

Characterization and Normalization of Rat Salivary Cholinesterase


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Abstract

Cholinesterase (ChE) is an enzyme which allows neurons to return to a resting state by hydrolyzing the neurotransmitter acetylcholine. Organophosphate pesticides, which are commonly used in agriculture, inhibit the activity of ChE. The inhibition of ChE activity in plasma and red blood cells is used as a biomarker for human pesticide exposure. Currently, salivary ChE activity is being examined as a non-invasive biomarker; however little is known about baseline ChE activity in saliva. In order to characterize baseline salivary ChE, saliva was collected from 5 adult male Sprague-Dawley rats, salivary ChE was measured using the Ellman assay, and total salivary protein was measured using the bicinchoninic acid (BCA) protein assay. Comparison of total ChE activity indicated that rat salivary ChE is variable amongst individuals at a given time point (coefficient of variation=30% to 67%) and over the 2 hour collection time (coefficient of variation=66.8% or 71.6%). Variation of total protein was also seen with coefficients of variation of 36% to 57% between individuals at each collection time and 66.2% over collection. The correlation coefficient between total protein and total ChE activity was determined to be 0.731. Normalization of ChE activity by total protein resulted in a coefficient of variation of 37% over the collection time. These results demonstrate the variability of salivary ChE activity and suggest that total protein may be used as a normalization measure in saliva biomonitoring.

Introduction

Pesticides are used extensively worldwide, especially in agriculture, to control damage to crops caused by insects and other pests. Organophosphate (OP) insecticides are compounds that control pests by inhibiting cholinesterase (ChE) through the binding of the OP's oxon metabolite to ChE (Kamatani *et al.*, 1976). The normal physiological function of ChE is the hydrolysis of acetylcholine (ACh), a neurotransmitter necessary for proper nervous system function, and the inhibition of this action results in accumulation of ACh in the synaptic gap (Miles *et al.*, 1998). In mammals, inhibition of ChE can cause salivation, lachrymation, convulsions, diarrhea, and even death as a result of high doses of or long-term exposure to OPs (World Health Organization, 1986). With almost 200,000 global deaths a year due to OP poisoning, concerns about exposure levels have been raised (Haywood and Karalliedde, 2000). Individuals who apply OP pesticides to crops are at risk for OP poisoning (Washington State Legislature, 2004). In several states, including California and Washington, ChE levels in plasma and red blood cells are monitored in agricultural workers that apply pesticides; however these methods are often impractical (Lu *et al.*, 2009). In general, when ChE activity in plasma has decreased 40% from original baseline levels, workers are removed from contact with the pesticide until their plasma ChE activity levels recover to 20% inhibition (Washington State Legislature, 2004). Unfortunately, if ChE activity levels cannot be measured in a rapid manner, hazardous levels may be reached and maintained without prompt detection, possibly causing poisoning. In addition, blood biomonitoring can be invasive, costly, and time consuming (Wilson *et al.*, 1996).

In an effort to make biomonitoring more practical, Lu *et al.* (2009) have proposed the use of saliva as a potential non-invasive biomarker, which could be easily obtained from humans in the field in order to determine real-time exposure to OP pesticides. Saliva has the advantage of being easily collected without the need for medical specialists or specialized equipment and the potential to be an accurate indicator of the availability of pesticide to tissues (Nigg and Wade, 1992).

Inhibition of salivary cholinesterase in response to organophosphates has also been reported in live rats (Abdollahi *et al.*, 2004). Novel electrochemical sensors, instruments which measure a current based on ions in a solution in a manner similar to a pH meter, allow for portability of biomonitoring using saliva ChE measurements as a biomarker (Liu *et al.*, 2008, Wang *et al.*, 2008, and Wang *et al.*, 2008). This strategy is being explored, however little research has been done to characterize the baseline salivary ChE activity and total protein in the model subjects (Nigg and Wade, 1992, Kousba *et al.*, 2003, and Henn *et al.*, 2006). Kousba *et al.* (2003) examined the relative functionality of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) in rat saliva using selective inhibition of the enzymes and reported high inter-rat variability in ChE activity. Henn *et al.* (2006) examined cholinesterase activity in human saliva using two different collection methods and reported high intra- and inter-individual variability. Normalization of ChE activity using total protein in saliva appeared to have no effect on reducing variability; however, limitations of this study exist due to the uncontrolled environment of the human subjects and potential complications with sampling techniques (Henn *et al.*, 2006)

The purpose of the present study was to analyze the potential of saliva for use in biomonitoring of OP pesticides by characterizing baseline salivary ChE activity and related measures in the Sprague-Dawley rat. Although saliva has been suggested for biomonitoring of OP pesticides, there are no known controlled investigations into the baseline of the ChE activity in this fluid. Saliva was collected from five adult male Sprague-Dawley rats with five collection intervals over two hours. Saliva samples were then analyzed for ChE activity using an Ellman assay (Ellman *et al.*, 1961) and total protein using the bicinchoninic acid (BCA) assay (Smith *et al.*, 1985). I hypothesized that ChE activity in saliva can be used for biomonitoring and that total protein is an effective normalization method for ChE activity from different individuals or collection intervals.

Materials and Methods

Chemicals

Bovine serum albumin standard (2 mg/mL), BCA reagent A (1% BCA-Na₂, 2% Na₂CO₃·H₂O, 0.16% Na₂ tartrate, 0.4% NaOH, and 0.95% NaHCO₃), and BCA reagent B (4% CuSO₄·5H₂O in deionized water) were acquired from Thermo-Fisher Scientific (Rockford, IL, USA). Aerrane (Isoflurane) was obtained from Baxter Pharmaceuticals (Deerfield, IL, USA). S-butyrylthiocholine chloride (BTCl), 5,5-dithio-bis(2-nitrobenzoic acid) (DTNB), acetylthiocholine chloride (ATCl), pilocarpine, and other general laboratory chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) and were reagent grade or better.

Animals

Adult male Sprague-Dawley rats (300-400 g) were obtained from Charles River Laboratories, Inc. (Wilmington, MA, USA). Rats were housed in solid-bottom cages with hardwood chips under standard laboratory conditions with free access to water and Purina Certified Rodent Chow[®] 5002 (Purina Mills, St. Louis, MO, USA). All procedures with animals were in compliance with protocols established in the NIH/NRC *Guide and Use of Laboratory Animals* and were reviewed by the Institutional Animal Care and Use Committee of Battelle, Pacific Northwest Division.

Saliva collection

Rats were anesthetized with isoflurane in conjunction with oxygen in an inhalation chamber. Once under anesthesia, the jugular vein was cannulated, and a tracheotomy was performed to facilitate breathing and anesthesia during saliva collection. Rats were then infused with 1 mg/mL of pilocarpine, a non-selective muscarinic receptor agonist, in physiological saline via the jugular vein cannula at a rate of 3 mL/hr for two hours. Pilocarpine induced salivation and saliva was collected continuously using glass capillary tubes draining into 2 mL microcentrifuge vials. Mixed saliva was collected in intervals from 0-15, 15-30, 30-60, 60-90, and 90-120 minutes, at which time, rats were euthanized. Saliva samples were stored at -80°C until time of analysis.

Determination of total ChE enzyme activity

Characterization of total ChE enzymatic activity and protein concentration was determined for each rat at each time point (n=25). Salivary cholinesterase (ChE)

activity was determined using a modified Ellman assay (Ellman *et al.*, 1961). Since 95% of total ChE activity in saliva is butyrylcholinesterase (BuChE), total ChE enzymatic activity was determined using both ATCl and BTCl (Kousba *et al.*, 2003). Generally, ATCl is a good substrate for both acetylcholinesterase (AChE) and BuChE, and BTCl is hydrolyzed by BuChE only (Lassiter *et al.* 1998 and Chuiko, 2000). Hydrolysis of either ATCl or BTCl results in the formation of a yellow compound, 5-thio-2-nitro-benzoic acid (Fig. 1).

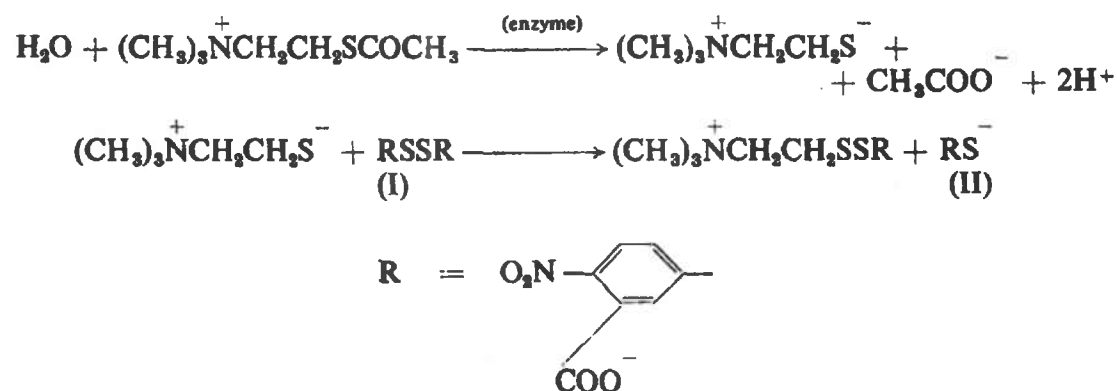


Figure 1: Acetylthiocholine is hydrolyzed by cholinesterase into acetic acid and thiocholine, which reacts with 5,5-dithio-bis(2-nitrobenzoic acid) (RSSR) to form 5-thio-2-nitro-benzoic acid (RS⁻), a yellow compound, used for detection in the colorimetric Ellman Assay for cholinesterase activity (Ellman *et al.*, 1961).

Saliva samples were thawed at room temperature and aliquots were diluted in 24 volumes of phosphate buffer, pH of 7.4. For each 200 μL aliquot of diluted sample, 20 μL each of both DTNB and ATCl or BTCl were added to each well of a 96-well plate. ChE activity, as described by the hydrolysis of ATCl or BTCl, was monitored by following the absorbance profile at 405 nm over a 20 min period with readings of absorbance in optical density (OD) taken every 2.5 min using a 96-well automated

microplate reader (Tecan GENios). Activity of ChE was defined as the linear change of OD over time.

Determination of total protein

A modification of the BCA protein assay (Smith *et al.*, 1985) in a 96-well a microplate was used to determine total protein in each sample. Aliquots of thawed saliva from each sample were diluted in 24 volumes of 0.1M phosphate buffer, pH of 7.4. A commercially available solution of bovine serum albumin (BSA) with a concentration of 2 mg/mL was serially diluted by half in 0.1M phosphate buffer, pH of 7.4, to create a standard curve with one blank (n=9). Twenty microliters of diluted sample or standard were loaded into wells.

The BCA working reagent was prepared by mixing 50 volumes of Reagent A with one volume of Reagent B. Two hundred microliter aliquots of this working reagent were then added to each well. Samples were incubated at 37°C for 30 min, and products of the resulting reaction (Fig. 2) were measured using the absorbance at 562nm in a 96-well microplate reader (Tecan GENios). A linear regression model of the standard curve was used to determine the protein concentrations of each sample.

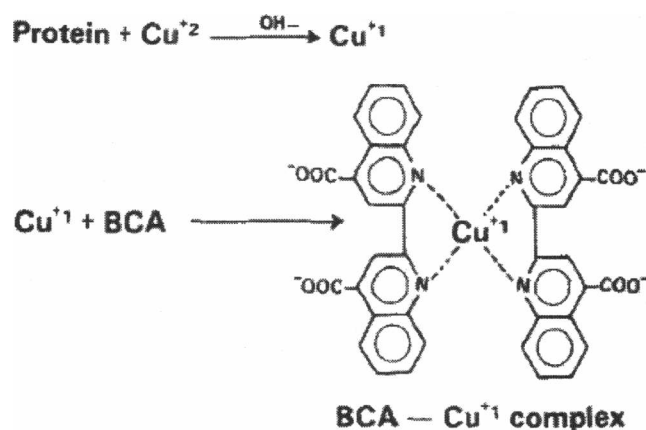


Figure 2: Protein in a saliva sample reacts with Cu²⁺ to form Cu¹⁺ which forms a complex with bichinchoninic acid which can be quantified by the absorbance at 562 nm (Smith et al., 1985).

Statistical analysis

The coefficient of variation or coefficients of variation (CV) were calculated as the standard deviation divided by the mean and describe the dispersion of a sample set. Intra-individual variability was determined for each collection time by calculating CVs using five collection interval values for each individual. Inter-individual variability at a given collection interval was determined by calculating the CV using values from each individual (n=5). The CV using all values from all rats at all collection intervals (n=25) also described inter-individual variability. Standard error, standard deviation divided by the square root of the sample size, was also used to indicate the inter-individual variability. The relationship between ChE activity and total protein was derived using simple linear regression. Pearson product-moment correlation coefficient (correlation coefficient) was calculated to describe the relationship between ChE activity and total protein. All statistical analyses were conducted using Excel.

Results

Variation in total salivary ChE activity

Total salivary ChE activity, as determined by the Ellman assay, decrease over the duration of collection and substantial inter-individual variability was observed (Fig. 3). CV values were calculated for each collection interval and for the duration of collection. CVs over the entire collection time were 68.8% and 71.6% for ATCl and BTCl, respectively. When the ATCl substrate was utilized, the coefficient of variation ranged from 29.8% at 30 minutes to 65.2% at 15 minutes. When BTCl was used, the coefficient of variation ranged from 31.52% at 30 minutes to 66.9% at 15 minutes. Variation over collection time was also observed in each rat, CVs varied from 16.8% in rat 1 to 104.6% in rat 3 when the ATCl substrate was used and from 15.3% in rat 1 to 114.0% in rat 3 when the BTCl was used.

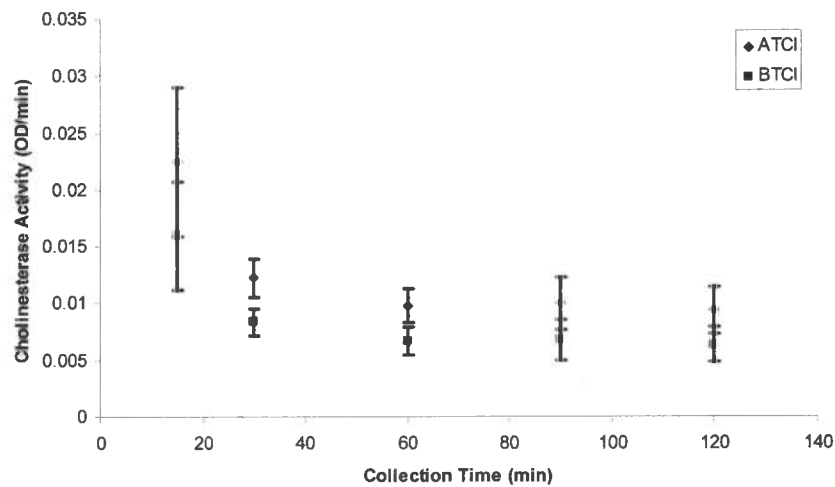


Figure 3: Mean (\pm standard errors) cholinesterase activity of saliva from rats infused with pilocarpine, as determined by an Ellman assay with either acetylthiocholine (ATCl) or S-butyrylthiocholine (BTCl) substrates.

Variation in total protein and salivary flow rate

Total protein, as determined by the BCA assay, was variable for both inter and intra-individual sampling over the collection period (Fig. 4). CV amongst individuals ranged from 36.0% at 30 minutes to 57.1% at 90 minutes, while the overall CV was 66.1%. Individual CVs ranged from 15.6% to 105.9%. Standard errors were also determined at each collection time and varied from 0.627 at 120 minutes to 1.910 at 15 minutes. Mean salivary flow rate decreased over collection (Fig. 5). Inter-individual variation was also seen, with a CV of 30.2%. Total protein increased with flow rate and had a correlation coefficient of 0.659 (Fig. 6).

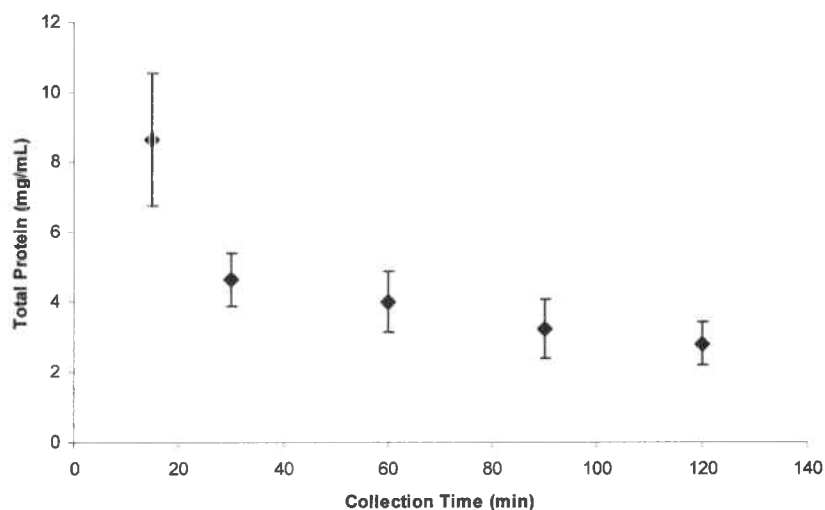
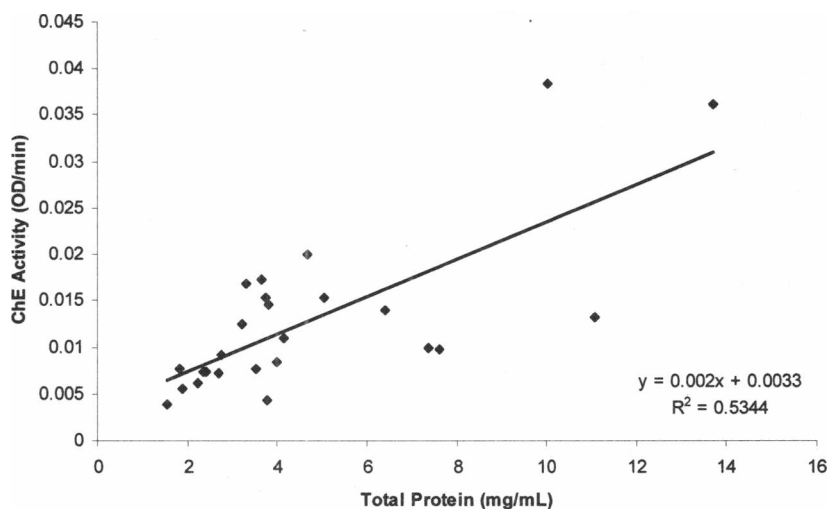


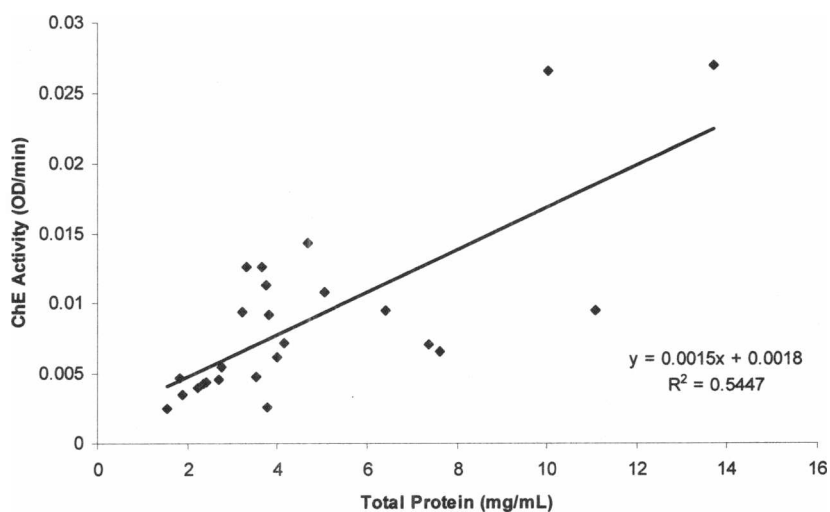
Figure 4: Total protein in rat saliva using a bicinchoninic acid protein assay showed inter-individual variation over the collection time.

Correlation of total ChE activity and total protein

ChE activity increased with total protein in saliva (Fig. 7). The correlation coefficient was 0.731 and 0.738 for the ATCl and BTCl substrates, respectively.



(A)



(B)

Figure 7: Salivary cholinesterase activity using (A) acetylthiocholine chloride or (B) S-butyrylthiocholine chloride in the Ellman assay is correlated with salivary protein concentration.

Since ChE activity and protein concentration were strongly correlated, normalization of ChE activity was explored using Equation 1 where A_n is normalized ChE activity, A_i is ChE activity measured by the Ellman assay, V_s is sample volume, and C_p is concentration of total protein determined by the BCA assay. Variation was not eliminated by this normalization process (Fig. 8); however protein normalized ChE activity does not change over time. Inter-individual CVs were then determined for the normalized ChE activity, 36.97% for ATCl and 40.81% for BTCl. Protein normalization resulted in a decrease in CVs from non-normalized values (Fig. 9).

$$A_n = \frac{A_i}{V_s * C_p} \quad \text{Equation 1}$$

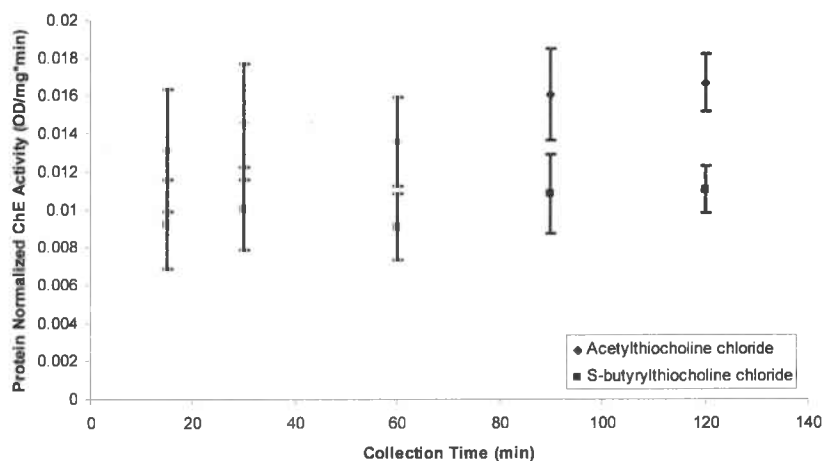


Figure 8: Protein normalization of salivary cholinesterase activity from five rats using two substrates in the Ellman Assay.

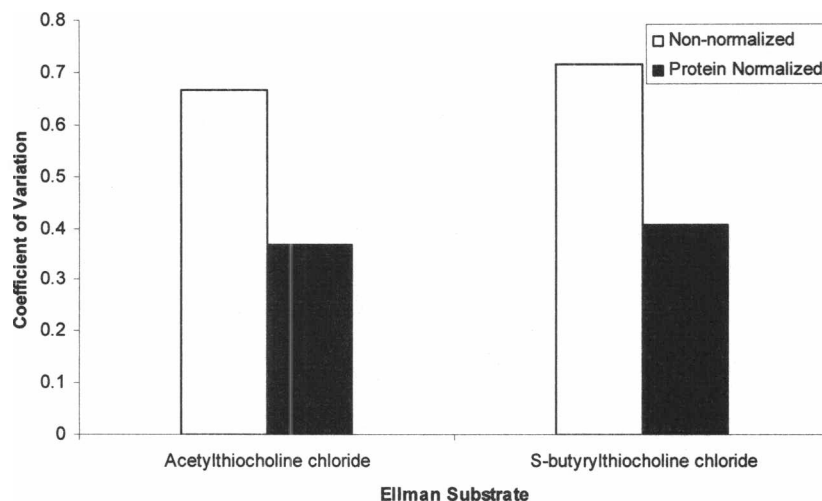


Figure 9: Non-normalized and protein normalized coefficients of variation of salivary ChE activity in rats determined by the Ellman assay using two substrates.

Discussion

In the current study, baseline rat salivary ChE activity was characterized using an Ellman assay along with total protein as determined using a BCA assay. Salivary ChE activity has been suggested as a non-invasive biomarker for pesticide exposure in humans due to the ease of its collection, potential for accurate OP pesticide availability to tissue estimates, and the advent of portable electrochemical sensors (Lu *et al.*, 2009; Nigg and Wade, 1992; Liu *et al.* 2008; Wang, H. *et al.*, 2008; Wang, J. *et al.*, 2008; Kousba *et al.* 2003; Henn *et al.*, 2006). However, few studies have examined characteristics of saliva as a potential biomarker in either humans or Sprague-Dawley rats (Lu *et al.*, 2009; Nigg and Wade, 1992; Kousba *et al.* 2003; Henn *et al.*, 2006).

In the present study, total protein was determined in an attempt to normalize ChE activity and reduce both the intra and inter-individual variability. Inter-individual

variability was reduced from 68.8% to 37.0% for ATCl and from 71.6% to 40.8% for BTCl. Intra-individual variation was also reduced in three of the five rats; however, two rats had an increase in intra-individual variation when ChE activity was normalized by total protein. Overall, protein normalization decreased variability in salivary ChE from 68.8% to 37.0%.

Other studies have reported high variability in ChE activity; however their measurements were taken from individual collections taken each day, rather than by continuous collection (Kousba *et al.*, 2003; Henn *et al.*, 2006). Kousba *et al.* (2003) reported inter-individual variation in ChE activity from saliva measured on four successive days which was also seen in this study. Henn *et al.* (2006) reported similar intra- and inter-individual variation to that seen in this study, which was not reduced by protein normalization, and may be the result of several limitations in their study. In Henn *et al.* (2006) human saliva samples were collected either using a pipette for unstimulated saliva or a Salivette[®] for stimulated saliva. When Salivette[®] collection was used, ChE became trapped in the wool roll even after centrifugation, requiring Henn *et al.* (2006) to measure ChE activity directly on the roll. Although surveys of participants' exposure to pesticides were conducted at each collection, individuals may not have reported all exposures which could introduce unknown variables to the study (Henn *et al.*, 2006). In contrast, rats in this study were in a controlled environment, not exposed to pesticides, saliva was stimulated using pilocarpine and wool rolls were not used in saliva collection. Thus, dissimilar study conditions could have lead to discrepancies between these studies.

A potential limitation of the present study was the co-administration of pilocarpine to induce salivation and isoflurane to induce and maintain anesthesia. Freitas *et al.* (2006) observed a reduction in brain ChE activity with a single pilocarpine dose of 400 mg/kg, while Kousba *et al.* (2003) saw no marked decrease in ChE activity with the administration of pilocarpine. In the present study, rats received a significantly lower dose of pilocarpine (~15 and 20 mg/kg infusion over two hours), compared to Freitas *et al.* (2006) therefore, ChE activity was unlikely affected. It has also been reported that the electrolyte composition of saliva changes with salivary flow rate, however no known studies have examined the effect of flow rate on protein concentrations in saliva (Schneyer and Schneyer, 1960; Dawes, 1966). No studies I know of have examined the effects of isoflurane on ChE activity. However, Lejus *et al.* (2002) examined the effect of sevoflurane, a halogenated anesthetic similar to isoflurane, on ChE activity in humans and saw no significant changes.

Comparison of the coefficients of variation from measured ChE activity and normalized ChE activity demonstrated a reduction in variation suggesting that total protein is a viable method for normalizing ChE activity in rat saliva. Further research may be warranted utilizing human samples to determine if a similar relationship exists. Western blots may be used to determine if the fraction of AChE and BuChE of total protein has a stronger correlation with ChE activity and may provide additional measures to further reduce variability. Reliability of ChE measurements in saliva will be a key achievement for use of ChE activity in human biomonitoring, and overall, this study supports the use of total protein as a normalization measure in salivary ChE biomonitoring to reduce variability. Thus, I accept my original hypothesis.

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