Comparison of Acetylcholinesterase Assays Run under Conditions Specified by the Standard Ellman Method and Conditions Specified by a Commercial Cholinesterase Reagent Kit

July 30, 1998
HS-1752

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Acknowledgment

Review and editing of this work was done with the assistance of Marylou Verder-Carlos, Kathy Orr, and Louise Mehler. Statistical support was provided by Sally Powell.

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Introduction

Cholinesterase (ChE) monitoring plays a major role in protecting pesticide application workers from subacute poisoning with organophosphate (OP) insecticides.[1] ChE enzymes include acetylcholinesterase (AChE) (red blood cell [RBC]) and pseudocholinesterase (plasma or serum cholinesterase) - also known as butyl cholinesterase (BuChE). Monitoring methods include ΔpH(Michel), Boehringer Mannheim Corporation Reagent Set or BMC kit (Ellman-Boehringer), DuPont ACA, and Kodak-Ektachem[2]. When using different methods, results obtained cannot be directly compared because of variations in reporting units and underlying test methodology. Table 1 illustrates the differences between the standard Ellman method and the commercial BMC kits (presently the most common method used in laboratories). These differences cause problems in clinical settings when one laboratory runs baseline samples using one method and another laboratory runs the follow-up samples (e.g. at time of illness). Based upon a review of OP-related illnesses reported to the California Pesticide Illness Surveillance Program, methods used to measure baseline and follow-up levels differed frequently. For the years 1982 through 1990, case review demonstrated that of the 277 records reporting multiple tests, 123 (44.4%) contained results from at least two different test methods.[3] These cases included a parathion fatality[f] investigated by several California agencies.[4 5] As that case illustrates (see footnote), assay conditions and reporting units may differ markedly between laboratories. To establish quality control procedures for laboratories, inter-method comparison is essential when using different methods to measure cholinesterase.[6] This paper describes a method for directly comparing results between whole blood and red blood cell assays run under the standard Ellman conditions and the conditions specified for a commonly used commercial test-kit.

Methods

Blood samples were drawn in heparinized tubes from 9 volunteer subjects. The samples were kept on ice and assayed the same day for cholinesterase activity (Figure 1).

Blood was assayed with the Biotek Automated Microplate Reader (model EL3401), according to the following standard Ellman procedure: 220 μl of pH 8 phosphate buffer and 10 μl DTNB

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[f] The deceased established a baseline RBC cholinesterase level taken 12/19/89, (31 days before his death, performed by a laboratory in Tarzana, Ca), which had a value of 7,955 compared to a normal range of 5,000-10,000 (midpoint of normal range = 7,500). The baseline plasma cholinesterase was 3633 mU/ml compared to a normal range of 1,500-3,800 (midpoint of the range = 2,650). The post-mortem RBC cholinesterase value of a sample preserved in sodium fluoride was 6.5 IU/ml, compared to the normal range of 9.9-16.0 (midpoint of the range = 12.95). No serum CHE was obtained, but pericardial fluid contained 1.2 IU/ml of pseudocholinesterase activity. No normal range is available for pseudocholinesterase activity in pericardial fluid, but the normal serum range for this method is 2.4-6.2 IU/ml, (midpoint of the range = 4.3). Results of tests performed on 10 coworkers January 21 (the day following the death) showed marginal cholinesterase depression, but could not be directly compared to the baseline values because they were run at a different laboratory.

were added to each well, followed by 30 μl of sample. The samples were incubated for 5 minutes, then 30 μl of acetylthiocholine were added.

The substrate, pH, and chromogen concentrations used for the commercial kit assay were as specified in the package insert for the BMC kit for manual AChE assays (Boehringer-Mannheim Corporation, Indianapolis, Indiana).

In lieu of separating plasma and red cells by centrifugation, we conducted both microplate procedures on whole blood samples in test wells containing 0.2 mM of quinidine after reconstitution. Quinidine is a specific inhibitor of plasma cholinesterase[7] (butyryl cholinesterase - BuChE).

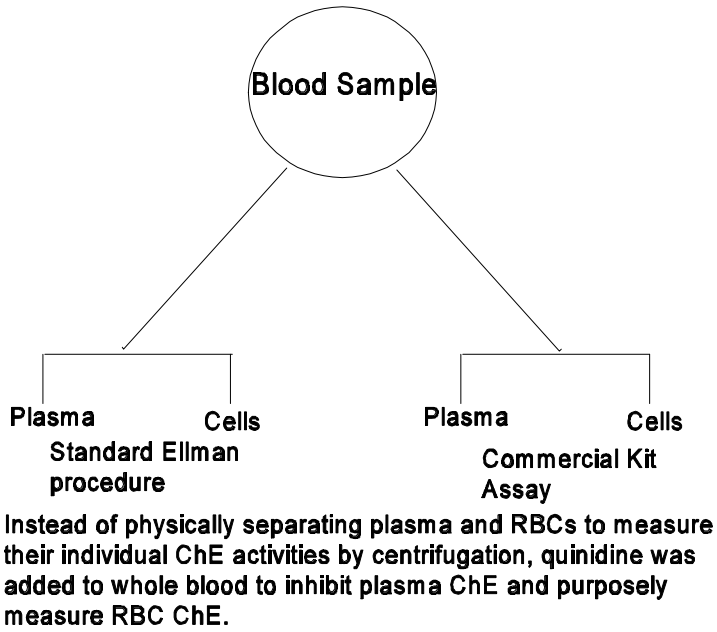

Figure 1 Method used for each blood sample collected.

Comparison of Test Methods

Initial evaluation of dilution versus inhibition

Multiple analyses were performed under both the BMC kit conditions and the standard Ellman conditions using blood from one subject. The cholinesterases were progressively inhibited with the organophosphate diisopropyl fluorophosphate (DFP) as follows: Fifty µl of DFP stock (varying concentrations) were added to 450 µl of whole blood, which was then incubated for 30 minutes at room temperature (24°C), then put on ice. The inhibited blood was diluted to 3%. The final concentrations of DFP in the assay solution for the progressive inhibition experiments were $1x10^{-7}$ mM, $3x10^{-7}$ mM , $1x10^{-6}$ mM,

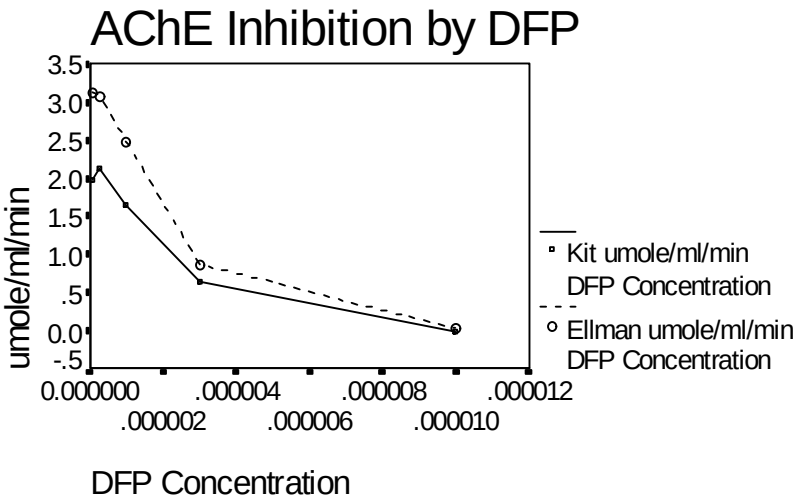

Figure 2 - Progressive inhibition of AChE with diisopropyl fluorophosphate (DFP)

$3x10^{-6}$ mM, and $1x10^{-5}$ mM (Figure 2).

Corresponding measurements were made of blood from the same subject following serial dilution (100%, 75%, 50%, 25%, and 10%) in diphosphate buffer. [Just prior to assay, blood was diluted to 3%: 30 μl of whole blood was added to 970 μl of 0.5% Triton X-100 in sodium phosphate buffer (50 mM and pH 7.2 for B-M kit, and 100 mM and pH 8.0 for Ellman). The 3% blood was diluted with the same buffer to give the following concentrations: 100%, 75%, 50%, 25% and 10%.] A plot of activities was constructed for both sets of experiments under BMC kit conditions versus those measured under Ellman conditions (Figure 3). Then, a multiple linear regression model was used to evaluate the difference between the slope and intercept of the lines derived from the two sets of experiments (Figure 4):

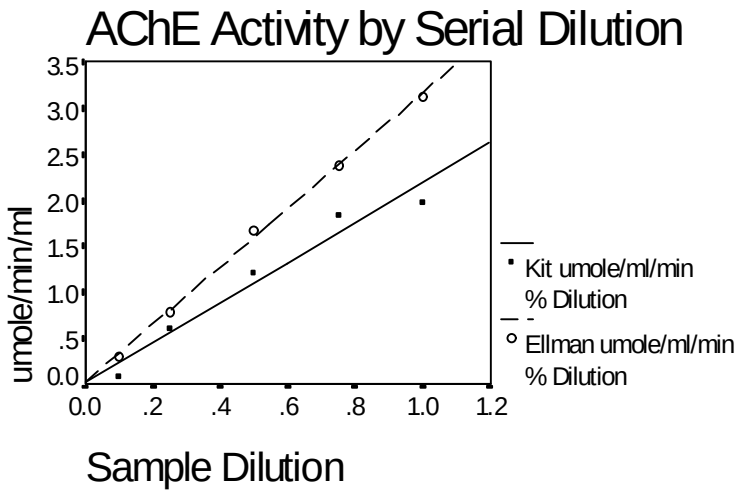

Figure 3- Serial dilution of AChE activity with diphosphate buffer

y1 = α + β1X = line derived from inhibition data:
y2= (α + β3) + (β1 + β2)x = (α + β3) + β1x + β2x = line derived from the serial dilution experiments;

β2 = hypothetical difference in slope between slope of line 1 (derived from the DFP inhibition experiments) and slope of line 2 (derived from the dilution experiments); represented by dummy variable D in SPSS model - set to value of x for group 1 (inhibition data) and to 0 in group 2 (dilution data);

β3 = hypothetical difference between intercept of line 1 and line 2; represented by dummy variable E in SPSS model - set to 0 for group 1 (inhibition) and to 1 in group 2 (dilution). The significance of the various terms in the model is evaluated in SPSS[8] by a t-test for each variable. The level of statistical signficance used for all analyses was 0.05.

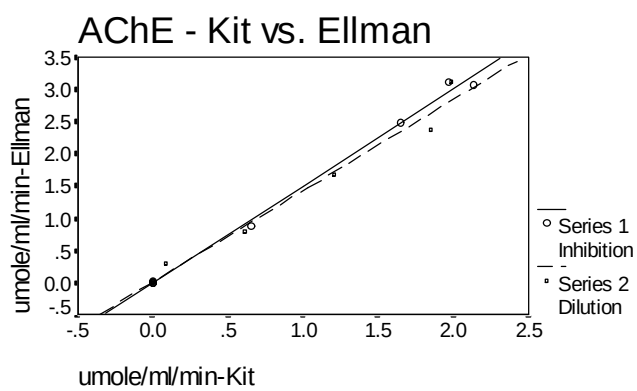

Figure 4 - Comparison of activity measured under Ellman conditions and under test kit conditions - series 1: samples prepared by progressive inhibition with diisopropyl fluorophosphate and series 2: serial dilution with diphosphate buffer

Comparison of multiple subjects with dilution methodology

Each test person's blood was diluted to 100, 75, 50, 25, and 10% in diphosphate buffer, then run on a microplate reader simultaneously in separate sample wells with reagents from each assay method. Each test was run in triplicate and the mean and standard deviation reported. We graphically plotted the relationship between the activities measured by the standard Ellman assay and the assays run with the BMC kits, then performed a linear regression analysis.

Evaluation of measurement errors

The coefficient of variance for the Ellman and kit assays was calculated from the mean and standard deviation for the triplicate assays performed on individual assays. The effect of measurement errors and of application of the conversion formula on calculated % inhibitions was also studied. Ellman activities predicted from the resulting regression equations were compared first to values actually measured and the per cent difference calculated. Consequently, a regression model tested the effect of the conversion factor on estimates of differential activity between samples. This involved calculating the differences in activities measured under the standard Ellman and BMC kit conditions for each subject at dilutions of 75%, 50%, 25% and 10% relative to the activity at the 100% solution. We performed the same calculations used to predict Ellman activities with the conversion formula from the activities measured under the BMC kit conditions. Differential activity was also calculated for predicted Ellman activities relative to the baseline measured under the Ellman conditions.

Results and Discussion

Initial evaluation of dilution versus inhibition

Although the relation between the DFP concentration and ChE activity was not linear (Figure 2), there was a linear relationship between activity and the % dilution in the second set of measurements (Figure 3). The relationship between measurements made under the standard Ellman conditions and those made under the BMC kit conditions appeared to be linear in both the dilution and inhibition experiments (Figure 4). Inhibition regression analysis of data in Table 2 showed that activities measured under the two conditions were highly correlated (R^2=0.994; regression formula: y=1.5X -0.018; SE of slope= 0.071, SE of intercept=0.13). When the activities measured under the Ellman and BMC kit conditions were compared by serial dilution, a similar result was obtained (R^2=0.965; regression formula: y=1.4 X +0.0498; SE of slope=0.1529, SE of intercept=0.25). Neither the slope nor the intercept of the two lines was statistically different when evaluated in the multiple regression model. Only the t value for $\beta 1$ was significant (p for t($\beta 1$)=0.000; p for t ($\beta 2$)=0.4779; p for t($\beta 3$)=0.78).

Comparison of multiple subjects using dilution analysis

Figure 5 summarizes the results for AChE assays for nine subjects tested on two sequential days. Linear regression analysis showed a strong correlation between assays run under standard Ellman conditions and those run under conditions specified for the BMC kit (R^2=0.983; conversion formula: y = 1.39 x-0.00979, with the SE of slope of regression line =0.028 and SE of the intercept = 0.15). This R^2 value compares favorably to those reported in the literature by Groff et al[9] and Mosca et al[10]. In Groff's study, interconversion of AChE assays performed with the manual ΔpH method and those performed by an automated version of the Ellman method had an R^2 value of 0.88. Similarly, Mosca's study achieved an R^2 value of 0.97 for interconversion between activities measured by the Ellman method and activities measured by a new method to monitor pH changes in capillary blood following hydrolysis of acetylcholine.

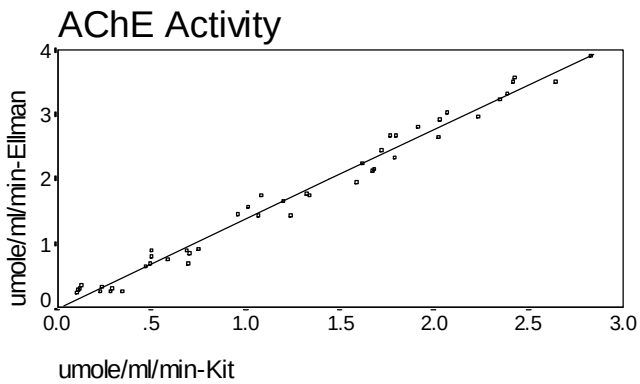

Figure 5 - Relationship between AChE activities measured under standard Ellman conditions and those measured under conditions specified by the BMC kit; R^2=0.983; regression formula: y = 1.39 x -0.00979

Evaluation of measurement errors

Coefficient of variance for Ellman and BMC kit activity measurements - In this series of experiments, the innate variability of the AChE assay under Ellman conditions appeared somewhat lower than under BMC kit conditions. The mean and standard deviation were calculated from results of the triplicate samples run for each of the nine subjects on undiluted samples and at 0.75, 0.5, 0.25, and 0.10 dilutions (total n=45). The average coefficient of variance for individual measurements of AChE was 5.34% for the Ellman assay and 12.17% for the kit assay. The mean value for each measurement was used in all of the regression analyses and in the calculations of % inhibitions.

Measured Ellman activities versus BMC kit activities transformed by conversion formula (predicted Ellman) - The difference between measured Ellman activities and BMC kit activities transformed by conversion formula (predicted Ellman activities) corresponds to the deviation of individual measurements from the line represented by the regression equation. The mean absolute difference was 0.1 µmole/ml/min (range: 0.0036 to 0.30 µmole/ml/min). As expected, the difference between predicted Ellman and measured Ellman activities was not statistically significant in a paired T-test. Expressed as % of total activity, the mean absolute difference between measured and predicted Ellman activities was 14.8%. However, these varied according to the degree of sample dilution. For undiluted samples, the mean absolute difference between

predicted and measured Ellman activities was 3.0% (range: 0.2 to 5.6%), but the mean absolute deviation for the 0.1 dilution was 32.6% (range: 2.7 to 90%).

Quantitative comparison between inhibitions calculated with Ellman measurements versus kit measurements and measurements calculated - Although the activities measured under standard Ellman conditions were approximately 40% higher than those measured under the BMC kit conditions, the % inhibitions calculated by the two methods were very similar (Figure 6). The mean arithmetic difference (sum of positive and negative differences/total observations) between inhibitions calculated using the Ellman activities and the kit activities was +1.8%. The mean absolute difference was 4.0% (range: 0.25 to 13.69%). Although small, this difference was of marginal statistical significance in a paired t-test (p=0.034). Inhibitions calculated from BMC kit measurements transformed by the conversion formula were very similar to those calculated directly from the kit measurements (mean individual difference = 0.15%; range: 0.0% to 0.32%).

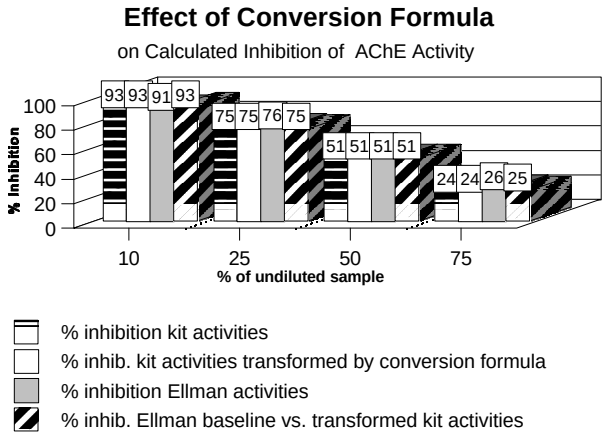

Figure 6 - Comparison of % inhibitions based on kit activities, kit activities transformed by conversion formula, Ellman activities, % inhibition Ellman baseline versus transformed kit activities

Effect of conversion formula on inhibitions calculated from differing baseline and follow-up methods - The activities measured on undiluted samples under Ellman conditions were chosen as the baselines and compared to predicted Ellman activities (activities measured under kit conditions transformed by conversion formula) for serial dilution samples. Inhibitions calculated from these simulated baseline and follow-up samples were similar to those calculated from kit activities. The mean arithmetic difference = -0.74% (range: -4.31 to 3.80%) and the mean absolute difference was 1.34% (range 0.01 to 4.31%). Inhibitions calculated from differing baseline and followup methods were also similar to those calculated from Ellman activities. The mean arithmetic difference = +1.2% (range -6.8 to+10.6%) and the mean absolute difference was 4.0% (range 0.12 to 10.6%).

Conclusions

In California, experience shows that many clinical facilities and laboratories provide services for individual agricultural businesses. The results of these experiments suggest that among laboratories currently performing AChE tests for the State of California's medical supervision

program, standardization of reporting is possible using the Ellman assay conducted at room temperature as a reference standard. Prior studies[11] have limited interassay comparisons to the normal enzyme activity range. To obtain a broader activity range for interassay comparisons, we simulated the effect of enzyme inhibition by diluting samples in diphosphate buffer. Preliminary experiments showed that the interassay regression line obtained from this procedure was equivalent to that obtained by serially inhibiting AChE with DFP (Figure 4).

AChE activity was approximately 40% lower under BMC kit conditions than under Ellman conditions in these experiments and had a higher coefficient of variance (5.34% vs. 12.17%). Nevertheless, correlation between the two methods was excellent ($R^2 = 0.983$). The mean difference between measured Ellman activities and activities predicted from kit activities and the conversion formula ($y = 1.39 x-0.00979$) was 0.1 µmole/ml/min and did not exceed 0.30 µmole/ml/min across the entire range of samples. Inhibitions calculated from the Ellman and BMC kit measurement also showed good agreement, on average, across the range of dilutions studied. The mean absolute difference was 4.0% (range: 0.25 to 13.69%). Expressing kit measurements in terms of the Ellman reference conditions by application of the conversion formula, altered the calculation of % inhibitions by less than 1% across the entire range of activities studied. However, inhibitions calculated from differing baseline (Ellman) and follow-up (kit) methods showed some variability when compared to inhibitions calculated from either method alone. The mean absolute difference was 1.34% (range 0.01 to 4.31%) compared to inhibitions calculated from the kit activities alone and 4.0% (range 0.12 to 10.6%) compared to inhibitions calculated from Ellman activities.

The inhibitions calculated from differing baseline and follow-up indicate that assay variability can be reduced by standardizing on pH, substrate concentration, and other assay conditions. Alternatively, standardizing the reporting with reference to the original Ellman assay conditions as done in this study, allows comparisons to be made between followup and baseline samples run in different laboratories. To standardize reporting of AChE tests used for medical surveillance and for evaluation of suspected poisonings due to ChE inhibitors, individual laboratories who conduct assays should carry out comparison studies similar to those described in this report. Additional work to be done includes study of sample handling variables (e.g. delay between time of blood draw and refrigeration). It will also be important to perform a direct comparison of the use of the quinidine inhibitor with physical separation of red cells and plasma as a means of measuring the AChE and BuChE activities.

Appendix 1 contains the proposed amendments in Titles 3 and 26, section 6728 of the California Code of Regulations. The amendments specify the cholinesterase test conditions the laboratories must follow in order to obtain approval from the State Department of Health Services.

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10. Mosca et al. *op.cit.*

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Table 1 - Comparison of Operating Conditions of Standard Ellman Assay Conditions with Boehringer-Mannheim Commercial Kits			
Parameter	**Ellman**	**Boehringer-Mannheim-Manual**	**Boehringer-Mannheim-Automated**
Spectophoto-metric wavelength (nanometers)	412	405	480
Substrate	Acetylthiocholine	Acetylthiocholine	Acetylthiocholine
Concentration (mM)	0.5 - 0.10	5.4	5.4
pH	8.0	7.2	7.2
DTNB concentration (mM)	0.32	0.24	0.24
Diphosphate buffer (mM)	100	50	50

Table 2 - Comparison of Ellman and commercial kit AChE activities in inhibition and dilution experiments		
DFP Concentration	*Ellman*	*Commercial Kit*
1×10^{-07}	3.13	1.97
3×10^{-07}	3.09	2.13
1×10^{-06}	2.49	1.65
3×10^{-06}	0.88	0.65
1×10^{-05}	0.03	0
R^2 = 0.994; slope = 1.52 SE = 0.071; intercept -0.018 SE = 0.13		
Dilution	Ellman	Commercial Kit
1	3.12	1.99
0.75	2.39	1.85
0.5	1.67	1.21
0.25	0.79	0.62
0.1	0.31	0.09
R^2 = 0.965; slope = 1.39 SE = 0.15; intercept 0.050 SE = 0.25		

Table 3 Comparison of AChE Activity for Standard Ellman Conditions versus Commercial Kit and Modeling of Effect of Conversion Factor

Subject	% Blood	Ellman Activity (umol/min/ml)	Kit Activity (umol/min/ml)	Predicted Activity	% Diff. obs. vs pred. ([O-P/P]x100)	% Depress. Trans-	% Depress. Kit	% Depress. Ellman	Ellman base vs. trans. kit % depression
1	100	2.92	2.03	2.83	3.1				
	75	2.16	1.68	2.34	-8.7	17.3	17.3	26.3	19.85
	50	1.43	1.07	1.50	-4.6	47.1	47.3	51.0	48.73
	25	0.65	0.47	0.66	-0.8	76.7	77.0	77.6	77.44
	10	0.25	0.28	0.40	-60.2	85.8	86.1	91.4	86.20
2	100	2.81	1.91	2.67	4.9				
	75	2.14	1.67	2.34	-9.1	12.4	12.5	23.7	16.74
	50	1.43	1.24	1.73	-20.9	35.2	35.3	49.0	38.38
	25	0.69	0.70	0.98	-41.9	63.4	63.6	75.5	65.17
	10	0.26	0.35	0.49	-90.0	81.6	81.9	90.8	82.51
3	100	3.03	2.07	2.89	4.7				
	75	2.24	1.62	2.26	-1.1	21.6	21.6	26.1	25.28
	50	1.45	0.96	1.34	7.9	53.6	53.8	52.1	55.82
	25	0.69	0.50	0.70	-0.5	75.8	76.1	77.1	76.96
	10	0.24	0.11	0.16	33.1	94.4	94.8	92.1	94.70
4	100	3.33	2.39	3.33	-0.3				
	75	2.45	1.72	2.40	1.9	28.0	28.1	26.4	27.79
	50	1.65	1.20	1.68	-1.5	49.7	49.8	50.3	49.53
	25	0.79	0.50	0.71	10.5	78.7	78.9	76.1	78.62
	10	0.29	0.11	0.17	41.5	95.0	95.3	91.4	94.97
5	100	3.25	2.35	3.28	-1.0				
	75	2.35	1.79	2.50	-6.5	23.9	23.9	27.7	23.06
	50	1.57	1.01	1.42	9.7	56.9	57.0	51.7	56.42
	25	0.76	0.59	0.83	-9.0	74.8	75.0	76.7	74.55
	10	0.27	0.23	0.33	-24.8	89.9	90.2	91.8	89.82
6	100	3.93	2.83	3.95	-0.5				
	75	2.97	2.23	3.11	-4.8	21.2	21.2	24.4	20.78
	50	1.96	1.59	2.22	-13.0	43.8	43.9	50.0	43.50
	25	0.91	0.75	1.05	-14.5	73.5	73.7	76.7	73.36
	10	0.30	0.29	0.41	-38.7	89.6	89.8	92.5	89.55
7	100	3.51	2.42	3.38	3.8				
	75	2.67	1.77	2.47	7.6	26.9	26.9	23.8	29.63
	50	1.76	1.34	1.87	-6.3	44.7	44.8	49.9	46.77
	25	0.89	0.69	0.97	-9.4	71.3	71.5	74.8	72.39
	10	0.35	0.13	0.19	45.5	94.3	94.6	90.0	94.54
8	100	3.51	2.65	3.69	-5.0				
	75	2.65	2.03	2.83	-6.7	23.3	23.4	24.6	19.49
	50	1.77	1.32	1.85	-4.7	49.9	50.0	49.7	47.38
	25	0.85	0.71	0.99	-16.3	73.1	73.3	75.7	71.76
	10	0.33	0.23	0.34	-2.7	90.9	91.1	90.7	90.44
9	100	3.59	2.43	3.39	5.6				
	75	2.69	1.80	2.51	6.5	25.8	25.9	25.1	29.99
	50	1.76	1.09	1.52	13.8	55.1	55.3	50.9	57.64
	25	0.90	0.50	0.71	21.3	79.1	79.3	74.9	80.27
	10	0.31	0.12	0.18	41.8	94.6	94.9	91.3	94.93

Table 4 Variations in error by % dilution of sample AChE Activity				
Measured versus Predicted Ellman Activities				
% of Undiluted Sample	Mean Ellman Activity	Mean Ellman Activity Predicted from Kit Activities & Conversion Formula	Mean % Individual Difference Measured vs. Predicted Ellman activity	Range % Difference
100	3.32	3.27	1.7	-5.0 to 5.6
75	2.48	2.53	-2.3	-9.1 to 6.5
50	1.64	1.68	-2.2	-20.8 to 13.8
25	0.79	0.84	-6.7	-41.9 to 21.3
10	0.29	0.30	2.7	-90.0 to 45.5
Total			-3.1	-90.0 to 45.5
Effect of Regression Formula on Inhibitions Calculated from Differing Baseline and Follow-up Methods				
% of Undiluted Sample	% Inhibition Kit Activity	% Inhibition Kit Activity Transformed by Conversion formula	Mean % Individual Difference	Range % Difference
75	22.3	22.2	-7.5	-34.1 to 16.5
50	48.4	48.6	-1.8	-8.7 to +5.2
25	74.3	74.0	+0.3	-2.5 to +2.1
10	91.0	90.7	0.1	-0.7 to 0.1
Inhibitions Calculated from Ellman Activities versus Inhibitions Calculated from Kit Activities				
% of Undiluted Sample	% Inhibition Kit Activities	% Inhibition Ellman Activites	Mean % Individual Difference Ellman vs Kit Activities	Range % Difference
75	22.3	34.0	34.3	+13.8 to 59.8
50	48.6	50.5	4.0	-10.2 to 27.9
25	74.3	76.1	2.4	-5.9 to 15.7
10	91.0	91.3	0.4	-5.1 to 9.8

Appendix 1

DEPARTMENT OF PESTICIDE REGULATION PROPOSED CHANGES IN THE REGULATION PERTAINING TO CHOLINESTERASE TESTING IN TITLES 3 and 26 OF THE CALIFORNIA CODE OF REGULATIONS

Amend section 6728 (f) to read:

6728. Medical Supervision.

(f) (1) To meet the requirements of these regulations, red cell and plasma cholinesterase tests ordered by a medical supervisor for occupational health surveillance shall be performed by a clinical laboratory currently approved by the State Department of Health Services to perform these tests.

(2) Reporting units shall be in International units per milliliter (IU/ml).

(3) Baseline and followup assays specified in 6728.2 (A) shall be conducted by the same laboratory method.

(3) The clinical laboratory shall report assay results according to the following reference conditions:

(a) The assay shall be performed on an ultraviolet spectrophotometer at a wavelength between 405 and 425 nanometers.

(b) The assay shall be performed at a temperature of 25°C.

(c) The followings, conditions regarding the buffer/chromogen shall apply.

(1) A sodium phosphate buffer shall be used at a concentration of 100 mM and a pH of 8.

(2) Dithiobisnitrobenzoic acid at a concentration of 0.32 mM shall be used with the sodium Phosphate buffer.

(d) The following conditions regarding the substrate shall apply:

(1) Acetylthiocholine shall be used at an assay concentration of 1.0 mM after reconstitution.

(e) A plasma cholinesterase inhibitor shall be used when performing a whole blood cbolinesterase assay. Quinidine shall be used at a concentration of 0.2 mM after reconstitution.

(f) Blood-samples shall be collected using EDTA or Heparin as an anticoagulant.

(g) Blood samples shall be kept in ice or at a temperature of 4° Celsius until time of assay. If sample is centrifuged to remove the erythrocytes from the plasma, the plasma may be stored frozen until the assay is performed. If whole blood is used, the assay shall be performed-within 24 hours after blood collection. Time of sample collection and analysis shall be specified on the report.

(3) Procedure for establishing equivalency of test methods shall be as specified in “Comparison of Acetylcholinesterase Assays Run under Conditions Specified by the Standard Ellman Method and Conditions Specified by a Commercial Cholinesterase Reagent Kit.”