Available online at www.sciencedirect.com
SCIENCE DIRECT
Chemico-Biological Interactions 157–158 (2005) 253–256
Chemico-Biological Interactions
www.elsevier.com/locate/chembioint

Monitoring cholinesterases to detect pesticide exposure

Barry W. Wilson*, Daniel E. Arrieta, John D. Henderson

Department of Animal Science and Environmental Toxicology, University of California, Davis, CA 95616, USA

Available online 18 November 2005

Abstract

Progress toward a standard blood cholinesterase assay to assess pesticide exposures in the agricultural workplace and to identify possible victims of chemical warfare agents is discussed. Examples given are drawn from collaborations with clinical laboratories in California and the Department of Defense Cholinesterase Reference Laboratory (CRL).

Keywords: ChE monitoring; Standardization; ChE assays

1. Introduction

Blood cholinesterases (ChEs), red blood cell acetylcholinesterases (RBC AChE, E.C. 3.1.1.7) and serum butyrylcholinesterases (BChE, E.C. 3.1.1.8), are enzyme biomarkers that detect potentially dangerous organophosphate (OP) and carbamate (CB) exposures. Methods in common use are designed to be rapid, one size-fits-all assays to monitor potential target populations and deal with pesticide exposure episodes or potential chemical warfare terrorist attacks. In the United States, California and Washington are the only states currently monitoring ChEs of OP/CB handlers. Elsewhere, ChEs are determined if exposures are suspected.

In California, regulations specify monitoring blood ChEs when using chemicals with toxicities <50 mg/kg (Class I pesticides), and ≥ 50 and ≤ 500 mg/kg (Class II pesticides). Examples are aldicarb, azinphosmethyl (Class I pesticides) and malathion (Class II pesticide). Mixers, loaders, applicators and flaggers, but not field workers themselves, are tested if they work with pesticides for 7 days or more in a 30-day period. Base lines are taken in duplicate from the same laboratory. The workplace is evaluated if blood ChE levels of RBC and/or plasma are <80% of baseline. Workers are required to leave the workplace if levels are <70% for RBC, and <60% for plasma ChEs and cannot return to work until enzyme activity has recovered to 80% of baseline. New employees are tested after 30 days, and then examined every 60 days thereafter [1,2].

A similar program was started recently in the state of Washington following a lawsuit. Blood ChEs of mixers, loaders and applicators are monitored by a single state laboratory. A 20% or greater depression of ChEs compared to baseline leads to investigation of the worksite; 30% or greater depression of RBC AChE, or 40% of plasma BChE require removal of workers from the field; they may return when their blood levels reach 80% of baseline. Thousands of workers have already been tested, a data base has been established, some ChE depressions have been reported and cases are being followed [3].

But, the assay method used in Washington has not been quantitatively standardized with that used in California. And, in California, there is no data base in which to store ChE values obtained since the California testing program was established in the 1970s. Indeed, until recently there was no requirement to standardize the assays themselves [4].

* Corresponding author. Tel.: +1 530 7523519; fax: +1 530 7520175.
E-mail address: bwwilson@ucdavis.edu (B.W. Wilson).

0009-2797/$ – see front matter
doi:10.1016/j.cbi.2005.10.043

2. Measuring ChE's

The several commonly used assays to determine blood ChEs were not designed to be interchangeable. One is the end point delta pH method of Michel [5]; it measures acetylcholine (ACh) breakdown with a pH meter. A modification used by the Department of Defense Cholinesterase Reference Laboratory (CRL) is precise and even though it is slow, and its throughput is low, the US Army monitors more than 15,000 subjects per year with it. Another end point assay is the radioactive ACh method of Johnson and Russell [6], in which an organic/water extraction separates 3H-labeled ACh from its products. This micro-assay is suitable for multiple determinations. It is accurate, but it is expensive and has a disposal problem because of radioactive waste. Neither of these endpoint assays is suited to kinetic analyses. Popular kinetic assays use thiocholine substrates. The most common is that of Ellman et al. [7] in which the breakdown of acetylthiocholine (ATCh) is detected colorimetrically with dithiobisnitrobenzoate (DTNB). The assay is reliable, accurate and inexpensive. Several clinical kits have been marketed and there are many published variations.

A number of clinical laboratories use a kit marketed by Roche suitable for automated analyzers. We used a multi-well plate modification of the Ellman assay appropriate for research laboratories [4] in our work with California clinical laboratories and the US Department of Defense to harmonize clinical and research methods.

3. Difficulties with ChE assays

Activity in umol/min/ml; mean ± S.D. (N). ATCh substrate: RBC: 0.8 mM; Plasma: 7 mM. Autotechnicon; Humiston and Wright [8].

No assay is free from problems, both practical and theoretical, and human and experimental [2,4]. One human problem is a penchant for investigators to implicitly assume ChE levels are readily transferable from one species to another. Examples of the relative activities of ChE in RBCs and plasma of humans, dogs and rats are shown in Table 1. Dogs and rats are much lower in total blood AChE activity than the human. Moreover, unlike the human, they (and other animals too) have relatively high levels of AChE in their sera, raising doubts about values from studies that assume AChE levels are restricted to RBCs alone. Another source of error is a transient high thiocholine "blank" activity in RBCs of some species such as the rat that is often not considered in designing the assays and interpreting the results.

Table 1
Comparative activities of human and other species

Species	RBC	Plasma
Human	135 ± 29 (60)	37 ± 9 (56)
Dog	18 ± 4 (18)	25 ± 6 (18)
Male rat	9.0 ± 1.3 (24)	4.3 ± 1.0 (45)

A third problem with focusing on RBCs is difficulties in accurately pipetting and reproducibly washing them to reduce serum contamination. A fourth difficulty is the potential interference from hemoglobin (Hb), which adsorbs at 410 nm, a wavelength similar to the maximum adsorption of DTNB.

Other problems stem from the nature of the commercial clinical kits themselves. For example, the popular Roche colorimetric kit is run at a pH and an ATCh concentration that is not optimal for the assay. The recently discontinued Sigma kit used a substrate that is not preferred by RBC AChE and is better suited to determining blood serum BChE [4].

A major problem in detecting depressions in ChE activity is reactivatibility of the inhibitor–AChE enzyme complex. Blood samples often are not iced, presumably on the notion that AChE inhibition by OPs is "irreversible". Unfortunately, this is not the case until a structural reorganization of the inhibitor–enzyme complex known as "aging" has occurred [2,4]. Carbamates and methyl-organophosphates are readily rehydrolyzed at ambient temperatures, spontaneously reactivating enzyme activity within a few hours or days. In the case of carbamates, special techniques are required to successfully determine ChE inhibitions [4]. Lack of icing blood samples to reduce such reactivations was a confounding factor in an otherwise thorough study of Yeary et al. [9].

4. Standardization

The State of California recently revised its AChE monitoring: Title 3, California Code of Regulations, Section 6728, Medical Supervision, specifies use of the Ellman assay for blood monitoring, and requires clinical laboratories to submit data to demonstrate the reliability of their assays and to derive correction factors if necessary. We were asked by the Department of Pesticide Registration (DPR) to help the clinical laboratories comply, examining reproducibility and interconvertibility of the assays.

In the first round of comparisons, 9 of the 25 clinical laboratories sent undiluted and 50% diluted blood samples to UCD for ChE analyses. Two laboratories did their own comparisons. All used ATCh as a substrate. The results were disappointing, especially when attempting

Table 2
Summary of blood ChE comparisons from UC Davis and Clinical Labs

Correlation (r^2)	RBC	Plasma
>90	0.98, 0.95	0.99, 0.94
	0.96, 0.93	0.97, 0.99
70 ⩾ 90	0.74, 0.79	0.81, 0.88
<70		0.61, 0.33

Adapted from Wilson et al. [4].

to convert results from one laboratory to those of another (Table 2).

In general, plasma values tended to be better correlated and of lower variability than were RBC values. One reason may be the relative ease in pipetting plasma compared to serum.

5. AChE standard

Because of difficulties in coordinating sample collecting, arranging for shipping and quality control between laboratories, we developed a bovine ghost RBC AChE standard [10].

Hb and other proteins within bovine RBCs were removed by osmotic shock and the AChE containing membranes washed and then solubilized to prepare a long-lasting AChE standard. The RBC ghost enzyme is stable when stored at low temperature; it provides a standard for use in our own laboratory and for distribution to other laboratories too.

Fig. 1 illustrates the reliability of the standard. Diluted ghost preparations were assayed at UC Davis and duplicate samples shipped to the EPA laboratory in Raleigh, NC where they were assayed by Dr. Stephanie Padilla.

6. Second standardization

For the second time, the California Department of Pesticide Regulation (DPR) asked laboratories to compare their ChE assay to the method outlined in the regulation, and demonstrate a correlation of 0.9 or better, and, again, our laboratory was asked to assist. Diluted bovine RBC ghost samples were sent to participating laboratories and human blood and plasma samples were provided to clinical laboratories to compare with assays performed at UC Davis. When bovine ghost standards were sent to 10 clinical laboratories, activities were below the detection level set for their instruments. Nevertheless, three yielded correlation coefficients of 0.97–0.98. Fourteen laboratories agreed to determine blood samples sent to them. The plasma BChE activities from the clinical laboratories compared favorably to those measured by UCD; correlations ranged from 0.96 to 0.99. Nine of the 10 laboratories reported whole blood activities that met the $0.9r^2$ criteria. Six of the eight laboratories reported RBC AChE activities with acceptable correlations [11].

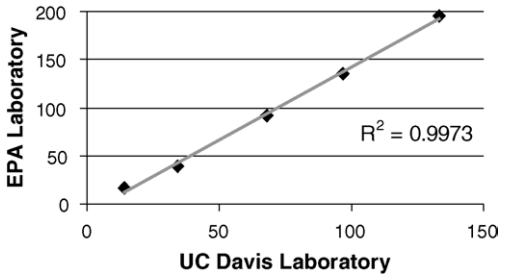

Fig. 1. Comparison of ghost RBC assays. Means of triplicate samples. Adapted from Wilson et al. [4].

7. Conclusions

Clinically determined plasma BChE correlated better than whole blood or RBC activities at UC Davis suggesting plasma might be preferable to RBC or whole blood for monitoring. But which pesticides prefer which enzyme, how long enzyme depressions last and what neural enzymes correspond to plasma BChE are a few of the questions still to be considered before recommending serum BChE in preference to RBC AChE. The RBC results were better than in the previous study when only two of the seven clinical laboratories "passed." In this trial, six of eight RBC and 9 of 10 whole blood AChE values were acceptable. Perhaps, the samples in this study were more uniform because they were prepared in a single laboratory, contributing the larger number of acceptable correlations. Although still a first approximation, the population-based correlations were sufficient to permit comparing one laboratory with another.

One outcome of the project was a letter from DPR on 8 July 2003 to the California Agricultural Commissioners listing nine clinical laboratories on the approved list for ChE testing under Section 3CCR 6728. The current list is available at: www.cdpr.ca.gov/docs/whs/lablist.htm.

8. Closing

Standardizing blood ChE monitoring is only one of the matters that would make this method of detecting OP exposures more useful [12]. Others are:

a. Extend the monitoring of mixer, loaders and applicators to include periodic examination of field workers too. These laborers in the agricultural workplace may

wear less protective clothing and receive training than the pesticide handlers.

b. Arrange for a central data storage, accessible to more than a single agency so that the results of exposure episodes may be rapidly disseminated to the public health community.
c. Provide the clinical findings to the persons monitored. It is surprising to a laboratory-based biochemist that clinical assays initiated by physicians are not routinely made available to their subjects and stored in a common data base.
d. Periodically carry out a formal review of the policies and procedures of the clinical laboratories and of the replicability of their results.

Finally, another important bit of unfinished business is to establish a normal human range for blood ChEs, providing 95% confidence intervals that may be used when individual baselines are lacking. Although commercial kits include ChE ranges with the instructions, they have not been formally validated, and much of the literature on this topic is more than 40 years old. Work in progress from our laboratory yields a 95% range of 6.3–10.7 umol/min/ml with a mean of 8.11 ± 0.067 (S.D.) umol/min/ul ACTh (Ellman units) for normal human blood AChE (Arrieta et al., 2005, in preparation).

This study illustrates how a bench laboratory biochemical quality assurance project can impact public health regulations and worker safety. The continuing use of antiChE pesticides and the fear of chemical terrorism underline the need for national standards and periodic quality assurance testing of clinical ChE monitoring. The recommendation by CA DPR that laboratories lose their approval if they do not meet state standards is a new and tougher policy requiring tighter scrutiny of performance.

Acknowledgements

The authors acknowledge Al Ramirez from the Wilson laboratory, Jim Sanborn (OEHHA), Joe Billitti (Aguron), Mike O'Malley (UCD Employee Health), Stephen McCurdy (UCD Epidemiology), Stephanie Padilla (US EPA), Matthew Keifer (U. Washington) and CA and Utah clinical laboratories. Support for the research was from UCD NIOSH Center for Agricultural Health and Safety; NIEHS Center for Agricultural Health Science; DOD; OEEHA; and CA DPR.

References

[1] B.W. Wilson, Cholinesterases, in: W.F. Loeb, F.W. Quimby (Eds.), The Clinical Chemistry of Laboratory Animals, second ed., Taylor and Francis, 1999, pp. 430–454.
[2] B.W. Wilson, Cholinesterases, in: R.I. Krieger (Ed.), Handbook of Pesticide Toxicology, vol. 2, Academic Press, 2001, pp. 967–985.
[3] M. Keifer, Cholinesterase monitoring in Washington state, in: American Public Health Association 133rd Annual Meeting and Exposition, 5–9 November 2005, New Orleans, USA (abstract 104518).
[4] B.W. Wilson, J.D. Henderson, A. Ramirez, M.A. O'Malley, Standardization of clinical cholinesterase measurements, Int. J. Toxicol. 21 (2002) 385–388.
[5] H.O. Michel, An ekectrometric method for the determination of red blood cell and plasma cholinesterase activity, J. Lab. Clin. Med. 34 (1949) 1564–1568.
[6] C.D. Johnson, R.I. Russell, Rapid simple radiometric assay for cholinesterase, suitable for multiple determinations, Anal. Biochem. 64 (1975) 229–238.
[7] G.L. Ellman, K.D. Courtney, V. Andres Jr., R.M. Featherstone, A new and rapid colorimetric determination of acetylcholinesterase activity, Biochem. Pharm. 7 (1961) 88–95.
[8] C.G. Humiston, G.W. Wright, An automated method for the determination of cholinesterase activity, Toxic. Appl. Pharmacol. 10 (1967) 467–480.
[9] R.A. Yeary, J. Eaton, E. Gilmore, B. North, J. Singell, A multiyear study of blood cholinesterase activity in urban pesticide applicators, J. Toxicol. Environ. Health 39 (1993) 11–25.
[10] D. Arrieta, A. Ramirez, E. DePeters, D. Bosworth, B.W. Wilson, Bovine red blood cell cholinesterase as a monitoring standard, Bull. Environ. Contam. Toxicol. 71 (2003) 447–452.
[11] B.W. Wilson, J.D. Henderson, D.E. Arrieta, M.A. O'Malley, Meeting requirements of the California cholinesterase monitoring program, Int. J. Toxicol. 23 (2004) 97–100.
[12] R. Das, A. Steege, S. Baron, J. Beckman, R. Harrison, Pesticide-related illness among migrant farm workers in the United States, Int. J. Occup. Environ. Health 7 (2001) 303–312.