<td>6.8 ± 0.8</td>
</tr>
<tr>
<td>Women (n = 6)</td>
<td>0.20 ± 0.04</td>
<td>9.0 ± 0.0</td>
<td>2.8 ± 0.6</td>
<td>16.5 ± 1.7</td>
<td>1.8 ± 0.4</td>
<td>6.6 ± 1.2</td>
</tr>
<tr>
<td>Men (n = 6)</td>
<td>0.28 ± 0.08</td>
<td>10.0 ± 1.0</td>
<td>3.5 ± 0.7</td>
<td>16.7 ± 1.1</td>
<td>2.3 ± 0.5</td>
<td>7.0 ± 1.2</td>
</tr>
<tr>
<td>3,5-DHBA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All (n = 12)</td>
<td>0.99 ± 0.08</td>
<td>8.3 ± 0.6</td>
<td>15.2 ± 1.4</td>
<td>15.9 ± 0.8</td>
<td>9.8 ± 0.9</td>
<td>24.5 ± 1.9</td>
</tr>
<tr>
<td>Women (n = 6)</td>
<td>1.0 ± 0.2</td>
<td>8.5 ± 0.5</td>
<td>15.2 ± 2.1</td>
<td>14.7 ± 1.2</td>
<td>9.8 ± 1.4</td>
<td>27.0 ± 3.2</td>
</tr>
<tr>
<td>Men (n = 6)</td>
<td>0.94 ± 0.07</td>
<td>8.2 ± 1.1</td>
<td>15.2 ± 1.9</td>
<td>17.2 ± 1.0</td>
<td>9.8 ± 1.2</td>
<td>21.9 ± 1.8</td>
</tr>
<tr>
<td>3,5-DHPPA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All (n = 12)</td>
<td>2.5 ± 0.2</td>
<td>7.4 ± 0.6</td>
<td>34.0 ± 2.4</td>
<td>14.8 ± 1.0</td>
<td>22.0 ± 1.5</td>
<td>65.0 ± 1.9</td>
</tr>
<tr>
<td>Women (n = 6)</td>
<td>2.1 ± 0.3</td>
<td>7.2 ± 0.9</td>
<td>30.2 ± 4.2</td>
<td>14.8 ± 1.7</td>
<td>19.5 ± 2.7</td>
<td>63.8 ± 3.0</td>
</tr>
<tr>
<td>Men (n = 6)</td>
<td>2.8 ± 0.2</td>
<td>7.7 ± 0.9</td>
<td>37.9 ± 0.8</td>
<td>14.8 ± 1.3</td>
<td>24.5 ± 0.5</td>
<td>66.2 ± 2.6</td>
</tr>
</tbody>
</table>

\(^1\) All values are means ± SEMs. Differences between each metabolite were evaluated by 1-factor ANOVA. Correlations with ages or BMI were conducted by Spearman rank correlation coefficients. ER\(_{\text{max}}\), maximum excretion rate; \( t_{\text{max}} \), time to reach maximum excretion rate; \( t_{1/2} \), apparent half-life; WG, whole grain; 3,5-DHBA, 3,5-dihydroxybenzoic acid; 3,5-DHBA glycine, 2-(3,5-dihydroxybenzamido)acetic acid; 3,5-DHPPA, 3,5-dihydroxyphenopropanoic acid; 3,5-DHPPA, 5-(3,5-dihydroxyphenyl)pentanoic acid.

\(^2\) Significantly different from women, \( P \leq 0.03 \) (unpaired t test); however, differences were not significant after correction for BMI.

metabolites was as follows: 3,5-DHPPTA (3.8%), 3,5-DHBA glycine (6.8%), 3,5-DHBA (24.5%), and 3,5-DHPPA (65.0%) (Table 2).

The concentrations of these 4 metabolites in the urine samples collected from the same group of participants (n = 12) who consumed RG wheat bread were also analyzed. Their mean urinary excretion rates at each time point are also presented in Fig. 2. Our results clearly showed that the excretion rates of these 4 metabolites dramatically increased after WG wheat bread consumption, suggesting that all 4 compounds are the metabolites of alkylresorcinols.
alkylresorcinols were not detected in this study. This may be due to the fact that these intermediates are not stable and can be rapidly further metabolized to generate small phenolic acids: 3,5-DHPPTA, 3,5-DHPPA, and 3,5-DHBA. In addition, both 3,5-DHBA and 3,5-DHPPA were identified as the metabolites of alkylresorcinol C19:0 and C21:0 via direct in vivo evidence, which is consistent with Marklund et al.’s (35) in vivo results reporting that 3,5-DHBA and 3,5-DHPPA are the metabolites of alkylresorcinols in rats.

Next, the specificity of 3,5-DHPPTA was investigated. With a longer side chain than that of 3,5-DHBA or 3,5-DHPPA, it is unlikely that 3,5-DHPPTA would have multiple sources. Only 1 study has suggested that 3,5-DHPPTA could be the metabolite of tea polyphenol (-)-epigallocatechin 3-gallate in vitro and in the feces of rats that received oral administration of (-)-epigallocatechin 3-gallate (36). However, 3,5-DHPPTA was not reported as the metabolite of tea polyphenols in humans (37,38).

We further confirmed this by searching for 3,5-DHPPTA in human urine samples collected at 0–2, 2–6, 6–9, 9–12, and 12–24 h after consumption of 3 g of tea polyphenol powder (1 g of powder derived from 6 g of dry green tea leaves), which is equal to 6–9 cups of tea. 3,5-DHPPTA was not detectable in these human urine samples (data not shown). Therefore, 3,5-DHPPTA has the potential to be used as a unique metabolite of alkylresorcinols to reflect WG wheat and rye intake. However, 3,5-DHBA and 3,5-DHPPA were excreted into human urine in a greater amount than the novel metabolites 3,5-DHPPTA and 3,5-DHBA glycine (Table 2). It is unlikely that these 2 novel metabolites alone can be used as exposure markers for occasional consumption of WG wheat and rye products, but it is likely that they can be used as exposure markers for habitual WG wheat and rye consumption. It is estimated that the average per capita intake of alkylresorcinols in Finland and Denmark is 39.8 and 37.1 mg/d, respectively, which is close to the dose used in this study (39). These 2 novel metabolites may be used in combination with 3,5-DHBA and 3,5-DHPPA as potential biomarkers to increase the accuracy of recording WG wheat and rye intake in epidemiologic studies.

Studies have shown that both urinary and plasma 3,5-DHBA and 3,5-DHPPA can be used as biomarkers for WG wheat and rye intake (18,19). Soderholm et al. (18) reported that t1/2 for 3,5-DHBA and 3,5-DHPPA was 9.9 and 11.9 h, respectively, and that tmax was 6.0 h for 3,5-DHBA and 5.4 h for 3,5-DHPPA in human urine after a single intake of rye bread. In the present study, 3,5-DHBA and 3,5-DHPPA showed longer t1/2 (15.9 h for 3,5-DHBA and 14.8 h for 3,5-DHPPA) and tmax (8.3 h for 3,5-DHBA and 7.4 h for 3,5-DHPPA) than those mentioned above. Many confounding factors can be considered for such a
difference, such as genetic differences between the participants of these 2 studies and the differences between chemical compositions of alkylresorcinols in wheat and rye. Our results also showed that the new metabolites 3,5-DHPPTA and 3,5-DHBA glycine had t_{1/2} (11.1 h for 3,5-DHPPTA and 16.6 h for 3,5-DHBA glycine) and t_{max} (6.3 h for 3,5-DHPPTA and 9.5 for 3,5-DHBA glycine) similar to those of 3,5-DHBA and 3,5-DHPPA (Table 2), suggesting that these 2 new metabolites may also serve as the exposure biomarker for WG wheat and rye consumption. Further investigation of plasma pharmacokinetics regarding 3,5-DHPPTA and 3,5-DHBA glycine must be conducted to shed light on the possibility that these 2 new alkylresorcinol metabolites could be used in combination with 3,5-DHBA and 3,5-DHPPA to increase the accuracy of WG intake estimation.

Our study had several limitations. First of all, this was a human feeding study with dietary restriction. The dose of alkylresorcinols (61 mg) used in this study is relatively high as a single dose but yet is achievable as a daily intake of alkylresorcinols in a WG habitual diet among consumers. Further research is needed to determine whether 3,5-DHPPTA and 3,5-DHBA glycine can be used as an exposure marker of WG wheat and rye intake in free-living populations. Second, the large variation between individuals can also be considered to limit the present study. Thus, a larger group of individuals would allow a more exact analysis of the mean t_{1/2} and t_{max} values. In addition, only urine samples were collected in this study. It would be worthwhile to determine the pharmacokinetics of 3,5-DHPPTA and 3,5-DHBA glycine in plasma.

In conclusion, the study of the metabolism of alkylresorcinols in mice and in humans led to the identification of 10 metabolites, including 2 novel metabolites, 3,5-DHPPTA and 3,5-DHBA glycine. The discovery of the new alkylresorcinol metabolite 3,5-DHPPTA in mice and in humans is consistent with the proposed metabolic pathway of alkylresorcinols, whereas the finding of the novel 3,5-DHBA glycine in humans at least broadens the possible metabolic pathway of alkylresorcinols. The pharmacokinetic study of 3,5-DHPPTA, 3,5-DHBA glycine, 3,5-DHBA, and 3,5-DHPPA showed that both 3,5-DHPPTA and 3,5-DHBA glycine have pharmacokinetic parameters similar to those of 3,5-DHBA and 3,5-DHPPA, indicating that they may be used as potential biomarkers to precisely evaluate the intake of WG wheat and rye in epidemiologic studies. However, this needs to be further validated in free-living populations.

Acknowledgments
S.S. designed the research and had primary responsibility for the final content; S.S., Y.Z., K.L.S., and X.C. conducted the research; S.S., Y.Z., and K.L.S. analyzed the data; and S.S., Y.Z., and K.L.S. wrote the manuscript. All authors read and approved the final manuscript.

Literature Cited


