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# Evaluation of a Fluorescent Method for Measuring Cholinesterase Activity in Mammalian Blood and Tissue

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Cholinesterase (ChE) activity has been widely used as a biochemical marker of cholinergic system function in laboratory, wildlife, and human studies (Fulton and Key 2001; Padilla et al 1996). Acetylcholinesterase (AChE, EC 3.1.1.7) prefers to hydrolyze acetylcholine (ACh), butyrylcholinesterase or non-specific cholinesterase prefers other choline esters (BChE, EC 3.1.1.8). Acute exposures to organophosphate and carbamate pesticides inhibit ChE activities, causing accumulation of ACh at synapses, and their disruption in organisms. Although critical to cholinergic transmission in the brain, ChEs are also present in several other tissues, including blood. Both red blood cell (RBC) and plasma ChE activities have been widely used as bio-indicators of exposure and intoxication to these pesticides (Wilson et al 1997). Reduced ChE activities in blood have been reported among spray-workers as a result of improper handling of pesticides (Baker et al 1978; Wilson et al 1997). Exposures to other environmental pollutants, including mercury, have been shown to cause alterations in ChE activity in humans (Zabinski et al 2000), wildlife (Lionetto et al 2003) and laboratory animals (Gill et al 1990; Hastings et al 1975; Lakshmana et al 1993).

There are several methods to measure ChE activity (St. Omer and Rottinghaus 1992, Wilson 2001). The most widely used is a colorimetric assay developed by Ellman et al (1961). While the Ellman assay is simple and inexpensive, the absorbance peak of hemoglobin (415 nm) can interfere with detection of its reaction end-product, 2-nitro-5-benzoic acid (405nm). Recently, a fluorescent based ChE assay kit was marketed for measurement of the purified enzyme (A12217, Molecular Probes, Eugene, OR, USA ( Zhou et al 2000). It detects choline, the ChE reaction product, through a multi-step process in which the oxidation of choline by choline oxidase results in the formation of $H_2O_2$, which is detected through a horseradish peroxidase-coupled reaction with 10-acetyl-3,7-dihydroxyphenoxazine (Amplex Red). The final product, resorufin, is detected by its fluorescence (ex. 563, em. 587) (Zhou et al 1997). It is said to provide high sensitivity (0.002 units/ml of purified AChE from electric eel, as reported by Molecular Probes) without hemoglobin interference.

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The objective of this study was to evaluate this fluorescent assay as a tool to measure ChE activities in biological specimens collected from humans and wildlife.

## MATERIALS AND METHODS

Reagents used in the assay were optimized to ensure maximal ChE activity in human plasma, American mink (*Mustela vison*) brain tissue and bovine red blood cell (RBC) ghost preparations (Arrieta et al 2003), and an inter-laboratory comparison test with the Ellman assay was also performed. Horseradish peroxidase (EC 1.11.1.7), resorufin, ACh, choline oxidase (EC 1.1.3.17), iso-OMPA, BW285c51, and all other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). 10-acetyl-3, 7-dihydroxyphenoxazine (Amplex Red) was acquired from Molecular Probes, Inc (Eugene, OR, USA). Approval to conduct this study was granted by the McGill University Ethical Review Committee. Human blood samples (10 mL) were drawn by a registered nurse into tubes containing EDTA-$K_2$ from 5 healthy volunteers between 25 and 35 years of age who had never been exposed to pesticides. Plasma was isolated by centrifugation of whole blood at 200 x g for 10 min at 4°C, pooled, and stored at –80°C. Brain tissues from captive mink (*Mustela vison*), having no history of exposure to environmental pollutants, were obtained from Michigan State University's Experimental Fur Farm (East Lansing, MI, USA). Brain tissues were dissected and stored at -80°C and shipped to the laboratory in Montreal. Upon thawing, brain tissues were sonicated in cold phosphate buffer (50 mM $NaH_2PO_4$, 5 mM KCl, 120 mM NaCl, pH 7.4) and 0.1% Triton X-100, and centrifuged at 15,000 x g for 10 min at 4°C and the supernatants collected. The protein concentration in the supernatants was determined by the Bradford method (Bradford, 1976). Bovine RBC ChE standards were prepared from a dairy herd of Holstein and Guernsey cows (Animal Science Department, UC Davis) as previously described (Arrieta et al 2003) and stored at –70°C until use. Briefly, plasma and red blood cells were separated by centrifugation, the red blood cells lysed by distilled water, the membranes collected by centrifugation, solubilized by treatment with Triton X-100 and stored at –70°C until needed.

ChE measurements using the Amplex Red assay, available as a kit from Molecular Probes (Eugene, OR, USA; Catalog No. A12217), were based on the procedure described by Zhou et al. (2000). Triplicate samples of human plasma (0.01-50 nL/well), mink brain preparations (0.1–3.0 µg/well) or bovine RBC ghost standards (0.01-1.0 µL/well) were added to the wells of microplates in 100 µL of phosphate buffer. The reaction was initiated with the addition of 100 µL reaction buffer (10 µM Amplex Red, 2 U/mL horseradish peroxidase, 0.2 U/ml choline oxidase and 50 µM acetylcholine; final concentration in 200 µL). Preliminary studies were performed with varying concentrations of substrate, ACh (25 – 2000 µM), and Amplex Red (1.5 to 200 µM) to determine optimal assay conditions. Fluorescence was measured every 5 min at 540/590 (excitation/emission) by a fluorometric plate reader (Wallac Victor 2, Perkin

Elmer, Boston, MA, USA) at 25°C. The reaction was generally linear between 10-90 min. The end product, resorufin (0 - 5 μM), was used as the standard, and specific activities of samples were expressed as μmol/min per mg (protein) or ml (plasma or RBC ghosts). Sodium potassium buffer was used as a blank and 10 μM $H_2O_2$ was used as a positive control. Inhibitor studies were performed by pre-incubating (15 min at 25°C) diluted samples with iso-OMPA (0.1 mM - BChE specific inhibitor) or BW285c51 (0.01 mM - AChE inhibitor) prior to the initiation of the reaction. Total ChE activity was determined in the absence of inhibitors.

For validation purposes, ChE activities were measured using the Ellman procedure (Ellman et al 1961) modified for a 96-well automated microplate reader (Padilla et al 1999). Assay volumes of 320 μl consisted of 250 μl 0.1 mM sodium phosphate buffer, pH 8.0, 10 μl of 322 μM dithiiobisnitrobenzoate (DTNB), 30 μl of enzyme sample and 30 μl of acetylthiocholine (final concentration of 1.0 mM). Activities were read at 405 nM at 25-27°C and reported as μmol/min/ml.

## RESULTS AND DISCUSSION

The Ellman assay typically uses the substrate acetylthiocholine (ATC) in concentrations ranging from 0.4 – 2.0 mM which allows for the determination of maximal enzymatic activity (Augustinsson et al 1978; Ellman et al 1961). Figure 1 shows that increasing concentrations of ACh (0 to 1 mM), in the absence of enzyme, resulted in a concentration dependent increase of background fluorescence interfering with the detection of ChE activity at ACh concentrations above 0.25mM. For this reason, a sub-maximal ACh concentration of 50 μM was used to detect ChE activity, permitting determination of relative rates.

ChE activity increased in a linear fashion as a function of the total amount of tissue in the reaction well (Figure 2) within approximately one order of magnitude. ChE activity could be determined from very small amounts of diluted sample (i.e. 0.05 nL/well of human plasma and 0.1 μg/well of brain supernatant protein) calculated from the linear region of the curves between 10 and 25 min. Saturation occurred when product formation was greater than 5 μM of resorufin.

Experiments were performed to determine the optimal concentration of Amplex Red for maximal ChE activity. The commercially available Molecular Probes kit suggested a final Amplex Red concentration of 200 μM. The present study indicated that Amplex Red concentrations between 3 μM and 12 μM yielded maximal activity when using RBC ghost standards (Table 1). Similar results were observed with mink and plasma samples (data not shown). Amplex Red concentrations below 3 μM did not yield detectable resorufin and final fluorescent concentrations above 25 μM progressively reduced enzymatic activity. Therefore, 10 μM of Amplex Red was chosen for the remaining tests.

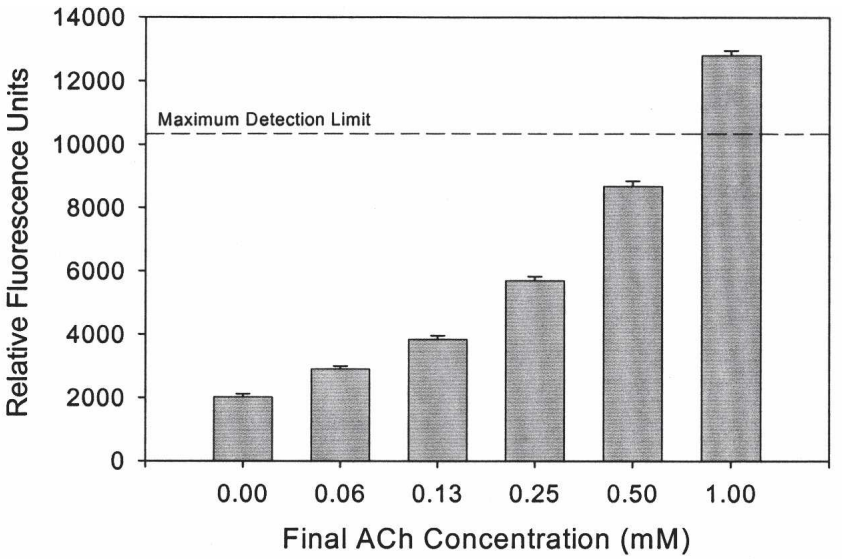


**Figure 1.** Effects of ACh concentration on background fluorescence in the absence of tissue sample. Averages of triplicate determinations.
Fluorescent detection became non-linear above 11,000 relative fluorescence units.

**Table 1.** Effects of Amplex Red concentration on ChE activity in bovine RBC ghost standards. Mean ± SD of triplicate determination.

| Amplex Red (μM) | ChE Activity (μmol/min/ml) |
|---|---|
| 1.5 | $0.1 \pm 0.5^{a}$ |
| 3.0 | $14.1 \pm 0.3^{b}$ |
| 6.0 | $13.9 \pm 0.3^{b}$ |
| 12 | $13.1 \pm 0.4^{b,c}$ |
| 25 | $12.9 \pm 0.3^{c}$ |
| 50 | $9.6 \pm 0.2^{d}$ |
| 100 | $5.9 \pm 0.3^{e}$ |
| 200 | $2.7 \pm 0.4^{f}$ |

Different letters represent significance (ANOVA, Tukey post hoc test; $p < 0.05$)

Both BW284c51 and iso-OMPA inhibitors were used to identify the AChE and BChE activity in plasma, bovine RBC, and mink brain tissue (Table 2). The activity in the RBC standard was associated with AChE; it was sensitive to BW284c51 but not iso-OMPA, consistent with previous findings (Arrieta et al 2003). Human plasma ChE activity was associated with BChE activity. Mink brain samples contained both enzymes, approximately 30% of total activity could be attributed to BChE and the remaining 70% to AChE activity. These data suggest that the fluorescent assay detected both enzymes.

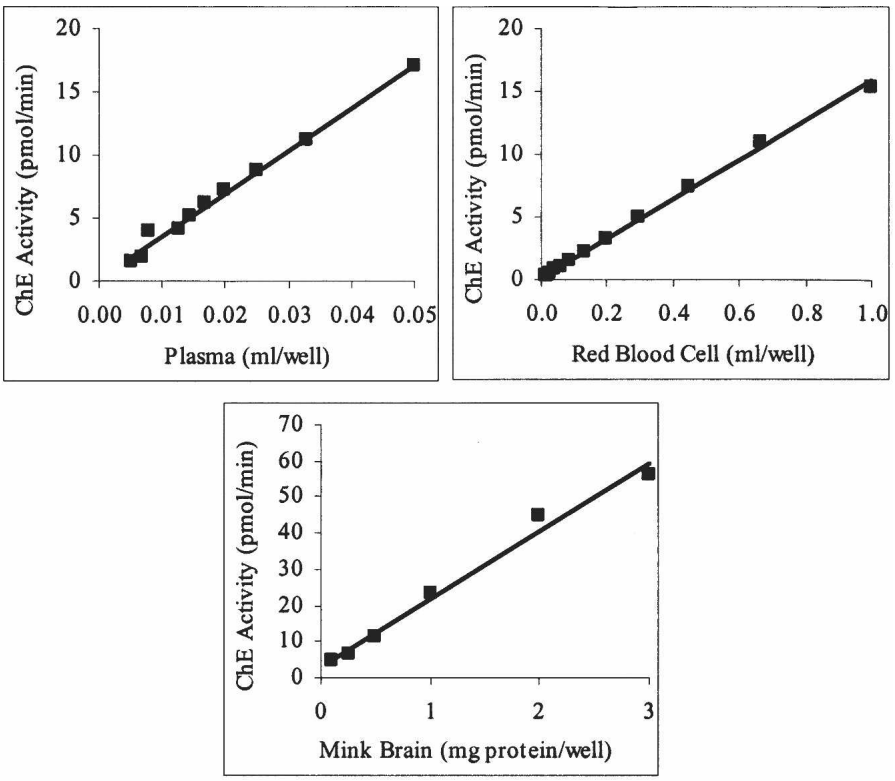


**Figure 2.** Linear relationship between ChE activity and tissue concentration (Mean of triplicate determination).

**Table 2.** Determination of AChE and BChE activities (Mean ± SD, n=3).

| *Inhibitor* | *Bovine RBC Ghosts (% Total ChE)* | *Human Plasma (% Total ChE)* | *Mink Brain (% Total ChE)* |
|---|---|---|---|
| iso-OMPA (0.1 mM) | 91 ± 4.5 | 2.4 ± 3.1 | 64 ± 8.6 |
| BW284C51 (0.01 mM) | -0.1 ± 1.4 | 92 ± 4.2 | 38 ± 7.3 |

The Amplex Red assay was compared to the established Ellman assay using RBC ghost standards (n = 15) and mink brain tissue (n = 9) analyzed at McGill University using the Amplex Red method and University of California, Davis using the Ellman assay. Linear regression analyses indicated a strong relationship between the two methods for both RBC standards ($r^2$ = 0.98) and mink brain tissue ($r^2$ = 0.93) (Figure 3). The slopes of the regression lines differed from 1.0 because different concentrations of substrate were used.

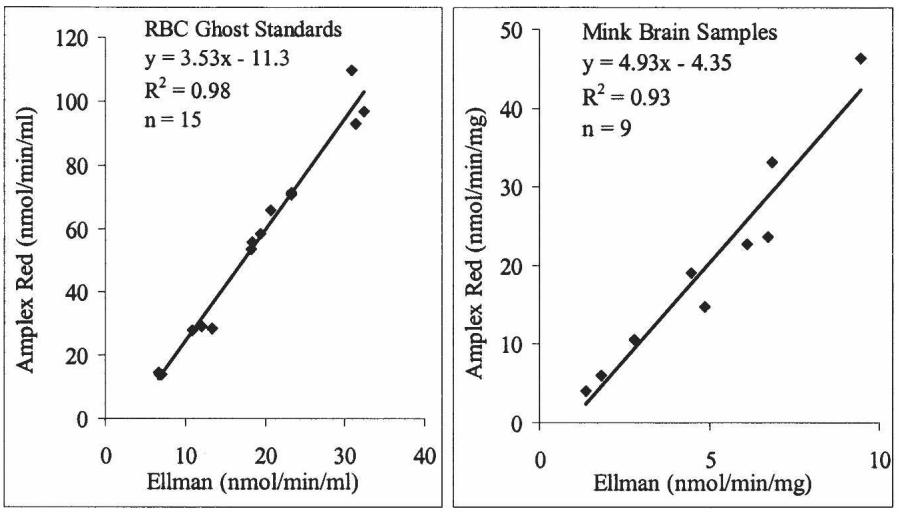


**Figure 3.** Comparison of ChE activity using mink and bovine tissue measured with Amplex Red and Ellman assays.

These results demonstrate that the Amplex Red-based assay is a sensitive method to measure both AChE and BChE in brain, RBC, and plasma. Inter-laboratory comparisons correlated well with the established Ellman assay. However, because of substrate interference and resulting high background fluorescence, this fluorescent assay is not useful for determining maximal ChE activity or developing substrate saturation curves. The assay may still be particularly useful in measuring ChE in samples contaminated with hemoglobin, and its high sensitivity may be of interest when amounts of tissue are limited.

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