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Development and Optimization of a Highly Specific and Sensitive Assay to Detect Acetylcholinesterase Activity and Its Inhibition by Donepezil

J. Thompson[1], R. Islam[1], M. Balasurya[2] and J. Knight[2]
[1]Celerion and [2]Somru BioScience

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Background

Acetylcholinesterase (AChE) is one of the most critical enzymes for nerve response and function. It is primarily found at neuromuscular junctions and cholinergic synapses in the central nervous system. Cholinesterases are enzymes responsible for cleaving cholinergic neurotransmitters; this action allows cholinergic neurons to return to their resting state after activation. If cholinergic neurons were to remain activated, then muscles could not relax once they had been tensed. AChE activity serves to terminate the synaptic transmission.

Vertebrates have two cholinesterases: acetylcholinesterase (AChE) is located mainly in chemical synapses and erythrocyte membranes while butyrylcholinesterase (BChE) is located mainly in plasma. AChE and BChE are differentiated based on their preferred substrate: AChE hydrolyzes acetylcholine (ATC) faster, while BChE hydrolyzes butyrylcholine (BTC) faster. However, both substrates can be hydrolyzed by both cholinesterases. This makes the specific measurement of AChE challenging.

Purpose and Methods

AChE inhibitors are among the key drugs approved for Alzheimer's disease (AD) and myasthenia gravis. In order to evaluate pharcodynamic properties of these drugs it is critical to measure the specific activity of AChE without interference from BChE.

Here we present an enzyme activity method based on the kit from Somru BioScience Inc. that is highly specific to AChE activity and sensitive enough to measure inhibition of AChE activity by donepezil.

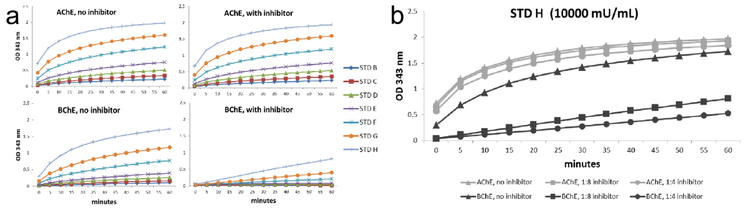

Figure 1: Eel acetylcholinesterase (AChE) and equine butyrylcholinesterase (BChE) activities were tested with or without BChE inhibitor. a) Kinetic calibration curves show diminished OD values for BChE but not AChE, proving specificity of the inhibitor. b) Inhibition is concentration dependent: STD H kinetic curves are shown for AChE, in which activity remains uninhibited, and BChE, in which activity is inhibited at both 1:8 and 1:4 dilutions of inhibitor.

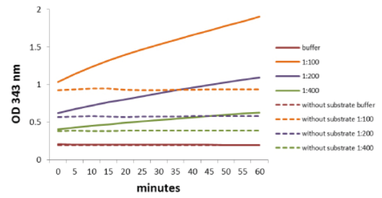

Figure 2: Contribution of background signal from whole blood and determination of optimal dilution for use in assay. Eel acetylcholinesterase (AChE) activity in human whole blood diluted 1:100, 1:200, or 1:400 was tested with or without substrate- dotted lines indicate background signal.

Results from 343 nm readout (Figures 1 and 2)

- Confirmed specificity of assay substrate is for AChE and not BChE
- High background noise, presumably from contribution of hemoglobin, necessitates high dilution of sample, possibly leading to loss of signal from AChE activity
- New chromophore using 670 nm wavelength will be tested

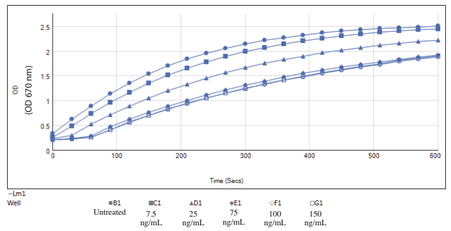

Figure 3: Area under curve for AChE activity in RBCs treated with donepezil at concentrations ranging from 7.5 – 150 ng/mL. Undiluted RBCs were analyzed at 670 nm in a kinetic assay with reads every 30 seconds. 2 minutes was chosen for subsequent method qualification and sample analysis.

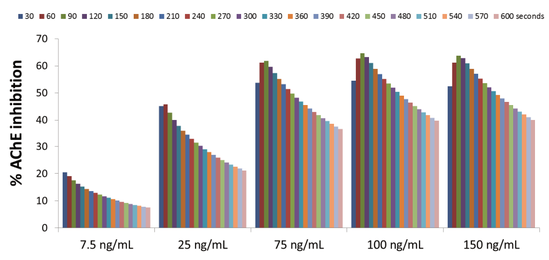

Figure 4: Inhibition of AChE activity in RBCs by donepezil concentrations ranging from 7.5 – 150ng/mL. Undiluted RBCs were analyzed at 670 nm in a kinetic assay with reads every 30 seconds.

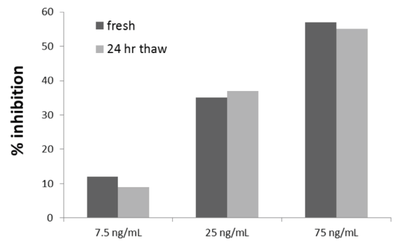

Figure 5: Sample stability in matrix. Inhibition of AChE activity in RBC was measured for various levels of donepezil treatment in samples assayed immediately after removal from -80°C, or in those that were allowed to sit at 5oC for 24 hours. Assays were performed at 670 nm 2 minutes post-addition of substrate.

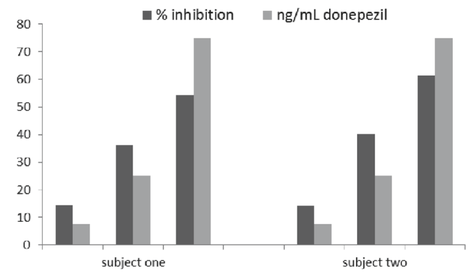

Figure 6: Inhibition of AChE activity in RBC correlates to concentration of donepezil. Samples from two individuals treated with donepezil were assayed at 670 nm for AChE activity 2 minutes post-addition of substrate. Assay is sensitive enough to detect inhibition of AChE activity at 7.5 ng/mL donepezil.

	untreated				7.5 ng/mL				25 ng/mL				50 ng/mL				100 ng/mL				150 ng/mL			
			mean	%CV			mean	%CV			mean	%CV			mean	%CV			mean	%CV			mean	%CV
	85	81	83	3	71	69	70	2	70	66	68	4	49	47	48	4	52	53	53	2	50	50	50	0
	85	79	82	6	76	73	75	3	65	65	65	0	46	47	46	2	51	52	51	1	48	47	47	2
	85	82	84	2	76	70	73	6	65	67	66	1	48	45	47	5	49	48	48	1	44	44	44	0
mean			83				73				66				47				51				47	
%CV			1				3				2				2				4				6	
background subtracted			59				49				42				23				27				23	
% inhibition							18				29				61				54				61	
			mean	%CV			mean	%CV			mean	%CV			mean	%CV			mean	%CV			mean	%CV
	78	75	76	3	64	64	64	0	66	60	63	7	46	95	71	49	43	45	44	4	43	46	45	5
	74	74	74	0	71	69	70	2	62	64	63	2	44	48	46	6	45	47	46	2	43	45	44	2
	71	66	68	5	65	63	64	2	60	60	60	1	45	46	45	2	43	45	44	4	44	43	43	2
mean			73				66				62				46				45				44	
%CV			6				5				3				1				2				1	
background subtracted			49				42				38				22				21				20	
% inhibition							14				23				56				58				59	
			mean	%CV			mean	%CV			mean	%CV			mean	%CV			mean	%CV			mean	%CV
	93	95	94	2	83	81	82	2	75	74	75	1	57	57	57	0	56	55	55	1	55	57	56	2
	90	92	91	2	84	83	84	1	76	78	77	2	58	55	56	3	55	58	57	3	55	55	55	0
	90	93	91	2	85	85	85	0	76	76	76	0	56	54	55	3	56	56	56	0	55	57	56	2
mean			92				84				76				56				56				56	
%CV			2				2				1				1				1				1	
background subtracted			68				60				52				32				32				32	
% inhibition							12				24				53				53				54	
			mean	%CV			mean	%CV			mean	%CV			mean	%CV			mean	%CV			mean	%CV
	86	84	85	2	72	67	70	5	62	63	63	0	52	52	52	1	45	47	46	3	48	48	48	0
	85	86	85	1	77	72	75	5	61	60	60	1	49	50	49	1	46	48	47	3	44	42	43	2
	81	77	79	4	79	74	76	5	66	67	66	1	48	49	49	1	45	44	44	2	47	49	48	2
mean			83				74				63				50				46				46	
%CV			4				5				5				4				3				6	
background subtracted			59				50				39				26				22				22	
% inhibition							16				34				56				63				62	
			mean	%CV			mean	%CV			mean	%CV			mean	%CV			mean	%CV			mean	%CV
	68	67	68	1	62	60	61	2	52	51	51	1	43	44	44	2	41	42	42	2	42	44	43	3
	64	66	65	2	61	61	61	0	48	49	48	1	42	41	42	0	44	43	44	1	41	42	41	3
	68	68	68	0	64	63	64	1	51	54	53	3	47	47	47	0	44	44	44	0	43	44	43	1
mean			67				62				51				44				43				43	
%CV			2				3				4				3				3				2	
background subtracted			43				38				27				20				19				19	
% inhibition							12				38				54				56				57	
			mean	%CV			mean	%CV			mean	%CV			mean	%CV			mean	%CV			mean	%CV
	66	63	65	3	59	60	60	1	53	50	52	5	43	43	43	0	39	38	38	2	39	40	39	2
	64	61	63	3	63	61	62	3	49	49	49	1	43	43	43	1	42	42	42	0	39	39	39	1
	65	65	65	1	61	57	59	5	51	52	52	1	42	41	41	1	35	35	35	1	40	39	40	0
mean			64				60				51				43				38				39	
%CV			2				3				3				3				8				1	
background subtracted			40				36				27				19				14				15	
% inhibition							10				33				54				64				62	

Table 1: Data for 6 runs are shown in which 3 sets of untreated RBC samples and 3 sets of 5 levels of donepezil fortified RBC samples were assayed in duplicate. Acceptance criteria (% CV for all levels of donepezil treatment must be equal to or less than 20.0%) was met, showing assay precision.

Conclusion

- Use of substrate coupled with 670 nm chromophore abrogates background noise, so no need to dilute RBC samples
- High assay reproducibility
- Samples can be re-assayed after freeze thaw cycles
- Demonstration of short-term stability in matrix
- Inhibition of AChE activity directly correlates to donepezil concentration

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