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RESEARCH ARTICLE

In vivo oxime administration does not influence Ellman acetylcholinesterase assay results

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Abstract

Organophosphorus compounds (OPs) are potent inhibitors of acetylcholinesterase (AChE). Treatment for OP poisoning is by administration of atropine sulfate, an oxime, and diazepam. Oximes such as 2-PAM are used to reactivate OP-inhibited AChE so as to restore normal enzymatic function and serve as a true antidote. There are reports of non-enzymatic hydrolysis by oximes of acetylthiocholine in *in vitro* preparations in the widely used Ellman assay for AChE activity, which may confound the interpretation of AChE activity by producing elevated results. The purpose of this experiment was to determine if there is appreciable interference by therapeutic levels of oximes on the results of the Ellman assay in assessing AChE reactivation by oxime compounds *in vivo*. When therapeutic doses of oximes (2-PAM, HI-6, MMB-4, or MINA) were administered intramuscularly to guinea pigs and samples collected 60 min later, there was no statistical difference between oxime and saline control groups in measured AChE activity in various tissue samples, including blood. With appropriate dilution of samples prior to spectrophotometric assay, the Ellman assay is an acceptable method to measure *in vivo* oxime reactivation of inhibited AChE. Inclusion of an oxime control group to insure that this particular type of interference is not causing false readings in the assay is a prudent step.

Keywords: *Acetylcholinesterase; acetylthiocholine; Ellman assay; in vivo; oxime*

Abbreviations: ACh, acetylcholine; AChE, acetylcholinesterase; ATChI, acetylthiocholine iodide; BBB, blood-brain barrier; BCA, Bicinchoninic acid; ChE, cholinesterase; CNS, central nervous system; DMS, dimethansulfonate salt; DTNB, 5,5'-dithiobis-2-nitrobenzoic acid; HI-6, 1-(4-carbamoylpyridino) methoxymethyl-2-(hydroxyiminomethyl) pyridinium; im, intramuscular; MINA, monoisonitrosoacetone; MMB-4, methoxime or 1,1'-methylene-bis[4-(hydroxyimino) methyl]pyridinium; OP, organophosphorus compound; 2-PAM, pralidoxime or pyridine-2-aldoxime methylchloride; RBC, red blood cell; TNB, 5-thio-2-nitrobenzoic acid; WB, whole blood.

Introduction

Organophosphorus compounds (OPs), pesticides, and nerve agents are potent inhibitors of cholinesterases (ChEs), in particular acetylcholinesterase (AChE), an enzyme that hydrolyzes the cholinergic neurotransmitter acetylcholine (ACh) in the peripheral and central nervous systems (Taylor 2001). The inhibition of AChE leads to an excess in ACh in the synapses of affected nerves, resulting in a variety of signs and symptoms such as hypersecretion, bronchoconstriction, miosis, muscular twitching, mental confusion, convulsive seizures, flaccid paralysis, respiratory distress, and death (McDonough and Shih 1997; Ecobichon 2001; Taylor 2001; Eddleston et al. 2004b). OP poisoning is treated

by administration of atropine sulfate, a drug used to antagonize muscarinic receptor binding by excess ACh, and 2-PAM (pralidoxime; pyridine-2-aldoxime methylchloride), an oxime drug used to reactivate inhibited AChE. Diazepam, a benzodiazepine drug, is also used in an effort to curb brain seizures and motor convulsions caused by exposure to OPs (Moore et al. 1995; Lallement et al. 1997; Taylor 2001; Eddleston et al. 2004a).

The mechanism of reactivation of OP-inhibited AChE by oxime compounds is a dephosphorylation of the enzyme's active site via a nucleophilic attack on the phosphorous atom, generating an oxime-phosphate or oxime-phosphonate, and reconstituting the active site serine (Taylor

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2001; Kassa 2002). This restores the activity of the enzyme, allowing normal hydrolysis of ACh to occur. The use of oximes to reactivate OP pesticide- or nerve agent-inhibited AChE is well established (Wilson and Ginsburg 1955; Ecobichon 2001; Eyer 2003; Eddleston et al. 2004a; Marrs et al. 2006), but there is disagreement over the most efficacious oxime in the event of nerve agent poisoning. Oximes differ in their ability to reactivate AChE inhibited by the different nerve agents (Antonijevic and Stojiljkovic 2007). As a result, there has been a concerted effort to investigate new oximes that have a broader spectrum of reactivating capacity.

To evaluate potential medical countermeasures, particularly AChE reactivators, against OPs it is necessary to measure AChE activity. At present, the Ellman method (Ellman et al. 1961) is the most commonly used, adapted, or modified method for measuring AChE activity. In the Ellman method, acetylthiocholine iodide (ATChI) is added as substrate and is hydrolyzed by AChE present in the sample to form thiocholine. The thiocholine reacts with the Ellman reagent 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) to form a 5-thio-2-nitrobenzoic acid (TNB) anion, the colored indicator, which absorbs at 412 nm. Enzyme activity is then determined by measuring absorbance and following the increase in yellow color resulting from TNB production as a function of time (Ellman et al. 1961). It has long been known that oximes can hydrolyze ATChI (Bergner and O'Neill 1958; Skrinjarik-Spoljar and Kralj 1980; Nadarajah 1992; Pannbacker and Oehme 2003). More recently, Petroianu et al. (2004) used human plasma (no AChE present) to examine the *ex vivo* concentration-effect relationship between pralidoxime (2-PAM) and what they described as 'pralidoxime-induced cholinesterase pseudoactivity'. They found that a false reading for cholinesterase activity would occur at oxime concentrations that exceeded 10 μ M in the cuvette. This potential interaction has fostered concerns that the Ellman method is unreliable for measuring AChE activity in oxime studies, due to the esterase-like activity of oximes (Sakurada et al. 2006; Sinko et al. 2007). However, the claim that physiological concentrations of an oxime could interfere with measurement of AChE activity in biological samples *ex vivo*, when the oxime is administered parenterally to animal or human subjects, has not been supported by any published data, including our previous studies (Shih et al. 1991; 2007; Shih 1993).

In this study we tested for oxime-Ellman substrate/reagent interference to determine if the Ellman assay is suitable for determining AChE activity *in vivo* when evaluating the efficacy of an oxime. Four oximes were administered individually by the intramuscular (im) route to guinea pigs at therapeutic doses in an experimental model for subcutaneous (sc) exposure of OP nerve agents (Shih et al., 1991; Shih et al. 2007; 2009; McDonough et al. 2009). The sc route was selected for two reasons: (1) sc injection allows for more accurate dosing of nerve agents, as opposed to dermal or percutaneous absorption, and (2) sc injection limits

exposure to laboratory personnel. Guinea pigs were used in nerve agent study models because, unlike the mouse or rat, guinea pigs possess little amounts of carboxylesterase that bind to nerve agents, and are more similar to non-human primates in their response to pyridostigmine bromide (PB) pre-treatment for protection against nerve agents (Inns and Leadbeater 1983; Maxwell et al. 1987; 1988). The oximes studied in this experiment (see Figure 1 for chemical structures) consisted of (1) 2-PAM, which is the standard US drug used for OP insecticide and nerve agent intoxication and is a reference mono-pyridinium oxime for research and development, (2) HI-6 (1-(4-carbamoylpyridino)methoxymethyl-2-(hydroxyiminomethyl)pyridinium dimethansulfonate), which is the oxime used by Canada and some European countries for nerve agent poisoning (Moore et al. 1995; Aas 2003) and is an effective bis-pyridinium oxime in an animal model for research investigation (Shih et al., 1991), (3) MMB-4 (methoxime; 1,1'-methylene-bis[4-(hydroxyimino)methyl]pyridinium dimethansulfonate), which is a bis-pyridinium oxime being considered for advanced development in nerve agent poisoning (Singh et al. 2007; Saxena et al. 2008), and (4) MINA (monoisoinitrosoacetone), a lipid soluble tertiary alkylloxime that penetrates the brain (Rutland 1958; Cohen and Wiersinga 1960) and has recently been revisited for its potential central nervous system (CNS) protection (Shih et al. 2007; 2009; McDonough et al. 2009). AChE activity in blood, brain regions, and peripheral tissues of guinea pigs was tested for a possible treatment effect of oxime on the results from Ellman assays.

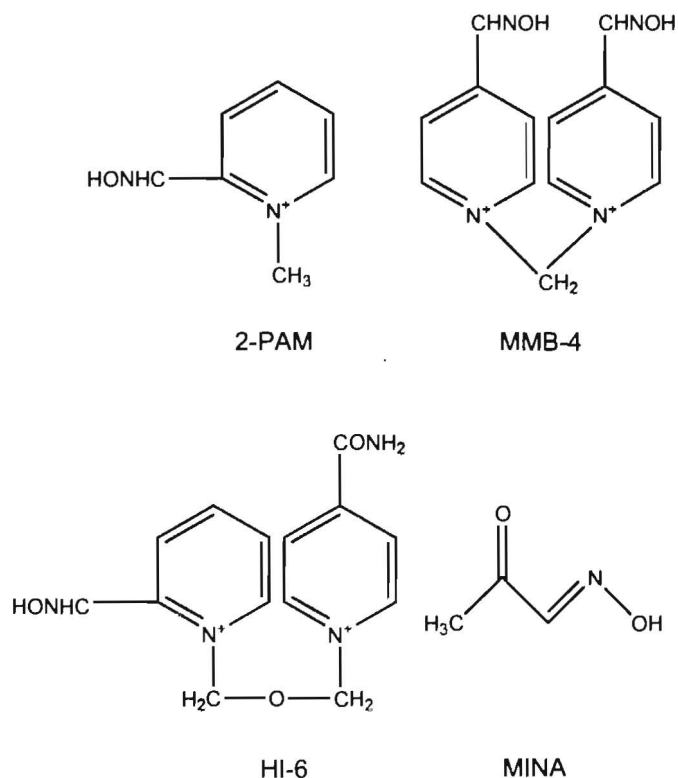


Figure 1. Structures of the various oximes used in the study.

Materials and methods

Animal welfare

Research was conducted in compliance with the Animal Welfare Act and other Federal statutes and regulations relating to animals and experiments involving animals and adhered to principles stated in the Guide for the Care and Use of Laboratory Animals, by the *Institute of Laboratory Animal Resources*, National Research Council (National Research Council Publication No. 85-23, 1996). The research environment and protocols for animal experimentation were approved by the Institutional Animal Care and Use Committee (IACUC). The facility where this research was conducted is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

Subjects

Male Hartley guinea pigs (250–300 g, Crl:(HA) BR COBS; Charles River Labs., Kingston, NY) were housed in individual cages in temperature ($21 \pm 2^\circ\text{C}$) and humidity ($50 \pm 10\%$) controlled quarters that were maintained on a 12-h light-dark schedule (lights on at 6:00 am) and allowed food and water ad libitum except during experimental periods. Animals were acclimated for 1 week prior to experimentation.

Materials

Saline (U.S.P.) was purchased from Braun Medical Inc. (Irvin, CA). Heparin sodium was purchased from U.S.P., Inc. (Rockville, MD). AChE from electric eel, bovine serum albumin, ATChI, and MINA were purchased from Sigma-Aldrich (St. Louis, MO). DTNB (5,5'-dithiobis(2-nitrobenzoic acid), bicinchoninic acid (BCA) Protein Assay Reagent A (sodium carbonate, sodium bicarbonate, BCA^- detection reagent, and sodium tartrate in 0.1 N sodium hydroxide) and BCA Protein Assay Reagent B (4% cupric sulfate) were purchased from Pierce Biotechnology, Inc. (Rockford, IL). 2-PAM was purchased from Ayerst Labs, Inc. (New York, NY). HI-6 and MMB-4 were obtained from the depository at Walter Reed Army Institute of Research (Silver Spring, MD). DTNB was prepared in Tris buffer (0.05 M, pH 8.2) to a concentration of 0.424 M. HI-6, 2-PAM, MMB-4, and MINA were prepared in saline and administered im with a volume of 0.5 ml/kg.

Experimental procedure

One-to-three days prior to the experiment, blood (0.25–0.5 ml) was drawn using the toenail clip method (Vallejo-Freire 1951) and collected into a 1-ml microcentrifuge tube containing 50 μl of heparin sodium (15 units/ml) to determine baseline AChE activity in whole blood (WB) and red blood cells (RBCs). On the day of the study, guinea pigs were injected subcutaneously (sc) with saline in place of nerve agent in this model (Shih et al. 2007; 2009; McDonough et al. 2009). Five minutes later, saline, MMB-4 (26.0 mg/kg), HI-6 (27.8 mg/kg), 2-PAM (25.0 mg/kg), or MINA (80.0 mg/kg) was administered im. The doses of oxime were chosen because they result in the highest therapeutic effectiveness

without any toxic effects in guinea pigs (Shih et al. 2007; 2009; McDonough et al. 2009). Sixty minutes after im saline or oxime administration, the animals were deeply anesthetized by isoflurane anesthesia (5% in oxygen) and euthanized. Blood was collected into a 1-ml microcentrifuge tube containing 50 μl of heparin sodium (15 units/ml). Brain regions (brainstem, cerebral cortex, and striatum) and peripheral tissues (diaphragm and heart) were dissected. Brain regions were diluted 1:20 (w/v), while peripheral tissues were diluted 1:5 (w/v) in 1% Triton-X100 solution (in 0.9% saline), and then homogenized. Homogenized samples were spun in a centrifuge at $31,000 \times g$ (brain for 20 min, peripheral tissue for 30 min). Supernatant was removed and kept frozen at -80°C until AChE analysis. Two dilutions were made from the blood samples. For the WB, 20 μl of collected blood was diluted 1:25 (v/v) in 1% Triton-X100 solution. For the RBCs, the original blood sample was centrifuged for 5 min at $16,000 \times g$ to separate the RBC and plasma. Ten microliters of the packed RBC were then re-suspended and diluted 1:50 (v/v) in 1% Triton-X100 solution. The dilute RBC and WB samples were kept frozen at -80°C until AChE analysis. The AChE activity was measured spectrophotometrically using a variation of the microplate method modified from Ellman et al. (1961), and a BCA protein assay was used to obtain protein concentrations in the tissue samples to standardized AChE levels among tissues, as was reported elsewhere (Shih et al. 2005).

AChE analysis

On the day of AChE analysis, the brain and peripheral tissue supernatant samples were thawed, and three 7- μl replicates of each sample were pipetted into a 96-well microplate (UV Star, Greiner, Longwood, FL). The dilute RBC and WB samples were thawed, and three 10- μl replicates of each sample were pipetted into the microplates. Standard curves were established by adding 7 or 10 μl (for brain and peripheral tissue samples or WB and RBC samples, respectively) AChE from electric eel at 3.75, 7.5, and 15.0 U/ml. Twenty microliters of de-ionized water was added to each well containing brain and peripheral tissue samples, and 17 μl of de-ionized water was added to each WB and RBC sample. Following the addition of water, 200 μl of DTNB (0.424 M, pH 8.2) was added to each sample well. Each microplate was then incubated and agitated for 10 min at 37°C . Immediately thereafter, 30 μl of the substrate ATChI (51.4 mM) was added to each well. The samples were read in a microplate reader (Spectramax Plus 384, Molecular Devices, Sunnyvale, CA) at 410 nm (at 20-s intervals). Blood plates were read for a total of 3.5 min and brain/peripheral tissue plates were read for 2.0 min. The AChE activity (μmol substrate hydrolyzed/ml/min) was determined from the slope of absorbance vs time using Softmax plus 4.3 LS software (Molecular Devices, Sunnyvale, CA).

Protein analysis

Protein levels in the brain and peripheral tissue samples were determined by a BCA protein assay method (Pierce

Biotechnology, Inc.). The standard curve was created using bovine serum albumin at the following concentrations: 0.5, 0.75, 1.0, 1.5, and 2.0 mg/ml. Three replicates of 10 μ l for each brain sample were added to individual microplate wells. To each well of brain samples 200 μ l of working reagent was then added. Three replicates of 5 μ l for each peripheral tissue sample were added to individual microplate wells. The peripheral tissue samples were further diluted by adding 5 μ l of de-ionized water before adding 200 μ l of BCA working reagent. The microplates were then incubated at 37°C for 30 min. The microplates were allowed to cool to room temperature (15 min) before being read using a microplate reader (Spectramax Plus 384, Molecular Devices, Sunnyvale, CA) and Softmax Plus 4.3 LS software. A single measurement of absorbance was made at 562 nm and protein concentrations were extrapolated from the standard curve.

Data analysis

AChE activity in the blood was initially expressed as μ mol ATChI hydrolyzed/ml/min (Table 1) and then converted to percentage of the individual animal's baseline AChE value that was obtained 1–3 days prior to the experimental study

(Figure 2). The coefficient of variance expressed as a percentage (CV%) was calculated and is provided in Table 1 to highlight lack of total variance and to allow for comparison of total variance (2.50–5.51%) among treatment groups of each tissue type (independent of mean activity). Statistical analysis was performed separately for each tissue type using a one-way ANOVA with treatment as the independent and AChE activity as the dependent variable. A post-hoc Tukey's HSD test was used for multiple comparisons of treatments. Statistical significance was defined as $p < 0.05$.

Results

AChE activity in blood

AChE activities in RBC and WB collected 60 min after im saline or oxime injection are shown in Table 1. The saline-treated control AChE activities in WB and RBC on the day of experiment were 2.19 ± 0.08 and 2.39 ± 0.14 μ mol/ml/min, respectively. Figure 2 shows these same data when they were expressed as a percentage of their individual baseline AChE activity obtained 1–3 days earlier. There was no significant difference (RBC: $F = 0.748$, $df = 5, 56$, $p = 0.591$; WB: $F = 0.284$,

Table 1. The effect of *in vivo* oxime administration on acetylcholinesterase (AChE) activity in various tissues of the guinea pig.

Treatments	Whole blood ^a	Red blood cells ^a	Diaphragm ^b	Heart ^b	Brainstem ^b	Cortex ^b	Striatum ^b
Saline ($n = 7$)	2.19 ± 0.08	2.39 ± 0.14	12.67 ± 0.64	17.35 ± 0.91	265.46 ± 7.12	57.82 ± 0.94	452.89 ± 19.40
MINA (80 mg/kg) ($n = 6$)	2.26 ± 0.09	2.63 ± 0.12	11.94 ± 0.28	16.46 ± 1.37	258.20 ± 7.87	59.36 ± 2.41	453.79 ± 25.01
HI-6 (27.8 mg/kg) ($n = 6$)	2.19 ± 0.09	2.41 ± 0.10	11.72 ± 1.10	18.07 ± 0.90	258.97 ± 12.93	57.92 ± 1.70	495.64 ± 21.13
2-PAM (25 mg/kg) ($n = 6$)	2.14 ± 0.10	2.32 ± 0.09	13.45 ± 1.14	17.61 ± 0.90	244.02 ± 9.51	55.68 ± 1.60	473.03 ± 27.49
MMB-4 (26 mg/kg) ($n = 6$)	2.28 ± 0.13	2.44 ± 0.16	12.74 ± 0.68	17.67 ± 0.78	264.14 ± 11.75	54.71 ± 1.04	449.18 ± 11.96
CV% ^c	2.50	4.78	5.51	3.43	3.30	3.28	4.20

^a AChE activity expressed as μ mol ATChI hydrolyzed/ml/min $\pm$ SEM on the day of experiment.

^b AChE activity expressed in μ mol ATChI hydrolyzed/g protein/min $\pm$ SEM on the day of experiment.

^c CV% (% coefficient of variance for each tissue type) = (mean standard deviation of treatment groups/mean of treatment group means) $\times$ 100.

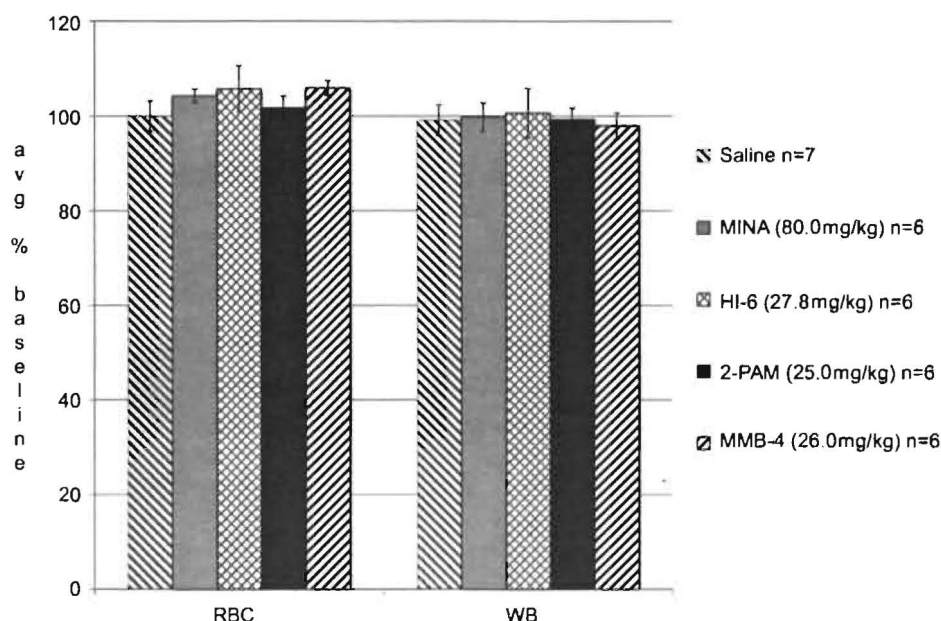


Figure 2. AChE activity in red blood cell (RBC) and whole blood (WB) as a percentage of individual baseline AChE activity (mean $\pm$ SEM). The baseline blood AChE activity was obtained 1–3 days prior to experimental treatment. No statistical difference was found among all tested groups.

df=5,56, $p=0.920$) in AChE activity in any of the oxime-treated WB or RBC samples when compared with their respective saline (Table 1) or with their baseline controls (Figure 2). The %CVs among treatments are 2.50 and 4.78 for WB and RBCs, respectively.

AChE activity in brain regions

Table 1 shows the AChE activities in the brainstem, cortex, and striatum in saline- and oxime-treated groups at 60 min after im administration. The AChE activities of the oxime-treated groups for each brain region were not significantly different from each other or their respective saline-treated controls (brainstem: $F=0.733$, df=4,26, $p=0.578$; cortex: $F=1.350$, df=4,26, $p=0.278$; striatum: $F=0.806$, df=4,26, $p=0.532$). The AChE activity in the striatum (452.89 ± 19.40 $\mu\text{mol/g protein/min}$ for saline control) was the highest, whereas the cortex had the lowest AChE activity (57.82 ± 0.94 $\mu\text{mol/g protein/min}$ for saline control) among all brain regions, which is about one eighth of that of the striatum (Table 1). The %CV's among treatments are 3.30, 3.28, and 4.20 for brainstem, cortex, and striatum, respectively.

AChE activity in peripheral tissues

Table 1 also shows the AChE activities in the diaphragm and heart in saline- and oxime-treated groups at 60 min after im administration. There was no significant difference between the AChE activities of any of the oxime treatment groups or saline controls in the peripheral tissues sampled (diaphragm: $F=0.678$, df=4,26, $p=0.613$; heart: $F=0.355$, df=4,26, $p=0.838$). The %CV's among treatments are 5.51 and 3.43 for diaphragm and heart, respectively.

Discussion

The use of oximes in the treatment of OP nerve agent or pesticide poisoning is critical, as oximes are the only drugs that directly act on OP-inhibited AChE to restore proper enzymatic function and thus cholinergic neural transmission. While there is a difference of opinion as to which oxime is best suited for use in OP poisoning, there is a strong agreement that an AChE reactivator should be used in treating OP intoxication (Taylor 2001; Eyer 2003). The research and development of more efficacious or broader spectrum AChE reactivators is heavily dependent on an accurate AChE assay.

The Ellman assay is the most widely used, and is an essential tool for researchers involved in studies of AChE inhibition and reactivation. However, there are reports in the literature that have indicated that the Ellman assay has some limitations. It has been demonstrated that non-enzymatic hydrolysis of ATChI catalyzed by oximes readily occurs in *in vitro* preparations (Sakurada et al. 2006; Petroianu 2007). It is important to point out that this interference is based primarily on ATChI and oxime concentrations in the assay medium where the interaction occurs (Skrinjarik-Spoljar and Kralj 1980; Nadarajah 1992; Pannbacker and Oehme 2003; Petroianu et al. 2004; Sinko et al. 2007).

Routine *in vitro* evaluating techniques involve adding the oxime directly to the assay medium containing pure enzyme or tissue preparations and an OP AChE inhibitor, and measuring the activity of AChE. In contrast, the *in vivo* models normally used in the laboratory involve administering the oxime into the animal either before or after OP exposure in an effort to determine AChE reactivating potential or derive an efficacy protective ratio. The critical difference between the two techniques is that there is absorption, distribution, metabolism, and elimination occurring *in vivo*. These parameters can have a significant impact on the concentrations of oxime available to interact with ATChI when the blood or tissue samples are obtained, prepared, and analyzed by the Ellman assay.

The current study was, therefore, designed to assess whether parenteral administration of oximes at therapeutic doses *in vivo* and subsequent assay of the prepared tissue samples would cause significant changes in AChE activity. In this study 2-PAM, MMB-4, HI-6, and MINA were administered intramuscularly at doses of 25, 26, 27.8, and 80 mg/kg, respectively. Pharmacokinetic and bioavailability data for these oximes from published reports are summarized in Table 2 and described below. In rats given a dose of 20 mg/kg 2-PAM, the maximal plasma concentration was 6.7 ± 1.5 $\mu\text{g/ml}$, with a half-life of 35.0 min and a clearance rate of 10.9 ± 4.4 ml/min (Green et al. 1986). In a study by Singh et al. (2007), in guinea pigs given 26 mg/kg doses of MMB-4 DMS, the maximal plasma concentration was 5.195 ± 3.092 $\mu\text{g/ml}$, with a half-life of 43.43 min and a clearance rate of ~ 5.9 ml/min. In the heart, diaphragm, and thigh muscle the maximum concentrations of MMB-4 were reached in 5–10 min to 0.281, 0.392, and 0.174 $\mu\text{g/ml}$, respectively. In rats given a dose of 20 mg/kg HI-6, the maximal plasma concentration was 46.1 ± 8.7 $\mu\text{g/ml}$, the

Table 2. Pharmacokinetic data reported for 2-PAM, MMB-4, HI-6, and MINA.

Oxime	Administered dose (mg/kg)	Species	Maximum plasma concentration ($\mu\text{g/ml}$)	Plasma half-life (min)	Clearance (ml/min)	Reference
2-PAM	20.0	Rat	6.7 ± 1.5	35.0	10.9 ± 4.4	Green et al. (1986)
MMB-4	26.0	Guinea pig	5.2 ± 3.1	43.4	~ 5.9 #	Singh et al. (2007)
HI-6	20.0	Rat	46.1 ± 8.7	59.5	4.7 ± 1.1	Simon and Briggs (1985)
MINA	35.0	Rat	~ 50	ND*	ND*	Rutland (1958)
DAM	200.0	Rat	ND*	137.0	ND*	Dultz et al. (1957)

* ND = not determined.

This value was calculated using the equation: $\text{CL} = \text{Dose}/\text{AUC}$ (Green et al. 1986).

half-life was 59.5 ± 25.5 min, and the clearance rate was 4.7 ± 1.1 ml/min (Simons and Briggs 1985). Very limited pharmacokinetic data are available for MINA; however, administration of 35 mg/kg, ip, MINA to rats resulted in a maximum WB concentration of ~ 50 μ g/ml, which was reached within 10–15 min. At the end of the 3-h sampling period, 70% of the MINA had been eliminated (Rutland 1958). Diaceetylmonoxime (DAM), an oxime structurally similar to MINA, at a dose of 200 mg/kg, has a half-life of 137 min in rats (Dultz et al. 1957).

Based on maximal plasma concentrations, half-life values and clearance rates, there appears to be an insufficient amount of oxime available in the tissue samples to react with ATChI. The samples used in the current experiment were collected 60 min after oxime administration. According to the elimination data cited above, it is clear that the oximes (with the exception of MINA) had undergone at least one half-life cycle before tissue collection. This halved any available oxime in the plasma for potential interference in the Ellman assay. Since the tissues (diaphragm, heart, or skeletal muscle) absorb the oxime at a slower rate and to a lesser degree than the plasma (Singh et al. 2007), the amount of oxime available to interact with ATChI in the assay is practically non-existent or reduced to a minimum. Furthermore, our routine assay processing of the ex vivo samples called for additional dilution in initial tissue preparations (brain, 20 $\times$; skeletal muscle, 5 $\times$; WB, 25 $\times$; RBC, 50 $\times$ in 1% Triton-X100 solution) to free the membrane bound AChE. The dilution of the samples ex vivo, when combined with the effects of oxime elimination *in vivo*, resulted in sample concentrations of oxime significantly lower than those found to produce pseudocholinesterase activity in the *in vitro* studies reported elsewhere (Petroianu et al. 2004; Sakurada et al. 2006; Sinko et al. 2007).

It has been reported that administration of OP nerve agents can alter the elimination kinetics of 2-PAM (Green et al. 1985a). The administration of sarin or soman caused a biphasic response in the maximal plasma concentration of 2-PAM. The administration of ≤ 2.2 LD₅₀ doses of these nerve agents caused a decrease in maximal plasma concentration of 2-PAM, and administration of ≥ 2.8 LD₅₀ doses of these nerve agents caused an increase in maximal plasma concentration of 2-PAM (Green et al. 1985b). However, it should be cautioned that this observation is only for 2-PAM vs sarin and soman. There may be a different effect for VX and other OPs, and a difference between mono- and bis-quaternary oximes elimination under these conditions as well. Consequently, in the event of co-administration of nerve agent (usually under 2.0 LD₅₀) and oxime *in vivo*, there is the potential for further decrease of oxime concentrations available for reaction with ATChI during assay.

Measuring oxime reactivation of OP-inhibited AChE activity in biological samples derived from *in vivo* studies may not be hindered by the oxime-ATChI reaction that can occur in *in vitro*. The doses of oximes used in the current study do not increase apparent AChE activity in oxime-

treated samples when compared with the saline control group, even in blood samples (WB and RBCs). This suggests that there is an insufficient amount of oxime to interfere with the Ellman substrate ATChI to produce elevated AChE activity, as reported by Sakurada et al. (2006). In fact, Sakurada et al. (2006) used excessive 2-PAM concentrations, giving a final 2-PAM concentration in the cuvet of up to 161 μ M, which resulted in an annoying oxime blank reaction (Worek and Eyer 2006). Our *in vivo* samples, particularly blood, had been diluted at least 625-fold before they were subjected to Ellman substrate. This contention is further supported by the MINA results, in which a relatively higher dosage (80.0 mg/kg) of MINA was injected and MINA had a longer elimination time from the body. Even though MINA is eliminated more slowly (i.e. stayed in the body longer) than the quaternary oximes, there was no significant difference in assay results of AChE activity between MINA and control group in any of the tissues tested, including brain regions where MINA has been shown to reactivate nerve agent-inhibited AChE (Rutland 1958; Shih et al. 2007; 2009).

In conclusion, the *in vitro* oxime studies demonstrating oxime interference with the Ellman assay substrate ATChI (Sakurada et al. 2006) are not applicable to *in vivo* studies, at least not under the conditions reported here for 60 min sample collection. However, it is advisable, when performing *in vivo* oxime reactivation studies, to measure oxime-only controls to insure that this particular type of interference as reported by others is not causing false readings in the Ellman assay.

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