

### 30. THE DETERMINATION OF HUMAN ERYTHROCYTE ACETYLCHOLINESTERASE (ERY-ACHE) IN A MODIFIED ELLMAN ASSAY

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#### ABSTRACT

The colorimetric Ellman method is the most commonly used assay for the determination of human Ery-AChE in occupational health screening and therapeutic monitoring of organophosphate poisoned patients. Nevertheless, the procedure is still in an experimental stage, and no certified method is available. Major confounding factors are the high hemoglobin absorption, the changes of methemoglobin absorption in the presence of detergents (particularly pronounced in lyophilized samples), the thermochromic shifts of the indicator absorbance, the interference of butyrylcholinesterase, and the appropriate sample storage when an anticholinesterase and reactivator are present. Here, we present a modified approach that requires only low-tech laboratory equipment (filter photometer with mercury lamp) and gives reliable results down to 3% residual activity. To increase the signal-to-noise ratio, the color development is measured at 436 nm, the acetylthiocholine concentration is reduced to 0.45 mM, and the pH is lowered to 7.4. Hemolysis is achieved by Triton X-100, with its final concentration not exceeding 0.01%. Ery-AChE activity is referred to the hemoglobin content, determined as cyanmethemoglobin at 546 nm. Butyrylcholinesterase activity is inhibited by 20  $\mu$ M ethopropazine. The within-run precision was 1 and 5% in native and inhibited (95%) samples. When diluted 1:100 and frozen at -20°C, paraoxon-inhibited samples were stable over 1 month even in the presence of 10  $\mu$ M obidoxime.

#### INTRODUCTION

The determination of acetylcholinesterase (AChE, EC 3.1.1.7) activity is important in 1) monitoring and studying of exposure to pesticides (1) and chemical warfare agents (2); 2) therapeutic monitoring of organophosphate poisoned patients (3); and 3) titrating the anticholinesterase dosage used in Alzheimer's disease (4).

While the most interesting muscular and neuronal AChE is not accessible to direct measurement, Ery-AChE can easily be obtained. This source is regarded to be a reliable surrogate marker (5), because of the structural and functional similarities due to the common genetic origin of the catalytic subunit (6).

A variety of methods (electrometry, pH-stat, tintometry, radiometry and colorimetry) have been developed, and each method has its distinct advocates (7; 8). The most widespread method is based on the hydrolysis of acetylthiocholine. The assay introduced by Ellman in 1961 (9) makes use of the thiocholine-mediated cleavage of the chromogenic disulfide DTNB. Although the method is rather rapid, simple, and cheap, the Ellman method has its limitations because the peak absorption around 412 nm of the colored indicator TNB<sup>-</sup> coincides with the Soret band of mammalian hemoglobins. Moreover, various side reactions may falsify the determination of Ery-AChE activity. For these reasons we felt it necessary to modify the original Ellman method and to develop a robust procedure for estimating AChE activity in whole blood samples.

The method was optimized for human blood to also allow the detection of small activities of Ery-AChE in intoxicated patients. Hence, major efforts have been undertaken to reduce annoying side-effects and to optimize the signal-to-noise ratio. To this end, we lowered the reaction pH to 7.4 instead of 8.0 and reduced the substrate concentration from 1.0 to 0.45 mM, i.e. 5 x  $K_m$ , resulting in 83%  $V_{max}$ . Thereby, the blank reaction was reduced to about 1% of normal Ery-AChE activity, which allows a detection limit of 3% of normal (10).

#### *Selection of a suitable wavelength for the Ellman assay*

The interference of hemoglobin absorption can be circumvented by measuring TNB<sup>-</sup> outside its absorbance maximum. We selected 436 nm wavelength, since this wavelength is available in simple filter photometers equipped with mercury lamps. Thereby exact reproducibility of wavelength and band width are guaranteed without the need of a sophisticated spectrophotometer that would be a prerequisite if absorbance measurements are undertaken at a steep flank. At 436 nm the hemoglobin absorption is reduced to one fourth compared to 412 nm, while the indicator absorption is still 80% of its maximum. Thus, the signal-to-noise ratio is increased by a factor of three (10).

#### *Minimizing of side reactions*

Background changes of the sample matrix are of major concern. Thiol groups in the blood sample, most of all in glutathione, hemoglobin, and albumin, react with DTNB at widely varying rates, also differing among species.

Because these reactions are complete in 5-6 min in human blood samples at 0.3 mM DTNB, pH 7.4 and 37°C, we have adopted a blank reaction period of 10 min (10). After this time also the temperature of the cuvette is equilibrated to 37°C, which improves the assay reproducibility. A reaction temperature of 37°C was chosen, because this temperature is generally recommended when determining human enzymes. Moreover, adjustment to this temperature usually does not require an additional cooling device (opposed to 25°C reaction temperature). This aspect may be most important in countries with hot climate.

If the sample contains methemoglobin, particularly high in frozen samples or in lyophilized specimens that may be used as standards, slow absorption change of this pigment is observed when detergents are present in order to facilitate hemolysis (10). Hence we keep the Triton X-100 concentration in the assay very low (final concentration 0.01%).

Last but not least, butyrylcholinesterase (BChE, EC 3.1.1.8), particularly in human plasma, markedly contributes to acetylthiocholine hydrolysis. Using packed or washed red cells, or inclusion of “selective” BChE inhibitors, are the most common means to tackle this problem (2). Since we are working with frozen and hence hemolysed samples, procedures to physically remove BChE are inappropriate. Hence, we include ethopropazine as a quite selective BChE inhibitor (11). At a final concentration of 20 µM, BChE is inhibited by 97% while AChE is reduced by approx. 5% (10). We regard this small, but reproducible effect insignificant in view of the advantages to preserve the activity in frozen samples.

#### *Sample storage*

Inadequate sample storage is another factor that may confound AChE determination. While the high stability of native AChE usually does not pose major problems, the presence of anticholinesterases and/or reactivators does. For example, paraoxon-inhibited Ery-AChE shows a reactivation *ex vivo* with a half-time of some min at therapeutic oxime concentrations (12). To obtain reliable results, such a reaction has to be stopped immediately after blood sampling.

In order to minimize reactions between AChE, inhibitor and reactivator, immediate dilution of whole blood samples after withdrawal was considered a convenient method, feasible also in a busy emergency room. An approx. 1:100 dilution of whole blood in the assay buffer slows down bimolecular reactions by a factor of 10.000. The samples can be kept in a freezer at -20°C, allowing transportation, storage, and analysis up to 1 month later without significant changes. To correct for dilution errors, the enzyme activity is referred to the hemoglobin content (13; 14), measured as cyanomethemoglobin by a modified Zijlstra method (15) at 546 nm (another mercury emission line). Care is to be taken to avoid cyanide loss in the transformation solution which can be minimized (10) by the addition of sodium bicarbonate, pH 8.8, instead of the primary phosphate as used in the original Zijlstra method (pH 7.3). All these considerations have led to the following SOPs (16).

## **MATERIALS AND METHODS**

### *SOP Sample Handling*

Mix 0.2 ml (preferably taken with an insulin syringe) of venous blood (EDTA or heparin) immediately with 20 ml diluting reagent and store in a freezer at -20°C until shipment (dry ice) or analysis. Ideally, the diluting reagent is composed of 0.1 M sodium phosphate buffer, pH 7.4 containing 0.03% Triton X-100 to facilitate complete hemolysis. We use buffer-containing vials prepared in advance (Super polyethylene vials® for liquid scintillation counting, Packard) that are stored in the refrigerator. If need be, saline can be used instead as diluting reagent. The samples are thawed by gentle shaking the vials in cold water (conveniently in a gyrotory water bath shaker) and kept on ice until analysis.

### *SOP Determination of AChE Activity*

Mix in polystyrene cuvettes:	final conc. (mM)
2.000 mL sodium phosphate buffer (0.1 M; pH 7.4)	100
0.100 mL DTNB (10 mM)	0.3
0.010 mL ethopropazine (6 mM)	0.02
1.000 mL hemolysate (whole blood 1:100)	
Equilibrate at 37°C for 10 min, then add:	
0.050 mL acetylthiocholine (28.4 mM)	0.45
Record color development for 3 min at 436 nm	
(e.g. in a filter photometer at 1.0 AUFS; $\square = 10.6 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ ).	
Correct for spontaneous substrate hydrolysis (substitute hemolysate by buffer).	
Typical readings: Hemolysate	170 mE/min
Blank	2 mE/min

### *SOP Total hemoglobin*

Mix 1.00 mL hemolysate (whole blood 1:100) with 1.00 mL modified Zijlstra reagent and incubate for 10 min at room temperature.

Read extinction at 546 nm (room temperature or 37°C)

(e.g. in a filter photometer at 1.0 AUFS;  $\epsilon = 10.8 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ ).

Typical reading: 0.48 E; corresponding to 44  $\mu\text{M}$  hemoglobin iron.

For calculation of the specific activity of Ery-AChE, presented as U/ $\mu\text{mol}$  Hb(Fe), calculate the quotient of AChE activity ( $\mu\text{M}/\text{min}$ ) and  $\mu\text{M}$  Hb as obtained from the measurements in hemolysate (whole blood 1:100) and multiply with 1.58 to correct for different dilutions in determining hemoglobin and AChE.

Typical activity of normal Ery-AChE: 0.6 U/ $\mu\text{mol}$  Hb(Fe).

By multiplying this value with 62 one gets the dimension U/g hemoglobin.

## RESULTS

### *Assay linearity*

The linearity of the assay response to different enzyme activities was tested in the presence of the same matrix concentration. This was achieved by mixing inhibited AChE with various proportions of active enzyme. As shown in Fig. 1 the correlation was linear and intersected the y-axis at a blank rate of roughly 2 mE/min. A linear correlation was found between AChE activity and hemoglobin content when the same sample of whole blood was diluted at 1:50 to 1:200 (Fig. 2). Hence, exact dilution of freshly withdrawn blood is not critical. The specific activity in these samples averaged 0.623 U/ $\mu\text{mol}$  Hb at a CV of 1.26%.

### *Within-run precision*

Repetitive measurements of native samples gave excellent reproducibility (Table 1). A comparable within-run precision was obtained with blood samples in the presence of paraoxon-ethyl, paraoxon-methyl and obidoxime at its therapeutic concentration of 10  $\mu\text{M}$  (3), resulting in inhibition between 10 and 94% of control.

### *Between-run precision*

The determination of AChE activities in a native sample over 5 consecutive days resulted in acceptable reproducibility with a CV of 3% (Table 2). Repetitive freezing and thawing of whole blood dilutions over 3 days had no effect on AChE activity (CV 1.35%) or hemoglobin content (CV 0.70%).

### *AChE stability*

AChE activity in diluted, frozen whole blood samples was stable for at least 7 days and decreased slowly to  $91.7 \pm 1.8\%$  within 5 weeks. The inhibition brought about by paraoxon-ethyl or paraoxon-methyl remained constant for a month even in the presence of free inhibitor and obidoxime (Fig. 3).

## CONCLUSIONS

The data presented show that reliable measurements of Ery-AChE activity can be easily performed with low-tech equipment and under conditions that are likely to be found at the scene where intoxications are to be expected. The sample handling is easy, exact dilution is not necessary, the only prerequisite is an effective cooling facility (freezer). Enzymatic analysis does not need a centrifuge but only a simple thermostated filter photometer to determine enzyme activity and to allow reference to the hemoglobin content. The described procedure is intended for manual use, but should be easily adapted to autoanalyzers or microtiter plate procedures if large quantities of samples are to be measured.

It should be noted that determination of plasma cholinesterase should be possible in the diluted whole blood samples on substituting acetylthiocholine by butyrylthiocholine and omission of the BChE inhibitor. Alternatively, BChE activity can be easily determined in serum or plasma, which, however, requires rapid physical separation from the blood cells (10).

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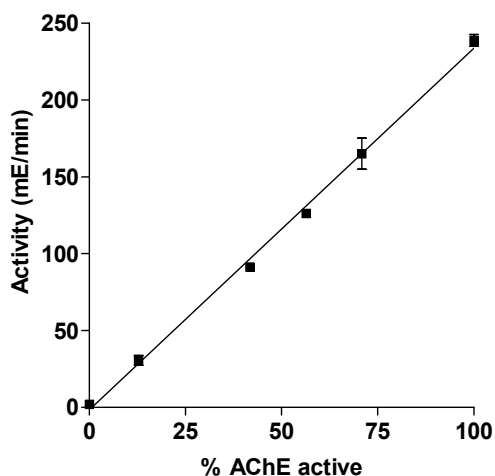
## KEYWORDS

Ellman assay, acetylcholinesterase, organophosphates, erythrocytes

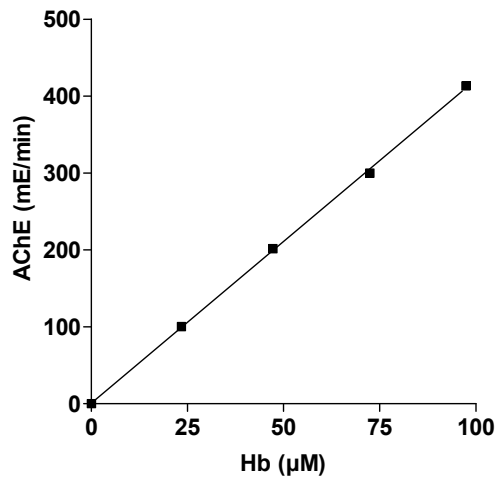
## FIGURES AND TABLES

**Figure. 1** Assay linearity of different enzyme activities at constant sample matrix.

Inhibited AChE samples were mixed with various proportions of native enzyme (n=2). The data are shown as mE/min (mean  $\pm$  SD;  $r^2 = 0.9975$ ).

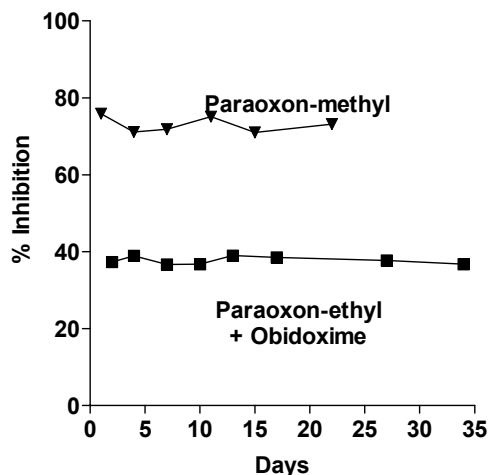


**Figure. 2** Correlation of AChE activity and hemoglobin content in differently diluted whole blood samples (1:50 to 1:200, n=2, mean  $\pm$  SD;  $r^2 = 0.9996$ ).



**Figure. 3** Persistence of AChE inhibition during storage at -20°C.

Whole blood was inhibited with paraoxon-methyl (3 μM) or paraoxon-ethyl (1 μM) in the presence of obidoxime (10 μM) for 15 min at 37°C, followed by immediate dilution of the blood samples (1:100). Samples (n=5) were thawed after different periods and analyzed in the modified Ellman assay (mean ± SD).



**Table 1** Within-run precision of Ery-AChE determination in whole blood

Paraoxon-ethyl (μM)	Obidoxime (μM)	Activity (mU/μmol Hb)	CV (%)	Inhibition (%)
0	0	586±5	0.95	0
0.2	0	32±1	4.26	94
0.2	10	524±2	0.50	11
1.0	10	224±2	1.05	64

Blood was incubated with inhibitor and oxime for 15 min at 37°C, as indicated (n=10).

Immediately thereafter blood was diluted (1:100) and all samples were frozen and analyzed the next day.

The specific activity is given as mean ± SD and the assay reproducibility as coefficient of variation (CV).

**Table 2.** Between-run precision of Ery-AChE determination in whole blood

Activity (mU/μmol Hb)	CV (%)
651±18	0

Whole blood dilutions (n=25) were assayed on five consecutive days. The specific activity is given as means ± SD and the assay reproducibility as coefficient of variation (CV).