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Award Number: DAMD17-01-1-0772

TITLE: Improving Blood Monitoring of Enzymes as Biomarkers of
Risk from Anticholinergic Pesticides and Chemical Warfare Agents

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REPORT DATE: October 2007

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT:

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REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
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1. REPORT DATE 01-Oct-2007		2. REPORT TYPE Final		3. DATES COVERED 20 AUG 2001 - 23 SEP 2007	
4. TITLE AND SUBTITLE Improving Blood Monitoring of Enzymes as Biomarkers of Risk from Anticholinergic Pesticides and Chemical Warfare Agents				5a. CONTRACT NUMBER DAMD17-01-1-0772	
				5b. GRANT NUMBER	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Barry W. Wilson, Ph.D. John D. Henderson				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of California Davis, California 95616-8671 Email: bwwilson@ucdavis.edu				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT Blood biomarkers are an important way to monitor exposure to anticholinergic pesticides and chemical warfare agents. The widely used colorimetric Ellman assay is based on the hydrolysis of acetylthiocholine; CHPPM uses an equally reliable but slower delta pH method to monitor approximately 15,000 DOD personnel each year. Two different approaches were used to calculate conversion factors for expressing delta pH AChE in terms of Ellman assay units. First, paired samples were assayed by the delta pH method at CHPPM and by the Ellman method at UC Davis. Second, DFP-treated whole blood was used to mimic OP exposure. RBC AChE activity was measured using the Ellman and delta pH assays at UCD. The normal range of AChE activities from the CHPPM delta pH assay was converted to Ellman units, generating benchmarks for clinical determinations in the absence of baseline data. Conversion factors between Test Mate kit cholinesterase measurements and the delta pH and Ellman methods were also determined. The normal range of AChE activities from the CHPPM delta pH assay was converted to Test-Mate					
15. SUBJECT TERMS Blood biomarkers, chemical warfare agents, pesticides, cholinesterases					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT UU	18. NUMBER OF PAGES 55	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U			19b. TELEPHONE NUMBER (include area code)

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INTRODUCTION

Intensive use of anticholinergic pesticides such as organophosphate esters (OPs) and threats of chemical warfare agents (CWAs) demonstrate the need for rapid, high throughput, reliable determinations of blood cholinesterases (ChEs) to provide warnings of exposures. Many clinical and research laboratories use the colorimetric Ellman assay based on the hydrolysis of acetylthiocholine (Ellman, *et al.*, 1961) and red blood cells (RBCs). However, the Cholinesterase Reference Laboratory (CRL) of CHPPM (US Army Center for Health Promotion and Preventive Medicine) uses a slower delta pH method based on that of Michel (1949) to monitor RBCs from more than 15,000 DOD personnel each year. Although this pH assay is reliable, it is slow and not readily adaptable for automation or field use.

One goal of this project was to establish a conversion factor between CRL's delta pH assay and the Ellman-based colorimetric assays. Another goal was to provide conversion factors for the portable Test-mate kit manufactured by EQM Research, Inc. Studies of ours and others have shown that past models did not adequately adjust for temperature, making them unsuitable for field use (Oliveira *et al.*, 2002). The manufacturer promised to make a custom model that would permit us to adjust assay parameters to optimize its response.

Still another goal focused on genetically sensitive individuals exposed to anticholinergic chemicals. Lowered serum BChE, a scavenger of antiChE agents, may put individuals at increased risk from OP and carbamate (CB) agents (reviewed by Wilson, 1999, 2001). A polymorphic form of paraoxonase (PON1), an enzyme that destroys selected OPs, was reported to be reduced in a cohort of veterans suffering from "Gulf War Syndrome" (Haley *et al.*, 1999) and there was evidence that low levels of BChE and PON1 affected sensitivity to OP exposures of experimental animals (Shih *et al.*, 1998, Broomfield *et al.*, 1991).

Materials and Methods

Materials

All chemicals were purchased from Sigma Chemical Co.

Methods

Sample Handling

RBC samples were shipped to UC Davis overnight on cold packs by CHPPM personnel. Upon receipt, the temperature of the samples was checked (CHPPM protocol states samples will be <10°C during shipping). Additional blood was obtained from volunteers at the University of California, Davis (UCD) under an approved Human Subjects Protocol, collected in EDTA vacutainers and kept on ice. Within 4 hours of collection, RBCs were separated by centrifugation at 1000 x g for 15 minutes. Samples were stored

at 4°C and kept on ice during use. Ghost RBC samples were stored at -70°C, and shipped overnight to CHPPM on dry ice by UC Davis personnel.

Ghost RBC Preparation

Bovine blood was centrifuged at 1000 x g, the plasma discarded, the RBCs resuspended in isotonic buffer and washed twice. RBC ghosts were prepared by lysing the cells with hypertonic buffer. After the membrane bound AChE was centrifuged at 100,000 x g, the pellet was solubilized in buffer with Triton X-100 detergent and the solution diluted and stored at -70°C until use.

Specific ChE Inhibitors

Specific inhibitors were used to distinguish ChE enzymes. Quinidine, a selective inhibitor of mammalian BuChE, was used at a final concentration of 2×10^{-4} M. BW285c51, a selective inhibitor of AChE, was used at a final concentration of 2×10^{-5} M.

ChE Determinations

Ellman Cholinesterase

RBCs were diluted 1/50 in a lysis buffer, 0.5% Triton X-100, 0.1 M sodium phosphate buffer, pH 8. AChE activity was measured using a modified colorimetric method of Ellman *et al.* (1961) in a 96 well plate reader at 25°C. The final concentrations of the substrate, acetylthiocholine, and the color reagent, dithiobisnitrobenzoate (DTNB), were 1 and 10.3 mM respectively. Activities were reported as umol/min/ml RBC. Activities were determined in the presence and absence of 0.02 mM quinidine sulfate, a selective BChE inhibitor.

Delta pH ChE

Delta pH measurements determined at UCD were performed according to Standard Operating Procedure # CRL40-2.7 provided by CRL. A 200 ul aliquot of RBCs was added to 4 ml of assay buffer (13 mM sodium barbital, 3 mM potassium phosphate monobasic, 510 mM sodium chloride, and 0.012% (w/v) saponin, pH 8.05). An initial pH measurement was recorded prior to adding acetylcholine bromide (10 mM final concentration), followed by a final pH measurement seventeen minutes later. The pH change of a substrate blank (no RBCs present) averaged 0.05 ± 0.02 delta pH/hr (n = 9). Assays were carried out at 25°C. Results were expressed as delta pH/hour.

Test-mate OP Kit

Test-mate measurements were made according to the instructions for the FDA approved kit (EQM Research, Inc., 2003). Centrifuged RBCs were resuspended in an equal volume of PBS. Ten ul of RBC mixture were added to the assay vial with an automatic pipetter (not with the provided glass capillary). The kit's reagent mixture was added to the vial and the assay carried out in the Model 400 Test-mate kit. Reagent concentrations were 1 mM ATCh and 3 mM DTNB. Activity was reported as umol/min/g hemoglobin (EQM Research, Inc., 2003). The Test-mate is designed to normalize activity determinations per hemoglobin (Hb) because of the inaccuracies inherent in pipetting small aliquots of blood (the kit supplies capillary tubes for transferring blood, we preferred to use micropipettors in the UCD assays).

Results

Task One. Task One was to compare the Ellman assay performed under optimum conditions to the CHPPM delta pH assay, examine the variability and reliability of both assays, establish baseline values and generate conversion factors to enable comparisons between them and other proposed or commercial assays.

Normal Range of AChE

The availability of a large number of RBC AChE samples from CHPPM provided an opportunity to establish a contemporary normal range of human ChE values. With the collaboration of epidemiologist-physician Dr. Stephen McCurdy (UCD), AChE determinations of blood specimens from 991 Department of Defense personnel not exposed to anti-ChE agents were statistically analyzed. The median age of the subjects was 42 years (range 18-76). The majority of specimens (823, 82.1 %) were from men. Men were on average older than women (median age 44 vs. 36 years, $p < 0.001$, Wilcoxon test). The mean $\pm$ SD for the CRL delta pH assay was 0.74 ± 0.06 units. Delta-pH values were slightly greater for men than for women (0.75 vs. 0.74, $p < 0.001$, Wilcoxon test). Multivariate linear regression analysis showed an association for delta pH with age (slope $+0.0008$ delta-pH units for each year of age, $p < 0.001$). There was a small, but statistically significant, reduction in delta pH ChE associated with time (-0.006 delta pH units per 100 days, $p < 0.001$). A multiple regression model incorporating age, gender, and test date explained only 3.4% of the observed variance. The small magnitude of these effects and their minimal role in accounting for the observed variability suggested it was appropriate to ignore them when evaluating delta-pH data (Arrieta *et al.*, 2009).

Interlaboratory RBC Comparison

Seven sets of 20 RBC samples each were sent by CHPPM to UC Davis. Each laboratory determined the ChE activity with its method. The comparisons are shown in Figures 1-7. Four of the seven sets (1, 2, 4 and 6) had good correlations ($r \geq 0.8$) and similar slopes (Table 1).

Table 1. Comparison of AChE Measurements at CHPPM and UC Davis

Sample Set	Slope	r^2
1	0.047	0.74
2	0.045	0.79
3	0.028	0.25
4	0.048	0.80
5	0.025	0.34
6	0.039	0.64
7	(-) 0.016	0.14

Slopes are ratio of delta pH/Ellman AChE activities.

A concern that whether a delay of one day before the samples were assayed made a difference was shown not to be the case (Table 2).

Table 2. Comparison of Same Day and Next Day AChE Measurements

Sample Set	Slope	r ²
6 - Same Day	0.039	0.64
6 - Next Day	0.046	0.67
7 - Same Day	(-) 0.016	0.14
7 - Next Day	(-) 0.027	0.24

Slopes are ratio of delta pH/Ellman AChE activities.

The interlaboratory comparison of set seven of the samples showed an anomaly: an inverse relationship (negative slope in the linear regression) and these values were not used in subsequent analyses. The mean AChE levels ($\pm$ SD, standard deviation) for the remaining 120 samples were: 8.29 ± 1.17 $\mu\text{mol}/\text{min}/\text{ml}$ RBC for the UC Davis determinations; and 0.76 ± 0.06 delta pH/hr for the CHPPM determinations.

Intralaboratory RBC Comparison

Comparisons of the CHPPM delta pH assay and the UCD Ellman assay carried out on split RBC samples shipped to UCD by CHPPM were inconsistent. Instead, to reduce variability, blood from volunteers was collected and both assays were run at UCD. RBCs were prepared following the procedures used by CHPPM (which did not include washing the RBCs after the plasma fractions were removed.)

The influence of the presence of plasma in the samples was checked by comparing washed and unwashed RBCs, and using specific ChE inhibitors (Figure 8). Washing had no effect on the ChE activity; the specific AChE inhibitor, BW284c51, inhibited the ChE activity in the RBC preparation ($p < 0.01$, ANOVA) and the specific BuChE inhibitor, quinidine, slightly inhibited RBC activity ($p < 0.01$, ANOVA), while inhibiting almost all ChE activity in plasma ($p < 0.01$, ANOVA).

Substrate concentration curves were generated to check whether the assays were being performed under optimal conditions. The UCD Ellman assay is run with 1 mM acetylthiocholine iodide substrate and the CRL delta pH assay is run with 10 mM acetylcholine bromide substrate. Under Ellman assay conditions, RBC AChE activity showed the expected excess substrate inhibition: activity decreased when the substrate concentration was 5 mM or greater (Figure 9). There was also a decrease in RBC AChE activity in the delta pH assay, but not until the substrate concentration exceeded 10 mM (Figure 10). There was no decrease in plasma BuChE activity with substrate concentrations up to 20 mM in the delta pH assay.

Since the delta pH is an end point assay and the Ellman assay is continuously recorded, the question arose whether the time course of the delta pH assay was linear during the seventeen minutes of the assay. The results of recording pH values during the time course of the assay (Figure 11) indicated the assay proceeded linearly.

We approached the conversion of RBC AChE activity units between assay methods in two ways:

Conversion of Activity Units via Interlaboratory Measurements

The paired results of AChE activities determined by UCD (Ellman) and CHPPM (delta pH) were plotted (Figure 12). Linear regression analysis yielded a conversion of:

$$\text{Ellman activity} = 15.0 \times \Delta\text{pH} - 3.06.$$

Conversion of Activity Units via Intralaboratory Measurements

RBC preparations were treated with varying concentrations of the ChE inhibitor diisopropyl fluorophosphate (DFP) to mimic OP-exposed samples. (DFP was chosen because it does not require metabolic activation to be an effective ChE inhibitor.) Whole blood from 5 volunteers was treated with DFP prior to centrifugation, and the RBCs were washed to remove residual DFP before being measured. The DFP inhibition curves for both assays are shown in Figure 13 and the resulting activities plotted against each other (Figure 14). Linear regression yielded a conversion of :

$$\text{Ellman activity} = 14.4 \times \Delta\text{pH} - 0.79.$$

The conversion factors derived from linear regression analysis are summarized in Table 3.

Table 3. AChE Assay Conversion Factors

Method	Regression	n	r ²
Delta pH vs Ellman (CHPPM /UCD)	$E = 15.0 \times \Delta\text{pH} - 3.06$	120	0.53
Delta pH vs Ellman (UCD)	$E = 14.4 \times \Delta\text{pH} - 0.79$	40*	0.92
Test-mate vs ΔpH	$T-M = 47.1 \times \Delta\text{pH} - 6.49$	40*	0.99
Test-mate vs Ellman	$T-M = 3.02 \times E - 2.07$	40*	0.91

AChE activity units: Delta pH ($\Delta\text{pH/hr}$); Ellman ($\mu\text{mol/min/ml RBC}$);

Test-mate (T-M; $\mu\text{mol/min/g Hb vs delta pH}$ and $\mu\text{mol/min/ml vs Ellman}$)

* 40 samples: 5 individual blood samples x treatment at 8 [DFP]

The factors were applied to the 991 CHPPM delta pH assay determinations to convert the activities into Ellman units ($\mu\text{mol/min/ml}$). The means and ranges of the converted CHPPM data are presented in Table 4.

Table 4. AChE Activity of DOD Workers Converted to Ellman Assay Units

Method	n	Mean	SD	Min	Max
Interlaboratory CHPPM vs UCD Conversion	991	8.11	0.67	6.3	10.7
Intralaboratory DFP Inhibition Conversion	991	9.94	0.89	6.8	15.6

AChE activity is $\mu\text{mol/min/ml}$.

Min and Max represent the 95% limits of the population.

The distribution of the normal AChE range (from 991 CHPPM delta pH determinations) was converted into Ellman AChE activity units by the Interlaboratory Conversion (Figure 15).

Task Two. Task Two was to test the stability of an RBC ghost standard and its usability for clinical standardizations.

RBC Standard Characterization

Bovine blood contains only AChE, which is mostly in the RBCs (unlike human blood which has AChE in the RBCs and BChE in the plasma). The lack of serum ChE activity was recognized as early as the 1950s (Hermenze and Goodwin, 1959) and confirmed by us by showing the insensitivity of bovine blood to iso-OMPA (a specific BChE inhibitor) and its sensitivity to BW 284c51 (a specific AChE inhibitor) (Table 5), suggesting bovine blood might be a good source of AChE for a standard.

Table 5. Cholinesterase Activity of Bovine Blood and Its Fractions

Treatment	Whole Blood	RBC	Plasma
None	2.23 $\pm$ 0.59	2.10 $\pm$ 0.62	0.107 $\pm$ 0.033
10-4 M iso-OMPA	2.17 $\pm$ 0.53	1.92 $\pm$ 0.54	0.103 $\pm$ 0.31
5 x 10-5 M BW284c51	-0.28 $\pm$ 0.01	-0.057 $\pm$ 0.01	0.0087 $\pm$ 0.018

Activity = umol/min/ml; mean $\pm$ SD; n = 6

We found that RBC ghost AChE activity prepared as described in the methods section fell off at 4°C after 50 days but was stable for at least a year when stored at -70°C (Figure 16; Arrieta *et al.*, 2003).

Laboratory Comparisons

We attempted to use the ghost RBC standard to compare the delta pH method at CHPPM with the Ellman method at UCD. The ghost RBC preparation was diluted to 100, 75, 50, 25 and 10% of stock concentration. Sets of samples were sent from UC Davis to CHPPM and also from UC Davis to UC Davis to control for shipping. The samples were assayed at each laboratory using their respective methods. Unfortunately, the activity level in the samples was too low to be tested at CHPPM, suggesting the delta pH method was less sensitive than the Ellman colorimetric method. Since 2002, a RBC standard has been included with each run conducted at UC Davis. The activity level of new preparations of the RBC standards has been increased so that they are within the sensitivity of clinical laboratory assays. Recently, we have been sending samples of the preparation to PAML, a clinical laboratory in Spokane, Washington, to be used as a standard for inclusion with their modified Ellman assays of blood from orchard workers (Wilson *et al.*, 2009). Unfortunately, an increased workload at CRL, in addition to turn-over of their personnel, led to the cessation of further testing of RBC standards between the two laboratories.

Task Three. Task Three was to conduct experiments with a specially designed Test-mate Kit with an uncorrected read out so we could establish the conditions needed for an optimum assay and conversion factors to harmonize its results with clinical laboratory assays.

Patrick Eberly, CEO of EQM Research, Inc informed us that a new, temperature regulated model (Test-mate CHE version D) was nearing the end of development and would be available for us to evaluate (letter attached as an appendix to this report). Unfortunately, the instrument never materialized. Eberly told us the instrument had not been manufactured due to issues related to designing it to meet FDA approval.

In place of the unavailable experimental Test-mate model, we studied the Model 400, the instrument available commercially today (Oliveira *et al.*, 2002). Results on the 400 model showed that, as indicated in its instruction manual, it will not function well under field conditions and should be used by a trained technician in a laboratory setting. Since the Model 400 is in use by the military, we proceeded to establish a conversion factor with the delta pH method.

We carried out our comparison using blood from 5 volunteers. The samples were inhibited by a range of DFP concentrations and assayed by the delta pH, Test-mate and Ellman methods.

Comparison of the Test-mate and delta pH methods is shown in Figure 17. Linear regression yielded a conversion of:

$$\text{T-M Activity} = 47.1 \times \Delta\text{pH} - 6.49.$$

When this conversion factor was combined with the 991 delta pH values from CHPPM used earlier in the project, we obtained a range of Test-mate values of 18.4 to 47.2 $\mu\text{mol}/\text{min}/\text{g Hb}$ with a mean of $28.6 \pm 2.90 \mu\text{mol}/\text{min}/\text{g Hb}$.

A comparison between the Test-mate and Ellman assays is shown in Figure 18. Linear regression yielded a conversion of :

$$\text{T-M Activity} = 0.409 \times \text{E} - 0.33.$$

The conversion factors derived from linear regression analysis are summarized in Table 3 (see Task One above).

Task Four. Task Four was to explore the feasibility of incorporating BChE variant and PON1 polymorphisms into a screen using a selected set of DOD personnel.

Unfortunately, the technical requirements were beyond the capabilities of CHPPM and the project was set aside. The conditions of blood collection specified by CHPPM are geared to RBCs and not whole blood or plasma as required for the BChE and PON1 analyses. We contacted Captain Gull, the director of CHPPM, and Major Lefkowitz, the former manager, to determine if such a screen could be practically implemented. One approach would be to use the colorimetric two substrate (diazinon-oxon and paraoxon) PON1 assay (Richter, *et al.*, 2004, Costa, *et al.*, 2005a). This would require procedural

changes in how blood was collected and handled at CHPPM before any new assays could be carried out. Plasma would need to be collected separately; EDTA collection tubes could not be used due to interference with the PON1 assay. Also, we do not know whether additional equipment might be necessary for CHPPM to carry out these assays. Given the status of the project and the workload of CHPPM we regretfully reached the conclusion that the project was not feasible within the scope of this grant.

KEY RESEARCH ACCOMPLISHMENTS

- ◆ The delta pH data stored by CHPPM was used to establish a normal range of human RBC AChE activity.
- ◆ A conversion factor was calculated between Ellman and delta pH determinations of ChE.
- ◆ The conversion factor was used to calculate a normal range of human RBC AChE values in Ellman units.
- ◆ The Ellman assay substrate inhibition concentration was confirmed to be above 2-3 mM ACTh.
- ◆ The delta pH assay demonstrated a substrate inhibition concentration in excess of 10mM.
- ◆ The end-point delta pH method of CHPPM was linear over time, but it was also less sensitive than the colorimetric kinetic Ellman-based AChE method used in our laboratory.
- ◆ A bovine RBC ghost AChE standard was developed.
- ◆ The RBC AChE standard was stable for more than a year when stored at -70°C.
- ◆ The standard is used routinely in our assays and by PAML, a commercial clinical laboratory in the state of Washington.
- ◆ A conversion factor for AChE activities was developed between the CHPPM delta pH and Test-mate assays, methods currently used by the military.
- ◆ The data were used to generate a normal range of human RBC values in Test-mate units that compared favorably to the values given in the EQM Instruction manual.

REPORTABLE OUTCOMES

Publications

D. Arrieta, A. Ramirez, E. DePeters, D. Bosworth, and B.W. Wilson. 2003. Bovine red blood cell ghost cholinesterase as a monitoring standard. *Bulletin of Environmental Contamination and Toxicology* 71(3), 447-452.

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Stamler C J, Arrieta D E, Basu N, Henderson J D, Wilson BW, Chan HM. 2006. Evaluation of a fluorescent method for measuring cholinesterase activity in mammalian blood and tissue. *Bulletin of Environmental Contamination and Toxicology* 77(6):785-792

Arrieta DE, McCurdy SA, Henderson JD, Lefkowitz LJ, Reitstetter R, and Wilson BW. 2009. Normal range of human red blood cell acetylcholinesterase activity. *Drug and Chemical Toxicology*. 32:182-185.

Presentations and Abstracts

Wilson BW. Monitoring Cholinesterase in Exposed Workers. Northwest Center for Occupational Health and Safety, Seattle, Washington March 8, 2002. (Invited Lecture)

Wilson BW, Henderson JD, Ramirez A, Kayton R and Spencer P. Low level effects of pyridostigmine bromide and delayed neuropathy organophosphates in experimental animals. *Bioscience 2002 Review*, June 2-7, 2002. (Poster)

Wilson BW, Henderson JD, Ramirez A, O'Malley MA and Arrieta D. Standardization of Clinical Cholinesterase Measurements. *Cultivating Collaborations: Health and Safety in Western Agriculture*, September 16-18, 2002 Coeur d'Alene, ID (Poster)

Wilson, B. Slippery Slopes on the Cholinergic Highway. North American Congress of Clinical Toxicology, Palm Springs September 24-29, 2002 (Invited lecture)

Wilson BW, JD Henderson, DE Arrieta, SA McCurdy and RE Reitstetter. Conversion of Delta pH and Ellman Values for Cholinesterase. Presented at the 42nd Annual Meeting of the Society of Toxicology; March 9-13, 2003; Salt Lake City, Utah.

S.A. McCurdy, J.D. Henderson, D.E. Arrieta, L.J. Lefkowitz, R.E. Reitstetter, and B.W. Wilson. 2003. Normal range of cholinesterase levels among US Defense Department personnel. Poster presented at NIOSH Conference, November, 2003 San Francisco, CA.

McCurdy SA, JD Henderson, DE Arrieta, LJ Lefkowitz, RE Reitstetter, and BW Wilson. Determining a Reference Value for Blood Cholinesterase using US Defense Department Personnel. Presented at the 43rd Annual Meeting of the Society of Toxicology; March 21-24, 2004; Baltimore, Maryland.

Arrieta DE, VM Nihart, JD Henderson, SA McCurdy, RE Reitstetter, LJ Lefkowitz and BW Wilson. Comparison of Delta pH and Ellman Colorimetric Cholinesterase Assays. Presented at the Bioscience 2004 Medical Defense Review; May 16-21, 2004; Hunt Valley, Maryland.

Arrieta DE, VM Nihart, JD Henderson, RE Reitstetter, LJ Lefkowitz and BW Wilson. Comparing Cholinesterase Assays used to Detect Pesticide Exposure and Chemical Terrorism. Presented at the Annual National Institute for Occupational Safety and Health

Meeting: “Cultivating a Sustainable Agricultural Workplace”; September 11-14, 2004; Troutdale, Oregon.

Wilson BW, JD Henderson, DE Arrieta, A Ramirez, VM Nihart, MA O’Malley, SA McCurdy and LJ Lefkowitz. Standardizing Clinical Cholinesterase Monitoring for Pesticide Applicators and Chemical Terrorist Episodes. Presented at VIII International Meeting on Cholinesterases; September 26-30, 2004; Perugia, Italy.

Wilson BW, JD Henderson, DE Arrieta, VM Nihart, MA O’Malley, SA McCurdy and LJ Lefkowitz. Monitoring Cholinesterases: Bench to Application. Presented at 25th Annual Meeting of the Southeast Pharmacology Society; November 4-5, 2004; Oxford, Mississippi.

Arrieta DE, VM Nihart, JD Henderson, SA McCurdy, LJ Lefkowitz and BW Wilson. A Conversion Factor Between Two Cholinesterase Assays and Its Application in Establishing Normal Range for Human RBC AChE. Presented at the 44th Annual Meeting of the Society of Toxicology; March 6-10, 2005; New Orleans, Louisiana. The Toxicologist, Vol 84, Number 5-1, Abstract No. 1278.

Wilson BW, JD Henderson and DE Arrieta. Cholinesterase Assays as Indicators of Exposure. Presented at 229th American Chemical Society National Meeting; March 13-17, 2005; San Diego, California.

Wilson BW, VM Nihart, JD Henderson, A Ramirez and DE Arrieta. Monitoring Cholinesterases: An Example of Translational Research. Presented at the 45th Annual Meeting of the Society of Toxicology; March 5-9, 2006; San Diego, California.

Arrieta DE, VM Nihart, JD Henderson, A Ramirez, LJ Lefkowitz and BW Wilson. Monitoring Cholinesterases: Progress Toward a Normal Human Range and Conversion Factors. Presented at the Bioscience Review; June 4-9, 2006; Hunt Valley, Maryland.

CONCLUSIONS

There has been much concern about possible terrorist biological warfare attacks on civilian such as with anthrax. Surprisingly, there has been little public alarm expressed concerning the possibility of using pesticides as chemical weapons even though hazardous pesticides such as parathion and aldicarb are readily available in quantity throughout the world and can be delivered to populated areas by means as simple as a crop duster. Military agents such as soman and sarin are not necessary to mount demoralizing and lethal attacks against civilian targets. Anti-ChE pesticides are already “weaponized” and ready to go, making it important to prepare first responders with sufficient means to treat more than a few farm workers at a time and to quickly detect that anticholinergic weapons have been used. Given such a scenario, there are concerns over the lack of standardization of ways to rapidly detect the use of anti-ChE pesticides as terrorist agents and to be prepared to respond to them.

“The devil is in the details” is a cautionary cliché that exemplifies the unglamorous tasks of this project, examining the methodology of ChE testing to see whether direct comparisons between Ellman and delta pH determinations provide bases for a first pass at standardization.

Comparison of ChE results from Ellman and delta pH human blood assays yielded an approximation of normal human ranges of ChE activity. This information should be of much value to government and clinical laboratories concerned with terrorism and public health. There are no national standards for blood AChE or BChE determinations suitable for use by first responders. Methodologies and facilities exist, but national standards do not.

We collaborated with the California (CA) Department of Pesticide Regulation (DPR) and the NIOSH Western Center for Agricultural Health & Safety at UC Davis. (Wilson *et al.*, 2004) to improve the long-standing formal blood ChE monitoring program of California for mixers, loaders and applicators performed by several clinical laboratories in the state. When CA specified use of the Ellman assay or its equivalent to continue in the program (Wilson *et al.*, 2004), the results were striking: of the fourteen laboratories that participated; 9 met the ChE criteria for whole blood, 14 for plasma and 6 for RBCs. Based on such data, on July 8, 2003, DPR notified the CA Agricultural Commissioners that 9 of the participating laboratories were approved for ChE testing. Later work resulted in acceptable RBC values for 2 more of the laboratories. Currently, discussions are underway to periodically retest the commercial laboratories.

The Ellman AChE assays performed here displayed a classic substrate concentration inhibition curve with acetylthiocholine concentrations of 2-5 mM or greater (Wilson *et al.*, 1995); a similar effect on the delta pH assay did not occur until higher concentrations. The reasons were unclear. Washing the RBC delta pH preparation ruled out a significant contribution of plasma to the overall ChE activity of the unwashed RBC delta pH preparation. Furthermore, the substrate concentration curve was not changed when acetylthiocholine (the Ellman substrate) was substituted for acetylcholine in the delta pH assay (data not shown). Perhaps the difference in the curves may be a problem of methodology: the Ellman assay is kinetic, the delta pH assay is an endpoint determination. Another possibility is that high acetylcholine concentrations may change the configuration of the enzyme itself.

Regardless, given the continuing dangerous state of international affairs, consideration should be given to CHPPM or an equivalent laboratory taking on the role of a national standard laboratory that can perform both Ellman based and delta pH assays and provide standards to state and clinical laboratories.

The ghost RBC standard we developed was useful in our ChE method comparisons, such as with the Sigma ChE kit (Wilson *et al.*, 2002), and in quality assurance routines. Currently, a sample of the standard is included with each run conducted at UC Davis and at PAML.

A research group at the WRAIR laboratory has patented a ChE method using the thiocholine biochemistry of the Ellman assay. (Feaster *et al.*, 2001). They have published a paper comparing it, the delta pH, the Test Mate and the Ellman assay (Haigh *et al.*, 2008) from which conversion factors could be derived.

The conversion values for delta pH and Ellman assays permit using the large CHPPM data base to establish a normal range for human RBC AChE in Ellman units, one of the goals of the project. Most clinical laboratories use assays based on the Ellman assay and rely either on unvalidated values listed in the commercial kits or their own records to establish a normal range to decide whether exposures have occurred in the absence of individual baseline data. It is interesting that our preliminary normal range of RBC AChE activity (converted from the CHPPM delta pH values) of 6.3 to 10.7 umol/min/ml is very close to the range of 6.71 to 10.0 quoted in the Roche cholinesterase kit (Roche Diagnostics, 2001). Yeary, *et al.* (1993) lists 7.86–12.9 umol/min/mL at 30°C. The range is expected to be higher when the assay is performed at a higher temperature, as with Yeary (30°C) compared to our range (25°C). The Roche range (37°C) is lower than might be expected, probably due to their suboptimal assay conditions which result in 40% lower activity (Wilson, *et al.*, 1995).

The conversion of the CHPPM delta pH normal range to Test-mate units gave a mean of 28.6 ± 2.9 umol/min/g Hb, and a range of 18.4 – 47.2. This compares favorably with the values for the Test-mate in its instruction manual of 27.1 ± 2.9 , and a range of 21.9 – 37.3 (n = 40; EQM Research, Inc., 2003).

So far as we know, the Army is continuing to use the Test-mate kit as a field kit. We used the current Model 400 kit at constant temperature as directed by the FDA approved instructions. The Army evaluation study of the Model 400 (Taylor *et al.*, 2003) used a mobile field laboratory with controlled temperatures. The Army's technical bulletin for the Test-mate (available on the USAMRICD website) describes the use of the older OP Kit model in the outdoors rather than in the specified field laboratory setting (TB MED 296).

The conversion factors derived from our delta pH and Ellman comparisons with the Test Mate should be used with caution. Sample sizes were small, and the Test-mate is a “black box” instrument. It does not display raw data basing its results on a complicated algorithm. For example, it includes displaying “activities” corrected to “room temperature” via factors that are not made clear. It is unfortunate that EQM Research, Inc. was unable to provide the instrument we needed to track down the sources of their results.

The conversion values for delta pH and Test-mate assays determined here permit using the large CHPPM data base to establish a normal range for human RBC AChE in Test-mate units. Whether or not the Test-mate is used to establish a baseline for each soldier in the field, this normal range is an important resource when no previous background measurements are available.

We appreciate the encouragement and assistance of the leadership of CHPPM, especially given the frequent turnover of personnel during the course of our project. We gratefully acknowledge the assistance of then Captain Lefkowitz and the continuing involvement of then Captain Reitstetter in our research and analyses. We thank Ms. Donna Goodman (CHPPM) for her able assistance during the project.

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APPENDICES

Figure 1. RBC Cholinesterase Assay Comparison: Sample Set 1
Figure 2. RBC Cholinesterase Assay Comparison: Sample Set 2
Figure 3. RBC Cholinesterase Assay Comparison: Sample Set 3
Figure 4. RBC Cholinesterase Assay Comparison: Sample Set 4
Figure 5. RBC Cholinesterase Assay Comparison: Sample Set 5
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Figure 17. Test-mate vs. Delta pH Overall Comparison
Figure 18. Test-mate vs. Ellman Overall Comparison
Letter from EQM, Inc.

D. Arrieta, A. Ramirez, E. DePeters, D. Bosworth, and B.W. Wilson. 2003. Bovine red blood cell ghost cholinesterase as a monitoring standard. *Bulletin of Environmental Contamination and Toxicology* 71(3), 447-452.

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Figure 1. RBC Cholinesterase Assay Comparison: Sample Set 1

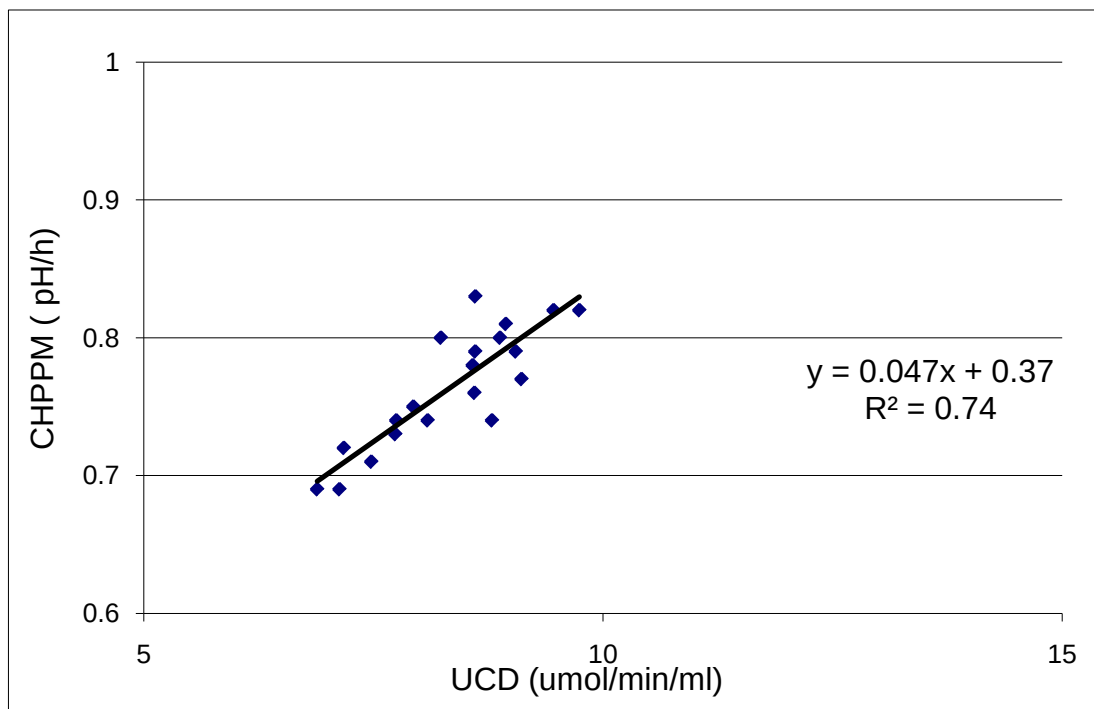


Figure 2. RBC Cholinesterase Assay Comparison: Sample Set 2

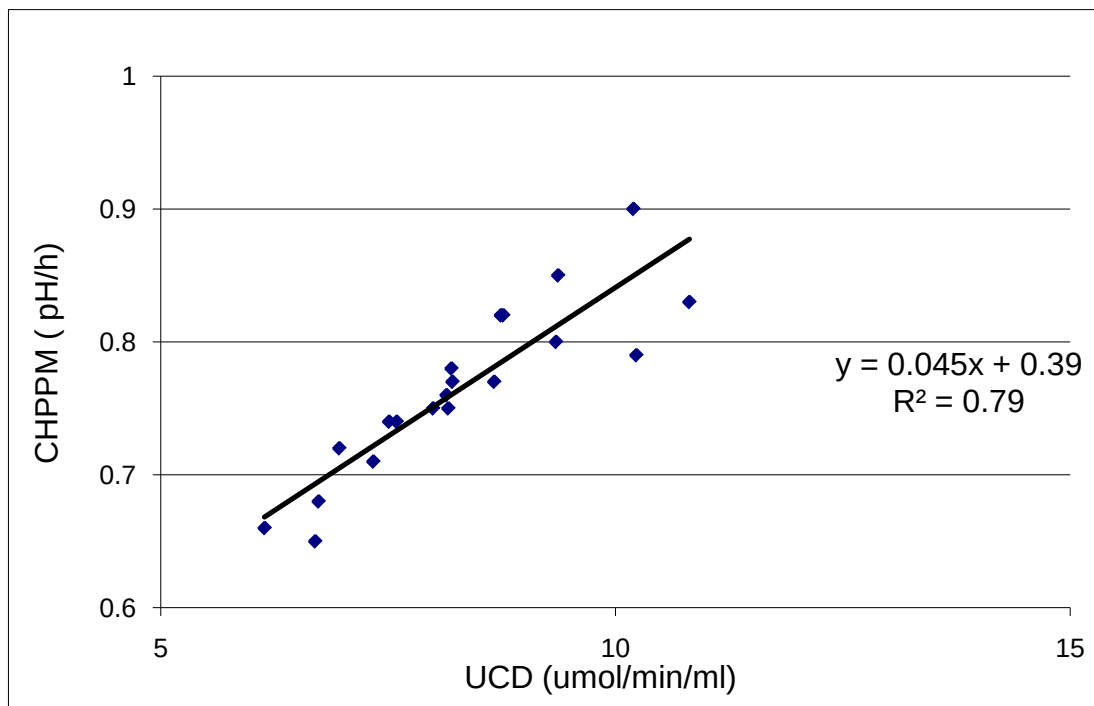


Figure 3. RBC Cholinesterase Assay Comparison: Sample Set 3

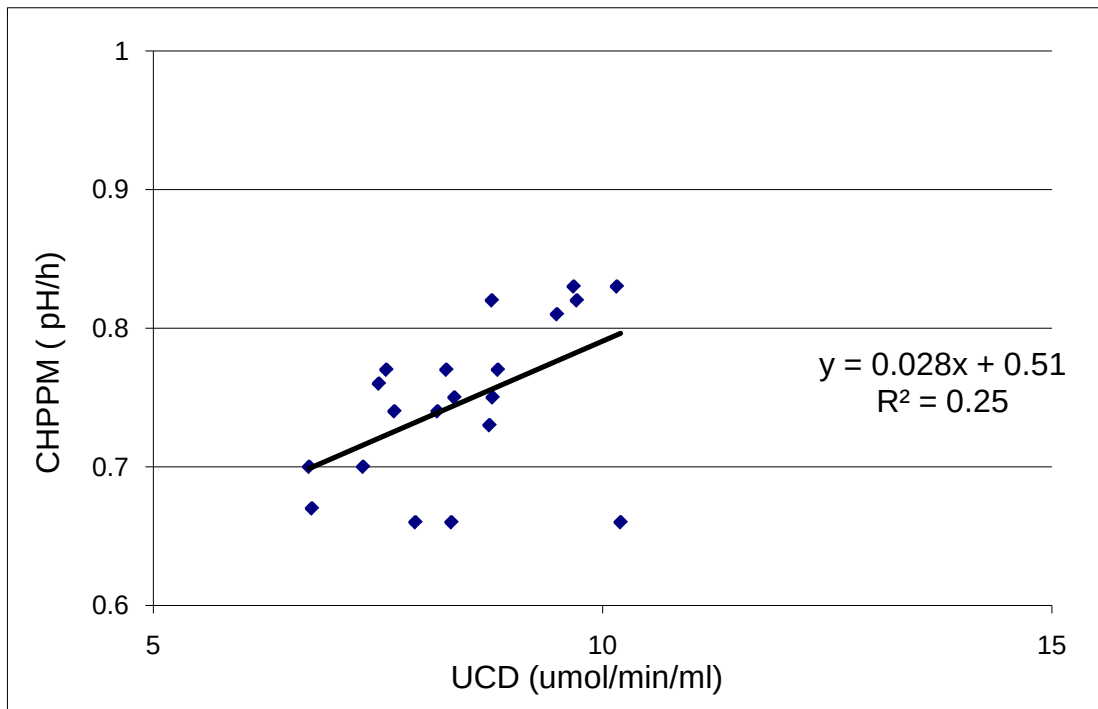


Figure 4. RBC Cholinesterase Assay Comparison: Sample Set 4

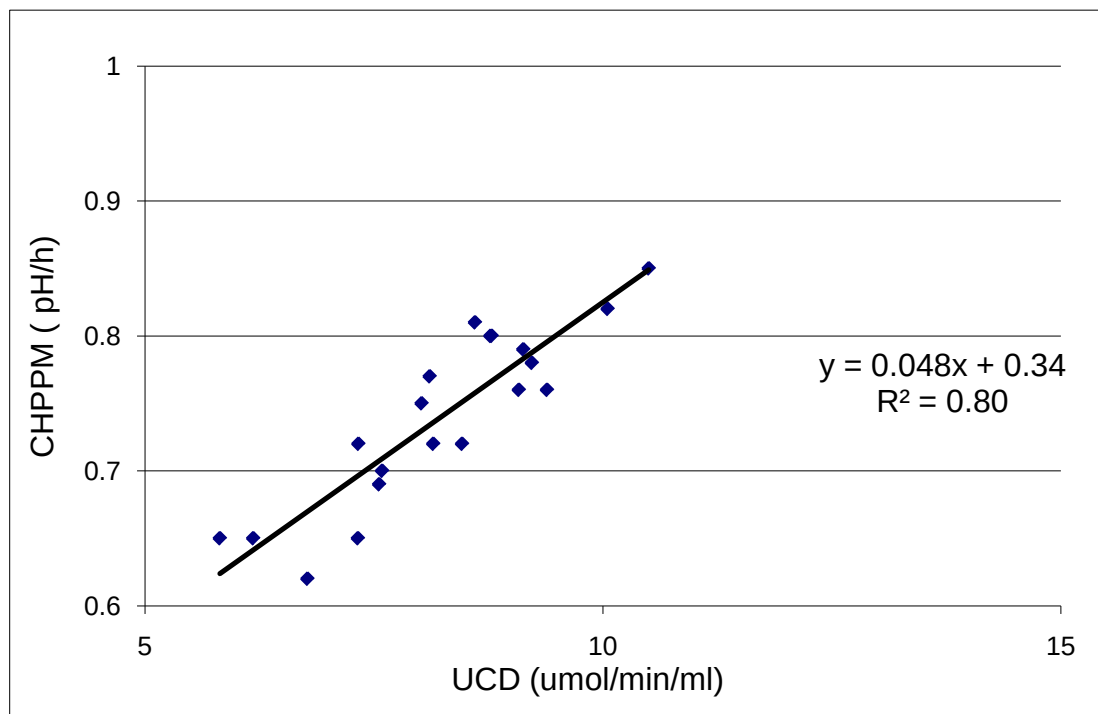


Figure 5. RBC Cholinesterase Assay Comparison: Sample Set 5

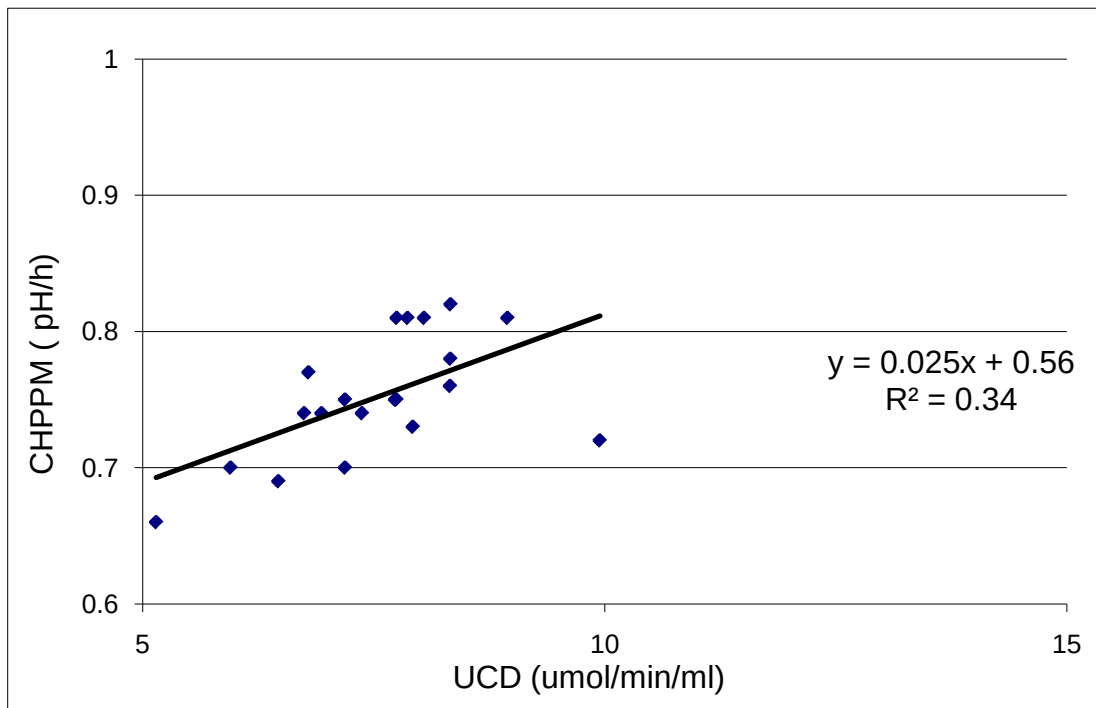


Figure 6. RBC Cholinesterase Assay Comparison: Sample Set 6

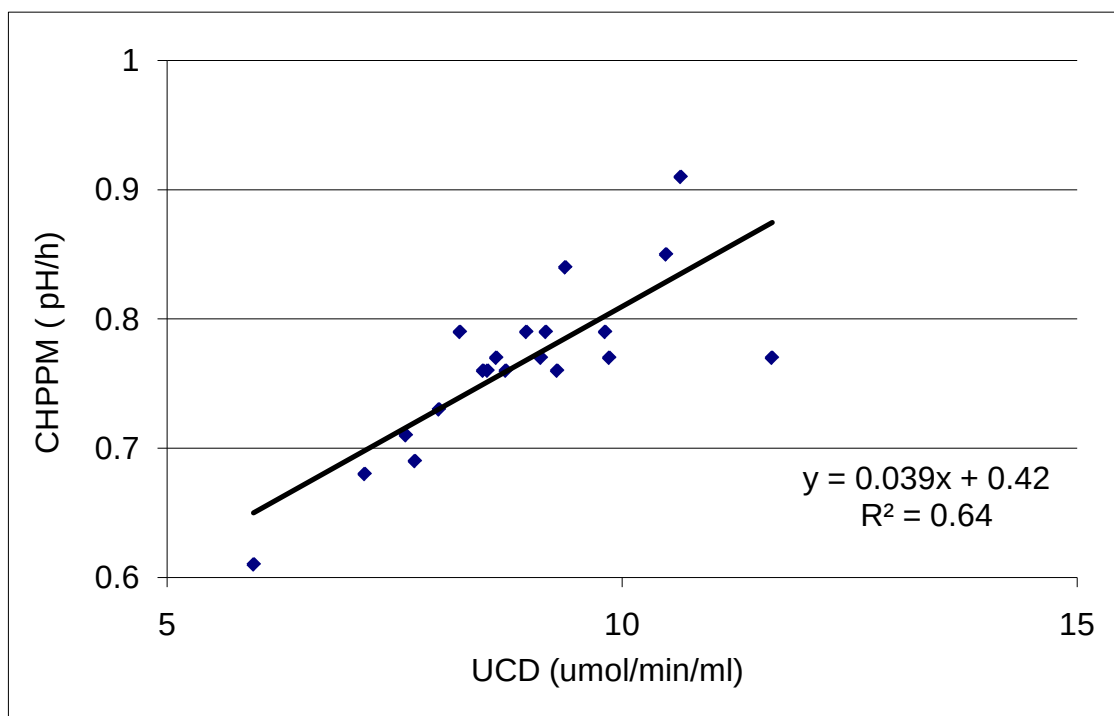


Figure 7. RBC Cholinesterase Assay Comparison: Sample Set 7

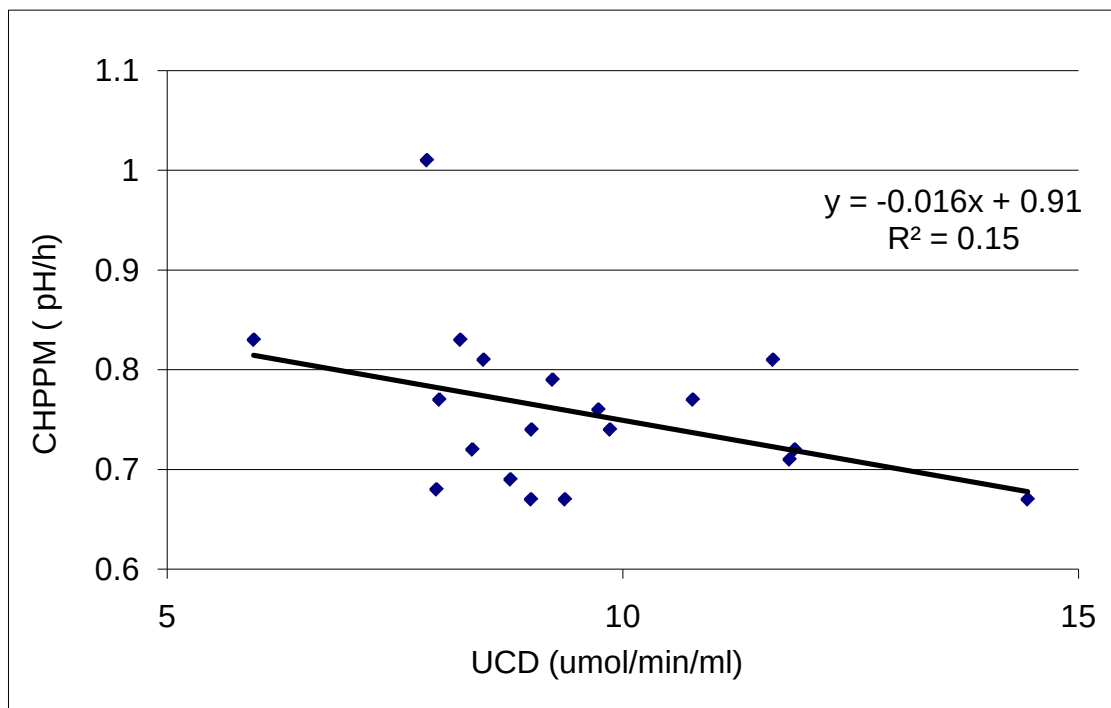
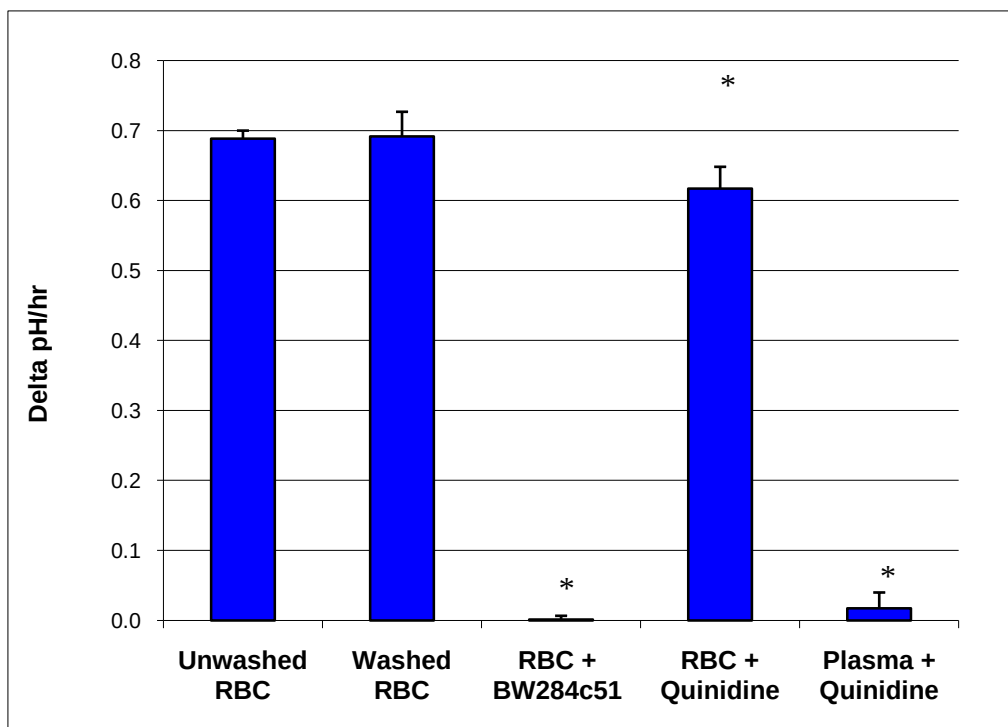


Figure 8. Effect of Specific ChE Inhibitors on Human Blood Fractions



ChE activity is mean $\pm$ sd; n = 3: washed/unwashed RBCs; 5: inhibitor-treated

* Different from unwashed RBC control (p < 0.01, ANOVA).

BW284c51 is a specific AChE inhibitor; Quinidine is a specific BChE inhibitor

Figure 9. Effect of Substrate Concentration in Ellman Assay

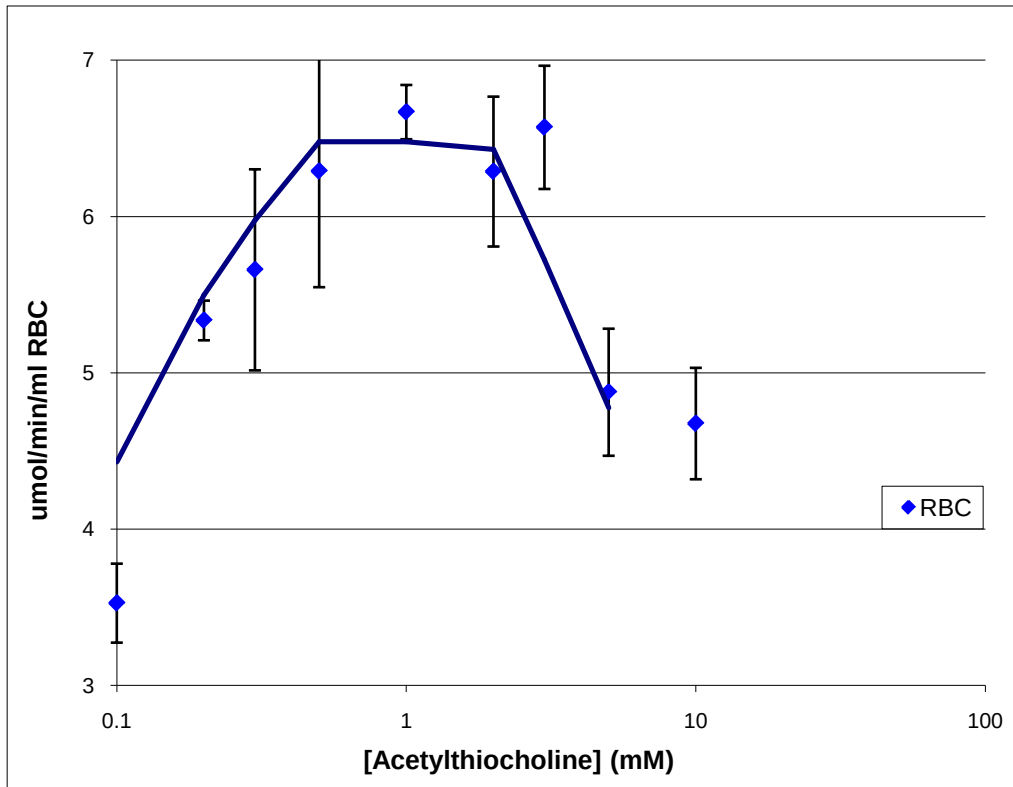


Figure 10. Effect of Substrate Concentration in Delta pH Assay

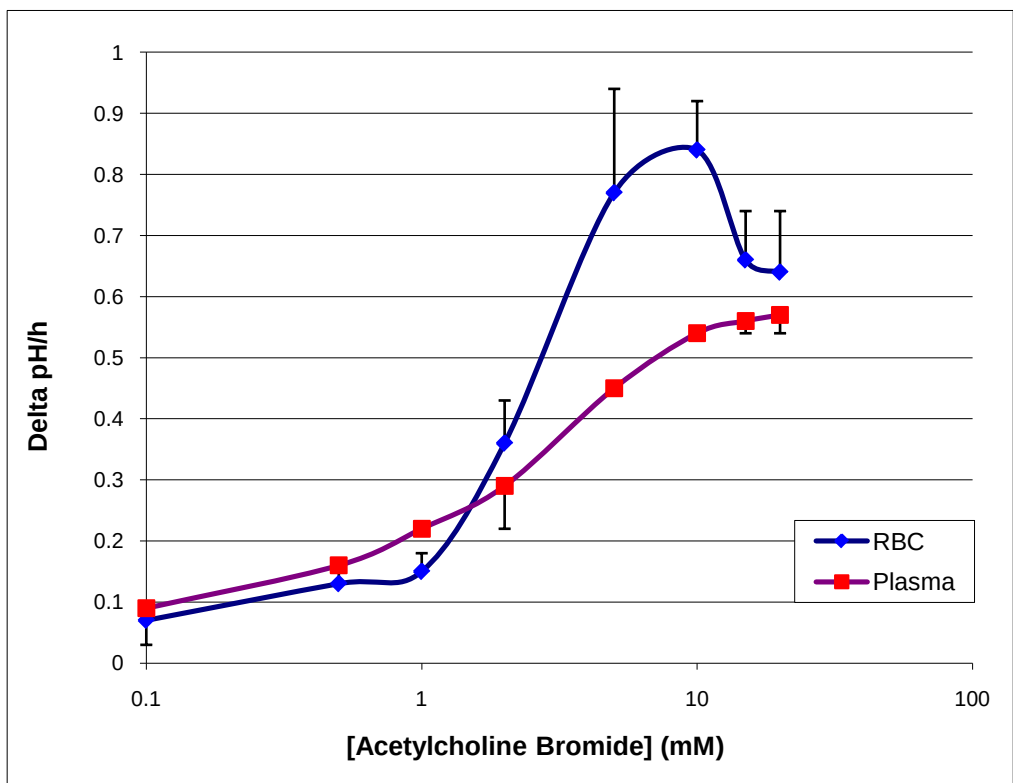


Figure 11. Kinetic Measurement of the Delta pH Assay

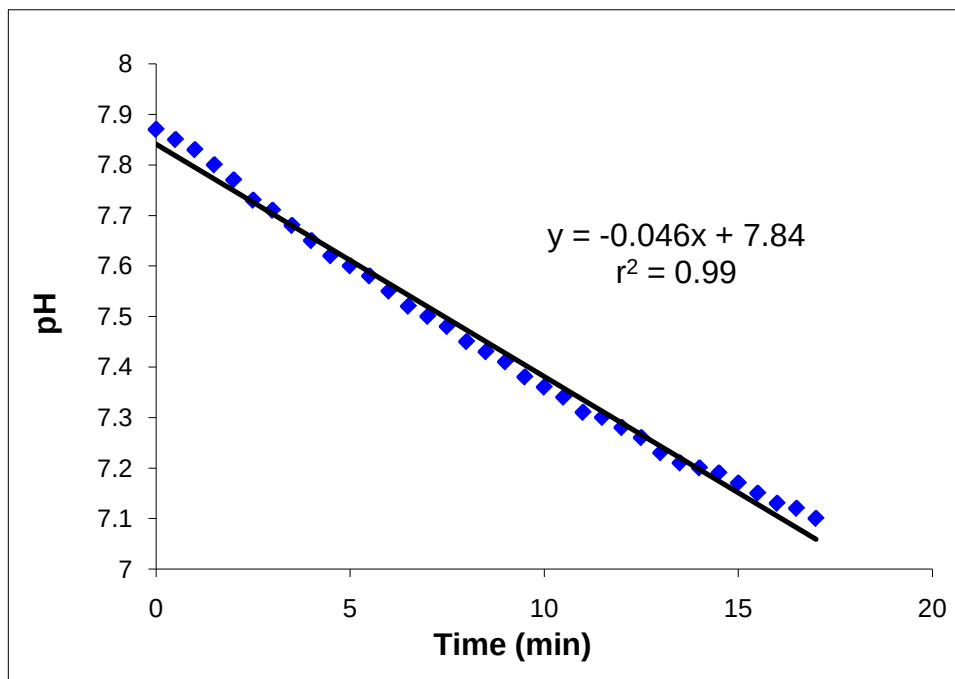
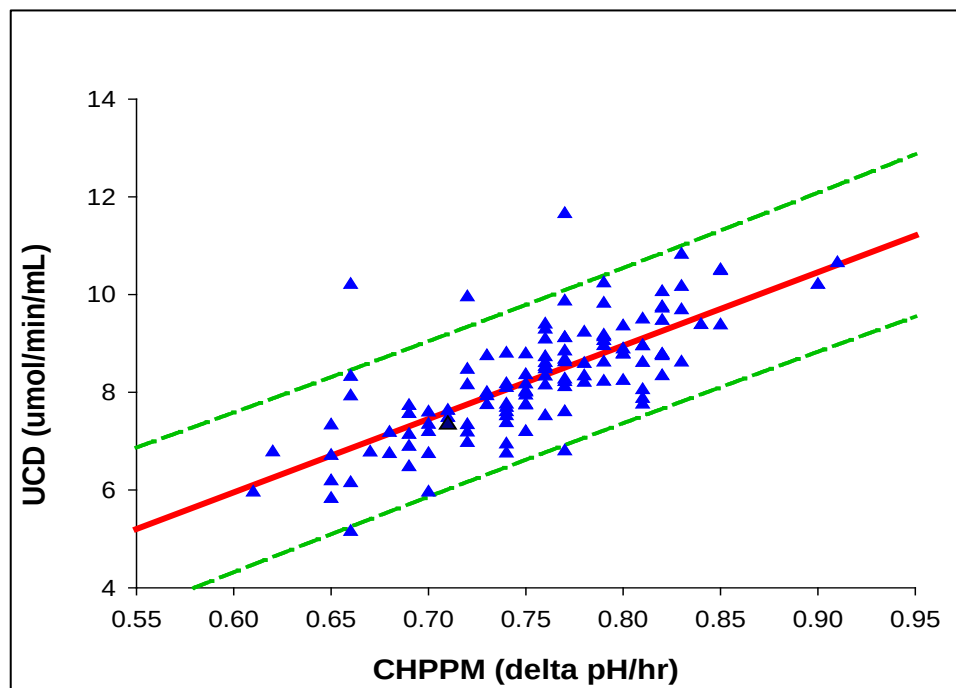


Figure 12. Comparison of CHPPM Delta pH Assay and UCD Ellman Assay



Split RBC samples, n = 120.

Figure 13. DFP Inhibition of RBC AChE

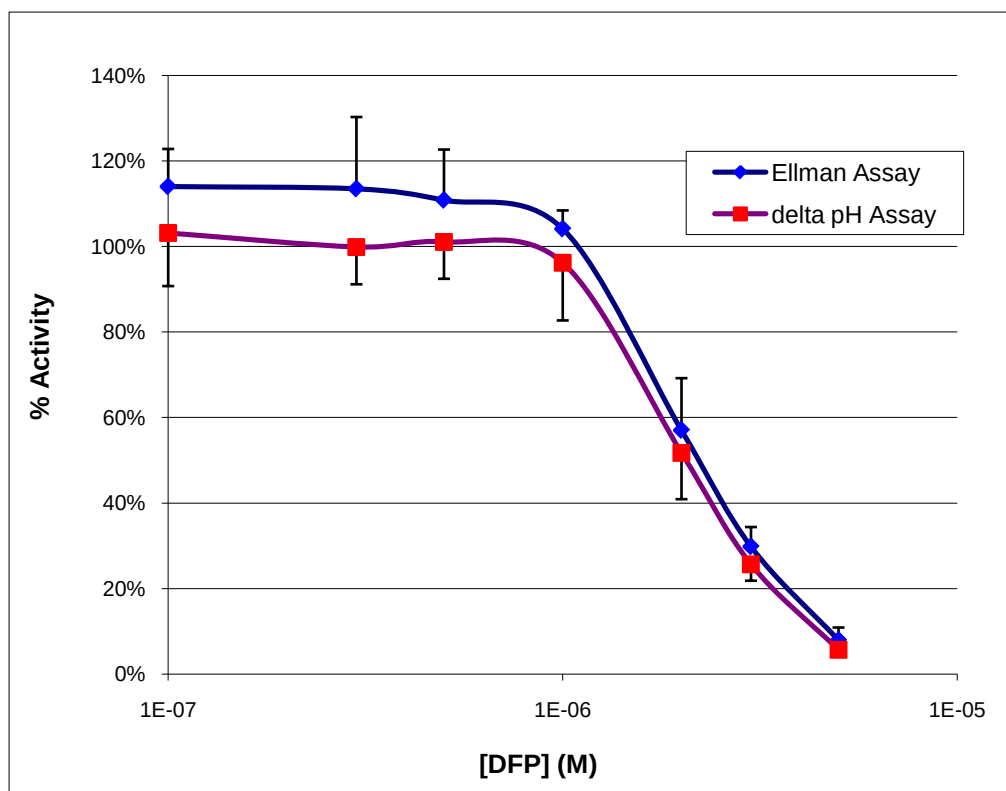


Figure 14. Ellman vs. Delta pH Intralaboratory Conversion

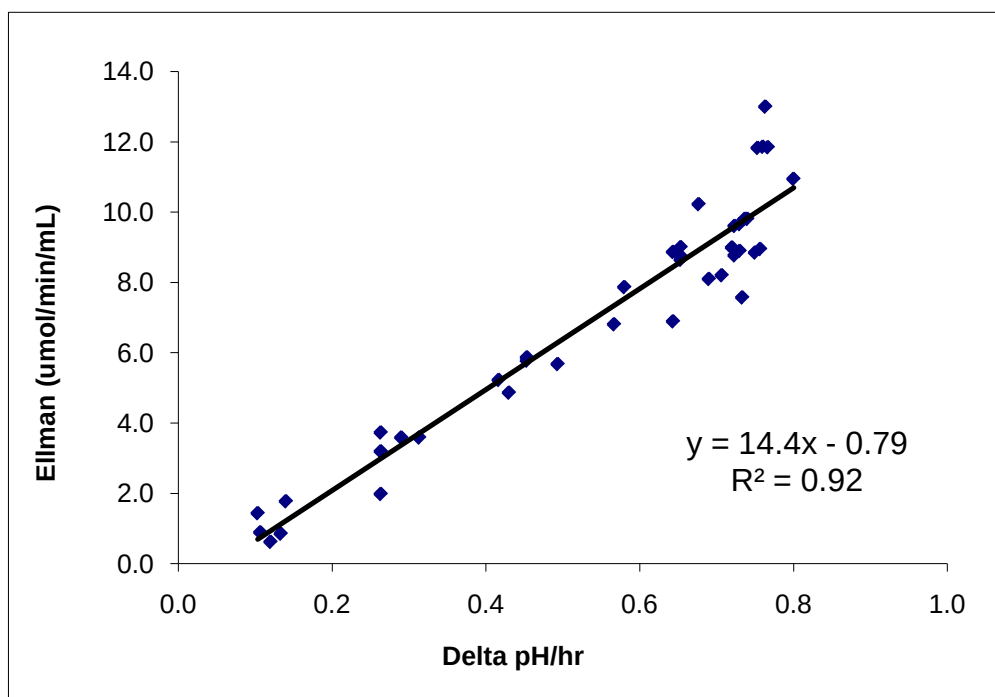
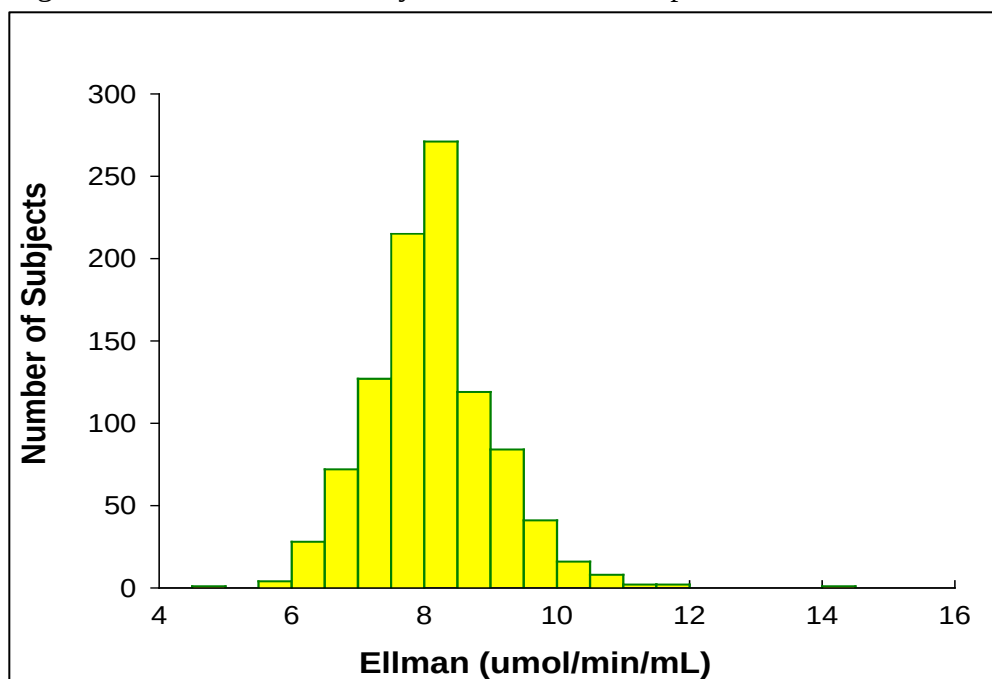


Figure 15. RBC AChE Activity Distribution: Delta pH to Ellman Conversion



ChE activity converted from delta pH to Ellman units:

$$\text{Ellman} = 15.0(\text{delta pH/hr}) - 3.06$$

Determinations from the DOD ChE monitoring program; n = 991.

Figure 16. Ghost RBC AChE Storage Stability

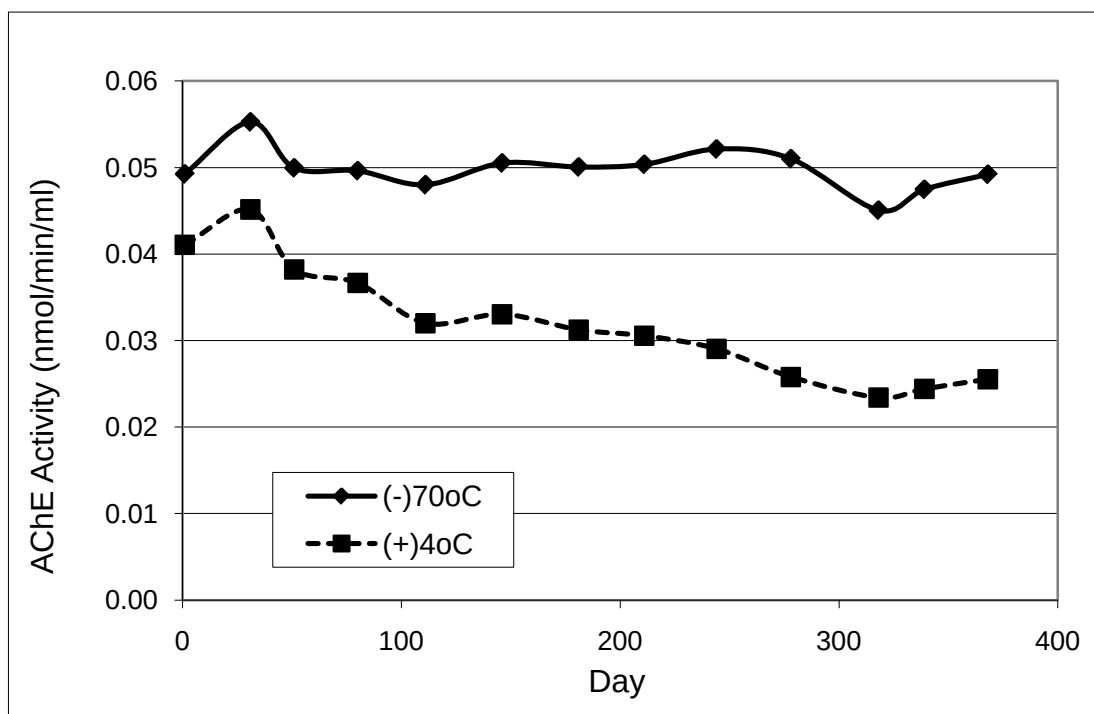
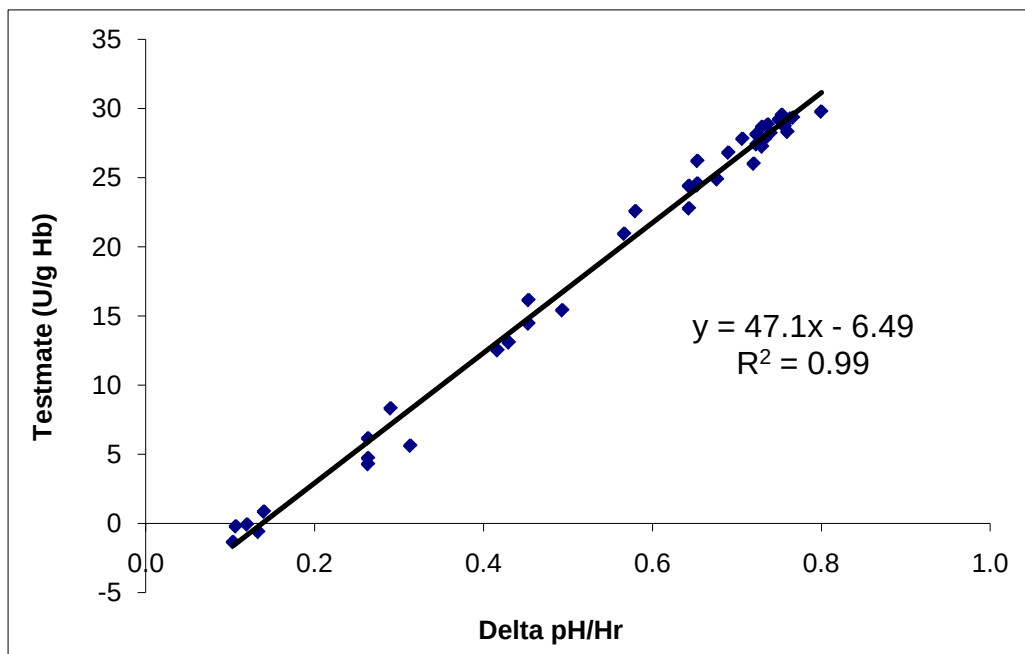
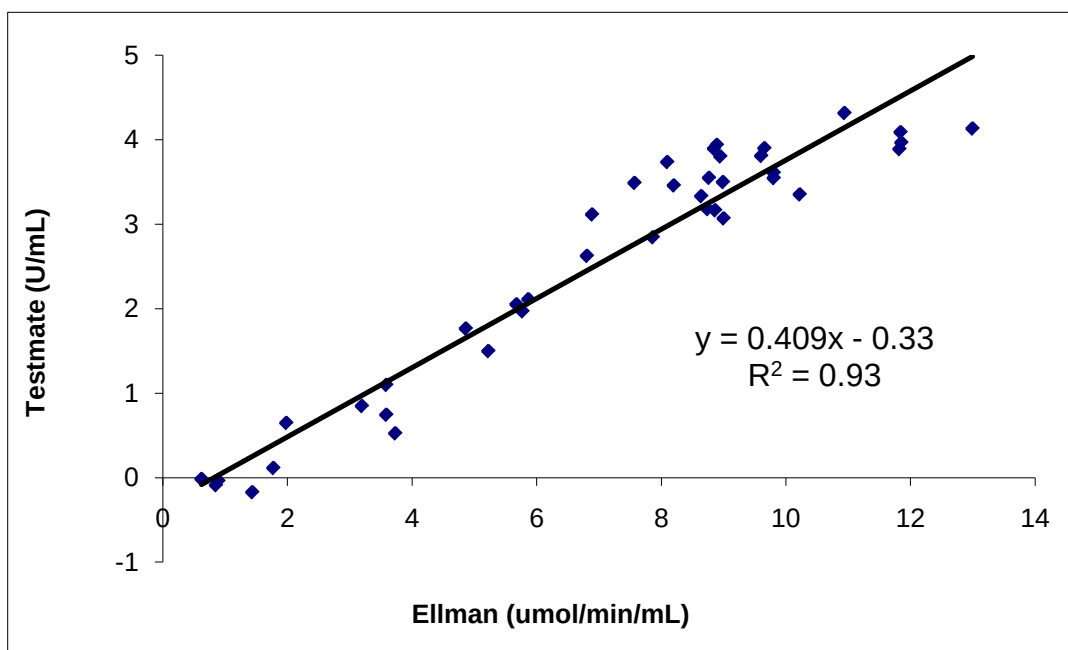


Figure 17. Testmate vs. Delta pH Comparison



Human RBCs treated with varying concentrations of DFP assayed at UCD.
n = 40: blood from 5 individuals, treated with 8 [DFP] *in vitro* .

Figure 18. Testmate vs. Ellman Comparison



Human RBCs treated with varying concentrations of DFP assayed at UCD.
n = 40: blood from 5 individuals, treated with 8 [DFP] *in vitro* .

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13 October, 2003

Dear Professor Wilson:

Thank you for your continued interest in the Test-mate ChE Cholinesterase Test System. As you requested, I am writing this letter to be included with your annual report that will be submitted to USACHPPM this week. The following is a brief summary of recent research, development, and engineering accomplishments.

The new Test-mate ChE (Version D) will be offered for commercial sale in the near future. The Version D instruments are far superior to the Version C instruments that are now being sold. The new instruments will contain several design improvements that include the extension of the operational temperature range for accurate measurements of AChE, Hgb, and Q (hemoglobin corrected erythrocyte cholinesterase) to allow operation between 10°C and 50°C. As in the older Version C instruments, the Version D instruments are currently programmed to display testing results automatically adjusted to 25°C. Like the previous versions of the Test-mate ChE, the new Test-mate ChE (Version D) instruments are intended for use only with human blood. Four such new prototype instruments have been tested - two by the German Armed Forces and two by USAMRICD.

The Cholinesterase Chemistry Set (manufactured by EQM Research, Inc.), is a moderately priced collection of reagents for use in the accurate determination of AChE, BChE, and Hgb using a Molecular Devices SpectraMax microplate reader, will soon be offered for sale. This system has been extensively tested and additional testing is being scheduled. The Cholinesterase Chemistry Set presents the final results as read at the assay reaction temperature of 37°C. A detailed manuscript will be submitted to the Bioscience 2004 Review Committee.

Prior to its formal release, I am currently engaged in changing the Test-mate ChE (Version D) to display results directly comparable to The Cholinesterase Chemistry Set microplate method. This will provide consistent laboratory cholinesterase measurements and field cholinesterase measurements. Once this composite package is fully completed and more thoroughly tested, I will be glad to arrange for both a Test-mate ChE (Version D) and The Cholinesterase Chemistry Set reagents to be available to you and your laboratory personnel for evaluation.

Best Regards,



Patrick Eberly, Ph.D.
President - EQM Research, Inc

Bovine Red Blood Cell Ghost Cholinesterase as a Monitoring Standard

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Received: 1 July 2002/Accepted: 12 May 2003

Monitoring human blood cholinesterase (ChE) activities is becoming increasingly important with the continued worldwide use of anticholinergic pesticides and fears of chemical warfare agents. The availability of standard operating procedures and ChE enzyme preparations are important elements of rapid responses to such life-threatening situations.

Mammalian blood has two main enzymes suitable for detecting the presence of ChE inhibitors. One is commonly referred to as “true” acetylcholinesterase (AChE, EC 3.1.1.7); the other as serum or pseudocholinesterase (BuChE, EC 3.1.1.8). AChEs prefer acetyl substrates, they are inhibited by substrates above 1–2 mM, by 0.5 mM BW284c51 and other selective inhibitors. BuChEs prefer butyryl, sometimes propionyl substrates depending on the species (Wilson, 2001). They are not inhibited by excess substrates at levels below 5–10 mM, and are inhibited by 0.1 mM iso-OMPA. AChE activity is limited to the formed blood elements in the human but not necessarily in other mammals. For example, up to half of the serum ChE activity may be AChE in rodents such as the adult rat and serum AChE may be even higher in the blood of embryos (e.g., fetal calf serum; see Wilson, 2001 for a recent review).

We conducted a comparison test at the request of the EPA with several clinical laboratories on blood ChE of rats exposed to various levels of an organophosphate pesticide (Wilson et al, 1996), we found that the conduct of the assay introduced inaccuracies into the clinical laboratory procedures. For example, not correcting for a transient thiol oxidase activity present in rodent red blood cells (RBC) introduced an indeterminate error in the results. Subsequently, while working with California state agencies and clinical laboratories licensed to monitor farm worker blood ChEs, we found conditions of a commonly used version of the Ellman assay (formerly Boehringer-Mannheim kit, Catalog No. 450035, Boehringer Mannheim Corporation, Indianapolis, IN) decreased the activity of the assay by approximately 40 percent (Wilson et al. 1997). In a comparison project with several clinical laboratories, only two of nine that participated achieved satisfactory results assaying ChE in human blood (Wilson et al. 2002). These findings led to a change in the California state regulations requiring ChE assays be convertible to values obtained from an optimized Ellman assay (Wilson et al.

2002). As part of a project to help standardize and optimize ChE assays for clinical laboratories, we developed a RBC ghost AChE standard using cows. This brief report describes the RBC ghosts and their enzymatic properties.

MATERIALS AND METHODS

Blood was drawn into heparinized tubes from a dairy herd of Holstein and Guernsey cows of the UC Davis Animal Science department. The blood iced and brought back to the laboratory where RBC ghosts were prepared according to a method modified from Hansen and Wilson (1999) for rabbits. Whole blood was added to a balanced Na-K-phosphate buffer, pH 8.0 and centrifuged for 15 minutes at 2500 xg, 4 °C. The supernatant was removed and the pellet incubated in 20 mOsm sodium phosphate buffer, pH 7.4 to hemolyze the RBCs. A cushion of 7% sucrose (w/v) was carefully added to the centrifuge tube and the preparation centrifuged for 20 minutes at 27,800 xg, 4 °C. The supernatant was removed and the packed RBC membranes solubilized adding 0.5% Triton-X-100 in 100 mM sodium phosphate buffer (v/v), pH 7.4.

ChE activities were assayed in triplicate using the standard Ellman assay (Ellman et al. 1961) and a 96 well automated microplate reader similar to that described by Padilla (Padilla et al. 1999). Assay volumes of 320 µl consisted of 250 µl 0.1 M sodium phosphate buffer, pH 8.0, 10 µl of 322 µM dithiobisnitrobenzoate (DTNB), 30 µl of enzyme sample and 30 µl of acetylthiocholine. Acetylthiocholine final concentrations were routinely 1.0 mM; other levels were used as required. Activities were read at 405 nm and 25-27 °C and reported as µmol/min/ml.

RESULTS AND DISCUSSION

The RBC ghost preparation may be stored at -70 °C for a year or more without loss in activity. For example, activity of one preparation kept at -70 °C averaged 0.050 ± 0.002 (n=13) µmol/min/ml over a period of 368 days stored in a low temperature freezer, but fell off over time in samples kept in the refrigerator (Figure 1). (Standard deviations of nine samples were ± 3 % of the means, too low to depict in the figure).

The data in Table 1 from 6 cows demonstrate ChE activity was confined to the RBC fraction. This was evidenced by its insensitivity to iso-OMPA and sensitivity to BW 284c51. (Approximately 5% of the enzyme activity was in the plasma fraction, and it, too, was sensitive to BW 284c51.)

Enzyme activities from 20 cows (including the six cows shown in Table 1) led to the same conclusion (Table 2). There was virtually no BuChE activity in whole blood or in the plasma fraction. RBC ghost enzyme activity was run with each experiment, a useful practice with such a standard. The mean activity of 8 trials was 0.896 ± 0.038 µmoles/min/ml of sample preparation (SEM).

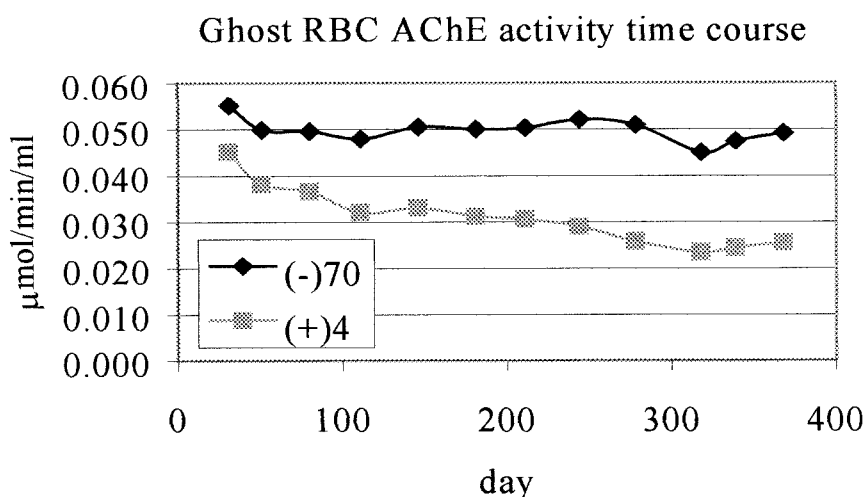


Figure 1. Bovine red blood cell ghost activity with storage. Means of six to nine samples.

Table 1. Acetylthiocholine hydrolysis of bovine blood and its fractions (μmoles/min/ml, mean ± st. dev.)

Treatment	Whole Blood	RBC Fraction	Plasma
None (n=6)	2.23 ± 0.59	2.10 ± 0.62	0.107 ± 0.033
10-4 M iso OMPA	2.17 ± 0.53	1.92 ± 0.54	0.103 ± 0.31
5 x10-5 M BW284c51	-0.28 ± 0.01	-0.057 ± 0.01	0.0087 ± 0.018

Table 2. Acetylthiocholine hydrolysis of 20 bovine blood samples (μmoles/min/ml, mean ± st. dev.)

Fraction	Activity
Whole Blood (No Treatment)	1.98 ± 0.37
With Iso-OMPA	1.95 ± 0.32
Plasma (No Treatment)	0.096 ± 0.031
With Iso-OMPA	0.092 ± 0.032

Enzyme activities from 20 cows (including the six cows shown in Table 1) led to the same conclusion (Table 2). There was virtually no BuChE activity in whole blood or in the plasma fraction. RBC ghost enzyme activity was run with each experiment, a useful practice with such a standard. The mean activity of 8 trials was 0.896 ± 0.038 μmoles/min/ml of sample preparation (SEM).

Table 3 illustrates the lack of serum BuChE by examining plasma and RBC fractions of one cow with increasing concentrations of acetyl, butyryl and

propionyl thiol substrates. The only appreciable activity present was from RBC acetylthiocholine hydrolysis; it was inhibited by substrate above 2 mM, one of the hallmarks defining “true” AChE.

Table 3. Effect of substrate concentration on ChE activity.

Substrate	μmoles/min/ml	± st. deviation
ACTC	RBC	Plasma
0.1	0.874 ± 0.090	0.052 ± 0.001
0.2	1.340 ± 0.023	0.071 ± 0.001
0.3	1.373 ± 0.164	0.081 ± 0.002
0.5	1.713 ± 0.449	0.092 ± 0.002
1	1.898 ± 0.564	0.098 ± 0.004
2	2.437 ± 0.454	0.092 ± 0.009
3	1.722 ± 0.260	0.108 ± 0.000
5	0.620 ± 0.615	0.109 ± 0.002
BuTC	RBC	Plasma
0.1	-0.047 ± 0.026	0.017 ± 0.001
0.2	-0.046 ± 0.018	0.022 ± 0.00
0.3	-0.083 ± 0.034	0.026 ± 0.001
0.5	-0.052 ± 0.011	0.034 ± 0.001
1	-0.052 ± 0.017	0.093 ± 0.006
2	0.010 ± 0.028	0.057 ± 0.001
3	0.041 ± 0.020	0.069 ± 0.005
5	0.068 ± 0.053	0.077 ± 0.001
PTC	RBC	Plasma
0.1	0.412 ± 0.066	0.033 ± 0.001
0.2	0.605 ± 0.063	0.045 ± 0.001
0.3	0.705 ± 0.019	0.053 ± 0.001
0.5	0.831 ± 0.086	0.063 ± 0.001
1	0.912 ± 0.040	0.076 ± 0.000
2	0.734 ± 0.225	0.077 ± 0.002
3	1.076 ± 0.284	0.080 ± 0.005
5	0.612 ± 0.219	0.091 ± 0.005

(ACTC: Acetylthiocholine; BuTC: butyrylthiocholine; PTC: propionylthiocholine in mM). Activities in μmoles/min/ml, n=3.

Unlike the human, bovine blood had little if any serum ChE activity, whether AChE or BuChE. The lack of serum ChE activity was recognized as early as the 1950s (e.g. Hermenze and Goodwin (1959) in their study of ChE RBC activity in cattle). Nevertheless, Harlin and Ross (1990) used whole bovine blood in a collaborative study of the Ellman assay to recommend a procedure subsequently adopted by the AOAC as a standard. Unfortunately, Harlin and Ross (1990) did not consider that the lack of BuChE in bovine blood may impair its usefulness for

monitoring inhibitors such as chlorpyrifos that prefer serum BuChE (Wilson, 2001).

The ghost preparation maintained activity at -70°C but declined after a few weeks at 4°C . Others (e.g. Stefan et al. 1977) found that bovine RBC activity was maintained for "several weeks" or "longer" at -24 to 3°C . The stability of the RBC ghosts at ordinary freezer temperatures was not investigated here and should be studied. Regardless, the ease with which bovine red blood cell ghosts can be prepared, their stability in storage, as well as the availability of a lyophilized commercial preparation (e.g. Sigma Chemicals Type XII-S Acetylcholinesterase, which is a bovine red blood cell, lyophilized powder) suggests they would be useful as a standard to compare tests between laboratories or within a single laboratory.

Acknowledgments. We thank Jack Henderson, Robert Ellis-Hutchings and Erika Wallender. Supported in part by NIEHS Center Grant P30-ES-05707, The UC Davis Agricultural Health and Safety Center, NIOSH Cooperative Agreement U07/CCU906162, the EPA Center for Ecological Health Research at UC Davis, and the Department of Defense (DAMD 17-01-1-0772).

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Meeting Requirements of the California Cholinesterase Monitoring Program

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California (CA) has a long-standing formal blood cholinesterase (ChE) monitoring program for mixers, loaders, and applicators of pesticides. When the authors found commercial clinical kits were not optimal for assaying blood ChEs, CA regulations were revised to specify use of the Ellman ChE assay or to demonstrate a conversion factor with a correlation (r^2) of 0.9 or better. The authors were enlisted to work with the clinical laboratories. Only two of seven participating laboratories generated an acceptable correlation for red blood cells (RBCs), whereas four of five laboratories had an acceptable correlation for plasma ChE. Subsequently, the CA Department of Pesticide Regulation (DPR) restated the need to meet this requirement and the authors worked with several of the clinical laboratories using a bovine ghost RBC ChE as a reference. Unfortunately, only 3 of 10 laboratories had acceptable correlations. Next, the authors provided all interested laboratories with human blood and plasma samples to perform the comparison study outlined in the regulation (Section 6728f). Fourteen laboratories participated; 9 met the ChE criteria for whole blood, 14 for plasma, and 6 for RBCs. Based on such data, on July 8, 2003, DPR notified the CA Agricultural Commissioners that nine of the participating laboratories were approved for ChE testing. Later work resulted in acceptable RBC values for two of the laboratories and their approval. The authors continue to work with laboratories interested in being on the approved list. The current list may be seen at www.cdpr.ca.gov/docs/whs/lablist.htm.

Keywords Acetylcholinesterase, Butyrylcholinesterase, Cholinesterase Monitoring, Clinical Laboratories, Nerve Agents, Organophosphates

Reports of the demise of organophosphate (OP) and organocarbamate (CB) pesticides may be said, with homage to Mark Twain (Clemens 1897), to be exaggerated. For example, although use in California declined by an impressive 21% from 2000 to 2001, there still were 9,226,936 pounds of active ingredient of these cholinesterase (ChE)-inhibiting chemicals applied in 2001, the latest year for which figures are available (California Department of Pesticide Regulation 2002). California is the only state to have a formal ChE monitoring program for mixers, loaders, and applicators as specified in Title 3, Section 6728 of the *California Code of Regulations*. Moreover, there is no national standard for determining the levels of these recognized sensitive biomarkers of exposure to select agrochemicals and chemical warfare agents.

We have shown that commonly used clinical ChE kits are not optimal for assaying blood ChEs (Wilson et al. 1997). These results, in part, led to the revision of California regulations. Section 6728 (f) specifies the methodology (modified from Ellman et al. 1961) for the required determinations of human erythrocyte (RBC) acetylcholinesterase (AChE, EC 3.1.1.8) and plasma cholinesterase (BChE, EC 3.1.1.7). However, because most clinical laboratories use commercial kits that do not follow the specified procedures to the letter, the regulation also provides for alternate ChE methods. Those using an alternate method must compare values from a set of samples assayed using both methods. An equation is derived from this comparison for converting ChE values assayed by the alternate method to values that would be obtained by the specified method. This comparison must meet the requirement of a correlation coefficient (r^2) equal to 0.9 or better, as stated in the regulation. In an earlier trial, only two of seven participating clinical laboratories achieved an acceptable

Received 28 October 2003; accepted 13 January 2004.

The data presented here were based on a report to the California Department of Pesticide Regulation, May 6, 2003. The authors thank the assistance of staff from the California Department of Pesticide Regulation, Department of Health Services, and Office of Environmental Health Hazard Assessment. Supported in part by the Western Center for Agricultural Health and Safety, NIOSH cooperative agreement U07/CCU906162, and the Center for Environmental Health Sciences, NIEHS center grant P30-ES-05707. Bovine RBC AChE preparation development was supported in part by USAMRMC DAMD17-01-1-0772.

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correlation (r^2) for RBC AChE and four of five laboratories for plasma BChE assay levels with our laboratory (Wilson et al. 2002).

This disappointing result led to a second round of comparisons with ChE monitoring clinical laboratories. The California Department of Pesticide Regulation (DPR) notified the laboratories on the List of Laboratories Approved to Perform Cholinesterase Testing for Occupational Health Surveillance (from the California Department of Health Services) that they needed to meet this requirement to remain on the approved list. Because research from our laboratory was one of the bases for the revision of the regulation (Wilson et al. 2002), we were enlisted to assist in meeting the conversion requirement.

The first step was to compare measurements of a bovine ghost RBC AChE preparation developed in our laboratory (Hanson and Wilson 1999; Arrieta et al. 2003) with results from a few selected clinical laboratories. The second step was to provide interested clinical laboratories with human blood and plasma samples to perform the comparisons outlined in the regulation.

MATERIALS AND METHODS

Bovine Ghost RBC AChE Preparation

Bovine blood was centrifuged at $1000 \times g$, the plasma discarded, and the RBCs resuspended and washed twice in isotonic buffer. Ghosts were prepared by lysing the cells with hypertonic buffer and centrifuging the membrane bound AChE at $100,000 \times g$. The pellet was solubilized in buffer with Triton X-100 detergent and the solution stored at -70°C (Hanson and Wilson 1999; Arrieta et al. 2003). Several dilutions were made with buffer yielding solutions of 100%, 75%, 50%, 25%, and

10% enzyme activity. These solutions were split into aliquots, frozen, and shipped on dry ice to participating laboratories.

Human Blood and Plasma Preparation

Up to 50 ml of blood was drawn from each of 10 volunteers at the University of California Davis (UCD) Employee Health offices, collected in heparinized vacutainers, iced, and hematocrit values were determined. Approximately two-thirds volume of each volunteer's blood was centrifuged at $1000 \times g$, the plasma removed, and saved. A portion of the remaining whole blood and plasma was diluted to 50% with buffer. The 100% and 50% whole blood and plasma samples from each volunteer donor were divided into aliquots; the blood was stored at 4°C and the plasma frozen at -70°C . Blood samples were shipped on ice and plasma shipped on dry ice within 24 h to participating laboratories (including our UCD research laboratory).

ChE Assay

A modified version of the Ellman colorimetric assay (Ellman et al. 1961) was used to perform the ChE measurements as described in Wilson et al. (2002), using a 96-well microplate reader. The final concentrations of acetylthiocholine substrate and dithiobisnitrobenzoate (DTNB) color reagent were 1.0 mM and 0.32 mM, respectively. Sample volumes were $10 \mu\text{l}$ of 1/50 dilutions of whole blood samples, and $30 \mu\text{l}$ of 1/10 dilutions of plasma. The final assay volume was $320 \mu\text{l}$. BChE activity in whole blood samples was inhibited with 0.02 mM quinidine (Wright and Sabine 1948). Six absorbance readings were made automatically at 1-min intervals to determine enzyme activity. Assays were carried out at $24.5^\circ\text{C} \pm 0.5^\circ\text{C}$.

TABLE 1
Participating laboratories and their ChE assay methodology

Laboratory	Instrument	Reagents	Temperature
A	Hitachi 717	Roche	37°C
B	<i>INP</i>	<i>INP</i>	<i>INP</i>
C	Dimension AR	Dade Dimension	37°C
D	Dimension RxL	Roche Cholinesterase (no. 124117)	37°C
E	Hitachi 717	Boehringer Mannheim	37°C
F	Hitachi 747 equivalent	Roche Diagnostics (catalog no. 1877763)	37°C
G	Beckman DU65 spectrophotometer	Boehringer Mannheim Reagent Kit	25°C
H	Olympus 640	Roche Diagnostics—Acetylthiocholine	37°C
I	<i>INP</i>	<i>INP</i>	<i>INP</i>
J	Bayer Opera	Sigma Diagnostics ChE (PTC)	<i>INP</i>
K	Beckman LX-20	Beckman CHE no. 443797	37°C
L	Dade Dimension RXL	Roche Cholinesterase	37°C
M	Blood: Olympus Plasma: Beckman	Blood: Roche Plasma: Beckman	<i>INP</i>
N	Hitachi 717	Roche	25°C

INP = information not provided.

TABLE 2

Research and clinical ChE measurements: Conversion comparison

Laboratory	Correlation coefficient (r^2)		
	Plasma	Whole blood	RBCs
A	0.99	0.90	0.90
B	0.98	—	—
C	0.98	—	—
D	0.98	0.91	—
E	0.99	—	0.79
F	0.98	0.03	—
G	0.99	0.92	0.92
H	0.98	0.93	0.94
I	0.98	0.91	0.93
J	0.99	0.88	0.87
K	0.98	—	—
L	0.98	0.92	0.92
M	0.98	0.92	0.88
N	0.96	0.90	0.91

sensitivity of the instruments or the acceptable ChE levels in the programming.

Conversions

Laboratories on the approved list that performed ChE assays were given an opportunity to participate in the conversion trial. Seventeen expressed interest in participating. Of those, three declined prior to shipment of samples. The remaining 14 laboratories (denoted by arbitrary alphabetical designations) are listed along with their assay methodologies in Table 1.

The correlation coefficients for the ChE activity comparisons between the UCD and the clinical laboratories are shown in Table 2. Corresponding line equations representing the conversions for each clinical laboratory are shown in Table 3.

Plasma BChE activities from the laboratories compared favorably to those measured by the UCD laboratory. Correlation coefficients ranged from 0.96 to 0.99. Ten laboratories reported activities of whole blood; nine met the 0.9 r^2 criteria (acceptable results include those rounded off to 0.9). Nine laboratories reported RBC AChE activities; eight had acceptable correlations. Examples of an acceptable and an unacceptable correlation are shown in Figure 1.

RESULTS

Bovine Ghost RBC AChE

AChE preparations were sent to 10 clinical laboratories as a test of shipping samples and performing assays. The activity levels ranged from 0.01 to 0.1 $\mu\text{mol}/\text{min}/\text{ml}$. These activities were below the levels found in human samples (1 to 2 $\mu\text{mol}/\text{min}/\text{ml}$ in plasma and 8 to 17 $\mu\text{mol}/\text{min}/\text{ml}$ in RBCs) and too low to be measured in some of the laboratories, possibly due to

DISCUSSION

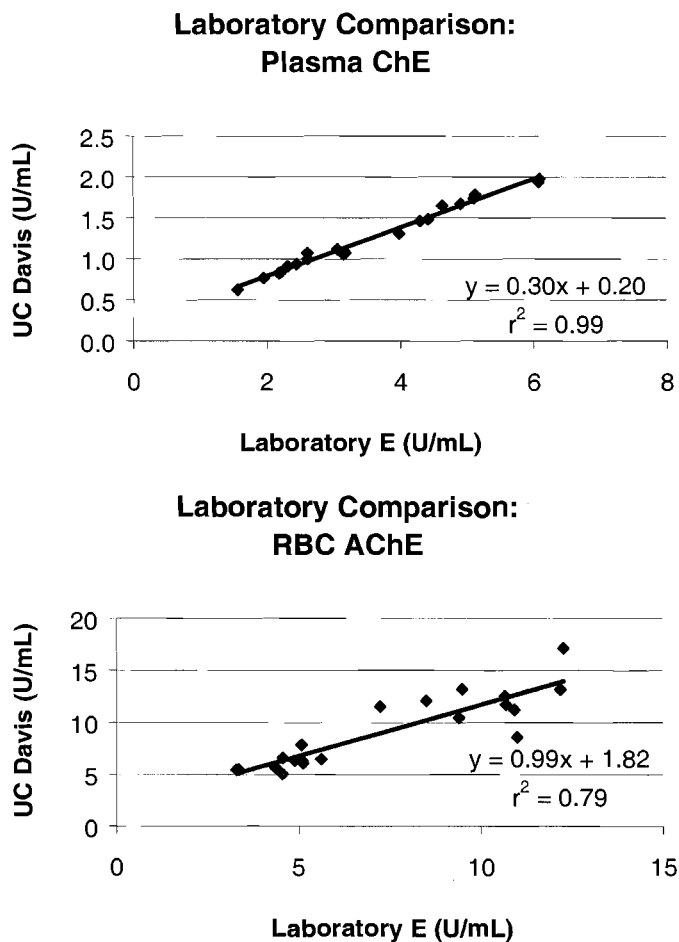
In an earlier study (Wilson et al. 2002), samples were sent to UCD by each clinical laboratory, rather than UCD shipping a matched sample set to each laboratory, as was done in this study. In both studies, plasma BChE activities showed a better correlation between laboratories than activities measured from whole blood or RBCs. Perhaps the viscous nature of whole

TABLE 3

Research and clinical ChE measurements: Conversion line equations

Laboratory	Plasma	Whole blood	RBC
A	$y = 0.29x + 0.19$	$y = 0.93x + 0.12$	$y = 0.92x + 0.082$
B	$y = 0.15x + 0.20$		
C	$y = 0.10x + 0.076$		
D	$y = 0.43x + 0.082$	$y = 0.81x - 0.93$	
E	$y = 0.30x + 0.20$		$y = 0.99x + 1.82$
F	$y = 0.29x + 0.22$	$y = 0.093x + 3.93$	
G	$y = 0.50x + 0.094$	$y = 0.79x + 0.22$	$y = 0.95x + 0.87$
H	$y = 0.34x + 0.11$	$y = 0.68x + 0.26$	$y = 0.83x + 1.55$
I	$y = 0.38x + 0.17$	$y = 1.61x + 1.18$	$y = 0.91x - 0.45$
J	$y = 0.20x + 0.15$	$y = 7.36x - 0.69$	$y = 1.07x + 0.73$
K	$y = 0.15x + 0.20$		
L	$y = 0.26x + 0.23$	$y = 0.58x + 0.66$	$y = 0.78x + 2.10$
M	$y = 0.14x + 0.20$	$y = 0.64x + 0.40$	$y = 0.79x + 2.71$
N	$y = 0.46x + 0.25$	$y = 1.00x - 0.41$	$y = 1.17x - 0.53$

Line equation: y (research value) = mx (clinical value) + C .

**FIGURE 1**

Examples of acceptable and unacceptable correlations.

blood samples made them more difficult to pipette accurately, contributing to the poorer correlations.

Overall, the RBC results were better in the present than in the earlier trial where only two of seven clinical laboratories achieved acceptable correlations. The more uniform preparation of the samples in the present as compared to the previous study may have contributed to the larger number of acceptable correlations: 6 of 8 for RBCs and 9 of 10 for whole blood values. Subsequent work with two of the laboratories resulted in two more acceptable correlations for RBCs. Parenthetically, it is surprising that all laboratories did not report RBC activity, because it is required in the state regulation. Regardless, after the disappointing results of the first study, it is heartening that most of the laboratories met the criteria for inclusion in the acceptable clinical laboratory list in this round.

The bovine ghost RBC AChE preparation of this batch was not useful in most clinical laboratory settings due to the lower detection limit of the clinical instruments compared to the microplate reader used in the UCD research laboratory. We plan to make a preparation with a higher AChE activity in the future.

Currently, so far as we know, California is the only state that requires monitoring of blood ChE levels in the agricultural workplace. Arizona had a program but it has been terminated. The state of Washington is setting up a similar monitoring program but it is not yet underway. It is important that such monitoring be performed so that the results are comparable between laboratories and between states to assure pesticide workers a safe workplace wherever they may labor. And it is sobering to realize that rapid, accurate, and transferable ChE assay reports are virtually a necessity in this time of concern about chemical terrorism.

During the preparation of this report, the Department of Pesticide Regulation sent a letter dated July 8, 2003, to California Agricultural Commissioners using the comparisons reported here as part of the bases for approving nine clinical laboratories for ChE testing under Section 3CCR 6728. The later work noted above led to the approval of two more of the laboratories. The approved list is available at www.cdpr.ca.gov/docs/whs/lablist.htm.

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Monitoring cholinesterases to detect pesticide exposure

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Available online 18 November 2005

Abstract

Progress toward a standard blood cholinesterase assay to assess pesticide exposures in the agricultural workplace and to identify possible victims of chemical warfare agents is discussed. Examples given are drawn from collaborations with clinical laboratories in California and the Department of Defense Cholinesterase Reference Laboratory (CRL).

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Keywords: ChE monitoring; Standardization; ChE assays

1. Introduction

Blood cholinesterases (ChEs), red blood cell acetylcholinesterases (RBC AChE, E.C. 3.1.1.7) and serum butyrylcholinesterases (BChE, E.C. 3.1.1.8), are enzyme biomarkers that detect potentially dangerous organophosphate (OP) and carbamate (CB) exposures. Methods in common use are designed to be rapid, one size-fits-all assays to monitor potential target populations and deal with pesticide exposure episodes or potential chemical warfare terrorist attacks. In the United States, California and Washington are the only states currently monitoring ChEs of OP/CB handlers. Elsewhere, ChEs are determined if exposures are suspected.

In California, regulations specify monitoring blood ChEs when using chemicals with toxicities <50 mg/kg (Class I pesticides), and ≥ 50 and ≤ 500 mg/kg (Class II pesticides). Examples are aldicarb, azinphosmethyl (Class I pesticides) and malathion (Class II pesticide). Mixers, loaders, applicators and flaggers, but not field workers themselves, are tested if they work with pesticides for 7 days or more in a 30-day period. Base lines are

taken in duplicate from the same laboratory. The workplace is evaluated if blood ChE levels of RBC and/or plasma are <80% of baseline. Workers are required to leave the workplace if levels are <70% for RBC, and <60% for plasma ChEs and cannot return to work until enzyme activity has recovered to 80% of baseline. New employees are tested after 30 days, and then examined every 60 days thereafter [1,2].

A similar program was started recently in the state of Washington following a lawsuit. Blood ChEs of mixers, loaders and applicators are monitored by a single state laboratory. A 20% or greater depression of ChEs compared to baseline leads to investigation of the worksite; 30% or greater depression of RBC AChE, or 40% of plasma BChE require removal of workers from the field; they may return when their blood levels reach 80% of baseline. Thousands of workers have already been tested, a data base has been established, some ChE depressions have been reported and cases are being followed [3].

But, the assay method used in Washington has not been quantitatively standardized with that used in California. And, in California, there is no data base in which to store ChE values obtained since the California testing program was established in the 1970s. Indeed, until recently there was no requirement to standardize the assays themselves [4].

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2. Measuring ChE's

The several commonly used assays to determine blood ChEs were not designed to be interchangeable. One is the end point delta pH method of Michel [5]; it measures acetylcholine (ACh) breakdown with a pH meter. A modification used by the Department of Defense Cholinesterase Reference Laboratory (CRL) is precise and even though it is slow, and its throughput is low, the US Army monitors more than 15,000 subjects per year with it. Another end point assay is the radioactive ACh method of Johnson and Russell [6], in which an organic/water extraction separates ³H-labeled ACh from its products. This micro-assay is suitable for multiple determinations. It is accurate, but it is expensive and has a disposal problem because of radioactive waste. Neither of these endpoint assays is suited to kinetic analyses. Popular kinetic assays use thiocholine substrates. The most common is that of Ellman et al. [7] in which the breakdown of acetylthiocholine (ATCh) is detected colorimetrically with dithiobisnitrobenzoate (DTNB). The assay is reliable, accurate and inexpensive. Several clinical kits have been marketed and there are many published variations.

A number of clinical laboratories use a kit marketed by Roche suitable for automated analyzers. We used a multi-well plate modification of the Ellman assay appropriate for research laboratories [4] in our work with California clinical laboratories and the US Department of Defense to harmonize clinical and research methods.

3. Difficulties with ChE assays

Activity in $\mu\text{mol}/\text{min}/\text{ml}$; mean $\pm$ S.D. (N). ATCh substrate: RBC: 0.8 mM; Plasma: 7 mM. Autotechnicon; Humiston and Wright [8].

No assay is free from problems, both practical and theoretical, and human and experimental [2,4]. One human problem is a penchant for investigators to implicitly assume ChE levels are readily transferable from one species to another. Examples of the relative activities of ChE in RBCs and plasma of humans, dogs and rats are shown in Table 1. Dogs and rats are much lower in total blood AChE activity than the human. Moreover,

unlike the human, they (and other animals too) have relatively high levels of AChE in their sera, raising doubts about values from studies that assume AChE levels are restricted to RBCs alone. Another source of error is a transient high thiocholine “blank” activity in RBCs of some species such as the rat that is often not considered in designing the assays and interpreting the results.

A third problem with focusing on RBCs is difficulties in accurately pipetting and reproducibly washing them to reduce serum contamination. A fourth difficulty is the potential interference from hemoglobin (Hb), which adsorbs at 410 nm, a wavelength similar to the maximum adsorption of DTNB.

Other problems stem from the nature of the commercial clinical kits themselves. For example, the popular Roche colorimetric kit is run at a pH and an ATCh concentration that is not optimal for the assay. The recently discontinued Sigma kit used a substrate that is not preferred by RBC AChE and is better suited to determining blood serum BChE [4].

A major problem in detecting depressions in ChE activity is reactivability of the inhibitor–AChE enzyme complex. Blood samples often are not iced, presumably on the notion that AChE inhibition by OPs is “irreversible”. Unfortunately, this is not the case until a structural reorganization of the inhibitor–enzyme complex known as “aging” has occurred [2,4]. Carbamates and methyl-organophosphates are readily rehydrolyzed at ambient temperatures, spontaneously reactivating enzyme activity within a few hours or days. In the case of carbamates, special techniques are required to successfully determine ChE inhibitions [4]. Lack of icing blood samples to reduce such reactivations was a confounding factor in an otherwise thorough study of Yeary et al. [9].

4. Standardization

The State of California recently revised its AChE monitoring: Title 3, California Code of Regulations, Section 6728, Medical Supervision, specifies use of the Ellman assay for blood monitoring, and requires clinical laboratories to submit data to demonstrate the reliability of their assays and to derive correction factors if necessary. We were asked by the Department of Pesticide Registration (DPR) to help the clinical laboratories comply, examining reproducibility and interconvertibility of the assays.

In the first round of comparisons, 9 of the 25 clinical laboratories sent undiluted and 50% diluted blood samples to UCD for ChE analyses. Two laboratories did their own comparisons. All used ATCh as a substrate. The results were disappointing, especially when attempting

Table 1
Comparative activities of human and other species

Species	RBC	Plasma
Human	135 $\pm$ 29 (60)	37 $\pm$ 9 (56)
Dog	18 $\pm$ 4 (18)	25 $\pm$ 6 (18)
Male rat	9.0 $\pm$ 1.3 (24)	4.3 $\pm$ 1.0 (45)

Table 2
Summary of blood ChE comparisons from UC Davis and Clinical Labs

Correlation (r^2)	RBC	Plasma
>90	0.98, 0.95	0.99, 0.94
	0.96, 0.93	0.97, 0.99
70 $\geq$ 90	0.74, 0.79	0.81, 0.88
<70		0.61, 0.33

Adapted from Wilson et al. [4].

to convert results from one laboratory to those of another (Table 2).

In general, plasma values tended to be better correlated and of lower variability than were RBC values. One reason may be the relative ease in pipetting plasma compared to serum.

5. AChE standard

Because of difficulties in coordinating sample collecting, arranging for shipping and quality control between laboratories, we developed a bovine ghost RBC AChE standard [10].

Hb and other proteins within bovine RBCs were removed by osmotic shock and the AChE containing membranes washed and then solubilized to prepare a long-lasting AChE standard. The RBC ghost enzyme is stable when stored at low temperature; it provides a standard for use in our own laboratory and for distribution to other laboratories too.

Fig. 1 illustrates the reliability of the standard. Diluted ghost preparations were assayed at UC Davis and duplicate samples shipped to the EPA laboratory in Raleigh, NC where they were assayed by Dr. Stephanie Padilla.

6. Second standardization

For the second time, the California Department of Pesticide Regulation (DPR) asked laboratories to compare their ChE assay to the method outlined in the regu-

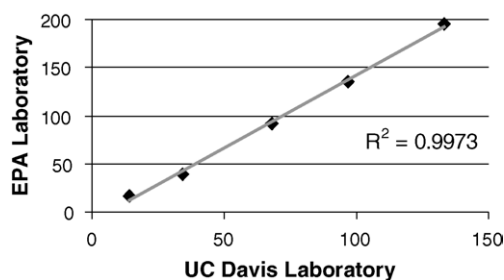


Fig. 1. Comparison of ghost RBC assays. Means of triplicate samples. Adapted from Wilson et al. [4].

lation, and demonstrate a correlation of 0.9 or better, and, again, our laboratory was asked to assist. Diluted bovine RBC ghost samples were sent to participating laboratories and human blood and plasma samples were provided to clinical laboratories to compare with assays performed at UC Davis. When bovine ghost standards were sent to 10 clinical laboratories, activities were below the detection level set for their instruments. Nevertheless, three yielded correlation coefficients of 0.97–0.98. Fourteen laboratories agreed to determine blood samples sent to them. The plasma BChE activities from the clinical laboratories compared favorably to those measured by UCD; correlations ranged from 0.96 to 0.99. Nine of the 10 laboratories reported whole blood activities that met the $0.9r^2$ criteria. Six of the eight laboratories reported RBC AChE activities with acceptable correlations [11].

7. Conclusions

Clinically determined plasma BChE correlated better than whole blood or RBC activities at UC Davis suggesting plasma might be preferable to RBC or whole blood for monitoring. But which pesticides prefer which enzyme, how long enzyme depressions last and what neural enzymes correspond to plasma BChE are a few of the questions still to be considered before recommending serum BChE in preference to RBC AChE. The RBC results were better than in the previous study when only two of the seven clinical laboratories “passed.” In this trial, six of eight RBC and 9 of 10 whole blood AChE values were acceptable. Perhaps, the samples in this study were more uniform because they were prepared in a single laboratory, contributing the larger number of acceptable correlations. Although still a first approximation, the population-based correlations were sufficient to permit comparing one laboratory with another.

One outcome of the project was a letter from DPR on 8 July 2003 to the California Agricultural Commissioners listing nine clinical laboratories on the approved list for ChE testing under Section 3CCR 6728. The current list is available at: www.cdpr.ca.gov/docs/whs/lablist.htm.

8. Closing

Standardizing blood ChE monitoring is only one of the matters that would make this method of detecting OP exposures more useful [12]. Others are:

- Extend the monitoring of mixer, loaders and applicators to include periodic examination of field workers too. These laborers in the agricultural workplace may

wear less protective clothing and receive training than the pesticide handlers.

- b. Arrange for a central data storage, accessible to more than a single agency so that the results of exposure episodes may be rapidly disseminated to the public health community.
- c. Provide the clinical findings to the persons monitored. It is surprising to a laboratory-based biochemist that clinical assays initiated by physicians are not routinely made available to their subjects and stored in a common data base.
- d. Periodically carry out a formal review of the policies and procedures of the clinical laboratories and of the replicability of their results.

Finally, another important bit of unfinished business is to establish a normal human range for blood ChEs, providing 95% confidence intervals that may be used when individual baselines are lacking. Although commercial kits include ChE ranges with the instructions, they have not been formally validated, and much of the literature on this topic is more than 40 years old. Work in progress from our laboratory yields a 95% range of 6.3–10.7 $\mu\text{mol}/\text{min}/\text{ml}$ with a mean of 8.11 ± 0.067 (S.D.) $\mu\text{mol}/\text{min}/\text{ul}$ ACTh (Ellman units) for normal human blood AChE (Arrieta et al., 2005, in preparation).

This study illustrates how a bench laboratory biochemical quality assurance project can impact public health regulations and worker safety. The continuing use of antiChE pesticides and the fear of chemical terrorism underline the need for national standards and periodic quality assurance testing of clinical ChE monitoring. The recommendation by CA DPR that laboratories lose their approval if they do not meet state standards is a new and tougher policy requiring tighter scrutiny of performance.

Acknowledgements

The authors acknowledge Al Ramirez from the Wilson laboratory, Jim Sanborn (OEHHHA), Joe Billitti

(Aguron), Mike O'Malley (UCD Employee Health), Stephen McCurdy (UCD Epidemiology), Stephanie Padilla (US EPA), Matthew Keifer (U. Washington) and CA and Utah clinical laboratories. Support for the research was from UCD NIOSH Center for Agricultural Health and Safety; NIEHS Center for Agricultural Health Science; DOD; OEEHA; and CA DPR.

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Evaluation of a Fluorescent Method for Measuring Cholinesterase Activity in Mammalian Blood and Tissue

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Received: 21 April 2006/Accepted: 16 November 2006

Cholinesterase (ChE) activity has been widely used as a biochemical marker of cholinergic system function in laboratory, wildlife, and human studies (Fulton and Key 2001; Padilla et al 1996). Acetylcholinesterase (AChE, EC 3.1.1.7) prefers to hydrolyze acetylcholine (ACh), butyrylcholinesterase or non-specific cholinesterase prefers other choline esters (BChE, EC 3.1.1.8). Acute exposures to organophosphate and carbamate pesticides inhibit ChE activities, causing accumulation of ACh at synapses, and their disruption in organisms. Although critical to cholinergic transmission in the brain, ChEs are also present in several other tissues, including blood. Both red blood cell (RBC) and plasma ChE activities have been widely used as bio-indicators of exposure and intoxication to these pesticides (Wilson et al 1997). Reduced ChE activities in blood have been reported among spray-workers as a result of improper handling of pesticides (Baker et al 1978; Wilson et al 1997). Exposures to other environmental pollutants, including mercury, have been shown to cause alterations in ChE activity in humans (Zabinski et al 2000), wildlife (Lionetto et al 2003) and laboratory animals (Gill et al 1990; Hastings et al 1975; Lakshmana et al 1993).

There are several methods to measure ChE activity (St. Omer and Rottinghaus 1992, Wilson 2001). The most widely used is a colorimetric assay developed by Ellman et al (1961). While the Ellman assay is simple and inexpensive, the absorbance peak of hemoglobin (415 nm) can interfere with detection of its reaction end-product, 2-nitro-5-benzoic acid (405nm). Recently, a fluorescent based ChE assay kit was marketed for measurement of the purified enzyme (A12217, Molecular Probes, Eugene, OR, USA (Zhou et al 2000). It detects choline, the ChE reaction product, through a multi-step process in which the oxidation of choline by choline oxidase results in the formation of H₂O₂, which is detected through a horseradish peroxidase-coupled reaction with 10-acetyl-3,7-dihydroxyphenoxazine (Amplex Red). The final product, resorufin, is detected by its fluorescence (ex. 563, em. 587) (Zhou et al 1997). It is said to provide high sensitivity (0.002 units/ml of purified AChE from electric eel, as reported by Molecular Probes) without hemoglobin interference.

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The objective of this study was to evaluate this fluorescent assay as a tool to measure ChE activities in biological specimens collected from humans and wildlife.

MATERIALS AND METHODS

Reagents used in the assay were optimized to ensure maximal ChE activity in human plasma, American mink (*Mustela vison*) brain tissue and bovine red blood cell (RBC) ghost preparations (Arrieta et al 2003), and an inter-laboratory comparison test with the Ellman assay was also performed. Horseradish peroxidase (EC 1.11.1.7), resorufin, ACh, choline oxidase (EC 1.1.3.17), iso-OMPA, BW285c51, and all other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). 10-acetyl-3, 7-dihydroxyphenoxazine (Amplex Red) was acquired from Molecular Probes, Inc (Eugene, OR, USA). Approval to conduct this study was granted by the McGill University Ethical Review Committee. Human blood samples (10 mL) were drawn by a registered nurse into tubes containing EDTA-K₂ from 5 healthy volunteers between 25 and 35 years of age who had never been exposed to pesticides. Plasma was isolated by centrifugation of whole blood at 200 x g for 10 min at 4°C, pooled, and stored at -80°C. Brain tissues from captive mink (*Mustela vison*), having no history of exposure to environmental pollutants, were obtained from Michigan State University's Experimental Fur Farm (East Lansing, MI, USA). Brain tissues were dissected and stored at -80°C and shipped to the laboratory in Montreal. Upon thawing, brain tissues were sonicated in cold phosphate buffer (50 mM NaH₂PO₄, 5 mM KCl, 120 mM NaCl, pH 7.4) and 0.1% Triton X-100, and centrifuged at 15,000 x g for 10 min at 4°C and the supernatants collected. The protein concentration in the supernatants was determined by the Bradford method (Bradford, 1976). Bovine RBC ChE standards were prepared from a dairy herd of Holstein and Guernsey cows (Animal Science Department, UC Davis) as previously described (Arrieta et al 2003) and stored at -70°C until use. Briefly, plasma and red blood cells were separated by centrifugation, the red blood cells lysed by distilled water, the membranes collected by centrifugation, solubilized by treatment with Triton X-100 and stored at -70°C until needed.

ChE measurements using the Amplex Red assay, available as a kit from Molecular Probes (Eugene, OR, USA; Catalog No. A12217), were based on the procedure described by Zhou et al. (2000). Triplicate samples of human plasma (0.01-50 nL/well), mink brain preparations (0.1-3.0 µg/well) or bovine RBC ghost standards (0.01-1.0 µL/well) were added to the wells of microplates in 100 µL of phosphate buffer. The reaction was initiated with the addition of 100 µL reaction buffer (10 µM Amplex Red, 2 U/mL horseradish peroxidase, 0.2 U/ml choline oxidase and 50 µM acetylcholine; final concentration in 200 µL). Preliminary studies were performed with varying concentrations of substrate, ACh (25 – 2000 µM), and Amplex Red (1.5 to 200 µM) to determine optimal assay conditions. Fluorescence was measured every 5 min at 540/590 (excitation/emission) by a fluorometric plate reader (Wallac Victor 2, Perkin

Elmer, Boston, MA, USA) at 25°C. The reaction was generally linear between 10-90 min. The end product, resorufin (0 - 5 μ M), was used as the standard, and specific activities of samples were expressed as μ mol/min per mg (protein) or ml (plasma or RBC ghosts). Sodium potassium buffer was used as a blank and 10 μ M H₂O₂ was used as a positive control. Inhibitor studies were performed by pre-incubating (15 min at 25°C) diluted samples with iso-OMPA (0.1 mM - BChE specific inhibitor) or BW285c51 (0.01 mM - AChE inhibitor) prior to the initiation of the reaction. Total ChE activity was determined in the absence of inhibitors.

For validation purposes, ChE activities were measured using the Ellman procedure (Ellman et al 1961) modified for a 96-well automated microplate reader (Padilla et al 1999). Assay volumes of 320 μ l consisted of 250 μ l 0.1 mM sodium phosphate buffer, pH 8.0, 10 μ l of 322 μ M dithiobisnitrobenzoate (DTNB), 30 μ l of enzyme sample and 30 μ l of acetylthiocholine (final concentration of 1.0 mM). Activities were read at 405 nM at 25-27°C and reported as μ mol/min/ml.

RESULTS AND DISCUSSION

The Ellman assay typically uses the substrate acetylthiocholine (ATC) in concentrations ranging from 0.4 – 2.0 mM which allows for the determination of maximal enzymatic activity (Augustinsson et al 1978; Ellman et al 1961). Figure 1 shows that increasing concentrations of ACh (0 to 1 mM), in the absence of enzyme, resulted in a concentration dependent increase of background fluorescence interfering with the detection of ChE activity at ACh concentrations above 0.25mM. For this reason, a sub-maximal ACh concentration of 50 μ M was used to detect ChE activity, permitting determination of relative rates.

ChE activity increased in a linear fashion as a function of the total amount of tissue in the reaction well (Figure 2) within approximately one order of magnitude. ChE activity could be determined from very small amounts of diluted sample (i.e. 0.05 nL/well of human plasma and 0.1 μ g/well of brain supernatant protein) calculated from the linear region of the curves between 10 and 25 min. Saturation occurred when product formation was greater than 5 μ M of resorufin.

Experiments were performed to determine the optimal concentration of Amplex Red for maximal ChE activity. The commercially available Molecular Probes kit suggested a final Amplex Red concentration of 200 μ M. The present study indicated that Amplex Red concentrations between 3 μ M and 12 μ M yielded maximal activity when using RBC ghost standards (Table 1). Similar results were observed with mink and plasma samples (data not shown). Amplex Red concentrations below 3 μ M did not yield detectable resorufin and final fluorescent concentrations above 25 μ M progressively reduced enzymatic activity. Therefore, 10 μ M of Amplex Red was chosen for the remaining tests.

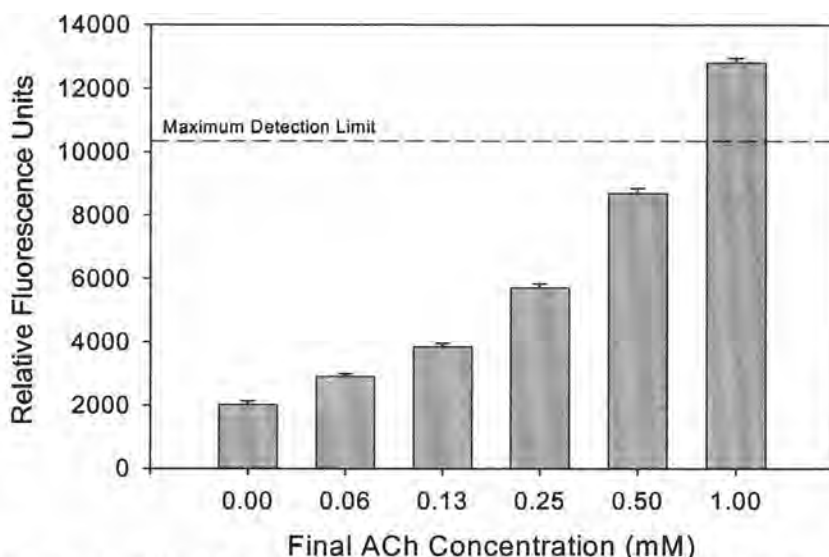


Figure 1. Effects of ACh concentration on background fluorescence in the absence of tissue sample. Averages of triplicate determinations.

Fluorescent detection became non-linear above 11,000 relative fluorescence units.

Table 1. Effects of Amplex Red concentration on ChE activity in bovine RBC ghost standards. Mean $\pm$ SD of triplicate determination.

Amplex Red (μ M)	ChE Activity (μ mol/min/ml)
1.5	0.1 ± 0.5^a
3.0	14.1 ± 0.3^b
6.0	13.9 ± 0.3^b
12	$13.1 \pm 0.4^{b,c}$
25	12.9 ± 0.3^c
50	9.6 ± 0.2^d
100	5.9 ± 0.3^e
200	2.7 ± 0.4^f

Different letters represent significance (ANOVA, Tukey post hoc test; $p < 0.05$)

Both BW284c51 and iso-OMPA inhibitors were used to identify the AChE and BChE activity in plasma, bovine RBC, and mink brain tissue (Table 2). The activity in the RBC standard was associated with AChE; it was sensitive to BW284c51 but not iso-OMPA, consistent with previous findings (Arrieta et al 2003). Human plasma ChE activity was associated with BChE activity. Mink brain samples contained both enzymes, approximately 30% of total activity could be attributed to BChE and the remaining 70% to AChE activity. These data suggest that the fluorescent assay detected both enzymes.

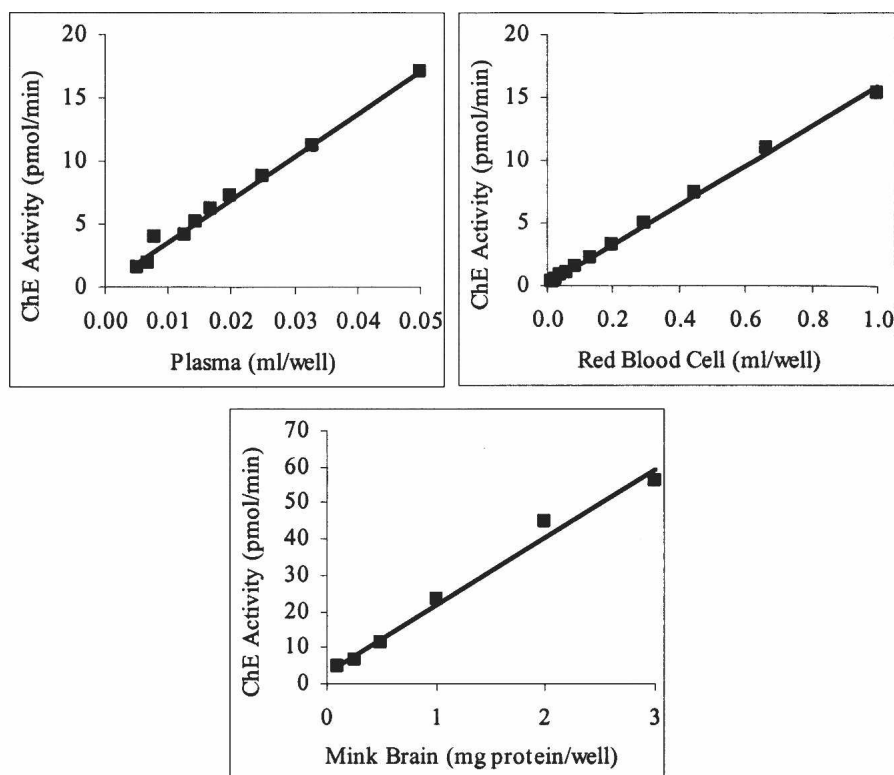


Figure 2. Linear relationship between ChE activity and tissue concentration (Mean of triplicate determination).

Table 2. Determination of AChE and BChE activities (Mean $\pm$ SD, n=3).

<i>Inhibitor</i>	<i>Bovine RBC Ghosts (% Total ChE)</i>	<i>Human Plasma (% Total ChE)</i>	<i>Mink Brain (% Total ChE)</i>
iso-OMPA (0.1 mM)	91 $\pm$ 4.5	2.4 $\pm$ 3.1	64 $\pm$ 8.6
BW284C51 (0.01 mM)	-0.1 $\pm$ 1.4	92 $\pm$ 4.2	38 $\pm$ 7.3

The Amplex Red assay was compared to the established Ellman assay using RBC ghost standards (n = 15) and mink brain tissue (n = 9) analyzed at McGill University using the Amplex Red method and University of California, Davis using the Ellman assay. Linear regression analyses indicated a strong relationship between the two methods for both RBC standards ($r^2 = 0.98$) and mink brain tissue ($r^2 = 0.93$) (Figure 3). The slopes of the regression lines differed from 1.0 because different concentrations of substrate were used.

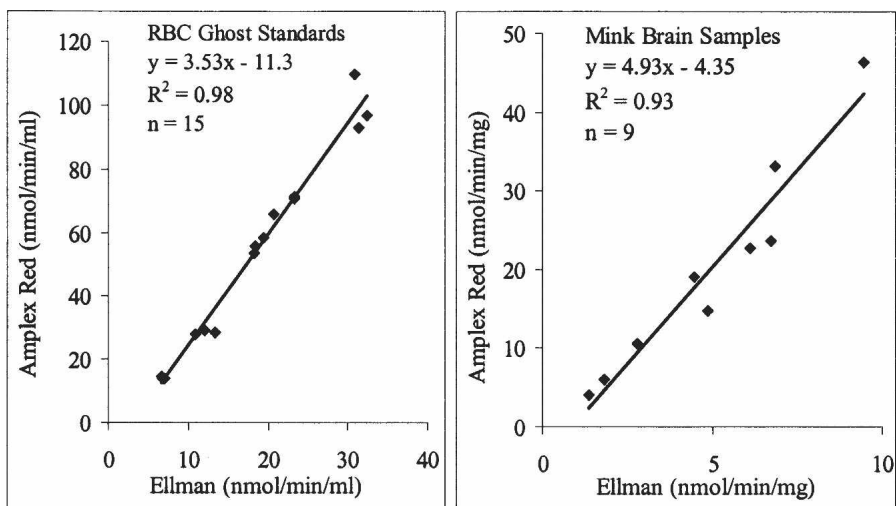


Figure 3. Comparison of ChE activity using mink and bovine tissue measured with Amplex Red and Ellman assays.

These results demonstrate that the Amplex Red-based assay is a sensitive method to measure both AChE and BChE in brain, RBC, and plasma. Inter-laboratory comparisons correlated well with the established Ellman assay. However, because of substrate interference and resulting high background fluorescence, this fluorescent assay is not useful for determining maximal ChE activity or developing substrate saturation curves. The assay may still be particularly useful in measuring ChE in samples contaminated with hemoglobin, and its high sensitivity may be of interest when amounts of tissue are limited.

Acknowledgments. Supported by the Collaborative Mercury Research Network (COMERN) and the Natural Science and Engineering Research Council (NSERC) of Canada. UC Davis's contribution supported in part by Western Center for Agricultural Health & Safety (1U50/OH07550), the NIEHS Center (ES05707) and the Department of Defense (DAMD17-01-1-0772). C.J. Stamler and N. Basu were recipients of NSERC Postgraduate Fellowships. We thank Dr. Steven J. Bursian (Michigan State University) for the donation of mink brain tissues. Technical assistance by Erin Tapley (UCD) and Donna Leggee (McGill) is greatly appreciated.

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RESEARCH ARTICLE

Normal range of human red blood cell acetylcholinesterase activity

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Abstract

The normal range of human erythrocyte acetylcholinesterase (RBC-AChE) activity is important when monitoring exposure to pesticides and chemical warfare agents. A modification of Michel's method measured RBC-AChE activities from 991 individuals (818 males and 173 females) presumably unexposed to nerve agents. Median age was 42 (range, 18–76) years. RBC-AChE (mean $\pm$ SD) was 0.74 ± 0.06 delta pH units/hour. Multivariate linear regression showed an association with age (slope $+0.0008$ delta pH units/hour for each year; $P < 0.001$) unlikely to be clinically significant. The findings represent the largest study of human RBC-AChE to date providing measures of central tendency and variation.

Keywords: *Acetylcholinesterase; human; red blood cell*

Introduction

Human blood contains two forms of cholinesterase (ChE). Erythrocytes contain acetylcholinesterase (RBC-AChE; E.C. 3.1.1.7) and plasma contains butyrylcholinesterase (BChE; E.C. 3.1.1.6). The level of RBC-AChE serves as a convenient biomarker for the cholinergic function of the nervous system (Wilson, 2001). Organophosphorus ester pesticides and chemical warfare agents disrupt cholinergic transmission by inhibiting cholinesterases and artificially increasing the levels of acetylcholine.

The Department of Defense (DOD) Cholinesterase Reference Laboratory (CRL) at the U.S. Army Center for Health Promotion and Preventive Medicine (USACHPPM) measures RBC-AChE activities for workers that participate in an occupational medical surveillance program. The CRL also manages a quality assurance program for 12 ChE testing laboratories

located at several U.S. army installations and maintains a database of RBC-AChE activities. All RBC-AChE activities are measured by using a modification (Ellin et al., 1973) of the delta pH method of Michel (1949). This assay measures the decrease in pH that occurs when RBC-AChE catalyzes the hydrolysis of acetylcholine to form choline and acetic acid, yielding measurements with low variability (Witter, 1963). Its advantages include cost, precision, and a database that extends over 50 years of use by the U.S. army. Disadvantages of the method include its relatively slow throughput and the fact that it is an endpoint assay precluding relatively simple rate determinations. A decade later, more sensitive spectrophotometric methods were developed to determine both RBC and sera cholinesterases, such as the Ellman assay (Ellman et al., 1961), that required more expensive chemicals and equipment. To date, the U.S. army has continued to utilize the Michel method in its

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(Received 13 May 2008; revised 10 February 2009; accepted 03 March 2009)

ISSN 0148-0545 print/ISSN 1525-6014 online © 2009 Informa UK Ltd
DOI: 10.1080/01480540902863440

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surveillance program because, in part, of its favorable cost-benefit ratio.

In this report, we used the Michel method to analyze RBC-AChE activities for 991 individuals to estimate the normal population values and evaluate the potential influence of age, gender, and test date, since normal population values for human RBC-AChE activities have not been reviewed in a number of years and many of the earlier studies analyzed relatively small data sets that contained little data on females.

Materials and methods

CRL measures RBC-AChE activities by using a modification (Ellin et al., 1973) of the delta pH method of Michel (1949) for personnel that work predominantly at chemical storage depots and chemical demilitarization facilities. RBC-AChE activities were analyzed for 991 individuals between January and December 2000 by using results stored in a CRL database and using analyses limited to one RBC-AChE determination per individual. (Many individual RBC-AChE activities were appropriate to determine a normal range. Baseline activities were critical when interpreting results for a given individual.) None of these individuals were considered exposed to anticholinesterase organophosphorus compounds. Informed consent was not obtained prior to initiation of this retrospective study and all individually identifiable information was removed. This study was reviewed and approved for data analysis as an exempt human use protocol by the Walter Reed Army Institute of Research Human Use Review Committee. Data were analyzed with Stata 7.0 (Stata Corporation, College Station, Texas, USA), using standard statistical techniques.

Results

The study population was normally distributed; it included 818 males (82.5%) and 173 females (17.5%). Median age was 42 years. On average, men were older than women (44 vs. 36 years; $P < 0.0001$; Wilcoxon test) (Table 1). Mean values and range of RBC-AChE activities were nearly identical for both men and women. The mean ($\pm$ SD) RBC-AChE activity was 0.75 ± 0.06 delta pH units/hour for men and 0.74 ± 0.07 delta pH units/hour for women. The distribution of results suggests that RBC-AChE activities were normally distributed (Figures 1–3). The 5–95th percentile range of RBC-AChE values was 0.65–0.85 delta pH units/hour for men and 0.63 to 0.86 delta pH units/hour

Table 1. Summary of delta pH measurements for 991 DOD personnel.

Measurement	All	Male	Female
Number of subjects	991	818	173
Median age	42	44	36
Age range	(18–76)	(18–76)	(18–67)
RBC-AChE (delta pH/hour)	0.74 ± 0.06	0.75 ± 0.06	0.74 ± 0.07
Delta pH/hour (range, 5–95th percentile)	(0.65–0.85)	(0.65–0.85)	(0.63–0.86)

RBC-AChE values are mean $\pm$ standard deviation.

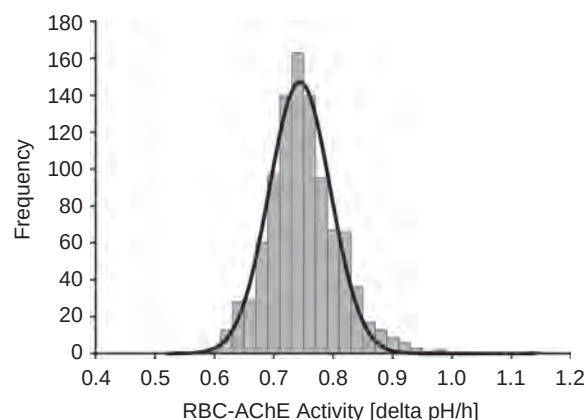


Figure 1. Distribution of RBC-AChE activities for DOD personnel obtained between January and December 2000. The mean $\pm$ SD was 0.74 ± 0.06 delta pH units/hour; $n = 991$.

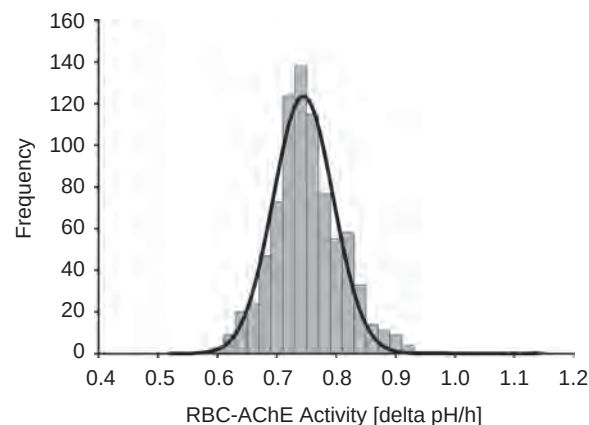


Figure 2. Distribution of RBC-AChE obtained from male DOD personnel between January and December 2000. The mean $\pm$ SD was 0.75 ± 0.06 delta pH units/hour; $n = 818$.

for women. A multivariate linear regression model determined the association between delta pH measurements in relation to age, gender, and test date (the date RBC-AChE was determined at the CRL). There was a significant association between delta pH and age (slope $+0.0008$ delta pH units/hour for each year of age; $P < 0.001$). No association was identified with gender. A small, but statistically significant, reduction

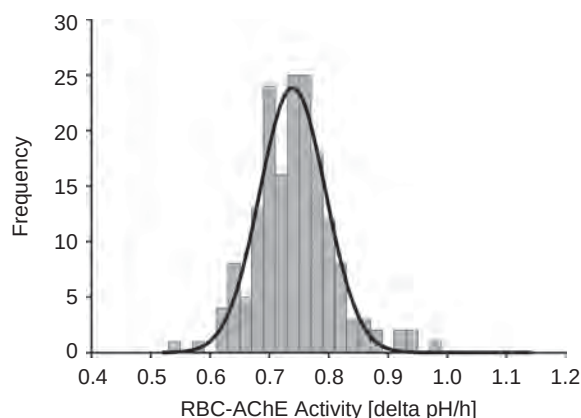


Figure 3. Distribution of RBC-AChE obtained from female DOD personnel between January and December 2000. The mean $\pm$ SD was 0.74 ± 0.07 delta pH units/hour; $n = 173$.

in delta pH measurements was associated with test date (-0.006 delta pH units per 100 days; $P < 0.01$). A small percentage of the variance was explained by the multivariate linear regression model incorporating age, sex, and test date.

Discussion

Blood ChEs, such as acetylcholinesterase (AChE, EC 3.1.1.7) and butyrylcholinesterase (BChE, EC 3.1.1.6), have been widely used as biomarkers to monitor exposure to anticholinesterase agents (Costa, 1998; Wilson, 2001; Wilson et al., 2005). Reliable normal population data for RBC-AChE activities are needed to establish the interindividual variation in these enzyme levels and compare results from different laboratories. Measurements of intraindividual variation, interindividual variation, and the precision of the analytical method can be used to calculate an index of individuality, which is a measure of biological variation (Harris, 1974). An index of individuality of 0.45 for male RBC-AChE activities was recently calculated; however, there were insufficient data to establish an index of individuality for female workers (Lefkowitz et al., 2007). (An index of individuality of less than 0.6 indicates that individual-specific reference ranges should be used instead of population reference intervals.) It has long been recognized that when RBC-AChE activities show sufficient interindividual variation, comparison of test results with a population reference interval is contraindicated. Instead, RBC-AChE activities must be compared to an individual's baseline. In this report, we demonstrated that the interindividual variation for RBC-AChE activities was essentially the same for both men and women. An understanding of the

normal biological variation in RBC-AChE activities is important because this information may be used to establish guidelines and policies for assessing exposures to anticholinesterase organophosphorus compounds. Currently, no observable effect levels (NOELs) have not been established for RBC-AChE inhibition, and there is a lack of information on the intraindividual variation of RBC-AChE activities in women.

Several earlier studies investigated normal population values (a measure of interindividual variation in RBC-AChE activities). These studies often included small numbers of subjects, used different methods to measure RBC-AChE levels, and were comprised mainly of males within a narrow age range (Freemont-Smith et al., 1952; Vorhaus et al., 1950; Wolfsie and Winter, 1952). The earliest population study was by Limperos and Ranta (1953). They reported mean RBC-AChE activities of 0.76 ± 0.03 delta pH units/hour for 52 male subjects. Identical results were obtained for 49 female subjects. A later study by Rider et al. (1957) established a normal human range of RBC-AChE activities by analyzing specimens from 800 healthy blood donors presumably not exposed to anticholinesterase organophosphorus compounds and analyzed by using Michel's delta pH method. The mean RBC-AChE activities for the 40-year-old age group were 0.766 and 0.750 delta pH units/hour, respectively, for men and women. Our current results (0.75 ± 0.06 delta pH units/hour for males and 0.74 ± 0.07 delta pH units/hour for females) are in excellent agreement with these earlier studies and highlight the reliability of Michel's delta pH method. Further, they closely match the reference range of 0.63–0.89 delta pH units/hour cited by the Department of the Army Technical Bulletin Medical 590 (2001).

Previous studies found that RBC-AChE levels did not change significantly with age in adults (Lefkowitz et al., 2007; Shanor et al., 1961; Witter, 1963). We report here an association between age and RBC-AChE activity (slope $+0.0008$ delta pH units/hour for each year of age; $P < 0.001$), but the magnitude of the change with age was small and was unlikely to be clinically significant. Based on this slope, it would take approximately 12 years for an individual's RBC-AChE activity to increase by 0.01 delta pH unit/hour. The lack of correlation between RBC-AChE and gender is also consistent with the works of others (Maroni et al., 2000).

These findings represent an important step in establishing an RBC-AChE index of individuality for women, although a larger, prospective study measuring intraindividual variability of RBC-AChE activity over time is needed. Since there has been an increase

in women working with pesticides and nerve agents, it is critical to ascertain whether there is a concomitant difference in the index of individuality for RBC-AChE activities, providing information for policy makers to establish the appropriate frequency of RBC-AChE testing for individuals that are enrolled in medical surveillance programs.

Conclusion

The data presented provide a normal human range of RBC-AChE activity based on a modified delta pH assay. While knowledge of such a reference range is important in the event of emergencies, it is not a substitute for the establishment of individual baseline values. Establishing a normal range for human RBC-AChE activities represents a first step toward providing a benchmark for military and civilian use. Work is under way to determine a conversion factor between the delta pH assay and the popular colorimetric assay (Ellman et al., 1961) to establish a normal range in Ellman units.

Acknowledgments

The authors thank USACHPPM for providing the delta pH measurements and demographic information. This work was supported by USAMRMC (DAMD17-01-1-0772), Western Center for Agricultural Health and Safety (NIOSH Cooperative Agreement U07/CCU906162), and the Center for Environmental Health Sciences (NIEHS Center Grant P30-ES-05707). Data were presented, in part, by Daniel E. Arrieta for partial completion of the PhD in pharmacology and toxicology (UCD). The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the Department of the Army or the Department of Defense.

Declaration of interest: The authors report no financial conflicts of interest.

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