at position 506 to change to a stop codon, producing a null allele and probably causing a 50% decrease in the measured factor V activity in a heterozygote. Unfortunately, quantitative assays for factor V activity or protein were not available.

Obviously, the premature stop codon that we identified would have effects on the coagulation pathway vastly different from those of a factor V Leiden mutation, as well as significantly different clinical and therapeutic implications. The C→T change at position 1690 would produce a factor V deficiency, whereas the factor V Leiden mutation (G→A at position 1691) causes a thrombophilic predisposition. Because of the proximity of the 2 melting peaks seen in the Roche LightCycler factor V assay, the results have the potential to be misinterpreted as positive for factor V Leiden mutation if the C→T 1690 mutation is present instead of the G→A 1691 mutation. Accordingly, based on our results and DNA sequence confirmation, the patient’s thrombotic condition was considered not related to a factor V Leiden mutation.

This report highlights the importance of recognizing the difference between these 2 peaks so that a patient with a potentially hemorrhagic condition is not mistakenly diagnosed with a thrombophilic condition. Although recognition of the C→T mutation at position 1690 by other methods has been described previously, to our knowledge this is the first report of this mutation detected with the LightCycler methodology. Given the increasing popularity of such assays, providers need to be cognizant of this potentially false-positive result. Furthermore, it underscores the value of quantitative QC measures for allele-specific assays that use melting-temperature analyses. We emphasize the importance of establishing and maintaining QC material and carefully examining the resulting data.

We wish to thank Dr. D. Haverstick and the staff of the molecular diagnostics laboratory.

Gas Chromatographic–Mass Spectrometric Analysis for Measurement of p-Cresol and Its Conjugated Metabolites in Uremic and Normal Serum, Henriette de Loor,1,2 Bert Bammens,3 Pieter Evenepoel,1 Vicky De Preter,1 and Kristin Verbeke1 (1 Laboratory of Digestion and Absorption, 2 Laboratory of Nephrology, and 3 Department of Medicine, Division of Nephrology, University Hospital Gasthuisberg K.U. Leuven, Leuven, Belgium; * address correspondence to this author at: Laboratory of Digestion and Absorption, University Hospital Gasthuisberg, Herestraat 49, B-3000 Leuven, Belgium; fax 32-16-344399, e-mail Kristin.Verbeke@uz.kuleuven.ac.be)

p-Cresol (4-methylphenol; 108 Da) is a protein-bound solute retained within the body in renal failure (1). p-Cresol is of interest because it has several toxic effects in vitro (2–6) and clinical correlates have been demonstrated (7, 8). In the absence of external exposure (9), p-cresol originates uniquely from bacterial tyrosine fermentation in the large intestine (10). During passage through the colonic mucosa and liver, it is detoxified by conjugation processes (sulfation and glucuronidation) (11–13). Thus, one might expect to find p-cresylsulfate and p-cresylglucuronide in the serum, but reports on conjugated p-cresol in renal failure patients are scarce (14–16). Most techniques to deproteinize serum samples (e.g., heat and acidification) may also partially hydrolyze sulfate esters and glucuronide bonds. Hence, the “total” (i.e., protein-bound and unbound) and “unbound” p-cresol reported in most studies probably reflect both unconjugated and (part of the) conjugated forms of the solute (17–21). We determined the extent of desulfation and deglucuronidation by deproteinization with heat and acid followed by gas chromatographic–mass spectrometric (GC-MS) analysis (19) with p-nitrophenylglucuronide and p-nitrophenylsulfate as model substrates. We also calculated exact amounts of unconjugated p-cresol, p-cresylsulfate, and p-cresylglucuronide in serum of hemodialysis patients and healthy controls.

Percentage desulfation and deglucuronidation by different methods (see below) was determined for random serum samples. Further analyses were performed on 9 serum pools from hemodialysis patients [n = 86; 49 male; mean (SD) age, 69.8 (1.5) years] and 5 serum pools from healthy controls [n = 29; 10 male; 31.0 (1.4) years; creatinine clearance, 87.1 (1.4) mL · min−1 · (1.73 m2)−1]. Serum pools were stored at −80 °C until analysis. The ethics committee of the University Hospital Leuven approved the study, and informed consent was obtained from all participants. p-Nitrophenylglucuronide, p-nitrophenylsulfate, and 2,6-dimethylphenol (all >98% purity) were from Sigma-Aldrich; p-cresol and p-nitrophenol were from Supelco; and β-glucuronidase (Escherichia coli K12) was from Roche. Other materials were of analytical grade.

Several methods were used for sample preparation. Method A [acid and heat deproteinization (19)] consisted of dilution of 500 μL of serum with 350 μL of water; addition of 100 μL of p-nitrophenylglucuronide (1.2 g/L)

References

2. von Ahsen N, Schutz E, Armstrong VW, Oellerich M. Rapid detection of prothrombotic mutations of prothrombin (G20210A), factor V (G1691A), and methylene tetrahydrofolate reductase (C677T) by real-time fluorescence PCR with the LightCycler. Clin Chem 1999;45:694–6.

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or 100 μL of p-nitrophenylsulfate (1 g/L) to determine percentage deglucuronidation or desulfation, respectively; adjusting to pH 1 with H₂SO₄ heating to 90 °C for 30 min; cooling to room temperature; addition of 50 μL of 2,6-dimethylphenol (200 mg/L) as internal standard; extraction with 1 mL of ethyl acetate; mixing for 30 s; centrifugation at 1583 g for 20 min; drying of 500 μL of supernatant over anhydrous sodium sulfate; and transfer of 100 μL to the GC-MS instrument.

Method B (enzymatic deglucuronidation + method A) included addition of 1 mL of phosphate buffer (0.1 mol/L; pH 7.0) to 500 μL of serum and 100 μL of p-nitrophenylglucuronide (1.2 g/L) or 100 μL of p-nitrophenylsulfate (1 g/L); addition of 20 μL of β-glucuronidase; incubation at 37 °C for 30 min; and further acidification, heating, and extraction according to method A, except that 2 mL of ethyl acetate was used in the extraction step.

Method C (acetone deproteinization + method A) consisted of the addition of 100 μL of p-nitrophenylglucuronide (7 g/L) or 100 μL of p-nitrophenylsulfate (6 g/L) to 500 μL of serum; addition of 3 mL of cold acetone; centrifugation at 1583 g for 10 min; separation of supernatant from pellet by decantation; adjusting to pH 5 with HCl (0.6 mol/L); addition of 100 μL of 2,6-dimethylphenol (200 mg/L) as internal standard; extraction with 3 mL of chloroform; mixing for 30 s; centrifugation at 1583 g for 20 min; drying of 500 μL of the lower layer over anhydrous sodium sulfate; and transfer of 100 μL to the GC-MS instrument.

Method D (ultrafiltration + method A) for determination of the unbound fraction of p-cresol included ultrafiltration of 1 mL of serum at 2000 g for 60 min at room temperature with ultrafiltration membrane (cutoff, 30 000 Da; Centrifree and MPS Micropartition UF Devices; Amicon); dilution of 500 μL of filtrate with 450 μL of water; and further acidification, heating, and extraction according to method A (19).

Sample preparation was followed by separation and identification by GC-MS (Trace GC-MS; ThermoFinnigan) as follows: splitless injection of 0.5 μL of sample on an AT5-ms analytical column [30 m × 0.32 mm (i.d.); 1-μm film thickness; Alltech Associates] with GC-grade helium (5.9 mL/min) as the carrier gas; oven programming from 75 °C (isotherm for 5 min) to 280 °C (15 °C/min); and identification by MS (electron impact full scan mode from m/z 59 to m/z 590 at 2 scans/s). Quantitative results were obtained by the internal standard method. The detection limit (LOD) for p-cresol was 0.15 mg/L and the quantification limit (LOQ) was 0.30 mg/L. The LOD and LOQ for p-nitrophenol were 0.5 and 2 mg/L, respectively. The LOD and LOQ were defined empirically (22). Values below the LOQ were considered zero for calculations. Three aliquots of each sample were measured, and mean values are reported. Interassay CVs were calculated from these triple measurements. The intraassay CV was determined by injecting a single sample 3 times.

The mean (SD) yield of hydrolysis of p-nitrophenylglucuronide by method A was 16.0 (0.2)% and that for p-nitrophenylsulfate was 97.9 (6.5)%. Thus, the amount of p-cresol measured by method A comprises the unconjugated p-cresol, 16% of glucuronidated p-cresol, and almost all p-cresylsulfate present in the serum (Eq. 1).

\[
A = \text{unconjugated p-cresol} + 0.16 \times \text{p-cresylglucuronide} + 0.98 \times \text{p-cresylsulfate}
\]  

The enzymatic reaction used in method B led to complete deconjugation of p-nitrophenylglucuronide [yield, 101.3 (3.0)%]. Hydrolysis of p-nitrophenylsulfate was 97.8 (2.2)%. Thus, the amount of p-cresol measured by method B comprises almost all forms of p-cresol in the serum (Eq. 2).

\[
B = \text{unconjugated p-cresol} + \text{p-cresylglucuronide} + 0.98 \times \text{p-cresylsulfate}
\]  

Method C was intended to avoid deconjugation and hence to measure only unconjugated p-cresol. Although there was no measurable deconjugation of the glucuronidated substrate (below the LOD), limited hydrolysis [7.2 (5.1)%] of p-nitrophenylsulfate was observed (Eq. 3).

\[
C = \text{unconjugated p-cresol} + 0.072 \times \text{p-cresylsulfate}
\]  

The p-cresol concentrations measured in serum pools of HD patients (pools I to IX) and healthy controls (pools X to XIV) when we used methods A, B, and C are shown in Table 1. Combining of Eqs. 1–3 allowed calculation of the exact amounts of unconjugated p-cresol, p-cresylglucuronide, and p-cresylsulfate in each pool (Table 1, middle portion). Total p-cresol was significantly higher in patients than in controls [24.25 (4.98) mg/L vs 1.86 (0.31) mg/L; P <0.0001]. In both groups, most p-cresol was in its sulfated form [95.5 (3.3)% vs 98.2 (1.6)%; difference not significant], and a small proportion was glucuronidated [3.8 (2.4)% vs 1.8 (1.6)%; difference not significant]. Unconjugated p-cresol was undetectably low in most of the participants. P-Cresol concentrations measured with method A constituted 90.2 (3.0)% of the total p-cresol [patients, 90.6 (3.6)%; controls 89.5 (1.1)%; difference not significant]. The median (range) concentration of unbound p-cresol (method D) was 3.22 (2.01–5.65) mg/L in the patients (nongaussian distribution). This concentration was 16.8 (5.4)% of the concentration found when method A was applied to unfiltered samples. Unbound p-cresol was below the LOD in the controls (Table 1, last rows). The interassay variability was low for all methods (Table 1, last column). The intraassay CV for measurement of p-cresol was 0.68%.

Three important conclusions can be made from our study. First, most p-cresol in human serum is present as its sulfated metabolite, and a small proportion is glucuronidated. Unconjugated p-cresol is undetectably low in most cases. p-Cresylsulfate has previously been reported to be the major metabolite of p-cresol (14, 15). This finding is not surprising because colonic mucosal cells, considered to be more important than the liver for detoxification of phenols originating from the intestinal lumen (12), conjugate phenols with a preponderance of sulfation over glucuronidation (12, 13). By contrast, the major p-cresol...
Table 1. Concentrations of p-cresol and its different fractions in patients (pools I to IX) and controls (pools X to XIV).

<table>
<thead>
<tr>
<th>Method</th>
<th>p-Cresol, mg/L</th>
<th>p-Cresylsulfate, mg/L</th>
<th>p-Cresylglucuronide, mg/L</th>
<th>Unconjugated p-cresol, % of total</th>
<th>p-Cresol obtained by method A, % of total</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>22.34</td>
<td>1.12</td>
<td>0.20</td>
<td>94.0</td>
<td>23.4</td>
</tr>
<tr>
<td>B</td>
<td>23.28</td>
<td>0.80</td>
<td>0.00</td>
<td>94.9</td>
<td>21.5</td>
</tr>
<tr>
<td>C</td>
<td>1.90</td>
<td>0.20</td>
<td>0.00</td>
<td>99.8</td>
<td>14.6</td>
</tr>
<tr>
<td>D</td>
<td>2.28</td>
<td>0.20</td>
<td>0.00</td>
<td>99.8</td>
<td>12.0</td>
</tr>
<tr>
<td>E</td>
<td>22.34</td>
<td>1.12</td>
<td>0.20</td>
<td>94.0</td>
<td>23.4</td>
</tr>
<tr>
<td>F</td>
<td>23.28</td>
<td>0.80</td>
<td>0.00</td>
<td>94.9</td>
<td>21.5</td>
</tr>
<tr>
<td>G</td>
<td>1.90</td>
<td>0.20</td>
<td>0.00</td>
<td>99.8</td>
<td>14.6</td>
</tr>
<tr>
<td>H</td>
<td>2.28</td>
<td>0.20</td>
<td>0.00</td>
<td>99.8</td>
<td>12.0</td>
</tr>
<tr>
<td>I</td>
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<td>0.00</td>
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</tr>
<tr>
<td>K</td>
<td>1.90</td>
<td>0.20</td>
<td>0.00</td>
<td>99.8</td>
<td>14.6</td>
</tr>
<tr>
<td>L</td>
<td>2.28</td>
<td>0.20</td>
<td>0.00</td>
<td>99.8</td>
<td>12.0</td>
</tr>
</tbody>
</table>

Notes:
- LOD, below the limit of detection for p-cresol (i.e., <0.15 mg/L).
- Amounts of p-cresylsulfate and p-cresylglucuronide are expressed as corresponding concentrations of p-cresol.

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