Meeting Requirements of the California Cholinesterase Monitoring Program

Barry W. Wilson,[1] John D. Henderson,[1] Daniel E. Arrieta,[1] and Michael A. O'Malley[2,3]

[1]*Departments of Animal Science and Environmental Toxicology, and* [2]*Employee Health, University of California, Davis, California, USA*
[3]*Department of Pesticide Regulation, California Environmental Protection Agency, Sacramento, California, USA*

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.

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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

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Address correspondence to Barry W. Wilson, PhD, Department of Environmental Toxicology, University of California, One Shields Avenue, Davis, CA 95616, USA. E-mail: bwwilson@ucdavis.edu

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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 μl of 1/50 dilutions of whole blood samples, and 30 μl of 1/10 dilutions of plasma. The final assay volume was 320 μl. 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	*INP*	*INP*	*INP*
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	Boerhinger Manheim Reagent Kit	25°C
H	Olympus 640	Roche Diagnostics—Acetylthiocholine	37°C
I	*INP*	*INP*	*INP*
J	Bayer Opera	Sigma Diagnostics ChE (PTC)	*INP*
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	*INP*
N	Hitachi 717	Roche	25°C

INP = information not provided.

TABLE 2
Research and clinical ChE measurements: Conversion comparison

	Correlation coefficient (r^2)		
Laboratory	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

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 μmol/min/ml. These activities were below the levels found in human samples (1 to 2 μmol/min/ml in plasma and 8 to 17 μmol/min/ml in RBCs) and too low to be measured in some of the laboratories, possibly due to 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.

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) $+C$.

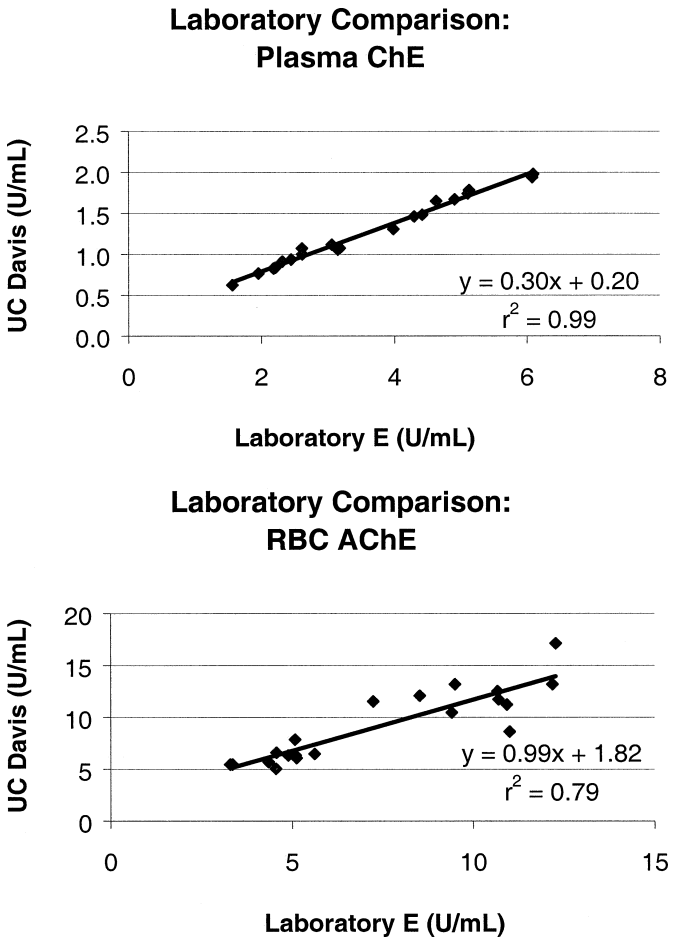

FIGURE 1
Examples of acceptable and unacceptable correlations.

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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