

76. RAPID, QUANTITATIVE, AND SIMULTANEOUS DETERMINATION OF AChE AND BChE LEVELS IN UNPROCESSED WHOLE BLOOD

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ABSTRACT

The concentration of acetylcholinesterase (AChE, EC 3.1.1.7) and butyrylcholinesterase (BChE, EC 3.1.1.8) in blood is a stable biomarker of suppressed and/or heightened central and peripheral nervous system activity. AChE and/or BChE activity is selectively altered after exposure to nerve agents, organophosphates (OPs), pesticides, anesthetics, terrorists' chemical agents, cocaine, and in some neurodegenerative disease states. Therefore, blood cholinesterase activity can be exploited as a tool for confirming exposure to the agents and diagnosis of diseases.

We developed a robust protocol to quickly and simultaneously provide accurate (greater than 99%) and precise (less than 1%) blood concentrations of both AChE and BChE. This method measures the activity of whole blood in the presence of three AChE and BChE substrates from which the individual AChE and BChE contributions are calculated. This is possible because each protein possesses a different affinity for each of the substrates, and a direct relationship exists between activity and enzyme concentration. To date, we have applied our technique to blood in the presence and absence of selective (huperzine-A and tetraisopropylphosphoramidate) and non-selective (pyridostigmine bromide) cholinesterase inhibitors. Furthermore, we have used this technique to successfully screen 320 samples in one four-hour time interval.

In conclusion, we developed a method capable of providing fast, precise, and accurate AChE and BChE measurements. Unlike the conventional clinical tests, our procedure provides a more detailed picture of the patient's cholinesterase profile (both AChE and BChE), produces results in less than five minutes, and is capable of screening thousands of samples using state of the art robotics.

INTRODUCTION

Acetylcholinesterase (AChE) is one of nature's most elegantly engineered proteins. The physiological role of the enzyme is the acceleration of hydrolysis of neurotransmitter acetylcholine at nerve-nerve and neuromuscular junctions. The enzyme is ideally suited for this role, because it possesses one of the fastest turnover rates known (1,2). Inhibitors of AChE have been used medicinally in the treatment of glaucoma, myasthenia gravis, and recently, with the FDA approval of tacrine (3) and Aricept (4), Alzheimer's disease. Other inhibitors have been used agriculturally and domestically as pesticides and insecticides. And unfortunately, some of the most potent inhibitors have been developed for chemical warfare. The most recent devastating demonstration of the availability, rapidity and lethality of these agents was the release of sarin into the Japanese commuter train system in Tokyo. Furthermore, since urban terrorism is on the rise, Federal, State and local authorities need a reliable, fast, inexpensive, standard method for confirming such an attack in order to initiate appropriate containment, decontamination and treatment measures.

Current clinical determination of cholinesterase levels within blood includes Michel, microMichel, pH stat, Ellman, and microEllman methodologies (5,6,7). These methods, however, suffer from wide statistical error, long clinical turn around times, lack standardization among labs, and normally determine only the serum or red blood cell cholinesterase concentration. As a result, the reliability of these results is often questioned, and inter-lab comparison is impossible. Worse yet, due to the abnormally long turn around times (up to seven days), physicians are required to treat a patient presenting cholinesterase poisoning long before receiving confirmation. In addition to the clinical methodologies, one field deployable unit is commercially available, the Test-Mate OP system (EQM Research Inc., Cincinnati, OH). Although this unit is fairly user friendly, it possesses several major limitations including numerous processing steps, the use of selective BChE inhibitors, and requires two blood samples and double the processing time for complete AChE and BChE screening.

Therefore, we have developed a new methodology that quickly and simultaneously determines the concentrations of AChE and BChE in unprocessed, whole blood. Unlike the conventional clinical tests or the Test-Mate OP unit, our method provides a full analysis of the patient's cholinesterase levels, does not rely on the addition of selective AChE or BChE inhibitors, uses a single non-invasive blood collection technique (finger prick), is not labor intensive, and produces results in less than five minutes. We have circumvented the aforementioned problems by simultaneously determining the levels of AChE and BChE in aliquots from the same blood sample.

This is possible because blood contains two cholinesterases that possess different affinities for any given substrate, and a linear correlation exists between enzyme activity and concentration. Thus, if one measures the activity of any given blood sample with two different substrates, then it is feasible to calculate the precise concentrations of both proteins (i.e., two equations with two unknowns). Furthermore, monitoring the activity with three substrates (equations 1-3) provides three fold degenerate data [i.e., three sets of two equations (equations 1&2, 2&3, 1&3) with two unknowns]. In equations 1-3, the rates of substrate hydrolysis are represented by R1, R2, or R3 and correspond to the turnover of substrate 1, substrate 2, or substrate 3, respectively. The [AChE] and [BChE] refer to the actual concentrations of AChE and BChE contained in the sample. Finally, the coefficients in each equation (i.e., x1, x2, x3, and y1, y2, y3) represent the sensitivity coefficients. These coefficients represent the contribution that AChE and BChE contribute to the overall rate of substrate hydrolysis (R1, R2, R3).

$$R1 = x1[AChE] + y1[BChE] \quad (1)$$

$$R2 = x2[AChE] + y2[BChE] \quad (2)$$

$$R3 = x3[AChE] + y3[BChE] \quad (3)$$

Simultaneously solving the three sets of degenerate equations provides three independent estimates for the concentrations of AChE and BChE. Therefore, determining the mean value and the standard deviation for the three independently derived values provide an excellent estimate of the authentic concentrations of each protein

MATERIALS AND METHODS

Acetylthiocholine iodide (ATC), propionylthiocholine iodide (PTC), butyrylthiocholine iodide (BTC), 4,4'-dithiopyridine (DTP), and tetraisopropylphosphoramidate (Iso-OMPA) were purchased from Sigma Chemical Co (St. Louis, MO). Racemic huperzine-A (Hup-A) was purchased from CalBiochemical-NovaBiochem Corporation (San Diego, CA). Water was polished to 18.2 mega-ohm by passage through a Millipore purification system (Millipore, Bedford, MA). Trunk blood obtained from 10 Hartley guinea pigs (Charles River Laboratories, Wilmington, MA) was stored in the presence of EDTA. All blood samples were refrigerated at 4 C until used.

The following stock reagents and buffer were prepared and stored at -20 C until needed, or stored at 4 C when in use. Thirty mM ATC (sATC), 30 mM PTC (sPTC), and 30 mM BTC (sBTC) were prepared in 18.2 Mega-ohm water, and 6 mM DTP (sDTP) was prepared in 10% HPLC grade methanol/buffer. The buffer consisted of 50 mM sodium phosphate, pH 8.0. Working reagents were prepared in buffer immediately prior to use and stored at room temperature. These reagents were 1.0 mM ATC and 200 DTP (A), 1.0 mM PTC and 200 μ M DTP (P), 1.0 mM BTC and 200 μ M DTP (B), and 200 μ M 4,4'-dithiopyridine (D). Solutions of the selective inhibitors Hup-A (0 to 900 nM), Iso-OMPA (0 to 5.12 μ M), and both (0 to 900 nM Hup-A and 0 to 5.12 μ M Iso-OMPA) were prepared in water. In addition, serial dilutions of each species whole blood were prepared in water.

All species' blood and dilutions were titrated using a series of inhibitors. 10 μ L of an inhibitor solution was transferred to a PCR tube followed by the quantitative addition of 150 μ L of a whole blood dilution. The contents were thoroughly mixed and incubated at room temperature for three hours. Then, 10 μ L aliquots from each PCR tube were transferred repetitively to one row of a standard 96 well microtiter plate (Corning, Acton, MA). Next, 290 μ L aliquots of D (control) were added to columns 1-3, 290 μ L aliquots of A were added to columns 4-6, 290 μ L aliquots of P were added to columns 7-9, and 290 μ L aliquots of B were added to columns 10-12. An assay was performed on the plate using a Molecular Devices SpectraMax Plus microtiter spectrophotometer (Sunnyvale, CA). The first was a four-minute kinetic assay in which the change in absorbance at 324 nm was monitored. Other parameters included sixty second pre-read mixing, three second shaking between reads, twelve second intervals, and linear least squares data analysis. Subsequent cholinesterase screening was performed as described above unless otherwise noted, except that the samples were prepared solely by dilution with water.

RESULTS

A representative titration of guinea pig blood with a selective inhibitor (Hup-A) is depicted in Figure 1A. In this Figure, titrations at several blood dilutions for ATC turnover are displayed. Each titration was fit via nonlinear least squares analysis to a simple binding isotherm possessing a residual velocity. Note that all rates were corrected for blood hydrolysis of DTP. The control velocities, V_c , and the residual rates, V_r , for each substrate (i.e., ATC, PTC, and DTP) were graphed as a function of blood concentration, Figure 1B. The resulting relationships were fit via linear least squares analysis then tabulated in Table 1. The slopes for each V_r relationship corresponds to the contribution that BChE contributes to the corresponding control reaction and are the sensitivity coefficients (SC). The SC for each substrate for AChE is calculated by subtracting the corresponding BChE SC coefficient from the control. The sensitivity coefficients for AChE and BChE for each substrate are contained in Table 2. Similar results were also obtained for the Iso-OMPA titration, except that the V_r slopes correspond to the AChE SC, and the BChE

SC are obtained by subtracting those for AChE from the corresponding Vc relationship. The SC determined from the Iso-OMPA titration, and the average values from both titrations are contained in Table 2. In reference to equations 1-3 above, x1, x2, x3 and y2, y2, y3 correspond to the sensitivity coefficients of AChE and BChE for ATC, PTC, and BTC, respectively. The observed rates for ATC, PTC, and BTC likewise correspond to R1, R2, and R3, respectively.

The observed rates of hydrolysis for the inhibitor solutions consisting of Hup-A, Iso-OMPA, and the combination of Hup-A and Iso-OMPA were converted into U/mL. This was accomplished via equations 1-3 and the average sensitivity coefficients for AChE and BChE (cf. Table 2). The processed AChE and BChE concentrations for the Hup-A and Iso-OMPA titrations are contained in Table 3. The results for the 8x diluted blood sample of guinea pig are plotted in Figure 2. In this Figure, panel A refers to the results from the Hup-A titration, panel B corresponds to the Iso-OMPA titration, panel C corresponds to the AChE results for titration by the combination mixture (Hup-A and Iso-OMPA), and panel D depicts the BChE results for the combination mixture. It is important to note that for ease of comparison with panels A and B the axis in panels C and D have been chosen to reflect the response due to Hup-A and Iso-OMPA, respectively. In other words, the AChE response was plotted as a function of Hup-A rather than Iso-OMPA, and BChE was plotted as a function of Iso-OMPA rather than Hup-A.

DISCUSSION

As clearly demonstrated by Table 2, the sensitivity coefficients for AChE and BChE are identical regardless of which titrant is used. This indicates that all observed activity for ATC, PTC, and BTC hydrolysis is due to the combined actions of AChE and BChE. Thus, use of the sensitivity coefficients as detailed in the background section is feasible. Although it is possible to use only two substrates for analysis, it is preferred that three be used. It is our opinion that the use of three substrates provides redundant and independent determinations of the concentration of both AChE and BChE. The average reflects a statistically better representation of the true value than any single determination. Additionally, the standard deviation associated with mean provides is very informative. First, it provides an internal validation of the assay. If the protocol were flawed, large fluctuating standard deviations would be expected. This does not occur as per Table 3, which possesses a nearly constant standard deviation of 0.003 U/mL. Second, it indicates how precisely the concentration of each protein is known. The average precision of this assay is 0.7% and 0.3% for AChE and BChE, respectively (cf. Table 3). For AChE and BChE, this is the average percent confidence value for the eight separately calculated concentrations of AChE in the Iso-OMPA and Hup-A titration data, respectively. Sample to sample variation (repetitive determinations) is also contained in this data. The precision for AChE is roughly 3% and for BChE the precision is about 1.4% (i.e., the standard deviation of the eight independently determined concentrations of AChE and BChE in the presence and absence of Iso-OMPA and Hup-A, respectively).

Since pure samples of guinea pig blood AChE and BChE were not available, the accuracy of the method was assessed indirectly. Since Hup-A is selective for AChE, the loss in activity with respect to a control reaction at infinite inhibitor concentration corresponds to the concentration of AChE contained in the sample. Likewise, the concentration of BChE in the sample can be extracted from Iso-OMPA titration. The uninhibited data in Table 3 was compared to the calculated theoretical concentrations of AChE (0.610 U/mL) and BChE (0.911 U/mL). The accuracy for AChE and BChE determined using this method is greater than 99% for both AChE and BChE. Although not included in this paper, similar results are obtained for other species over several titrations. Obviously, obtaining pure AChE and BChE for spiking blood samples with known amounts would be more desirable; however, an indication of the accuracy has nonetheless been presented.

Finally, blood samples inhibited by mixtures of Hup-A and Iso-OMPA produced identical results to those obtained independently using pure Hup-A and pure Iso-OMPA solutions. This is clearly demonstrated comparing the data and theoretical curves of AChE titrated by Hup-A alone (2A) to those in the presence of both inhibitors (Figure 2C). In addition to visual inspection, the calculated inhibition constants are 1.6 +/- 0.1 nM and 1.7 +/- 0.1 nM in the absence and the presence of Iso-OMPA, respectively. Comparing panels B (Iso-OMPA alone) and D (Hup-A and Iso-OMPA) in Figure 2 provides similar results for BChE. The calculated IC50 values are 13 +/- 3 nM and 10 +/- 3 nM in the absence and the presence of Hup-A, respectively. Obviously this method is capable of discriminating individual effects on AChE and BChE activities in the presence of selective and non-selective cholinesterase inhibitors.

CONCLUSION

In conclusion, a methodology capable of screening unprocessed whole blood samples has been presented. The unprecedented precision (<1%) and accuracy (>99%) of this procedure is unmatched by current clinical practices,

and unlike the current methods, does not suffer from large statistical fluctuations nor long turn around times. Additionally, this protocol provides a much more detailed picture of an individual's cholinesterase profile using non-invasive sampling techniques. Although not discussed in this paper, initial validation with the COBAS/FARA, TestMate OP, and Michel methods is underway, and the preliminary results demonstrate linear correlations between all methods at a high confidence level (an average regression coefficient of about 0.99 for both AChE and BChE). In addition, the sensitivity coefficient method has been applied to several species including human, Rhesus monkey, and Sprague Dawley rats. Finally, the simplicity, precision and accuracy of this protocol will allow clinicians to screen and confirm suspected ChE intoxication with unprecedented confidence, and therefore be able to initiate, follow, and dynamically adjust treatment. It is anticipated that further refinement of this technique will provide a universally accepted, real time, inexpensive method for simultaneously screening whole blood samples for AChE and BChE.

REFERENCES

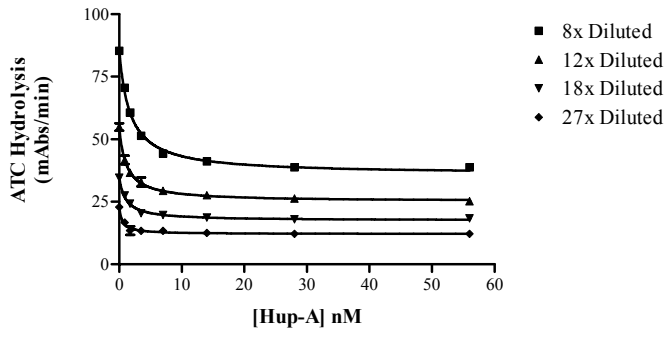
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KEYWORDS

Cholinesterase, screening, chemical warfare agents, biomarker, inhibitor

FIGURES AND TABLES

A



B

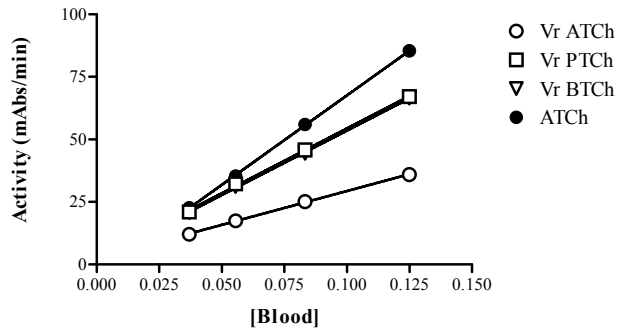
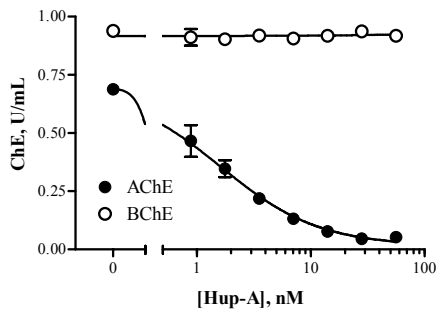


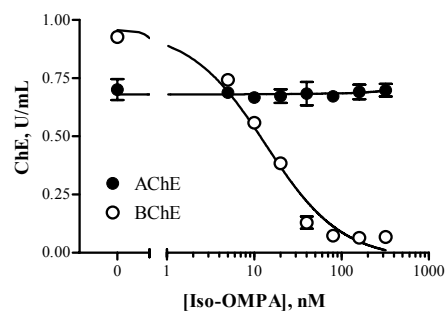
Figure 1. A representative titration of guinea pig blood with a selective inhibitor (Hup-A).

Figure 2. The processed AChE and BChE concentrations for the Hup-A and Iso-OMPA for the 8x diluted blood sample of guinea pig.

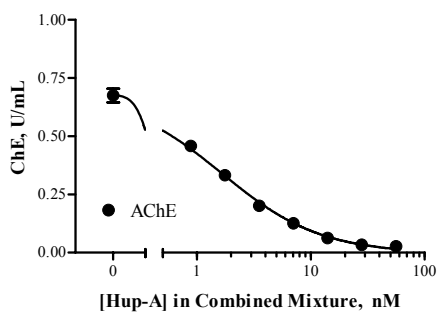
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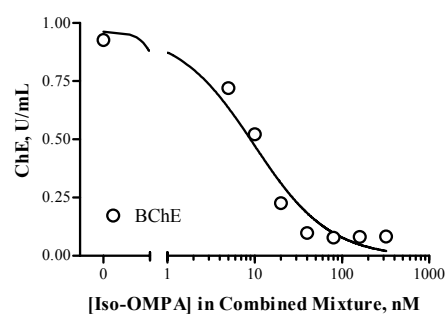


Table 1. Slopes of Control Reactions and Residual Activities

	Control		Hup-A	
	mAbs/min/[Blood]	Error	mAbs/min/[Blood]	Error
ATC Slopes	715	7	270	4
PTC Slopes	744	11	518	12
BTC Slopes	557	10	515	9

Table 2. Hartley Guinea Pig Blood Sensitivity Coefficients

		AChE	BChE
		mAbs/min/[Blood]	mAbs/min/[Blood]
Hup-A Titration	ATC	444	270
	PTC	226	518
	BTC	43	515
Iso-OMPA Titration	ATC	418	297
	PTC	194	550
	BTC	0	557
Average	ATC	431	284
	PTC	210	534
	BTC	21	536

Table 3. Processed Titration Results (Activity to U/mL)

[Hup-A], nM	[AChE]		[BChE]			[Iso-OMPA], nM	[AChE]			[BChE]	
	Value	STD	Value	STD	% CV		Value	STD	% CV	Value	STD
56	0.006	0.004	0.907	0.004	0.4	320	0.605	0.007	1.2	0.048	0.003
28	0.019	0.002	0.929	0.002	0.2	160	0.63	0.003	0.5	0.071	0.001
14	0.055	0.003	0.912	0.001	0.1	80	0.616	0.001	0.2	0.08	0.001
7	0.136	0.002	0.892	0.002	0.2	40	0.635	0.004	0.6	0.143	0.001
4	0.214	0.002	0.907	0.002	0.2	20	0.617	0.004	0.6	0.279	0.005
2	0.334	0.002	0.93	0.003	0.3	10	0.585	0.004	0.7	0.565	0.003
1	0.462	0.001	0.913	0.002	0.2	5	0.597	0.005	0.8	0.699	0.001
0	0.61	0.007	0.925	0.004	0.4	0	0.582	0.008	1.4	0.945	0.004
			1.4		0.3		3.2		0.7		
			%CV		Average		%CV		Average		