Liquid-Chromatographic Measurement of Phenylalanine and Tyrosine in Serum

F. W. Splierto, W. Whitfield, M. Apetz, and W. Harry Hannon

With phenylalanine ammonia-lyase (EC 4.3.1.5) we converted phenylalanine (Phe) and tyrosine (Tyr) to trans-cinnamic acid and p-coumaric acid, respectively. These were separated by “high-performance” liquid chromatography and detected at 280 nm. We measured the Phe and Tyr content of human serum by adding 100 mU of the enzyme to a 20-μL serum aliquot, mixing for 2 h at 24 °C, then stopping the reaction with 1 mL of cold methanol. Precipitated proteins were removed by centrifugation, and the separated clear supernates were stored at −20 °C. For chromatographic separation, detection, and quantification, we used a system equipped with a C-18 reversed-phase column, a variable-wavelength spectrophotometer, a printer–plotter, and a microcomputer. The mobile phase was a mixture of dilute aqueous (50 g/L) acetic acid and CH₃CN (80/20, by vol). CVs for specimens containing 100 mg of Phe or Tyr per liter varied from 5 to 10%. Analytical recoveries were near 100%.

Additional Keyphrases: phenylketonuria • tyrosinemia • chromatography, reversed-phase • enzymic methods • radioenzymic assay • heritable disorders

Serum phenylalanine (Phe) and tyrosine (Tyr) must be reliably estimated in the detection of phenylketonuria and tyrosinemia. These two amino acids are usually measured by ion-exchange chromatography (1) or fluorometry (2), but both methods have their shortcomings. Fluorometric procedures cannot be used to measure serum Phe and Tyr simultaneously because chemical methods for the two are different. Moreover, the fluorometric assays are sensitive to small changes in pH, and depend on the measurement of poorly characterized fluorescent products. Ion-exchange chromatography measures the amino acids simultaneously, but is expensive to set up, requires a complicated gradient-elution scheme for chromatographic separation, and depends on a post-column derivatization reaction for detection.

We report a new method for measuring serum Phe and Tyr, in which they are enzymically de-aminated and the products chromatographically separated and detected at 280 nm. This method is more convenient than the fluorometric procedures because it allows for the simultaneous detection of both amino acids. Unlike the ion-exchange procedures, our method involves simple isocratic elution and does not require post-column derivitization.

The basis for our procedure rests upon two reports. One (3) describes the use of phenylalanine ammonia lyase (PAL; EC 4.3.1.5) to de-amine Tyr and Phe to p-coumaric and trans-cinnamic acids, respectively. The other (4) describes a “high-performance” liquid-chromatographic (HPLC) procedure for isolating and quantifying trans-cinnamic and p-coumaric acids from complex biological materials. Our method incorporates the essentials of both reports. After the Phe and Tyr in serum are incubated with PAL, the enzymically produced trans-cinnamic and p-coumaric acids are separated by reversed-phase HPLC and are detected on-line at 280 nm. We describe here the development of the method, its linearity and precision, and how it compares with the fluorometric assays. We also report on the use of [³H]Phe and [³H]Tyr to monitor the enzymatic reaction.

Materials and Methods

Instrumentation. We used an Analyst Series 7800 liquid-chromatographic system (Laboratory Data Control, Riviera Beach, FL 33404): it consisted of a CCM minicomputer, two Constametric III high-pressure pumps, a Spectromonitor wavelength detector, a thermal printer–plotter, and a 50-μL sample-injection loop. A 10-μm particle size column of C-18 octadecylsilane, 250 × 4.6 mm i.d. (Excalibur column packed with Spherisorb; Applied Science Labs., Inc., State College, PA 15168), was used for all separations. Retention times, peak areas, and peak heights were calculated automatically.

We measured the radioactivity of the tritiated amino acids with an LS-350 Scintillation Spectrometer (Beckman Instruments Inc., Fullerton, CA 92639).

Reagents. Acetonitrile, methanol, and acetic acid designated for use with HPLC were purchased from several commercial suppliers. We obtained Tris, Tris HCl, L-phenylalanine, L-tyrosine, and p-coumaric acid from Sigma Chemical Co., St. Louis, MO 63178.

We used the PAL enzyme from Sigma Chemical Co., who gives its specific activities for Phe and Tyr, respectively, as 1.3 and 0.3 kU per gram of protein. We obtained tritiated Phe (L-[ring-2,6-³H(N)]; 46.7 kCi/mol), tritiated Tyr (L-[ring-2,6-³H(N)]; 30 kCi/mol), and the scintillation solution, Aquasol, from New England Nuclear Corp., North Billerica, MA 01862.
<table>
<thead>
<tr>
<th>PHE (mg/L)</th>
<th>0</th>
<th>20</th>
<th>40</th>
<th>60</th>
<th>80</th>
</tr>
</thead>
<tbody>
<tr>
<td>TYR (mg/L)</td>
<td>0</td>
<td>20</td>
<td>40</td>
<td>60</td>
<td>80</td>
</tr>
</tbody>
</table>

**Absorbance at 280 nm:**

<table>
<thead>
<tr>
<th>Time (Minutes)</th>
<th>0</th>
<th>2</th>
<th>7</th>
<th>8.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absorbance</td>
<td>2.70</td>
<td>2.63</td>
<td>7.30</td>
<td></td>
</tr>
</tbody>
</table>

**Fig. 1.** Chromatograms of methanol extracts of enzyme-treated sera to which various concentrations of Phe and Tyr were added.

Retention times for p-coumaric and trans-cinnamic acids were, respectively, 2.7 and 7.5 min; 50-μL samples; other conditions as in text.

Fluorometry of Phe and Tyr. In one study we measured serum Phe and Tyr with kits purchased from Sigma Chemical Co., according to the manufacturer's instructions (5). Both methods depend upon fluorometric detection. The Phe method is based on that of McCaman and Robins (6), the Tyr method on that of Wong et al. (2).

**Chromatographic procedure for Phe and Tyr.** To 100 μL of enzyme solution (1000 U/L of 0.1 mol/L Tris buffer, pH 8.0), add 20 μL of Tris buffer, standard dissolved in Tris buffer, or sample; mix the solution, then incubate it for 2 h at about 25 °C (±3 °C). Add 1 mL of chilled methanol to stop the reaction. Store the specimens at -20 °C if they cannot be assayed promptly. Centrifuge thawed specimens at 3000-4000 × g for 10 min to remove precipitated proteins. Apply 50-μL aliquots of the supernate to the column; elute isocratically at 2.6 mL/min (pressure: 10.3 MPa, or 1500 lb./in.²) with freshly prepared eluent: acetic acid (50 g/L of H₂O) and acetonitrile, 80/20 by vol. Monitor the absorbance of the eluate at 280 nm.

We adjusted full-scale deflection of the recorder pen to correspond to 0.01 A and calculated serum Phe and Tyr concentrations by using a least-squares method relating absorbance peak area to known concentration of amino acid in the calibrator.

**3H assay.** We used [3H]Phe and [3H]Tyr to measure recovery and the PAL-catalyzed conversions of Phe to trans-cinnamic acid and Tyr to p-coumaric acid. The tritiated amino acids were added to serum pools, then incubated with or without enzyme. Methanolic extracts were prepared and injected onto the column as described above. Column effluent...
was collected at 15-s intervals, then added to scintillation vials containing 10 mL of Aquasol. The tritium content of each fraction was measured by liquid scintillation spectrometry.

**Results and Discussion**

Enzymic deamination of Phe and Tyr produces reaction products that absorb strongly in the ultraviolet part of the spectrum. The procedure reported by Shen and Abell (3) is based on this fact, but in their spectrophotometric assay, absorbance must be measured at two different wavelengths. p-Coumaric acid, derived from Tyr, has an absorbance maximum at 315 nm, whereas trans-cinnamic acid does not absorb at this wavelength. Both trans-cinnamic and p-coumaric acids absorb strongly at 290 nm. Thus, to calculate the absorbance at 290 nm due to trans-cinnamic acid (and, ultimately, Phe) concentration, the following equation must be used:

\[ A_{\text{trans-cinnamic}} = A_T - (\epsilon_{290}/\epsilon_{315})(A_{315}) \]

where \( A_{\text{trans-cinnamic}} \) = absorbance at 290 nm due to trans-cinnamic acid, \( A_T \) = absorbance at 290 nm due to both trans-cinnamic and p-coumaric acids, \( \epsilon_{290} \) = molar absorptivity for p-coumaric acid at 290 nm, \( \epsilon_{315} \) = molar absorptivity for p-coumaric acid at 315 nm, and \( A_{315} \) = absorbance at 315 nm.

For sera that contain unusually large concentrations of Tyr, the error associated with the subtraction may be large. Our method offers an alternative to the spectrophotometric procedure. By using chromatography we can physically separate the two acids, obviating the above correction. Several publications deal with separating trans-cinnamic and related aromatic acids. The above-described system consisting of a reversed-phase C-18 column, an acetic acid/H2O/CH3CN mobile phase, and detection at 280 nm was adequate. Chromatograms presented in Figure 1 show that trans-cinnamic and p-coumaric acids are well separated and readily detected in this system. Because of its phenolic hydroxyl group, p-coumaric acid is more polar than trans-cinnamic acid and hence, having less affinity for the nonpolar column packing, elutes before trans-cinnamic acid.

Other experiments showed that the retention times for both compounds were greatly influenced by the composition of the mobile phase. For our preliminary experiments we used the acetic acid/water (5/95 by volume) system described by Wulf and Nagel (4). Although separation and quantification were satisfactory, we soon abandoned this system because retention times \( (R_t) \) were too long. At a flow rate of 2.6 mL/min, the \( R_t \) for trans-cinnamic acid was 12 min; for p-coumaric acid, 30 min. Besides Wulf and Nagel, others (7–9) also report that \( R_t \)'s can be shortened by including an organic solvent such as methanol in the mobile phase. We tried this, but had problems with unstable baseline. Acetonitrile proved to be a better solvent, as indicated by the data in Figure 1. Calibration was conducted in two ways. We used calibrator solutions containing either Phe or Tyr and solutions containing both amino acids. Table 1 lists standard-curve parameters (slope, y-intercept, and correlation coefficients) for both approaches. Mean values for the slopes and correlation coefficients were about the same for both. But variability, as measured by the CV of the slope and y-intercepts, was less for calibration curves obtained with the second system, probably because fewer steps are necessary for calibration when solutions containing both amino acids are used.

Table 2 lists results of repeated analysis of the Phe and Tyr content of serum samples supplemented with these amino acids. Precision estimates again showed less analytical variability when we used calibrators containing both Phe and Tyr. Furthermore, measured Phe and Tyr concentrations were within 2 to 4 mg/L of the expected values. By comparison, Shen et al. (10), using the enzymic procedure, report CVs of approximately 10%.

We used tritiated amino acids to follow the PAL-catalyzed reaction. In one experiment \(^3\)H-Phe was added to a serum specimen, then incubated with enzyme or buffer as described in Materials and Methods. After 2 h, methanol extracts were

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**Table 1. Linear-Regression Statistics for Standard Curves Obtained by Using Calibrators Containing Phe, Tyr, or Both**

<table>
<thead>
<tr>
<th>Peak area</th>
<th>Separate calibrators</th>
<th>Both amino acids</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( (n = 5) )</td>
<td>( (n = 4) )</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Slope*</td>
<td>51 130</td>
<td>4276</td>
</tr>
<tr>
<td>y-intercept*</td>
<td>-10 398</td>
<td>6872</td>
</tr>
<tr>
<td>( r )</td>
<td>0.9841</td>
<td>0.012</td>
</tr>
</tbody>
</table>

| Tyrosine |               |               |               |
| Slope* | 30 724 | 4414 | 14 | 28 621 | 755 | 3 |
| y-intercept* | -1 990 | 4318 | 200 | -752 | 2253 | 300 |
| \( r \) | 0.9974 | 0.003 | 0.3 | 0.9974 | 0.003 | 0.3 |

* Slope and y-intercepts expressed in arbitrary peak area units.
prepared and chromatographed. Figure 2 summarizes
the data. In the absence of enzyme, 95% of the radioactivity eluted
with a retention time similar to that for Phe. We also observed
two other peaks, one with an Rf of 2 min, and the other with
an Rf of about 3 min. After incubation with PAL, radioactive
Phe disappeared, and 83% of the [3H] appeared as trans-cin-
namic acid. The peak that eluted at 2 min also increased
substantially. In other studies we found that for the first 10
min there was a linear relationship between the amount of 3H
eluting at 2 min and the duration of the incubation. However,
the maximum amount of early eluting peak never exceeded
13% of the original dose, even after prolonged incubation. We
have been unable to identify the peak. Perhaps it arises as a
result of a competing reaction catalyzed by a contaminating
enzyme.

Although the conversion of [3H]Tyr to [3H]p-coumaric acid
was more straightforward, chromatographic separation was
more difficult. We found that with our standard system it was
difficult to separate Tyr from p-coumaric acid. Separation
could be effected, however, by changing the mobile phase. In
the chromatographic separation portrayed in Figure 3,