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# Fluorometric Determination of Pseudocholinesterase Activity in Postmortem Blood Samples

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**ABSTRACT:** A fluorometric assay using 3-(*p*-hydroxyphenyl) propionic acid (HPPA) was conducted to determine the activity of pseudocholinesterase (ChE) [Enzyme Commission (EC) No. 3.1.1.8] in postmortem blood samples so as to test for organophosphate poisoning. By the enzymatic reaction of ChE, its substrate, benzoylcholine, produces choline, which is oxidized by choline oxidase to generate hydrogen peroxide. HPPA is oxidized by hydrogen peroxide and peroxidase to become the fluorogenic dimer whose concentration is measured fluorometrically at an excitation emission wavelength of 320 nm and an elimination emission wavelength of 404 nm. The selectivity and sensitivity of the present method were found to be superior to those of conventional pH and spectrophotometric methods.

**KEYWORDS:** toxicology, cholinesterase, blood, fluorometric determination, cholinesterase activity, determination of ChE activity

Organophosphorus compounds are frequently used for suicidal and homicidal purposes owing to their accessibility. Since intoxication by organophosphorus insecticides, which act as cholinesterase inhibitors, leads to loss of pseudocholinesterase (ChE) activity in the blood [*1*], its determination in postmortem blood can be used to confirm the diagnosis of intoxication, which is important for determining the cause of death for legal purposes.

Although various methods for the determination of this activity have been proposed, their application is sometimes difficult since postmortem blood samples have an acidity that is higher and a color that is richer than flesh blood, as a result of rotting and hemolysis. In this study, a fluorometric method using the enzymatic reactions of ChE was carried out to determine the presence of ChE in postmortem blood.

## Materials and Methods

### *Chemicals and Enzymes*

Pseudocholinesterase (acylcholine acylhydrolase) [Enzyme Commission (EC) No. 3.1.1.8] from human plasma was purchased from Boehringer Mannheim Co. Choline oxidase [choline oxygen 1-oxidoreductase (COD) (EC.1.1.3.17)], isolated from *Alcaligen*

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species; peroxidase (POD) (EC.1.11.1.7) from horseradish; and hemoglobin from bovine erythrocytes were obtained from Sigma Chemical Co. The compound 3-(*p*-hydroxyphenyl)propionic acid (HPPA) was obtained from Nakarai Chemicals Ltd. Unless otherwise specified, all chemicals were of reagent grade and were obtained from Wako Pure Chemical Industries, Ltd. Standard serum was purchased from Nihon Shoji Co. The serum and blood samples were obtained from healthy controls. Cadaver blood was provided through the courtesy of Gunma prefectural Criminal Science Laboratory (Gunma, Japan).

*Instrumentation*

Fluorometric measurements were conducted using a Jasco spectrofluorometer (Japan Spectroscopic Co.). Controlled incubation was carried out using an incubator (Taiyo Kagaku Kogyo Co., Ltd.)

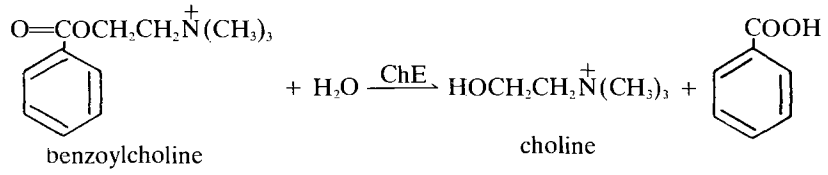
*Assay of ChE Activity in Whole Blood*

A 10-μL aliquot of a 100-fold dilution of whole blood was added to 0.9 mL of 50m*M* Tris/hydrochloric acid (HCl) buffer (pH 7.4), containing 45 μmol of sodium azide ($NaN_3$) and 0.9 μmol of benzoylcholine chloride, followed by incubation at 37°C for 30 min. One hundred microlitres of 0.05*M* Tris-HCl buffer (pH 7.4), containing 5 μmol of neostigmine methylsulfate and 0.15 μmol of HPPA were added to a solution containing choline as the reaction product. After standing for 1 min, the solution mixture was added to a reagent mixture of 2.0 mL of 0.13*M* Tris-HCl buffer (pH 8.7), containing 13 units of COD and 0.4 units of POD. After thoroughly mixing the solution mixture and letting it stand for 3 min, the fluorescence intensity was determined at 404 nm, with excitation at 320 nm.

## Results and Discussion

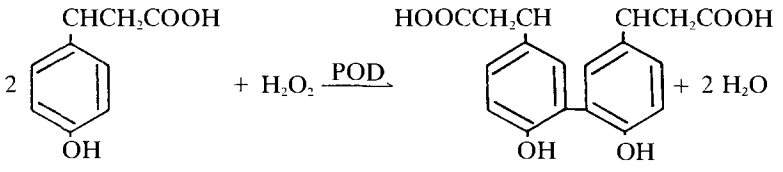
*Determination of ChE Activity in Healthy Control Blood*

By the enzymatic reaction of ChE, its substrate, benzoylcholine, produced choline (Eq 1), which was oxidized by choline oxidase to generate hydrogen peroxide (Eq 2). HPPA was oxidized by the hydrogen peroxide and peroxidase to become the fluorogenic dimer (Eq 3), whose concentration was measured fluorometrically by the method described above.




benzoylcholine choline (1)

$$HOCH_2CH_2\overset{+}{N}(CH_3)_3 + 2\ O_2 + H_2O \xrightarrow{COD} 2\ H_2O_2 + HOOCCH_2\overset{+}{N}(CH_3)_3 \quad (2)$$




HPPA (3)

Zaitsu and Ohkura found that HPPA served as an excellent fluorogenic substrate for conducting the POD-mediated reaction with hydrogen peroxide, and devised a rapid and sensitive method for determining hydrogen peroxide and POD activity [6]. To determine under what conditions the fluorometric assay of cholinesterase activity should be carried out using HPPA, the characterization of choline was first carried out. The reaction of choline chloride (7 nmol) with 2.5 units of COD was completed within 10 min in a pH 7.4 Tris-HCl buffer. A solution of $NaN_3$ (6m*M*, 100 μL) was also added in the solution of choline chloride to prevent the decomposition of hydrogen peroxide by catalase present in the choline oxidase reagent as contaminant. The standard curve of the choline chloride obtained was linear from 0.05 to 7 nmol.

The enzymatic method using absorption spectroscopy to determine serum ChE activity has been described by Okabe et al. [5]. Benzoylcholine is the best substrate for this purpose. Using the conditions used by Zaitsu and Ohkura and our own for measuring choline chloride by fluorescence, we assayed the serum ChE activity. The effects of substances present in serum were examined. Physiological substances, such as amino acid, ascorbic acid, bilirubin, glucose, and sodium chloride (NaCl), present at concentrations ten times those in normal human blood, and certain inorganic substances, such as manganous chloride ($MnCl_2$) and ferric chloride ($FeCl_3$), were found to exert no significant effect on the fluorometric determination of ChE activity.

ChE activity in the human serum samples ($n$ = 50) was determined by both the present and a conventional spectrophotometric method (the 4-aminoantipyrine-phenol-peroxidase method using a cholinesterase B test by Wako Pure Chemical Industries, Japan), followed by a comparison of the results obtained (Fig. 1). The linear regression equation, $Y = 1.267x + 34.9$, and a correlation coefficient of 0.988 were obtained for each method, with $y$ as the activity by the present method and $x$ as that by the spectrophotometric method. From the correlation coefficient, the present method is shown to be useful for determining ChE activity in human serum. However, the values for this activity by the present method were found to exceed those by the spectrophotometric method considerably. This discrepancy may possibly have been caused by the inhibitory effect of phenol present in the reagents used in the latter method. For confirmation of this point, the

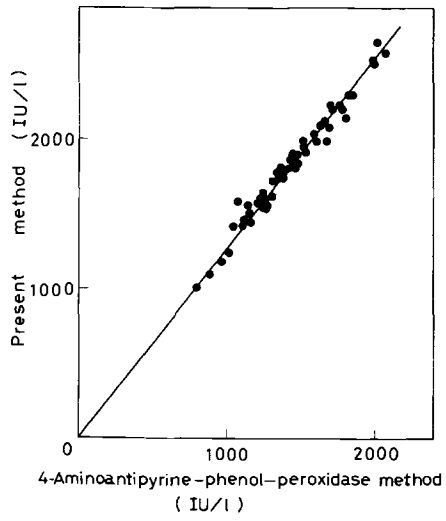



FIG. 1—*Correlation of ChE activity in serum obtained by the 4-aminoantipyrine-phenol-peroxidase method and the present method.*

activity of ChE in serum containing 21 μmol of phenol was determined by the present method. The activity was found to be much less than that in serum containing no phenol. This clearly demonstrates that the present method is superior to the conventional method.

Before the present method was used for analysis of postmortem blood specimens, it was used for the determination of activity in fresh whole blood.

The hemolysis of a whole blood sample for assay would result in increased concentrations of biological substances, such as hemoglobin and catalase. Both of these would prevent an accurate determination of ChE activity in such a sample because of their catalysis of hydrogen peroxide decomposition. However, this would not be a problem at a sample hemoglobin concentration less than 3 g/dL. The normal adult hemoglobin value lies between 12 and 18 g/dL. Diluting a blood sample 10 times would cause the concentration to be less than 2 g/dL.

One hundred microlitres of 0.45*M* $NaN_3$ were added to a whole blood sample to inhibit catalase, so that it would have no significant effect on the ChE activity measurement. The assay procedure was thus established for a whole blood sample, as described above. When benzoylcholine was used as a substrate for determination of the activity of true cholinesterase, no activity was measured. Therefore, the present method is useful for selective determination of ChE in blood. ChE activity determined for a whole blood sample was in good agreement with that for its serum. A whole blood sample whose ChE activity was 2220 IU/L was assayed ten times by the present method. The coefficient of variation was less than 1.7%.

### *Determination of ChE Activity in Postmortem Blood*

ChE activity was determined for several postmortem blood samples, whose viscosity and acidity were higher than those of flesh blood because of putrefaction of the former. The results are shown in Table 1. The ChE activity in the case of intoxication from organophosphorus insecticide was clearly far less than that in cases of death arising from other causes. The ChE activity values for the latter cases were almost same as those in serum samples of healthy controls (see Fig. 1 and Table 1), although the accuracy of determination was not very good. The present method was found to be useful for routine analysis for a diagnosis of intoxication by organophosphorus compounds.

The present method has been shown to be superior in sensitivity to other methods. For instance, it is 100 times more sensitive than any spectrophotometric methods, such as the 4-aminoantipyrine-phenol-peroxidase method, and 500 times more sensitive than

TABLE 1—*Pseudocholinesterase activity in postmortem blood.*

| Sample No. | ChE Activity, IU/L | Cause of Death |
|---|---|---|
| 1 | >10 | intoxication by organophosphorus insecticide |
| 2 | 1990 ± 110 | intoxication by carbon monoxide |
| 3 | 2100 ± 240 | burning |
| 4 | 1760 ± 230 | burning |
| 5 | 1850 ± 190 | burning |
| 6 | 1670 ± 40 | intoxication of paraquat |
| 7 | 2130 ± 60 | intoxication of paraquat |
| 8 | 1210 ± 120 | drowning |
| 9 | 1560 ± 210 | congestive heart failure |
| 10 | 1130 ± 170 | congestive heart failure |
| 11 | 1340 + 180 | congestive heart failure |
| 12 | 1600 ± 160 | congestive heart failure |

the pH method. Considering its high sensitivity, the present method should readily find application in the determination of ChE in postmortem blood for forensic science purposes.

Most United States agencies add sodium fluoride (NaF) to postmortem blood as a preservative. However, it is recommended that NaF not be added to samples, since NaF is known to be a deactivator of ChE.

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