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13. ABSTRACT (Maximum 200 Words)

Blood biomarkers are an important way to monitor exposure to anticholinergic pesticides and chemical warfare agents and to establish whether some are at greater risk than others from exposure to them. Many clinical and research laboratories use the colorimetric Ellman assay based on the hydrolysis of acetylthiocholine. CHPPM (US Army Center for Health Promotion and Preventive Medicine) uses a slower delta pH method based on that of Michel to monitor 16,000 DOD personnel each year. This year OP exposure was mimicked by treating whole blood from UCD (University of California, Davis) with DFP, to give a range of AChE activity. RBC AChE activity was measured using both the Ellman and delta pH assays at UCD. An estimated conversion factor was calculated. A normal range of AChE activities from the CHPPM delta pH assay was converted to Ellman units. These ranges are important benchmarks for clinical laboratory ChE determinations in the absence of baseline data.

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INTRODUCTION

There is a need for rapid, high throughput, reliable and transferable determinations of blood cholinesterase (ChE) levels to provide early warning of exposures due to the intensive use of pesticides such as organophosphate esters (OPs) and threats of chemical warfare agents. The colorimetric Ellman assay based on the hydrolysis of acetylthiocholine (Ellman, *et al.*, 1961) is used by many clinical and research laboratories. A slower delta pH method, based on that of Michel (1949), is used to monitor the erythrocyte (RBC) acetylcholinesterase (AChE) of over 15,000 DOD personnel involved with chemical nerve agent and demilitarization operations each year, by the Cholinesterase Reference Laboratory (CRL) at the US Army Center for Health Promotion and Preventive Medicine (CHPPM). Although pH assays are reliable and have low variability, they are not readily adaptable for automation or field use.

One goal of this project is to establish a conversion factor between the pH and colorimetric assays applicable to monitoring studies and field tests. Another goal is to provide conversion factors for the portable Test-Mate kit manufactured by EQM, Inc. since studies have shown that the current model does not adequately adjust for temperature to be useful for field use. Plans agreed to by the manufacturer are to use a new model from the manufacturer with improved assay parameters.

Another issue is that of genetically sensitive individuals exposed to anticholinergic chemicals. Lowered plasma butyrylcholinesterase (BuChE), a scavenger of antiChE agents, may put individuals at increased risk to OP and CB agents (reviewed by Wilson, 1999). A polymorphic form of paraoxonase (PON1), which destroys selected OPs, has been reported to be reduced in a cohort of veterans suffering from "Gulf War Syndrome" (Haley *et al.*, 1999). There is evidence that low levels of BuChE and PON1 affect sensitivity to OP exposures of experimental animals (Shih *et al.*, 1998, Broomfield *et al.*, 1991). Whether human PON1 isoforms may yield increased sensitivity or protection to specific OPs has not been clearly demonstrated (reviewed by Costa *et al.*, 2003). Following completion of the cholinesterase tasks, plans are to investigate the incidence of lowered BuChE and PON1 in the blood samples.

BODY

Materials

All chemicals were purchased from Sigma Chemical Co.

Methods

Sample collection

Blood was obtained from volunteers at the University of California, Davis (UCD) under an approved Human Subjects Protocol. Blood was 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 and stored at 4°C.

Ellman Cholinesterase Assay

RBCs were diluted 1/50 in 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 96 well plates at 25°C. The final concentrations of the substrate acetylthiocholine and the color reagent dithiobisnitrobenzoate (DTNB) were 1 and 10.3 mM respectively. Activity was reported as umol/min/ml.

Delta pH Cholinesterase Assay

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.

Specific Cholinesterase 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.

Task One. Conduct a careful comparison of the Ellman assay performed under optimum conditions and the DOD pH assay to examine the variability and reliability of both assays, to establish baseline values and to generate conversion factors to enable comparisons between them and other proposed or commercial assays.

Previous tests comparing the CHPPM delta pH assay and the UCD Ellman assay were carried out on split RBC samples collected by CHPPM and shipped to UCD. The results were inconsistent. To reduce variability, volunteer blood was collected at UCD and both assays were run at UCD. The RBCs were prepared following the procedures used by CHPPM, which did not include washing the RBCs after the plasma fraction was removed. The presence of plasma in the sample was checked by comparing washed and unwashed RBCs, and by using specific ChE inhibitors (Figure 1). 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). 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 Ellman assay displayed decreased RBC AChE activity when the substrate concentration was 5 mM or greater (Figure 2). There was also a decrease in RBC AChE activity in the delta pH assay, but not until the substrate concentration exceeded 10 mM (Figure 3). There was no decrease in plasma BuChE activity with substrate concentrations up to 20 mM in the delta pH assay.

The RBC preparations were treated with varying concentrations of a ChE inhibitor to mimic OP-exposed samples and broaden the range of observed activities. Diisopropyl fluorophosphate (DFP) was chosen because it does not require metabolic activation to be an effective ChE inhibitor. Whole blood was treated with DFP prior to centrifugation. The RBCs were washed to remove residual DFP before being measured by each assay system. This was repeated using blood from 3 volunteers. The DFP inhibition curves for both assays are shown in Figure 4. The resulting activities from each assay were plotted against each other (Figure 5). Linear regression yields an estimated conversion factor between the two assays: delta pH = (0.091 x Ellman) + 0.0052.

We reported a normal range of delta pH ChEs from the DOD monitoring program in last year's report. The distribution of these activities converted into Ellman units is shown in Figure 6. These values are from 991 unexposed individuals ranging from 18 to 76 years of age. There was no influence of age or gender. The average activity was 8.11 $\pm$ 0.67 umol/min/ml (mean $\pm$ sd). The range of activity was 6.3 to 10.7 umol/min/ml (95% confidence interval).

Task Two. Test the stability and usability of a red blood cell ghost standard suitable for clinical standardizations.

Task Two has been accomplished and the results published (Arrieta *et al.*, 2003). The activity of the preparation on hand was too low the delta pH method. We plan to increase the activity level in a new preparation. The ghost RBC standard is included in each Ellman microplate assay conducted at UCD.

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

Unfortunately, the new instrument did not become available for study this year. The Model 400 available commercially is the one we had studied in the past. We are still waiting for EQM to provide the next model for our collaborative study (see letter from company president Patrick Eberley from 2003 appended to the report).

Task Four. Explore the feasibility of incorporating BuChE variant and PON1 polymorphisms into a screen of workers for whom blood ChE baselines are required using a selected set of DOD personnel.

Currently, the conditions of blood collection performed by CHPPM are geared to RBC and not whole blood or plasma studies. We plan to discuss setting aside some blood samples specifically for this task with Captain Lefkowitz perhaps through satellite laboratories of CHPPM near UCD.

KEY RESEARCH ACCOMPLISHMENTS

- The characteristic substrate inhibition of AChE is altered under the delta pH assay conditions.
- Mimicked OP exposure by treating whole blood with $1x10^{-6}$ M to $5x10^{-6}$ M DFP.
- Calculated an estimated conversion factor between Ellman and delta pH determinations of ChE.
- Applied the conversion factor to CHHPM delta pH data to produce a normal range of human RBC AChE values in Ellman units, useful for clinical laboratories.

REPORTABLE OUTCOMES

Arrieta DE, SA McCurdy, JD Henderson, LJ Lefkowitz, RE Reitstetter and BW Wilson. 2004. Normal Range of RBC Cholinesterases in a DOD Monitoring Program. In Preparation.

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.

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.

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.

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. Presented at NIOSH Conference, November, 2003 San Francisco, CA.

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.

CONCLUSIONS

The Ellman assay displayed a classic substrate inhibition curve associated with AChE at substrate concentrations of 5 mM or greater (Wilson *et al.*, 1995) but the delta pH assay did not. Plasma BuChE is known to have a higher substrate optimum than AChE, and its

presence could have been a factor in the delta pH result. The test of washing the RBC preparation ruled out any significant contribution of plasma to the overall ChE activity. The substrate concentration curve was not changed when acetylthiocholine (the Ellman substrate) was substituted for acetylcholine in the delta pH assay (data not shown). The difference in the curves may be a problem of methodology: the Ellman assay is kinetic, whereas the delta pH assay is an endpoint determination. Our next step is to determine the linearity of the activity rates for the delta pH method, especially at low substrate concentrations by measuring pH over time, making the delta pH test into a kinetic assay for the sake of the experiment. Another possibility is that high acetylcholine concentrations are changing the configuration of the enzyme itself.

Our first approach to mimic OP-exposed blood samples was the addition of DFP directly into the assay (data not shown). We were concerned about inherent differences in the degree of ChE inhibition due to concentrations of RBCs present in each assay (the more dilute RBCs in the Ellman assay could be more inhibited by an equal concentration of DFP than the RBCs in the delta pH assay). This lead to the second approach where whole blood was treated with DFP prior to centrifugation and the resulting RBCs were assayed by both ChE methods. The same inhibited sample could be assayed by both methods, reducing variability.

The preliminary conversion values for delta pH and Ellman assays permit using the large 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 the 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 10.02 quoted in the Roche cholinesterase kit.

A research group at the WRAIR laboratory has developed a new ChE method (Feaster *et al.*, 2001). We look forward to working with WRAIR and CHPPM on comparing this new method with the delta pH and Ellman assays, and how they can be made compatible with each other.

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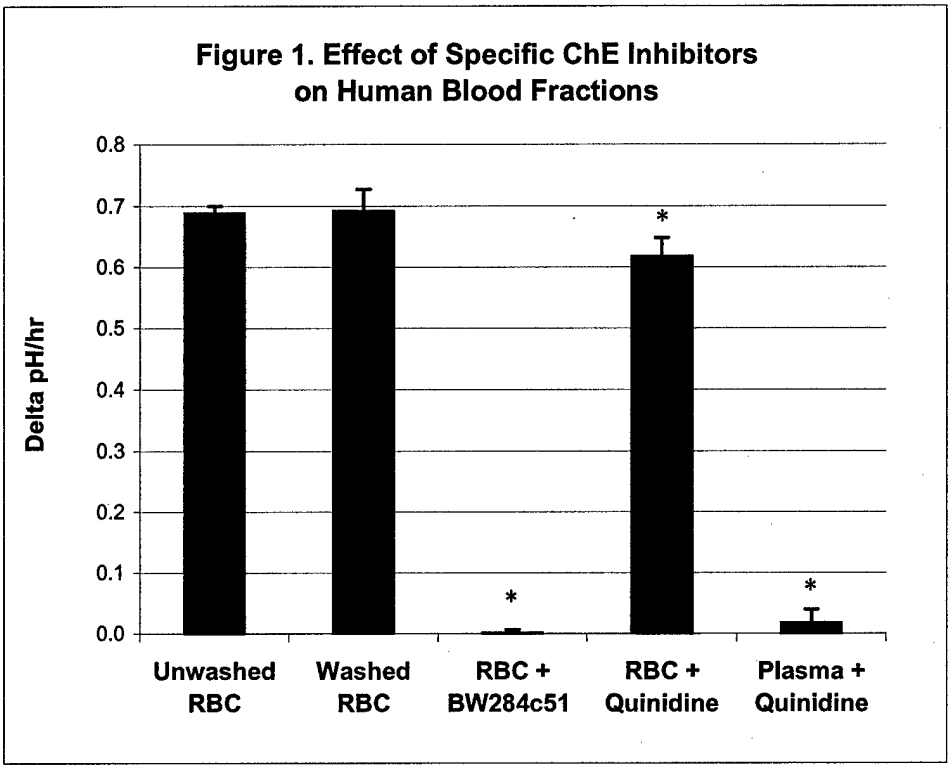
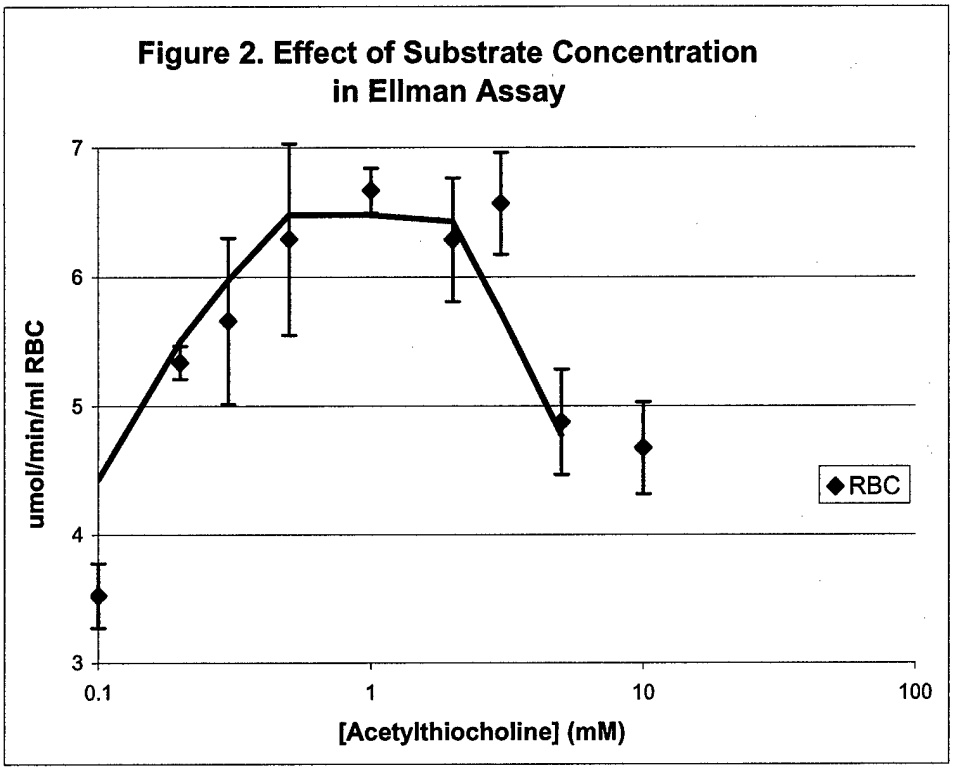
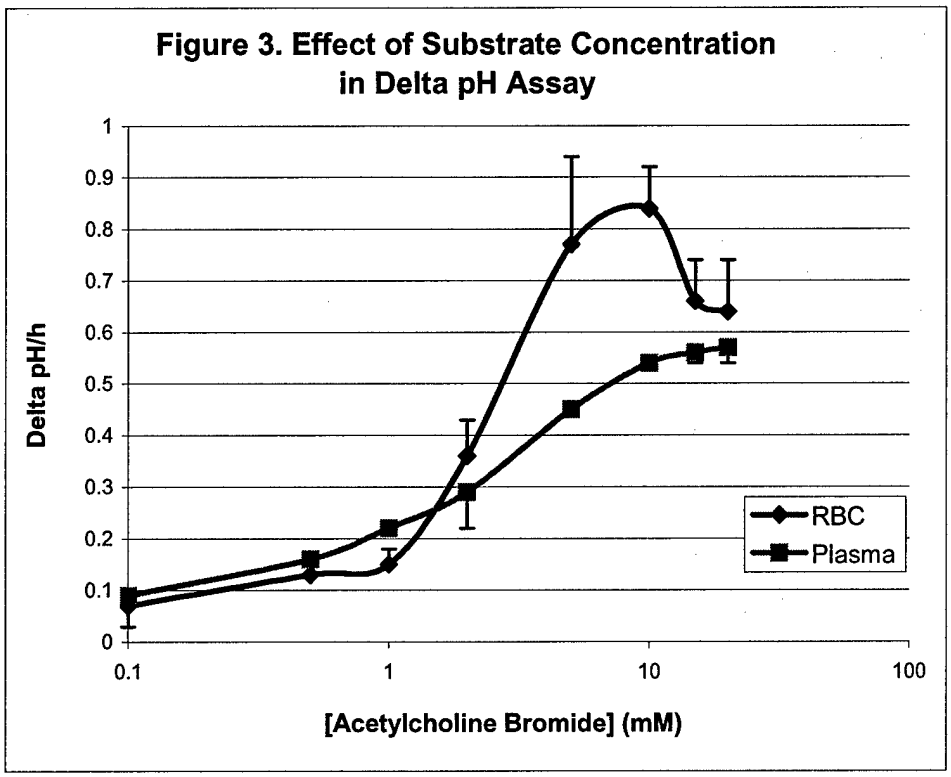
APPENDICES



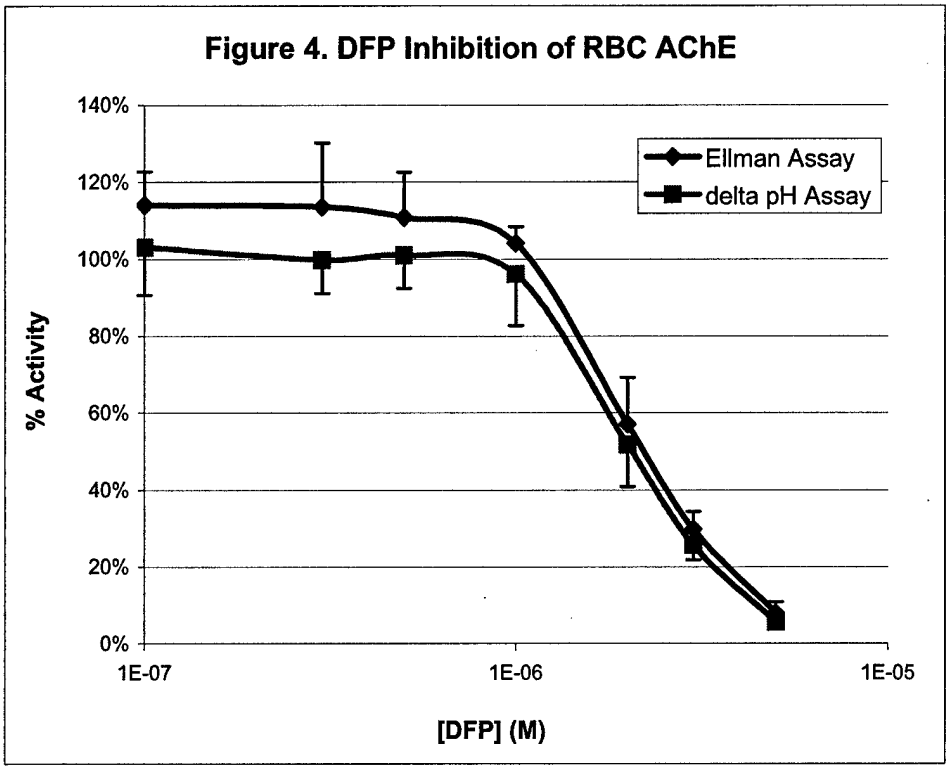
ChE activity is mean $\pm$ sd; n = 3 for washed and unwashed RBCs;
n = 5 for inhibitor-treated samples.
* Different from unwashed RBC control ($p<0.01$, ANOVA).
BW284c51 is a specific AChE inhibitor; AChE is associated with human RBCs.
Quinidine is a specific BChE inhibitor; BChE is associated with human plasma.



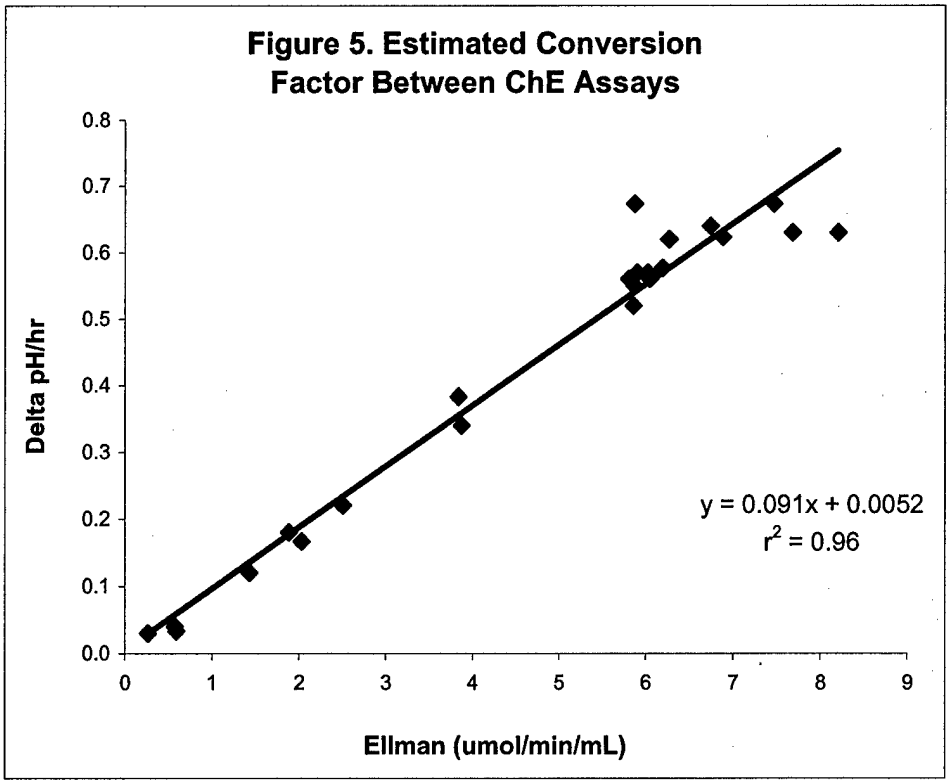
ChE activity is mean ± sd; n = 3.



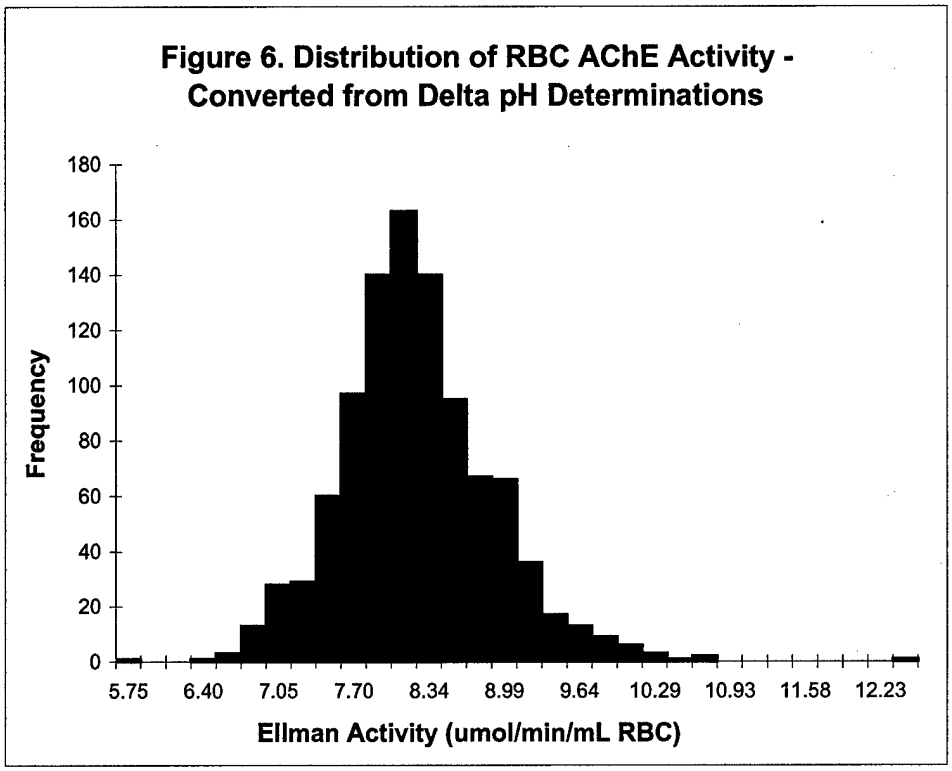
ChE activity is mean ± sd; n = 3.



ChE activity is mean ± sd of RBCs from 3 individuals.



ChE determined in RBCs from 3 individuals treated with varying concentrations of DFP.



ChE activity converted from delta pH to Ellman units using the estimated conversion factor y = 0.091x + 0.0052.
Determinations from the DOD ChE monitoring program; n = 991.

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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 10ºC and 50ºC. As in the older Version C instruments, the Version D instruments are currently programmed to display testing results automatically adjusted to 25ºC. 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 37ºC. 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,

J. Patrick Eberly

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