Colorimetric Determination of Serum Cholinesterase and Its Genetic Variants by the Propionylthiocholine-Dithiobis(nitrobenzoic Acid) Procedure

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Introduction
Measurement of serum cholinesterase (EC 3.1.1.8, acylcholine acyl-hydrolase) has been used to assess liver function, monitor excessive exposure to the anticholinesterase organophosphorus insecticides, predict susceptibility to prolonged apnea after administration of the muscle relaxant succinylcholine, and investigate the inheritance of variants of the enzyme.

There are many methods for measuring serum cholinesterase, but the colorimetric method based on the Ellman reaction (1) and pioneered by Garry and Routh (2) has recently become popular, because of its simplicity, speed, and sensitivity. A version of the method outlined here has been described (3).

Principle
Cholinesterase hydrolyzes propionylthiocholine (equation 1) to yield free sulphydryl, which reacts with 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB)1 to yield the 5-thio-2-nitrobenzoate ion (equation 2).

\[
\text{C}_3\text{H}_5\text{C} \equiv \text{S} - \text{CH}_2 - \text{CH}_2 - \text{N(CH}_3\text{)}_3 + \text{H}_2\text{O} \xrightarrow{\text{cholinesterase}} \text{C}_3\text{H}_6\text{CO}_2\text{H} + \text{propionic acid} \]

\[
2\text{CH}_3\text{N} - \text{CH}_2 - \text{CH}_2 - \text{SH} + \text{thiocholine} \rightarrow \text{O}_\text{NO}_2 \text{S} - \text{S} - \text{(CH}_3\text{)}_3\text{N} - \text{CH}_2 - \text{CH}_2 - \text{SH} + \text{2CH}_3\text{N} - \text{CH}_2 - \text{CH}_2 - \text{SH} \quad \text{DTNB} \]

The yellow nitrobenzoate has a useful absorption maximum at 410 nm; DTNB absorbs maximally at 320 nm. For detection of cholinesterase variants the standard reaction is run with and without the inhibitors dibucaine and sodium fluoride.

Materials and Methods
Reagents
1. Phosphate buffer, pH 7.6, \( \mu = 0.1 \). Prepare separate solutions of 4.73 g of Na\(_2\)HPO\(_4\) per liter and 13.61 g of KH\(_2\)PO\(_4\) per liter and mix to give a final

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1 Nonstandard abbreviations and symbols used: DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); PTCI, propionylthiocholine iodide; Tris, tris(hydroxymethyl)aminomethane; and \( \mu \), ionic strength.
pH of 7.6. About 60 ml of the monobasic potassium salt is required per liter of the dibasic sodium phosphate.

2. **Substrate**, propionylthiocholine iodide, 20 mmol/liter. Dissolve 6.064 mg of PTCI per milliter of solution (see Note 2).

3. **Color reagent**, DTNB, 0.423 mmol/liter. Dissolve 167 mg of DTNB (Eastman Kodak Co., Rochester, N. Y. 14650; other sources are also satisfactory) per liter of buffer. Store in a brown glass bottle at room temperature or at 4 °C (see Note 3).

4. **Dibucaine**, 0.3 mmol/liter. Dissolve 57 mg of dibucaine • HCl (“Nupercaine hydrochloride,” Ciba) in water to make 500 ml of solution. This solution is stable indefinitely at room temperature.

5. **Sodium fluoride, 40 mmol/liter**. Prepare daily by dissolving 84 mg of NaF in water and diluting to 50 ml.

6. **Quinidine sulfate solution**, 0.5 g/100 ml. Dissolve 0.5 g of quinidine sulfate (Sigma Chemical Co., St. Louis, Mo. 63178) in water to make 100 ml of solution.

**Serum**

Serum cholinesterase is extraordinarily stable in undiluted serum. Unrefigerated sera can be mailed for long distances without refrigeration without significant change in activity or inhibition. Sera are usually stored frozen at −20 °C, but sera left at 4 °C or room temperature for weeks, even with gross bacterial contamination, have given results consistent with those from the fresh specimens. Moderate hemolysis does not interfere if the serum is well centrifuged to remove erythrocyte ghosts.

*Note:* When a patient has prolonged apnea it is not wise to study serum obtained during the paralysis; anomalous results are commonly obtained, probably because of circulating succinylcholine or succinylmonocholine or other pharmacologic agents. Because the analytical results are of no use in management of the apneic patient, it is better to get a fresh serum the next day for enzymatic assay.

**Procedure (Table 1)**

The procedure described is for the assay of cholinesterase activity in the presence and absence of dibucaine as an inhibitor.

1. Depending on the number of assays, prepare dilutions of stock PTCI with an equal volume of (a) water and (b) stock dibucaine.

2. Pipet 3 ml of DTNB-buffer (reagent 3) into each of six tubes (three for activity alone and three for inhibition by dibucaine). Place in a water bath set at 37 °C.

3. After 5 min, add 1 ml of substrate (1a) to each of three tubes and 1 ml of substrate plus inhibitor (1b) to each of the other set of three tubes.

4. Add 1 ml of a 100-fold dilution of serum in deionized water to two tubes of each set. If low activity is expected, the serum should be diluted less. Water is the best diluent, because salts alter the activity. It is best to dilute the serum shortly before the assay, because the cholinesterase often slowly loses activity after dilution.

5. Exactly 3 min later, add 1 ml of quinidine reagent to all tubes, followed by 1 ml of diluted serum to the blanks (the third tube in each set). With experience, the timing may be varied to give an optimum color intensity (A = 0.4 to 0.6) in tubes without inhibitor.

6. Read the absorbance of the unknown against the corresponding blank at 410 nm within a short time, preferably within 30 min. Because quinidine does not completely inhibit the reaction, the color will slowly deepen, but does so equally in both blanks and unknowns.

The concentrations, in millimoles per liter, of the ingredients in the final assay mixtures are: PTCI 2, dibucaine 0.03, NaF 4, DTNB 0.254, and phosphate 25.

*Notes:* 1. It is advisable to store all solutions in glass, because inhibitors are extracted from some plastic bottles. Should results be inconsistent, check the cleanliness of the glassware and the quality of the water.

2. Estimate the daily requirement for substrate and prepare that amount. Excess substrate may be frozen, but it is more convenient to prepare it as needed.

3. For purposes of cholinesterase assay, the DTNB solution is stable for long periods if stored in the dark at room temperature. If the DTNB solution is to be used for standardization (see below), the solution must be prepared freshly or if stored in a brown bottle in the dark at 4 °C it is stable for at least 12 months.

4. When using a spectrophotometer with a narrow waveband the instrument should be set at 410 nm. With the Coleman II instrument, setting at 425 nm gives the greatest difference between the blank and the assay mixture. With other wideband spectrophotometers, the optimum wavelength should be experimentally determined.

5. For fluoride inhibition, dilute the stock PTCI with an equal volume of stock NaF solution to yield the final substrate–inhibitor solution, and proceed as described for dibucaine inhibition. Temperature has a large effect on fluoride inhibition, and the results given in Table 2 can be obtained only if the assay is at 37 °C.

6. In methods based on the Ellman reaction as described in the literature, both phosphate and Tris buffers have been used. The ratio of cholinesterase activity in the two buffers is different in sera of normal subjects and in

<table>
<thead>
<tr>
<th>Table 1. Protocol for the Procedure</th>
<th>Uninhibited</th>
<th>Inhibited</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum Substrate</td>
<td>1 → 100</td>
<td>1 → 100</td>
</tr>
<tr>
<td>1 ml</td>
<td>Blank</td>
<td>Blank</td>
</tr>
<tr>
<td>2 ml</td>
<td>Test</td>
<td>Test</td>
</tr>
<tr>
<td>DTNB in buffer</td>
<td>3 ml</td>
<td>3 ml</td>
</tr>
<tr>
<td></td>
<td>Warm to 37 °C (5 min)</td>
<td></td>
</tr>
<tr>
<td>Substrate serum</td>
<td>1 ml</td>
<td>1 ml</td>
</tr>
<tr>
<td></td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Quinidine serum</td>
<td>1 ml</td>
<td>1 ml</td>
</tr>
<tr>
<td></td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

Read A at 410 nm within 30 min.
sers from cholinesterase variants (see Discussion). Total activity decreases with increasing concentrations of either buffer. Thus it is important to maintain a constant, rather dilute buffer system and avoid high concentrations of salts. Phosphate buffer has advantages in that nonenzymatic hydrolysis of substrate is slower than in Tris, and buffer changes with temperature are smaller.

Calculations

The cholinesterase activity is expressed in International Units (U/ml = μmoles/min/ml) at 37 °C and is calculated from:

\[
U/ml = \frac{x \cdot y \cdot \Delta A_{\text{unknown}}}{13.6 \cdot z} = 14.71 \cdot \Delta A_{\text{unknown}} \quad (3)
\]

(for above conditions) where \(x\) is the volume in milliliters at which the absorbance is read, \(y\) is the serum dilution (100 for normal serum), \(\Delta A_{\text{unknown}}\) the increase in absorbance corrected for the blank, 13.6 the millimolar absorptivity of the 5-thio-2-nitrobenzoate for a 1-cm cuvette, and \(z\) the number of minutes of incubation. The molar absorptivity may be determined by the standardization procedure given below. When using cuvettes that have light-paths that are not 1 cm, the activity may be calculated from:

\[
U/ml = \frac{c \cdot x \cdot y \cdot \Delta A_{\text{unknown}}}{A_{\text{standard}} \cdot z} \quad (4)
\]

where \(c\) is the micromolar concentration of the standard in terms of sulfhydryl, \(A_{\text{standard}}\) the absorbance of the standard solution, and the other symbols as for equation 3.

Inhibition by dibucaine or fluoride is calculated:

\[
\text{Percent inhibition} = 100 \left(1 - \frac{A_{\text{with inhibitor}}}{A_{\text{without inhibitor}}} \right) \times 100
\]  

(5)

Standardization

Standard solutions of cysteine or glutathione may be used as a source of sulfhydryl, but are not stable on storage. The following procedure is satisfactory.

Dilute 0, 1, 2, 3, 5, and 10 ml of the DTNB-buffer (see Note 3) to 100 ml with buffer. These solutions correspond respectively to 0, 8.42, 16.8, 25.3, 42.1, and 84.2 μmol of sulfhydryl per liter. Pour convenient volumes of each dilution into cuvettes, add a small crystal of cysteine to each, and read at 410 nm. The color gradually fades but can be restored by adding more cysteine. The molar absorptivity of the 5-thio-2-nitrobenzoate can be calculated from the absorbance values found. The results are identical to those obtained with freshly prepared standard cysteine solutions (4).

Results and Discussion

Although other systems of nomenclature have been used, the system outlined by Motulsky (5) for describing the phenotypes and genotypes at serum cholinesterase locus 1 is presently preferred. The most common, or "normal," gene is termed \(E_1^u\) and the "usual" phenotype, \(U\), denotes homozygosity for this gene, \(E_1^u E_1^u\). Other alleles known for this system are the dibucaine-resistant, or "atypical" gene, \(E_1^s\); the fluoride-resistant, or "fluoride" gene, \(E_1^f\); and the "silent" gene, \(E_1^d\) (see Table 2). The atypical and fluoride genes are recognized by lesser inhibition with dibucaine and fluoride, respectively, than is the usual gene. The silent gene is recognized by the virtual absence of cholinesterase activity when in the homozygous state and by the aberrant inheritance when co-existing with the atypical or fluoride genes. The silent gene itself exists in several slightly variant forms, \(S_1, S_2\) and \(S_4\) (6).

Table 2 gives the phenotypes and the inferred genotypes thus far observed in this system and the cholinesterase activities and inhibitions by the present method. The "vulnerable" phenotypes, those definitely or probably associated with prolonged apnea after the administration of succinylcholine, are given

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Genotype</th>
<th>No. sera</th>
<th>Activity at 37 °C, μmol/min/ml</th>
<th>Percent inhibition by Dibucaine</th>
<th>Fluoride</th>
</tr>
</thead>
<tbody>
<tr>
<td>U</td>
<td>(E_1^u E_1^u)</td>
<td>63</td>
<td>8.44 ± 1.78</td>
<td>83.6 ± 1.3</td>
<td>79.7 ± 1.2</td>
</tr>
<tr>
<td>A</td>
<td>(E_1^u E_1^u)</td>
<td>13</td>
<td>1.90 ± 0.61</td>
<td>19.9 ± 8.7</td>
<td>84.0 ± 1.8</td>
</tr>
<tr>
<td>AS</td>
<td>(E_1^u E_1^u)</td>
<td>26</td>
<td>1.80 ± 0.37</td>
<td>20.7 ± 4.1</td>
<td>82.3 ± 3.4</td>
</tr>
<tr>
<td>S_1</td>
<td>(E_1^u E_1^u)</td>
<td>16</td>
<td>0.08 ± 0.01</td>
<td>5.3 ± 4.3</td>
<td>35.7 ± 6.1</td>
</tr>
<tr>
<td>S_2</td>
<td>(E_1^u E_1^u)</td>
<td>8</td>
<td>0.18 ± 0.05</td>
<td>67.6 ± 4.3</td>
<td>67.7 ± 1.7</td>
</tr>
<tr>
<td>F</td>
<td>(E_1^u E_1^u)</td>
<td>1</td>
<td>3.57</td>
<td>71.8</td>
<td>53.6</td>
</tr>
<tr>
<td>AF</td>
<td>(E_1^u E_1^u)</td>
<td>4</td>
<td>3.65 ± 0.47</td>
<td>60.2 ± 8.1</td>
<td>68.3 ± 1.0</td>
</tr>
<tr>
<td>FS</td>
<td>(E_1^u E_1^u)</td>
<td>1</td>
<td>3.47</td>
<td>76.7</td>
<td>64.9</td>
</tr>
<tr>
<td>UA</td>
<td>(E_1^u E_1^u)</td>
<td>59</td>
<td>5.84 ± 1.76</td>
<td>72.7 ± 3.1</td>
<td>80.1 ± 1.6</td>
</tr>
<tr>
<td>UF</td>
<td>(E_1^u E_1^u)</td>
<td>17</td>
<td>5.99 ± 1.26</td>
<td>79.8 ± 1.2</td>
<td>73.0 ± 1.7</td>
</tr>
<tr>
<td>US</td>
<td>(E_1^u E_1^u)</td>
<td>6</td>
<td>4.61 ± 0.57</td>
<td>84.4 ± 0.8</td>
<td>79.3 ± 1.4</td>
</tr>
</tbody>
</table>

* Values are mean ± SD.
in boldface. The most important values used for identification of each variant are italicized.

Table 2 shows that virtually all vulnerable phenotypes have low cholinesterase activity. This is better shown in Figure 1; there is little overlap with normal values. An activity greater than 4 U/ml makes the existence of a vulnerable phenotype unlikely and obviates the need for measurement of dibucaine and fluoride inhibitions. If 4 U/ml is used as the dividing line, of the 69 subjects with a vulnerable phenotype given in Table 2, only one (AF) would have been missed. On the other hand, 12 of the 59 UA and one of the 6 US phenotypes gave values less than 4 U/ml, although the latter phenotypes are unlikely to develop prolonged apnea after the use of succinylcholine.

There are acquired causes for low cholinesterase activity; in such circumstance the dibucaine and fluoride inhibitions are normal. Parenchymal liver disease regularly lowers cholinesterase activity in serum, and the measurement of serum cholinesterase is sometimes used as part of a battery of liver-function tests. Serum cholinesterase activity is decreased after the tenth week of pregnancy, in women on estrogen therapy, and is variably low in chronic renal disorders and in a wide variety of cachectic and wasting states. It is lowered if there has been excessive exposure to the anticholinesterase organophosphorous insecticides (sometimes used in the treatment of glaucoma), and serum cholinesterase activity can be monitored as an index of excessive exposure in workers involved in the manufacture or use of such compounds.

If pre-anesthesia screening is used, it would be wise to prohibit the use of succinylcholine if the serum cholinesterase activity is less than 4 U/ml—although there will be, for reasons outlined above, some patients with values below 4 U/ml who could probably tolerate succinylcholine well.

A low value should be followed up by the estimation of the inhibition by one or more inhibitors. If a vulnerable phenotype is found, the patient should be notified and a family study recommended; often other family members at risk of a similar occurrence can be found and warned. When the silent gene coexists with the atypical or fluoride gene, a family study is the only means at present for assigning the proper phenotypes.

Note (Evaluator F.M.-O.): All surgical cases in our institution have their total serum cholinesterase measured before surgery. If the total activity is less than 4 U/ml, the dibucaine inhibition is determined. In a six-month period, 2756 cases were tested of whom 170 (6.2%) had low activities. Of those with low activities, less than 5% had a decreased dibucaine inhibition.

Reproducibility of the present method is satisfactory; typical results are shown in Table 3.

Other investigators have used thiocohinesterase methods for the detection of the serum cholinesterase variants. Garry et al. (7) use the differences in activity in Tris and phosphate buffers and the inhibition of cholinesterase by fluoride in each buffer to identify the atypical and fluoride variants. Das and Liddell (8) use “RO2-0683” (Hoffmann-La Roche, Inc., Nutley, N. J. 07110) and 6% butanol for the same purpose. Both of these methods give good precision in the identification of the cholinesterase phenotypes, but lack the versatility of the present assay.

Note: Evaluator P. J. G. does not agree with this last statement, as he finds his procedure (7) sufficiently versatile.

Other inhibitors used in detecting the atypical gene can be used in the procedure outlined here. The final molar concentrations of several useful inhibitors are: “RO2-0683,” 3.5 × 10⁻⁶; solanidine, 4 × 10⁻⁷; and mytelase, 5 × 10⁻⁵. NaCl, certain other salts, and n-butanol can be incorporated into the assay mixture to help distinguish various phenotypes, but we have not found them useful.

The method can readily be automated. If the AutoAnalyzer is used, RO2-0683 should replace dibucaine because dibucaine accumulates in the tubing of the automated system. The concentration of the ingredients at the incubation point should approximate those described in this paper, and a series of normal and known variant sera should be used to obtain baseline values.

The acetylcholinesterase activity of the erythro-

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**Table 3. Reproducibility of Cholinesterase Assays and Inhibitions**

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>No. assays</th>
<th>Activity, μmol/min/ml</th>
<th>Percent inhibition by</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Dibucaine</td>
<td>Fluoride</td>
</tr>
<tr>
<td>Pool</td>
<td>12</td>
<td>5.95 ± 0.18</td>
<td>84.5 ± 1.3</td>
<td>80.5 ± 1.2</td>
</tr>
<tr>
<td>U</td>
<td>7</td>
<td>9.73 ± 0.66</td>
<td>82.9 ± 0.4</td>
<td>78.6 ± 1.2</td>
</tr>
<tr>
<td>U</td>
<td>8</td>
<td>8.11 ± 0.30</td>
<td>84.8 ± 3.1</td>
<td>80.1 ± 1.3</td>
</tr>
<tr>
<td>U</td>
<td>17</td>
<td>7.67 ± 0.32</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>UA</td>
<td>10</td>
<td>6.38 ± 0.39</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

* Each assay was done on a different day. Values are mean ±1 SD.

# The serum pool was derived from random excess serum from the clinical laboratory.

| N.D. = not determined. |   |

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**Note:**

- I. Activity, period, 10
- II. influenza
- III. cholinesterase
- IV. fibrosis
- V. wasting
- VI. diseases
- VII. growth
- VIII. factors
- IX. inhibitors
- X. inhibitions
- XI. activity
- XII. inhibition
- XIII. substrate
- XIV. sensitive
- XV. fluorides
- XVI. fluoride
- XVII. usual
- XVIII. usual
- XIX. usual
- XX. usual
- XXI. usual
- XXII. usual
- XXIII. usual
- XXIV. usual
- XXV. usual
- XXVI. usual
- XXVII. usual
- XXVIII. usual
- XXIX. usual
- XXX. usual
- XXXI. usual
- XXXII. usual
- XXXIII. usual
- XXXIV. usual
- XXXV. usual
- XXXVI. usual
- XXXVII. usual
- XXXVIII. usual
- XXXIX. usual
- XL. usual
- XLI. usual
- XLII. usual
- XLIII. usual
- XLIV. usual
- XLV. usual
- XLVI. usual
- XLVII. usual
- XLVIII. usual
- XLIX. usual
- L. usual
- LI. usual
- LII. usual
- LIII. usual
- LIV. usual
- LV. usual
- LV. usual
- LX. usual
- LXI. usual
- LXII. usual
- LXIII. usual
- LXIV. usual
- LXV. usual
- LXVI. usual
- LXVII. usual
- LXVIII. usual
- LXIX. usual
- LXX. usual
- LXXI. usual
- LXXII. usual
- LXXIII. usual
- LXXIV. usual
- LXXV. usual
- LXXVI. usual
- LXXVII. usual
- LXXVIII. usual
- LXXIX. usual
- LXXX. usual
- LXXXI. usual
- LXXXII. usual
- LXXXIII. usual
- LXXXIV. usual
- LXXXV. usual
- LXXXVI. usual
- LXXXVII. usual
- LXXXVIII. usual
- LXXXIX. usual
- C. usual
- D. usual
- E. usual
- F. usual
- G. usual
- H. usual
- I. usual
- J. usual
- K. usual
- L. usual
- M. usual
- N. usual
- O. usual
- P. usual
- Q. usual
- R. usual
- S. usual
- T. usual
- U. usual
- V. usual
- W. usual
- X. usual
- Y. usual
- Z. usual
cytes can be assayed by the same method by washing the cells with NaCl (9 g/liter) and hemolyzing them by a 200-fold dilution with water. PTCI can be used as substrate, but acetylthiocholine iodide is more rapidly hydrolyzed. Such assays are useful in following the effects of exposure to anticholinesterase chemicals and in the diagnosis of paroxysmal nocturnal hemoglobinuria.

References

No reprints of this paper will be available. For policy on papers in this series, see the Editorial in the October 1973 issue. Criticisms are invited from readers, and should be addressed to the Submitters, with a copy to the Editor-in-Chief.