CLINICAL BLOOD CHOLINESTERASE MEASUREMENTS FOR MONITORING PESTICIDE EXPOSURES

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INTRODUCTION

Measurement of blood cholinesterase (ChE) activity, especially acetylcholinesterases (AChE, EC 3.1.1.7) and butyrylcholinesterases (BChE, EC 3.1.1.8), is of worldwide interest and importance. They are one of the few biomarkers that function both as an indicator of exposure and also as evidence of an adverse effect. Determining blood ChEs is required for monitoring farmworkers in the state of California in the United States. In addition, ChE determinations are used in emergency wards to diagnose exposure to anti-ChEs, as part of the submissions to regulatory agencies to set safe levels of pesticides, to monitor wildlife and their exposure to dangerous anti-ChE chemicals, and to provide evidence of the use or escape of nerve gases.

Recently the reliability of ChE data submitted for regulatory and diagnostic purposes has been examined by the US and California Environmental Protection Agencies. Issues raised included variability of clinical laboratory results (Christenson et al., 1994; Wilson et al., 1992) and lack of validated and interlaboratory standard operating procedures.

This paper reviews our recent experiences in examining the reliability of thiocholine ester colorimetric assays, modified from the assay of Ellman et al. (1961), in the monitoring of blood ChEs from humans, domestic animals and wildlife.

ASSAYS AND CONDITIONS

Three common assays for ChE activities are: the Michel test, based on pH changes following hydrolysis of choline esters (Michel, 1949); the Johnson and Russell assay, in

Enzymes of the Cholinesterase Family, Edited by Daniel M. Quinn et al.
Plenum Press, New York, 1995

Table 1. Selected assays for ChE activity

Test	Basis	Type	Substrate	Plus/minus
Michel, 1949	Change in pH	Kinetic	ACh	Natural Substrate, Cheap / Slow
Johnson & Russell, 1976	Radiometric	End Point	ACh	Micro / Costly, Disposal
Ellman et al., 1961	Colorimetric	Kinetic	AcTh	Cheap / Unnatural Substrate

ACh = acetylcholine; AcTh = acetylthiocholine.

which radioactive acetate is separated into the toluene phase following hydrolysis of tritiated acetylcholine (Johnson and Russell, 1975); and the Ellman assay, based on the reaction of dithiobisnitrobenzoate (DTNB) with the thiocholine released by hydrolysis of thiocholine esters (Ellman et al., 1961). Some strengths and weaknesses of the assays are indicated in Table 1.

REGULATORY BACKGROUND

EPA Workshop

When an internal study by the Office of Pesticide Programs of the Environmental Protection Agency (EPA) revealed problems in their large data base of reports submitted to set safety levels for pesticides in food, a workshop on ChE methodologies was held (Wilson et al.,1992) bringing together government agency, industry and university scientists. The presentations documented the lack of standards and guidelines for the submission of data to the agency. Following the meeting a round robin test was conducted to see how well clinical and research laboratories could determine ChE activity in blood and brain by using the hydrolysis of thiocholine esters. Each laboratory used its own version of the basic assay with samples of tissues inhibited with organophosphate (OP) and carbamate (CB) pesticides. Several of the clinical laboratories used an automated Hitachi spectrophotometer with a reagent kit and instructions from Boehringer-Mannheim. Research laboratories used microassays run on multiple well plate readers (Doctor et al., 1988). The results (Wilson et al.,1993; Wilson et al., Submitted) revealed a lack of reproducibility of results from laboratory to laboratory, suitability of the instructions for reagent kits and the programming of the automated instrument, especially when measuring the AChE activity of the rat erythrocyte.

ChE Monitoring in California

At the same time, the Department of Pesticide Regulation of the California EPA surveyed clinical laboratories approved to monitor blood ChE of farmworkers. They also found several methods in use and a lack of guidelines and standard operating procedures (Choi et al., 1993). Approximately half of the laboratories used the Michel pH method, others used manual thiocholine/colorimetric and automated thiocholine-based assays. As a result of the survey, a project was started to develop a "gold standard" guideline to help laboratories demonstrate the reliability of their assays.

Testmate Kit

A third project began when US National Institute of Occupational Safety and Health (NIOSH) epidemiologist Kyle Steenland called our attention to the Test-Mate, a portable

Table 2. Thiocholine assay conditions

	Assay				
	Ellman	B/M manual	B/M automatic	Sigma	Testmate
Wavelength	412 nm	405 nm	480 nm	405 nm	470 nm
Substrate/conc	AcTh/0.5 mM*	AcTh/5.4 mM	AcTh/5.4 mM	PropTh/4 mM	AcTh/1 mM
Buffer pH	8.0	7.2	7.2	7.2	7.4
DTNB conc	0.32 mM	0.24 mM	0.24 mM	0.25 mM	0.3 mM

AcTh = Acetylthiocholine (*1 mM in Round Robin); PropTh = Propionylthiocholine; DTNB = Dithionitrobenzoate

colorimeter developed by Magnotti and colleagues (Magnotti et al., 1988). The instrument is hard-wired for a thiocholine-based assay and programmed to monitor human blood ChE levels from a drop of blood. The instrument first was used by occupational medicine physicians to examine exposure to pesticides in Nicaragua (Magnotti et al., 1987). We compared the performance of the instrument with that of a portable multiwell plate reader, determining enzyme activity with both instruments. Differences in the conditions of the several thiocholine assays discussed here are listed in Table 2.

BIOCHEMICAL BACKGROUND

Although all ChEs are equal in the sense that they rely upon serine-catalytic sites and hydrolyze choline esters, some are more equal than others with regard to their substrate specificities and the extent to which they have been studied. Different ChEs have distinctive substrate preferences and other biochemical specificities. For example, AChEs are inhibited by excess acetylthiocholine (AcTh), above approximately 1-2 mM, but BChEs continue to increase their rate of substrate hydrolysis with increasing substrate concentration. Birds and some other vertebrates (unlike mammals) have no AChE activity on their red blood cells. The plasma ChEs are another difference between species. Rat plasma ChE activity is 40-50 % AChE whereas human plasma ChE is virtually all BChE.

Although there have been a number of excellent comparative studies, few have involved the number of animals, including the human, necessary to establish "normal" ranges. Normal ranges of values for the human published in the instructions that accompany commercial kits (see Boehringer-Mannheim Catalog #450035 and #124117 and Sigma Diagnostics Procedure No. 422) do not address differences in optimum conditions for the determination of AChE and BChE.

EPA ROUND ROBIN

The EPA Round Robin test was designed to compare results between clinical and research laboratories. Rats were dosed with organophosphate (chlorpyrifos) and carbamate (carbaryl) pesticides. Blood and brain samples were taken: RBCs and plasma were separated, and the RBCs were treated with Triton X-100; brains were homogenized; and frozen samples were shipped to participating laboratories. The clinical laboratories used an assay kit and an automated, programmable multisample spectrophotometer of Boehringer-Mannheim/Hitachi. In the case of the RBC, the more inhibited was the sample, the more in error were the

Table 3. Round robin enzyme activities

	Plasma activity		RBC activity	
Assay conditions	Low Dose	High Dose	Low Dose	High Dose
410 nm, 25 C	67.6 ± 2.8	34.8 ± 3.5	51.7 ± 21.2	1.8 ± 2.0
410 nm, 37 C	60.5 ± 7.3	27.7 ± 5.4	46.8 ± 11.6	18.7 ± 17.8
480 nm, 37 C	54.0 ± 5.6	24.0 ± 1.7	71.0 ± 12.5	43.3 ± 7.5
Grand Mean	60.7 ± 6.8	28.8 ± 5.5	56.5 ± 12.8	21.3 ± 20.9

Mean ± Standard Deviation.

results from the clinical laboratories. Inhibitions were often much less than those found by the plate reader assays.

A major reason for the discrepancy was that the assays conducted according to the instructions and programming of Boehringer-Mannheim did not take into account oxidation of DTNB by the rat RBCs in the absence of AcTh. Tissue blanks were not recommended by the manufacturer, nor were they possible without reprogramming the machines. Even after correcting for blanks (Table 3), RBC values were more variable than were those from plasma and brain.

The high blank of rat RBCs is discussed in some detail by Loof at the ChE Methodology Workshop (Wilson et al., 1992) in which he concludes "Depending on the time set for reading, the background reaction, which cannot be inhibited by any phosphorus ester or carbamate, becomes the dominant part of the overall reaction. *If a sample blank is not introduced, false high results ...are found.* (Italics added.)

Other major differences between the assays of the clinical laboratories and the research laboratories were the pH of the assay buffers, substrate concentrations and the wavelength of the readings (Table 2). The peak absorbance of DTNB is between 405-415 nm, wavelengths that correspond to the Soret band of hemoglobin absorption. To avoid such interference, the automated assay of Boehringer-Mannheim/Hitachi determines DTNB absorption at 480 nm, significantly reducing the sensitivity of the assay (Wilson and Henderson, 1992).

OPTIMUM CONDITIONS FOR HUMAN ChEs

Conditions appropriate for assaying human ChEs were investigated in collaboration with the California EPA. Experiments were performed to examine the conditions of the Ellman et al. (1961) assay for the determination of AChE and BChE of humans and to compare the optimum conditions for the microplate reader with those recommended in the instructions for the kits of Boehringer-Mannheim Corporation and Sigma Chemical Company. Assays were performed with whole blood, washed and hemolyzed RBCs and plasma. Experiments such as the one shown in Figure 1 comparing the effect of acetylthiocholine concentration and pH on total AChE activity established that the assay conditions of Ellman et al. (1961) were optimal for the human enzymes.

Problems with the two commercial kits tested included choice of substrate, concentration of substrate and pH. For example, the Boehringer-Mannheim kit does not employ optimal conditions for AChE activity by using pH 7.2 buffer and a substrate concentration of 5.4 mM; both conditions reduced the activity of the enzyme. Assays lost BChE activity by running at rate-limiting substrate concentrations. It is not possible to recommend a single optimal AcTh concentration for both RBC AChE and plasma BChE; AChE enzymes are

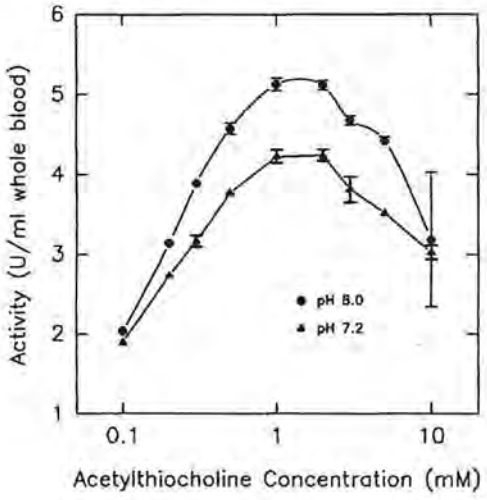

Figure 1. Effect of substrate concentration on AChE of human blood.

inhibited by AcTh in excess of 1-2 mM and BChE enzymes are not. Choosing another substrate does not help. The Sigma kit used propionylthiocholine, a substrate that is not optimal for the RBC enzyme (instructions with the Sigma kit indicate that the manufacturer focuses on using it to screen for plasma BChE variants to prevent adverse responses to succinylcholine and similar drugs during surgery, rather than emphasizing its use to monitor human blood enzymes for pesticide exposures). The need for a "tissue blank" to correct for the high endogenous DTNB oxidation by the rat RBC was not necessary with human RBC samples because normal activity in the human is 10-fold higher than in rats, minimizing the effect of background DTNB oxidation.

TESTMATE

The performance of the Testmate kit was examined in collaboration with the State of California. Workers harvesting peaches in an orchard previously sprayed with azinphos-methyl (Guthion) were examined with the Testmate procedure and with the microplate assay using blood drawn by venous puncture. The blood was iced and brought to the laboratory where it was assayed for AChE activity. The data showed a better than 90 percent correlation between assay methods (Figure 2), even though, like the automated Boehringer-Mannheim assay, absorption was determined at a wavelength removed from the optimum. In addition to validating the assays, the data provided evidence for reduced ChE activity in the blood of some of the workers.

The Testmate measures enzyme activity one sample at a time, a major inconvenience for large scale sample processing. One alternative is to adapt a multiwell plate reader to field studies by using a generator or a voltage converter and an automobile 12 volt battery, taking the instrument to the samples, rather than taking the samples to the laboratory. To accomplish this in a way useful to field biologists we lyophilized reagents (with sucrose as a filler) in 96 well plates. In this way, approximately thirty samples can be run in triplicate in less time than it would take to run a dozen samples with the Testmate.

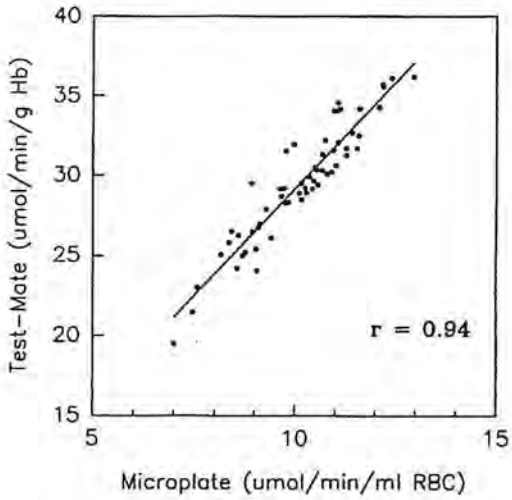

Figure 2. Comparison of AChE assay methods.

CONCLUSION: RESPONSIBILITIES

The work reported here supports several recommendations:

- *Pesticide levels.* The procedures used to set safe levels of pesticides need to be modified in so far as ChE measurements are concerned as was recommended by Wilson et al.(1993) in their report to the EPA Scientific Advisory Panel. Standard Operating Procedures and Good Laboratory Practices should be implemented for the participating laboratories.
- *Optimization of assay conditions.* Assays should be optimized for the pesticides and test animals under study including the human.
- *Manufacturer's instructions.* Companies that manufacture the kits for ChE assays used in clinical laboratories by personnel not trained in enzyme kinetics or biochemical toxicology should write instructions that spell out the limitations of their assays. By the same token, clinical laboratories undertaking such work should employ staff with sufficient biochemical expertise to understand the limitations of the assays and to be able to modify the recommendations of the manufacturer.
- *Conversion factors.* Baseline data obtained by assays that have been optimized for the samples concerned and run by documented standard operating procedures are needed to determine conversion factors between assays. This would permit transfer of information from one study to another, assisting in the detection of potentially harmful exposures. A farmworker in California may move from a region where the Michel pH test is performed to one where the clinical laboratory uses the Boehringer-Mannheim automated instrument to one using a manual Sigma propionylthiocholine procedure. Because the values derived from different methods cannot be compared directly, a way is needed to convert from one assay condition to another. Conversion factors can readily be derived so long as the dose/activity curves are linear. Preliminary conversion factors for some of the

Table 4. Conversion factors between assays

Sample	Boehringer/Mannheim	Sigma Diagnostic
RBC	1.43	3.04
Plasma	0.59	0.38

Factor converts measured activity to Ellman assay activity.

conditions discussed here (established by diluting rather than inhibiting the enzyme activity) are given in Table 4.

- *Testmate.* The results of these and other studies (Magnotti et al., 1988, Magnotti et al., 1987, Wilson and Henderson, 1992) attest to the reliability of the Testmate instrument. Our experience suggests that it, and similar instruments, are useful for field studies where more expensive and elaborate instruments are unavailable. Examples are state agency personnel immediately responding to a report of workers in a field suspected of being exposed to harmful levels of a pesticide, and wildlife care center personnel examining the blood of animals brought into the center with muscle weakness and convulsions. Because of the "hard-wired" program of the Testmate, it does not replace instruments that can be set to different wavelengths, sampling times and calculations of activities. Nevertheless, its relatively low cost makes it a cholinesterase "Best Buy" when used as directed for what it was designed to do. If funds are available, and multiple sample runs are necessary, 96 well plate readers can be hooked up to voltage converters or generators and moved to the field.

CLOSING

Bench scientists know that enzyme assays must be adjusted to the jobs they are called upon to perform, but we found that clinical laboratories submitting data for diagnostic and regulatory purposes relied upon generic instructions when assaying ChE activity for government agencies. Specific standard operating procedures for ChE assays applicable to the conditions and to the needs of the agencies are needed. Perhaps the lesson to be learned from the widespread use of assays performed under less than optimal conditions for diagnostic and regulatory purposes is the recognition of what may happen when a gap opens between bench laboratory research and those that apply it.

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