Measurement of Acetylcholinesterase in Erythrocytes in the Field

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We describe here a field method we developed for colorimetry of erythrocytic acetylcholinesterase (EC 3.1.1.7) in capillary blood samples. Three stable, premixed assay reagents and de-ionized water (but no centrifuge or balance) are required. This method, adapted for a microplate format, is essentially that of Ellman et al. (Biochem Pharmacol 1961;7:88-95) as modified by George and Abernethy (Clin Chem 1983;29:365-8). Assays were quantified and corrected for hematocrit by using a battery-powered colorimeter with a silicon carbide (blue) light-emitting-diode source. Advantages over existing field methods include better portability, ruggedness, greater precision, and lower cost per sample.

Additional Keyphrases: toxicology • screening • organophosphate pesticides • colorimetry • economics of laboratory operation • reference interval

Although the Third World accounts for only 15% of the world’s pesticide consumption (1), half of the estimated annual 500 000 to 1 million pesticide-associated poisonings and over half of the ensuing 19 000 deaths occur in developing countries (1).

Most of these poisonings are from organophosphate insecticides, which inhibit mammalian neuronal acetylcholinesterases. In developed countries, erythrocyte acetylcholinesterase (EC 3.1.1.7) is routinely measured because it is functionally similar (2) to the neuronal acetylcholinesterase. Its measurement is specified in existing legally mandated screening programs in the United States (3). California law requires determination of a baseline pre-exposure concentration and subsequent monitoring for anyone in certain job categories (4). A decrease in a worker’s concentration of acetylcholinesterase indicates over-exposure to such pesticides; the affected worker (often asymptomatic) must be removed from exposure, to prevent acute poisoning (3).

In developing countries, the activity of acetylcholinesterase in blood samples is often not monitored to prevent poisoning because collection, transport, and storage of samples is difficult and costly, and because appropriate laboratory facilities do not always exist.

The World Health Organization (WHO) currently endorses two field methods for use under conditions where laboratory facilities are not readily accessible (5): a colorimetric (“tintometric”) method first developed by Edson (6), and a field-type spectrophotometric method based on the assay of Ellman et al. (7, 8). Both methods have disadvantages: the former is only semiquantitative; the latter is cumbersome. In addition, these methods measure whole-blood cholinesterase, which is a mixture of plasma cholinesterase (EC 3.1.1.8) and erythrocyte cholinesterase, rather than only the preferred erythrocytic acetylcholinesterase. The spectrophotometric assay involves initial costs that are potentially prohibitive for many Third World public-health authorities and calls for use of costly disposable pipettes, and the colorimetric reagents are considerably more costly than those used in the Ellman method.

We have developed a modified Ellman-type field method that accurately, rapidly, and inexpensively measures erythrocyte acetylcholinesterase in uncentrifuged blood samples. We evaluated its utility by using it to assay blood from normal health-care workers, poisoned hospitalized patients, and workers at a primitive rural fumigation airstrip (where pesticides are formulated and loaded into airplanes used for pesticide application) on the northern Pacific coastal plain of Nicaragua, an area where extraordinarily high rates of insecticide poisoning are reported.

Materials and Methods

Zephiran chloride (benzalkonium chloride, a mixture of alkyl dimethylbenzylammonium chlorides varying in the alkyl moiety from C6H17 to C15H31) was obtained from Winthrop-Breon, division of Sterling Drug, Inc., New York, NY. "RBS-35" detergent was from Pierce Chemical Co., Rockford, IL. The 5.6-ML rubber-stoppered amber-colored bottles were from American Diagnostica, Inc., Greenwich, CT. The polystyrene 96-well microtiter plates, Brinkmann "Ultramicro" 5-µL pipetter, and Bausch & Lomb Minispectrophotometer 20 were from Fisher Scientific Co., Pittsburgh, PA. Reagent reservoirs and "Octapettes" (25-µL and 200-µL sizes) were from Hyclose, Logan, UT. Precalibrated measuring scoops were from McDonald’s, Inc., Vienna, Austria. All other reagents, including purified human erythrocyte acetylcholinesterase, were from Sigma Chemical Co., St. Louis, MO.

For spectrophotometric measurements we used a Model 2250 microplate reader (Bio-Rad Labs., Richmond, CA) or a Model PM II spectrophotometer (Carl Zeiss, New York, NY).

For colorimetric assay measurements we used a colorimeter designed by two of us (J.P.E. and R.A.M.); the light source was a blue light-emitting diode (LED; Siemens LB5410; silicon carbide). The prototype for this instrument has been described elsewhere (9, 10). A production unit, the Model 176 colorimeter (patent pending), is marketed by EQM Research, Inc., Cincinnati, OH. The sample chamber of this instrument was modified to use cuvettes with a 3.2-mm pathlength, fabricated from black acrylic and
clear acrylic (Cincinnati Plastics, Cincinnati, OH). The response of the LED-source colorimeter to the assay chromophore was determined by diluting, to give a final volume of 2.00 mL, 0.10 to 2.00 mL of a solution containing, per liter: 0.44 mmol of DTNB [5,5'-dithiobis(2-nitrobenzoic acid)], 0.22 mmol of cysteine, 5 g of benzothiazonium chloride, and 50 mmol of Tris HCl, pH 7.6. The diluent was the same except that it lacked cysteine.

Assay Reagents

_Erythrocyte acetylcholinesterase reagent_. We used a modified Ellman-type formulation (7, 11, 12): 2.9 g of acetylthiocholine iodide, 1.6 g of DTNB, 200 mg of quinidine sulfate dihydrate, 15.0 g of anhydrous potassium dihydrogen phosphate, and 78.1 g of anhydrous disodium hydrogen phosphate. The concentrations of the first three ingredients and of sodium potassium phosphate in the assay reagent mixtures were, respectively: 0.59 mmol, 0.35 mmol, 0.022 mmol, and 59 mmol per liter of solution, pH 7.5. After mixing by rotation or with use of a mortar and pestle, we lyophilized the mixture overnight and stored it in 5-mL rubber-stoppered amber-colored bottles at room temperature.

This reagent was stable at room temperature for at least six months until opened, after which it was stored in a desiccator (a jar containing anhydrous CaSO₄).

Using a precalibrated scoop, add about 200 (±50 mg (two level scoops)) of this reagent to a 20-mL volumetric vial or bottle (this is sufficient for 100 assays). Dilute the reagent in, and dilute to volume with, de-ionized water.

_Sample buffer_. This was a 10-fold concentrate containing 1 mol of Tris HCl, 10 g of Triton X-100 surfactant, and 1 g of sodium azide per liter, pH 7.6. The concentrate, stored at room temperature in polyethylene bottles, was diluted immediately before use.

*Stop* reagent. We stopped the assay reaction by using either a 100 g/L aqueous solution of benzethionium chloride (Hyamine 1622) or a 85 g/L solution of benzalkonium chloride.

Erythrocyte Acetylcholinesterase Assay

Sample buffer and stop solution can be prepared beforehand and stored either refrigerated or at room temperature (20–35 °C) for at least a year.

Blood samples are collected with use of sterile lancets and a 5-µL air-displacement pipette. Pipette tips and microplates can be re-used after first rinsing them with 100-fold-diluted RBS-35 detergent, then rinsing with de-ionized water, and boiling. Because the enzyme activity of acetylcholinesterase is extremely sensitive to the stop reagent, the pipette tips and bottles used to handle this solution should be carefully segregated from those used with the assay solution.

The assay microplates should be shielded from sunlight with an opaque cover during incubation steps.

We use the following protocol, assaying 16 samples. Place the samples in columns 1 and 8 (the plates contain 12 rows, with eight wells per column) for dilution, and perform the assay in columns 2, 3, 9 and 10, with blanks in columns 4, 5, 11, and 12. When one is assaying large numbers of samples, only a few blanks are necessary: see "Results".

1. Using a multipipette, pipette 200 µL of sample buffer into each well of columns 1 and 8.

2. Using a air-displacement pipettor, add 5 µL of freshly drawn capillary blood to the wells in columns 1 and 8 and mix thoroughly by using the pipette tip. (Place the used tip in a bottle of RBS-35 detergent.)

3. After all blood samples are taken and mixed, pipette 200 µL of de-ionized water into columns 1 and 8, mixing the samples.

4. While blood samples are sitting in diluent, make up the acetylcholinesterase reagent solution.

5. Using a 25-µL multipipette, transfer 25 µL of diluted blood from each well of columns 1 and 8 to wells in the adjacent "reaction" columns (2 and 3, 9 and 10, respectively).

6. Add 200 µL of the acetylcholinesterase reagent solution to each well of "reaction" columns 2, 3, 9, and 10 and allow the reaction to incubate at ambient temperature for 30 min. Also add 200 µL of assay solution to each well of columns 4, 5, 11, and 12 ("blanks"), followed immediately by 25 µL of the stop solution.

7. At the end of the incubation add 25 µL of the stop solution to each assay mixture in columns 2, 3, 9, and 10.

8. Adjust the colorimeter to zero with water, and measure the absorbance of the assay mixtures (at 440 nm) within 1 h of adding the stop reagent (to avoid fading). Combine the contents of duplicate wells and transfer this to a 3.2-mm cuvette by using a one-piece plastic transfer pipet. Measure the absorbance of the sample wells (also at 440 nm) for hemoglobin concentration (correction for hematocrit).

Calculation of Enzyme Activity

Multiply the absorbance readings (obtained with the blue LED) by 7.1 to obtain millimoles of substrate hydrolyzed per minute per liter of whole blood (kU/L) at pH 7.5 and ambient temperature. This is based on an absorbivity for the thionitrobenzoate–benzalkonium complex of 14 000 L · mol⁻¹ · cm⁻¹ at 440 nm (11), an integrated bandwidth response of 84% for the blue LED, a 3.2-mm pathlength, a 30-min incubation, and a volume proportion of 0.31 µL of whole blood in a final volume of 0.25 mL.

To adjust acetylcholinesterase activities determined at temperatures ranging from 24 °C to 30 °C to what results would have been if determined at 25 °C, multiply by the following factors (11, 13): 0.92 (27 °C), 0.81 (30 °C), 0.74 (33 °C), 0.69 (35 °C), 0.61 (37 °C). Intermediate values can be obtained by interpolation.

Enzyme activities are expressed in arbitrary units by calculating the quotient of the net absorbance (A) of enzyme activity divided by the hemoglobin absorbance, multiplied by 1000: activity, arb. units = 1000 (Aenzyme/Ahbg).

Results

Colorimetry with Use of the LED-Source Colorimeter

With six freshly charged "C" batteries (alkaline type) the LED-source colorimeter could be run continuously for >200 h without changes in its response characteristics. After the machine had "warmed up" for 10–15 min the blank transmittance fluctuated by <0.002 A and drifted from its initial water blank reading by <0.005 A per hour. By comparison, a fully charged Bausch & Lomb Minispectrophotometer 20 equipped with a digital voltmeter readout and set to 440 nm exhibited a fluctuation of approximately 0.005 A and a downward drift in blank transmittance readings of approximately 0.1 A per hour for an identical sample cell.

The response of the LED-source colorimeter was linear (<1% deviation from ideality) with increasing concentration of chromophore (Figure 1) to at least 0.75 A, approximately twice the range of the assay. The lower slope of the LED plot
relative to readings taken with a spectrophotometer reflects both the relative sizes of the sample cells (3.2 mm vs 10 mm) and a slightly decreased response (84%) for the filtered blue LED.

We verified the correction of enzyme activities for hematocrit by determination of oxyhemoglobin with the LED-source colorimeter by plotting the absorbance of the diluted blood in the sample wells as a function of volume (in μL) of added whole blood. This plot was linear between 0.1 and 0.6 A (n = 4, r = 0.999, y = 0.0761x + 0.0575). The enzyme activity (net absorbance) was divided by the hemoglobin absorbance, correcting for both sampling error and hematocrit. This normalized activity can be used to assess exposure to pesticide.

Erythrocyte Cholinesterase Assay

We evaluated the effect of semiquantitative measurement of premixed assay reagent on the kinetic response of the assay, using purified human erythrocyte acetylcholinesterase. To 50-μL samples containing 1 mU of purified acetylcholinesterase in sample buffer was added 100 μL of the acetylcholinesterase reagent solution in concentrations ranging from 2 to 25 g/L (acetylthiocholine iodide concentration range of reaction mixtures: 0.13–1.63 mmol/L). As shown in Figure 2, the assay measured the same amount of activity over a fivefold range of reagent concentration. Use of reagent concentrations <4 g/L resulted in a significantly decreased response; higher reagent concentrations resulted in proportionately higher blank values. Under direct bright fluorescent lighting the absorbance faded at a rate of approximately 10% per hour. However, when kept in the dark, even at 37 °C, the rate of fading was less than 1% per hour. Thus, the assays were performed in a shaded area and the plates were placed under opaque covers after the stop reagent was added.

Using samples of whole blood, the absorbance of the assay was linearly related to both time (0–60 min, n = 9, r = 0.998, y = 0.0011x − 0.016) and enzyme concentration (1–7 μL blood, n = 8, r = 0.998, y = 0.085x + 0.034). We found that, owing to the extremely low variation in the blank (CV about 5%), the assay capacity of the microplate could be conveniently increased to 32 by running several blanks from normal donors on a separate assay plate.

Precision

For the precision study, we drew four blood samples (runs) from each of five normal donors, and assayed each blood sample five times. Table 1 gives the results in arbitrary units for between-run and within-run precision. Within-run precision (CV) ranged from 0.8 to 5.0%, with an overall value of 2.8%. Between-run precision ranged from 1.5 to 4.9%, with a mean of 3.2%.

To assess day-to-day variation, we assayed blood from one individual in quadruplicate on four consecutive days. Erythrocyte cholinesterase was 834 arb. units (CV 5.0%). This individual was tested again after three and six months. The values were 896 and 803 arb. units, respectively.

Normal Reference Interval

The erythrocyte acetylcholinesterase activities of 16 apparently normal Nicaraguan men and women health-care workers, ages 18–35 years, ranged from 2.2 to 3.5 kU/L with a mean of 2.9 kU/L (CV 11.3%). In arbitrary units, a range of 644 to 1068 was found, with a mean of 892 (CV 12.2%).

To establish an occupational baseline range, we assayed samples from 44 agricultural workers for erythrocyte acetylcholinesterase during the winter season, when pesticides are not used (about three to four months after previous potential exposure). Enzyme activities ranged from 1.8 to
2.9 kU/L, with a mean of 2.4 kU/L (CV 11.4%). Enzyme activity in arbitrary units ranged from 492 to 826, with a mean of 661 (CV 10.1%).

Pesticide Exposure: a Case Study

Three patients admitted to the local hospital for acute organophosphate pesticide intoxication were assayed for cholinesterase during several weeks. Table 2 shows the pattern of increase in cholinesterase activity over time after interruption of exposure by hospitalization.

Discussion

The characteristics of an ideal field cholinesterase assay should include:
- Ability to accurately and specifically measure erythrocyte cholinesterase
- Stability of reagents to varying temperature and humidity for at least several months
- Precision to determine a baseline pre-exposure value for erythrocyte cholinesterase, for subsequent comparison during exposure
- Portability
- Being operational without need for line voltage, a balance, or a centrifuge
- Low cost
- Capacity to analyze, reasonably quickly, a large number of samples in the field

Erythrocyte cholinesterase activities obtained by this proposed field method (corrected for pH and temperature) correlated well with reported values (7, 12, 13), confirming its accuracy. Concentrations of DTNB, substrate, and pH were chosen to avoid significant inhibition of enzyme by DTNB (14), substrate (14), and acidity (13, 14). Nonenzymatic hydrolysis of DTNB (14) and substrate (13) was minimized by using a pH slightly lower than that needed for optimal activity, and interference from plasma cholinesterase was eliminated by the inclusion of quinidine sulfate (7, 11, 12).

A stable Ellman reagent was obtained by using anhydrous buffers and sealed vials. Assay blanks remained stable over a six-month period, indicating that premature substrate hydrolysis did not occur during extended storage at ambient temperatures.

For optimal precision, three operations were identified as being critical: reagent preparation, blood sampling, and colorimetry. The assay reagents were formulated to give a response that was relatively independent of small variations in the amount used, minimizing this source of error. The most accurate and precise, yet inexpensive method of blood collection was found to be air-displacement pipettors.

Although the field spectrophotometric method reportedly allows precision such that the CV is within 5% (8), our initial attempts at field measurements using the same commercially available spectrophotometer (fitted with a digital display) with our assay resulted in between-run error greater than 10%, primarily owing to instrument instability and the need for frequent recharging. Conventional colorimetry, which depends on the absorption by the analyte of visible black-body radiation emitted from filament tubes (wavelength filament light bulbs) has significant disadvantages such as "flicker" noise from the filament, less power efficiency, owing to broadband emission, and mechanical fragility.

To circumvent these problems, we developed an LED-source colorimeter. The source used in this instrument is filtered to minimize polychromatic error. Although 410 nm is the usual wavelength at which the Ellman chromophore is measured, in the presence of benzalkonium chlorides the peak wavelength undergoes a bathochromic shift to 440 nm, considerably overlapping the emission of the blue LED. Despite the low luminous intensity of the LED at this wavelength (50% of peak, approximately 1 mcd), a virtually noise- and drift-free linear response was obtained with this instrument. Such quality of performance is largely ascribable to recent developments in the gain and stability of the operational amplifiers used in its design.

Design of a field monitoring kit for portability is essential, because both instruments and reagents often must be transported for considerable distances and over rugged terrain. Line voltage is usually not available, which makes battery operation of electronic instrumentation essential. Use of a balance or centrifuge is usually impractical. The need for replacement parts may pose problems, because field sites are often inaccessible to resupply.

We made the equipment practically portable by using an LED-source colorimeter and a premixed assay system. The useful lifetime of the battery in this instrument permits daily use for nearly a year, and no other replacement parts are required for its operation. The use of whole blood eliminates the need for a centrifuge. The need for a balance is obviated by using a precalibrated measuring scoop and judicious formulation of the assay reagent to allow for semiquantitative reagent measurement. With the microplate assay format, hundreds of tests can be performed with a few grams of reagent and a minimum of other supplies, facilitating compact storage and convenient transport of the assay system.

Perhaps most importantly for developing countries, LED-source colorimeters are relatively inexpensive (US $500). By

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Table 1. Precision Study: Acetylcholinesterase Assay Enzyme Activity in Blood from Five Normal Subjects

<table>
<thead>
<tr>
<th>Run</th>
<th>Run 1</th>
<th>Run 2</th>
<th>Run 3</th>
<th>Run 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activity, mean (n = 5 each) arb. units (and CV, %)</td>
<td>649 (2.5)</td>
<td>613 (1.5)</td>
<td>609 (2.8)</td>
<td>610 (2.9)</td>
</tr>
<tr>
<td></td>
<td>838 (3.2)</td>
<td>777 (2.5)</td>
<td>781 (2.1)</td>
<td>729 (2.7)</td>
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<tr>
<td></td>
<td>652 (2.9)</td>
<td>637 (3.7)</td>
<td>601 (4.4)</td>
<td>618 (4.3)</td>
</tr>
<tr>
<td></td>
<td>849 (3.9)</td>
<td>813 (5.0)</td>
<td>783 (3.5)</td>
<td>771 (4.0)</td>
</tr>
<tr>
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<td>748 (1.8)</td>
<td>731 (0.8)</td>
<td>725 (1.7)</td>
<td>720 (1.8)</td>
</tr>
<tr>
<td>Between run</td>
<td>2.7</td>
<td>2.2</td>
<td>4.1</td>
<td>3.7</td>
</tr>
<tr>
<td>Within run</td>
<td>4.1</td>
<td>2.6</td>
<td>3.8</td>
<td>4.1</td>
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Table 2. Acetylcholinesterase Monitoring in Three Patients Exposed to Organophosphate Pesticide

<table>
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<th>Enzyme activity, arb. units</th>
<th>Day 2</th>
<th>Day 4</th>
<th>Day 10</th>
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<th>Day 39</th>
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<td>294</td>
<td>384</td>
<td>—</td>
<td>514</td>
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<td>—</td>
<td>172</td>
<td>244</td>
<td>268</td>
<td>406</td>
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<td>—</td>
<td>156</td>
<td>286</td>
<td>294</td>
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adapting the assay to a microplate format, less reagent was needed, lowering the cost. A kit, including colorimeter, pipettes, tips, reagents, lancets, microplates, and bottles currently can be assembled for less than US $1000, and will do 10 000 tests (pipette tips can be washed and sterilized, but lancets should not be re-used). Reagent costs are less than 0.1 cent per test, and all other components of the assay are re-usable (except lancets).

A field measurement of 46 samples was conducted in 3 h, including reading of hemoglobin. With practice, we believe up to 150 samples could be measured in a single day. This compares favorably with the colorimetric method used world wide in monitoring of vector-control workers (6, 15). The technician with the regional health authority in Leon, Nicaragua, can comfortably do 150 samples daily by the present method. In contrast, a recent description of the field spectrophotometric method (16) states that only 40 samples can be processed in one day.

The within-run and between-run CV of approximately 3% compares favorably with published laboratory results (12) for erythrocyte acetylcholinesterase (3.2% within-run, 5.4% between-run). Because the intra-individual variability over a few months (a typical growing season) for both men and women is approximately 6% for erythrocyte cholinesterase (17), a decrease of 18% (three standard deviations) or more in the activity of the erythrocyte enzyme from baseline values owing to external influence may be accurately discerned (18) with this method.

The validity of this method was demonstrated on hospital patients afflicted with acute pesticide exposure. Consistent with previous studies (15, 16, 19), exposure of these patients to pesticides resulted in severe depression of cholinesterase activity, followed by a gradual recovery of enzyme activity concentrations to normal values.

This field method is certainly precise enough to detect the 30% decrease from an individual’s baseline erythrocyte cholinesterase, recommended by public-health authorities as the criterion for medical removal from exposure (19). We are using this method in an ongoing epidemiological study of pesticide exposure in farmworkers. In addition, we are investigating the utility of both erythrocyte and plasma cholinesterase measurement as an index of such exposure.

We deeply appreciate the cooperation and support of the Nicaraguan Ministry of Health, Region II, and the American Friends Service Committee. We are also grateful to Kathryn Dowling for technical assistance with the field studies.

References