Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
Accessible at: www.pvj.com.pk

RESEARCH ARTICLE

A New Assay for Measurement of Acetylcholinesterase and Butyrylcholinesterase in Canine Whole Blood Combining Specific Substrates and Ethopropazine Hydrochloride as a Selective Butyrylcholinesterase Inhibitor

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ARTICLE HISTORY

Received: April 13, 2013
Revised: April 17, 2013
Accepted: April 24, 2013

Key words:
Acetylcholinesterase
Butyrylcholinesterase
Dog
Ethopropazine
Substrate
Whole blood

ABSTRACT

In the present report, a new assay combining specific substrates and a selective BChE inhibitor (ethopropazine hydrochloride) was used to measure both AChE and BChE in canine whole blood samples. Acetylthiocholine iodide (ATCI) and butyrylthiocholine iodide (BTCI) were used as substrates, whereas 2,2'-dithiodipiridine was used as chromophore. Ethopropazine concentration inhibiting over 95% BChE with minimum AChE inhibition was fixed at 0.3mM. The results confirmed that whole blood cholinesterase activity measured with BTCI in absence of ethopropazine corresponded with serum BChE, whereas whole blood cholinesterase analysed with ATCI in presence of ethopropazine reflected mainly erythrocytes and plasma AChE activity. This procedure showed good repeatability, it was easy and fast, and can be routinely used in veterinary laboratories.

To Cite This Article: Tecles F, A Tvarijonaviciute and JJ Cerón, 2013. A new assay for measurement of acetylcholinesterase and butyrylcholinesterase in canine whole blood combining specific substrates and ethopropazine hydrochloride as a selective butyrylcholinesterase inhibitor. Pak Vet J, 33(4): 458-461.

INTRODUCTION

Cholinesterase (ChE) includes a group of enzymes present in different organic tissues that hydrolyze choline esters. The main purpose of this enzyme activity is to hydrolyze the neurotransmitter acetylcholine in nervous tissue, although its function in other tissues such as blood is not well understood. Interest on blood cholinesterase determination started several years ago as a marker for anti-ChE insecticides exposure in humans (Jiang and Lockridge, 2013), livestock (Karanth and Pope, 2003) and wildlife (Santos *et al*., 2012). But more recently this enzyme has been studied in other disorders such as Alzheimer disease (García-Ayllón *et al*., 2010), inflammation (Das, 2007; Costa *et al*., 2012), cardiovascular disease (Calderon-Margalit *et al*., 2006), diabetes (Iwasaki *et al*., 2007) or liver cirrhosis (García-Ayllón et al 2012). Even ChE has been proposed to predict short-term outcome after hepatic resection for hepatocellular carcinoma (Donadon *et al*., 2013) and as a marker of oxidative stress in human lung cancer patients (Zanini *et al*., 2013). In dogs, ChE activity in blood seems to be influenced by obesity (Tvarijonaviciute *et al*., 2013).

There are at least two ChE isoenzymes in blood: acetylcholinesterase (AChE; EC 3.1.1.7), also known as erythrocyte or true ChE, which is found in red blood cells; and butyrylcholinesterase (BChE; EC 3.1.1.8), also known as plasma ChE or pseudo-ChE, which is present in blood serum (Das, 2007). Methods for their determination include spectrophotometric measurement in whole blood, plasma or erythrocytes. Whole blood is preferred by many authors (Harlin and Ross, 1990; Munro *et al*., 1991) since ChE activity can be measured more quickly and with less effort being not necessary to separate plasma and erythrocytes for individual monitorization of both isoenzymes (Meuling *et al*., 1992).

Using whole blood, some strategies can be used to avoid interference between isoenzymes determination: (a) Use of specific substrates for each enzyme. This procedure is supported by the existence of differences in substrate affinity. AChE hydrolyses acetylcholine and propionylcholine but not butyrylcholine, whereas BChE hydrolyses butyrylcholine at higher rate than acetyl and propionylcholine (Tecles and Cerón, 2001). This method has been satisfactorily applied in dogs, and it has demonstrated to be very simple, easy and useful in routine practice to monitor exposure to anti-ChE compounds (Tecles *et al*., 2000). However, the overlapping between isoenzymes affinity to substrates could mask subtle changes in whole blood AChE or BChE produced by other pathologies. (b) Use of specific inhibitors for one of the isoenzymes (i.e. ethopropazine hydrochloride, tetraisopropylpyrophosphoramide or phenothiazine derivatives are selective BChE inhibitors) (Reiner *et al*., 2004).

Both approaches have been applied for AChE and BChE measurement in whole blood by independent assays. The purpose of this paper was to develop an assay for AChE and BChE determination in canine whole blood by combining the use of specific substrates and the specific BChE inhibitor ethopropazine hydrochloride (Etho) that can be adapted to an automated analyzer.

MATERIALS AND METHODS

Reagents and apparatus: Acetylthiocholine iodide (ATCI), butyrylthiocholine iodide (BTCI), 2,2'-dithiodipyridine (2-PDS) and Etho were obtained from Sigma Chemical Co (St Louis, USA). Analyses were performed in a multiparametrical autoanalyzer (Olympus AU600, Olympus Diagnostica).

Sample preparation: Blood samples were obtained from five adult Beagle dogs owned to the University of Murcia. All dogs were apparently healthy after physical examination and free of organophosphate or carbamate compound exposure in the previous five months. Blood samples were collected by cephalic venipuncture and placed in tubes containing ethilenediaminetetraacetic acid (EDTA) as anticoagulant (BD Vacutainer). All haematocrit ranged between 37-45% (mean 40.20± 3.42%). The procedure involving animals were approved by the Murcia University Ethics Committee.

Following the guidelines of Cerón *et al*. (1999) whole blood dilutions from each sample were prepared at 1:50 ratio with distilled water. Rest of blood was then centrifuged at 1225g for 10 minutes to separate plasma and erythrocytes. Red blood cells were washed with saline three times and then diluted at 1:100 ratio using distilled water. Plasma was diluted in distilled water at 1:50 before analysis.

Method for ChE analysis: To determine the best Etho concentration, three whole blood, erythrocyte and plasma dilutions from three different dogs were used for this analysis. Each whole blood, erythrocyte and plasma dilution was divided in 6 aliquots and incubated at room temperature with the following final concentrations of Etho: 0, 0.05, 0.1, 0.2, 0.3 and 0.5mM. After 30 minutes of incubation, samples were analysed for ChE activity.

ChE in whole blood, erythrocyte and plasma dilutions was analysed by the automated method previously described (Cerón *et al*., 1996) and adapted for an Olympus AU600 autoanalyzer. The temperature of analyses was 37ºC. Final substrate concentration was $1x10^{-3}$ M. 2-PDS was used as chromophore. In all samples, the activity of a blank containing buffer-chromophore and substrate, and another blank consisting of sample and buffer-chromophore were calculated and subtracted from final activity. Cholinesterase activity was expressed as µmol of substrate hydrolysed/mL sample/ min.

Blood obtained from 5 healthy dogs was processed as above to obtain whole blood, erythrocytes and plasma dilutions. Then ChE was analyzed as follows: (1) two aliquots of each sample were prepared; (2) one aliquot was incubated during 30 minutes with Etho at a selected concentration (aliquot A), whereas the second aliquot was incubated with similar volume of distilled water (aliquot B); (3) ChE was measured in: a) whole blood aliquots A and B using ATCI and BTCI as substrates, respectively, b) in erythrocytes of the aliquots A and B using only ATCI, and c) in plasma aliquots A and B using only BTCI as substrate.

Suitability of the assay in whole blood was assessed by comparing results with those obtained separately in erythrocytes analysed with ATCI (corresponding with erythrocytes AChE) and in plasma analysed with ATCI (equivalent to plasma AChE) and with BTCI (equivalent to plasma BChE). Results obtained in erythrocytes and plasma was corrected by PCV in order to be compared with whole blood.

To assess repeatability of the assay, three whole blood samples from different animals were diluted in distilled water (1:50 dilution ratio). Whole blood dilutions from each dog were divided in six aliquots and AChE and BChE activities were analysed in each aliquot as described previously. All analyses were performed in the same batch to avoid any other interference.

Statistical analysis: Arithmetic means and standard deviations were calculated using routine descriptive statistical procedures. To assess differences between Etho concentrations a Kolmogorov–Smirnov test was used to assess normality of data, giving a nonparametric distribution. Normality was assumed by logarithmic transformation and 2-way ANOVA of repeated measures with Bonferroni post-test was performed. For the precision study, nested ANOVA was used to estimate the interindividual, intraindividual, and analytical components of variance (S^2_{inter}, S^2_{intra}, and S^2_{anal}, respectively). CV was calculated as $\sqrt{S^2_{anal}}$/arithmetic mean * 100. A p value < 0.05 was considered statistically significant. Graph Pad (Graph Pad Software Inc., La Jolla, CA) was used as statistical software for calculations.

RESULTS

Figure 1 shows the magnitude of inhibition observed in ChE activities in whole blood, erythrocytes and plasma after incubation with different Etho concentrations.

Results obtained in whole blood samples varied according with the substrate: when BTCI was used as substrate a significant inhibition of 80% was observed at the lowest Etho concentration (P<0.001), achieving over 95% with Etho concentration equal or higher than 0.3mM. ChE activity in erythrocytes measured with ATCI was not significantly affected by Etho. Plasma ChE measured with BTCI showed a significant inhibition even at lowest Etho concentration (P<0.01), achieving over 95% inhibition with Etho concentration equal or higher than 0.3mM (P<0.001).

Based on these results, Etho concentration that achieved maximum plasma BChE inhibition with minimal influence on erythrocyte AChE was fixed at 0.3mM.

Table 1 compares results obtained in whole blood with those obtained separately in erythrocytes and plasma

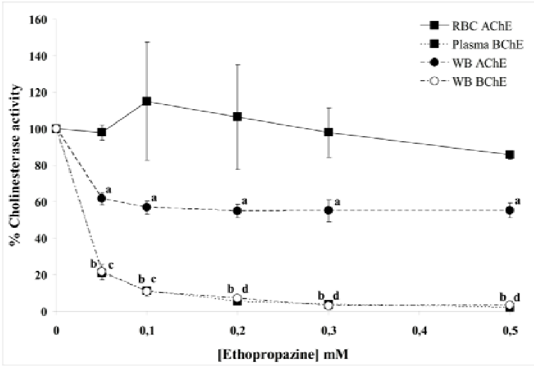

Fig. 1: Percent of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) activities obtained in whole blood (WB), erythrocytes (RBC) and plasma incubated with increasing Ethopropazine concentrations. Statistical analysis for WB AChE: a=P<0.001; statistical analysis for WB BChE: b=P<0.001; statistical analysis for Plasma BChE: c=P<0.01 and d=P<0.001.

Table 1: Acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) activities measured in whole blood and compared with erythrocytes AChE and plasma AChE and BChE (corrected by PCV). Results are given in µmol of substrate hydrolyzed/mL/min.

Measured ChE activity	N	Substrate	[Etho]	Activity (Range)
Whole blood AChE	5	ATCI	0.3mM	1.06 (0.87-1.31)
Whole blood BChE	5	BTCI	0µM	1.81 (0.48-2.54)
RBC AChE (PCV corrected)	5	ATCI	0.3mM	0.68 (0.44-1.34)
Plasma AChE (PCV corrected)	5	ATCI	0.3mM	0.22 (0.14-0.32)
Plasma BChE (PCV corrected)	5	BTCI	0µM	1.84 (0.90-2.80)

Table 2: Mean, S^2_{anal} and CV (%) of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) measurements in three whole blood samples. ChE activity is expressed in µmol substrate hydrolyzed/mL/min.

		ChE activity (SD)	Mean	S^2_{anal}	CV
AChE	Sample 1	0.87 (0.03)	1.00	5.68×10^{-4}	2.38%
	Sample 2	0.99 (0.03)			
	Sample 3	1.15 (0.03)			
BChE	Sample 1	0.52 (0.02)	1.79	172.51×10^{-4}	7.34%
	Sample 2	2.26 (0.08)			
	Sample 3	2.59 (0.22)			

after PCV correction. In presence of Etho, AChE activity observed in whole blood was very close to the sum of AChE observed in erythrocytes and plasma. In absence of Etho, BChE activity measured in whole blood was similar to that observed in plasma.

Table 2 shows the precision of AChE and BChE measurement in whole blood using the new assay which was assessed analysing three whole blood samples from different dogs. CVs were lower than 8%, indicating a good reproducibility of the assay.

DISCUSSION

Several reports regarding ChE activity determination can be found in literature. Traditionally this enzyme has been used to detect exposure to organophosphates and carbamates insecticides due to their action as ChE inhibitors (Dass *et al*., 1994). Since both serum BChE and erythrocyte AChE can be selectively inhibited by some insecticides, determination of both isoenzymes is indicated. But in recent years possible relationship between ChE and other pathologies has been reported. Serum BChE has been proposed as a marker of low-grade systemic inflammation (Das, 2007) and as a marker of cardiovascular risk factor being even capable to predict mortality (Calderon-Margalit *et al*., 2006; Ben Assayag *et al*., 2010). Serum BChE activity can be depressed by acute starvation in mice (Morris *et al*., 2011) and by chronic stress in rats (Tagliari *et al*., 2010). Blood ChE activities could be also affected with lipoprotein levels in obese humans and with degree of insulin resistance (Iwasaki *et al*., 2007). Serum BChE activity is increased in overweight dogs and correlate with other physical and biochemical markers of obesity (Tvarijonaviciute *et al*., 2010; Tvarijonaviciute *et al*., 2013). Moreover, the effects of these disorders in ChE isoenzymes could be different. For example, serum AChE but not BChE is increased in human Alzheimer's disease patients (García-Ayllón *et al*., 2010), and different response for erythrocyte AChE and serum BChE in obese dogs was observed, indicating that both isoenzymes could have different role in obese canine patients (Tvarijonaviciute *et al*., 2013).

The use of whole blood allows the measurement of both isoenzymes (AChE and BChE) without the necessity to separate plasma and erythrocytes. Reiner *et al*. (2004) used Etho as a selective BChE inhibitor and ATCI as substrate achieving satisfactory results. But in author's opinion the use of specific substrates would reflect the activity of each isoenzyme better than using only a unique substrate. No other reports combining Etho as a selective BChE inhibitor and specific substrates for simultaneous AChE and BChE measurement have been found in literature.

Etho is a selective BChE inhibitor (Tasso et al., 2011). It was chosen based on the results obtained by Naik *et al*. (2008), who studied the in vitro effect of different ChE inhibitors in purified human isoenzymes. These authors concluded that Etho at 20µM provided almost completely BChE inhibition with minimum effect on AChE (maximum inhibition was 10%). Other inhibitors traditionally used for this purpose such as tetraisopropylpyrophosphoramide produced higher influence in AChE. Our assays performed in erythrocytes and plasma dilutions demonstrated that Etho inhibits only BChE and AChE was not significantly affected. The best Etho concentration was fixed at 0.3mM, being much higher than reported by Naik *et al*. (2008). This fact could be related with the use of blood dilutions instead of whole blood or maybe with a higher resistance of canine ChE inhibition to Etho exposure. It is important to note that 45% inhibition was observed in whole blood when ATCI was used as substrate. Since BChE hydrolyzes also this substrate, this rate of inhibition after Etho exposure can be explained by the inhibition of BChE that cannot hydrolyze ATCI.

The new assay for AChE and BChE measurement in whole blood samples was established in three steps: (1) whole blood was diluted with distilled water at 1:50 ratio and dilution is separated in two aliquots; (2) one aliquot was incubated during 30 min with Etho at a final concentration of 0.3mM to inhibit serum BChE, then is analysed with ATCI as substrate (measuring only erythrocyte AChE activity); (3) the second aliquot was incubated with the same volume of diluent and analysed with BTCI as substrate (measuring only plasma BChE activity). Our results demonstrated that whole blood can be easily used for both isoenzyme determinations. AChE in whole blood (measured in presence of Etho and using ATCI as substrate) reflects combination between erythrocyte AChE and plasma AChE. BChE activity (measured with BTCI as substrate in absence of Etho) is equivalent to BChE obtained in plasma. The procedure showed good repeatability, it was easy and cheap, it can be adapted to an automated analyzer and used in routine practice.

It is important to note that AChE can be detected in plasma samples after inhibition with Etho. In this situation, BChE was almost completely inhibited (activity was 0.13 µmol/ml/min, range 0.04-0.23); and a low AChE activity was detected by using ATCI as substrate (0.38 µmol/ml/min, range 0.23-0.53). This low AChE activity seemed to be too low to have any relevant significance on total whole blood AChE. However, this low serum AChE activity could have clinical relevance as it has been found in humans affected by Alzheimer's disease (García-Ayllón *et al*., 2010). Although these authors postulated that immunodepletion of BChE in serum samples is needed to measure AChE in serum samples (García-Ayllón *et al*., 2010), the assay described in this paper could be also used to measure the low AChE activity present in plasma by just using plasma instead of whole blood.

Conclusion: This study demonstrated that ethopropazine hydrochloride can be used as a selective inhibitor of plasma BChE activity in dogs, and therefore can be used for a selective measurement of erythrocyte AChE and plasma BChE in canine whole blood samples using specific substrates for each isoenzyme. This procedure avoids centrifugation to separate plasma and erythrocytes, and the use of specific substrates reflects better each isoenzyme activity than using only one substrate. The method showed good reproducibility and could be routinely applied in laboratory dealings. Finally, this assay can be adapted to plasma samples for AChE measurements.

REFERENCES

Ben Assayag E, S Shenhar-Tsarfaty, K Ofek, L Soreq, I Bova, L Shopin, RM Berg, S Berliner, I Shapira, NM Bornstein and H Soreq, 2010. Serum cholinesterase activities distinguish between stroke patients and controls and predict 12-month mortality. Mol Med, 16: 278-286.

Calderon-Margalit R, B Adler, JH Abramson, J Gofin and JD Kark, 2006. Butyrylcholinesterase activity, cardiovascular risk factors and mortality in middle-aged and elderly men and women in Jerusalem. Clin Chem, 52: 845-852.

Cerón JJ, MJ Fernández, LJ Bernal and C Gutiérrez, 1996. Automated spectrophotometric method using 2, 2'-dithiodipyridine acid for determination of cholinesterase in whole blood. JAOAC Int, 79: 757-763.

Cerón, F Tecles and JC Espín, 1999. Comparison of different diluents and chromophores for spectrophotomeric determination of livestock blood cholinesterase activity. Res Vet Sci, 67: 261-266.

Costa MM, AS Silva, FC Paim, R França, GL Dornelles, GR Thomé, JD Serres, R Schmatz, RM Spanevello, JF Gonçalves, MR Schetinger, CM Mazzanti, ST Lopes and SG Monteiro, 2012. Cholinesterase as inflammatory markers in a experimental infection by Trypanosoma evansi in rabbits. An Acad Bras Cienc, 84: 1105-1113.

Das UN, 2007. Acetylcholinesterase and butyrylcholinesterase as possible markers of low-grade systemic inflammation. Med Sci Monit, 13: 214-221.

Dass P, M Mejia, M Landas, R Jones, B Stuart and J Thyssen, 1994. Cholinesterase: Review of methods. Clin Chem, 34: 135-157.

Donadon M, M Cimino, F Procopio, E Morenghi, M Montorsi and G Torzilli, 2013. Potential role of cholinesterases to predict short-term outcome after hepatic resection for hepatocellular carcinoma. Updates Surg, 65: 11-18.

García-Ayllón MS, I Riba-Llena, C Serra-Basante, J Alom, R Boopathy and J Sáez-Valero, 2010. Altered levels of acetylcholinesterase in Alzheimer plasma. PLoS One, 14: e8701.

García-Ayllón MS, C Millán, C Serra-Basante, R Bataller and J Sáez-Valero, 2012. Readthrough acetylcholinesterase is increased in human liver cirrhosis. PLoS One, 7: e44598.

Harlin KS and PF Ross, 1990. Enzymatic-spectrophotometric method for determination of cholinesterase activity in whole blood: collaborative study. JAOAC Int, 73: 616–619.

Iwasaki T, M Yoneda, A Nakajima and Y Terauchi, 2007. Serum butyrylcholinesterase is strongly associated with adiposity, the serum lipid profile and insulin resistance. Intern Med, 46: 1633-1639.

Jiang W and O Lockridge, 2013. Detectable organophosphorus pesticide exposure in the blood of Nebraska and Iowa residents measured by mass spectrometry of butyrylcholinesterase adducts. Chem Biol Interact, 203: 91-95.

Karanth S and C Pope, 2003. In vitro inhibition of blood cholinesterase activities from horse, cow, and rat by tetrachlorvinphos, Int J Toxicol, 22: 429-433.

Meuling WJA, MJM Jongen, H Emmen and JJ Van, 1992. An automated method for the determination of acetyl and pseudo cholinesterase in hemolized whole blood. Am J Indus Med, 22: 231–241.

Morris HJ, OV Carrillo, G Llauradó, ME Alonso, RC Bermúdez, Y Lebeque, R Fontaine, NE Soria and G Venet, 2011. Effect of starvation and refeeding on biochemical and immunological status of Balb/c mice; an experimental model of malnutrition. Immunopharmacol Immunotoxicol, 33: 438-446.

Munro NB, LR Shugart, AP Watson and RS Halbrook, 1991. Cholinesterase activity in domestic animals as a potential biomonitoring for nerve agent and other organophosphate exposure. J Am Vet Med Assoc, 199: 103–115.

Naik RS, BP Doctor and A Saxena, 2008. Comparison of methods used for the determination of cholinesterase activity in whole blood. Chem Biol Interact, 175: 298-302.

Reiner E, A Bosak and V Simeon-Rudolf, 2004. Activity of cholinesterases in human whole blood measured with acetylthiocholine as substrate and ethopropazine as selective inhibitor of plasma cholinesterase. Arh Hig Rada Toksikol, 55: 1-4.

Santos CS, MS Monteiro, AM Soares and S Loureiro, 2012. Characterization of cholinesterases in plasma of three Portuguese native bird species: application to biomonitoring. PLoS One, 7: e33975.

Tagliari B, TM dos Santos, AA Cunha, DD Lima, D Delwing, A Sitta, CR Vargas, C Dalmaz and AT Wyse, 2010. Chronic variable stress induces oxidative stress and decreases butyrylcholinesterase activity in blood of rats. J Neural Transm, 117: 1067-1076.

Tasso B, M Catto, O Nicolotti, F Novelli, M Tonelli, I Giangreco, L Pisani, A Sparatore, V Boido, A Carotti and F Sparatore, 2011. Quinolizidinyl derivatives of bi- and tricyclic systems as potent inhibitors of acetyl- andbutyrylcholinesterase with potential in Alzheimer's disease. Eur J Med Chem, 46: 2170-2184.

Tecles F, S Ubiela, S Martínez, L Bernal and JJ Cerón, 2000. Use of whole blood for spectrophotometric determination of cholinesterase activity in dogs. Vet J, 160: 242–249.

Tecles F and JJ Cerón, 2001. Determination of whole blood cholinesterase in different animal species using specific substrates. Res Vet Sci, 70: 233-238.

Tvarijonaviciute A, F Tecles and JJ Cerón, 2010. Relationship between serum butyrylcholinesterase and obesity in dogs: A preliminary report. Vet J, 186: 197-200.

Tvarijonaviciute A, JJ Cerón and F Tecles, 2013. Acetylcholinesterase and butyrylcholinesterase activities in obese Beagle dogs before and after weight loss. Vet Clin Pathol, doi: 10.1111/vcp.12032. [Epub ahead of print]

Zanini D, R Schmatz, LP Pelinson, VC Pimentel, P da Costa, AM Cardoso, CC Martins, CC Schetinger, J Baldissareli, M do Carmo Araújo, L Oliveira, J Chiesa, VM Morsch, DB Leal and MR Schetinger, 2013. Ectoenzymes and cholinesterase activity and biomarkers of oxidative stress in patients with lung cancer. Mol Cell Biochem, 374: 137-148.