Laboratory and field microassay of cholinesterases in whole blood, plasma, and erythrocytes

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A straightforward method is described for determination of the activities of erythrocyte acetylcholinesterase and plasma cholinesterase from single 10-μl samples of highly diluted whole blood. The procedure is a spectrophotometric microassay adapted for use with microtiteration plates. The use of an enzyme immunoassay reader facilitates the rapid analysis of large numbers of samples. An inexpensive adaptation of the method was also devised that requires no instrumentation and was evaluated under field conditions in Haiti. Either the field or laboratory versions of the method can accurately determine increments of 10% or less in the level of inhibition of acetylcholinesterase or cholinesterase in whole blood, plasma, or erythrocytes.

Exposure of workers to organophosphate insecticides is generally monitored using tintometric or spectrophotometric methods (18). In this way, the activities of plasma cholinesterase and/or erythrocyte acetylcholinesterase are determined, and individuals whose enzyme function is depressed beyond a designated value (generally 50% of normal) are restricted from further exposure. Wilhelm (22) described a modification of Ellman's method for determining plasma cholinesterase inhibition, and a spectrophotometric kit has been produced for measuring cholinesterase activity in whole blood and plasma.1,2

Both the spectrophotometric kit and the tintometric method3 facilitate assessment of depression of cholinesterase levels, but require expensive instrumentation. Furthermore, financial restraints on malaria control programmes in developing countries may preclude purchase of even basic equipment and supplies. In these countries, pesticide intoxication usually occurs in rural areas, where health care for diagnosis and treatment may be limited (10). Also, in more developed countries, continuous monitoring of acetylcholinesterase and cholinesterase levels in persons exposed to pesticides, though impractical with current methods, is desirable, since for some compounds the degree of exposure tolerated is low (14).

We have reported a spectrophotometric microassay for the routine determination of enzyme activity in a large number of small-volume samples in microtiteration plates (1). This method has also been adapted for use without instrumentation, thereby facilitating its use in the field for the simultaneous determination of acetylcholinesterase and cholinesterase activity in whole blood, plasma, or erythrocytes. Here, we describe the results obtained using the microassay to determine the blood cholinesterase activity of spray personnel in Haiti.

MATERIALS AND METHODS

Selection of subjects, blood collection, and preparation of blood fractions

In order to establish control baselines, blood was collected from 12 volunteers from the Centers for Disease Control who had not recently been exposed to cholinesterase inhibitors. In Haiti, four malaria spray-team foremen served as controls for the tests on 10 spraymen and insecticide mixers. The blood cholinesterase levels of the spraymen and mixers had been determined 1 month previously by tintometry and were less than 50% of normal values. For purposes of comparison, tintometric assays were re-run on the same day as the microassay.

Samples of blood (10 μl) were collected by fingerstick from fingers cleaned with an alcohol wipe. The first drop of blood was routinely discarded. Each
sample was diluted with 3 ml of buffer (0.05 mol/l potassium phosphate, pH 7.4) for the assays of whole blood.

No further preparation is required for routine laboratory or field microassays; and the fractionation of blood samples described below was carried out only for the purposes of the present study. Erythrocytes were sedimented by centrifugation (13 000 g, 3 minutes) of 1-ml portions of diluted whole blood. The supernatant was collected and recentrifuged, while supernatant from the second centrifugation served as the plasma enzyme fraction. For determination of the red blood cell fraction, erythrocytes were washed with buffer and recentrifuged. The resultant pellet was taken up in 1 ml of buffer and haemolysed by sonication. For microassay of blood fractions, the total volume used was the same as the amount of whole blood (10 μl).

**Laboratory microassay**

Solutions of acetylthiocholine iodide (ATCH, 0.75 g/l) and 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB, 0.13 g/l)* were prepared in phosphate buffer (see above). For the microassay, 50-μl aliquots of diluted whole blood suspension were transferred to the microtiter plate wells. Solutions of ATCH (100 μl) and DTNB (100 μl) were then added to each well and the absorbance at λ=410 nm was determined at 10-minute intervals using an enzyme immunoassay (EIA) reader.

Two methods for expressing enzyme activity were compared: Ellman's value for molar absorptivity (4) was used to calculate the substrate hydrolysis rate in nmol/min; and the procedure described by Garry & Routh (7), in which cholinesterase activity is expressed as the concentration of thiol groups liberated from the acetylthiocholine substrate obtained by comparison with a glutathione calibration curve. Final assay values were correlated to blood protein levels, determined using the microassay method described by Brogdon & Dickinson (7). For the cholinesterase and protein assays, an instrument blank containing 250 μl buffer, reagent blanks containing buffer instead of diluted blood, and a control using buffer instead of ATCH and DTNB were run. Since the microassay for cholinesterases is especially amenable to multiple replications, eight assay repetitions were routinely run simultaneously for each sample.

Standard curves, which were linear (correlation coefficient >0.99) over the concentration range 1-10 μg/l, were obtained for blood protein using bovine serum albumin and for glutathione using the reactions with DTNB (7). Cholinesterase inhibitors were also evaluated. For this purpose, solutions of quinidine sulfate (20 μmol/l) and of eserine (10 μmol/l) were prepared at concentrations that cause maximum inhibition of plasma cholinesterases and all cholinesterases, respectively (7). Aliquots (10 μl) of these inhibitors were pipetted into the blood suspension prior to addition of ATCH and DTNB, and an equivalent volume of buffer was then added to the control assay wells.

**Field microassay**

The field microassay is a simplified version of the laboratory procedure. However, for the field procedure one set of control blood replicates is initiated simultaneously with the test blood samples, and a second set from the same control samples is started 20 minutes later. Results are read 40 minutes after initiation of the first set of samples.

The intensity of the yellow chromophore in the test wells is compared visually with that of the 20-minute control. Since the intensity of the latter is half that of the 40-minute control, solutions whose intensities are greater or less than that of the 20-minute control inhibit cholinesterase activity by greater or less than 50%, respectively. Control blood microassays may be run at additional intervals to estimate other blood inhibition levels. For example, test samples compared at 40 minutes with control replicates that have run for 10 minutes can be used to estimate cholinesterase inhibition greater or less than 75%.

* Sigma, St Louis, MO, USA.

Fig. 1. Course of hydrolysis of acetylthiocholine iodide in microassays of diluted whole blood at 25 °C and 37 °C. Values shown are the means for eight replicate assays of samples from 12 laboratory volunteers (standard deviations within individual samples, <0.02 absorbance units (r>0.99)).
Table 1. Rates of hydrolysis of acetylthiocholine iodide [ATCH] and of formation of thiol groups in the laboratory microassay of diluted whole blood, erythrocytes, and plasma

<table>
<thead>
<tr>
<th>Substance</th>
<th>Rate of hydrolysis*</th>
<th>Rate of formation of thiol groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole blood</td>
<td>µmol/min per assay</td>
<td>µmol/min/ml</td>
</tr>
<tr>
<td>Erythrocytes</td>
<td>0.383±0.009</td>
<td>2.29±0.06</td>
</tr>
<tr>
<td>Plasma</td>
<td>0.215±0.015</td>
<td>1.29±0.07</td>
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</tbody>
</table>

* Values are mean ± standard deviation for eight replicates.

The accuracy of the field procedure was assessed both in the laboratory and under realistic field conditions in Haiti. The results obtained by technicians newly trained in the method, who estimated the results visually, were compared with those obtained for the same samples using a portable enzyme immunoassay reader. Although the reagents are the same as those used in the WHO spectrophotometric field kit, the stability of pre-weighed reagents was evaluated at ambient temperatures over a 2-week period in the field.

RESULTS

Laboratory studies

Hydrolysis of ATCH by blood cholinesterase from the 12 laboratory volunteers produced microassay plots that were linear (correlation coefficient >0.99) for at least 60 minutes, with only slight temperature variation in the results between 25 °C and 37 °C (Fig. 1). Rates of hydrolysis of ATCH, expressed in various ways, and of the rate of production of thiol groups in microassays of whole blood, erythrocytes, or plasma cholinesterase for these volunteers are shown in Table 1.

Under the reaction conditions used, erythrocyte cholinesterase accounted for nearly two-thirds of the total cholinesterase activity of whole blood (Fig. 2), the remainder being associated with plasma cholinesterase. As reported by Garry & Rouch (7), a 20-mol/l solution of quinidine sulfate completely inhibits plasma cholinesterase activity but has no effect on erythrocyte cholinesterase (Fig. 3). The relative contribution of erythrocyte cholinesterase in the microassay described here is lower than that in the WHO spectrophotometric kit test. Also, the amount of blood (0.167 µl per 250 µl assay volume) produced low blank values at λ = 410 nm.

In a separate experiment, untrained technicians were able routinely to classify (to ±10%) using samples as greater or less than 50% inhibited using paraoxon, a powerful cholinesterase inhibitor.

Field studies

Field personnel from the Haitian malaria control programme visually correctly interpreted blood cholinesterase levels in the 14 Haitian spray personnel evaluated in the field. The same microassay wells
Fig. 3 Course of hydrolysis of acetylthiocholine iodide in microassays of diluted whole blood, erythrocytes, and plasma in the presence of a solution of quinidine sulfate (20 μmol/l). Plots are regressions (r > 0.99) for eight replicate assays of samples from 12 laboratory volunteers (standard deviations within individual samples, <0.02 absorbance units).

were also read using a portable EIA reader, and the data are summarized in Table 2, together with the results of the tintometric assay from the same individuals. The results obtained by both methods were in good agreement. In addition, the rates of hydrolysis of ATCH determined in the field were very similar to those measured in the laboratory. It should be noted that, since the microassay results are quantitated using control samples instead of an external standard, adjustment of the results for temperature variations is not necessary.

The results of the microassays conducted in the field using reagents stored at ambient temperature over Drierite for at least 2 weeks were indistinguishable from those performed in the laboratory using freshly prepared reagents.

DISCUSSION

Blood cholinesterase can be determined accurately and precisely (between-run and within-run precision in the laboratory (standard deviation <0.01 mmol/min)) using the procedures described, which also permit simultaneous measurement of the activity of erythrocyte and plasma cholinesterase. The assay is also more straightforward than other methods for the determination of these enzymes.

The results of the microassay are comparable to those reported for other methods of determining plasma and erythrocyte cholinesterases, and its precision is similar to that of Ellman's method (3, 7, 22). The precision of the method can be increased further by diluting the samples, thereby reducing the effects.

Table 2. Rates of hydrolysis of acetylthiocholine iodide (ATCH) and proportion of normal enzyme activity in whole blood from test subjects and controls in Haiti

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Rate of hydrolysis a (μmol per assay)</th>
<th>Absorbance b (0.410 nm)</th>
<th>Enzyme activity (%) c, d</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Microassay</td>
</tr>
<tr>
<td>Controls</td>
<td></td>
<td></td>
<td>100</td>
</tr>
<tr>
<td>1</td>
<td>26</td>
<td>1.4</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>26</td>
<td>1.4</td>
<td>100</td>
</tr>
<tr>
<td>3</td>
<td>23</td>
<td>1.3</td>
<td>90</td>
</tr>
<tr>
<td>4</td>
<td>26</td>
<td>1.4</td>
<td>100</td>
</tr>
<tr>
<td>Test subjects</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>18</td>
<td>1.0</td>
<td>70</td>
</tr>
<tr>
<td>2</td>
<td>16</td>
<td>0.8</td>
<td>60</td>
</tr>
<tr>
<td>3</td>
<td>21</td>
<td>1.1</td>
<td>80</td>
</tr>
<tr>
<td>4</td>
<td>18</td>
<td>1.0</td>
<td>70</td>
</tr>
<tr>
<td>5</td>
<td>16</td>
<td>0.8</td>
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<tr>
<td>6</td>
<td>18</td>
<td>1.0</td>
<td>70</td>
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<td>7</td>
<td>23</td>
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<td>0.4</td>
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<td>9</td>
<td>16</td>
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<td>60</td>
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<tr>
<td>10</td>
<td>18</td>
<td>1.0</td>
<td>70</td>
</tr>
</tbody>
</table>

a Values are mean of four replicates for each sample.
of uneven sampling of suspended red cells (21). The microassay causes minimal haemolysis, while its low variability is characteristic of automated assays (14). A further advantage of the assay is that addition of 10 μl quinidine sulfate (20 μmol/l) to some of the wells permits simultaneous determination of erythrocyte acetylcholinesterase and plasma cholinesterase, with replicates, on a 10-μl sample of whole blood. Addition of 10 μl of 10 μmol/l eserine to the wells causes maximum inhibition of blood cholinesterase and this is consistent with results reported for animal tissues (17). Concurrent determination of both enzymes is advantageous (16) since plasma cholinesterase levels are depressed in certain pathological and physiological conditions, e.g., malnutrition (8, 9, 16); genetically determined variants occur in which cholinesterase activity is decreased (2, 15); and both enzymes may be inhibited to different degrees and lengths of time following exposure to insecticides (23). The conditions used for development of the microassay slightly favour determination of the erythrocyte enzyme (10, 11, 24); however, the concentration of the acetylthiocholine substrate can be adjusted to favour plasma cholinesterase (7).

The method is straightforward, rapid, and inexpensive (chemicals for 100 tests, US$ 0.25; reusable items, less than US$ 20 per set). Up to 60 replicate assays can be made from a 10-μl fingerstick sample of blood, and it is thus a simple matter to rapidly re-assay samples. The low rate of chromophore development and use of automatic pipettes allow the simultaneous evaluation of the cholinesterase activity in the blood of 40-60 individuals. Automated determinations of blood cholinesterase are currently performed in many hospital laboratories (10), but require instrumentation not routinely available in developing countries. In addition, the increasing incidence of human exposure to pesticides has made it necessary to routinely monitor more individuals. Furthermore, as the resistance of disease vectors to pesticides becomes more widespread, more toxic compounds have to be employed in areas where cholinesterase monitoring using conventional methods is difficult, if not impossible. For example, in Haiti fenitrothion was introduced for malaria control following a comparative study with malathion and DDT. Fenitrothion is potentially more toxic than malathion (6), but field trials in Kenya (5) and Thailand (12, 13) indicated that it could be used safely. After careful study, protective measures, including assay of blood cholinesterase by tinometry, were introduced to monitor exposure of spray personnel (19, 20). Nevertheless, with the tinometric method it is difficult for one field laboratory to perform weekly tests on several hundred spray personnel, all of whom must be transported to a central location. Also, tinometers are expensive (US$ 85), and this precludes their use in field testing stations. In contrast, the microassay is considerably cheaper. The results of the microassay are also less ambiguous to read (lighter or darker than a standard solution) than those of the tinometric test (a series of subtle colour variations). The microassay therefore readily facilitates determination of the levels of erythrocyte and plasma cholinesterase in whole blood and is suitable for use in the field in developing countries.

ACKNOWLEDGEMENTS

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RÉSUMÉ

MICROTITRAGE EN LABORATOIRE ET SUR LE TERRAIN DES CHOLINESTÉRASES DANS LE SANG TOTAL, LE PLASMA ET LES ÉRYTHROCYTES

L'article décrit un microtitrage simple et direct pour le dosage simultané de l'acétylcholinestérase et de la cholinestérase plasmatique dans le sang total, le plasma et les érythrocytes. Le titrage est basé sur la méthode d'Elman et peut être utilisé au laboratoire avec un lecteur pour titrage immuno-enzymatique ou sur le terrain sous une forme simplifiée n'exigeant aucun appareillage. Pour établir les valeurs de référence, on a suivi l'hydrolyse de l'acétylthiocholine par la cholinestérase sanguine de 12 volontaires n'ayant pas d'antécédents récents d'exposition à des inhibiteurs de la cholinestérase. Le taux d'hydrolyse déterminé au laboratoire grâce à un lecteur pour titrage
immune-enzymatique était linéaire pendant la première heure, et on n’observait que peu de variations dues à la température lorsque l’analyse était effectuée entre 25 °C et 37 °C. Dans les conditions de l’essai, l’activité de la cholinestérase érythrocytaire était d’environ les deux tiers de celle de la cholinestérase du sang total, le tiers restant étant dû à la cholinestérase plasmatique. Lors d’une autre expérience, on a examiné en routine des échantillons de sang, par cette technique de microtitrage, pour les classer selon le degré d’inhibition par le paraoxon, un puissant inhibiteur de la cholinestérase, en échantillons inhibés à plus de 50% ou à moins de 50% (± 10%).

En utilisant la version simplifiée du titrage, le personnel du programme haïtien de lutte antipaludique a correctement interprété les taux de cholinestérase sanguins de 14 employés affectés à la pulvérisation d’insecticide en Haïti, le titrage étant effectué sur le terrain. Comme la valeur quantitative des résultats du microtitrage est déterminée par rapport à un échantillon témoin et non par rapport à un étalon externe, il n’est pas nécessaire d’effectuer une correction pour tenir compte des effets de la température. Le microtitrage est à la fois exact et précis et permet de déterminer simultanément l’activité enzymatique érythrocytaire et plasmatique par addition de sulfate de quinidine à certains des goûters de titrage. Cette méthode est facilement adaptable à la mesure en double de l’activité de la cholinestérase, puisque plus de 60 titrages peuvent être exécutés à partir d’un échantillon de 10 µL de sang prélevé par piqûre au doigt, et elle convient à l’application sur le terrain dans les pays en développement.

REFERENCES