Spectrophotometric Micromethod for Measuring Cholinesterase Activity in Serum or Plasma

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A simple ultraviolet method has been developed for determining serum or plasma cholinesterase activity. The cholinesterase acts on acetyl- or butyrylthiocholine to release thiocholine, which reacts with 2,2'- or 4,4'-dithiodipyridine, showing absorbance at 324 or 343 nm, respectively. The activity can be measured kinetically and also after incubation for 3 to 5 min at 20°C to 37°C. A volume of 10 μl of serum or plasma is sufficient for the determination. This method is well suited for clinical investigation as well as experimental studies.

Additional Keyphrases release of thiocholine from acetyl- or butyryl-choline • kinetic measurement • normal values

Thiocholine esters such as acetylthiocholine or butyrylthiocholine have been widely used as substrates for measuring cholinesterase activity. The SH group of the thiocholine liberated by the enzyme action is determined by the reaction with nitroprusside (1) or 5,5-dithiobis-(2-nitrobenzoic acid) (2-6), or thiocholine itself can also be determined fluorometrically by the reaction with o-phthalaldehyde (6).

A simple, rapid, kinetic micromethod for measuring the activity of cholinesterase in serum or plasma was developed, with use of acetylthiocholine or butyrylthiocholine as substrate and 2,2'- or 4,4'-dithiodipyridine as the chromogenic agent. This highly sensitive method requires less than 10 μl of plasma or serum for duplicate analysis, and the enzymatic reaction can be followed kinetically. Furthermore, this method can be used for automated as well as manual analysis.

Materials and Methods
Reagents

Acetylthiocholine iodide and butyrylthiocholine iodide, 50 mmol/liter. This substrate solution is stable for 1 week if kept refrigerated.

2,2'- or 4,4'-Dithiodipyridine (2,2'-PDS or 4,4'-PDS), 0.2 mmol/liter of phosphate buffer (pH 7.4, 0.2 molar).

Sodium fluoride, 30 mg/ml of water. This is the inhibitor.

Thiocholine was prepared in this laboratory from acetylthiocholine by hydrolysis with crystalline cholinesterase.

Reduced glutathione.

Procedure

Method 1. In two cuvets, one for the test sample and the other for the blank, 1 ml of 2,2'-PDS or 4,4'-PDS and 1 ml of the substrate solution are mixed. Then, 5 μl of serum and an equivalent amount of water are added to the sample and blank cuvets, respectively. The absorbance is measured at 343 nm for 2,2'-PDS and 324 nm for 4,4'-PDS every minute for 5 to 10 min against the blank with a Hitachi double-beam spectrophotometer. Assay of the enzyme activity is carried out at 37°C. However, any controlled temperature between 20°C and 37°C can be used for the assay.

Method 2. Pipet 1 ml of 2,2'- or 4,4'-PDS solution and 1 ml of substrate solution into the sample test tube and the blank tube. Add 5 μl of plasma or serum to the tubes, followed by 0.3 ml of inhibitor solution (NaF) to the blank tube and mix. After the incubation of 5 min at 37°C, the enzyme reaction is stopped in the test sample by adding 0.3 ml of inhibitor solution. The absorbance is measured as described in Method 1.

Definition of unit. The cholinesterase activity is expressed as micromoles of sulfhydryl groups liberated in 1 min from 1 ml of plasma or serum at 37°C. The difference in absorbance between the test sample and the blank is calibrated from a thiocholine calibration curve. Reduced glutathione can also be used for the calibration. It is also possible to use the molecular extinction coefficient of 2- or 4-thiopyridine to calculate the activity.

Results

Absorption Spectra

Reaction product of reduced glutathione with 2,2'- or 4,4'-dithiodipyridine. Figure 1 shows absorption spectra of 2,2'-PDS and 4,4'-PDS with reduced glutathione. When 2,2'-PDS reacts with reduced glutathione, forming 2-thiopyridine, an absorption peak is observed at 343 nm. The formation
of 2-thiopyridine can be followed by the change in absorbance. Similarly, 4,4′-PDS reacts with reduced glutathione, forming 4-thiopyridine with an absorption peak at 324 nm. Formation of 4-thiopyridine can be followed by the change in absorbance at 324 nm. (Thiocoline and glutathione react equally, on a molecular basis, with the dithiopyridine.)

Absorption spectra of the reaction mixture of acetyl- or butyrylthiocholine and serum with 2,2′- or 4,4′-dithiodipyridine. Absorption spectra of 2,2′- or 4,4′-PDS, with the reaction mixture of thiocholine ester and human serum under the conditions of Method 2 are presented in Figure 2. When 2,2′-PDS reacts with the thiocholine liberated from acetyl- or butyrylthiocholine by serum cholinesterase, formation of 2-thiopyridine can be followed by the change in absorbance at 343 nm, because neither the corresponding thiocholine ester nor 2,2′-dithiodipyridine absorbs importantly at that wavelength. Conversely, the 2,2′-PDS consumed can be estimated from its absorption at 233 nm. An analogous situation applies to 4,4′-PDS: the formation of 4-thiopyridine is followed by the change in absorbance at 324 nm, which measures the enzyme’s ability to liberate thiocholine from thiocholine esters.

4-Thiopyridine is the more sensitive reagent (8, 9).

Effect of Variables in Procedure

Effect of duration of the reaction of 2,2′- or 4,4′-dithiodipyridine with thiols. The reduction of 2,2′- or 4,4′-PDS with reduced glutathione at 20° and 37°C and pH 7.5 takes place momentarily. The 2-thiopyridine formed is stable for at least 1 h. Similarly, when 2,2′- or 4,4′-PDS reacts with thiocholine, the 2-thiopyridine is stable for at least 1 h under the conditions of this assay.

Effect of the concentration of acetyl- or butyrylthiocholine on the serum cholinesterase activity. The enzyme reaction rate was increased following the increase in the concentration of acetyl- or butyrylthiocholine. The enzyme reaction plateaus at the concentrations of acetylthiocholine and butyrylthiocholine of about 0.012 mol/liter.

Relation between incubation time and observed enzyme activity in serum. (Figure 3). With 5, 10, and 15 μl of serum, units of enzyme activity are linearly correlated with incubation time for as long as 10 min either with acetyl- or butyrylthiocholine, under the conditions of this method.

Relation of quantity of serum to enzyme activity in serum. When the enzyme activity of 5, 10, or 15 μl of serum is measured, with use of acetyl- or butyrylthiocholine and 2,2′- or 4,4′-dithiodipyridine, the units of enzyme activity and the amount of serum used are linearly correlated under the conditions of the present assay (Figure 4).

Temperature and activity of serum cholinesterase. When the enzyme activity is measured at various temperatures from 20° to 37°C, greater enzyme activity is observed at the higher temperature (Figure 5). The enzyme reaction is easily followed, even at room temperature, because the reaction of 2,2′- or 4,4′-PDS with thiols is not so much influenced by temperature within the above range.

Effect of sodium fluoride on the reaction of serum cholinesterase and acetyl- or butyrylthiocholine. When 0.3 ml of sodium fluoride (30 mg/ml) is added to the incubation system, cholinesterase activity in serum is completely inhibited (Figure 6). Therefore, this concentration of sodium fluoride can be used to stop the enzyme reaction in the manual method.

Correlation of results obtained by the DTNB colorimetric method, the present method, and the titration method. Results obtained by the colorimetric method of Garry and Routh (4) and by the present method correlate well (Figure 7). However, results obtained by the pH titration method (7) do not correlate with the results obtained by either the present method or the DTNB method, as shown in Figure 8.

Cholinesterase Activity in Serum of Healthy Individuals

These results are presented in Table 1. The
cholinesterase activity in serum of healthy subjects, as determined by the present method at 37°C with use of acetyl- and butyrylthiocholine, is 1.8–4.4 and 4.0–7.8 units, respectively.

**Table 1. The Values of Serum Cholinesterase Activity in Healthy Individuals**

<table>
<thead>
<tr>
<th>Case no.</th>
<th>Sex</th>
<th>Age</th>
<th>Acetylthiocholine</th>
<th>Butyrylthiocholine</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>25°C 37°C</td>
<td>25°C 37°C</td>
</tr>
<tr>
<td>1</td>
<td>M</td>
<td>37</td>
<td>1.7   3.1</td>
<td>3.0    5.8</td>
</tr>
<tr>
<td>2</td>
<td>M</td>
<td>37</td>
<td>1.6   2.8</td>
<td>1.9    4.8</td>
</tr>
<tr>
<td>3</td>
<td>F</td>
<td>23</td>
<td>0.9   1.8</td>
<td>2.2    4.4</td>
</tr>
<tr>
<td>4</td>
<td>M</td>
<td>23</td>
<td>1.1   2.5</td>
<td>3.0    6.0</td>
</tr>
<tr>
<td>5</td>
<td>M</td>
<td>23</td>
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<td>2.6    4.8</td>
</tr>
<tr>
<td>6</td>
<td>F</td>
<td>60</td>
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</tr>
<tr>
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<td>M</td>
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<td>1.7   2.5</td>
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</tr>
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</tr>
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<tr>
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<td>2.8    4.9</td>
</tr>
<tr>
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<td>M</td>
<td>36</td>
<td>2.6   4.2</td>
<td>4.7    6.9</td>
</tr>
<tr>
<td>Av</td>
<td></td>
<td></td>
<td>1.8   3.0</td>
<td>3.3    5.5</td>
</tr>
</tbody>
</table>

*SH liberated, μmol/min/ml of plasma or serum (37°C).

**Discussion**

The principle of this method is as follows: acetyl- or butyrylthiocholine is hydrolyzed by cholinesterase and liberates thiocholine. The SH group of thiocholine reacts with 2,2'-PDS or 4,4'-PDS to give the corresponding 2- or 4-thiopyridine (Figure 9). The 2-thiopyridine and the 4-thiopyridine are almost exclusively in the tautomeric thioform, with the mobile hydrogen attached to the

![Fig. 3. Relationship between incubation time and units of enzyme activity in serum](image1)

![Fig. 4. Correlation between units of enzyme activity and amounts of serum](image2)

![Fig. 5. Effect of temperature on the determination of activity of serum cholinesterase](image3)
Fig. 6. Effect of sodium fluoride on the reaction of serum cholinesterase with thiocholine esters.

Fig. 7. Correlation between results obtained by the DTNB colorimetric method and the present uv method.

Fig. 8. Correlation between results obtained by the DTNB colorimetric, the present uv, and the titration method.

Fig. 9. The reactions for measuring cholinesterase activity with acetylthiocholine and 2,2'- or 4,4'-dithiodipyridine.

nitrogen (8). This causes the ultraviolet absorption spectra of these thiopyridines to be quite different from those of the corresponding disulfides. It is thus possible to follow the course of the reaction spectrophotometrically and kinetically by indirectly measuring the thiocholine liberated from the acetyl- or butyrylthiocholine by the cholinesterase, from measurement of the production of 2- or 4-thiopyridine.

Grassetti et al. (8, 9) studied the reaction of a number of sulfur-containing pyridines with thiols. They reported that the extinction of 4-thiopyridine at 324 nm is almost three times as great as the extinction of 2-thiopyridine at 343 nm; thus 4,4'-dithiodipyridine is the more sensitive reagent. We obtained a similar result with thiocholine in this study. Grassetti et al. (8) also studied the reaction of various thiols such as cysteine, reduced glutathione, reduced lipoic acid, mercaptopyruvic
acid, and coenzyme A; they found that the range of pH at which 2,2’-dithiodipyridine can be effectively used as an SH reagent is broader, 3.4 to 8.1. Therefore, these reagents are quite suitable for use in the assay of thiocholine at pH 7–8, within the physiological range.

With time, a mixture of acetyl- or butyrylthiocholine and 2,2’- or 4,4’-dithiodipyridine slightly increases its absorbance at 324 or 343 nm in the absence of cholinesterase, possibly forming the tautomeric thioform caused by spontaneous hydrolysis of thiocholine ester. This reaction is minimized when a lower concentration of 2,2’- or 4,4’-dithiodipyridine, such as 0.2 mmol/liter, is used. However, when the higher concentration (2 mmol/liter) is used, a considerable amount of 2- or 4-thio- pyridine is formed. Therefore, a blank must be used even in the kinetic method and the use of a double-beam spectrophotometer is desirable.

In the present method, the quantity of serum or plasma required for the determination is 5 to 20 μl; the time required is not longer than 10 min. The results of this method agree well with the results of the colorimetric method of Garry and Routh (4). Therefore, this extremely sensitive and simple method is well suited for use in the routine clinical chemistry laboratory.

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References