

# Oral administration of pyridostigmine bromide and huperzine A protects human whole blood cholinesterases from ex vivo exposure to soman<sup>☆</sup>

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## Abstract

Cholinesterases (ChEs) are classified as acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) according to their substrate specificity and sensitivity to selected inhibitors. The activities of AChE in red blood cells (RBC-AChE) and BChE in serum can be used as potential biomarkers of suppressed and/or heightened activity in the central and peripheral nervous systems. Exposure to organophosphate (OP) chemical warfare agents (CWAs), pesticides, anesthetics, and a variety of drugs such as cocaine, as well as some neurodegenerative and liver disease states, selectively reduces AChE or BChE activity. In humans, the toxicity of pesticides is well documented. Therefore, blood cholinesterase activity can be exploited as a tool for confirming exposure to these agents and possible treatments.

Current assays for measurement of RBC-AChE and serum BChE require several labor-intensive processing steps, suffer from wide statistical variation, and there is no inter-laboratory conversion between methods. These methods, which determine only the serum BChE or RBC-AChE but not both, include the Ellman, radiometric, and  $\Delta$ pH (modified Michel) methods. In contrast, the Walter Reed Army Institute of Research Whole Blood (WRAIR WB, US Patent #6,746,850) cholinesterase assay rapidly determines the activity of both AChE and BChE in unprocessed (uncentrifuged) whole blood, uses a minimally invasive blood sampling technique (e.g., blood from a finger prick), and is semi-automated for high-throughput using the Biomek 2000 robotic system.

To date, the WRAIR whole blood assay was used to measure AChE and BChE activities in human blood from volunteers in FDA clinical trials. In the first FDA study, 24 human subjects were given either 30 mg PB orally ( $n = 19$ ) or placebo ( $n = 5$ ). Blood samples were obtained pre-dosing and 2.5, 5, 8, and 24 h post-dosing. The samples were analyzed for AChE and BChE activity using the WRAIR WB robotic system, and for PB concentration by HPLC. We found that maximal inhibition of AChE (26.2%) and concentration of PB (17.1 ng/mL) occurred at 2.5 h post-PB dosing. AChE activity returned to almost 100% of pre-dose values by 6 h. A dose-dependent linear correlation was found between the amount of PB measured in the blood and the inhibition of AChE. Following soman (GD) exposure, recovered AChE activity was similar to levels that were reversibly protected by the PB administration. Therefore, the WRAIR ChE WB data clearly supports the conclusion that PB is an effective pre-treatment drug for nerve agent exposure (GD).

<sup>☆</sup> The opinions or assertions contained herein are the private views of the author, and are not to be construed as official, or as reflecting true views of the U.S. Department of the Army or the Department of Defense.

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In the second FDA human study for the treatment of Alzheimer's disease, the WRAIR ChE WB assay was used to determine the RBC-AChE and serum BChE profile of healthy elderly volunteers receiving Huperzine A. Huperzine A is a plant-derived reversible and selective AChE inhibitor compared to BChE, and is a more potent inhibitor of AChE than PB. Huperzine A is available as a nutraceutical, a natural supplement reported to improve memory, and has a variety of neuroprotective effects. Individuals received an increasing dose regimen of huperzine A (final dose 200 µg after 4 weeks), which produced more than 50% inhibition of RBC-AChE. Huperzine A was well tolerated by these patients at doses that sequestered more RBC-AChE than PB, and thus warrants further study as a prophylaxis for OP poisoning in addition to Alzheimer's therapy.

Due to the documented use of OPs by terrorists and in warfare around the globe, Federal, State, and local authorities need a reliable, fast, inexpensive, and standard method for confirming such an assault in order to initiate appropriate containment, decontamination, and treatment measures. This assay is ideal for prescreening military personnel for atypical ChE activities that would preclude their deployment to areas of potential CWA exposure. The WRAIR WB ChE assay will fulfill the requirement for rapid and reliable monitoring of such exposure in military and civilian populations.

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## 1. Introduction

Cholinesterases are highly polymorphic carboxylesterases that display broad substrate specificity and are involved in the termination of neurotransmission in cholinergic synapses and neuromuscular junctions of the central nervous system (CNS). ChEs are classified as acetylcholinesterase and butyrylcholinesterase according to their substrate specificity and sensitivity to selected inhibitors [1]. The concentration of AChE and BChE in blood is potentially a stable biomarker of suppressed and/or heightened central and peripheral nervous system activity. Exposure to nerve agents, OPs, pesticides, anesthetics, terrorists' chemical agents, cocaine, and some neurodegenerative disease states and their treatments selectively reduces AChE or BChE activity. In humans, the toxicity of pesticides is well documented [2]. Therefore, blood cholinesterase activity can be exploited as a tool for confirming exposure to the agents and possible therapeutics [3].

The current tests used to determine cholinesterase levels in blood, however, are not U.S. Food and Drug Administration approved and have significant drawbacks including the lack of standardization, long turn-around times, and difficulty in comparing results between alternate laboratories utilizing different ChE assays that report values in different or non-standard units. In part, this is because clinical determination of ChE levels in blood typically utilize three different techniques: Ellman, Michel ( $\Delta$ pH), and pH stat, and normally determine either RBC-AChE or serum BChE concentrations, but not *both* [4]. In addition, radioactive assays for AChE activity, although very sensitive, require special handling and disposal and are not suitable for field use.

Therapeutic drugs have a wide effect on cholinesterases [5] and minor inhibition is observed

with a variety of anesthetics. High inhibition is observed (purposely) with pyridostigmine bromide, its therapeutic advantage being to increase the muscle strength in myasthenia gravis patients by inhibiting acetylcholinesterase that results in an accumulation of acetylcholine at cholinergic synapses [6]. Yet it is the organophosphate chemical warfare agents that are some of the most potent and irreversible inhibitors that can produce excessive accumulation of acetylcholine, and ultimately a cholinergic crisis in man leading to death [3]. Although the physiological state of an individual, drugs, pesticides, and chemical warfare agents affect cholinesterases, only for the latter two are routine cholinesterase measurements utilized. Studies on the toxicology of nerve agents and on treatment of intoxications have predominantly focused on lethal and supra-lethal doses of the agent. However, the possible relationship between the so-called Gulf War Syndrome and accidental exposure to trace amounts of sarin and cyclohexyl sarin [7] has made clear that knowledge on the acute and delayed effects of low or trace exposure to nerve agents, insecticides, and a variety of environmental chemicals is scarce [8,9]. Several military and terrorist scenarios can be envisioned in which low or trace exposures become significant.

Given the potential increase in urban terrorism that may include the use of chemical warfare organophosphate agents, Federal, State, and local authorities need a reliable, fast, inexpensive, and standard method for confirming such an assault in order to initiate appropriate containment, decontamination, and treatment measures. To this end, we have developed a new methodology – The Walter Reed Army Institute of Research Whole Blood (WRAIR WB) cholinesterase assay – that quickly and accurately determines the activities of AChE and BChE simultaneously in unprocessed, whole blood

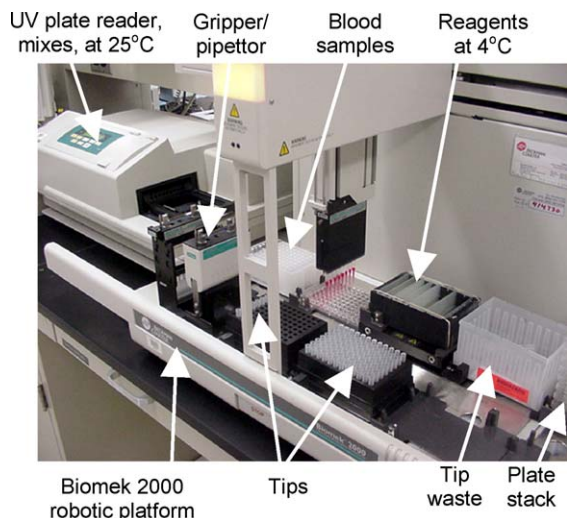


Fig. 1. WRAIR whole blood robotic assay, consisting of the Biomek 2000, UV microplate reader, and computer processing equipment (see Section 2).

[10–12]. The WRAIR WB assay measures the activity of whole blood in the presence of three substrates for AChE and BChE, which provide redundancy and independent determination of both AChE and BChE activities. This is possible because: (1) each protein possesses a different affinity and sensitivity for each of its substrates and (2) a direct relationship exists between activity and enzyme concentration.

Currently, the WRAIR WB high throughput assay (Fig. 1) has been used to support two FDA clinical trials. The first study was to demonstrate that pyridostigmine bromide pretreatment protected RBC-AChE against the OP chemical warfare agent soman (GD). In the second study, huperzine A, a potential therapeutic for Alzheimer's disease [13], was shown to specifically inhibit RBC-AChE without adverse effects in normal elderly volunteers. The methods and results described below support the conclusion that the WRAIR WB assay is ideal for rapid and reliable monitoring of OP exposure in military and civilian populations and also monitoring therapeutic regimens for neurological diseases, e.g., myasthenia gravis or Alzheimer's, where ChE inhibitors are used.

## 2. Methods

### 2.1. The WRAIR whole blood (WB) assay theory and design

Current clinical determination of cholinesterase levels includes the Michel, pH stat, Ellman, and micro-

Ellman methodologies [1]. These methods require several processing steps, normally determine either the serum or RBC cholinesterase activities but not both, and suffer from statistical error. For instance, in order to determine the RBC-AChE levels, the Michel method ( $\Delta\text{pH}$ ) requires centrifugation to pellet RBCs followed by washing to remove serum BChE from the pellet prior to analysis of the sample for AChE. Since the different assay techniques measure different parameters (e.g.,  $\Delta\text{pH}$  per hour for the Michel assay in comparison to absorbance change per minute for the Ellman assay), inter-lab comparison has not been established. In addition to the clinical methodologies, a field deployable unit is commercially available, the Test-Mate OP<sup>TM</sup> system (EQM Research Inc., Cincinnati, OH). Although this unit is designed for field use, a selective BChE inhibitor is required to measure AChE. Therefore, two blood samples are necessary, which results in longer processing times for complete AChE and BChE screening.

The WRAIR WB method does not rely on the addition of selective AChE or BChE inhibitors, uses a single minimally-invasive blood collection technique of a finger prick (equally acceptable would be a small blood sample from a tube of *uncentrifuged* blood kept at 4°C or frozen), is not labor intensive, and produces rapid results due to automation. Thus, we have circumvented the aforementioned problems and simultaneously determined the levels of both AChE and BChE. This is possible because blood contains two cholinesterases that possess different affinities for any given substrate, and a linear correlation exists between enzyme activity and concentration. Thus, if one determines the activity in any given blood sample with two different substrates, then it is feasible to calculate the precise concentrations of both proteins (i.e., two equations with two unknown variables [14]). Furthermore, monitoring the activity with three different substrates (Fig. 2) provides three-fold degenerate data (i.e., three sets of two equations with two unknown variables). In Fig. 2, the rates of substrate hydrolysis are represented by  $R_1$ ,  $R_2$ , and  $R_3$  and correspond to the turnover of substrate 1, substrate 2, and substrate 3, respectively. The [AChE] and [BChE] refer to the actual activities of AChE and BChE in the sample. Finally, the coefficients in each equation (i.e.,  $x_1$ ,  $x_2$ ,  $x_3$ , and  $y_1$ ,  $y_2$ ,  $y_3$ ) represent sensitivity coefficients and are the contribution that AChE and BChE independently contribute to the overall rate of hydrolysis of each substrate ( $R_1$ ,  $R_2$ ,  $R_3$ ). Simultaneously solving these three sets of degenerate equations provides three independent estimates for the activities of AChE and BChE. Therefore, determining

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

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

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

The [BChE] can be calculated in a similar manner.

Fig. 2. Mathematical representation of the sensitivity coefficients described in Section 2.

the mean value and the standard deviation for these independently derived values provides the activities of each protein, AChE and BChE.

## 2.2. WRAIR WB assay conditions

The final concentrations of the substrates for the 96-well microtiter plate in the WRAIR WB assay were 1 mM each of acetylthiocholine iodide (ATC), propionylthiocholine iodide (PTC), butyrylthiocholine iodide (BTC), and 0.2 mM 4,4'-dithiopyridine (DTP), the indicator for the hydrolyzed thiocholine (UV absorbance at 324 nm). To perform the ChE assays, human whole blood was collected from subjects and stored with heparin at 4 °C or frozen at –80 °C. Note that hemoglobin from lysed blood does not interfere with the assay at this wavelength, thus providing a greater signal to noise ratio (data not shown [11]). A small aliquot of blood, typically 10 µL, which was diluted 20-fold in distilled water, was placed in the well to give a final volume of 300 µL using 50 mM sodium phosphate buffer, pH 8.0 containing the appropriate substrate and DTP, and performed in triplicate. Following a 60-s pre-read shaking to mix the contents thoroughly, a 4-min kinetic assay was performed (at 25 °C) on each plate using a Molecular Devices SpectraMax Plus<sup>384</sup> microtiter spectrophotometer (Sunnyvale, CA), interfaced to a Beckman-Coulter Biomek 2000 robotic station (Fullerton, CA), that performed all the plate and sample handling steps. Each well was read at 14-s intervals, interspersed with 3 s shaking. The data were subjected to linear least squares analysis, from which the activities of AChE and BChE (U/mL) were calculated using SoftMax v4.6 and an Excel spreadsheet, which also documents substrate batches for GLP (Good Laboratory Practices) record keeping.

## 2.3. Measurement of pyridostigmine bromide induced ChE inhibition

Aliquots of whole blood from 24 volunteers (19 given a single oral dose of pyridostigmine bromide (30 mg tablet) and five placebos) were withdrawn for the determination of RBC-AChE and serum BChE activity. A blood sample was taken before the PB dose (0 time or prescreen) and then at 2.5, 5, 8 and 24 h after dosing. Blood samples were frozen at –80 °C, and thawed immediately prior to the WRAIR WB cholinesterase assay to limit decarbamylation (i.e., reversal of ChE inhibition by PB).

## 2.4. Measurement of huperzine A-induced ChE inhibition (RBC-AChE)

Aliquots of whole blood from 12 healthy elderly volunteers given an increasing dose regimen of huperzine A (50 µg twice daily for 1 week, then increasing doses of 100, 150, and finally 200 µg twice daily in Week 4) were withdrawn at various time intervals up to 24 h for RBC-AChE and serum BChE activity determination. Blood was sampled immediately prior to the first huperzine A dose and 24 h after the last (200 µg) dose. Three individuals served as controls and were given a placebo. As for PB-containing blood, samples were frozen (–80 °C) and thawed immediately prior to the WRAIR WB cholinesterase assay.

## 2.5. Measurement of protection afforded to RBC-AChE by PB or huperzine A to ex vivo soman exposure

To evaluate the protection afforded to RBC-AChE by PB or huperzine A, blood samples stored at –80 °C

were thawed and then exposed to the irreversible OP soman ( $1 \mu\text{M}$ ) for 10 min at room temperature. These experiments were conducted at USAMRICD, Aberdeen Proving Ground, MD. Free PB or huperzine A and GD were removed from the blood by using Biorad spin columns (6 cm) containing 300 mg of  $\text{C}_{18}$  (Waters, Milford, MA). After adding  $2.5 \mu\text{L}$  of saponin (50 mg/mL) and vortexing for 1 min,  $180 \mu\text{L}$  of the lysed whole blood was added to the column and centrifuged at  $1000 \times g$  for 2 min. In addition to removing free ligands from the blood, almost 100% of the AChE/BChE activity from the original blood sample is recovered. Although these samples were not exposed to oximes, interfering compounds including oximes such as 2-PAM and HI-6 can also be removed (data not shown). Thus, after PB or huperzine A removal, we are able to monitor the time taken to achieve full return in activity (decarbamylation for PB or dissociation from the active site by huperzine A), and how much of the RBC-AChE is protected from GD exposure. The percentage of recovery of ChE activity is calculated as follows: percentage of inhibited (PB/huperzine A or placebo samples) =  $100 \times (\text{ChE U/mL of samples at times post-dosing}) / (\text{ChE U/mL pre-dosing})$ . The percentage of recovery (PB/huperzine A or placebo samples exposed to GD) =  $100 \times (\text{ChE U/mL of GD samples at times post-dosing}) / (\text{ChE U/mL pre-dosing})$ .

## 2.6. HPLC assays of PB and huperzine A

To correlate AChE-RBC inhibition with blood concentrations of PB or huperzine A, or to verify compliance of PB consumption (for example troops anticipating potential OP exposure), we have developed a sensitive HPLC technique to quantify PB [15] and huperzine A in human blood (unpublished). The PB HPLC technique is based on solid phase extraction, lyophilization for concentration, and HPLC of the reconstituted samples using strong-cation exchange chromatography, isocratic elution, and absorbance measurement. Huperzine A was resolved with F-substituted benzene reverse phase chromatography and measurement by HPLC-MS. The linear dynamic range of sensitivity covers at least 500–0.5 ng of PB and 250–0.038 ng of huperzine A (not shown).

## 3. Results

### 3.1. PB and huperzine A inhibition of ChEs in blood

Fig. 3 illustrates the inhibition of whole blood AChE and BChE in the 19 human volunteers who received PB. Each solid circle represents data from a single individual. Maximal AChE inhibition is observed at 2.5 h post

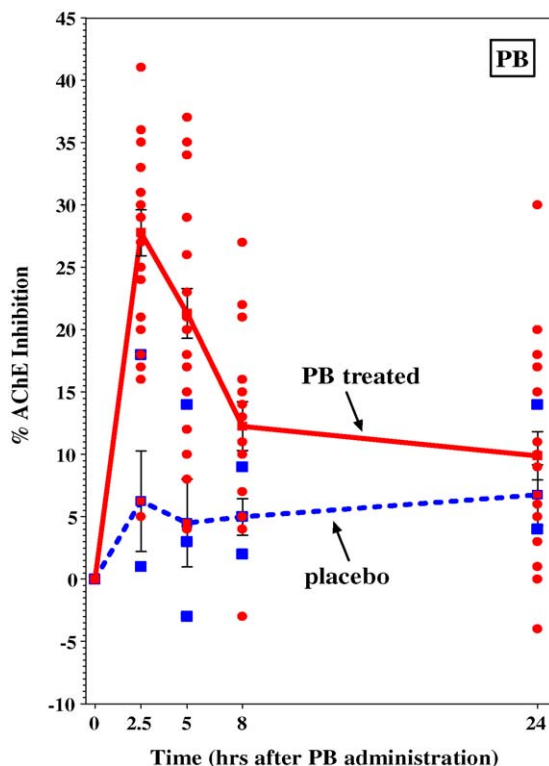


Fig. 3. Time course of inhibition of RBC-AChE in humans given a single 30 mg dose of pyridostigmine bromide (PB). Nineteen volunteers received PB (solid line with circles), with five receiving a placebo (dashed squares). ChE were measured using the WRAIR WB assay.

PB dosing, with return to placebo levels achieved after 24 h. At 2.5 h, 19 PB-dosed individuals yielded a mean inhibition of AChE of 27%, with a range of inhibition of approximately 16–40%. The solid squares in Fig. 3 represent the individuals receiving only the placebo with little change in AChE activity being observed. Serum BChE levels were less affected by PB, approximately 12% overall inhibition observed after 2.5 h (not shown). PB-induced reversible AChE inhibition is variable, and could be due to a number of factors including individual PB absorption, weight, gender, age, or other variables including food intake.

Analogously, Fig. 4 illustrates the inhibition of whole blood AChE by huperzine A in healthy elderly human volunteers in a FDA trial to evaluate its safety for Alzheimer's disease therapy. The inhibition of RBC-AChE is seen for the 12 individuals (solid circles) given the increasing doses of huperzine A (200  $\mu\text{g}$  dose shown). The closed squares represent data from the three individuals given a placebo. With a 50  $\mu\text{g}$  dose, a maximal reduction in RBC-AChE activity of about 18% is seen after 1 h with activity returned to normal about 12 h later. In contrast, the 200  $\mu\text{g}$  dose (given in Week 4) pro-

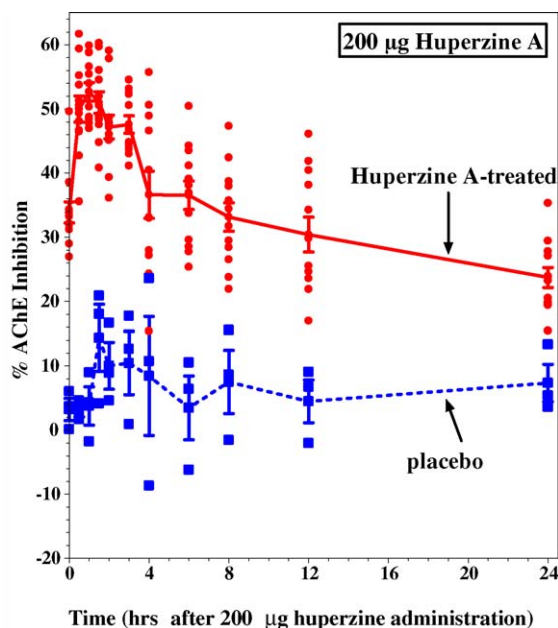


Fig. 4. Time course of inhibition of RBC-AChE in humans given an increasing dose of huperzine A. Twelve volunteers received the drug (solid circles), and three receiving a placebo (dashed squares). ChEs were measured using the WRAIR WB assay.

duced a much larger reduction in activity (approximately 53%) 2 h after the last dose was administered. It can also be seen that RBC-AChE activity returns to normal rather slowly, with about 25% inhibition still being observed 24 h later (Fig. 4). Huperzine A, even at 200  $\mu\text{g}$ , had no effect on BChE levels (data not shown).

### 3.2. RBC-AChE protection assays to *ex vivo* GD by PB and huperzine A

PB carbamylates and huperzine A reversibly binds to AChE, and thereby protects the enzyme from reaction with OPs. The activity of the PB or huperzine A-protected but inhibited AChE will be restored once the drug-AChE complex spontaneously decarbamylates (PB) or dissociates (huperzine A), which occurs after GD is cleared from the blood. To illustrate this, the maximally PB-inhibited RBC-AChE (2.5 h post-dose, Fig. 3) or huperzine A-inhibited RBC-AChE (1.5 h, Fig. 4) were exposed to GD (soman) *ex vivo*. Next, the blood samples were rapidly centrifuged through a column to remove any free GD and drug, which bind to the column matrix while allowing all the ChEs to pass through the column. Under these circumstances, any RBC-AChE not protected by PB or huperzine A would be irreversibly inhibited by GD. In contrast, the RBC-AChE protected by PB/huperzine A would spontaneously decarbamy-

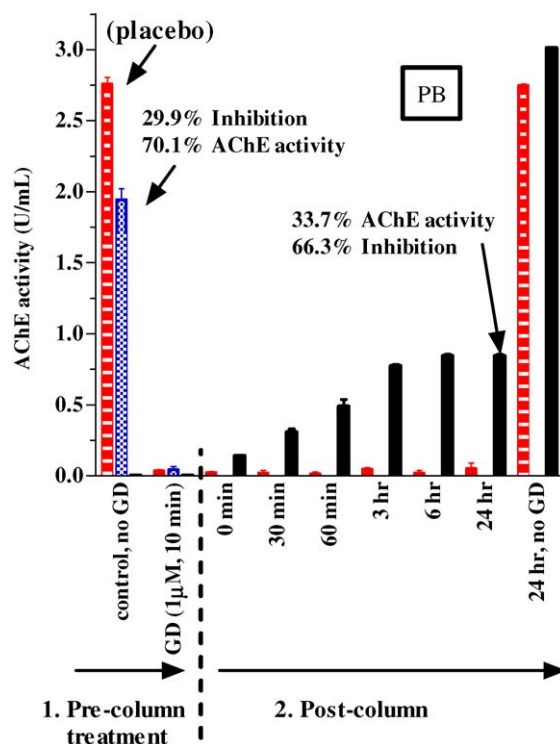


Fig. 5. Effects of GD on AChE activity in whole blood from human volunteers who had taken PB (30 mg tablet). Blood was drawn 2.5 h post-dose when RBC-AChE is maximally inhibited by PB (hatched bar (blue), left of dashed line). After GD exposure, PB and GD were removed as described in Section 2 (post-column treatment). The PB-protected AChE activity returned by 6 h post-column. Solid bars show the correlation between the initial percentage of inhibition of RBC-AChE by PB and the subsequent return of AChE activity due to decarbamylation of the protected enzyme after removal of PB and GD.

late/dissociate over time, and this enzyme's activity would be restored. In Fig. 5, the striped bars (red) represent RBC-AChE without any PB (placebo), while the hatched bar (blue) represents AChE from an individual receiving the 30 mg PB tablet. In the first part, after GD treatment, no AChE activity is observed by the WRAIR assay in either the placebo or drug treated volunteers (solid bars close to 0 U/mL). However, after the spin column removal of free PB and GD, and a 24 h period to allow for complete decarbamylation, the PB-inhibited AChE is restored to the level that was initially inhibited by PB (29.9% inhibition by PB before the column versus 33.7% returned AChE activity post-column; Fig. 5). Also shown in Fig. 5 is that the recovery of AChE activity is almost complete after 3 h. These results definitively demonstrate that RBC-AChE is protected by PB from *ex vivo* GD.

Similar results were observed for huperzine A protection of RBC-AChE. In this case, huperzine A (200  $\mu\text{g}$ )

was a more potent inhibitor than PB, yielding 52% inhibition (Fig. 4). However, after the column for removal of free huperzine A and GD, and a 4-h period to allow for complete dissociation, the huperzine A-inhibited AChE was almost restored to the level that was initially inhibited by the drug (52% inhibition by the drug before the column versus 54% returned AChE activity post-column; data not shown). This suggests that huperzine A is highly effective in protecting RBC-AChE from ex vivo GD exposure.

### 3.3. Correlation of PB and huperzine A concentration in blood and RBC-AChE inhibition

The PB/huperzine A concentrations in the same blood samples used to determine RBC-AChE inhibition were analyzed by our HPLC or HPLC/MS analysis (see Methods). A linear correlation ( $r^2=0.98$ ) was established between the amount of PB measured in the blood in all 19 treated subjects and the percentage of inhibition of RBC-AChE. The amount of PB in the blood ranged between 0 and 17 ng/mL. A linear correlation ( $r^2=0.87$ ) was also established between the amount of huperzine A in the blood and percentage of RBC-AChE inhibition. Levels of huperzine A ranged between 0.3 and 3.5 ng/mL (not shown). It is clear that huperzine is a more potent RBC-AChE inhibitor than PB since smaller concentrations of the drug were required to achieve greater RBC-AChE inhibition.

## 4. Conclusions

The WRAIR whole blood cholinesterase assay is capable of providing fast, precise, and accurate AChE and BChE measurements, and can be directly correlated for cholinesterase activity with the classical measurement techniques. Unlike the conventional clinical tests, the WRAIR WB procedure provides a more detailed picture of the patient's cholinesterase levels (i.e., by measuring both AChE and BChE simultaneously), produces rapid results, and is capable of high-throughput screening of whole blood by employing state-of-the-art robotics with associated precision. This method can thus rapidly establish a military or civilian personnel's exposure to insecticides, nerve agents, medicinally administered drugs (such as PB and huperzine A), narcotics, and anesthetics.

The recommended dose of PB [16] as a pretreatment for OP exposure is 90 mg per day ( $3 \times 30$  mg at 8 h intervals). After a single 30 mg dose to human volunteers, we have demonstrated that the maximal inhibition of RBC-AChE (average 27%) occurs after 2.5 h, and that

this reversibly inhibited PB-AChE complex can spontaneously decarbamylate resulting in the loss of PB inhibition and the restoration of the original AChE activity. In contrast, there was no return of AChE activity after GD exposure ex vivo in the human blood of volunteers receiving the placebo.

Huperzine A is a reversible and highly selective AChE inhibitor compared to BChE [13], and we demonstrated that it is a more potent inhibitor of RBC-AChE than PB. Huperzine A is available as a "nutraceutical", a natural supplement reported to improve memory, and has a variety of neuroprotective effects [17]. Huperzine A is presently undergoing clinical trials as a potential treatment for Alzheimer's disease, which provided us the opportunity to measure huperzine A inhibition of RBC-AChE of healthy elderly volunteers using the WRAIR WB assay. Huperzine A exhibited no apparent side effects, even when given a 200  $\mu$ g dose of huperzine A. This produced more than 50% inhibition of RBC-AChE (Fig. 5). Like PB, we demonstrated that the huperzine A-AChE complex spontaneously dissociated following ex vivo exposure to GD after spin column treatment, and the original protected RBC-AChE activity was restored. In contrast to PB, huperzine A passes the blood brain barrier and would protect both the CNS and peripheral AChE. We established a good correlation between the percentage of RBC-AChE inhibition by PB and huperzine and percentage of brain and guinea pig diaphragm muscle AChE inhibition (not shown). Therefore, the WRAIR RBC-AChE assay is a reliable surrogate for CNS and peripheral exposure to OPs. Using a sensitive HPLC method to quantify drug levels in plasma, we found excellent agreement between the levels of the two drugs and the RBC-AChE inhibition observed using the whole blood assay.

These data support the premise that pyridostigmine bromide is an effective drug for prophylaxis against the lethal effects of GD nerve agent poisoning. Given the extensive cumulative experience with the use of PB in patients with myasthenia gravis [6] and the significantly higher doses prescribed over many years (up to 1.5 g/day), PB is a safe drug when used as a pretreatment for OP poisoning. Likewise, huperzine A is a potential drug for prophylaxis for organophosphate poisoning. It displays higher specificity for AChE than PB and has a longer biological half-life [18].

In conclusion, given the potential increase in urban terrorism that may include the use of chemical warfare organophosphate agents, Federal, State, and local authorities need a reliable, fast, inexpensive, and standard method for confirming such an assault in order to initiate appropriate containment, decontamination, and

treatment measures. The WRAIR whole blood assay fulfills these requirements.

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Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals, NRC Publication, 1996 edition. These studies were approved by the WRAIR and GU human use review committees.

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