Quantification of Alkylresorcinol Metabolites in Urine by HPLC with Couloic Electore Array Detection, Anja Koskela, Anna-Maria Linko-Parvinen, Perttu Hiisvuori, Adile Samaletdin, Afaf Kamal-Eldin, Matti J. Tikkanen, and Herman Adlercreutz

Background: Whole-grain rye and wheat cereals contain high amounts of alkylresorcinols (ARs), phenolic lipids. ARs can be quantified in plasma. Two recently identified urinary AR metabolites, 3,5-dihydroxyphenylbenzoic acid (DHBA) and 3-(3,5-dihydroxyphenyl)-1-propanoic acid (DHPPA), may be useful as biomarkers of intake of whole-grain rye and wheat.

Methods: We evaluated 4 pretreatment protocols for quantifying urinary DHBA and DHPPA using HPLC coupled with a couloic electrode array detector. Syringic acid was used as the internal calibrator.

Results: Measured urinary concentrations of DHBA and DHPPA were 0.8–115 μmol/L. The mean recoveries of all added concentrations were 85%–104% for DHBA and 86%–99% for DHPPA, depending on the degree of the purification. The protocol versions with less purification correlated well with the protocol including highest purification. The correlation coefficients (r²) were 0.9699–0.8153 for DHBA and 0.9854–0.8371 for DHPPA.

Conclusion: Although the protocol with the most purification steps was most specific, all protocols were suitable for measuring DHBA and DHPPA in urine. The rapid protocol with simple hydrolysis could be used in large-scale clinical studies. Additional investigation is needed to clarify whether these metabolites are useful biomarkers of whole-grain intake and helpful in the exploration of its association with human diseases.

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Quantitative studies indicate that the consumption of whole-grain cereal products is beneficial, possessing health-promoting effects and decreasing the risk of several chronic diseases (1, 2). Partly owing to a lack of reliable biomarkers, investigation of the association of whole-grain intake with disease prevalence has been difficult. Alkylresorcinols (ARs) are found in high concentrations in rye and wheat whole-grain cereals and have been proposed to function as plasma markers of whole-grain intake (3–5). ARs are absorbed via the lymphatic system and have a rather short elimination half-life (6, 7). According to Ross et al. (8) the urinary secretion of intact ARs is minor. The urinary metabolites identified as 3,5-dihydroxybenzoic acid (DHBA) and 3-(3,5-dihydroxyphenyl)-1-propanoic acid (DHPPA) (8) were suggested by these authors to function as biological markers of whole-grain intake in humans. We describe a method for quantification of these metabolites in human urine.

We purchased acetonitrile, ethyl acetate, and methanol from Rathburn Chemicals; ortho-phosphoric acid from Riedel-de Haën; acetic acid, formic acid, potassium dihydrogenophosphate, and sodium acetate from Merck; syringic acid (SyrA), and sulfatase from Sigma-Aldrich; β-glucuronidase from Fluka; DHBA from Aldrich; and DHPPA from IsoSep AB.

We obtained 30 urine samples from 15 volunteers who consumed whole-grain wheat or rye bread for 1-week periods (9) and 3 urine samples from 3 volunteers with celiac disease who consumed no wheat or rye products. The Ethics Committee of the Department of Medicine, Helsinki University Central Hospital, Helsinki, Finland approved the study. All study participants gave informed consent.

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We used an HPLC system (ESA Biosciences) equipped with a model 540 autosampler, 2 model 580 solvent pumps, and a model 5600 coulometric electrode array detector (CEAD) with 8 electrode pairs. DHBA was quantified at 670 mV, DHPPA at 570 mV, and SyrA at 380 mV.

The analytes were separated using mobile phases consisting of 50 mmol/L phosphate buffer pH 2.3/methanol 90/10 (by volume) (phase A), and 50 mmol/L phosphate buffer pH 2.3/methanol/acetonitrile 40/40/20 (by volume) (phase B), with a 25-min linear gradient from 0% to 100% phase B (see Fig. 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol53/issue7). The analytical column was an Inertsil ODS-3 (GL Sciences) 3 x 150 mm, connected to a Quick Release RP-18 (Upchurch Scientific) 3 x 10 mm guard column. Retention times, retention time variation, detection potentials, and detector response variation are presented in Table 1 in the online Data Supplement.

We developed sample preparation protocol C, with the highest amount of purification, and tested 3 modified protocols (A, B, and D) with different degrees of sample purification (Fig. 1).

To 100 μL of urine we added 600 ng of internal calibrator SyrA in 8 μL of methanol. The sample was hydrolyzed overnight at 37 °C with equal volume (100 μL) of hydrolysis solution containing 0.1 mol/L Na-acetate buffer pH 5, 0.2 kU/L β-glucuronidase, and 2 kU/L sulfatase. After incubation, 3 50-μL aliquots A, B, and C (equal to 25 μL of urine) were removed and treated as follows (aliquots A–C correspond to the protocols A–C):

For protocol A, 50 μL each of methanol and HPLC mobile phase 20% phase B/phase A were added to the sample and then analyzed with HPLC-CEAD.

For protocol B, 500 μL of methanol was added to the sample, and further purified using DEAE-Sephadex ion-exchange chromatography in the free base form (10). The sample was applied to the column with 50 μL of methanol. Neutral steroids were eluted with 6 mL of methanol and discarded. DHBA and DHPPA were eluted with 8 mL of 0.5 mol/L formic acid in methanol. The fraction was evaporated, reconstituted with 50 μL of methanol, 100 μL of HPLC mobile phase 20% phase B/phase A was added, and the sample was analyzed with HPLC-CEAD.

For protocol C, a 50 μL aliquot was extracted twice with 300 μL of ethyl acetate. The combined organic phase was evaporated, reconstituted with 500 μL of methanol, and further purified as described for protocol B.

For protocol D we added 60 μL of methanol and 120 μL of HPLC mobile phase 20% phase B/phase A to 20 μL of nonhydrolyzed urine, and analyzed the sample directly with HPLC-CEAD.

For all 4 protocols (A–D) 10 μL of the sample was injected into HPLC.
To test if deconjugation of the metabolites occurs during standing in the acidic environment before injection, we diluted urine followed by immediate injection. Nine additional injections from the same vial were performed during the following 8 h.

We measured the recoveries of DHBA and DHPPA in triplicate by supplementing 5 different concentrations of both compounds into low endogenous concentration urine samples. To avoid interference with the enzymatic hydrolysis from too high concentration of methanol, the added solutions were evaporated to dryness and reconstituted with a few microliters of methanol before addition of the urine. Six-point calibration curves (20–670 μg/L) were used. The mean recoveries of all added concentrations were 85%–104% for DHBA and 86%–99% for DHPPA, depending on the protocol used. Sample treatment protocol C yielded the lowest recoveries (see Table 2 in the online Data Supplement).

Reference calibrators were used to calculate limits of detection and to test the linearity. The limit of detection, using a signal:noise ratio of 3:1, varied from 28 to 80 pg for DHBA and from 20 to 70 pg for DHPPA, per injection, depending on the treatment protocol used, and corresponding to urine concentrations 0.17–0.48 mol/L and 0.13–0.45 mol/L, respectively. Protocol C, including highest purification, gave the lowest limit of quantification. Linearity ranged from the limit of detection to 20 000 pg per injection for both analytes (the upper limit not tested), with correlation coefficients of 0.9990 for DHBA and 0.9994 for DHPPA.

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

The specificity of the method was based on the retention times and the oxidation patterns compared with DHBA, DHPPA, and SyrA. Eleven other phenolic acids were tested: 3,4-dihydroxymandelic acid, 3,4-dihydroxyphenylpropionic acid, 4-hydroxy,3-methoxyxymandelic 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 had similar retention times and oxidation patterns.

All urine samples (n = 33) were quantified with all protocol versions. The quantitative results of samples analyzed with protocols A and B showed good correlations with the sample results of protocol C, using the highest purification (see Fig. 2 in the online Data Supplement). The correlation data for protocol D showed a slope of 0.74 for DHPPA, which we presume resulted from the lack of sample hydrolysis in protocol D, whereby the possible conjugates were not measured.

After 10 injections of diluted urine (protocol D) from the same sample vial, as described earlier, no change in DHPPA concentration was observed. The measured DHBA concentration, however, increased 15% within the first 2 h, possibly because of initial deconjugation, after which no change was observed (see Fig. 3 in the online Data Supplement). In all analysis sequences, 1 blank and 1 calibrator were placed first (total run time ~2 h), so all samples were waiting at least for 2 h before injection. Therefore no further change in DHBA concentrations in the analyzed samples occurred, and the results did not depend on what position in the sequence they were located.

The calibration linearity was greater than the concentrations of any of the samples analyzed, and we assumed that the linearity was sufficient for most normal urine samples. Protocol C was first chosen to get rid of the possible interfering compounds. When DHBA and DHPPA were found to occur in higher than anticipated concentrations, protocols A, and B, with less purification, were tested, as well as simple dilution of the urine (protocol D). The results using protocol D indicated that

<table>
<thead>
<tr>
<th>DHBA*</th>
<th>Protocol A</th>
<th>Protocol B</th>
<th>Protocol C</th>
<th>Protocol D</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Straight from hydrolysis</td>
<td>No extraction</td>
<td>Most purified</td>
<td>Direct urine dilution</td>
</tr>
<tr>
<td>Low</td>
<td>4.1 (0.4)</td>
<td>2.4 (0.5)</td>
<td>1.0 (0.2)</td>
<td>4.1 (0.7)</td>
</tr>
<tr>
<td></td>
<td>Intra-/inter assay CV %</td>
<td>3.6/8.8</td>
<td>12.5/21.8</td>
<td>9.5/16.7</td>
</tr>
<tr>
<td>Medium</td>
<td>15.9 (1.8)</td>
<td>15.9 (1.8)</td>
<td>14.1 (1.0)</td>
<td>13.9 (1.5)</td>
</tr>
<tr>
<td></td>
<td>Intra-/inter assay CV %</td>
<td>3.4/11.2</td>
<td>6.5/11.1</td>
<td>4.7/7.4</td>
</tr>
<tr>
<td>High</td>
<td>34.7 (4.4)</td>
<td>36.9 (3.2)</td>
<td>32.6 (2.5)</td>
<td>46.4 (4.8)</td>
</tr>
<tr>
<td></td>
<td>Intra-/inter assay CV %</td>
<td>9.7/12.7</td>
<td>4.2/8.5</td>
<td>3.3/7.7</td>
</tr>
<tr>
<td>DHPPA*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>8.0 (1.1)</td>
<td>6.2 (0.7)</td>
<td>5.7 (0.6)</td>
<td>8.0 (1.0)</td>
</tr>
<tr>
<td></td>
<td>Intra-/inter assay CV %</td>
<td>1.5/13.2</td>
<td>5.7/11.5</td>
<td>4.4/10.1</td>
</tr>
<tr>
<td>Medium</td>
<td>23.2 (2.4)</td>
<td>23.4 (2.0)</td>
<td>23.0 (2.0)</td>
<td>15.1 (1.7)</td>
</tr>
<tr>
<td></td>
<td>Intra-/inter assay CV %</td>
<td>2.5/10.1</td>
<td>4.2/8.5</td>
<td>6.0/8.7</td>
</tr>
<tr>
<td>High</td>
<td>40.4 (3.2)</td>
<td>44.5 (3.8)</td>
<td>43.3 (4.1)</td>
<td>26.3 (2.9)</td>
</tr>
<tr>
<td></td>
<td>Intra-/inter assay CV %</td>
<td>11.1/7.9</td>
<td>4.9/8.5</td>
<td>6.7/9.5</td>
</tr>
</tbody>
</table>

*Values are expressed as mean (SD) μmol/L. Intraassay (n = 10), interassay (n = 5).
DHBA and DHPPA mainly exist in the unconjugated form in urine. The protocols A, B, and C correlated well with each other (see Fig. 2 in the online Data Supplement), yielding similar results. Low concentrations (<7.1 μmol/L) of DHBA and DHPPA were found in urine samples from the volunteers with celiac disease. Gluten-free cereals, such as corn, millet, rice, and buckwheat, in addition to nuts and some varieties of peas, have a central role in the diets of patients with celiac disease. The low urinary concentrations of DHBA and DHPPA found in the celiac patients may be attributable to intake of millet, corn, cashew nuts, and garden pea varieties reported to contain minute amounts of ARs (3, 11, 12). Other dietary sources for minor formation of DHBA and DHPPA may be flavonoids (13), although these form mainly monohydroxylated and 2,3-, 2,4- or 3,4-dihydroxylated metabolites (14–16). The formation of 3,5-DHBA and 3,5-DHPPA metabolites is also possible. Further investigations are needed to find out if DHBA and DHPPA could be used as biomarkers of whole-grain intake.

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