Phenylalanine and Tyrosine in Serum and Eluates from Dried Blood Spots as Determined by Reversed-Phase Liquid Chromatography

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We have developed a reversed-phase liquid-chromatographic procedure for simultaneously determining phenylalanine and tyrosine in serum and eluates of dried blood spots. Batch derivatization with phenylisothiocyanate and a 10-min linear gradient chromatographic assay with ultraviolet absorbance detection provide rapid sample throughput. Interrun precision (CV) is <12%; analytical recovery (from blood spot samples) exceeds 85%. Results for patients' samples correlate well with those from an amino acid analyzer and we encountered no apparent interferences. The speed and specificity of this assay facilitate the rapid diagnosis and monitoring of patients with phenylketonuria.

Additional Keyphrases: amino acid • phenylketonuria • heritable disorders • screening

Assay of phenylalanine (Phe) and tyrosine (Tyr) is used to establish the diagnosis of phenylketonuria (PKU), and Phe concentrations in blood are measured to follow the course of treatment. 1 Nonstandard abbreviations: PKU, phenylketonuria; HPLC, "high-performance" liquid chromatography; PTC, phenylisothiocyanate; Phe, phenylalanine; Tyr, tyrosine.

Methods

Chemicals. Phe, Tyr, and norvaline (internal standard) were obtained from Sigma Chemical Co., St. Louis, MO 63178. Triethylamine and PTC were obtained from Pierce Chemical Co., Rockford, IL 61105. Sodium acetate, acetic acid, and acetonitrile were from J.T. Baker Chemical Co., Phillipsburg, NJ 08865. Reagent-grade water was prepared in-house from a "Milli-Q" water system (Millipore Corp., Bedford, MA 01730).

Reagents: Stock acetate buffer, 1.0 mol/L, was prepared from sodium acetate, adjusted to pH 6.5 with concentrated acetic acid, then diluted to 0.1 mol/L for use. Coupling reagent (prepared within 1 h of use) consisted of acetonitrile/triethylamine/water (5.0/2.5/1.5 by vol) to which we added 20 μL of 20 mmol/L norvaline solution. A fresh vial of PTC was opened each day.

Standards and controls: Stock standards were prepared by dissolving Tyr and Phe in water to give concentrations of 2.0 and 20 mmol/L, respectively. Stored at 3–5 °C, the stock standards have been stable for a year. The Tyr–Phe working standard, 250 μmol/L each, prepared by diluting the respective stock standards with water, is stable at least six months at 3–5 °C. The Tyr–Phe spot standard was prepared by saturating a PKU test card with Tyr–Phe working standard, and allowing it to air dry (10). Control materials for the serum assay were Sigma Metabolic Control and Monitrol II from Dade Reagents, Miami, FL 33152. For blood-spot controls an aliquot of pooled whole EDTA-anti-coagulated blood was centrifuged and its Tyr and Phe content determined by six replicate analyses with an amino acid analyzer (Model 119; Beckman Instruments, Fullerton, CA). The remaining pooled whole blood was then divided into two portions for the low- and high-concentration blood-spot controls. We added Tyr and Phe standards to give final concentrations of the low and high controls of 150–175 and 300–350 μmol/L, respectively. Normal concentrations in serum of the neonate are Phe 29–164 μmol/L and Tyr 20–361 μmol/L. After mixing each control pool for 1 h, aliquots were spotted onto PKU test cards and allowed to air dry. The control and standard test cards are stable for at least six months when stored desiccated at 3–5 °C.

Specimens: Whole blood obtained by venous or skin puncture was allowed to clot. After centrifugation, the serum was removed and stored at 3–5 °C. Dried blood spots were prepared by saturating standard PKU test cards (no. 903; Schleicher & Schuell, Keene, NH 03431) with free-flowing whole blood obtained by skin puncture (10). The cards were air dried at room temperature, then stored desiccated at 3–5 °C until assay.

Sample preparation: Aqueous working standards, controls, and patients' sera, 0.02 mL each, were mixed with 0.2 mL of coupling reagent and centrifuged (1500 × g, 5 min) until clear. From the test cards containing standards, controls, and patients' blood we obtained two spots (about 0.02 mL of sample), using a standard-diameter paper punch (10). After the spots were transferred to glass test tubes, we added 0.2 mL of coupling reagent and left the samples for 30–60 min on a standard serological rotator set to 60–100 rpm. The eluate was removed and reserved for derivatization.

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**Derivatization:** To the supernates (from the aqueous samples) and the eluates (from the dried blood spots), we added 0.02 mL of PITC. After 20–30 min at ambient temperature, we extracted the derivatized amino acids from the reaction mixture by vortex-mixing with 0.5 mL of the 0.1 mol/L acetic buffer for 30 s. To this extract containing the derivatized amino acids, we added 2.5 mL of methylene dichloride, centrifuged, and removed the clear aqueous layer, which was reserved for chromatography.

**Chromatography:** We used an HPLC system consisting of a Series 4 pump, ISS automatic sampler, LC 95 UV-Vis detector, and Sigma 15 data collection station (all from Perkin-Elmer Corp., Norwalk, CT 06856). The system was equipped with a "Zorbax" (Du Pont, Wilmington, DE 19890) 5-μm particle size, 4.6 mm × 15 cm reversed-phase C-18 analytical column encased in a water jacket kept at 45 °C.

The solvent system consisted of acetonitrile (solvent A) and 0.1 mol/L acetic buffer (solvent B). The chromatograph was equilibrated with a mixture of solvent A and solvent B (15/85 by vol). After sample injection (50 μL), equilibration conditions were maintained for 1 min, followed by a linear gradient for 7 min to a 28/72 by vol mixture of solvent A and solvent B. The proportion of solvent A was stepped to 60/40 by vol for 5 min to purge late-eluting amino acids and potential interfering peaks, then returned to equilibration conditions during 2 min via a linear gradient. The column effluent was monitored at 254 nm and data were recorded to 0.05 A full scale.

**Calculations:** Amino acid concentrations were calculated by using std/Int std. peak height ratios compared with patient's amino acid/int. std. peak height ratios. The spot standards were used to calculate results for blood spots, and aqueous standards were used for serum. Spots prepared from aqueous standards gave the same degree of response as dried blood spots in the amino acid analyzer.

**Precision:** Within-run precision was determined by using the Sigma metabolic control for serum and the high-concentration blood spot control. Ninety-day run-to-run precision was determined by replicate analysis of four different pools (n = 30).

**Analytical recovery:** Sufficient Phe and Tyr standard was added to a pooled specimen of normal blood, by the method described for preparation of spot controls, to increase the Phe and Tyr concentrations by 300 μmol/L each. Spots were prepared in the manner described previously; the remainder of the supplemented pool was centrifuged and the serum was used for serum recovery studies.

**Comparison:** Results for patients' specimens (spots and serum) by the present method were compared with results for serum as determined by ion-exchange chromatography in the Beckman 119 amino acid analyzer.

**Results**

The chromatogram (Figure 1) demonstrated well-resolved peaks, free from apparent interferences. Early-eluting amino acids appear concurrently at the chromatogram front; late-eluting amino acids are washed from the column with the 60% acetonitrile after the assay.

The standard curve is linear to at least 2000 μmol/L for Phe and Tyr in both dried blood spots and serum. The lower limit of detection is <10 μmol/L, and we could discriminate between concentration differences of 10 μmol/L for both analytes in either blood spots or aqueous samples. Precision (Table 1) was acceptable. The target values for the Sigma standard for phenylalanine and tyrosine were 222 and 290 μmol/L.

**Table 1. Precision Data**

<table>
<thead>
<tr>
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<th>Phenylalanine concn, μmol/L</th>
<th>Tyrosine concn, μmol/L</th>
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<tr>
<td></td>
<td>n</td>
<td>Mean</td>
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<td><strong>Serum control (Sigma)</strong></td>
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<td>Intra-run</td>
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<td>Dried blood-spot control</td>
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<td>Run to run</td>
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<td><strong>Dried blood-spot control</strong></td>
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<td>Serum-based control</td>
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<td>Monitrol II</td>
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<td>Sigma</td>
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**Fig. 1. Typical chromatogram of a patient’s dried blood spot**

- **TYR:** tyrosine (245 μmol/L) at 3 min and 40% full-scale response, **N-VAL:** norvaline (int. std., 200 μmol/L) at 4.5 min, and **PHE:** phenylalanine (285 μmol/L) at 8 min μmol/L.

During the course of this study, 15 dried blood spots with Phe concentrations ranging from 60 to 1300 μmol/L were forwarded to us by the Centers for Disease Control, Atlanta, GA, as part of their neonatal-screening proficiency-test samples. The analysis was performed by the PITC technique. The correlation between the PITC method and the CDC target values was r = 0.95, the slope 1.08, the intercept −0.6, and the standard error of the estimate 63.

Samples used in the correlation study contained between 28 and 1098 μmol of Tyr and 38 to 1814 μmol of Phe per liter. Good correlation was found among results by the three methods: serum HPLC, blood-spot HPLC, and serum amino acid analyzer (Table 2). Clinically, all three methods were in agreement except for one sample that demonstrated an
Table 2. Correlations with Amino Acid Analyzer Results

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<th>Phenylalanine</th>
<th>Tyrosine</th>
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<tr>
<td>n r Slope Intercept SEE</td>
<td>r Slope Intercept SEE</td>
</tr>
<tr>
<td>PITC (serum) vs PITC (spots)</td>
<td>37 .96 .85 +13 82.7 .99 .87 +15 34.5</td>
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<tr>
<td>PITC (serum) vs AAA (serum)</td>
<td>57 .96 1.05 +8 38.6 .99 1.09 -17 24</td>
</tr>
<tr>
<td>PITC (spots) vs AAA (serum)</td>
<td>37 .96 1.19 +1 82.8 .99 1.23 -30 29.5</td>
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AAA, Beckman amino acid analyzer; SEE, standard error of the estimate; PITC, HPLC separation of derivatized amino acids.

abnormally low value for Phe by HPLC serum analysis while values by HPLC blood-spot analysis and ion-exchange were both normal.

Analytical recovery of added Phe and Tyr for blood spots and serum, respectively, were 82% and 85% for Phe and 87% and 92% for Tyr.

The good correlation between results by HPLC and those from the amino acid analyzer implies lack of interference by other physiologically present amino acids. Other common interfering substances such as lipids or bilirubin and excess and decomposed reagent are effectively removed by the methylene dichloride wash. We saw no interference by carbamazepine, ethoxuximide, phenobarbital, phenytoin, or primidone at high therapeutic concentrations.

Discussion

Determinations of abnormally high serum phenylalanine in neonates with hyperphenylalaninemia as determined by the Guthrie bacterial inhibition method confirms the diagnosis of PKU (II). Variants exist, some of which have only moderately increased Phe concentrations. Transient neonatal tyrosinemia, in contrast to PKU, produces high concentrations of both phenylalanine and tyrosine in serum. The clinical laboratory therefore must differentiate between these other causes of hyperphenylalaninemia and classic PKU. PKU can be successfully managed by adherence to a low phenylalanine diet, and adjustments in diets and monitoring of compliance are based on serum phenylalanine determinations, which thus must be accurate (II). Phenylalanine concentrations of mothers with successfully treated PKU must be maintained within narrow limits during their pregnancy, to avoid adverse effects on the development of the fetus (II). Our analysis procedure should be useful in all these circumstances.

This method combines the microsampling advantage inherent in blood collection on filter paper, with an accurate, precise, and specific HPLC method. Samples may be collected at remote sites by nurses, public health personnel, or even parents, then forwarded by regular mail. With minor change, the same procedure is used to analyze serum samples.

Major advantages of PITC derivatizations over other derivatives for HPLC analysis include stability at room temperature (>48 h) and lack of sensitivity to light, thus allowing easy adaptation to large-scale automated analysis.

Only one other study has been reported in which chromatography is used for blood-spot Phe analysis (II2). It employed, however, traditional ion-exchange methods with their inherent disadvantages.

Our procedure could serve as a model for the analysis for physiologically amino acids other than Tyr and Phe. This would allow general amino acid analysis by versatile standard HPLC equipment, rather than requiring the use of expensive dedicated ion-exchange chromatographs. The analysis verification data we developed and our in-house experience for one year demonstrate the validity of this method for determining phenylalanine and tyrosine.

References