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Comparison of methods used for the determination of cholinesterase activity in whole blood☆

Ramachandra S. Naik*, Bhupendra P. Doctor, Ashima Saxena

Division of Biochemistry, Walter Reed Army Institute of Research, 503 Robert Grant Ave, Silver Spring, MD 20910-7500, USA

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ABSTRACT

Cholinesterases (ChEs) are classified as either acetylcholinesterase (AChE) or butyrylcholinesterase (BChE) based on their substrate and inhibitor specificity. Organophosphate and carbamate compounds commonly represented by herbicides, pesticides, and nerve gases irreversibly inhibit ChEs. Therefore, exposure to organophosphates and carbamates is normally assessed by measuring ChE activity in blood. There are two approaches for measuring AChE and BChE activity present in whole blood: (1) separating blood into erythrocytes, which contain only AChE, and plasma which contains only BChE, to measure their activity individually, or (2) use a BChE-specific inhibitor to measure the activity of AChE in whole blood. A number of studies have reported the use of different inhibitors for the simultaneous measurement of AChE and BChE activities. However, the inhibitors used for completely inhibiting BChE activity also inhibited AChE activity leading to errors in reported values. The goal of this study was to find the most accurate and simple method for the simultaneous determination of AChE and BChE activity in animal whole blood. Solutions containing human AChE and BChE in various proportions were prepared and AChE and BChE activities were measured using three reported methods. Results demonstrate that ethopropazine and (−) huperzine A appear to be the most specific ChE inhibitors. Preliminary results with human and animal whole blood suggest that 20 μM ethopropazine and 500 nM (−) huperzine A can be used for measuring AChE and BChE activities across species.

1. Introduction

Several methods have been reported for the determination of acetylcholinesterase (AChE, EC 3.1.1.7) and butyrylcholinesterase (BChE, EC 3.1.1.8) levels in whole blood. While some of these methods use multiple substrates and require the separation of plasma and erythrocyte fractions for the measurement of erythrocyte AChE and plasma BChE activities [1–3], others use ChE-specific inhibitors [4–7]. Three methods, that use inhibitors, were recently reported for the simultaneous measurement of AChE and BChE activities. The WRAIR whole blood ChE assay uses three substrates – acetylthiocholine (ATC), butyrylthiocholine (BTC), and propionylthiocholine (PTC) – for measuring ChE activity in whole blood [5,6]. It also requires the use of an AChE-specific inhibitor (huperzine A) and a BChE-specific inhibitor (Iso-OMPA) to determine the sensitivity coefficients of AChE and BChE for each substrate. The second method to determine the relative proportions of AChE and BChE in animal plasma uses multiple ChE-specific inhibitors [7]. The third method uses ethopropazine for the determination of AChE activity in human whole blood [8]. The purpose of this study was to compare these three methods and to determine the most specific inhibitors that can be used for the accurate deter-

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* Corresponding author. Tel.: +1 301 319 9946; fax: +1 301 319 9150.
E-mail address: ramachandra.naik@us.army.mil (R.S. Naik).

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mination of AChE and BChE activities in whole blood. Since we wanted to determine the most suitable method for the estimation of ChE activities in the blood of animals that were exposed to organophosphates as well as animals that were administered ChE as bioscavengers, these methods were evaluated using enzyme mixtures containing different amounts of human (Hu) AChE and Hu BChE, rather than whole blood. Results demonstrate that ethopropazine and (−) huperzine A appear to be the most specific ChE inhibitors.

2. Materials and methods

2.1. Materials

Purified recombinant Hu AChE expressed in Chinese Hamster Ovary cells [9] and plasma-derived native Hu BChE [10] were available in our laboratory. ATC, BTC, DTNB [5,5′-dithiobis(2-nitrobenzoic acid)], dibucaine, edrophonium, eserine, ethopropazine, (−) huperzine A and Iso-OMPA were obtained from Sigma Chemical Co. (St. Louis, MO). BW284c51 [1,5-bis(4-allyldimethylammoniumphenyl) pentan-3-one dibromide] was purchased from Burroughs Wellcome (Research Triangle Park, NC). Stock solutions of all inhibitors and substrates were prepared in water and stored at −20 °C.

2.2. Preparation of enzyme mixtures

Stock solutions (0.5 U/ml) of Hu AChE and Hu BChE were prepared in 0.05% bovine serum albumin in phosphate buffered saline. Different volumes of these stock solutions were mixed to obtain the following mixtures: (1) 0.5 ml of Hu AChE + 9.5 ml of Hu BChE, (2) 2.5 ml of Hu AChE + 7.5 ml of Hu BChE, (3) 5.0 ml of Hu AChE + 5.0 ml of Hu BChE, (4) 7.5 ml of Hu AChE + 2.5 ml of Hu BChE, and (5) 9.5 ml of Hu AChE + 0.5 ml of Hu BChE.

2.3. Determination of AChE and BChE activities

AChE and BChE activities in the enzyme mixtures were determined using three methods, which are described below:

2.3.1. WRAIR whole blood ChE assay

AChE and BChE activities in the enzyme mixtures were measured as described [5,6]. Briefly, five serial dilutions of each enzyme mixture were prepared in water. Seven dilutions of (−) huperzine A (ranging from 14 nM to 900 nM) and Iso-OMPA (ranging from 0.08 μM to 5.12 μM) were prepared in water by serial double dilution. Each enzyme mixture (150 μl) was mixed with 10 μl of each inhibitor dilution, and incubated for 3 h at 25 °C. To determine the sensitivity coefficient, 10 μl of each enzyme-inhibitor mixture was dispensed into the wells of a 96-well microtiter plate followed by the addition of 290 μl of a substrate solution, and the plates were read at 412 nm for 4 min using a SPECTRAmax PLUS plate reader (Molecular Devices Corp., Sunnyvale, CA). Substrate solutions were prepared by mixing 8.4 ml of 50 mM sodium phosphate buffer, pH 8.0 (PB) with 300 μl each of 30 mM ATC (or PTC or BTC)

$$x_1[AChE] + y_1[BChE] = R_1 \quad \text{Substrate 1}$$
$$x_2[AChE] + y_2[BChE] = R_2 \quad \text{Substrate 2}$$
$$x_3[AChE] + y_3[BChE] = R_3 \quad \text{Substrate 3}$$

$$[AChE]_{1,2} = \frac{\begin{vmatrix} x_1 & R_1 \\ x_2 & R_2 \end{vmatrix}}{\begin{vmatrix} x_1 & y_1 \\ x_2 & y_2 \end{vmatrix}} \quad \text{and} \quad [AChE]_{1,3} = \frac{\begin{vmatrix} x_1 & R_1 \\ x_3 & R_3 \end{vmatrix}}{\begin{vmatrix} x_1 & y_1 \\ x_3 & y_3 \end{vmatrix}} \quad \text{and} \quad [AChE]_{2,3} = \frac{\begin{vmatrix} x_2 & R_2 \\ x_3 & R_3 \end{vmatrix}}{\begin{vmatrix} x_2 & y_2 \\ x_3 & y_3 \end{vmatrix}}$$

$$\text{Mean } [AChE] = \frac{[AChE]_{1,2} + [AChE]_{1,3} + [AChE]_{2,3}}{3}$$

where x1= sensitivity coefficient of AChE determined using ATC as the substrate, x2= sensitivity coefficient of AChE determined using PTC as the substrate, x3= sensitivity coefficient of AChE determined using BTC as the substrate, y1= sensitivity coefficient of BChE determined using ATC as the substrate, y2= sensitivity coefficient of BChE determined using PTC as the substrate, y3= sensitivity coefficient of BChE determined using BTC as the substrate. The [BChE] was calculated in a similar manner.

Fig. 1. Method for calculating AChE and BChE activities by WRAIR whole blood ChE assay.

and DTNB. Control solution contained only PB and DTNB. For each diluted enzyme mixture, the hydrolysis rates (V_{max}) for all three substrates were measured twice, once with serial dilutions of (−) huperzine A and once with Iso-OMPA. V_{max} values were used to obtain sensitivity coefficients for AChE and BChE that in turn were used in the equations shown in Fig. 1, to obtain activities of AChE and BChE in U/ml. AChE and BChE activities were also measured by titrating the dilutions of the enzyme mixtures against higher concentrations (that inhibits BChE activity completely) ranging from 1.56–100 μM Iso-OMPA.

2.3.2. Method described by Bartels et al.

In the method described by Bartels et al., AChE and BChE in plasma were estimated by measuring AChE and BChE activities in the presence of several BChE- and AChE-specific inhibitors, respectively [7]. The following inhibitor solutions were prepared in water: 300 μM eserine, 3 μM BW284c51, 3 mM edrophonium, 3 mM dibucaine, 300 μM ethopropazine, and 3 mM Iso-OMPA. Eserine was used as a general ChE inhibitor, and at 10 μM concentration it inhibited both AChE and BChE completely. A solution of DTNB (30 mM) and PB (v/v, 1:22) was prepared to yield a solution containing DTNB at a final concentration of 1.3 mM (PB-DTNB). In a 96-well microtiter plate, 230 μl of PB-DTNB solution was added to the first nine columns, followed by 10 μl of each enzyme mixture. Each inhibitor solution (10 μl) was added to a row, except that water instead of inhibitor was added to the control row. After incubating for 30 min at 25 °C, 50 μl of substrate solution was added in triplicate. Substrate solutions were prepared by mixing 9.6 ml of PB with 2.4 ml of 30 mM ATC or BTC. ATC was added to columns 3–6, BTC was added to columns 7–9, and only PB was added to columns 1–3. V_{max} were measured by reading the plates at 412 nm for 5 min. V_{max} values were corrected for 1 cm path length, dilution fac-