Modified Erythrocyte Uroporphyrinogen I Synthase Assay, and Its Clinical Interpretation

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Assay of erythrocyte uroporphyrinogen I synthase is an accepted diagnostic test for acute intermittent porphyria, particularly in those individuals who are asymptomatic or in whom the disease is not biochemically manifested by excretion of excess porphyrin precursor. The assay described is based upon a coupled-enzyme procedure in which added δ-aminolevulinic acid and its dehydratase present in erythrocytes are used to generate porphobilinogen as substrate for uroporphyrinogen synthase. Zinc and dithiothreitol are added with preincubation to give maximum activity and reproducibility. These agents also prevent inhibition by lead. Healthy young women had a mean activity of 40 nmol of porphyrin formed per milliliter of erythrocytes per hour, men an activity of 38 nmol/ml/h.

Preparation of control specimens is described. This assay gave within-day CVs ranging from 1.9 to 2.8%. Precautions in interpretation of results are discussed.

Additional Keyphrases: acute intermittent porphyria · diagnostic aids · lead poisoning · normal values · enzyme kinetics

Enzyme abnormalities seen in acute intermittent porphyria (AIP) consist of increased activity of δ-aminolevulinic synthase (EC 2.3.1.37), decreased activity of uroporphyrinogen I synthase (URO-S), and decreased activity of steroid Δ4,5α-reductase (no EC no. assigned) (1, 2). High urinary excretion of δ-aminolevulinic acid, porphobilinogen, and uro- and coproporphyrin are found in overt clinical disease, but these findings may be absent in the latent form. By contrast, erythrocyte URO-S assay shows consistently decreased activity in the gene carriers without overt disease (3–5), thereby permitting identification of those individuals who are subject to a potentially lethal reaction precipitated by drugs, liver toxins, hormonal abnormalities, diet, and other factors.

Several precautions with interpretation of erythrocyte URO-S assays are noteworthy. For example, enzyme activities between normal individuals and those with AIP may overlap (3–6), especially if maximum precision and accuracy are not maintained. Our recently described method in which δ-aminolevulinic acid is used as substrate may pose an additional problem of falsely positive tests for AIP in lead-intoxicated individuals due to inhibition of δ-aminolevulinic dehydratase (ALA-D), which normally is present in erythrocytes at 18-fold the activity of URO-S and as such is not normally rate limiting (2). Persons with congenitally low ALA-D may also pose problems with this original method (in preparation).

This report describes modifications of our original assay in which porphobilinogen is enzymatically generated (2). Improvements include enhanced specificity, greater accuracy and precision, elimination of lead interference, availability of quality control, and a documented sex-related effect on URO-S activity.

Materials and Methods

Reagents

Blood diluted: Dissolve 0.2 ml of Triton X-100 surfactant in 100 ml of de-ionized water, and dissolve 28.8 mg of ZnSO₄·7H₂O in 100 ml of this solution to yield a 1 mmol/liter solution, which is stable indefinitely.

Dithiothreitol: Immediately before the assay, add 7.7 mg DTT to 50 ml of the ZnSO₄·Triton X-100 solution to yield a 1 mmol/liter solution. The instability of this solution precludes storage.

δ-Aminolevulinic acid substrate: Dissolve 6.70 g of Na₂HPO₄·7H₂O in 100 ml of de-ionized water (0.25 mol/liter) and titrate to pH 7.5 with a citric acid solution prepared by dissolving 5.25 g of the dihydrate in 100 ml of water (0.25 mol/liter). Prepare ALA substrate by dissolving 67.04 mg of δ-aminolevulinic acid hydrochloride (Sigma Chemical Co., St. Louis, No. 63178) in 100 ml of this buffer to yield 4 mmol/liter. Store at −20 °C in convenient aliquots, which are stable for at least six weeks.

Trichloroacetic acid: Dissolve 10 g of trichloroacetic acid in 100 ml of water. This solution is stable indefinitely.

Coproporphyrin standard: Prepare a standard concentration of 76 nmol/liter by dissolving 5.0 μg of coproporphyrin (Sigma Chemical Co., or Porphyrin Products, Logan, Utah 84321) in 5.0 ml of 1.5 mol/liter HCl, heating, and then diluting to 100 ml with 1.5 mol/liter HCl. This solution is stable for at least a year when kept in a tightly stoppered container in the dark at 4 °C.

Control: For quality control, centrifuge a suitable quantity of heparinized whole blood, remove the plasma, and restore the original volume with 2 g/liter Triton X-100 solution containing neither zinc nor DTT. This specimen can be assayed repetitively to obtain between-batch mean and SD values. Assay individual aliquots concomitantly with diagnostic specimens. Well-sealed aliquots stored at −20 °C are stable for at least four months.

Specimen: Heparinized whole blood, obtained by venipuncture or by capillary puncture, sufficient to perform an
primary filter (405 nm) and a No. 25 secondary filter (595 nm).

**Calculations**

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nmol \text{ porphyrin formed/ml erythrocytes/h} = \frac{(\text{fluor. of test}) \times (76.4) \times (4.55) \times (100)}{(\text{fluor. of std.}) \times (1000) \times (0.05) \times (\text{hematocrit})}
\]

which reduces to

\[
\frac{(\text{fluor. of test}) \times 695}{(\text{fluor. of std.}) \times \text{HCT}}
\]

where:

- fluor. = fluorometric reading in arbitrary units
- 76.4 = concentration of coproporphyrin std. in nmol/liter
- 4.55 = final volume of the trichloroacetic acid supernatant solution in ml
- 1000 = conversion factor for nmol/liter to nmol/ml
- 0.05 = volume of specimen in ml

**Results**

**Analytical Variables**

_DTT concentration:_ A concentration range of 10⁻² to 10⁻⁷ mol/liter in the preincubation mixture was studied. Figure 1 shows a nonlinear, low-sloped curve, with maximum activity at 1 nmol/liter. A mean enhancement in enzyme activity of about 8% was found in the assay of 36 different specimens. Fluorescence of the blank was enhanced in some cases by DTT, 0.01 mol/liter.

_Zinc sulfate concentration:_ With zinc sulfate concentrations of 10⁻² to 10⁻⁷ mol/liter in the Triton reagent, disparate activities were obtained, depending upon preincubation. Without preincubation, little or no stimulation was seen from 10⁻⁶ to 10⁻⁴ mol/liter, but at higher concentrations of 10⁻³ and 10⁻² mol/liter there was a marked decrease of activity to 20% of control values, not appreciably altered by adding 1.0 mmol of DTT per liter. After 30-min preincubation, zinc sulfate at 10⁻⁴ to 10⁻³ mol/liter enhanced activity by 12% (Figure 2). The latter concentration was thus chosen for routine use.

_Preincubation:_ The effect of preincubation periods up to 60 min and in the presence of 1 mmol of DTT and zinc sulfate per liter contained in the Triton X-100 diluent were determined. Maximal enhancement of 8% was found with the 30-min preincubation period.

_Storge of specimens:_ Addition of 1 mmol of DTT and zinc sulfate per liter to enzyme incubations resulted in complete restoration of URO-S activity in specimens that had been stored for as long as one week at room temperature and for at least one month at 4 °C (Figure 3). The frozen (−20 °C) aliquots of control lyase were restored to original activity after periods of as long as four months.

**Normal Values**

URO-S activity in 63 healthy college-age men and 67 healthy college-age women (Figure 4) averaged 39 nmol of porphyrin formed per milliliter of erythrocytes per hour (SD = 5.1; range = 24–54). The mean activity for the women was 40 (range = 30–54), for the men 38 (range = 24–49). Our test results gave within-day coefficients of variation ranging from 1.9 to 2.8%.

**A Case Study of Lead Interference**

A patient with a past history of hemolytic anemia was admitted to University Hospital with severe abdominal pains. Results for intravenous pyelogram, barium enema, and pelvic
ultrasound examinations proved to be within normal limits. The hematologic work-up gave the following results: hematocrit 30%, hemoglobin 11.2 g/dl, mean cell volume 92 fl, reticulocyte index 8.5%. Erythrocyte morphology showed poikilocytosis and basophilic stippling. Iron-loaded sideroblasts and a few ringed sideroblasts were noted in the bone marrow biopsy. Subsequent investigation for lead poisoning showed a blood lead level of 880 μg/liter, protoporphyrin/heme ratio 460 (reference mean = 10.8), urinary ALA 86 mg/24 h (range = 1.5–7.5 mg/24 h), and a urinary lead excretion of 54 μg/24 h. The URO-S activity in the absence of DTT and zinc was 7.0 nmol of porphyrin formed per milliliter of erythrocytes per hour, but this was restored to 85 by the addition of these two reagents. The low activity found without DTT and zinc suggests a falsely positive diagnosis of AIP. However, the abnormally high activity is consistent with the increased URO-S activity found in young erythrocytes that accompany an increased reticulocyte index.

Discussion

The incorporation of diithiothreitol as a reducing agent at a concentration of 1.0 mmol/liter in the Triton reagent (final concentration, 5 × 10⁻⁴ mol/liter) was found in this study to augment activity by approximately 8%, and an additional increment of approximately equal magnitude was found with 30-min preincubation. Although one report of a URO-S assay utilizing porphobilinogen as substrate failed to find enhanced porphyrin yield with the use of glutathione as the reducing agent (7), others have found a salutary effect of reducing agents on ALA-D activity. In one study, glutathione exerted maximum stimulation at 3.3 mmol/liter (8), and in others glutathione (9) and DTT at 20 mmol/liter (10) increased the activities of this enzyme up to twice the control values. DTT also restored lead-inhibited enzyme activity to normal. These reducing agents were postulated to remove lead by chelation from the sulphydryl groups of the enzyme (9).

Other reports have noted the enhanced activity of ALA-D by zinc (11, 12). Whereas this enzyme activity in erythrocytes and liver of rats varied directly with zinc intake, in vitro addition of zinc yielded only slight augmentation of activity, suggesting a requirement for zinc as a cofactor at the site of apoenzyme synthesis (13). Others have found that addition of zinc to the ALA-D assay yields maximal activation at 0.1 mmol/liter (9). In our experience, this concentration of zinc gives a rather constant 20% stimulation of ALA-D activity.⁵

Even though the stimulation of activity afforded by DTT and zinc in fresh blood was slight, assurance of complete activation improves both precision and accuracy and for these reasons are important components of the procedure.

ALA-D and ferrochelatase (EC 4.99.1.1) are considered to be the enzymes in the heme biosynthetic pathway that are most sensitive to lead inhibition (14). Lead chloride at a concentration of 0.1 mmol/liter also inhibits erythrocyte URO-S activity in a reversible, non-competitive manner (15). The potential for spurious positive diagnosis of AIP in lead-poisoned individuals prompted these studies of the in vitro effects of zinc and DTT on the lead-inhibited assay. A related report described the inadvertent ingestion of zinc oxide by a 7-year-old girl lead-poisoned from pica: after the zinc oxide ingestion, a 10-fold increase in erythrocyte ALA-D developed after initial subnormal values were found (16). Reportedly, suppression of ALA-D activity is maximal with a blood lead concentration of 10⁻⁵ mol/liter (17), whereas we have found marked progressive suppression of porphyrin production with lead concentrations ranging from 10⁻⁷ to 10⁻³ mol/liter and with 30-min preincubation. Lead does not change the Kₘ of ALA-dehydratase, but Vₘₐₓ is decreased, suggesting noncompetitive inhibition (10, 12). With rats receiving 200 mg of lead per kilogram of diet, the original decrease in erythrocyte ALA-D activity was restored to control values by adding zinc chloride in vitro, with maximal effect at 0.1 mmol/liter, suggesting that there was competitive binding of lead and zinc to the sulphydryl groups of the enzyme (18).

We could correct lead-induced suppression of URO-S activity to near-control values of Vₘₐₓ by adding equimolar (1.0 mmol/liter) concentrations of zinc ions to the 30-min preincubation mixture. The apparent Kₘ for URO-S and the Vₘₐₓ for controls were found to be 7.8 × 10⁻⁶ mol/liter and 33.3 nmol, respectively, whereas with lead alone the respective values were 1.4 × 10⁻⁵ mol/liter and 0.5 nmol, suggesting a combination of competitive and noncompetitive inhibition. The addition of zinc to the reaction mixture that also contained lead resulted in near-normalization of Vₘₐₓ to 28.6 nmol, but the Kₘ remained increased at 1.9 × 10⁻⁵ mol/liter.

⁵ Unpublished data.
suggesting continued competitive inhibition; however, under the conditions of the test with 1-h incubation, the $V_{max}$ is reached and lead inhibition is obviated. Our kinetic measurements for URO-S in normal subjects were similar to those noted by others, with a mean $K_m$ of 12.3 $\mu$mol/liter and a mean $V_{max}$ of 35.7 nmol (6). The respective values in AIP were 6.2 $\mu$mol/liter and 18.0 nmol.

In interpreting URO-S activities, one must bear in mind the effect of mean age of the erythrocytes. Thus, any hematological abnormality that could shorten mean cell age could mask a decreased activity that would be diagnostic for AIP.

Brocklehurst (19) has recently suggested using ALA in a substrate concentration of 20 $\mu$mol/liter, to decrease the cost of the assay. We surmise that even a 10% increase in enzyme activity would justify the greater cost if optimum assay conditions are obtained as a result.

References


