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13. ABSTRACT *(Maximum 200 Words)*

Blood biomarkers are an important way to monitor exposure to anticholinergic pesticides and chemical warfare (CW) 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 more than 25,000 DOD personnel each year. This year blood drawn under the appropriate regulations by CHPPM was centrifuged and hydrolyzed before being assayed by the delta pH method with acetylcholine as substrate. Paired samples were sent to UC Davis to be assayed with acetylthiocholine by the Ellman method. UC Davis samples were diluted with buffer and run with and without quinidine to separate activities due to acetylcholinesterase (AChE) and non-specific cholinesterase (BuChE). Slopes of pH vs Ellman results for three of five sets of samples had similar slopes and yielded correlations of r^2 of 0.74 to 0.8. Work continues on comparisons to establish critical assay conditions and Ellman equivalents of the delta pH assay.

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INTRODUCTION

Intensive use of anticholinergic pesticides such as organophosphate esters (OPs) and threats of chemical warfare agents establish the need for rapid, high throughput, reliable and transferable determinations of blood cholinesterase (ChE) to provide early warnings of exposures. Many clinical and research laboratories use the colorimetric Ellman assay based on the hydrolysis of acetylthiocholine (Ellman, *et al.*, 1961). CHPPM (US Army Center For Health Promotion and Preventive Medicine) uses a slower delta pH method based on that of Michel (1949) to monitor more than 25,000 DOD personnel each year. Although it is a reliable assay of low variability, pH assays are not readily adaptable for automation or field use. A major 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. Studies have shown that the current model does not adequately adjust for temperature to be useful for field use. Future work will use a custom model from the manufacturer which will allow us to adjust assay parameters. A bovine RBC AChE standard preparation we developed will be used in assay comparisons. Another issue is that of genetically sensitive individuals exposed to anticholinergic chemicals. Lowered BChE, a scavenger of antiChE agents, may put individuals at increased risk from OP and CB agents (reviewed by Wilson, 1999, 2001). 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 BChE and PON1 affect sensitivity to OP exposures of experimental animals (Shih *et al.*, 1998, Broomfield *et al.*, 1991).

BODY

Material

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

ChE Determinations

RBC samples received from CHPPM were diluted 1/50 in Lysis Buffer (0.5% Triton X-100, 0.1 M sodium phosphate, pH 8) and measured using the colorimetric method of Ellman, *et al.* (1961), modified for use with an automatic microplate reader. Activity was determined in the presence and absence of 0.02 mM quinidine sulfate, a selective BChE inhibitor.

Ghost RBC Preparation
Bovine blood was centrifuged at 1000 x g and the plasma discarded. The RBCs were resuspended in isotonic buffer and washed 2 times. RBC ghosts were prepared by lysing the cells with hypertonic buffer. The membrane bound AChE was centrifuged at 100,000 x g. The pellet was solubilized in buffer with Triton X-100 detergent, the solution diluted and stored at -70°C.

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.

RBC sample comparison.
Five sets of 20 RBC samples were sent by CHPPM to UC Davis. Each laboratory determined the ChE activity with its respective method. The comparisons between laboratories are shown in Figures 1-5. Three of the five sets (1, 2 and 4 have good correlations and similar slopes (Table 1). More sets will be sent from CHPPM and run at UC Davis.

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

Sample Set	Slope	Correlation, 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

Slopes are ratio of delta pH/Ellman AChE activities.

Preliminary Baseline Levels
The mean AChE levels ($\pm$ standard deviation) for the 100 samples in the comparison above are: 8.17 ± 1.11 umol/min/ml RBC for the UC Davis determinations; 0.75 ± 0.06 delta pH/hr for the CHPPM determinations. The CHPPM value is in agreement with the AChE values from a previous CHPPM AChE data set. This set of 1,443 values (from 991 individuals) has a mean AChE level of 0.74 ± 0.06 delta pH/hr. Statistical analysis of the data set reveals no difference in AChE levels due to age or date of testing. There is a small statistical difference due to gender ($p < 0.001$, Kruskall-Wallis non-parametric test) (Figure 6).

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

RBC Standard Characterization
Bovine blood contains only AChE, which is mostly in the RBC fraction (unlike human blood which has AChE in the RBCs and BChE in the plasma fraction). The lack of serum

ChE activity was recognized as early as the 1950s (Hermenze and Goodwin, 1959). This is confirmed here by the insensitivity of bovine blood to iso-OMPA (a specific BChE inhibitor) and its sensitivity to BW 284c51 (a specific AChE inhibitor) (Table 2). This makes the blood a good AChE source for a standard.

Table 2. Cholinesterase Activity of Bovine Blood and Its Fractions

Treatment	Whole Blood	RBC	Plasma
None	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 x 10-5 M BW284c51	-0.28 ± 0.01	-0.057 ± 0.01	0.0087 ± 0.018

Activity = umol/min/ml; mean ± sd; n = 6

The ghost RBC standard AChE activity was stable for a year stored at -70°C, but fell off at 4°C after 50 days (Figure 7).

Laboratory Comparison

The ghost RBC standard was diluted 100, 75, 50 25 and 10% of stock concentration. Sets of samples were sent from UC Davis to CHPPM and also to UC Davis (to control for shipping). The samples were assayed at each laboratory using its respective method. The activity level in the samples was too low to be determined at CHPPM, indicating that the delta pH method is less sensitive than the Ellman colorimetric method.

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.

This task will be addressed later in the project. Patrick Eberly, CEO of EQM Inc., informs us that a new, temperature regulated model is nearing the end of development.

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

This task will be addressed later in the project.

KEY RESEARCH ACCOMPLISHMENTS

- Acceptable correlations of RBC activity measured by the Ellman and the delta pH methods were demonstrated.
- AChE activity levels in the current samples are in agreement with the existing CHPPM data set. Older delta pH data will be useful in establishing normal ranges of human RBC AChE activity, an important product of this research project.
- A bovine ghost RBC AChE standard was stable for more than a year when stored at -70°C.

REPORTABLE OUTCOMES

Publications

Wilson B.W., Henderson J.D., Ramirez A. and O'Malley M.A. Standardization of clinical cholinesterase measurements. Int. J. Toxicology (2002) 21(5): In Press

Arrieta D,, Ramirez A, DePeters E, Bosworth D and Wilson BW. Bovine Red Blood Cell Ghost Cholinesterase as a Monitoring Standard. Bulletin of Environmental Contamination and Toxicology, Accepted for Publication.

Oliveira G.H., Henderson J.D. and Wilson B.W. 2002. Cholinesterase measurements with an automated kit. Am. J. Indust. Med. Supplement 2:49-53.

Presentations and Abstracts

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

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

CONCLUSIONS

Although we did not foresee current chemical terrorism concerns when the grant was written, the comparisons of Ellman and delta pH human blood ChE levels provide a valuable opportunity to establish normal human ranges for ChE enzymes. This is something that is lacking for the use of government and clinical laboratories to rapidly establish whether exposure to anticholinesterase agents has occurred. The data provided to us by Cpt. Reitstetter, a former director of the CHPPM labs is being analyzed by Dr. McCurdy and Mr. Arrieta and prepared for publication. The difference between genders in AChE levels is small, there is considerable overlap of the range of activity and it is not known whether such a difference would be of physiological significance.

The direct comparisons between Ellman and delta pH measurements that have been accomplished are resulting in analyses of methodology that will lead to further standardization and more accurate conversions. There were three directors of CHPPM in the past year since the project began; the recent appointment of Cpt. Lefkowitz to CHPPM and his enthusiasm for the project, and the continuing involvement of Cpt. Reitstetter will materially expedite the work in the coming year.

The poor correlation of AChE activity in two of the RBC sample sets is being investigated. The only common factor so far is that sets 1, 2, 4 were assayed the day they were received at UC Davis; set 3 and 5 were assayed the day after and stored at 4°C. The history of sample handling and assay at CHPPM is being checked. Future sample sets will be assayed at UC Davis on the day received and the day after receipt to see if there is any effect on activity levels.

Examination of the conditions of the delta pH procedure and those of the Ellman procedure suggest that the sensitivity of the delta pH assay may be improved by lowering its initial acetylcholine substrate concentration. The concentration now in use is within the inhibitory range (Wilson 1999, 2001). This matter will soon be put to the test, perhaps improving sensitivity to where we can use the current bovine RBC ghost standard preparation without concentrating it further. The ghost RBC standard was useful in previous work on ChE method comparisons, such as with the Test-mate Kit (Oliveira, *et al.*, 2002), and a sample is included with each run conducted at UC Davis as a routine quality control.

We thank Ms. Donna Goodman (CHPPM) for her able assistance in getting this project underway, Captains Reitstetter, Paulus and Lefkowitz for their courteous assistance in coordinating samples from CHPPM and UC Davis and their thoughtful suggestions for implementing the project.

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Ellman GL, Courtney KD, Andres V Jr., Featherstone RM. 1961. A new rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharm. 7:88-95.

Haley RW, Billecke S, La Du BN. 1999. Association of low PON1 type Q (type A) arylesterase activity with neurologic symptom complexes in Gulf War veterans. Toxicol. and Appl. Pharm. 157(3):227-333.

Hermenze F, Goodwin WJ (1959) Normal cholinesterase activity in the erythrocytes of cattle. J. Econ. Entomol. 52:66-68.

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Shih DM, Gu L, Xia Y-R, Navab M, Li W-F, Hama S, Castellani LW, Furlong CE, Costa LG, Fogelman AM, Lusis AJ. 1998. Mice lacking serum paraoxonase are susceptible to organophosphate toxicity and atherosclerosis. Nature (London) 394(6690):284-287.

Wilson BW. 1999. Cholinesterases. In Clinical Chemistry of Laboratory Animals. Quimby F and Loeb W (Eds). pp 430-440. Taylor and Francis Inc., Philadelphia.

Wilson BW 2001. Cholinesterases. In Hayes Handbook of Pesticide Toxicology, Vol 2, Agents, 2nd edition, R. Krieger Ed. Academic Press pp 967-985.

APPENDICES

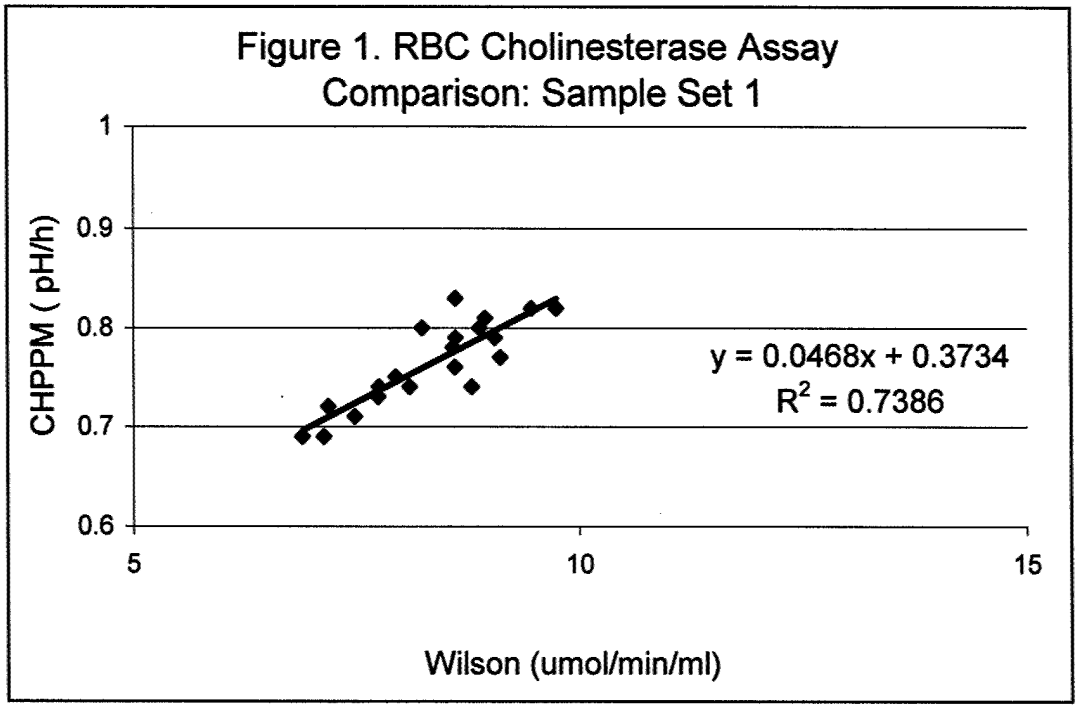


Figure 1. RBC Cholinesterase Assay
Comparison: Sample Set 1
CHPPM (pH/h)
1
0.9
0.8
0.7
0.6
5
10
15
y = 0.0468x + 0.3734
R2 = 0.7386
Wilson (umol/min/ml)

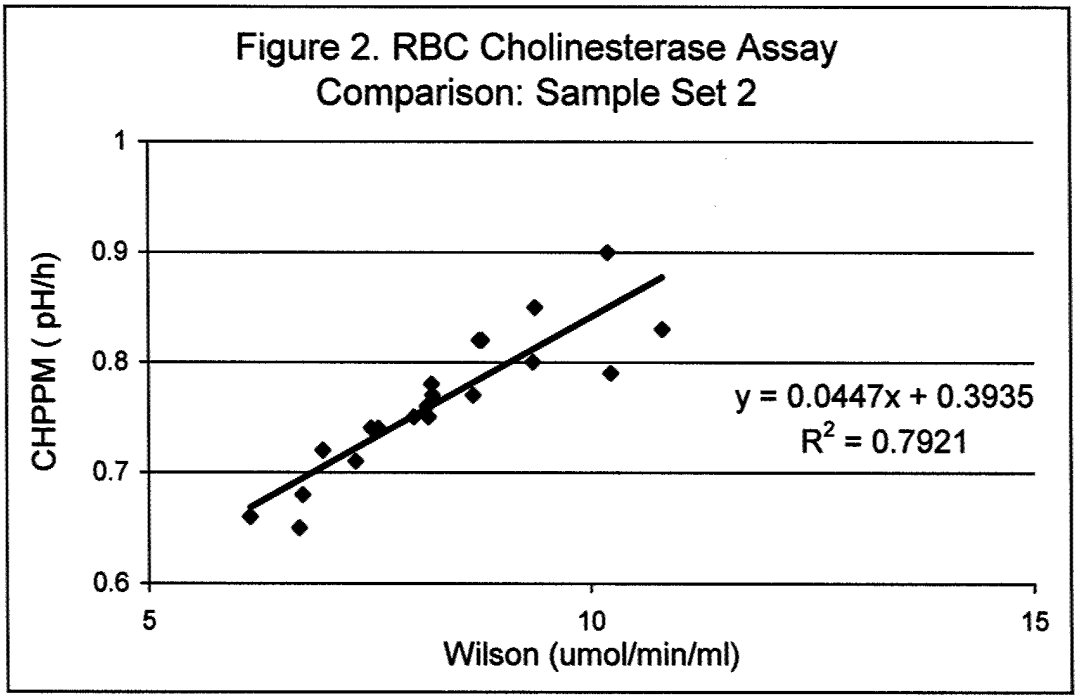


Figure 2. RBC Cholinesterase Assay
Comparison: Sample Set 2
CHPPM (pH/h)
1
0.9
0.8
0.7
0.6
5
10
15
y = 0.0447x + 0.3935
R2 = 0.7921
Wilson (umol/min/ml)

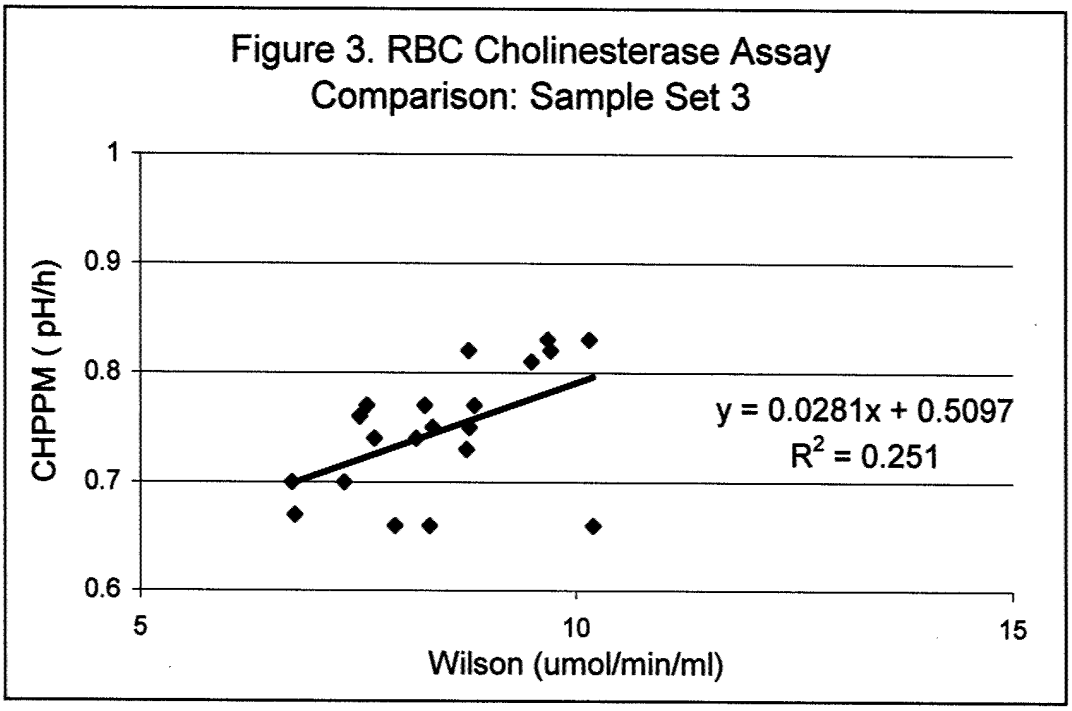


Figure 3. RBC Cholinesterase Assay
Comparison: Sample Set 3
CHPPM (pH/h)
1
0.9
0.8
0.7
0.6
5
10
15
Wilson (umol/min/ml)
y = 0.0281x + 0.5097
R2 = 0.251

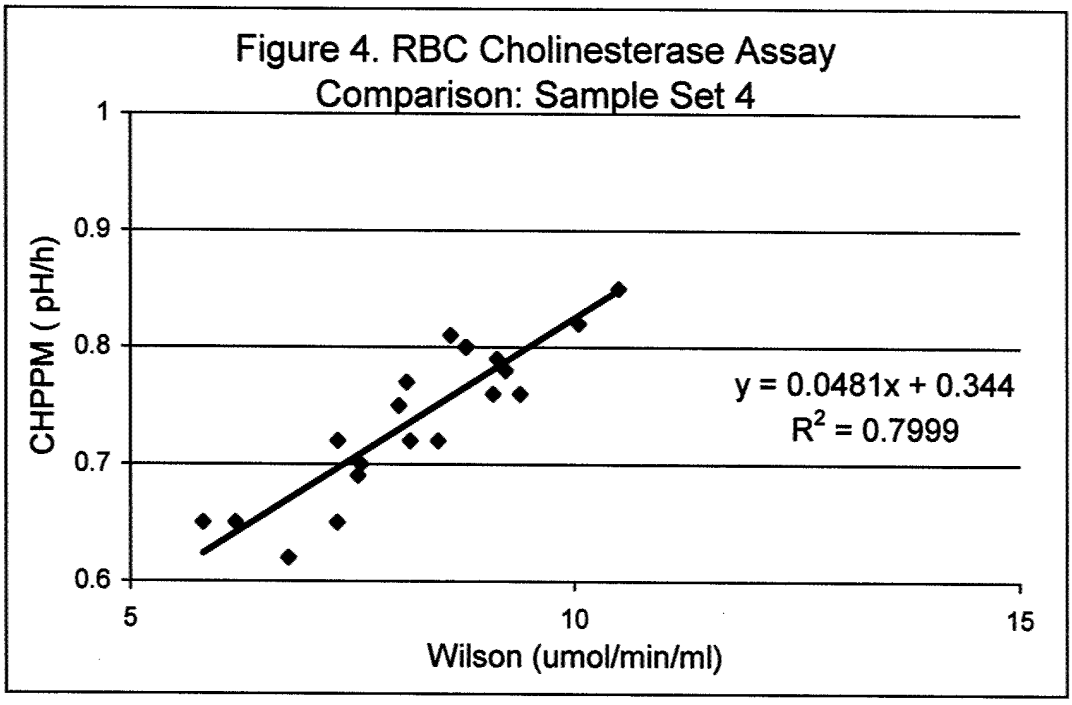


Figure 4. RBC Cholinesterase Assay
Comparison: Sample Set 4
CHPPM (pH/h)
1
0.9
0.8
0.7
0.6
5
10
15
Wilson (umol/min/ml)
y = 0.0481x + 0.344
R2 = 0.7999

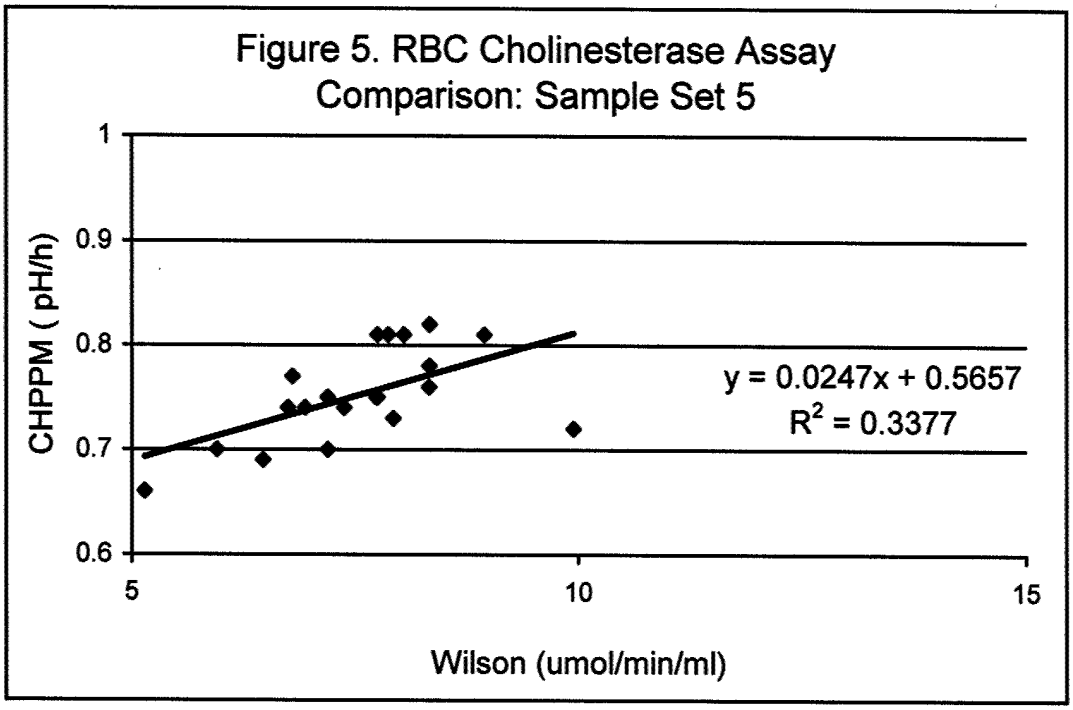
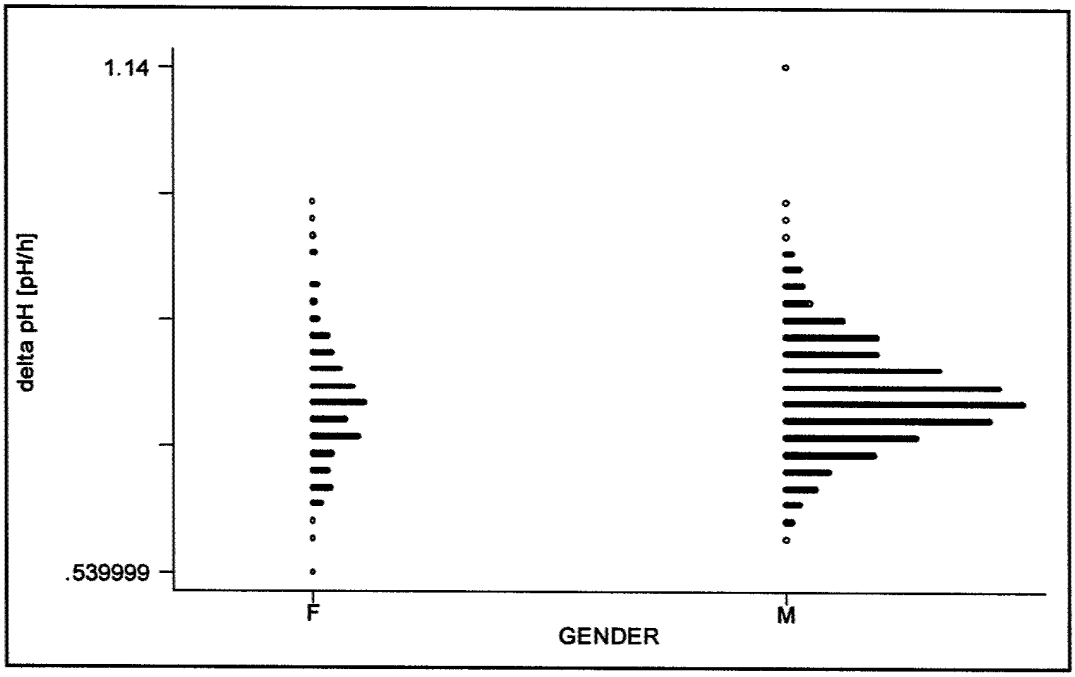


Figure 6. Gender Differences in CHPPM AChE Data Set



AChE Levels: Female = 0.733 ± 0.068 umol/min/ml (n = 269)
Male = 0.746 ± 0.059 umol/min/ml (n= 1174)

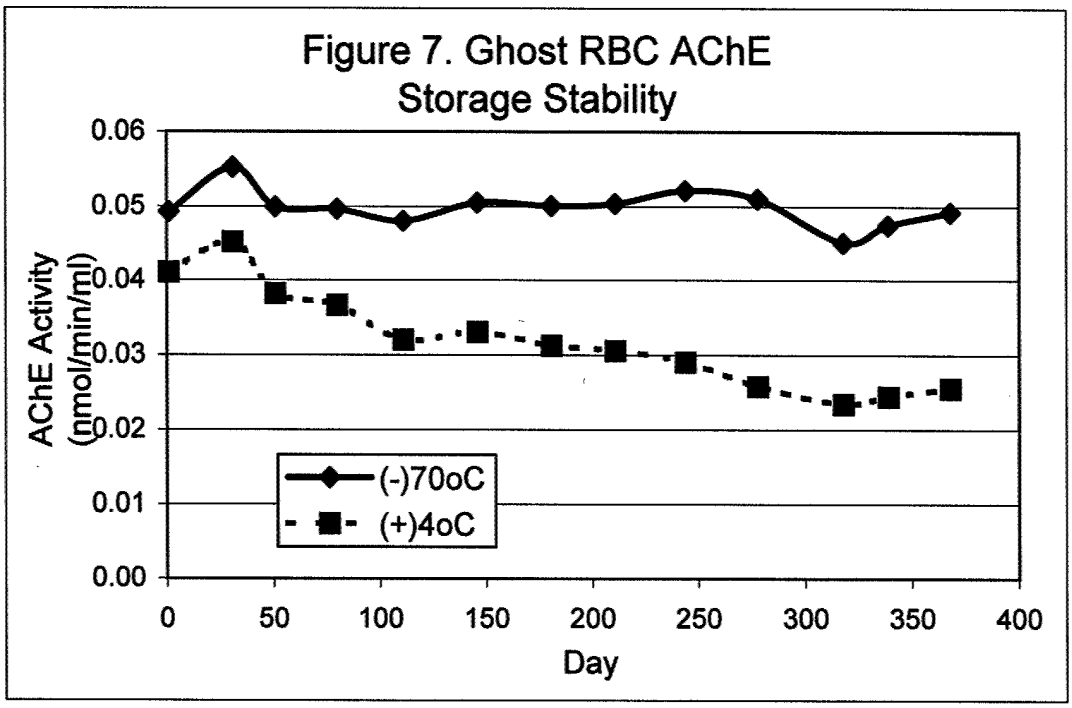


Figure 7. Ghost RBC AChE Storage Stability
AChE Activity (nmol/min/ml)
0.06
0.05
0.04
0.03
0.02
0.01
0.00
0
50
100
150
200
250
300
350
400
Day
(-)70oC
(+)4oC