age site for MboII improves the accuracy of the test, controlling the efficiency of the restriction reaction. MboII restriction analysis of the 5′PR polymorphism of the CYP3A4 gene, as described here, is less complex than conformation-sensitive gel electrophoresis and DNA sequencing (5, 7, 8). Therefore, it can easily be introduced into a large number of laboratories. The proposed method is rapid, does not require confirmatory tests, and can be useful for screening the CYP3A4 polymorphism in pharmacogenetic research.

This study was supported by grants from CAPES Brazil. We thank Dr. Maria Aparecida Nagai for technical assistance, and Luis A. Salazar and Elizabeth C. R. Guzmán for assistance in collecting samples. Selma A. Cavalli is the recipient of a fellowship from CAPES-Brazil.

References


Quantification of Benzoic, Phenylacetic, and Phenylbutyric Acids from Filter-Paper Blood Spots by Gas Chromatography–Mass Spectrometry with Stable Isotope Dilution, Xiaolin Yu, Mark M. Thompson, Dashuang Shi, and Mendel Tuchman (Children’s National Medical Center and the George Washington University, 111 Michigan Ave. NW, Washington DC 20010-2970; * author for correspondence: fax 202-884-6014, e-mail mtuchman@cnmc.org)

An important treatment of hyperammonemia in urea cycle disorders is the stimulation of alternative pathways for nitrogen excretion (1). Salts of benzoic acid (BA), phenylacetic acid (PAA), and phenylbutyric acid (PBA) have been found to be useful vehicles for the elimination of nitrogen by alternative pathways (2–4). BA is conjugated with glycine to form hippurate, which is efficiently excreted in the urine. PBA is metabolized in the liver to phenylacetyl-CoA, which is then conjugated with glutamine to form phenylacetylglutamine and subsequently excreted in the urine. Furthermore, benzoate has been used to treat nonketotic hyperglycinemia (5), salts of PBA and PAA are used in clinical trials as anticancer agents (6–9), and PBA has been found to stimulate fetal hemoglobin production and can potentially be used for treatment of hemoglobinopathies (10, 11). However, two patients have died in the US after being given the wrong doses of sodium benzoate and sodium phenylacetate (12), documenting the risk of toxicity, which could potentially be reduced by drug concentration monitoring.

HPLC assays for PAA and PBA in plasma have limited sensitivity (4). A gas chromatographic–mass spectrometric method had improved sensitivity (13), but the method did not use stable isotopes as internal standards and the clinical utility of the method was not documented. Here, we describe a rapid, precise, and convenient method to measure blood concentrations of these drugs using dried filter-paper blood spots.

BA, PAA, and PBA were purchased from Sigma-aldrich Co. [ring-d4]-BA, [ring-d2]-PAA, and [2,2,3,3-2H4]-PBA were purchased from Cambridge Isotope Laboratories. Regis RC-3 [N,O-bis-(trimethylsilyl)trifluoroacetamid containing trimethylsilylchlorosilane (101 by volume; BSTFA-TMS)] was purchased from Regis Chem Co. Blood collection cards (cat. no. 10538414) were purchased from Schleicher & Schuell.

Blood was blotted onto absorbent filter paper and dried at room temperature. The analytical samples consisted of four punched-out discs (6.3 mm in diameter), each of which contained ~10.6 µL of blood. When less blood was available, smaller discs were used. The method was sensitive enough to measure peak drug concentrations in a single 3-mm disc (2.4 µL of blood). The blood was eluted from the paper discs by sonication for 5 min in 1 mL of deionized water containing 100 µg of d2-BA, d2-PAA, and d2-PBA, respectively. The blood-spot extract was filtered through an Amicon Centrifree YM-30 filter (Millipore), yielding a clear filtrate. The filtrate was saturated with 1 g of NaCl and acidified to pH <1 with 0.2 mL of 5 mol/L HCl. The acids were extracted twice with 3 mL of ethyl
Fig. 1. Electron impact mass spectra (A, B, and C) and typical chromatogram (D) for the native and isotopic BA-, PAA-, and PBA-TMS derivatives. (A), the prominent m/z 179 (native) and 184 (isotopic) ions for BA were selected for quantification and calculation. (B), the prominent m/z 164 (native) and 169 (isotopic) ions for PAA were selected for quantification and calculation. (C), the prominent m/z 221 (native) and 225 (isotopic) ions for PBA were selected for quantification and calculation. (D), a typical chromatogram obtained from a blood-spot sample supplemented with 20.0 µg each of native BA, PAA, and PBA and 100 µg each of their respective isotopes. The elution times were 7.37 min for native BA-TMS, 7.34 min for d5-BA-TMS, 8.50 min for native PAA-TMS, 8.47 for d5-PAA-TMS, 13.85 min for native PBA-TMS, and 13.80 for d5-PBA-TMS.
acetate for 5 min on a mechanical shaker. The organic layers were combined and dried under a stream of nitrogen at 50 °C. Trimethylsilyl (TMS) derivatives of the organic acids were made by incubation with 100 µL of BSTFA-TMCS (10:1 by volume) for 20 min at 60 °C. The derivatized extract (2 µL) was injected into the gas chromatograph (Hewlett Packard Model 5890 II; Agilent Technologies) by an automated injector. The acids were separated on a cross-linked 5% diphenyl-95% dimethylsiloxane capillary column [Ultra 2; 30 m × 0.2 mm (o.d.); film thickness, 0.33 µm; Agilent Technologies].

The column temperature began at 100 °C and was ramped to 270 °C at 5 °C/min. The flow rate of the helium carrier gas was ~1 mL/min, and the linear velocity was 35 cm/s. Column-head pressure was 22.5 psi. The ions were detected by a quadrupole mass selective detector (Model 5971A; Hewlett Packard) equipped with an electron impact ion source that was autotuned and run in high-resolution mode with selected-ion monitoring. The following ions were determined within the 15-min runtime: m/z 179 for native BA-TMS, m/z 184 for d₅-BA-TMS, m/z 164 for native PAA-TMS, m/z 169 for d₅-PAA-TMS, m/z 221 for native PBA-TMS, and m/z 225 for d₅-PBA-TMS. The elution times were 7.37 min for native BA-TMS, 7.34 min for d₅-BA-TMS, 8.50 min for native PAA-TMS, 8.47 for d₅-PAA-TMS, 13.85 min for native PBA-TMS, and 13.80 for d₅-PBA-TMS. Dwell time for each ion was 50 ms, and the resolution was 0.5 atomic mass units. Quantification was performed with the calibration curves of m/z 179/184, m/z 164/169, and m/z 221/225 for BA, PAA, and PBA, respectively, representing the ratios of known amounts of native acids (0–10 µg) to isotopic acids (100 µg).

The extraction efficiency was calculated from six 100-µg aliquots of BA, PAA, and PBA spotted on filter paper. Reproducibility was determined by analysis of blood spots with 4.0 µg of added BA, PAA, and PBA. The stability of BA, PAA, and PBA on filter-paper blood samples over time was tested by analyzing nine blood spots, each containing 4.0 µg of the three acids, stored at room temperature and subsequently analyzed over a 1-month period. The heat stability of BA, PAA, and PBA in blood spots at 80 °C was examined by placing the samples in an oven for 4 and 24 h.

Fifteen patients with urea cycle disorders and 1 with propionic acidemia treated with one or more of these medications were tested using this method. The prominent m/z 179 and 184 ions (native and isotopic, respectively) for BA, m/z 164 and 169 ions for PAA, and m/z 221 and 225 ions for PBA (Fig. 1) were selected for quantification. The calibration curves were linear from 0 to 100 µg. The equations of the response curve were: y = 0.0131x + 0.0003 for BA; y = 0.0135x + 0.0002 for PAA; and y = 0.0121x + 0.0006 for PBA.

To determine the detection limits for BA, PAA, and PBA from filter-paper blood spots, samples with 0.01–10 µg of the acids added were prepared, followed by analysis as described above. The detection limits (signal-to-noise ratio >10) for the blood-spot samples were 0.01, 0.06, and 0.04 µg for BA, PAA, and PBA, respectively. When four 6.3-mm (diameter) discs (42.4 µL of blood) were used, the acids could be detected at concentrations

### Table 1. Peak and trough concentrations of BA, PAA, and PBA from dried filter-paper blood specimens of 16 individuals with urea cycle disorder treated with BA and/or PBA.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age, years</th>
<th>Diagnosis</th>
<th>Drug</th>
<th>Dose, mg/kg</th>
<th>Peak concentration, µmol/L</th>
<th>Trough concentration, µmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>BA</td>
<td>PAA</td>
<td>PBA</td>
</tr>
<tr>
<td>1</td>
<td>11.2</td>
<td>Propionic acidemia</td>
<td>PBA</td>
<td>120</td>
<td>15.2</td>
<td>371.6</td>
</tr>
<tr>
<td>2</td>
<td>22.5</td>
<td>Arginase deficiency</td>
<td>PBA</td>
<td>73.5</td>
<td>12.4</td>
<td>204.2</td>
</tr>
<tr>
<td>3</td>
<td>16.9</td>
<td>Argininosuccinic aciduria</td>
<td>PBA</td>
<td>122</td>
<td>31.2</td>
<td>307.0</td>
</tr>
<tr>
<td>4</td>
<td>20.2</td>
<td>Argininosuccinic aciduria</td>
<td>BA</td>
<td>49</td>
<td>49.6</td>
<td>&lt;10</td>
</tr>
<tr>
<td>5</td>
<td>20.2</td>
<td>Argininosuccinic aciduria</td>
<td>PBA</td>
<td>74</td>
<td>13.6</td>
<td>195.3</td>
</tr>
<tr>
<td>6</td>
<td>13.7</td>
<td>Citrullinemia</td>
<td>PBA</td>
<td>118</td>
<td>21.2</td>
<td>385.3</td>
</tr>
<tr>
<td>7</td>
<td>12.2</td>
<td>Citrullinemia</td>
<td>PBA</td>
<td>128</td>
<td>15.7</td>
<td>427.3</td>
</tr>
<tr>
<td>8</td>
<td>9.5</td>
<td>Argininosuccinic aciduria</td>
<td>BA</td>
<td>323</td>
<td>70.2</td>
<td>&lt;10</td>
</tr>
<tr>
<td>9</td>
<td>5.2</td>
<td>Citrullinemia</td>
<td>PBA</td>
<td>125</td>
<td>15.1</td>
<td>473.0</td>
</tr>
<tr>
<td>10</td>
<td>3.9</td>
<td>Argininosuccinic aciduria</td>
<td>PBA</td>
<td>158.5</td>
<td>32.0</td>
<td>86.7</td>
</tr>
<tr>
<td>11</td>
<td>10.9</td>
<td>OTC deficiency</td>
<td>PBA</td>
<td>99.2</td>
<td>31.8</td>
<td>94.3</td>
</tr>
<tr>
<td>12</td>
<td>10.1</td>
<td>OTC deficiency</td>
<td>BA</td>
<td>180</td>
<td>600.2</td>
<td>&lt;10</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>PAA</td>
<td>11.2</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>14.2</td>
<td>CPS deficiency</td>
<td>PBA</td>
<td>71</td>
<td>58.1</td>
<td>405.3</td>
</tr>
<tr>
<td>14</td>
<td>1.2</td>
<td>Citrullinemia</td>
<td>PBA</td>
<td>105</td>
<td>21.0</td>
<td>418.5</td>
</tr>
<tr>
<td>15</td>
<td>4.3</td>
<td>Citrullinemia</td>
<td>PBA</td>
<td>100</td>
<td>26.6</td>
<td>333.3</td>
</tr>
<tr>
<td>16</td>
<td>12.0</td>
<td>OTC deficiency</td>
<td>PBA</td>
<td>157.3</td>
<td>11.8</td>
<td>664.7</td>
</tr>
</tbody>
</table>

*Dose = 4.6 g.

b Collected 3.5 h after the dose.

* Collected 4 h after the dose.
as low as 1.9, 10.4, and 5.7 µmol/L for BA, PAA, and PBA, respectively. This method was sensitive enough to monitor the concentrations of the three drugs in the blood of the patients. To monitor the peaks of PAA and PBA (typically 200–500 µmol/L), a single 3-mm disc (2.4 µL of blood) was sufficient.

An analysis of the mass spectra of BA, PAA, and PBA at various concentrations showed spillover to the stable isotopes of 0.07%, 0.06%, and 0.25% from native BA (m/z 184), PAA (m/z 169), and PBA (m/z 225), respectively. Even at peak blood concentrations, these spillovers were inconsequential. To increase the sensitivity and overcome the potential problems of spillover in measuring these acids, the column temperature could be ramped at a slower rate (e.g., 3 °C/min) to better separate the native and isotopic peaks.

The extraction efficiencies for BA, PAA, and PBA were 74% ± 2%, 76% ± 3%, and 74% ± 3%, respectively (mean ± SD; n = 6). Repeated analysis of blood supplemented with 4.0 µg of BA, PAA, and PBA gave results of 3.9 ± 0.2, 4.0 ± 0.1, and 4.0 ± 0.1 µg (mean ± SD; n = 6), respectively. Dried filter-paper blood samples that were supplemented with 4.0 µg of BA, PAA, and PBA and stored up to 1 month at room temperature before analysis gave results of 4.0 ± 0.2, 4.0 ± 0.1 and 3.9 ± 0.1 µg (mean ± SD; n = 9), respectively. The results for blood spots heated at 80 °C for 24 h were within 2.6% (BA), 2.5% (PAA), and 5.2% (PBA) of those not heated.

We determined the peak (1.5 h postdose) and trough (predose) concentrations of BA, PAA, and PBA in 16 treated patients (Table 1). In six patients, PBA and PAA (predose) concentrations of BA, PAA, and PBA in 16 patients were 220–551 µmol/L. In six patients, PBA and PAA were much lower. In patient 13, PBA was 1542.4 µmol/L, and the highest postdose PBA concentration measured was 1542.4 µmol/L, and the highest postdose PBA concentration was 646.7 µmol/L. The BA concentration in patients not taking BA was 12–62 µmol/L, which likely reflects values in healthy individuals.

The method described here is sensitive, specific, and accurate. It allows easy collection and delivery to the testing site and should promote good monitoring of therapy. We also documented that the three analytes in the dried blood were stable to heat, alleviating concerns of heat stability if the sample was placed in an outside mailbox. Although extraction efficiency was ~75%, the high reproducibility and the use of stable isotopes as internal standards compensated for incomplete extraction. In addition, the use of selected-ion monitoring and the highly reproducible retention times of a capillary column allowed this method to detect the three acids with virtually no interference from other compounds.

This work was supported in part by a grant from Medicis Pharmaceuticals and by Grant RR 13297 from the National Center for Research Resources.

References