Assay for Erythrocyte Uroporphyrinogen I Synthase Activity, with Porphobilinogen as Substrate

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We describe a convenient, economical procedure for measuring uroporphyrinogen I synthase (EC 4.3.1.8) activity in erythrocytes, the results of which can be used to diagnose acute intermittent porphyria, in either its latent or acute stage. By limiting the test reaction sequence to the conversion of porphobilinogen to porphyrins, we eliminated several disadvantages of alternative methods in which δ-aminolevulinate is used as substrate. The latter assay was inhibited by lead; our procedure was not. Our procedure also gave greater porphyrin synthesis with less substrate. Erythrocytes of healthy women presented a mean activity of 12.4 nmol of porphyrin formed per liter per second; erythrocytes of healthy men presented a mean activity of 11.0 nmol/L per second. The within-run coefficient of variation (CV) for our assay was 1.8%; the between-run CV was 3.1%.

Additional Keyphrases: acute intermittent porphyria • heritable disorders • δ-aminolevulinate dehydratase • reference intervals • uroporphyrin

Acute intermittent porphyria (AIP)² has been an identifiable metabolic problem for more than four decades (1). Identification of individual cases has been mainly by recognition of increased urinary excretion of porphobilinogen (PBG). Most persons who have the trait for AIP excrete PBG excessively only during acute episodes of this disorder; therefore, the usefulness of the urinary PBG test is very limited in the identification of affected persons. Undoubtedly, the trait for AIP exists unrecognized in many persons—and remains unrecognized because it has not been expressed as clinically significant episodes.

During the last decade, several studies (1–3) have implicated a generalized deficiency of uroporphyrinogen I synthase (UPG-I-S) as the primary cause of AIP. Accordingly, the activity of UPG-I-S is always markedly decreased in erythrocytes of persons who have the AIP trait; in affected persons, the activity is reportedly less than half of the average (mean) activity shown by a cross-section of unaffected persons (4). Therefore, an assay for this enzyme in erythrocytes can be particularly useful.

Because acute episodes of AIP can precipitate fatal respiratory paralysis, and because uremia and various other metabolic complications can be induced during acute episodes, identification of persons having the AIP trait is important, whether the trait is latent or has already been expressed in terms of a medically significant disturbance. Identification of affected persons is especially important because acute episodes may be provoked by any of several commonplace chemical agents, including some commonly used drugs. Siblings and children of persons with AIP should be tested for the AIP trait in childhood if possible. Although the likelihood of a crisis of AIP in childhood is very small, detection of the AIP trait during childhood should be helpful in any case, to minimize risk of exposure of an affected person to environmental agents that could provoke a life-threatening crisis at any time.

Previously described procedures with PBG as substrate for measuring UPG-I-S activity have included some complicated technical details, such as lysing the erythrocytes by freezing and thawing, that could lead to imprecise measurements of the enzyme activity (4–6). The procedure we describe here excludes as many such details as possible.

δ-Aminolevulinic acid (ALA) has been used as the substrate in measuring UPG-I-S activity (7, 8); however, its use presents certain avoidable problems. Although it is less expensive and more stable than PBG, procedures in which it is used are susceptible to the variability of δ-aminolevulinate dehydratase (ALA-D) activity. Furthermore, the use of ALA as the substrate presents other biochemical disadvantages, including lead inhibition of ALA-D activity (9, 10).

We describe an improved method for routinely measuring erythrocyte UPG-I-S activity, with PBG as substrate. In addition, through comparison with a method with ALA as substrate (8), we validate the use of PBG rather than ALA.

Methods and Materials

Reagents

Triton X-100 solution: Dilute 2.0 mL of Triton X-100 surfactant to 1 L with distilled water. This solution is stable for at least six months.

Triton X-100/zinc sulfate/dithiothreitol solution: Dissolve 14.4 mg of ZnSO₄·7H₂O and 7.7 mg of dithiothreitol in 45 mL of Triton X-100 solution and dilute to 50 mL with Triton X-100 solution. This solution must be prepared immediately before assay.

Phosphate/citrate buffer: Dissolve 6.7 g of Na₂HPO₄·7H₂O in 95 mL of distilled water and adjust the pH to 7.5 with a citrate solution prepared by dissolving 1.21 g of citric acid monohydrate in 20 mL of distilled water and diluting to 25 mL. Dilute the buffer to a final volume of 100 mL with distilled water. Adjust the buffer pH immediately before preparing the substrate. This solution is stable for at least six months at room temperature.

Porphobilinogen substrate: Prepare a 1.4 mmol/L solution by dissolving 1.0 mg of porphobilinogen (Porphyrin Products, Logan, UT 84321) in 3.2 mL of phosphate/citrate buffer immediately before the assay.

δ-Aminolevulinate substrate: Prepare a 4 mmol/L solution by dissolving 67.04 mg of δ-aminolevulinic acid hydrochloride (Sigma Chemical Co., St. Louis, MO 63178) in 95 mL of phosphate/citrate buffer before diluting to 100 mL with buffer.

Trichloroacetic acid solution: Dissolve 100 g of trichloroacetic acid in 900 mL of distilled water and dilute to 1 L. This solution is stable for at least six months at room temperature.
**Uroporphyrin fluorescence standard**: Prepare a 100 μg/L standard by diluting 0.5 mL of a 500 μg/L solution of uroporphyrin I in 1 mol/L HCl (Porphyrin Products) to 2.5 mL with HCl, 1 mol/L. The 500 μg/L standard is stable for at least 30 days when stored in the dark at 4 °C.

**Specimens**: Centrifuge a 2.0-mL aliquot of heparinized whole blood for 10 min at 2500 rpm in a 12-mL calibrated, graduated centrifuge tube. Aspirate the plasma and wash the erythrocytes three times with 1-mL aliquots of a pre-chilled sodium chloride solution (9 g/L). Discard the rinse fluid after each rinse. Record the volume of packed cells and resuspend the cells to a final volume of 2.0 mL with saline. These specimens are stable at −20 °C for at least two months.

**Controls**: Appropriate control samples consist of washed erythrocytes from 2.0-mL samples of whole blood from persons ascertained to be healthy by Mayo Clinic internists. Stored at −20 °C, these samples should be stable for at least two months. One control sample should be assayed with each set of unknown samples.

**Procedure**

Add 1.0 mL of Triton X-100 solution to each of three 10-mL glass-stoppered centrifuge tubes for each specimen. Add 100 μL of specimen, stopper, and vortex-mix each sample, then incubate in a covered water bath for 15 min at 37 °C. Add 0.5 mL of phosphate/citrate buffer to one tube, which serves as the specimen blank. Also, add 0.5 mL of 1.4 mmol/L PBG substrate to each of the other two tubes. Stopper, vortex-mix, and incubate these reaction mixtures for exactly 30 min at 37 ± 0.1 °C. End the reaction by adding 2.5 mL of trichloroacetic acid solution to each tube, then mix and centrifuge for 10 min at 2000 rpm. Measure the fluorescence of the supernatant solution (we used an Amino-Bowman spectrophotofluorometer, American Instrument Co., Silver Spring, MD 20910), using the 100 μg/L uroporphyrin I standard. Set the excitation wavelength at 408 nm and the emission wavelength at 602 nm.

The procedure with ALA as the substrate was from Piepkorn et al. (9).

**Calculations**

The nanomoles of porphyrin formed per second per liter of erythrocytes are

\[(4.10/0.100)(120.4/1800)(V_{TS}/V_E)(F_X/F_{STD}),\]

or

\[2.74(V_{TS}/V_E)(F_X/F_{STD})\]

where

- 4.10 = final volume (mL) of the assay solution
- 0.100 = specimen volume (mL)
- 120.4 = concentration (nmol/L) of the uroporphyrin calibration standard
- 1800 = duration (s) of incubation with PBG substrate
- \(V_{TS}\) = total volume (mL) of erythrocyte suspension
- \(V_E\) = volume (mL) of washed, packed erythrocytes
- \(F_X\) = fluorescence reading of the test sample (arbitrary units)
- \(F_{STD}\) = fluorescence reading of the calibration standard (arbitrary units)

**Results and Discussion**

**Analytical Variables**

Blood specimens were drawn into evacuated blood tubes that contained heparin, ethylenediaminetetraacetate, or potassium oxalate. The latter two slightly inhibited UPG-I-S activity, so we recommend only heparin as a suitable anticoagulant for this test.

Specimens stored at room temperature or refrigerated at 4 °C showed a slightly decreased UPG-I-S activity after 24 h. Therefore, we recommend that all specimens be processed immediately by washing and resuspending the packed erythrocytes in cold saline, then either assaying immediately or storing them at −20 °C until analyses can be performed.

Various concentrations of PBG, ranging from 0.05 to 2.0 mmol/L in the substrate solution, were used to determine the optimum concentration of the substrate. As shown in Figure 1, the enzyme activity reaches a plateau when the concentration of PBG in the substrate solution exceeds 0.7 mmol/L. To ensure that the effect of substrate concentration will be negligible, we recommend use of a substrate solution containing 1.4 mmol of PBG per liter.

Figure 2 indicates that the product yield is linearly related to duration of incubation for at least 90 min. Because the procedure reported herein includes a 30-min incubation, the slope of our curve is assuredly proportional to the product yield for all samples. Our test results gave a within-run CV of 1.8% (n = 20). We also determined a between-run CV of 3.1% (n = 22).

**Reference Intervals**

The mean activity of uroporphyrinogen I synthase for 49 ostensibly healthy women ranging in age from 20 to 60 years was 12.4 nmol of PBG converted per second per liter (SD = 2.2, range = 8.1–16.8). The mean for 45 ostensibly healthy men ranging in age from 23 to 69 years was 11.0 nmol/s per liter (SD...
Table 1. Comparison of Substrates

<table>
<thead>
<tr>
<th>Lysate reagent, 1 mL</th>
<th>Substrate concn in reaction mixture, mmol/L</th>
<th>Enzyme activity, nmol/s per liter</th>
</tr>
</thead>
<tbody>
<tr>
<td>TZD c</td>
<td>1 mL of buffer</td>
<td>0.00 (specimen blank)</td>
</tr>
<tr>
<td>TZD c</td>
<td>1 mL of 4 mmol/L ALA</td>
<td>1.95 4.45 (0.22)</td>
</tr>
<tr>
<td>TX d</td>
<td>1 mL of 2 mmol/L PBG</td>
<td>0.98 9.51 (0.27)</td>
</tr>
<tr>
<td>TX d</td>
<td>1 mL of 0.1 mmol/L PBG</td>
<td>0.049 8.67 (0.19)</td>
</tr>
<tr>
<td>TX d</td>
<td>1 mL of 0.05 mmol/L PBG</td>
<td>0.024 8.19 (0.22)</td>
</tr>
</tbody>
</table>

TZD 0.5 mL of 8 mmol/L ALA
TX 0.5 mL of 4 mmol/L ALA
TX 0.5 mL of 1.4 mmol/L PBG
TX 0.5 mL of 0.1 mmol/L PBG

Table 2. Inhibition of \( \delta \)-Aminolaevulinate Dehydratase by Lead in Whole Blood

<table>
<thead>
<tr>
<th>Concn of whole-blood lead, ( \mu g/L )</th>
<th>Lysate reagent, 1 mL</th>
<th>Substrate concn in reaction mixture, mmol/L</th>
<th>Enzyme activity, nmol/s per liter</th>
</tr>
</thead>
<tbody>
<tr>
<td>850</td>
<td>TZD c</td>
<td>1 mL of 4 mmol/L ALA</td>
<td>5.43</td>
</tr>
<tr>
<td>570</td>
<td>TZD c</td>
<td>0.5 mL of 0.1 mmol/L PBG</td>
<td>8.13</td>
</tr>
<tr>
<td>620</td>
<td>TZD c</td>
<td>0.5 mL of 0.1 mmol/L PBG</td>
<td>6.63</td>
</tr>
</tbody>
</table>

* All samples were processed by the present procedure, except where noted.
* The accepted (11, 12) upper limit of the normal range for whole blood lead is 400 \( \mu g/L \).
* Abbreviations as in Table 1.

= 1.6, range = 7.9–14.7). For both women and men, values less than 7.0 nmol/s per liter should indicate AIP. The range of 7.0 to 8.0 nmol/s per liter is suspect; we consider values lying in this range to be indeterminate.

Five persons known to have AIP presented values of 6.2, 4.3, 3.3, 5.3, and 4.1 mmol/L per liter. A son of one of those persons presented a value of 5.3 mmol/L per second; so far, he has not experienced symptoms of AIP.

Substrate Comparison

Table 1 summarizes our comparison of PBG and ALA as substrates. Because 2 mol of ALA are required to synthesize 1 mol of PBG, we compared the amount of porphyrin synthesized in a reaction mixture containing 1.95 mmol of ALA per liter with that in a mixture containing 0.98 mmol of PBG per liter; the yield of porphyrin from PBG was 2.13-fold the yield from ALA. Piepkorn et al. (8) indicated that conversion of ALA to PBG might be rate-limiting in the synthesis of uroporphyrinogen from ALA. The enhanced porphyrin synthesis observed with PBG reinforces that suggestion. Even a reaction mixture containing only 24 \( \mu g \) of PBG per liter gave a greater porphyrin synthesis in 30 min than did a mixture containing 1.95 mmol of ALA per liter.

Our procedure avoids the difficulties inherent when ALA is used as substrate. By using PBG as substrate, we eliminate ALA-D as a factor in the reaction sequence that could preclude accurate measurement of UPG-I-S activity: if the ALA-D activity was low, it would probably result in an abnormally slow conversion of ALA to PBG and, therefore, in a falsely low value for UPG-I-S activity. Furthermore, by limiting the reaction sequence to the conversion of PBG to porphyrins, we are able to ensure that the activity measured represents the UPG-I-S activity.

In an effort to make the routine assay more economical and more accurate, we doubled the volume of erythrocytes in the reaction mixture and halved the amount of substrate used; PBG still gave a greater porphyrin yield than ALA (Table 1).

Our procedure was sufficiently sensitive to measure UPG-I-S activity below 1.0 nmol/s per liter, more than adequate to measure the low erythrocyte activities characteristic of AIP.

Lead Toxicity Study

Lead is known to interfere with ALA-D (9, 10), and has also been reported to interfere with UPG-I-S (7, 11). Therefore, we used two different approaches, to attempt to determine whether lead would affect our assay for UPG-I-S. In addition, we observed in comparison studies that lead inhibited porphyrin formation from ALA.

In one approach, we used a specimen from a patient whose whole-blood lead value was sufficiently high to reflect a toxic condition (12, 13) (Table 2). Replicate samples were incubated

Table 3. Lack of Lead Inhibition of Uroporphyrinogen I Synthase

<table>
<thead>
<tr>
<th>Concen of PbCl₂, ( mg/L )</th>
<th>Enzyme activity, nmol/s per liter</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.000 (specimen blank)</td>
<td>11.6</td>
</tr>
<tr>
<td>0.153</td>
<td>11.5</td>
</tr>
<tr>
<td>0.305</td>
<td>11.2</td>
</tr>
<tr>
<td>0.381</td>
<td>11.5</td>
</tr>
<tr>
<td>0.763</td>
<td>11.6</td>
</tr>
<tr>
<td>1.525</td>
<td>11.6</td>
</tr>
<tr>
<td>3.050</td>
<td>11.5</td>
</tr>
<tr>
<td>30.500</td>
<td>11.7</td>
</tr>
</tbody>
</table>

* All samples processed by the present procedure.
* Each value is the average of two determinations.
after lysing the cells with a Triton X-100 solution, either alone or with ZnSO₄ and dithiothreitol. The latter solution has been reported to reactivate lead-inhibited ALA-D (6). Two samples were incubated with ALA and two others with PBG as substrate. ZnSO₄ and dithiothreitol were essential for maximum synthesis of porphyrin when ALA was used as substrate, but not when PBG was used; therefore, although inhibition of ALA-D was demonstrable readily by toxic concentrations of lead, inhibition of UPG-I-S was not. Two additional specimens from other persons with lead intoxication were incubated with PBG with and without ZnSO₄ and dithiothreitol (Table 2). In those cases also, ZnSO₄ and dithiothreitol caused no increase in the conversion of PBG to porphyrins. ZnSO₄ and dithiothreitol, therefore, apparently do not provide any favorable effect in the UPG-I-S-catalyzed reaction in our phosphate/citrate buffer.

In the second approach, we used blood from a healthy young woman; aliquots were supplemented with various amounts of PbCl₂ (Table 3). This study confirmed that even very high concentrations of lead (up to 30.5 mg/L) do not inhibit the activity of UPG-I-S.

The basis of an apparent discrepancy with an earlier report (11) of UPG-I-S inhibition by lead is not clear. That inhibition by lead was observed with use of a different buffer, tris(hydroxymethyl)methylamine - HCl; otherwise, the conditions of incubation were similar to ours, where no inhibition occurred. Apparently, phosphate/citrate buffer is an advantageous support medium for the UPG-I-S reaction.

As a final note, careful study of the final assay solution with both a scanning spectrophotofluorometer and a “high-performance” liquid chromatograph indicated that the product formed during incubation of erythrocytes with PBG is almost entirely uroporphyrin. Our chromatographic system separated porphyrins having 8, 7, 6, 5, 4, and 2 carboxyls (manuscript in preparation).

Testing of UPG-I-S activity in erythrocytes is clearly a necessary step in the evaluation of persons suspected of having the trait for AIP. The documented history of this disorder now appears to be sufficient to indicate the mode of inheritance to be autosomal dominant (14); therefore, most persons with the trait for AIP should be recognizable through testing the UPG-I-S activity of their erythrocytes. Our observations have established that this activity can be assayed reliably and with good precision through incubation of erythrocytes with PBG; moreover, our observations have established that, to assay UPG-I-S activity, incubating erythrocytes with PBG is superior to incubating with ALA.

References