Taylor & Francis
Taylor & Francis Group

Journal of Toxicology and Environmental Health Part A

ISSN: 0098-4108 (Print) (Online) Journal homepage: http://www.tandfonline.com/loi/uteh19

FACTORS IN STANDARDIZING AUTOMATED CHOLINESTERASE ASSAYS

Barry W. Wilson

To cite this article: Barry W. Wilson (1996) FACTORS IN STANDARDIZING AUTOMATED CHOLINESTERASE ASSAYS, Journal of Toxicology and Environmental Health Part A, 48:2, 187-196, DOI: 10.1080/009841096161429

To link to this article: https://doi.org/10.1080/009841096161429

FACTORS IN STANDARDIZING AUTOMATED CHOLINESTERASE ASSAYS

Barry W. Wilson

Departments of Environmental Toxicology and Avian Sciences, University of California, Davis, California, USA

Stephanie Padilla

Neurotoxicology Division, U.S. Environmental Protection Agency, Research Triangle Park, North Carolina, USA

John D. Henderson

Departments of Environmental Toxicology and Avian Sciences, University of California, Davis, Davis, California, USA

with the assistance of the following round robin participants:

Stephan Brimijoin

Mayo Clinic, Rochester, Minnesota, USA

Proveen D. Dass

Agriculture Division, Miles, Inc., Stilwell, Kansas, USA

Glenn Elliot

DuPont Haskell Laboratory for Toxicology and Industrial Medicine, Newark, Delaware, USA

Bruce Jaeger

U.S. Environmental Protection Agency, Washington, DC, USA

David Lanz

U.S. Army Medical Research Institute of Chemical Defense, Aberdeen Proving Ground, Maryland, USA

Renee Pearson

CORNING Hazleton, Inc., Vienna, Virginia, USA

Rachel Spies

Pharmaco-LSR., East Millstone, New Jersey, USA

Received 17 August 1994; accepted 5 September 1995.

Supported in part by NIOSH University of California Agricultural Health and Safety Center at Davis (U07-CCU906162-02), U.S. Air Force (AFOSR-91-0226), NIEHS Center for Environmental Health Sciences (P30-ES-05707), and the U.S. Environmental Protection Agency (R814709) Center for Ecological Health Research at University of California, Davis. The authors thank the following for their contribution: K. Baetcke, J. Bool, B. P. Doctor, G. Flores, R. Gaul, K. Hickey, S. Jones, A. Kaminskis, M. Landes, M. Landis, R. Larrison, G. Lau, M. Mejia, K. Schalk, J. VanDenbussche, G. Williams, and S. Willig. The research described in this article has been reviewed by the Health Effects Research Laboratory, U.S. Environmental Protection Agency, and approved for publication. Approval does not signify that the contents necessarily reflect the views and policies of the agency, nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

Address correspondence to Dr. Barry W. Wilson, Department of Avian Sciences, University of California, Davis, CA 95616, USA.

Journal of Toxicology and Environmental Health, 48:187–195, 1996

0098-4108/96 $12.00 + .00

A scientific panel assembled by the U.S. Environmental Protection Agency (EPA) determined that variability in cholinesterase (ChE) activities in the agency's pesticide/animal study database likely was due to a lack of accepted guidelines for ChE methodology. A series of trials was held in which participating laboratories measured ChE activity in blood and brain samples from untreated and pesticide-treated rats using a colorimetric assay method. The degree of inhibition of ChE activity in plasma and brain samples compared to controls was consistent among most of the laboratories. The ChE activity in erythrocyte samples differed more between laboratories due to a high blank, low erythrocyte AChE activity and hemoglobin absorption at the wavelength of the assay. Strategies are suggested for minimizing the variability of ChE activity in hemoglobin-rich samples.

A panel of scientists was convened by the U.S. Environmental Protection Agency (EPA) after the agency determined that its database on animal studies of organophosphate (OP) and organocarbamate (OC) pesticides showed little correlation between the doses of pesticide used and the levels of cholinesterase (ChE) activity in tissues taken from the treated animals. The panel held a Workshop on Cholinesterase Methodologies (Wilson et al., 1992); one outcome of the meeting was the recognition that there were no generally accepted guidelines for standard operating procedures (SOPs) for ChE determinations, although ChE measurements are commonly taken during testing of anticholinesterases. This led to a series of collaborative "round robin" tests participated in by several research and clinical laboratories. This report summarizes the findings to date comparing automated and plate reader assays of ChE activity using acetylthiocholine hydrolysis and colorimetric techniques to determine the extent of ChE inhibition in rat blood and brain, with the subsequent development of a tentative SOP. Some of these findings have been presented in preliminary form (Wilson et al., 1995a, 1995b).

METHODS

Three pilot experiments led to the trial reported here. This report is derived from two written reports and one oral short progress report to the U.S. EPA.

Participants were instructed to run the thiocholine assay (Ellman et al., 1961) with dithiobisnitrobenzoate (DTNB) at 25–27°C and/or 37°C for at least 3 min, collecting a minimum of 6 data points at 410–412 nm. The recommended assay buffer was 0.1 *M* Na phosphate, pH 8, the final concentration of acetylthiocholine iodide was to be at least 0.5 m*M*, and the final concentration of DTNB at least 0.33 m*M*. These conditions are optimal for rat acetylcholinesterase activity (S. Padilla, personal communication). Substrate (substrate, buffer, DTNB, no tissue) and tissue (tissue, buffer, DTNB, no substrate) blanks were to be run and both subtracted to obtain the final activities. The "DTNA" assay, which replaced DTNB with dithionicotinic acid (DTNA), was read at 340 nm. The "automated" assay used DTNB, 480/660

nm, and the conditions specified by Boehringer-Mannheim (B-M) for their analysis kit (catalog number 124117 or 450035).

Memoranda were sent and conference calls were held before the trial. Detailed instructions and forms were sent to each voluntary participant. The results of each participant were sent to them to recheck for accuracy together with specific questions concerning data or conditions that were unclear from their report. Nevertheless, several participants did not use the conditions requested. For example, the concentration of substrate indicated by one participant was high enough to raise concerns that activities may have been inhibited. Some results were omitted from the test after discussions with the laboratory heads.

Participants were asked to determine the ChE activities of plasma and erythrocytes of rats dosed with two levels of an OP (chlorpyrifos), and the ChE activity in brain homogenates inhibited in vitro with a carbamate pesticide (carbaryl), comparing triplicate samples of each treatment with untreated controls.

Rats were dosed with chlorpyrifos (0, 30, or 120 mg/kg,sc) at the U.S. EPA laboratories, Research Triangle Park, NC. Four days after dosing (time of peak cholinesterase inhibition), the animals were anesthetized with carbon dioxide and killed by decapitation. Trunk blood was collected in heparinized tubes and plasma and packed erythrocytes were separated by centrifugation. The plasma was collected and frozen (–80°C) undiluted, while aliquots of the packed erythrocytes were diluted 1 : 20 (initial volume : final volume) with 1% Triton X-100 in Na phosphate buffer (pH 8), vortexed and frozen (–80°C). Whole brain was also removed from each animal and stored frozen (–80°C) until homogenization (1 : 25 v/v with 1% Triton in pH 8 buffer, on ice). Before the samples were shipped out, the samples for plasma and erythrocytes for each dosage level were pooled to create one sample and aliquots were sent out to each laboratory.

For the in vitro carbaryl experiments, brains from untreated rats were homogenized with a Dounce homogenizer (glass on glass, on ice, 1 : 9 in 0.1 *M* Na phosphate buffer, pH 8). Then the brain homogenates were incubated with the appropriate concentration (vehicle, 1 μ*M* or 10 μ*M* final concentration) of carbaryl (in acetone vehicle) for 2 h at 37°C.

Samples were packed in dry ice and shipped by air express to the other participants. The laboratories were instructed to store them at –80°C until analyzed.

Although the rat erythrocyte contains only true acetylcholinesterase (EC 3.1.1.7, AChE), rat plasma that contains ChE activity contains approximately one-third AChE activity in addition to its nonspecific cholinesterase (EC 3.1.1.8, BChE) activity (Aldridge, 1953).

Conditions chosen for the ChE assay were (a) 412 nm, 25°C or

37°C, (b) 480 nm, 37°C with DTNB, and (c) 340 nm, 25°C or 37°C with DTNA as the color reagent. All investigators were instructed to run tissue and substrate blanks and to use both of them in their calculations. A 5- to 10-min preincubation of all assay components except substrate was recommended to decrease the tissue blank values. Manual assay conditions for microtiter plates generally included 0.5 m*M* substrate and readings at 412 nm. The automated assay instructions called for 5.4 m*M* acetylthiocholine and 0.25 m*M* DTNB, read at 480 nm, and run at 37°C. The data were not corrected for temperature. The data were sent to Wilson and Padilla, who analyzed and compiled the results.

RESULTS

The three pilot experiments established the following results.

The majority of clinical laboratory participants (three of four) used a Hitachi automated instrument and a B-M "kit" for automated instruments containing all reagents (B-M/H procedure). The research laboratories prepared their own reagents and the majority (three of four) used 96-well microtiter plate readers. All laboratories used one or more versions of the acetylthiocholine/DTNB assay of Ellman et al. (1961).

Values for control samples were comparable between the research and the clinical laboratories for plasma and brain. However, the clinical laboratories using the B-M/H procedure tended to underestimate inhibitions in samples with low endogenous activity, in this case rat erythrocytes. ChE values for hemoglobin-rich, low activity tissues determined using the B-M/H automated procedure were often less than two times background, significantly reducing the sensitivity of the assays.

Blanks

As expected, the blanks (which remain fairly constant) represented more of the total activity of inhibited samples than of controls. This introduces inaccuracies if both blanks are not used in the calculations. (Preincubating the samples for 5–10 min before starting the assays with addition of substrate will reduce the tissue blank values and minimize this error.)

Inhibited Samples

Regardless of the specific methodology used, enzyme activities of inhibited samples were fairly consistent when corrected for blanks (Table 1). Plasma ChE levels were 28.5 ± 5.5 (mean ± standard deviation) and 60.7 ± 7.2% of controls (n = 14). Brain ChE samples were 18.5 ± 6.8 and 79.4 ± 4.7% of controls (n = 14). Erythrocyte

TABLE 1. Percent ChE activity compared to untreated sample

Conditions	Number of laboratory runs	Percent activity, 30 mg/kg	Percent activity, 120 mg/kg
	Plasma: treated in vivo with chlorpyrifos		
410 nm, 25°C, DTNB	3	67.6 ± 2.8	34.8 ± 3.5
410 nm, 37°C, DTNB	7	60.5 ± 7.3	27.7 ± 5.4
480 nm, 37°C, DTNB	3	54.0 ± 5.6	24.0 ± 1.7
340 nm, 37°C, DTNA	1	62.0	28.0
Grand total	14	60.7 ± 7.2	28.5 ± 5.5
	Red blood cells: treated in vivo with chlorpyrifos		
410 nm, 25°C, DTNB	3	51.7 ± 21.2	1.8 ± 2.0
410 nm, 37°C, DTNB	5	46.8 ± 11.6	18.7 ± 17.8
480 nm, 37°C, DTNB	3	71.0 ± 12.5	43.3 ± 7.5
340 nm, 25°C, DTNA	1	47.7	7.6
340 nm, 37°C, DTNA	2	50.1	13.1
Grand total	14	53.6 ± 15.1	18.8 ± 18.1
Conditions	Number of laboratory runs	Percent activity, 1 μ*M*	Percent activity, 10 μ*M*
	Brain: treated in vitro with carbaryl		
410 nm, 25°C, DTNB	4	81.3 ± 3.7	19.9 ± 7.6
410 nm, 37°C, DTNB	6	77.9 ± 2.5	16.8 ± 3.5
480 nm, 37°C, DTNB	3	78.0 ± 8.7	19.0 ± 12.8
340 nm, 37°C, DTNA	1	85.0	22.0
Grand total	14	79.4 ± 4.7	18.5 ± 6.8

Note. Percent activities are means ± standard deviations; samples were run in triplicate.

AChE values were more variable; the grand total of activities averaged 18.8 ± 18.1 and 53.6 ± 15.1% of controls (n = 14). The automated assays read at 480 nm assays tended to be the most inaccurate for erythrocyte samples, averaging 43.3 and 71%, levels much higher than those from assays run at the recommended wavelength for the Ellman assay. The few assays run with DTNA as a chromogen and read at 340 nm gave values similar to DTNB run at 410–412 nm.

CONCLUSIONS

The Ellman assay, and its modifications for multiple plate readers and automated instruments (such as Technicon and B-M/H), gave reasonably reproducible results when performed according to the wavelength, buffer, and substrate recommendations of the original Ellman report, insofar as measurements of plasma and brain ChE activities were concerned. However, the relatively low AChE activity of the rat erythrocyte, a relatively high DTNB tissue blank, and the

absorption of hemoglobin at the wavelength of the assay all contributed to reduced reproducibility and sensitivity. A recent study of Christenson et al. (1994) also found that AChE determinations in rat erythrocytes were more variable than in plasma or brain in an interlaboratory comparison.

Conducting the assay at 480 nm, as recommended by Boehringer-Mannheim, led to inaccuracies that resulted in overestimations of the activity of OP inhibited samples. Conducting the assay at 340 nm and using DTNA as the color reagent, as recommended by Loof (1992), gave results similar to assays using DTNB in one laboratory reported here, and in other studies of the participants (personal communications). Even though the tests were carefully discussed beforehand, several of the laboratories substituted conditions of their own or did not adequately report what was done.

DISCUSSION

The determination of cholinesterase levels is important in the monitoring and study of exposure to pesticides and chemical warfare agents and disorders such as Alzheimer's disease. Early methods of measuring ChE activity were reviewed by Witter (1963). There are several basic methods used to determine the activity of these widespread hydrolases (for a recent review see Wilson & Henderson, 1992). These include (a) detection of the release of a thiol group by hydrolysis of acetylthiocholine or other thiol esters with either DTNB or DTNA (Ellman et al., 1961; Loof, 1992), (b) measuring the change in pH that accompanies hydrolysis of acetylcholine (Hestrin, 1949), and (c) determinations of the hydrolysis of radiolabeled acetylcholine (Johnson & Russell, 1975). Each has its recent advocates. For example, the Association of Official Analytical Chemists sponsored two studies: One compared the ability of 10 laboratories to analyze duplicate samples of OP-inhibited whole bovine blood (Harlin & Ross, 1990) according to Ellman et al. (1961). In another study, 10 laboratories analyzed similar samples with a pH assay (Imerman, 1993). A third recent study used a radiometric assay to examine exposure of pesticide and fumigant applicators (Potter et al., 1993). Thiocholine hydrolysis assays were chosen for the investigations reported here because of their combination of sample handling capability, versatility, and ease. Limitations of the other other methods include a low "throughput" with assays based on pH changes, and increased difficulty and expense to dispose of radioactive chemicals used in radiometric assays.

One of the limitations of thiocholine-based assay methods that use DTNB is that the peak absorption of the color reagent corresponds to a major band of mammalian hemoglobin absorption. A solution is to sacrifice sensitivity and measure absorption at some other wavelength.

For instance, the portable Test-Mate kit (EQM Research Inc.; Magnotti et al., 1988; McConnell et al., 1990) determines human blood AChE activity at 470 nm and the B-M/H ChE automated technique uses 480 nm. Although sensitivity of the assay is reduced by 70% or more under these conditions (Wilson & Henderson, 1992), this approach is practical for samples with sufficient activity, such as human erythrocytes. Reading at a suboptimal wavelength to minimize hemoglobin interference may become extremely important, however, when analyzing rat erythrocytes, which have one-tenth of the activity of human erythrocytes.

Background changes in absorbance are another concern. The B-M/H procedure designated a high substrate concentration, approximately 10-fold that recommended by Ellman et al. (1961), and a concentration that inhibits acetylcholinesterase activity. In addition, this high substrate concentration doubles the substrate blank, further reducing the sensitivity of the assay. Also, the Hitachi machine automatically subtracted a substrate blank, but not a tissue blank unless specially instructed to do so. If tissue blanks were high, as with the rat erythrocyte, or if experimental activities were low, significant inaccuracies could result. It is especially important in these cases to make corrections for background tissue hydrolysis of the color reagent. Loof (1992) proposed that the high endogenous reduction of DTNB of the rat erythrocyte may be due to a high glutathione reductase activity.

The trial presented here used carbaryl as the test compound for brain homogenates because of a concern for the determination of ChE activity in tissues taken from carbamate-treated animals. Carbamate pesticides are unstable inhibitors of ChE and are more readily rehydrolyzable than OP esters. Results were satisfactory in this trial, but more study is needed to examine the variability of the assays when the carbamate pesticide is added in vivo. Reactivation of most carbamylated ChEs is enhanced when samples are diluted and/or are incubated for an extended time. A previous study of Padilla and colleagues (Nostrandt et al., 1993) suggested modifications to minimize spontaneous reactivation of enzyme activity, including preincubation of concentrated tissue with concentrated chromogen (i.e., DTNB), dilution to final reaction volume immediately before measurement, and measurement of ChE over a short period of time (5–10 min).

Past studies by others have found high and often unacceptable variability in Ellman-based assays between laboratories (Fleming et al., 1992; Mineau & Peakall, 1987). In the present study we found acceptable consistency among laboratories for tissues with low hemoglobin content and relatively high endogenous ChE activity. However, this study was designed to focus on the ChE assay itself, and problems inherent in tissue preparation and storage, animal dosing, tissue selection, and study design have yet to be addressed. Such factors

may need to be considered before a standard operating procedure for ChE assays can be initiated.

The data presented here led the investigators to recommend the following to the U.S. EPA Scientific Advisory Panel (Wilson et al., 1993):

1. Clinical pathology laboratories carrying out biochemical assays for diagnostic or regulatory purposes should have biochemists available to advise the pathologists that run the units on optimizing the assay procedures.
2. The U.S. EPA should consider requiring conformance to FIFRA guidelines (or a modification thereof) of good laboratory practices for cholinesterase measurements submitted to the agency.
3. Standard operating procedures submitted to the agency should be reviewed by scientists selected by the Agency with the advice of the Scientific Advisory Panel.
4. One or two more tests should be done on the ChE measurements using a few laboratories before protocol guidelines are finalized.
5. Other tests should focus on the use of DTNA to avoid hemoglobin interference in the assay, on the use of whole blood instead of separating red blood cells from plasma, and on the accuracy of determining ChE inhibition in animals treated with carbamate pesticides.

It is safe to say that no single specific assay will satisfy the requirements of those needing assays of ChE activity, considering the variety of species, chemicals, and enzymes concerned. What is important is to establish criteria for acceptance of techniques that conform to biochemical principles and to optimize the conditions of the assays.

Steps in the right direction are underway. The Clinical Laboratory Improvement Act (CLIA) provides for coordination between clinical laboratories and proficiency testing for serum analysts (*Federal Register*, 1992). Recently the College of American Pathologists (CAP) has been named an accrediting organization for clinical laboratories, permitting them to work with CAP on a voluntary basis to demonstrate they meet CLIA requirements (*Federal Register*, 1995). However, the regulations are restricted to human subjects and, regardless of species, do not require AChE or ChE testing. Unlike other activities of clinical pathology laboratories, there is, as yet, no agreed upon standard operating procedure for cholinesterase assays.

REFERENCES

Aldridge, W. N. 1953. The differentiation of true and pseudo cholinesterase by organo-phosphorus compounds. *Biochem. J.* 53:62–67.

Christenson, W. R., Van Goetham, D. L., Scroeder, R. S., Wahle, B. S., Dass, P. D., Sangha, G. K., and Thyssen, J. H. 1994. Interlaboratory cholinesterase determinations and the effect on the results of statistical evaluation of cholinesterase inhibition. *Toxicol. Lett.* 71:139–150.

Ellman, G. L., Courtney, K. D., Andres, V., Jr., and Featherstone, R. M. 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem. Pharmacol.* 7:88–95.

Federal Register. 1992. Feb. 28, 57:7157.

Federal Register. 1995. Feb. 9, 60:7774–7780.

Fleming, R. A., Holmes, S. B., and Busby, D. G. 1992. An interlaboratory comparison of data on brain cholinesterase activity in forest songbirds exposed to aerial application of Zectran®. *Arch. Environ. Contam. Toxicol.* 22:228–237.

Harlin, K. S., and Ross, P. F. 1990. Enzymatic-spectrophotometric method for determination of cholinesterase activity in whole blood: Collaborative study. *J. Assoc. Off. Anal. Chem.* 73:616–619.

Hestrin, S. 1949. The reaction of acetylcholine and other carboxylic acid derivatives with hydroxylamine, and its analytical application. *J. Biol. Chem.* 189:249–261.

Imerman, P. M. 1993. pH method for determination of cholinesterase in whole blood: Collaborative study. *J. AOAC Int.* 76:899–901.

Johnson, C. D., and Russell, R. L. 1975. A rapid simple radiometric assay for cholinesterase, suitable for multiple determinations. *Anal. Biochem.* 64:229–238.

Loof, I. 1992. Experience with the Ellman method: Proposal for a modification and an alternative method (PAP). *Proc. U.S. EPA Workshop on Cholinesterase Methodologies,* Arlington, VA, December 4–5, 1991, eds. B. W. Wilson, B. Jaeger, and K. Baetcke, pp. 119–142. Washington, DC: Office of Pesticide Programs, U.S. EPA.

Magnotti, R. A., Jr., Dowling, K., Eberly, J. P., and McConnell, R. S. 1988. Field measurement of plasma and erythrocyte cholinesterases. *Clin. Chem. Acta* 315:315–332.

McConnell, R., Pacheco Anton, A. F., and Magnotti, R. 1990. Crop duster aviation mechanics: high risk for pesticide poisoning. *Am. J. Public Health* 80:1236–1239.

Mineau, P., and Peakall, D. B. 1987. An evaluation of avian impact assessment techniques following broad-scale forest insecticide sprays. *Environ. Toxicol. Chem.* 6:781–791.

Nostrandt, A. C., Duncan, J. A., and Padilla, S. 1993. A modified spectrophotometric method appropriate for measuring cholinesterase activity in tissue from carbaryl-treated animals. *Fundam. Appl. Toxicol.* 21:196–203.

Potter, W. T., Garry, V. F., Kelly, J. T., Tarone, R., Griffith, J., and Nelson, R. L. 1993. Radiometric assay of red cell and plasma cholinesterase in pesticide appliers from Minnesota. *Toxicol. Appl. Pharmacol.* 119:150–155.

Wilson, B. W., and Henderson, J. D. 1992. Blood esterase determinations as markers of exposure. *Rev. Environ. Contam. Toxicol.* 128:55–69.

Wilson, B. W., Jaeger, B., and Baetcke, K., eds. 1992. *Proc. U.S. EPA Workshop on Cholinesterase Methodologies,* Arlington, VA, December 4–5, 1991. Washington, DC: Office of Pesticide Programs, U.S. EPA.

Wilson, B. W., Padilla, S., and Henderson, J. D. 1993. Progress report on round robin cholinesterase tests to the U.S. EPA Scientific Advisory Panel, U.S. EPA, Crystal City, VA.

Wilson, B. W., Padilla, S., Sanborn, J. R., Henderson, J. D., and Billitti, J. E. 1995a. Clinical blood cholinesterase measurements for monitoring pesticide exposure. In *Enzymes of the cholinesterase family,* eds. A. S. Balasubramanian, B. P. Doctor, P. Taylor, and D. M. Wuinn. New York: Plenum Publishing Corp., in press.

Wilson, B. W., Padilla, S., Sanborn, J. R., O'Malley, M. A., Henderson, J. D., and Billitti, J E. 1995b. Reliability of blood cholinesterase measurements for monitoring pesticide exposures. *Proc. Second National Conf. NIOSH-Sponsored Centers for Agricultural Health and Safety,* March 27 and 28, 1995, Colorado State University, Fort Collins, CO, pp. 152–158. High Plains Intermountain Center for Agricultural Health and Safety (HI-CAHS).

Witter, R. F. 1963. Measurement of blood cholinesterase. *Arch. Environ. Health* 6:537–563.