Arh. hig. rada, *19* (1968) 199.

DETERMINATION OF HUMAN PLASMA CHOLINESTERASE ACTIVITY BY ADAPTED ELLMAN'S METHOD

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(Received for publication March 19, 1968)

The spectrophotometric method described by Ellman and co-workers for determining erythrocyte and tissue cholinesterase activity was modified for measuring human plasma cholinesterase. The activity of plasma cholinesterase was determined in 108 healthy human subjects. A symmetric distribution of enzyme activity was observed with the arithmetic mean of the rate of substrate hydrolysis of 1.06 μmoles/min/ml and standard deviation of 0.23.

The activity of serum cholinesterase can be determined by various relatively simple and accurate methods (1, 2). Most of these methods which are applicable for evaluation of exposure to organophosphorus compounds are not satisfactory for measuring the degree of cholinesterase inhibition in persons exposed to carbamate insecticides. This is accounted for by the instability of carbamoylated cholinesterase causing a spontaneous reactivation of the inhibited enzyme (3, 4, 5) in the course of the measurement. The degree of influence of spontaneous reactivation depends upon experimental conditions of the particular method.

The spectrophotometric method with disulphide reagent described recently by *Ellman* and co-workers (6) for the determination of cholinesterase activity of erythrocytes and tissue homogenates provides adequate experimental conditions and yields reliable results. The method is based on the measurement of the enzyme activity by determining the rate of thiocholine formation due to enzyme hydrolysis of acetylthiocholine. Thiocholine reacts with 5,5-dithiobis(nitrobenzoic acid) (DTNB) producing a yellow anion. The intensity of the colour is measured spectrophotometrically. The production of the colour is based on the following two reactions:

$$\text{acetylthiocholine} \xrightarrow{\text{cholinesterase}} \text{thiocholine} + \text{acetate}$$

$$\text{thiocholine} + \text{DTNB} \longrightarrow \text{yellow anion}$$

MATERIALS AND METHOD

Reagents

Phosphate buffers 0.1 M, ph 7.4 and pH 7.0 were used.

Acetylthiocholine iodide was dissolved in redistilled water in concentrations ranging from 6×10^{-1} M to 6×10^{-3} M and kept refrigerated up to 14 days.

DTNB solution /5,5-dithiobis(2-nitrobenzoic acid)/ 0.01 M was prepared in 0.1 M phosphate buffer pH 7.0 containing 1.5 $NaHCO_3$/ml.

Method

Blood samples were obtained by a finger-prick. Samples were collected in glass capillaries as described by *Stubbs* and *Fales* (7). Plasma was separated by centrifuging the capillaries for 3–5 minutes at 2000 r. p. m.

100 μl DTNB solution was pipetted into a test tube and 3.0 μl phosphate buffer pH 7.4 was added. From a capillary tube 20 μl plasma was transferred into the test tube, the pipette being rinsed three times. The mixture was shaken and 50 μl substrate solution was added. After shaking, the contents of the test-tube were transferred into a photocell. The extinction was read at 412 mμ – the first reading being taken within one minute after the addition of the substrate, the second reading following exactly two minutes after the first.

The blank for each run consisted of the same reagents except plasma, and was prepared – and transferred to the photocell – before plasma was added to the experimental tube.

The assays were done at room temperature (22–25 °C). The final concentration of the thiol reagent was 3.2×10^{-4} M, while the concentrations of the substrate ranged from 1.0×10^{-2} M to 1.0×10^{-4} M.

Calculation

Cholinesterase activity is expressed as the change in extinction (ΔE) per minute, per ml plasma according to equation:

$$\frac{E_2 - E_1}{t_2 - t_1} \cdot 50 = \Delta E/\text{min/ml}$$

$E_2 - E_1$ is the difference between the second and the first reading taken at t_2 and t_1 minutes respectively. In our case $t_2 - t_1 = 2$.

Using the molar extinction coefficient of the yellow anion it is possible to express the enzyme activity in μmoles of substrate hydrolysed per minute, according to equation:

$$\mu\text{moles/min} = \frac{\Delta E}{\text{min}} \cdot \frac{\mathcal{V} \cdot 100}{E_M}$$

where ΔE/min is the change of extinction per minute, $\mathcal{V}$ is the volume of reaction mixture in ml and E_M is the molar extinction coefficient which is 13600 according to *Ellman* (8).

RESULTS AND DISCUSSION

Determination of experimental conditions

Effect of enzyme concentration. – A linear relationship between cholinesterase activity (expressed as ΔE/min) and the enzyme concentration (5–30 μl) was found (Fig. 1).

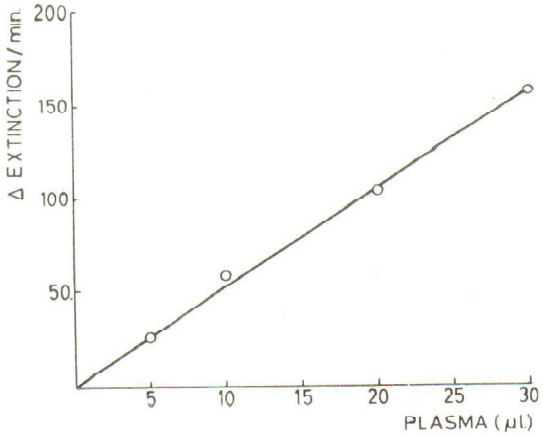

Fig. 1. *Relation of plasma cholinesterase activity to enzyme concentration. The acetylthiocholine concentration 1 × 10—³ M*

As shown in Fig. 2 the relationship between activity and time observed during the 6 minute reaction period was also found to be linear.

Effect of substrate concentration. – The enzyme activity was determined at different acetylthiocholine concentrations employing 20 μl plasma. The results indicate that the plasma cholinesterase activity is a linear function of time at each substrate concentration (Fig. 3), while the relation of the enzyme activity and the concentration of acetylthiocholine is represented as a curve (Fig. 4). This agrees with the results of other investigators (9, 10).

Normal plasma cholinesterase activity distribution

On the grounds of the above results optimum experimental conditions were chosen (20 μl plasma/3.15 μl reaction mixture, 1.0×10^{-3} M substrate, and 2 minute time of assay) and plasma cholinesterase activity was determined in 108 healthy persons.

Subjects were divided in 5 groups according to age as shown in Table 1.

No variations in cholinesterase activity were observed within the age groups studied, except in the youngest group (Fig. 5) with the smallest number of subjects, where one woman showed an extraordinary high

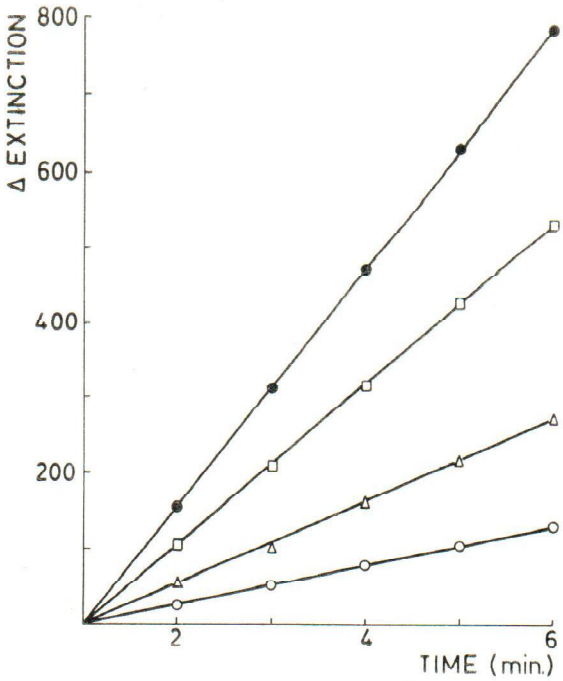

Fig. 2. *Plasma cholinesterase activity as a function of time of reaction with substrate* (1×10^{-3} *M*). *Quantity of plasma used:* (○) *5 μl,* (△) *10 μl,* (□) *20 μl and* (●) *30 μl*

Table 1

Age distribution of subjects

Age groups	Male	Female
Under 19	3	2
20 to 29	35	11
30 to 39	25	14
40 to 49	5	5
50 to 59	6	2
Total	74	34

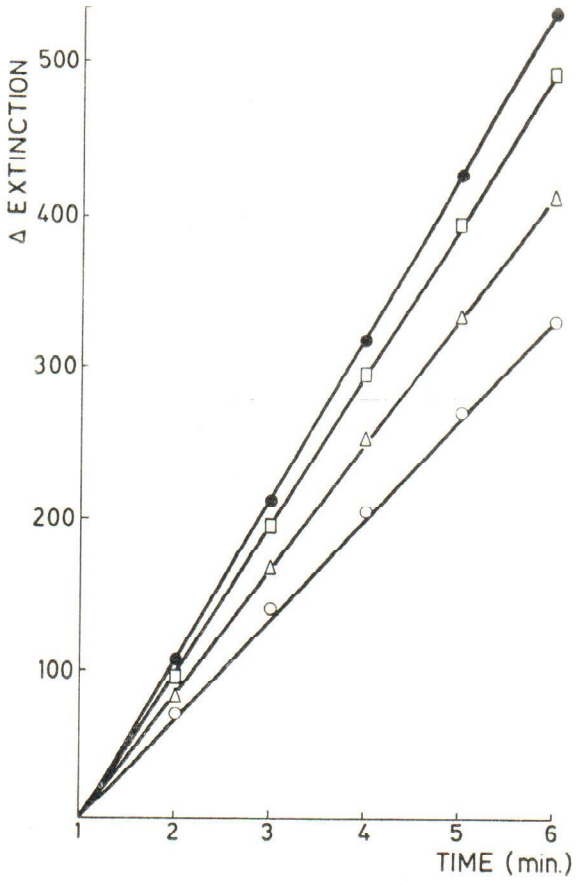

Fig. 3. *Effect of acetylthiocholine concentration on enzyme activity as a function of time. Quantity of plasma 20 µl, substrate concentration:* (○) 1×10^{-4} *M,* (△) 5×10^{-4} *M,* (□) 1×10^{-3} *and* (●) 1×10^{-2} *M*

plasma cholinesterase activity (1.94 µmoles/min/ml). In their study *Wetstone* and *LaMotta* (11) did not find age dependence variations either.

The frequency distribution curve of plasma cholinesterase activity of 108 individuals with no known previous exposure to anticholinesterase agents is shown in Fig. 6.

Symmetric distribution of enzyme activity was observed with the arithmetic mean of 1.06 µmoles/min/ml. The standard deviation of the mean was calculated to be 0.23 µmoles/min/ml.

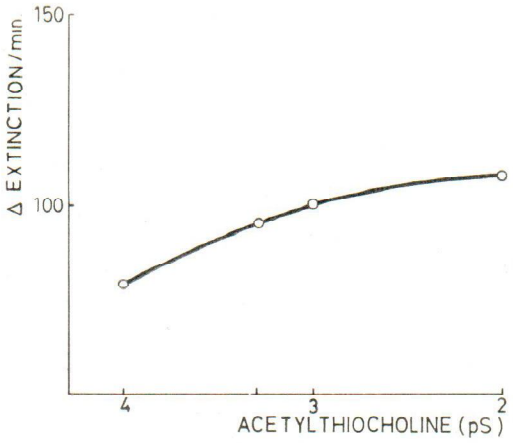

Fig. 4. *Effect of acetylthiocholine concentration on enzyme activity (20 µl of plasma)*

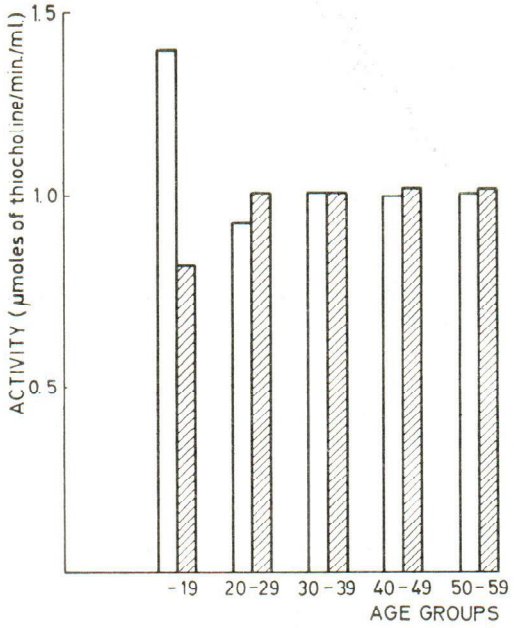

Fig. 5. *Plasma cholinesterase activity in relation to age and sex (□) female (■) male. The columns represent the mean value of enzyme activity of each group*

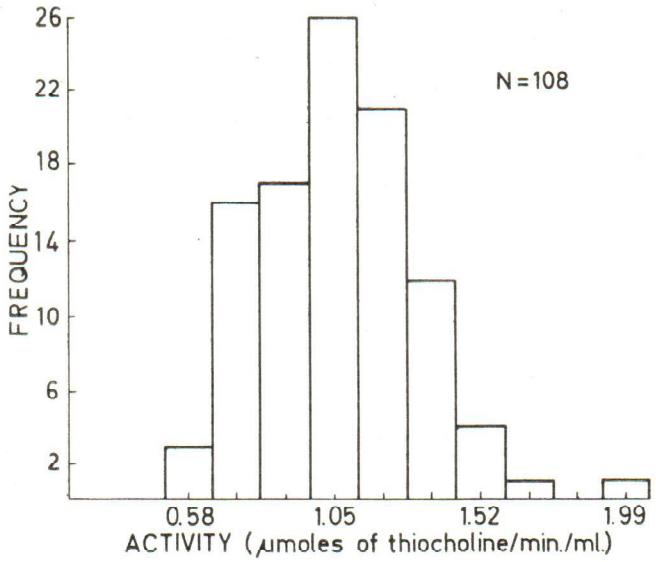

Fig. 6. *Distribution of plasma cholinesterase activity in 108 healthy persons*

The degree of variations expressed by a coefficient of variations of 21.7% agrees with the results of some other investigators who applying a similar method obtained the coefficient of 25.2% (10), 21.5% (12) and 16.7% (13).

Precision of the method was tested on a set of samples by two subsequent measurements of the activity of individual samples, the correlation between the two pairs of measurements being $r = 0.99$. Such a high correlation permits precise determination of the sample activity by only one measurement.

CONCLUSION

The method is characterized by 1. a relatively low substrate concentration (10^{-3} M — 10^{-4} M), 2, measurement of the initial activity of the enzyme, 3. a small amount of plasma necessary for one assay (20 μl), 4. a short time of measurement (2 minutes).

Owing to the simplicity of the analytical procedure the method is very convenient for estimation of human exposure to anticholinesterase poisons, carbamates in particular. Carbamoylated cholinesterase is relatively unstable and shows fast spontaneous reactivation. Due to short duration of the analytical procedure the carbamoylated cholinesterase reactivated in the course of the measurement is so small that may be neglected.

The method was used to determine normal plasma cholinesterase activities in 108 healthy subjects. The mean value was 1.06 ± 0.23 μmoles/min/ml. The precision of the method was tested on the same samples assessing the activity of a particular sample by two subsequent measurements. The correlation between 108 pairs of results was very high (r = 0.99).

With lesser modifications the method can be used as a reliable field method.

References

1. *Witter, R. F.:* Arch. Envir. Hlth, *6* (1963) 537.
2. *Simeon, V.:* Arh. hig. rada, *18* (1967) 383.
3. *Wilson, I. B., Hatch, m. A., Ginsburg, S.:* J. Biol. Chem., *235* (1960) 2312.
4. *Simeon, V., Reiner, E.:* V kongresni sastanak jugoslavenskih fiziologa, Sarajevo 1967, Zbornik kratkih sadržaja referata, 99.
5. *Reiner, E., Simeon-Rudolf, V.:* Biochem. J., *98* (1966) 501.
6. *Ellman, G. L., Courtney, K. D., Andres, V., Featherstone, R. M.:* Biochem. Pharmacol., *7* (1961) 88.
7. *Stubbs, J. L., Fales, J. T.:* Amer. J. med. Technol., *26* (1960) 25.
8. *Ellman, G. L.:* Arch. Biochem. Biophys., *74* (1958) 443.
9. *Garry, Phil. J., Routh, J. I.:* Clin. Chem., *11* (1965) 91.
10. *Humiston, G. C., Wright, G. J.:* Toxicol. Appl. Pharmacol., *10* (1967) 467.
11. *Wetstone, H. J., LaMotta R. V.:* Clin. Chem. *11* (1965) 653.
12. *Voss, G., Schuler, J.:* Bull. Environ. Cont. Toxicol., *2* (1967) 357.
13. *Weber, H.:* Deutsche Med. Wschr., *43* (1966) 1927.

Sadržaj

ODREĐIVANJE AKTIVNOSTI SERUMSKE KOLINESTERAZE LJUDI MODIFICIRANOM ELLMANOVOM METODOM

Spektrofotometrijska metoda po Ellmanu i suradnicima, 1961, adaptirana je za mjerenje aktivnosti kolinesterase plazme čovjeka. Glavna obilježja metode su ova: (1) koncentracija supstrata je relativno niska (10^{-3} M – 10^{-4} M); (2) metodom se mjeri početna aktivnost enzima; (3) za jedno određivanje potrebna je mala količina plazme (20 μl) i (4) vrijeme mjerenja je kratko (2 minute).

Zbog jednostavnosti analitičkog postupka metoda je vrlo prikladna za ocjenu ekspozicije ljudi eksponiranih antikolinesteraznim otrovima, naročito karbamatima. Karbamilirana kolinesteraza je relativno nestabilna, i brzo se spontano reaktivira. Zahvaljujući kratkom analitičkom postupku te metode karbamilirana kolinesteraza, koja se reaktivira u toku samog mjerenja, tako je malena da se može zanemariti.

Opisanom metodom određene su normalne aktivnosti kolinesteraze plazme ljudi u 108 zdravih osoba. Srednja vrijednost iznosila je 1.06 ± 0.23 μmola/min/ml. Pouzdanost metode testirana je na istim uzorcima odredivši aktivnost pojedinog uzorka sa dva uzastopna mjerenja. Korelacija između 108 parova rezultata bila je vrlo visoka (r = 0.99).

Uz manje adaptacije metoda se može korisno upotrijebiti i kao pouzdana terenska metoda.

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Jugoslavenske akademije znanosti i umjetnosti,
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Primljeno 19. III 1968.