A Sensitive Fluorometric Assay of Cholinesterase Activity with N-(9-Acridinyl)maleimide

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Cholinesterase (ChE) (acylcholine acylhydrolase, EC 3.1.1.8) occurs in many organs, mainly in the liver and blood. It catalyzes the hydrolysis of acetylcholine and butyrylcholine and controls the nervous system. It is important to determine the serum ChE activities for the diagnosis of liver failure, geriatric diseases, poisonings, etc. The enzyme activities have been determined based on the pH change, colorimetry of 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) with acetylcholine or butyrylcholine (BTC) as a substrate, and an enzymatic method using choline oxidase with benzoylcholine. Fluorometries of o-phthaldehyde or 3-(p-hydroxyphenyl)propionic acid, were also reported.

Recently, we reported on a highly sensitive fluorometry used to determine the activities of glutathione reductase with N-(9-acridinyl)maleimide (NAM), an SH reagent developed by Nara in our laboratory. In this paper, we describe our application of thiol fluorometry to measure the ChE activity in order to trace the thiol from BTC (Fig. 1).

Materials and Methods

Reagents and enzyme sources

Purified ChE was purchased from Sigma Chem. Co., BTC iodide, DTNB and quinidine sulfate purchased from Wako Chem. Co. were of analytical grade. Most of the reagents, enzyme preparations and their protein contents have been described in preceding papers.

Liver homogenates were stored for about one half year and human plasmas for one year at -20°C.

Determination of ChE activity by DTNB colorimetry

BTC and DTNB were dissolved in 0.1 M sodium phosphate at pH 8.0 and human plasmas were diluted 10 fold with 10 mM Tris. A mixture of 0.05 ml of 82 mM BTC and 0.25 ml of 50 mM DTNB was added to 0.1 ml of diluted enzyme sources. After 10 min of incubation at 37°C the reaction was stopped by the addition of 3 ml of 0.05% quinidine sulfate. The increase in the optical density (ΔOD) of the system at 470 nm was measured against that of the blank at zero time.

Fig. 1 Schema of a fluorometric assay of cholinesterase activity with N-(9-acridinyl)maleimide.
**Determination of ChE activity by NAM fluorometry**

Liver homogenates were diluted 10 fold and human plasma 300 to 1200 fold with 10 mM Tris. The substrate of 0.4 ml of 3.2 mM BTC in 0.1 M sodium phosphate at pH 7.5 was introduced to 0.1 ml of diluted enzyme sources. The mixture was incubated at 37°C for 10 min and supplied with 0.1 ml of a 0.25 mM NAM acetone solution and 3.0 ml of a 0.5 M boron carbonate buffer at pH 8.8. NAM stopped the enzyme reaction rapidly and formed a fluorescent complex with thiocholine up to 20 nmol. The fluorescence intensity (FI) at 435 nm (excitation at 360 nm) was measured using HITACHI 204 Fluorescence Spectrophotometer after standing for 45 min at room temperature in the dark. Background FI at zero time was assayed simultaneously as a blank. The hydrolysis of 1 µmol BTC/min was used as a unit of ChE activity under the abovementioned conditions. The specific activity is expressed as unit/mg protein.

**Results and Discussion**

**Background in the NAM and DTNB methods**

The principle of ChE assay is based on the determination of the released thiol from BTC in both the NAM and DTNB methods. The background fluorescence (or absorption) also results from the NAM (or DTNB) reaction with thiols in the sample. The sensitivities were greatly improved in the NAM method so as to eliminate any background due to the higher dilutions. It should be noted that NAM acted as a ChE inhibitor, since the same FI was obtained both with and without a ChE inhibitor, such as 2-isopropoxyphenyl methylcarbamate, at the end of the enzyme reaction. On the other hand, DTNB did not inhibit the enzyme activity.

**Optimal conditions for determining the ChE activity**

Commercially available BTC contained 0.45% thiocholine, which increased the blank value and caused serious errors in any sample with small ΔFI. For sensitive fluorometry, BTC added as a substrate should be minimized. Figure 2 shows the effect of the substrate concentration on the Δfluorescence intensity with 0.5 to 3.0 milli unit purified cholinesterase in 0.1 M sodium phosphate at pH 7.5 and 37°C for 5 min. •, 3.0 milli unit; △, 2.0 milli unit; ■, 1.0 milli unit; ○, 0.5 milli unit.

![Fig. 2 Effect of the substrate concentration on the Δfluorescence intensity with 0.5 to 3.0 milli unit purified cholinesterase in 0.1 M sodium phosphate at pH 7.5 and 37°C for 5 min. •, 3.0 milli unit; △, 1.5 milli unit; ○, 0.5 milli unit.](image1)

![Fig. 3 Relationship between the Δfluorescence intensity and pH of the incubation medium (0.1 M sodium phosphate at pH 6.0 to 9.0) with 0.5 to 3.0 milli unit purified cholinesterase at 37°C for 5 min. •, 3.0 milli unit; △, 2.0 milli unit; ■, 1.0 milli unit; ○, 0.5 milli unit.](image2)
concentrations on $\Delta F$ (FI—background $F$) with 0.5 to 3.0 milli unit purified ChE in the standard procedure. We used 2.5 mM BTC of the cuvett concentration for routine assays.

We compared two reaction mediums, 0.1 M sodium phosphate and 0.1 M Tris–malate, and selected the former, which gave a higher $\Delta F$ than that of the latter over broad pH ranges. Figures 3 and 4 show the relationship between $\Delta F$ and the pH of the incubation buffer, and $\Delta F$ and the incubation temperature, respectively, using 0.5 to 3 milli unit purified ChE in the 0.1 M phosphate buffer. The maximum $\Delta F$ was observed at a pH of 7.5 at 37°C. $\Delta F$ increased linearly with the enzyme concentration.

To follow the time course, $F$ was measured at 2 min intervals using diluted human plasma and bovin liver homogenates (Fig. 5). Since $\Delta F$ increased linearly during a 10 min period after starting, $F$ was measured within 10 min, and $F$ at zero time was used as a blank test in this work. The rate of $F$ increased linearly with the concentrations of human plasma diluted 300 to 1200 fold.

**Calibration curves**

The ChE activities were determined by measuring the released SH using the calibration curves of the standard thiocholine solutions (Fig. 6). The $\Delta OD$ in DTNB colorimetry increased linearly with the concentration up to 1000 nmol and $\Delta F$ in the NAM fluorometry up to 20 nmol. The standard solutions were prepared by the hydrolysis of 1 ml of 10 mM BTC with 1 ml of 1 unit/ml

![Graph](image_url)

**Fig. 5** Linear increase in the fluorescence intensity with the incubation time and enzyme concentration under the optimal conditions in NAM fluorometry. Dilution of human plasma (●, 300 fold; ▲, 600 fold; ■, 1200 fold) and bovin liver homogenate (○, 10 fold).

![Graph](image_url)

**Fig. 6** Calibration curves of thiocholine by NAM fluorometry (●) and the DTNB colorimetry (○) using the same stocked solution. The thiol content was checked by a GSH solution, which gives the same value by both the NAM and DTNB methods.

**Table 1** Specific ChE activities in human plasma and liver homogenates of four types of animals determined by the NAM fluorometry and the DTNB colorimetry

<table>
<thead>
<tr>
<th>Sample</th>
<th>Protein content/ mg ml$^{-1}$</th>
<th>NAM fluorometry</th>
<th>DTNB colorimetry</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Activity$^a$</td>
<td>Sample size$^b$</td>
</tr>
<tr>
<td>Plasma$^c$ A</td>
<td>37.9</td>
<td>120±2</td>
<td>(6.3)</td>
</tr>
<tr>
<td>B</td>
<td>40.9</td>
<td>139±3</td>
<td>(6.8)</td>
</tr>
<tr>
<td>C</td>
<td>57.5</td>
<td>68±2</td>
<td>(9.5)</td>
</tr>
<tr>
<td>Liver</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pig</td>
<td>18.2</td>
<td>0.9±0.1</td>
<td>(182)</td>
</tr>
<tr>
<td>Bovin</td>
<td>14.8</td>
<td>1.2±0.2</td>
<td>(148)</td>
</tr>
<tr>
<td>Sheep</td>
<td>18.7</td>
<td>2.1±0.1</td>
<td>(187)</td>
</tr>
<tr>
<td>Chicken</td>
<td>14.9</td>
<td>0.9±0.5</td>
<td>(149)</td>
</tr>
</tbody>
</table>

$^a$ Specific ChE activity (milli unit/mg protein) are shown Mean±SD for 8 samples.

$^b$ Sample size means protein weight (µg) in enzyme sources.

$^c$ Human plasmas of three individuals for blood transfusion.

$^d$ Under the lower limit of the standard curve.
purified ChE in 3 ml of 0.1 M sodium phosphate at pH 7.5 and 37°C for 60 min, and diluted with 10 mM Tris to suitable concentrations. The thiocholine stock solution was checked by comparing the ΔFI and ΔOD of GSH solutions of known concentrations.

Determination of the ChE activity by the NAM and DTNB methods

Table 1 gives the specific ChE activities in plasmas and livers by the NAM and DTNB methods along with the sample sizes and the standard deviations. The DTNB method showed about 10% higher values than did those by the NAM method. This should represent the influence of the higher background absorptions based on the higher protein concentrations. A linear relationship was observed between the measurements by the NAM method (x) and the DTNB method (y) in the range of 10 milli unit/mg protein and over (y=0.995x+15.8, r=0.995). Although the coefficient of deviation by the NAM method was about 4.5% (n=8), equal to the DTNB method, the NAM method improved the sensitivity by about 50 to 100 times compared to conventional colorimetry. The NAM fluorometry is sensitive, reproducible and simple, and is sufficient to allow us to use it for a mass screening test.