CHROM. 5322

# A SENSITIVE GAS CHROMATOGRAPHIC METHOD FOR HUMAN CHOLINESTERASE DETERMINATION

M. F. CRANMER AND A. PEOPLES

*Perrine Primate Research Branch, Division of Pesticide Chemistry and Toxicology, Food and Drug Administration, Department of Health, Education, and Welfare, P. O. Box 490, Perrine, Fla. 33157 (U.S.A.)*

(Received February 8th, 1971)

SUMMARY

A rapid and sensitive method is described for the determination of cholinesterase. Plasma or red cells are incubated with 3,3-dimethylbutyl acetate as the substrate. The reaction product, 2,2-dimethyl butanol, is extracted into carbon disulfide and determined by GLC. The method is suitable for the analysis of samples that are quite low in enzyme activity.

## INTRODUCTION

There are many useful and accurate methods for measurement of acetyl-cholinesterase[1-3]. However, to measure the effects of inhibitors on the cholinesterase of plasma and red cells, a rapid, accurate method is needed that requires only a few microliters of sample.

In several methods[4-6], enzyme activity is assayed by using 3,3-dimethylbutyl acetate (DMBA) as substrate. This paper presents a method in which the enzyme is incubated with the DMBA substrate and a buffer for a specified time (usually 30 min). The reaction is then stopped and the reaction product, 2,2-dimethyl-1-butanol (DMB) is extracted into carbon disulfide and measured by gas chromatography (GC). An additional advantage is that the $CS_2$ layer can be analyzed immediately or stored in the refrigerator for later analysis.

## EXPERIMENTAL

### *Apparatus*

A MicroTek MT-220 gas chromatograph equipped with a single hydrogen flame ionization detector and a Westronics dual pen recorder was used in this study.

The incubation of the reaction was carried out with a Thermolyne Dri-Bath permanently set at 37°. Components of the assay were added with Oxford pipettors and the reaction was carried out in a 5-ml glass-stoppered test tube. Blood samples were introduced into the reaction vessel either with Oxford pipettors or contained in heparinized capillary tubes.

*Reagents*

Reagents were obtained from the following sources: 3,3-dimethylbutyl acetate and 2,2-dimethylbutanol, K&K Laboratories; true cholinesterase, Type I, and pseudocholinesterase, Type IV, Sigma Chemical Co.; carbon disulfide, SpectrAR grade, Mallinckrodt Chemical Works; paraoxon, Farben Fabriken-Bayer-A.G.; diisopropylfluorophosphate, Aldrich Chemical Co.; Tween 20® emulsifier, Biscayne Chemical Laboratories; and control serum (Special Clinical Chemistry Control Serum), Hyland Laboratories. All other reagents were ACS grade.

*Preparation of reagents*

A stock solution of buffer is prepared by dissolving 44.73 g of potassium chloride, 4.12 g of sodium barbital, and 0.55 g of potassium monobasic phosphate in 200 ml of water[7]. The working solution is prepared by adding 20 ml of stock solution to 75 ml of water, adjusting the pH to 8.0 with 0.1 *N* HCl, and diluting the solution to a final volume of 100 ml with water.

To remove butanol impurities from DMBA, 5 parts of DMBA are mixed with 1 part of acetic anhydride. The mixture is kept at 37° for 24 h and then washed once with water to remove the acetic anhydride and impurities. A 0.2 *M* solution is prepared by diluting the DMBA with working buffer and adding emulsifier to a concentration of 0.2 %[8]. The pH is then adjusted to pH 8.0 with sodium hydroxide.

For gas chromatographic standards, DMB solutions containing 50, 100, 150, and 200 ng/$\mu$l are prepared in water containing 0.1 % emulsifier. 2 ml of $CS_2$ are added to 2 ml of each standard solution, and the mixtures are shaken well and centrifuged. The top layer is discarded and an appropriate volume of the $CS_2$ layer is injected into the gas chromatograph. Standard curves are prepared by plotting DMB concentration *vs.* peak height or peak area.

Concentrated formic acid (88 %) is diluted 1:1 with distilled water.

*Chromatography*

Column: a glass U-tube, 1/4 in. × 6 ft., was packed with Johns-Manville Chromosorb 101, mesh size 80–100, and conditioned overnight at 250°. Instrument conditions: column oven, 215°; detector, 265°; inlet, 245°; air, 300 ml/min; hydrogen, 20 ml/min; nitrogen, 40 ml/min.

*Procedure*

*Pseudocholinesterase.* To 1 ml of working buffer in a 5-ml ground glass-stoppered test tube, add 20 $\mu$l of plasma and warm to 37°. Add 0.2 ml of the 0.2 *M* DMBA solution and incubate for 30 min at 37°. Carry a blank, containing all reagents except plasma, throughout the procedure. After 30 min, stop the reaction with 0.1 ml of 44 % formic acid and then add 2 ml of $CS_2$. Shake the tube vigorously and centrifuge, and remove the top aqueous layer by aspiration. Inject an appropriate volume of the $CS_2$ into the gas chromatograph for DMB determination.

*True cholinesterase.* To 0.2 ml of water in a 5-ml ground glass-stoppered test tube, add 20 $\mu$l of packed red cells. Allow the cells to hemolyze, then add 0.5 ml of working buffer and warm to 37°. Proceed as in the determination for pseudocholinesterase, carrying through the procedure a blank containing all reagents except red cells.

*Calculation*

Compare the DMB concentration of the unknown samples, after subtracting the reagent blank, with standard curves determined each day with $CS_2$ extracts of the aqueous DMB standards. Calculate the results as $\mu$moles of DMBA hydrolyzed per ml of plasma or red cells per min as follows:

$$\frac{\mu\text{g DMB produced}}{\text{time of assay} \times \text{volume of plasma or red cells} \times 102\ \mu\text{g}/\mu\text{mole}}$$

## RESULTS AND DISCUSSION

The pH of the reaction solution used for the more common substrates, *i.e.*, acetylcholine perchlorate and butyrylcholine chloride, has been 8.0. The optimum pH for the commercial pseudocholinesterase and the cholinesterases in Hyland control serum, human plasma, and red cells with DMBA as a substrate is between 7.5 and 8.0 (Fig. 1). A pH of 8.0 was selected as the most appropriate pH for the GLC method.

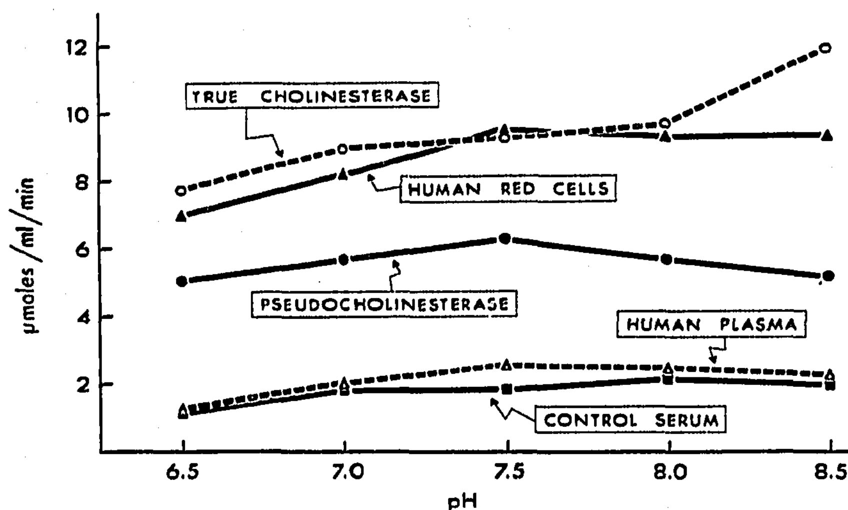


Fig. 1. pH effect on cholinesterase assay with 3,3-dimethyl butyl acetate as substrate.

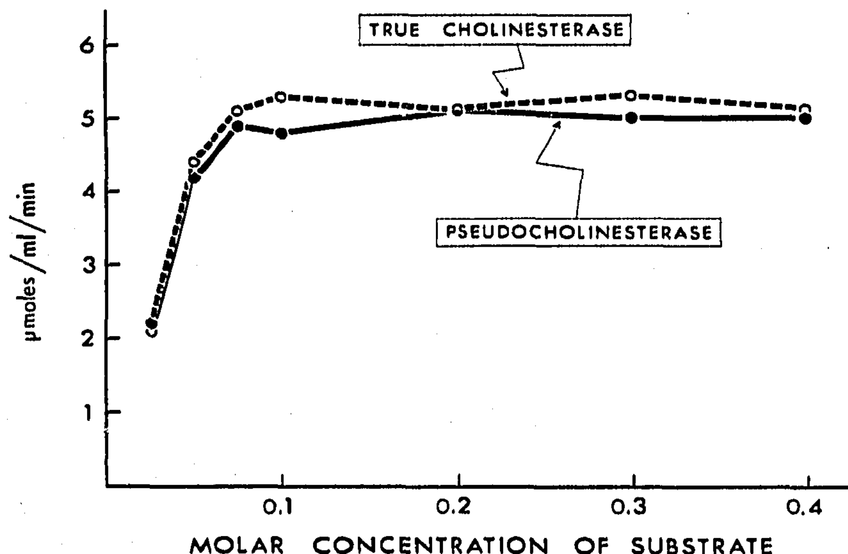


Fig. 2. Effect of substrate concentration on cholinesterase activity.

Fig. 2 shows the effect of substrate concentration on enzyme activity. At a DMBA concentration of 0.1 $M$ the enzyme has reached peak activity; increased concentration of substrate does not inhibit either the true cholinesterase or pseudocholinesterase. A substrate concentration of 0.2 $M$ was chosen to assure excess substrate during the entire reaction.

Varying dilutions of the commercial true cholinesterase and pseudocholinesterase were incubated with DMBA and the results were plotted as µmoles of DMB formed per ml of enzyme per min *vs.* relative concentration of enzyme present. Fig. 3 demonstrates that the sensitivity of the method is sufficient to yield linear results for enzyme activities down to 0.2 µmole/ml/min. As shown in Fig. 4, the reaction is linear when incubated for up to 50 min.

For the recovery studies, DMB was added to buffer and substrate in µg quantities so that the pH was not altered. After a 30-min incubation, formic acid and $CS_2$ were added as in the usual procedure. Table I shows that recovery of DMB from the $CS_2$ layer varied from 98 to 106% over the concentration range examined.

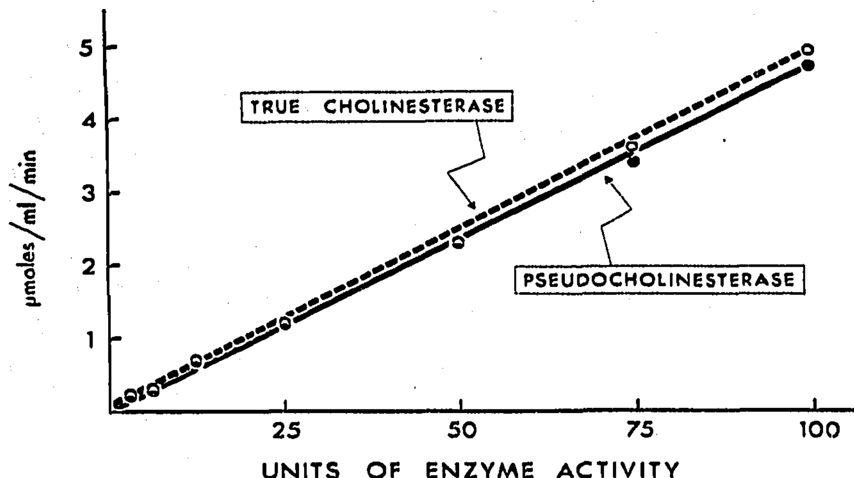


Fig. 3. Effect of enzyme concentration in the cholinesterase assay.

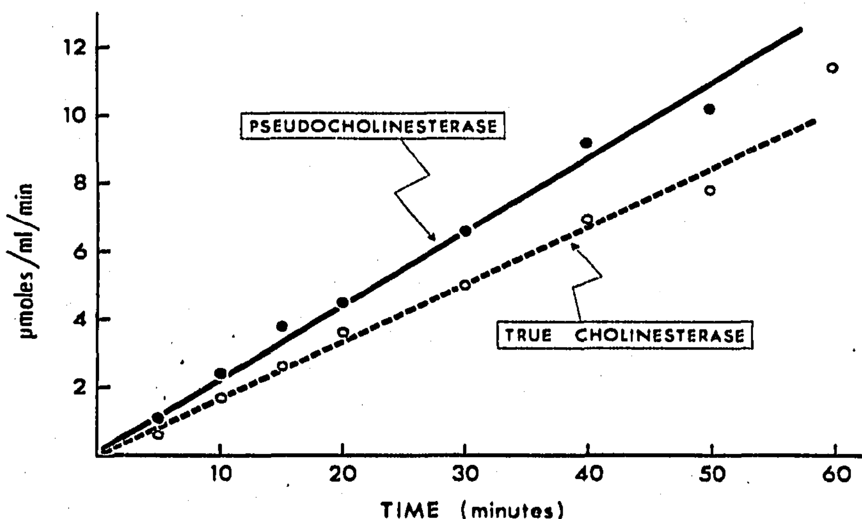


Fig. 4. Linearity of cholinesterase reaction with DMBA.

The reproducibility of the method is shown in Table II. Results of replicate analyses with the commercial true cholinesterase ranged from 5.4 to 5.6 μmoles/ml/min with a mean of 5.5 and a standard deviation of 0.08. Six analyses of the commercial pseudocholinesterase gave a range of 5.7 to 6.3 μmoles/ml/min with a mean of 6.0 and a standard deviation of 0.20.

Table III shows the results obtained with the present method on blood samples of the general population. Analyses of blood samples of 25 males and females gave a

TABLE I

RECOVERY OF 2,2-DIMETHYLBUTANOL FROM REACTION MIXTURE

| *2,2-Dimethylbutanol* | | *Recovery (%)* |
|---|---|---|
| *Added (μg)* | *Recovered (μg)* | |
| 102 | 102 | 100 |
| 102 | 102 | 100 |
| 204 | 214 | 105 |
| 204 | 200 | 98 |
| 408 | 435 | 106 |
| 408 | 410 | 100 |

TABLE II

REPLICATE ANALYSES OF SINGLE SAMPLES

| *Sample* | *μmoles of 3,3-DMBA hydrolyzed/ml/min* | |
|---|---|---|
| | *True cholinesterase* | *Pseudo-cholinesterase* |
| 1 | 5.4 | 6.3 |
| 2 | 5.4 | 6.1 |
| 3 | 5.4 | 6.0 |
| 4 | 5.4 | 5.9 |
| 5 | 5.5 | 5.7 |
| 6 | 5.6 | 5.9 |
| Mean | 5.45 | 5.98 |
| Standard deviation | 0.08 | 0.20 |

TABLE III

CHOLINESTERASE VALUES OF GENERAL POPULATION

| *Sample* | *Number of subjects* | *Mean 3,3-DMBA (μmoles/ml/min)* | *Standard deviation* | *Observed range (μmoles/ml/min)* |
|---|---|---|---|---|
| Plasma, males | 18 | 3.4 | 0.6 | 2.5– 4.6 |
| Plasma, females | 7 | 2.9 | 0.5 | 2.1– 3.7 |
| Plasma, combined results | 25 | 3.2 | 0.6 | 2.1– 4.6 |
| RBC, males | 18 | 9.8 | 0.9 | 8.2–11.8 |
| RBC, females | 7 | 10.0 | 0.6 | 9.2–10.9 |
| RBC, combined results | 25 | 9.8 | 0.8 | 8.2–11.8 |

TABLE IV

COMPARISON OF GLC AND pH STAT DETERMINATION OF CHOLINESTERASE INHIBITION BY PARAOXON AND DIISOPROPYLFLUOROPHOSPHATE

| *Paraoxon* (*μmoles*) | *Enzyme units* | *% Inhibition* | | *Diisopropyl-fluorophosphate* (*μmoles*) | *Enzyme units* | *% Inhibition* | |
|---|---|---|---|---|---|---|---|
| | | *GLC* | *pH Stat* | | | *GLC* | *pH Stat* |
| *True cholinesterase* | | | | | | | |
| $5 \times 10^{-5}$ | 6.3 | 98 | 98 | $2 \times 10^{-4}$ | 3.1 | 87 | 91 |
| $2.5 \times 10^{-5}$ | 6.3 | 82 | 84 | $1 \times 10^{-4}$ | 3.1 | 61 | 65 |
| $1.2 \times 10^{-5}$ | 6.3 | 51 | 48 | $5 \times 10^{-5}$ | 3.1 | 38 | 35 |
| $6 \times 10^{-6}$ | 6.3 | 26 | 24 | $2.5 \times 10^{-5}$ | 3.1 | 18 | 17 |
| $r^a$ | | 0.9270 | 0.9190 | $r$ | | 0.9373 | 0.9718 |
| *Pseudocholinesterase* | | | | | | | |
| $4 \times 10^{-4}$ | 45 | 85 | 89 | $4 \times 10^{-4}$ | 22 | 74 | 79 |
| $2 \times 10^{-4}$ | 45 | 51 | 44 | $2 \times 10^{-4}$ | 22 | 38 | 41 |
| $1 \times 10^{-4}$ | 45 | 29 | 27 | $1 \times 10^{-4}$ | 22 | 23 | 19 |
| $5 \times 10^{-5}$ | 45 | 17 | 19 | $5 \times 10^{-5}$ | 22 | 9 | 10 |
| $r$ | | 0.9970 | 0.9971 | $r$ | 22 | 0.9972 | 0.9995 |

[a] Correlation coefficient between cholinesterase inhibition and concentration of inhibitor.

range of 2.1 to 4.6 μmoles/ml/min for plasma cholinestrase, with a mean of 3.2 and a standard deviation of 0.6; for the red cell cholinesterase, the range was 8.2 to 11.8 with a mean of 9.8 and a standard deviation of 0.8. The relationship between the cholinesterase activities of human plasma and red cells as mesaured by GLC is similar but not identical to the relative activities as measured by the pH Stat method[2] (red cells 11.1–16.0; plasma 3.6–6.8).

For the inhibitor studies, the commercial enzymes were incubated at 37° with various concentrations of freshly prepared paraoxon or diisopropylfluorophosphate. At the end of 2 h, cholinesterase activity was determined; results are shown in Table IV. Absolute values for cholinesterase activity obtained by the pH Stat method[2] and this methods were not identical; however, the percent inhibition caused by the compounds compared very favorably, demonstrating a correlation between the two methods.

The cholinesterase values obtained by using Oxford pipettors were compared with those obtained by using the capillary tube technique[9]. The blood was collected in heparinized capillary tubes and spun in a micro hematocrit centrifuge. A 20-mm section containing plasma or red cells was cut from the capillary tubes and the entire section was added to the reaction vessel. The mean plasma pseudocholinesterase value with the capillary tube technique was 3.8 μmoles/ml/min (range 3.4–4.1; S.E. 0.09) and the mean red cell cholinesterase value was 14.1 (range 13.6–14.9; S.E. 0.15). The corresponding values obtained by using the Oxford pipettor were 3.5 and 14.0.

DMBA has been shown to be a suitable substrate for both true cholinesterase and pseudocholinesterase[4–6]. It is superior to acetylcholine and other commonly used substrates because its spontaneous decomposition and hydrolysis at the reaction pH are very low; in addition, both DMBA and DMB have good chromatographic qualities with Johns-Manville Chromosorb 101. DMBA has a retention time of about 6 min and can be used as an indicator of the presence of excess substrate at the end of

the reaction time. DMB, under the GC conditions described, has a retention time of approximately 4 min (Fig. 5).

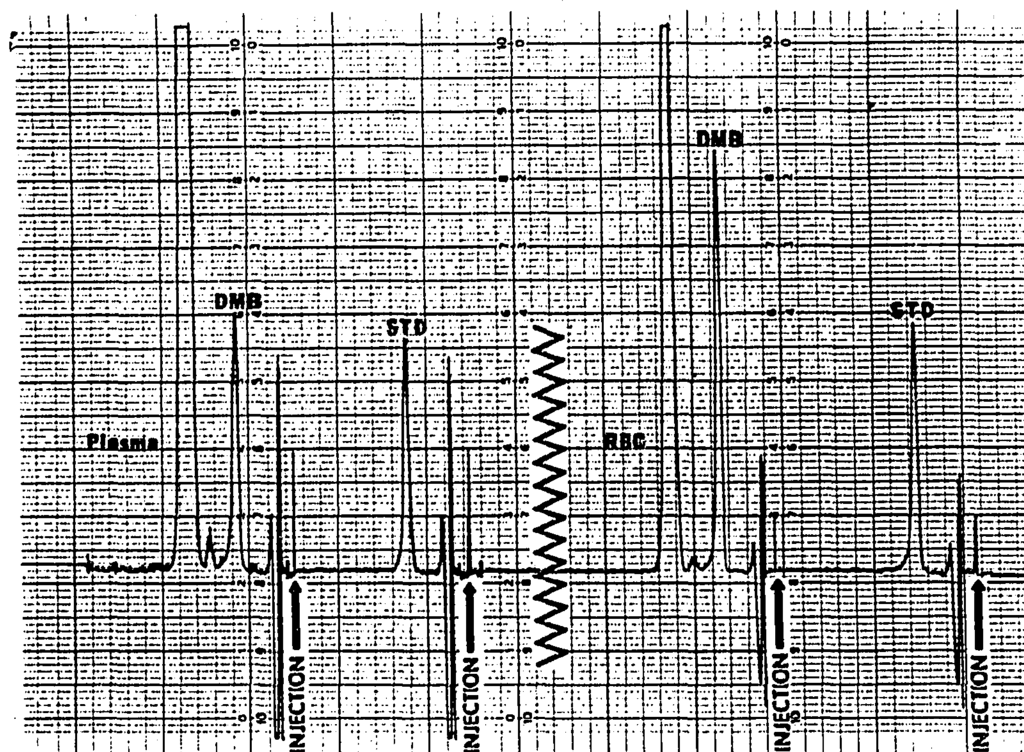


Fig. 5. Gas chromatogram of $CS_2$ layers from plasma and red cell cholinesterase assays.

Commercially available DMBA contains a small amount of DMB which interferes with the sensitivity of the method at very low activity. The reagent blank, also an indication of non-enzymatic hydrolysis of substrate, can be decreased by reacting the DMB with acetic anhydride to form DMBA. We found that the substrate remained stable at room temperature for about 2 months.

This method has been applied only to strong inhibitors of cholinesterase and not to reversible inhibitors.

## REFERENCES


1 H. O. Michel, *J. Lab. Clin. Med.*, 34 (1949) 1564.
2 D. P. Nabb and F. Whitfield, *Arch. Environ. Health*, 15 (1967) 147.
3 E. R. Larson, *Microelectric Method for Blood Cholinesterase Determination*, Division of Laboratories, California State Department of Public Health, 1957.
4 D. H. Adams, *Biochim. Biophys. Acta*, 3 (1949) 1.
5 D. H. Adams and V. P. Whittaker, *Biochim. Biophys. Acta*, 3 (1949) 358.
6 L. A. Mounter and V. P. Whittaker, *Biochem. J.*, 47 (1950) 525.
7 R. J. Henry, *Clinical Chemistry: Principles and Techniques*, Hoeber Medical Division, New York, 1964, p. 494.
8 A. Todrick, K. P. Fellowes and J. P. Rutland, *Biochem. J.*, 48 (1951) 360.
9 F. D. Aldrich, G. F. Walker and C. A. Patase, *Arch. Environ. Health*, 19 (1969) 617.