Assay of δ-Aminolaevulinate Dehydratase in 10 μL of Blood

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The action of lead on the enzyme δ-aminolaevulinate dehydratase (ALA-D; porphobilinogen synthase, EC 4.2.1.24) in erythrocytes is manifested by an apparent shift in the pH of maximum enzyme activity to lower pH values and by an overall decrease in enzyme activity (1). These effects are readily measurable. For the purpose of assessing exposure to lead, measurements are usually performed at pH 6.4 in phosphate buffer (2,3) and lead concentrations in blood and concomitant decreases in enzyme activity are correlated. The assay at pH 6.4 thus provides clinicians with a sensitive and early biological index of exposure to this ubiquitous environmental and industrial pollutant (4–7).

Several methods proposed for measuring ALA-D activity in erythrocytes are based upon the enzyme’s conversion of two molecules of aminolevulinic acid (ALA) to porphobilinogen, which is then measured spectrophotometrically by reaction with Erlich’s reagent (2–4, 8–11). In general, these have involved modifications of the original Bonsignore method (8) and usually suggest a change in the buffer system, for better control of the reaction pH. Assay methods vary from study to study, making intercomparison of results almost impossible. In an effort to achieve some uniformity in the assay of the enzyme, Berlin and Schaller (3) in 1974 proposed the “European Standardized Method,” which has since become widely accepted.

The methodology as it stands, however, has several drawbacks, one of which is the requirement for venipuncture to obtain a sufficient quantity of blood. In some instances, it may be substantially preferable to collect blood for ALA-D assay by skin puncture than by venipuncture, such as when there is: a need for frequent blood sampling to monitor occupational exposure to heavy metals; reticence on a donor’s part to submit to venipuncture; or veins difficult to tap, owing to their size and location. With children or infants, venipuncture can be distressful and excessively hazardous (12) and skin puncture is definitely preferable (13). There may be other reasons to opt for finger- or prick-blood collection, such as difficulties encountered in collecting, transporting, storing, and shipping large numbers of blood samples from remote and relatively inaccessible sites. For several of these reasons and because several other microtests could be performed on the same 250-μL blood sample, we considered it important to develop a micro-scale method for ALA-D assay, requiring 10 μL of whole blood or less. This paper reports such a method.

Materials and Methods

Blood Samples

Macro-scale blood samples were obtained by venipuncture from volunteer non-smokers with no known unusual exposure to heavy metals and from workers with known occupational exposure to lead. The blood samples were drawn with use of a disposable 10-mL plastic syringe (Becton Dickinson and Co., Rutherford, N.J. 07070) and immediately transferred to polystyrene culture tubes (no. 2570; Corning Glass Works, Corning, NY 14835) containing enough sodium heparinate to yield a final concentration of 500 USP units per milliliter of blood.

Micro-scale blood samples were obtained from volunteers via finger pricks, the blood being collected with heparinized micro hematocrit tubes (DADE Div., American Hospital Supply Corp., Miami, FL 33152). The blood samples were transferred to disposable 400-μL polypropylene tubes. All blood samples were kept at 4°C until used. The hematocrit of each blood sample was determined with a microcapillary centrifuge.

Reagents

Na2HPO4·12 H2O, NaH2PO4·2H2O, citric acid, mercuric chloride, and p-dimethylaminobenzaldehyde, all “AnalaR” grade chemicals, were from British Drug House Chemicals Ltd., Toronto, Canada. Triton X-100, δ-aminolevulinic acid, and dithiothreitol were obtained from Sigma Chemical Co., St. Louis, MO 63178. Trichloroacetic acid was from Fisher Scientific Co., Ottawa, Ontario, Canada.

Buffer Systems

All glassware and plastic containers were cleaned of possible heavy metal and organic contamination by soaking in a solution of dilute (100 mL/L) nitric acid and dilute (50 mL/L) perchloric acid for two days and rinsed five times with deionized water. Dibasic sodium phosphate–citrate and dibasic sodium phosphate–monobasic sodium phosphate buffers were prepared from 0.3 mol/L solutions of the components at 0.1 pH unit intervals between pH 5.8 and 7.2. The pH of each buffer was determined with an Accumet Model 520 digital pH/ion meter (Fisher) equipped with a microprobe combination electrode (Fisher), calibrated with a 0.25 mol/L sodium phosphate buffer reference standard pH 6.87 ± 0.005 (20°C) (U.S. National Bureau of Standards Reference Material no. 186 I and II-C). Buffer solutions were stored at 4°C and freshly prepared each month. Atomic absorption spectrometry indicated that lead, cadmium, and mercury concentrations were <10 μg/L and zinc and copper concentrations were <100 μg/L.

Preparation of Reagents

De-ionized water was used.

Sodium phosphate buffer, pH 6.4. Dissolve 53.72 g of Na2HPO4·12H2O in 500 mL of water (solution A) and 23.40 g of NaH2PO4·2H2O in 500 mL of water (solution B). Mix 100

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1These references are only samples from a large literature on the subject.

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mL of solution A with 168 mL of solution B. Verify the pH periodically and prepare freshy each month.

**Triton X-100 solution.** Dissolve 0.5 mL of Triton X-100 surfactant (Sigma) in 500 mL of water.

**δ-Aminolevulinic acid, 125 mmol/L.** Dissolve 299.5 mg of this reagent in 100 mL of water and store at 4 °C. Prepare weekly.

**Dithiothreitol, 20 mmol/L.** Place 154.7 mg in 50 mL of water; store at 4 °C. Prepare weekly.

**Trichloroacetic acid (60 g/L)/mercuric chloride (60 mmol/L).** Dissolve 15 g of trichloroacetic acid and 4 g of mercuric chloride in 250 mL of water. Prepare monthly.

**Modified Erlich’s reagent.** Place 2 g of p-dimethylaminobenzaldehyde in 60 mL of glacial acetic acid, add 32 mL of perchloric acid (700 g/L), and dilute to 100 mL with glacial acetic acid. Prepare freshly each month.

**Assay Procedures**

**Inactivated enzyme.** The ALA-D activity of blood is measured by determining the amount of porphobilinogen formed under specific conditions, by use of a modification of a method presented earlier (1) to accommodate micro amounts of blood per assay.

For the assay, pipet 10 μL of each blood sample into four 400-μL microtubes with an SMI-C micro pipet (Scientific Manufacturing Industries, Emeryville, CA 94608), add 50 mL of Triton X-100 solution, mix for 15 s, and place the mixture in an ice bath for 3 min, to complete lysis.

To the hemolysate add 100 μL of a freshly prepared mixture containing equal volumes of the sodium phosphate buffer and the substrate (δ-aminolevulinic acid) incubated at 37 °C for 30 min. To the control sample, immediately add 100 μL of trichloroacetic acid/mercuric chloride mixture and mix well. Incubate all samples at 37 °C for 60 min. At the end of the incubation period, stop the reaction by adding 100 μL of the trichloroacetic acid/mercuric chloride solution with a Dispensette (Brinkmann Instruments, Rexdale, Ontario, Canada). Mix vigorously and centrifuge at 11,500 × g for 5 min in an Eppendorf microcentrifuge Model 5413 (Brinkmann Instruments). Transfer 200 μL of the supernate to a micro cell (1-cm light path, 2 mm wide), add 400 μL of the modified Erlich reagent, cap, mix well, and measure the absorbance within 5 min at 555 nm vs a blank containing 400 μL of modified Erlich reagent and 200 μL of water. A Beckman Model 25 spectrophotometer, calibrated with liquid absorbance standards (U.S. National Bureau of Standards Standard Reference Material 931b) was used in this work.

**Activated enzyme.** For enzyme activation, add 25 μL of the dithiothreitol solution to the hemolysate and incubate at 37 °C for 30 min prior to the addition of the buffer–substrate solution. The remainder of the assay is conducted as detailed above.

**Calculations.** The enzyme activity is expressed as micromoles of porphobilinogen (PBG) generated per hour, per liter of erythrocytes, at 37 °C.

To calculate the enzyme units, multiply the dilution factor X (100/percent hematocrit) X (PBG absorptivity)⁻¹ X A₅₅₅, where A₅₅₅ = absorbance of the test sample minus absorbance of the control sample.

For the inactivated enzyme, the dilution factor is 78, so:
units = 78 X (100/percent hematocrit) X (6.2 X 10⁻²)⁻¹ X A₅₅₅ = 125.8 X 10⁻³ X A₅₅₅/percent hematocrit.

For the activated enzyme, use 85.5 as the dilution factor.

**Evaluation of Assay**

Within-day assay precision was determined by analyzing blood samples from normal and lead-exposed individuals. Because ALA-D activity decreases with time after collection (14), we attempted to assess between-day precision by measuring the enzyme’s activity periodically in the blood of control subjects over a six-month period.

**Method Comparison**

The activity of ALA-D, inactivated and activated by dithiothreitol, in blood samples from individuals having no known unusual exposures to heavy metal and from persons with known exposure to lead was assayed by both the micro-scale method described above and the widely accepted “European Standardized Method” (3). The correlation between assays was tested by linear correlation. The standard error of estimate was determined by the equation $S_{yc} = S_y \sqrt{1 - r^2}$ where $S_y$ = standard deviation of y, and r is the correlation coefficient.

**Results and Discussion**

We considered the following objectives in developing this micro-scale method: (a) the quantity of blood required needed to be 10 μL or less; (b) the results obtained needed to be reproducible and comparable with those obtained with an accepted method, such as the “European Standardized Method”; (c) the method needed to be developed in such a way as to avoid the procedural pitfalls (1) and interferences (15) we have recently documented. These factors are discussed in turn.

**Blood collection.** As noted by ourselves and others, the contamination of blood samples by metals such as zinc (15–17) and chelating substances (4, 18) in an evacuated collection tube can severely affect the ALA-D activity of blood. Zinc, for instance, activates the enzyme in vitro (19–21). For this reason, all blood samples assayed must be collected with plastic disposable syringes and stored in plastic tubes or microtubes whose contamination has been thoroughly investigated.

**Sample size.** Some difficulties were encountered in obtaining reproducible results with sample volumes <10 μL. However, using a positive displacement micro-pipette, we achieved good reproducibility with 10-μL blood specimens and adopted this approach for the assay.

**Hemolysate preparation.** Although the freeze–thaw method of preparing hemolysates tended to yield marginally higher activity values (15), we used instead the non-ionic detergent Triton X-100 because it afforded greater ease in the preparation of the lysates for large numbers of blood samples. Lysis was assumed to be virtually complete within minutes of adding the reagent, and there appeared to be no purpose in incubating the lyase at 37 °C for 10 or more minutes as recommended by others (2, 3, 9). As was indicated by Granick et al. (14) and ourselves (1), this practice needlessly reduces the enzyme activity.

**Buffer system.** As we noted in an earlier paper (1), the commonly used citrate buffer, possibly because of its chelating properties, consistently yielded ALA-D values 15 to 20% lower than the sodium phosphate buffer. We therefore selected the sodium phosphate buffer, 0.3 mol/L, being sufficient to overcome the buffering capacity of blood. The ALA-D activity curves for individuals having 50 to 100 μg of lead per liter of blood peaked at pH 6.40 ± 0.10. This value is considerably different from the 6.10 ± 0.05 value we reported earlier for this buffer system. We attribute this difference to the contamination of blood samples by substances in the evacuated collection tubes used during the earlier study.

**Reaction conditions.** The optimum δ-aminolevulinic acid substrate concentration (125 mmol/L), incubation temperature (37 °C), and duration of incubation (60 min) have been fully established in previous studies conducted by others, and we made no attempts to modify these conditions.

**Color formation.** According to our protocol, the reaction
mixture supernate is reacted with modified Erlich’s reagent in the optical micro cell. To speed the assay, we sought a more rapid development of the Erlich’s colored salt than the 15 min usually required. When we doubled the aliquot of the reagent added to the supernate, color development was essentially complete between 2 and 5 min and tended to diminish slightly thereafter. The resulting mixture had no noticeable precipitate and thus did not require the filtration suggested by others (3).

**Precision and Reproducibility**

ALA-D activity determined with the micro-scale method in a finger-prick blood sample collected from a single individual on 14 consecutive days was 1588 (SD 78) μmol of PBG per hour per liter of erythrocytes (CV, 4.9%). Twenty replicate determinations of the enzyme activity in the blood of an unexposed worker (lead, 100 μg/L) and of an exposed worker (lead, 420 μg/L) yielded results of 1065 (SD 54; CV, 5.1%) and 308 (SD 11; CV, 3.6%) enzyme “units,” respectively.

**Method Comparison**

ALA-D activity in the blood of 24 workers exposed to heavy metals was determined simultaneously by the proposed micro-scale method and by the “European Standardized Method.” The results obtained were evaluated by the least-squares linear regression method. The scattergram shown in Figure 1 demonstrates a good correlation, a negligible y-intercept, and a proportionality error (slope) in favor of the micro-scale method. This disparity between the two sets of values was expected since, as mentioned earlier, incubation of the hemolysate as required by the protocol of the “European Standardized Method” will reduce ALA-D activity significantly. To negate possible bias, we determined the ALA-D activity in split blood samples collected from 49 children living in the vicinity of a lead smelter by the “European Standardized Method” at the Centre de Toxicologie de Laval, Quebec, laboratory, and by the micro-scale method in our laboratory. An unavoidable delay in receiving the blood samples constrained our laboratory to perform the assay two days later than the Laval laboratory. Notwithstanding the loss of 5% or more in the enzyme’s activity (14) that such a delay can entail, the results obtained (r = 0.87, y = 0.63x – 388, S_y|x = 152) demonstrate a larger positive bias in favor of our micro-scale assay (x).

The ALA-D activity loss through incubation of the hemolysate can be restored by treatment with a chemical agent such as dithiothreitol, which provides exogeneous thiol groups. If incubation of the hemolysate is the main cause of the disparity between the two methods, determination of the activated enzyme by both methods should remove this difference. The results for 33 blood samples obtained from persons with no known unusual exposure to heavy metals (r = 0.97, y = 1.04x + 130, S_y|x = 135) indicate that this was indeed the case. However, even though a good correlation was again obtained, the regression line slope of 1.04 now indicates a positive bias in favor of the “European Standardized Method” (y). Inter-laboratory comparison of the two methods as described above for the assay of the activated enzyme confirmed these results (r = 0.90, y = 1.01x + 179, S_y|x = 131). Hence, it would appear that treatment of blood with Triton X-100 does not lead to total lysis. Nevertheless, when faced with the possible alternative procedures for performing lysis, such as incubating the hemolysate or the more cumbersome freeze-thawing technique, we have no reservation in recommending the use of Triton X-100 to achieve lysis for the determination of inactivated ALA-D with our micro-scale method.

The statistically significant correlation of results obtained from inter- and intra-laboratory comparison of analysis performed on aliquots of the same samples indicates that the micro-scale method can function as a sensitive and precise alternative to the widely accepted “European Standardized Method” when only small quantities of blood are available for ALA-D assay. We do not consider the positive bias of some 10% or less obtained with our method on the same blood samples to be a significant difference. However, if the use of an (activated/non-activated) enzyme activity ratio is contemplated as an indicator of lead exposure (4, 14, 22), one should be aware that a decidedly lower ratio will be obtained with our micro-scale method because of the combination of lower activated and higher inactivated ALA-D values obtained.

The major changes involved in the development of the micro-scale method are the use of 10 μL of blood instead of 200 μL, combined with the use of microtubes and small path-width spectro-photometric cells. In addition, the abandonment of the hemolysate incubation step and the increase in buffer concentration to 0.3 mol/L are incorporated for reasons previously documented (1). Triton X-100 rather than water is used at the hemolysis step. Other conditions, e.g., the pH of measurement for enzyme activity, the substrate concentration, and the reaction time, remain unchanged from macro-methodology.

The micro-scale method we describe requires relatively inexpensive equipment, small quantities of reagents, and a relatively short period of training. An inexperienced technician usually can perform 100 to 150 assays per day after two or three days of training. This method also includes important safeguards against certain factors that can drastically influence assay results.

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