A Rapid and Simple Ultramicromethod for the Estimation of Serum Pseudocholinesterase with Butyrylthiocholine as Substrate

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Measurement of the serum pseudocholinesterase activity has become a popular examination in routine laboratory work and its value in various condition, such as liver disease, malnutrition, fluorophosphate ester poisoning, nephrotic syndrome, hyperthyroidism, burn, post operative condition, and in pseudocholinesterase anomaly related to succinyl dicholine apnea has been described by several authors.[1)–6)]

Several methods has been developed for the determination of this enzyme.[8)–21)] At present, the most favored methods in clinical laboratory depend on measurements of fall of pH caused by the enzymatic release of acetic acid from the substrate (acetylcholine), which require the use of pH meter or some indicator such as phenol red or m-nitrophenol.[9)11)13)14)] These methods are suitable for the performance of tests with relatively large number of samples at a time.

However, acetylcholine as a substrate is very labile, being rapidly autolyzed, and the drop of pH in some buffer solutions extends occasionaly over optimal pH for the measurement of pseudocholinesterase activity of highly active sera. Other methods are not adequate for routine use since they require complex instrumentation and difficult technique, and demand much time for incubation and handling. Nowadays, a rapid, simple and accurate method is becoming necessary particularly in anesthesiology and in poisoning by organic phosphorus insecticides.[4)7)] Recently it was reported by several outhors[18)19)20)] that butyrylthiocholine would be an excellent substrate for the measurement of pseudocholinesterase activity. The S H group of the thiocholine enzymatically released from this substance can be determined colorimetrically by coupling with 5 : 5′–dithiobis–(2–nitrobenzoic acid) (DTNB) at 410mμ. The method to be described in this paper has been developed to provide a rapid method which is more convenient than those used previously. Its principle consists in direct reading of the initial velocity of enzymatic reaction. It requires only 0.02ml of plasma or serum, and can be finished within one minute and a half for one sample of the enzyme action.

METHOD

Principle

Serum pseudocholinesterase liberate thiocholine from butyrylcholine. The thiocholine thus liberated reacts spontaneously with DTNB which is contained in the reagent medium, forming the yellow dye, 5–mercapto–2–nitrobenzoic acid. The speed of increasing yellow coloration (absorption maximum 410 mμ) is directly measured with a stop watch in a spectrophotometer (Spectronic 20) equipped with a cuvette kept at constant temperature (37°C).

Instruments (General view, Fig. 1)

1. Spectronic 20 colorimeter (Shimadzu–Bausch and Laumb) : Cuvette and its holder are made to be constantly held at 37°C by a water and air jacket and equipped with a flow system to discard colored solution after reading its optical density by aspiration. (Fig. 2)

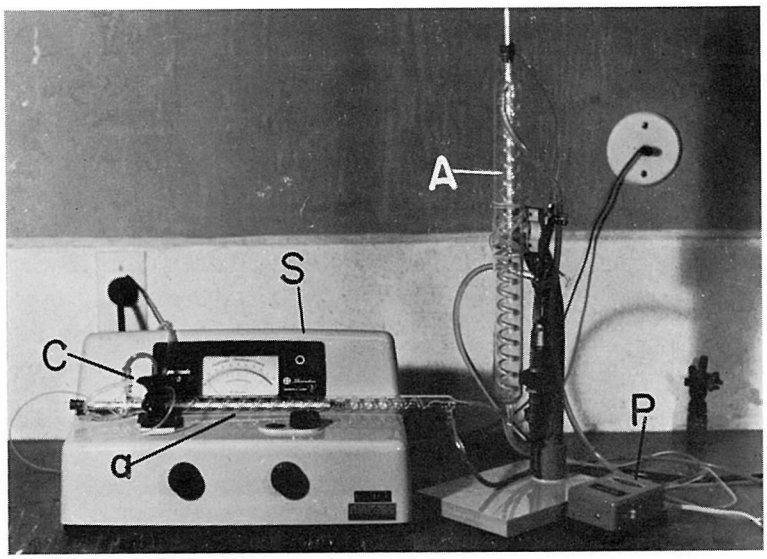

Fig. 1. General view of the assembly of apparatuses for the determination of serum pseudocholinesterase.

A : Automatic pipet to measure 1.0 ml solution at 37°C (See fig. 3).
a : A glass tube introducing warm air to the space surrounding the cuvette from the air pump (P).
C : Cuvette and its hoder (See fig. 2).
P : Air pump sending warm air (37°C) to the space surrounding the cuvette through a glass tube (a).
S : Spectrophotometer (Shimadzu-Bausch and Laumb).

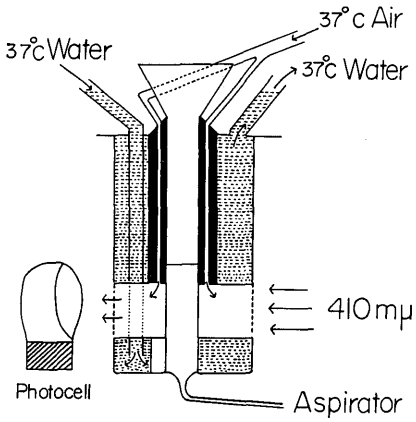

Fig. 2. Cuvette and its holder which enable to maintain constant temperature at 37°C, equipped with a water and air jacked of 37°C and a flow system to drain the colored solution.

2. Automatic pipets: (Fig. 3)

One (A) is an instrument which enables to measure 1.0 ml of substrate buffer solution exactly and rapidly at 37°C. This can be handled by the right hand only. Another (B) is a minipett (wihtout warming) which is able to measure 1.0 ml of chromogen buffer solution.

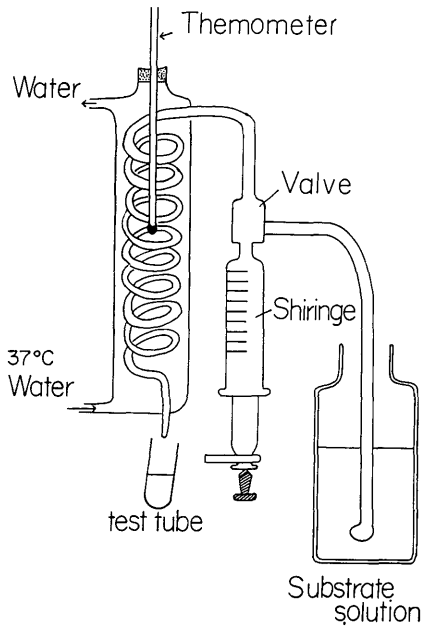

Fig. 3. Automatic pipet (A) to measure 1.0 ml solution at 37°C.

3. Stopwatch
4. Sanz ultramicropipet,[21] 20 μl
5. Test tube (7.0×1.2 cm)
6. Warm water bath (37°C)

Reagents

1. Tris-HCl buffer solution. pH 7.5.

Dissolve 6.05 g of Tris (hydroxymethyl) aminomethane in 100 0ml of distilled water. Adjust the pH of the solution to pH 7.5 by addition of an approprite amount (about 500 ml) of 50 mM HCl solution.

2. Chromogen buffer solution.

Dissolve 10 mg of DTNB in 100 ml of Tris-HCl buffer accurately.

3. Substrate buffer solution.

Dissolve 150 mg of butyrylthiocholine in 100 ml of Tris-HCl buffer.

Procedure

Calibration: Table I and (Fig. 4)

Prepare the 6 test tubes A, B, C, D, E, and Sc, and introduce the reagents as described in Table I. The absorbances of the solutions (37°C) B, C, D, and E are measured at 410 mμ in the specially designed Spectronic 20 colorimeter (Fig. I), setting the reading of the pointer to zero with solution A, which serves as the blank.

Table I. Preparation of standard solution for plotting a calibration.

Test tube	A	B	C	D	E	Sc
Mixed pooled serum (ul)	20	20	20	20	20	0
Chromogen buffer (ml)	0	0.1	0.2	0.3	0.4	1.0
Tris-HCl buffer (ml)	1.0	0.9	0.8	0.7	0.6	0
Substrate buffer (ml)	1.0	1.0	1.0	1.0	1.0	1.0

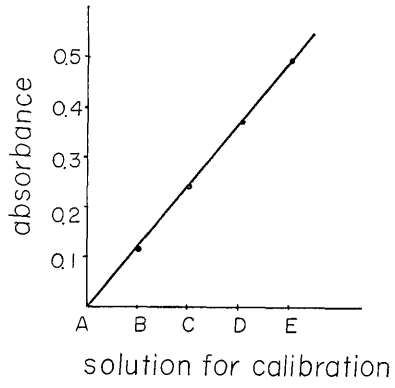

Fig. 4. Calibration curve.

Substrate check :

Sc solution (Table I) is checked everyday by measuring its absorbance with distilled water as the blank for reading zero at 410 mμ. If the absorbance is larger than that of solution C, the reagent solution can not be used because of apparent excessive autolysis of substrate (butyrylthiocholine).

Measurement :

1) Put 20 μl aliquots of sera in sample tubes individually.

2) Add 1.0 ml of chomogen buffer with an automatic pipet (B) to each of the sample tubes, mix, and warm in a 37°C water bath.

3) Take one of the sample tubes from the bath, introduce 1.0 ml of substrate buffer solution warmed at 37°C from the automatic pipet (A), mix quickly and transfer the mixture into the cuvette of a spectrophotometer (Spectronic 20).

4) Measure the time ($\triangle$ t), necessary for witnessing an increase of absorbance at 410 mμ by 0.10 in terms of second with a stopwatch exactly.

5) Discard the colored solution contained in the cuvette by aspiration system.

6) Repeat these procedure (3—5) for each of other sample tubes.

Calculation :

The cholinesterase activity is expressed in terms of micromoles sulfhydryl group liberated in 1 minute by 20 μl of plasma or serum.

The time $\triangle$ t required for yielding an increase of 0.10 in absorbance is compared with the calibration curve which has been prepared before-hand. This enables the reading of the concentration of 5-mercapto-2-nitrobenzoic acid which has been produced by the reaction of thiocholine enzymatically released with DTNB. The pseudocholinesterase activity is then calculated by the following equation.

$$\frac{0.1}{\triangle t} \times \frac{\text{d } \mu\text{M DTNB}}{S} \times 60 \quad (\mu\text{M}/1 \text{ min}/20 \mu\text{l}/37°\text{C})$$

where S refers to the absorbance of 5-mercapto-2-nitrobenzoic acid produced from dμM of DTNB. The term $\frac{\text{d } \mu\text{M DTNB}}{S}$ is easily obtained from the calibration curve. (Fig. 3)

For example, if a S value of 0.120 is read corresponding to 2.52×10^{-2} μM DTNB (referring to solution B) from the calibration curve,

$\frac{\text{d } \mu\text{M DTNB}}{S}$ will be $\frac{2.52 \times 10^{-2}}{0.120}$ equaling to 21×10^{-2}.

If $\triangle$ t is 30 seconds, the activity is calculated as $\frac{0.1}{30} \times 21 \times 10^{-2} \times 60$ or 4.2×10^{-2} μM/min/20 μl/ 37°C.

DISCUSSION

Several factors that may affect the accuracy and precision of the measurement of serum pseudocholinesterase activity were studied to evaluate the method described in this paper.

Increase in absorbance in relation to time (Fig. 5): The time-absorbance relationship in the coloration of this procedure was always linear over the range of increment of 0—0.3 irrespective of the activity (high, middle, and low) of the sera examined. In the present method concentration of substrate in the reagent solution was adjusted to the optimum range so that lineality of time–absorbance relationship might be obtained.

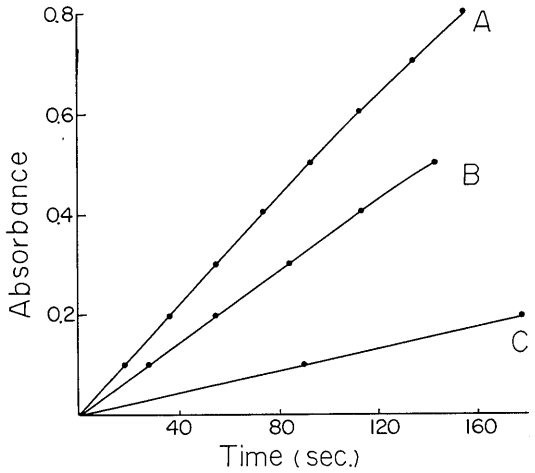

Fig. 5. I ncrease in absorbance with lapse of time (A, B and C refer to serum of high, middle and low pseudocholinesterase activity, respectively).

Temperature (Fig. 6): The temperature of cuvette was varied systematically from 15 to 60°C, in order to examine the relationship between temperature and apparent reading of activity. The experiment disclosed a dome-like curve with its summit (maximal reading of activity) at about 50°C, which was significantly higher than the so-called optimum temperature (45°C) for serum pseudocholinesterase activity propounded by other authors.[12)19)] The heating time for enzymatic activity is very short, being of the order of scores of seconds in our procedure, whereas in the previously proposed conventional methods the substrate solution is incubated for more than half an hour. Incubation for a long time at a temperature exceeding a certain limit (45°C) may cause spontaneous deterioration of the enzyme, and thus results in a temperature-activity curve with its summit dislocated to the side of lower temperature. As seen in Fig. 5 the slope of the curve is so steep around 37°C, especially in the case of serum

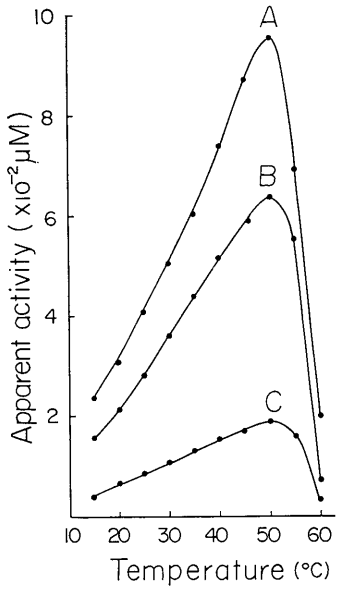

Fig. 6. Relation between temperature and reading of apparent activity in sera of high (A), middle (B) and low (C) Pseudocholinesterase level.

of high enzymatic activity, that even a slightest fluctuation of the temperature may influence the reading of enzymatic activity. Rigid control of the temperature of the cuvette is therefore essential. In our experience the cuvette system illustrated in Fig. 1 was entirely satisfactory for the maintainance of constant temperature.

pH of the medium (Fig. 7): The optimum pH of the medium for serum pseudocholinesterase was at 7.6. However, the pH of the reagent solution was made to be 7.5, taking the autolysis of the substrate (Fig. 5–D) into consideration.

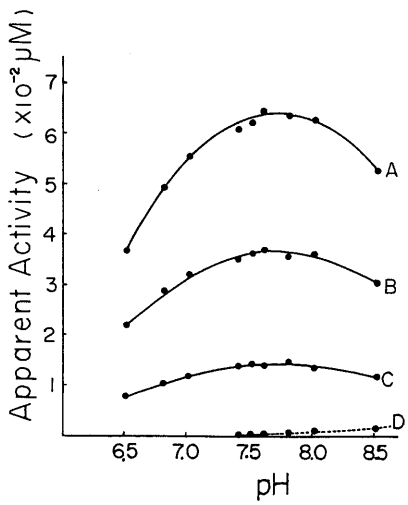

Fig. 7. Relation of the pH of the medium with the enzymatic activities, high (A) middle (B) and low (C) and with substrate autolysis (D).

Enzymatic activities in relation to amounts of serum (Fig. 8): Portions of pooled and mixed serum of moderate cholinesterase activity were measured serially from 0 to 100 μl in duplication and then analyzed for activity of pseudocholinesterase according to the procedure described in this paper. The results revealed a linear relationship of activity directly proportional to the amounts of enzyme over the range from 0 to 60 μl, which covers every possible activity encountered in routine clinical chemistry.

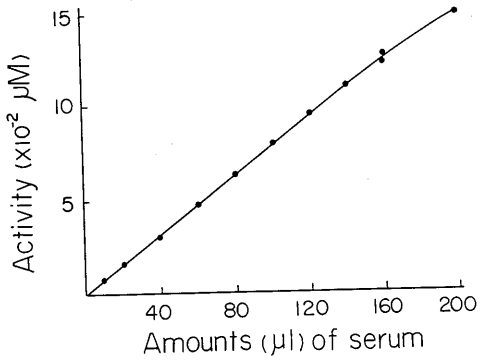

Fig. 8. Relation between the amounts of serum and their enzymatic activities.

Comparison of the present method with the conventional Sasaki's △pH method (Fig. 9): Sasaki's method is based on the principle of measuring colorimetrically the fall of the pH of the medium (△pH) caused by the enzymatically released acetic acid from the substrate (acetylcholine) with m-nitrophenol as indicator. One-hundred and twenty-eight comparisons were carried out for the period of 7 days. The correlation between them was satisfactory (coefficient of correlation was + 0.917) with day-to-day variation of as small as 2.0 %.

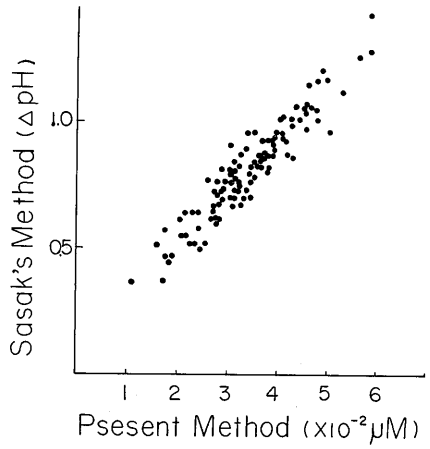

Fig. 9. Comparison of the present method with Sasaki's method.

Substrate : Butyrylthiocholine is not susceptible to hydrolysis by true cholinesterase contained in erythrocytes in contrast to acetylcholine, which is decomposed readily by either of the true and the pseudo cholinesterases. One of the experiments demonstrating such different aspect between butyrylthiocholine and acetylcholine is presented in Fig. 9. The activity of pseudocholinesterase in a pooled non-hemolysed serum was determined by Sasaki's method which employed acetylcholine and the present method which used butyrylthiocholine as substrate. Artificially hemolyzed sera were prepared by mixing the pooled serum with a fresh hemolysate containing intraerythrocytic true cholinesterase (Hb concentration 10 g/dl) in various proportions (10 : 90, 20 : 80, ..., 50 : 50). Their apparent cholinesterase activity, which is expressed in percentage of the activity of non-hemolyzed original serum, was measured by these two methods.

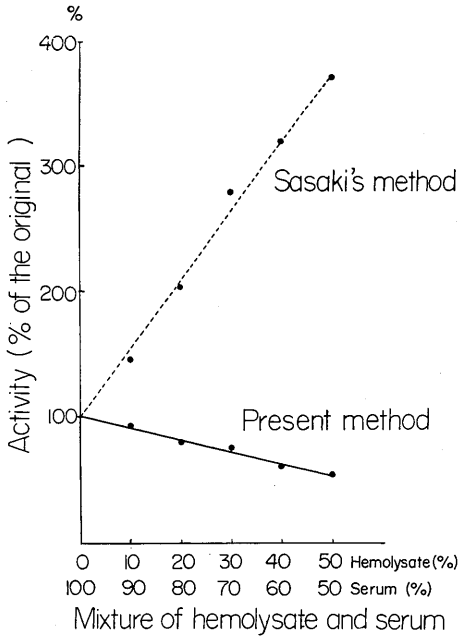

Fig. 10. Interference of the (true) cholinesterase originating from hemolysed erythrocytes in determination of serum pseudocholinesterase. Note that the estimation is not affected by hemolysis when butylthiocholine is used as substrate.

As seen in Fig. 10, it increased sharply with the increase in the degree of hemolysis (content of hemolysate) when it was measured by Sasaki's method (acetylcholine), whereas it decreased exactly in accordance with the degree of dilution effect with hemolysate when the present precedure (butyrylthiocholine) was employed. The apparent activity was in good agreement with that calculated

on the assumption that the hemolysate had no cholinesterase activity. This is interpreted as an evidence for that butyrylthiocholine is not hydrolysed by intraerythrocytic true cholinesterase. Acetylthiocholine is hydrolyzed by true cholinesterase as well as by pseudocholinesterase, though it is an analogue of butyrylthiocholine as thiocholinester.[19] Accordingly, it is thought that hemolysis does not interfere significantly with estimation of serum pseudocholinesterase activity when butyrylthiocholine is used as substrate. Butyrylthiocholine is superior to other substrates in stability. Moreover, the present method which employs butyrylthiocholine has an advantage of being checked for the substrate integrity everyday by the simple procedure described in "substrate-check".

Calibration curve: Calibration curve was constructed on the basis of the following principle. A series of different amounts of DTNB are brought into reaction with excess quantity of thiocholine which is liberated from a sufficient amount of butyrylthiocholine by enzyme action supplied by the pooled serum, in order to produce 5-mercapto-2-nitrobenzoic acid proportionally.

In our experience, calibration curve constructed with different amounts of glutathione, which is recommended by other authors,[16][17] was not reliable on account of day-to-day fluctuation and variation caused by difference in the lots of reagent samples.

Technical problems: The present procedure is a simple ultramicro method, which measures pseudocholinesterase activity with 20 μl of serum dispensing with the use of blank solution and inhibitor of enzyme whithin a time as short as one minute without incubation of substrate-serum mixture. The procedure is straight without transfer of substrate-serum mixture from a test tube to another. This is therefore free of estimation error caused by contamination. Sixty determinations can easily be carried out per hour. In addition, it will be adapted to a self-recording automatic apparatus to enhance its efficiency and convenience.

Precision: The day-to-day variation of the estimation by the present procedure with control serum did not exceed 2.0%.

The discrepancy in duplicate determinations was almost zero, being less than 2.0 %. The precision is therefore satisfactory for routine clinical chemistry.

SUMMARY AND CONCLUSION

A simple and rapid ultramicromethod for the determination of serum pseudocholinesterase with butyrylthiocholine as substrate was devised. Its principle consists in reading the initial velocity of the reaction of DTNB with thiocholine which is enzymatically released from butyrylthiocholine at 37°C. The production rate of the chromogenic substance (5-mercapto-2-nitrobenzoic acid), namely the

time (in seconds) necessary for causing an increment of 0.10 in absorbance is measured at 410 mμ in a photoelectric colorimeter equipped with a cuvette holder and a drain system warmed at constant temperature (37°C).

The method is suitable not only routine clinical chemistry but also for emergency clinic. It requires minimal equipment, specimens, pipetting and time, but has satisfactory precision and accuracy.

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