Standardization of Clinical Cholinesterase Measurements

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Previous studies showed that commonly used kits for measuring cholinesterases were not optimal for determining acetylcholinesterase (AChE) activity. Clinical use of different kits and methodologies resulted in AChE levels being reported in different units and activities that were not reproducible among laboratories. Findings such as these led to a revision in California regulations (covering AChE measurements for pesticide worker safety) calling for clinical laboratories to standardize their findings. The laboratories were contacted and invited to participate in a split-sample study of human blood AChE and nonspecific cholinesterase (BChE) assays. Participating laboratories measured erythrocyte (RBC) AChE and/or plasma BChE from undiluted and 50% diluted blood, according to their practices. Aliquots of blood samples were shipped to University of California Davis for measurement, using an optimized semiautomated plate reader version of the method of Ellman. Nine of 25 laboratories sent samples for comparison. Two others performed their own comparisons and submitted data to the state. Best correlations were obtained with BChE activity. Correlations (r^2) were .88 or above for four of five laboratories for BChE, and above .9 for two of seven laboratories for AChE. Reasons for poor correlations may include difficulties in pipetting RBCs, storage, and processing. A bovine AChE RBC ghost "standard" was devised and tested. Activity of the preparation was maintained at −70°C for approximately 11 months. A test with an East coast laboratory resulted in a high correlation, demonstrating the reliability of the RBC ghost standard and that one laboratory can replicate the AChE findings of another. The overall poor correlation of interlaboratory cholinesterase results points to the need to further standardize sample handling and assay methods.

Keywords Acetylcholinesterase, AChE, Assay, Clinical, Measurement, Standardize

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Cholinesterase (ChE) measurements are important indicators for detecting exposure to organophosphate (OP) and, with care, carbamate (CB) pesticides, and OP chemical warfare agents (Hoffmann, Solter, and Wilson 1999; Wilson et al. 1998, 2000; Wilson 2001). But, there is no agreement on standards for their determination. In collaboration with the Department of Pesticide Regulation (DPR) and the Office of Environmental Health and Hazard Assessment (OEHHA) of California Environmental Protection Agency (Cal EPA), this project has been optimizing conditions for sampling, storage, and assay of blood for red blood cell (RBC) acetylcholinesterase (AChE, EC 3.1.1.7) and plasma butyrylcholinesterase (BChE, EC 3.1.1.6). The improved tests are being validated in field studies, standard operating procedures are being refined, problems inherent in the clinical laboratory assays are being identified, and laboratory standards and new assays are being developed. Here, we report a comparison study with several clinical laboratories licensed by the state of California and the development of a simple, stable AChE standard suitable for clinical use.

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Twenty-five clinical laboratories were invited to participate in a split-sample study of human blood ChE assays. The intent was to establish the reproducibility of the assays performed by clinical laboratories, devise "correction" factors to enable them to compare results with each other, and satisfy the requirement of a recently revised state regulation (Title 3, California Code of Regulations, section 6728, Medical Supervision) specifying use of the procedure of Ellman et al. (1961), except "If an assay different from that described above is used, the method shall be shown comparable with the foregoing conditions and a conversion equation prepared."

METHODS

Participation in the project was voluntary. Clinical laboratories worked with Dr. Barry W. Wilson's laboratory or performed their own comparisons, submitting their results to Dr. Michael A. O'Malley (University of California Davis [UCD] Employee Health and DPR). Clinical laboratories were instructed to prepare samples of whole blood and blood diluted 50%. The samples were split into two sets of aliquots and one set was sent to Dr. Wilson's UCD laboratory. Some submitted samples more than one time. Sample sets were assayed for ChE activity by both laboratories. Results were sent to Dr. O'Malley, who compiled and compared the results.

Standard ChE Assays

The standard AChE assay in Dr. Wilson's UCD laboratory is a modification of Ellman's assay (1961) designed for a 96-well plate reader and discussed in a recent article (Wilson et al. 1997). Ten microliters of whole blood was hemolyzed and diluted 50-fold with 0.1 M sodium phosphate buffer, pH 8.0, and 0.5% Triton X-100 (tests have shown that this level of Triton is not inhibitory to human RBC activity). Final assay volume was 310 μl. The substrate was acetylthiocholine iodide (final concentration 1.0 mM). Dithio-bis-nitrobenzoate (DTNB; 0.32 mM final concentration) was the color reagent. Quinidine (0.02 mM) was used to inhibit BChE activity. Six readings were taken at 2-minute intervals at 410 nm. Temperature was 25°C, determined in the microplate wells with a probe. To estimate plasma BChE activity, 30 μl of blood diluted 10-fold with 0.1 M sodium phosphate buffer, pH 8, was used with the same assay conditions. Activities were expressed on per mg hemoglobin (Hb) or per milliliter blood bases.

RBC Ghosts

RBC ghosts are prepared from fresh bovine blood using a modification of the method of Hansen and Wilson (1999). Cows are maintained at the University of California Animal Science Department Dairy according to Animal Care Protocols approved by the Institutional Animal Care and Use Committee following National Institutes of Health (NIH) and United States Department of Agriculture (USDA) guidelines and under American Association for the Accreditation of Laboratory Animal Care (AAALAC) accreditation. Blood is collected in heparinized Vacutainer tubes, stored on ice, and transported to the laboratory. There they are centrifuged at low speed (1000 × g) in an RC2-B centrifuge with SS-34 head for 10 minutes to separate the plasma from the formed elements. The plasma layer is discarded. The RBC pellet is washed with buffer A (20 mM sodium phosphate, 135 mM NaCl, 3 mM KCl, pH 8) and then centrifuged for 10 minutes at 1000 × g. The red blood cell pellet is transferred to 30-ml centrifuge tubes using buffer A and centrifuged again at 1000 × g for 10 minutes. The supernatant is removed and 30 ml of buffer B (20 mOsm sodium phosphate, pH 7.4) is added to each tube with stirring. Five milliliters of a 7% (w/v) sucrose solution in buffer B is carefully pipetted into the bottom of each tube, forming a layer. The tubes are centrifuged at 27,000 × g for 20 minutes and the hemoglobin-containing supernatant discarded. Thirty milliliters of buffer A is added to the pelleted ghosts and centrifuged again at 27,000 × g for 10 minutes. The supernatant is discarded and 10 ml of buffer C (0.5% Triton X-100, in 0.1 M sodium phosphate buffer, pH 8.0) is added and the preparation put on ice. Aliquots of the solubilized ghosts are diluted 1:5 with buffer C, divided into 1-ml aliquots in microfuge tubes, and stored at 4°C or −70°C until assayed.

RESULTS

Nine laboratories sent samples to UCD for assay. Several laboratories did not determine plasma BChE activity. Two laboratories performed their own comparisons. The results of the split assays are summarized in Table 1 and the participating clinical laboratories are given alphabetical designations. Where data were available from both the UCD and clinical laboratories, correlations (r^2) were above .9 for two of seven laboratories for RBC AChE; .88 and above for four of five laboratories for plasma BChE. Correlations were .95 and above in comparisons from clinical laboratories performing in-house tests (two RBC AChEs and one plasma BChE). A satisfactory correlation and an unsatisfactory one are shown in Figures 1 and 2 respectively.

TABLE 1
Summary of blood ChE comparisons

	r^2		
Laboratory	RBC	Plasma	Comment
A	.98	—	In house tests, acceptable
B	.95	.99	In house tests, acceptable
C	.93	.94	2nd set, acceptable
D	.61	—	Poor, unacceptable
E	.74	—	Unacceptable
F	.004	.81	Unacceptable
G	.79	.88	Assay details lacking
H	.33	.97	RBC dilutions may be awry
I	.96	.99	Acceptable

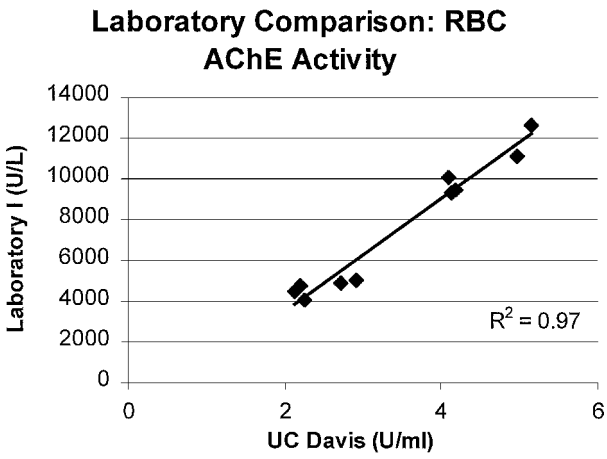

FIGURE 1
Example of an acceptable correlation.

Problems in making comparisons included the state not receiving data from one laboratory and data received from another clinical laboratory not matching the UCD data set. RBC activities submitted by a third clinical laboratory were approximately the same for the 100% and 50% dilutions, perhaps indicating a problem with dilution.

Most of the laboratories used the Boehringer/Mannheim (Roche) kit, but Sigma and Beckman kits were also used. Temperatures of clinical laboratory assays were often 37°C. Some laboratories provided little detail on their methods.

A stable RBC AChE standard was successfully devised from bovine blood (a species that lacks plasma BChE; Wilson 2001). AChE activities remained stable at ultralow temperatures (−70°C) for at least 328 days and at refrigerated temperatures (4°C) for at least 150 days. For example, up to 150 days, mean activities were 0.68 ± 0.04 U/ml at −70°C and 0.66 ± 0.03 U/ml at 4°C. There was a high correlation ($r^2 > .99$) between measurements of the diluted samples made in Dr. Padilla's laboratory at the EPA in North Carolina and those made at the UCD laboratory (Figure 3).

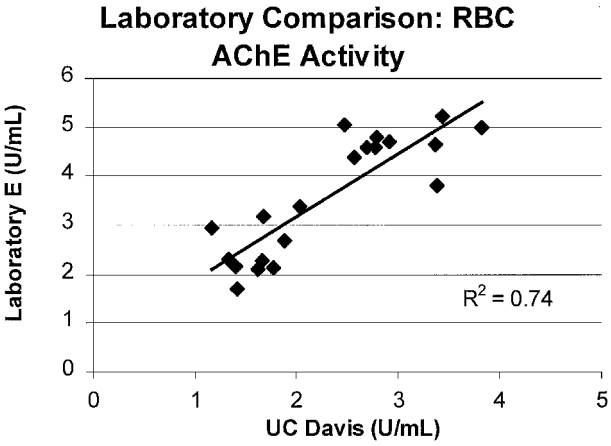

FIGURE 2
Example of an unacceptable correlation.

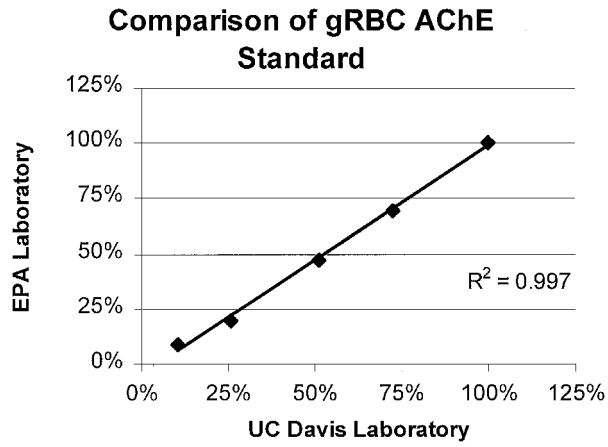

FIGURE 3
AChE activity comparison of a dilution series of a RBC ghost preparation measured at EPA and University of California Davis laboratories. Assay temperature was 27°C at EPA and 25°C at U.C. Davis.

DISCUSSION

Best results in the laboratory comparisons were from plasma BChE assays. One reason may be the difficulties encountered in pipetting thick RBC preparations. A solution would be to solubilize the whole blood sample with a detergent such as Triton X-100 and rely upon selective inhibitors (e.g., BW284c51 for AChE, quinidine for BChE) to distinguish RBC AChE and plasma BChE activities. Hematocrits could be avoided by expressing the results on a per mg Hb basis. Although satisfactory results were achieved for plasma assays, the state's instructions required that the same substrate (acetylthiocholine) be used for the plasma as for the RBC assays, even though the plasma enzyme prefers butyrylthiocholine and some OPs preferentially inhibit BChE instead of AChE (Hoffmann, Solter, and Wilson 1999; Wilson 2001).

An important issue is storage of the samples. For example, a US EPA–sponsored cholinesterase methodologies workshop found that sample storage and assay conditions for ChEs were often biochemically inappropriate (US EPA 1992). Some laboratory spokespersons told us that they did not require samples be iced before and during shipping. Perhaps this is a result of a common impression that OP inhibitions are "irreversible" and do not require storage on ice. Unfortunately, methyl OP inhibitions are spontaneously reactivatable with reactivation times of a few hours (Hoffmann, Solter, and Wilson 1999). Storage conditions need to be studied to demonstrate the differences between reactivations of inhibitions with carbamates, methyl, ethyl, and other OPs.

The availability of a reliable RBC AChE standard provides an objective test for clinical laboratories when performing the

assays and establishes conversion factors for their assay conditions. Commercial preparations are available. A comparison of the UCD bovine ghost RBC AChE preparation with a lyophilized bovine preparation of Sigma (catalog no. C5021) is in progress. The mean activity of the Sigma preparation was 0.582 ± 0.043 U/ml and 0.588 ± 0.043 U/ml for to 128 days at −70°C and 4°C, respectively.

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