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Quantification of Alkylresorcinol Metabolites in Plasma by High-Performance Liquid Chromatography with Coulometric Electrode Array Detection

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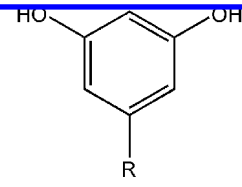
This study presents the optimization and validation of a rapid protocol for quantifying alkyresorcinol (AR) metabolites 3,5-dihydroxybenzoic acid (DHBA) and 3-(3,5-dihydroxyphenyl)-1-propanoic acid (DHPPA) in plasma, using high-performance liquid chromatography (HPLC) coupled with a coulometric electrode array detector. Syringic acid (SyrA) serves as the internal standard. The new method is simple and could be used in large epidemiological studies. The summed AR metabolite concentrations measured in plasma correlate significantly with the summed urinary AR metabolite concentrations ($R = 0.613$; $p < 0.001$) and with the summed intact AR (C17:0−C25:0) concentrations in plasma ($R = 0.686$; $p < 0.001$). Additional investigation is needed to clarify whether the two plasma AR metabolites are useful as biomarkers of whole-grain intake and helpful in the exploration of the association between whole-grain cereal intake and human diseases.

INTRODUCTION

Epidemiological studies indicate that the consumption of whole-grain cereal products is beneficial, having health-promoting effects and reducing the risk of several chronic diseases (*1, 2*). Whole-grain rye, wheat, and triticale cereals contain high amounts of alkylresorcinols (ARs), which are phenolic lipids. Chemically ARs are 1,3-dihydrobenzene homologues, with an odd-numbered, mainly saturated alkyl chain, with the range of 17−25 carbons at the 5 position of the benzene ring, (C15:0 is only found in small amounts in rye) (**Figure 1**). ARs are absent in highly refined products and in many of the commonly consumed cereal foods. They are however found in high amounts in the outer layers of rye and wheat. Low levels of ARs are found in barley, millet, and corn, and minute amounts are found in cashew nuts and some garden pea varieties (*3–5*). ARs are absorbed via the lymphatic system and have a rather short elimination half-life (*6, 7*). ARs are shown to have several biological effects (e.g., antibacterial, antifungal, and antioxidative activities), and they interact with some proteins, thoroughly discussed by Kozubek and Tyman (*4*) and Ross et al. (*3*). ARs do incorporate into human erythrocyte membranes (*8*) and are transported in human plasma lipoproteins (*9*). According to Ross et al., ARs were suggested to be metabolized via shortening of the alkyl chain by β-oxidation and formation of glucuronide and sulfate conjugates (*10*). Accumulating evidence both in human subjects and pigs suggests that the intact ARs, measured in plasma, are good biomarkers of whole-grain intake (*6, 11, 12*). We first developed a GC-MS method for the main individual alkylresorcinols in plasma (serum) (*13*), which has been used in recent studies (*7, 9*). The intact ARs do not occur in urine in significant amounts (*10*), whereas the two AR metabolites, identified as 3,5-dihydroxybenzoic acid (DHBA) and 3-(3,5-dihydroxyphenyl)-1-propanoic acid (DHPPA), are found in large amounts. The recently published method for urinary AR metabolites (*14*), based on HPLC combined with coulometric



Alkylresorcinol	Abbreviation used	R
5-n-Heptadecylresorcinol	AR C17:0	$C_{17}H_{35}$
5-n-Nonadecylresorcinol	AR C19:0	$C_{19}H_{39}$
5-n-Heneicocylresorcinol	AR C21:0	$C_{21}H_{43}$
5-n-Tricocylresorcinol	AR C23:0	$C_{23}H_{47}$
5-n-Pentacocylresorcinol	AR C25:0	$C_{25}H_{51}$
3,5-dihydroxybenzoic acid	DHBA	COOH
3-(3,5-dihydroxyphenyl)-1-propanoic acid	DHPPA	$(CH_2)_2$ COOH

Figure 1. Chemical structures of alkylresorcinols and their metabolites DHBA and DHPPA.

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electrode array detection (CEAD), is very simple and is intended to be used in large epidemiological studies. Most collections of biological materials for human epidemiological studies however do not contain urine samples. Consequently, we developed a modified HPLC-CEAD method to quantify AR metabolites in plasma. This communication presents the method and its validation. The quantitative measurement of DHBA and DHPPA in plasma is based on the method for the urinary DHBA and DHPPA developed by Koskela et al. (*14*).

MATERIALS AND METHODS

Instrumentation. We used an HPLC system from ESA Biosciences, Inc. (Chelmsford, MA) equipped with a model 540 autosampler, two model 580 solvent pumps, and a model 5600 coulometric electrode array detector with eight electrode pairs.

Reagents. Acetonitrile and methanol (MeOH) were from Rathburn Chemicals Ltd. (Walkenburg, Scotland, U.K.). *o*-Phosphoric acid was from Riedel-de Haën (Seelze, Germany). Acetic acid, diethylether, potassium dihydrogenphosphate, and sodium acetate were from Merck GmbH (Darmstadt, Germany). β-glucuronidase was from Roche Diagnostics GmbH, (Mannheim, Germany) and sulfatase was from Sigma-Aldrich Co. (St. Louis, MO).

Reference Compounds. DHBA was from Aldrich (Steinheim, Germany), DHPPA from IsoSep AB (Tollinge, Sweden), and syringic acid (SyrA) from Sigma-Aldrich Co. (St. Louis, MO).

Sample Protocol. We obtained 3-day pooled plasma and 72-h urine samples from 64 Finnish free living female volunteers. Two pairs of samples were collected from each subject, one in the spring and one in the autumn (from one subject, only the spring sample was collected), resulting in 127 of both plasma and urine samples. The subjects consumed their habitual diets without any dietary changes throughout the study. Age (46 ± 13 years), weight (60 ± 9 kg), and BMI (22.5 ± 2.7 kg/m^2) were recorded by questionnaire during the screening visit. One-third of the subjects were vegetarians (i.e., vegans (not consuming animal products), lacto-vegetarians (milk products included in the diet), or lacto-ovo-vegetarians (additionally eggs included)).

During the urine collection (72 h), the overnight fasting blood samples were taken each morning. Thus, plasma and urinary samples were obtained simultaneously. Urine was collected in bottles containing 1 g of ascorbic acid. After finishing the collection, 0.1% sodium azide was added. The 3-day plasma samples were pooled, and 0.1% ascorbic acid and 0.1% sodium azide were added. These additions were made to avoid deterioration of the compounds studied. All samples were stored at -20 °C until analyzed (*15, 16*). The Ethics Committee of the Helsinki University Central Hospital, Helsinki, Finland, approved the study. All study participants gave informed consent prior to the study.

Analytical Method. The previously described urinary analysis method was slightly modified for the analysis of plasma samples. Diethylether was chosen for the extraction to avoid the massive emulsions formed when ethyl acetate was used. Furthermore, acidification of the sample was necessary to achieve proper recoveries. After reconstituting the sample with MeOH and mobile phase, filtering was needed to remove the formed precipitate.

In detail, the procedure was as follows: The internal standard, 20 ng of SyrA in 2 μL of MeOH, was added to 100 μL of plasma. The sample was hydrolyzed overnight at 37 °C with an equal volume (100 μL) of hydrolysis solution (0.1 mol/L Na-acetate buffer pH 5, 0.2 U/mL β-glucuronidase, and 2 U/mL sulfatase). After incubation, the sample was acidified by adding 10 μL of glacial acetic acid to reach a pH of about 3. The sample was extracted with 2 mL of diethylether by vigorously shaking for 2 min. The organic phase was collected, and the procedure was repeated three times. The combined organic phases were evaporated to dryness and reconstituted with 50 μL of MeOH and 100 μL of HPLC mobile phase (20% phase B/80% phase A; for compositions, see below). The sample was filtered through a Gelman GHP 0.2 μm filter, and 10 μL was analyzed with HPLC-CEAD. The analytes were separated using mobile phases consisting of 50 mmol/L phosphate buffer pH 2.3/MeOH (90/10, v/v) (phase A) and 50 mmol/L phosphate buffer pH 2.3/MeOH/acetonitrile (40/40/20, v/v/v) (phase B), with a 25 min linear gradient from 0 to 100% phase B with a total flow of 0.3 mL/min. The analytical column was an Inertsil ODS-3 (GL Sciences Inc., Japan) 3 mm $\times$ 150 mm with particle size 3 μm, connected to a Quick Release RP-18 (Upchurch Scientific Inc., WA) 3 mm $\times$ 10 mm guard column. DHBA was quantified at 670 mV, DHPPA at 570 mV, and SyrA at 380 mV.

Table 1. Intra- and Interassay Precision, with Three Control Sample Concentrations[a]

control sample level	DHBA[b]	DHPPA[b]
low intra/interassay CV %	39.4 (2.5) 3.5/6.4	62.8 (8.2) 2.5/13.1
medium intra/interassay CV %	95.0 (12.0) 3.4/12.6	92.4 (6.8) 3.3/7.4
high intra/interassay CV %	358.4 (11.7) 5.9/3.3	184.4 (21.7) 5.7/11.8

[a] Values are expressed as mean (SD) nmol/L. Intraassay ($n = 10$), interassay (low $n = 5$; medium $n = 7$; high $n = 7$). [b] DHBA, 3,5-dihydroxyphenylbenzoic acid; DHPPA, 3-(3,5-dihydroxyphenyl)-1-propanoic acid.

Table 2. Recoveries of the Method[a]

analyte added	DHBA[b]	DHPPA[b]
ng	Recovery % (cv%)	Recovery % (cv%)
1	94 (6.4)	96 (10.3)
2	78 (3.5)	76 (3.5)
4	82 (1.0)	82 (3.2)
8	83 (0.1)	82 (1.6)
16	92 (5.0)	88 (6.6)

[a] Mean obtained by triplicate analyses. [b] DHBA, 3,5-dihydroxyphenylbenzoic acid; DHPPA, 3-(3,5-dihydroxyphenyl)-1-propanoic acid.

Precision. Precision was evaluated by measuring 10 replicate control samples with 3 different concentrations in a single analysis (intraassay), and in 5 or 7 separate occasions (interassay; **Table 1**).

Accuracy. Reference standards were used in calculating the limits of detection (LOD) and in testing the linearity. The LOD, using a signal-to-noise ratio of 3:1 and expressed as the amount of analyte per injection, was 5 pg per injection for both analytes. The limit of quantification, using a signal-to-noise ratio of 10:1, was 15 pg per injection for both analytes, corresponding to plasma concentrations of about 15 nmol/L. The linearity ranged from the LOD to 20 000 pg per injection (0.5−2000 ng/mL) for both analytes (the upper limit was not tested). The correlation coefficients were 0.999 for both DHBA and DHPPA.

The recoveries of DHBA and DHPPA were measured in triplicate by supplementing five different concentrations of both compounds into plasma samples with low concentrations of endogenous metabolites. Eight-point calibration curves (2−400 ng/mL, corresponding to 20−4000 pg per injection) were used. The mean recovery of all added concentrations, after correction based on the internal standard, was 85.7% (CV 7.9%) for DHBA and 84.7% (CV 10.0%) for DHPPA (**Table 2**). The internal standard had a recovery of 87.0% (CV 6.5%). The recoveries of the metabolites were not completely corrected using the internal standard SyrA, probably because of the difference in their polarity, and therefore in their extractability in diethylether. Finding an internal standard better than SyrA is however difficult because of numerous similar interfering natural compounds occurring in plasma. Without the addition of internal standard, no SyrA peak was detected in the samples tested ($n = 5$).

Specificity. The specificity of the method was based on the retention times and the oxidation patterns compared with the reference compounds DHBA, DHPPA, and SyrA. Quantification of DHBA was done on channel 7, at 670 mV, DHPPA on channel 6, at 570 mV, and SyrA on channel 4, at 380 mV. The peaks were considered pure when the channel ratio accuracies were $\geq$70% compared to the channel ratios of the reference compound (*17, 18*). Eleven other phenolic acids were tested: 3,4-dihydroxyphenylacetic acid, 3,4-dihydroxyphenylpropionic acid, 4-hydroxy-3-methoxymandelic acid, 4-hydroxyphenyl-2-propionic acid, caffeic acid, *o*-coumaric acid, *m*-coumaric acid, *p*-coumaric acid, ferulic acid, tannic acid, and sinapic acid. None of them had retention

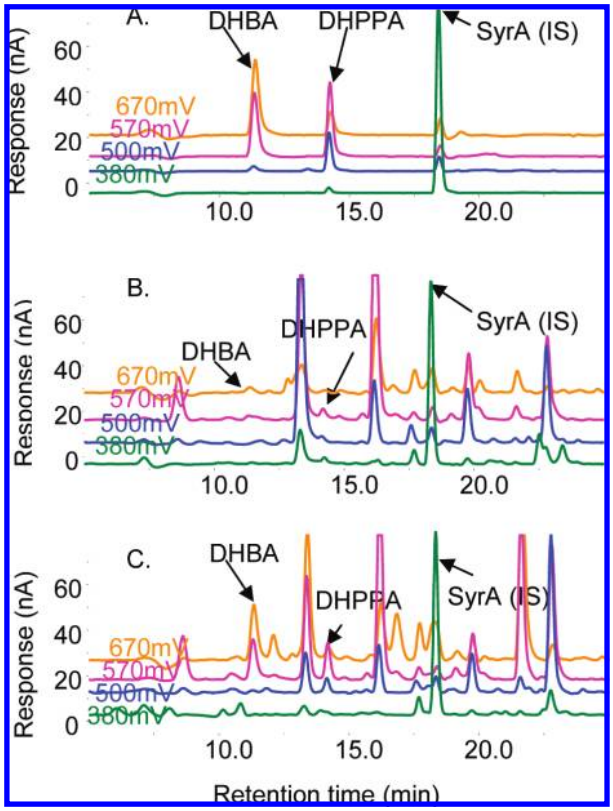

Figure 2. Chromatograms of the reference standards and two plasma samples. Channels from 4 to 7 are presented. SyrA (IS), DHPPA, and DHBA were detected on channels 4 (380 mV), 6 (570 mV), and 7 (670 mV), respectively. (**A**) Chromatogram of a reference standard. (**B**) Chromatogram of a low concentration sample (DHBA, 27 pg/injection; DHPPA, 48 pg/injection). (**C**) Chromatogram of a moderate concentration sample (DHBA, 270 pg/injection; DHPPA, 190 pg/injection).

times and oxidation patterns similar to DHBA, DHPPA, or SyrA (*14*). Chromatograms of the reference standards and two plasma samples are presented in **Figure 2**.

Statistical Analysis. Normality of distribution was determined using Kurtosis test. The data was log transformed for the correlation analyses. The analyses were performed using the SPSS 15.0 program (Chicago, IL).

RESULTS AND DISCUSSION

All plasma samples ($n = 127$) were quantified for both AR metabolites DHBA and DHPPA. The measured concentrations in plasma were 12−610 nmol/L for DHBA and 12−465 nmol/L for DHPPA. The intact ARs (C17:0−C25:0) in plasma and the AR metabolite levels in urine from the same subjects were measured for the purpose of another study (Aubertin-Leheudre et al., CEBP, accepted). The measured concentrations for the summed intact plasma ARs (C17:0−C25:0) were 20−230 nmol/L, for urinary DHBA 4−108 μmol/24 h, and for urinary DHPPA 7−188 μmol/24 h. The measured concentrations in plasma for DHBA and DHPPA were found to be about 200−500 times lower than the corresponding concentrations in the 24-h urine samples in the same subjects. The detection of phenolic compounds with coulometric electrode array detector (CEAD) is more sensitive and specific than with the conventionally used UV/vis detector, as discussed by Gamache and Acworth (*17*) and in the review article by Peñalvo and Nurmi (*18*).

Pearson's test was used for evaluating the correlations between AR metabolites in plasma with AR metabolites in urine and the summed intact plasma ARs (C17:0−C25:0) (**Figure 3**). The summed plasma AR metabolite concentrations correlated significantly with the summed urinary AR metabolite concentrations ($R = 0.613$) and with the summed intact plasma AR (C17:0−C25:0) concentrations ($R = 0.686$). Plasma DHBA correlated significantly with urinary DHBA ($R = 0.591$), as did plasma DHPPA with urinary DHPPA (R = 0.655); ($p < 0.001$ for all correlations).

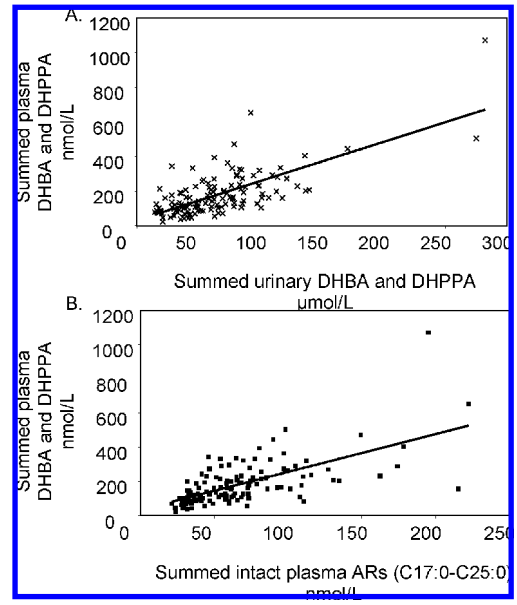

Figure 3. Correlations between plasma and urinary DHBA and DHPPA alkylresorcinol metabolites and the intact plasma alkylresorcinols. (**A**) Correlation between the summed plasma AR metabolites and the summed urinary AR metabolites: $R = 0.702$; $p < 0.001$. (**B**) Correlation between the summed plasma AR metabolites and the summed intact plasma ARs (C17:0−C25:0): $R = 0.650$; $p < 0.001$.

Because of a lack of AR metabolite conjugate reference compounds, it was impossible to determine the recovery during enzymatic hydrolysis. Instead, we analyzed the same plasma samples with and without hydrolysis. We found the hydrolysis of the sample necessary because about 10−30% of DHBA and 60−90% of DHPPA measured in plasma appeared to be in conjugated form (results not shown). In urine, however, almost all AR metabolites were found unconjugated (*14*).

The calibration linearity range clearly exceeded the concentrations of any of the samples analyzed, and we assume that the linearity is sufficient for all plasma samples.

The two AR metabolites DHBA and DHPPA, identified in urine by Ross et al. (*10*), were suggested by the authors to function as biomarkers of whole-grain intake in human subjects. The significant correlation of the summed plasma AR metabolites with the summed intact ARs (C17:0−C25:0) in plasma indicates that DHBA and DHPPA, measured in plasma, could serve the same purpose. We conclude that the new, validated method is specific, reproducible, and suitable for measuring DHBA and DHPPA in plasma. The analysis protocol is simple and can be performed with small plasma volumes. The new method could be used in large epidemiological studies to evaluate whether the plasma AR metabolites are appropriate biomarkers of whole-grain intake and to study the association between whole-grain consumption and human diseases.

ABBREVIATIONS USED

AR, alkylresorcinol; DHBA, 3,5-dihydroxyphenylbenzoic acid; DHPPA, 3-(3,5-dihydroxyphenyl)-1-propanoic acid; CEAD,

coulometric electrode array detector; SyrA, syringic acid; MeOH, methanol; LOD, limit of detection.

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