Journal of Coastal Life Medicine

journal homepage: www.jclmm.com

Original article doi:10.12980/JCLM.3.2015J5-11

Effect of ions on the activity of brain acetylcholinesterase from tropical fish

Caio Rodrigo Dias Assis[1*], Amanda Guedes Linhares[1], Vagne Melo Oliveira[1], Renata Cristina Penha França[1], Juliana Ferreira Santos[2], Elba Verônica Matoso Maciel Carvalho[3], Ranilson Souza Bezerra[1], Luiz Bezerra Carvalho Jr[1]

[1]*Laboratory of Enzymology-LABENZ, Biochemistry Department and Laboratory of Immunopathology Keizo Asami, Federal University of Pernambuco, Recife-PE, Brazil*

[2]*Academic Unit of Serra Talhada, Federal Rural University of Pernambuco, Serra Talhada-PE, Brazil*

[3]*Laboratory of Glycoproteins, Biochemistry Department, Federal University of Pernambuco, Recife-PE, Brazil*

ARTICLE INFO

Article history:
Received 9 Feb 2015
Received in revised form 28 Feb 2015
Accepted 22 Apr 2015
Available online 9 Jun 2015

Keywords:
Ions
Acetylcholinesterase
Biomarkers
Fish

ABSTRACT

Objective: To investigate the effect of ions on brain acetylcholinesterase (AChE; EC 3.1.1.7) activities from economic important fish [pirarucu, *Arapaima gigas*; tambaqui, *Colossoma macropomum*; cobia, *Rachycentron canadum* (*R. canadum*) and Nile tilapia, *Oreochromis niloticus* (*O. niloticus*)] comparing with a commercial enzyme from electric eel [*Electrophorus electricus* (*E. electricus*)].

Methods: The *in vitro* exposure was performed at concentrations ranging from 0.001 to 10 mmol/L (except for ethylene diamine tetraacetic acid; up to 150 mmol/L). Inhibition kinetics on *R. canadum* and *O. niloticus* were also observed through four methods (Michaelis-Menten, Lineweaver-Burk, Dixon and Cornish-Bowden plots) in order to investigate the type of inhibition produced by some ions.

Results: Hg^{2+}, As^{3+}, Cu^{2+}, Zn^{2+}, Cd^{2+} caused inhibition in all the species under study. Ca^{2+}, Mg^{2+} and Mn^{2+} induced slight activation in *R. canadum* enzyme while Pb^{2+}, Ba^{2+}, Fe^{2+}, Li^{+} inhibited the AChE from some of the analyzed species. The lowest IC_{50} and Ki values were estimated for *E. electricus* AChE in presence of Hg^{2+}, Pb^{2+}, Zn^{2+}. Under our experimental conditions, the results for *R. canadum* and *O. niloticus*, As^{3+}, Cu^{2+}, Cd^{2+}, Pb^{2+} and Zn^{2+} showed a non-competitive/mixed-type inhibition, while Hg^{2+} inhibited the enzyme in a mixed/competitive-like manner.

Conclusions: *E. electricus* AChE activity was affected by ten of fifteen ions under study showing that this enzyme could undergo interference by these ions when used as pesticide biosensor in environmental analysis. This hindrance would be less relevant for the crude extracts.

1. Introduction

Acetylcholinesterase (AChE, EC 3.1.1.7) is a crucial enzyme for the development and functioning of the nervous system and play an important role in hematopoietic differentiation and neural development[1]. Its classical function is to modulate the nerve impulse through the hydrolysis of the neurotransmitter acetylcholine in the synaptic cleft[2]. AChE inhibition is the mechanism of action of organophosphorus and carbamate pesticides, as well as the mode of action of the drugs used in treatment of Alzheimer's disease[2]. Therefore, AChE has been also used for monitoring these pesticide exposures *in vivo*[3] and *in vitro*[4] and even as a biocomponent of biosensors[5].

The investigation of AChE inhibitors and interfering substances is relevant to identify the usefulness of this enzyme as a tool in environmental and food monitoring[5-7]. Monitoring at biochemical level can specifically detect the presence of contaminants in the environment before they reach higher organizational levels[8].

Several studies reported inhibition of AChE activity by ions[9-11]. AChE activation by Ca^{2+}, Mg^{2+}, Al^{3+} has also been reported[12,13]. Therefore, high content of these ions in water samples from rivers, lakes and other environments can influence the detection of anticholinesterasic pesticides. These findings must be taken into account when biosensors based on AChE activity are proposed to analyze pesticide presence under some environment conditions. This fact can lead to false positives or negatives and misinterpretations in the analysis of results. Cholinesterase inhibition has been assayed in several species, including aquatic organisms, since the event effectively

*Corresponding author: Caio Rodrigo Dias de Assis, Laboratory of Enzymology-LABENZ, Biochemistry Department and Laboratory of Immunopathology Keizo Asami, Federal University of Pernambuco, Recife-PE, Brazil.
Tel: + 55 81 21268540
Fax: + 55 81 21268576
E-mail: caiodias2@hotmail.com

Foundaton Project: Supported by Fundação de Apoio à Ciência e Tecnologia do Estado de Pernambuco (FACEPE) for financial support (Grant numbers: IBPG-1301-2.08/08 FACEPE, IBPG-0523-2.08/11 FACEPE, BFP-0036-2.08/13 FACEPE and BFP-0111-2.08/13).

mirrors environmental impact even when these compounds are not present in the water due to the fact that they frequently remain attached to the enzyme.

This study investigated the effect of different ions (Al^{3+}, As^{3+}, Ba^{2+}, Ca^{2+}, Cd^{2+}, Cu^{2+}, $EDTA^{2-}$, Hg^{2+}, K^{+}, Li^{+}, Fe^{2+}, Mg^{2+}, Mn^{2+}, Pb^{2+} and Zn^{2+}) that could influence/interfere on the activity of brain AChE from three freshwater species of economic importance in aquaculture: Nile tilapia [*Oreochromis niloticus* (*O. niloticus*)], tambaqui [*Colossoma macropomum* (*C. macropomum*)], pirarucu [(*Arapaima gigas* (*A. gigas*)]; one saltwater farmed species: cobia [*Rachycentron canadum* (*R. canadum*)] and a commercial enzyme from electric eel [*Electrophorus electricus* (*E. electricus*)], providing information about their inhibitory behaviour and their potential interference in the use of AChE from these species as a biomarker for the presence of anticholinesterase compounds. In our previous studies, AChE from the same species was physicochemical and kinetically characterized and used to investigate the effect of organophosphorus and carbamate pesticides showing sensitivity comparable to a commercial and purified enzyme[14].

2. Materials and methods

2.1. Materials

AChE from electric eel *E. electricus* type VI-S, Acetylthiocholine iodide, bovine serum albumin, 5,5'-dithiobis(2-nitrobenzoic) acid (DTNB), tris (hydroxymethyl) aminomethane e magnesium sulphate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Hydrogen chloride, aluminium chloride, barium chloride, calcium chloride, lithium chloride and sodium arsenite were obtained from Merck (Darmstadt, Germany). Cadmium chloride, copper chloride, ferrous chloride, manganese chloride, lead chloride and zinc chloride were acquired from Vetec (Rio de Janeiro, Brazil). Disodium EDTA, mercuric chloride and potassium chloride were from Reagen (Rio de Janeiro, Brazil). The microplate spectrophotometer used was Bio-Rad xMark™ (Hercules, CA, USA) whereas the tissue disrupter was IKA RW-20 digital (Staufen, Germany). The juvenile specimens of *C. macropomum* [(30.0 ± 4.2) cm; (512.5 ± 123.7) g], *A. gigas* [(76.8 ± 8.7) cm; (4 118.0 ± 207.9) g] and *O. niloticus* [(12.0 ± 3.0) cm; (7.9 ± 1.2) g] were supplied by the Department of Fisheries and Aquaculture of the Universidade Federal Rural de Pernambuco (Recife, PE, Brazil). *R. canadum* [(51.67 ± 1.50) cm; (1 575.0 ± 329.6) g] was supplied by Aqualider Ltda. (Recife, PE, Brazil).

2.2. Enzyme extraction

The juvenile fishes were cultured under appropriate conditions and were sacrificed in ice bath (0 °C). The whole brains were immediately removed, pooled (from 5 per pool for *R. canadum* to 30 per pool for *O. niloticus*) and homogenized in 0.5 mol/L Tris-HCl buffer, pH 8.0, maintaining a ratio of 20 mg of tissue per mL of buffer. The homogenates were centrifuged for 10 min at 3 320 r/min (4 °C) and the supernatants (crude extracts) were frozen at -20 °C for further assays.

2.3. Enzyme activity and protein determination

Enzyme activity was evaluated using an adaptation of Ellman's method according to Assis *et al.*[14]. Briefly, 0.25 mmol/L DTNB (200 μL) prepared in 0.5 mol/L Tris-HCl buffer pH 7.4 was added to the crude extract (20 μL), and the reaction started by the addition of 62 mmol/L acetylthiocholine iodide (20 μL) except for the *C. macropomum* assays (125 mmol/L). Enzyme activity was determined by reading the absorbance increase at 405 nm for 180 seconds. A unit of activity (IU) was defined as the amount of enzyme capable of converting 1 μmol/L of substrate per min. A blank was prepared with the buffer instead crude extract sample. Protein content was estimated according to Sedmak and Grossberg[15], using bovine serum albumin as the standard.

2.4. Activity in presence of ions

AChE activity was assayed at 25 °C in presence of fifteen ions: Al^{3+} ($AlCl_3$), Ba^{2+} ($BaCl_2$), Ca^{2+} ($CaCl_2$), Cd^{2+} ($CdCl_2$), Cu^{2+} ($CuCl_2$ and $CuSO_4$), Fe^{3+} ($FeCl_3$), Hg^{2+} ($HgCl_2$), K^{+} (KCl), Li^{+} (LiCl), Mg^{2+} ($MgSO_4$), Mn^{2+} ($MnCl_2$), As^{3+} ($NaAsO_2$), Pb^{2+} ($PbCl_2$ and $Pb(C_2H_3O_2)_2$), Zn^{2+} ($ZnCl_2$) and the complex chelating ion $EDTA^{2-}$ as $C_{10}H_{14}N_2Na_2O_8$. The ions were diluted to five concentrations ranging from 0.001 to 10 mmol/L (each concentration 10-fold higher than the previous one) excepting $EDTA^{2-}$ which was assayed in concentrations up to 150 mmol/L. The ions solutions (10 μL) were incubated with crude extract (10 μL) for 40 min[6]. In order to minimize false negatives through thiobis-nitrobenzoate (TNB) and thiocholine reactions with some inhibitory ions, the incubation were performed only with the ions and the enzymatic extract and the blanks were performed with buffer instead of enzymatic preparation subtracting these interferences and spontaneous substrate hydrolysis. After the incubation, DTNB (200 μL) was added right before the substrate acetylthiocholine (20 μL) and the mixture was read at 405 nm for 180 second. The controls were performed with distilled water in the incubation instead of the ions solutions. The activity in the absence of the ions was considered as 100%.

Some assays were also carried out with activator ions in order to verify false positive occurrence by an eventual binding to DTNB: before DTNB and substrate addition, 10 μL of the samples were incubated for 40 min with 10 μL of 10 mmol/L neostigmine bromide (a total cholinesterase inhibitor) and with 10 μL of each of these ions (10 mmol/L). Blanks were performed replacing the samples by buffer and following the same procedure.

2.5. Inhibition kinetics

Samples of *O. niloticus* and *R. canadum* preparations were incubated with the most inhibitory ions (As^{3+}, Cu^{2+}, Cd^{2+}, Hg^{2+}, Pb^{2+} and Zn^{2+}) at six concentrations (0 to 10 mmol/L) and hyperbola model curves were produced with fourteen substrate concentrations ranging from 0 to 20.83 mmol/L to obtain the kinetic parameters in presence or absence of ions (k_{mapp}, V_{mapp} and k_m, V_{max}, respectively). Then, data were transformed to double reciprocal (1/*v vs* 1/*s*), Dixon (1/*v vs i*) and Cornish-Bowden (*s*/*v vs i*) plots in order to investigate the kinetic behaviour of the ions towards AChE and to distinguish unambiguously the types of inhibition[16,17].

The dissociation constant of the enzyme-inhibitor complex (ki) was estimated for competitive, mixed and non-competitive inhibitor ions using the intersection of linear regression curves from different concentrations of substrates in the Dixon plots[17] and also using Cheng and Prusoff equation[18]:

$$K_i = \frac{IC_{50}}{1 + \frac{[S]}{k_m}}$$

Where IC_{50} is the concentration capable of inhibiting 50% of enzyme activity. [S] represents substrate concentration and k_m is the Michaelis-Menten constant.

The dissociation constant of the enzyme-substrate-inhibitor ternary complex (k'_i) was estimated for non-competitive and mixed inhibitors

from the intersection of linear regression curves generated from different concentrations of substrates in the Cornish-Bowden plots[16].

2.6. Statistical analysis

In the previous sections, means of treatments and means of kinetic parameters in presence or absence of inhibitory ions were statistically analyzed using One-way ANOVA followed by Tukey's test. In section 2.4., data were fitted to linear and non-linear regression through sigmoidal (Boltzmann) or exponential decay ($P < 0.05$) modelling using MicroCal® Origin® version 8.0 in order to estimate the concentration capable to inhibit enzyme activity in 50% (IC_{50}).

3. Results

3.1. Activity in presence of ions

Table 1 reports the results referring to 1 mmol/L concentration of ions. Activation effect was only observed for the ions Mg^{2+} (13%) and Mn^{2+} (38%) on the *R. canadum* AChE. Ca^{2+} induced an increase of approximately 30% in *R. canadum* AChE activity at 10 mmol/L (data not shown). The inhibitions found here for Cu^{2+} and Zn^{2+} at 1 mmol/L were, respectively, 75% and 23% (*R. canadum*), 75% and 78% (*E. electricus*). Cu^{2+} inhibited (*A. gigas*) 23% of enzymatic activity, behaviour not induced by Zn^{2+} in this species. Zn^{2+} induced 35 (*C. macropomum*) and 29% (*O. niloticus*) inhibition. At 1 mmol/L, Pb^{2+} was able to inhibit the enzyme from *A. gigas* (32%), *R. canadum* (15%), *E. electricus* (71%). Cadmium induced inhibitions of 33% (*R. canadum*), 49% (*E. electricus*) and 35% (*O. niloticus*). As^{3+} inhibited *C. macropomum* (57%), *R. canadum* (63%), *E. electricus* (57%) and *O. niloticus* (61%) enzyme activities at 1 mmol/L. Ba^{2+}, Fe^{2+} and Li^{+} induced, under our experimental conditions, similar pattern (Table 1) and only *E. electricus* was significantly sensitive to these ions at 1 mmol/L. The chelating ion $EDTA^{2-}$ only inhibited *R. canadum* (6%) and *E. electricus* (28%) at 1 mmol/L. The enzymes from the other species under study were significantly inhibited only on the range of 50-100 mmol/L by this ion.

Among the fifteen ions analyzed, the most inhibitory ion was Hg^{2+}, which completely inactivated AChE from all the species under study when they were exposed to 1 mmol/L. However, the enzyme activity from *A. gigas* was less inhibited (71%) than the others.

No statistical difference was observed between activator ions (Al^{3+}, Ca^{2+}, Mg^{2+}, Mn^{2+} and K^{+}) action on brain AChE from *O. niloticus*, *C. macropomum*, *A. gigas* and *R. canadum* incubated with neostigmine bromide and their respective blanks in order to investigate interferences in the colorimetric readings by them.

Figure 1 displays an example of typical inhibitions plots showing the effect of Hg^{2+} ion on AChE of the species under study and from which were estimated the IC_{50} values for this ion as well as all other ions.

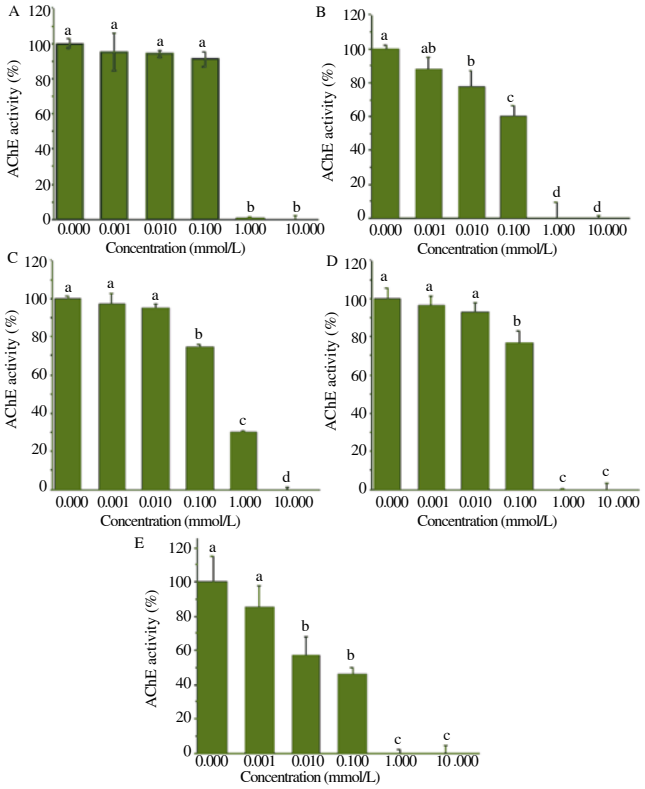

Figure 1. AChE activity from five species exposed to Hg^{2+}.
A: *O. niloticus*; B: *C. macropomum*; C: *A. gigas*; D: *R. canadum*; E: *E. electricus*; Data were compared using ANOVA and Tukey's test ($P < 0.05$).

3.2. Inhibition kinetics

Table 1 also shows the IC_{50} related to the ions towards the species under study. *E. electricus* was the most sensitive species presenting the lowest values for Cu^{2+}, Hg^{2+}, Pb^{2+}, Zn^{2+} and was the only species here to present this parameter for Ba^{2+}, $EDTA^{2-}$, Fe^{2+} and Li^{+}. *C. macropomum* and *R. canadum* presented low IC_{50} values for As^{3+} and

Table 1
Inhibition and IC_{50} of AChE activity by ions and heavy metals from several freshwater and marine species at 1 mmol/L.

Species	*O. niloticus*		*C. macropomum*		*A. gigas*		*E. electricus*		*R. canadum*	
	Inhibition (%)	IC_{50} (mmol/L)	Inhibition (%)	IC_{50} (mmol/L)	Inhibition (%)	IC_{50} (mmol/L)	Inhibition (%)	IC_{50} (mmol/L)	Inhibition (%)	IC_{50} (mmol/L)
Al^{3+}	ne	-	ne	-	ne	-	ne	-	13	-
As^{3+}	61	0.58	57	0.32	ne	-	57	0.98	63	0.21
Ba^{2+}	ne	-	25	-	ne	-	60	0.05	ne	-
Cd^{2+}	35	-	ne	6.30	ne	-	50	1.00	33	1.10
Cu^{2+}	ne	-	ne	4.13	23	5.77	75	0.05	75	0.37
$EDTA^{2-}$	ne	-	ne	-	ne	-	28	21.25	6	-
Fe^{2+}	ne	-	20	-	15	-	43	1.16	ne	-
Hg^{2+}	100	0.24	100	0.13	71	0.38	100	0.01	100	0.12
K^{+}	ne	-	ne	-	ne	-	26	-	ne	-
Li^{+}	ne	-	ne	-	ne	-	57	0.38	ne	-
Mn^{2+}	ne	-	ne	-	ne	-	24	-	38*	-
Pb^{2+}	ne	-	ne	-	32	-	71	0.01	15	-
Zn^{2+}	23		35		ne		78		23	

ne: No effect at 1 mmol/L ($P < 0.05$); –: No IC_{50} estimated at 1 mmol/L; *: Activation.

Hg^{2+} whereas brain AChE activity from *A. gigas* was less affected by the ions. The ki values using Cheng and Prusoff equation[18] followed the same trend on Table 2 where the lowest values occurred with *E. electricus* exposed to Hg^{2+}, Pb^{2+} and Zn^{2+}. From now on the results are related to only two species: *R. canadum* and *O. niloticus*. The other species behaved similarly.

Tables 3 and 4 show kinetic parameters k_m, V_{max} and their inhibited analogues k_{mapp} and V_{mapp} based on hyperbola model for brain AChE activity from *R. canadum* and *O. niloticus*. Using this model, Hg^{2+} showed a competitive-like trend of behaviour for both species. Cu^{2+} and Cd^{2+} presented non-competitive inhibition whereas As^{3+}, Pb^{2+} and Zn^{2+} showed mixed-type inhibition.

Figures 2 and 3 allow comparison between inhibitory effect of ions on *R. canadum* and *O. niloticus* brain AChE activity using Lineweaver-Burk regression plots. These results corroborate Tables 3 and 4 in relation to As^{3+}, Pb^{2+} and Zn^{2+}. However, Cd^{2+}, Cu^{2+} and Hg^{2+} ions showed mixed-type inhibition according to Lineweaver-Burk plots. The behaviour was similar between both species, excepting Cu^{2+}.

Figures 4-7 present a comparison between Dixon and Cornish-Bowden regression plots in which the types of inhibitory effects were confirmed by both graphical methods. In these figures, Hg^{2+} presents a competitive-like inhibitory effect. Table 5 provides another estimate of

Table 2

k_i (μmol/L)* of fifteen ions and heavy metals from several freshwater and marine species.

Species	Al^{3+}	As^{3+}	Ba^{2+}	Ca^{2+}	Cd^{2+}	Cu^{2+}	$EDTA^{2-}$	Fe^{2+}	Hg^{2+}	K^{+}	Li^{+}	Mg^{2+}	Mn^{2+}	Pb^{2+}	Zn^{2+}
O. niloticus	-	38.76	-	-	-	-	-	-	16.00	-	-	-	-	-	408.32
C. macropomum	-	24.59	-	-	484.00	317.30	-	-	10.00	-	-	-	-	-	301.18
A. gigas	-	-	-	-	-	433.80	-	-	28.57	-	-	-	-	-	-
E. electricus	-	78.50	4.01	-	100.90	4.01	1 702.50	92.90	0.80	-	30.40	-	-	0.80	0.80
R. canadum	-	25.37	-	-	132.90	44.70	-	-	14.50	-	-	-	-	-	759.90

k_i: The dissociation constant of the enzyme-inhibitor complex; *: Estimated by Cheng and Prusoff equation (1973); –: No k_i estimated in the range of concentration under study.

Table 3

Kinetic parameters of AChE from *R. canadum* concerning several concentrations of six inhibitory ions using hyperbola model.

Concentration (mmol/L)	Hg^{2+}		Cu^{2+}		Zn^{2+}		Pb^{2+}		Cd^{2+}		As^{3+}	
	K_m (mmol/L)	V_{max} (mIU/mg protein)	K_m (mmol/L)	V_{max} (mIU/mg protein)	K_m (mmol/L)	V_{max} (mIU/mg protein)	K_m (mmol/L)	V_{max} (mIU/mg protein)	K_m (mmol/L)	V_{max} (mIU/mg protein)	K_m (mmol/L)	V_{max} (mIU/mg protein)
0.000	0.731 ± 0.104^{a}	230.039 ± 5.082^{ab}	0.373 ± 0.081^{a}	251.481 ± 6.286^{a}	0.509 ± 0.031^{a}	204.087 ± 1.748^{a}	0.309 ± 0.052^{a}	226.95 ± 4.055^{a}	0.371 ± 0.091^{a}	259.181 ± 7.589^{a}	0.307 ± 0.073^{a}	258.556 ± 6.891^{a}
Concentration (mmol/L)	K_{mapp} (mmol/L)	V_{mapp} (mIU/mg protein)	K_{mapp} (mmol/L)	V_{mapp} (mIU/mg protein)	K_{mapp} (mmol/L)	V_{mapp} (mIU/mg protein)	K_{mapp} (mmol/L)	V_{mapp} (mIU/mg protein)	K_{mapp} (mmol/L)	V_{mapp} (mIU/mg protein)	K_{mapp} (mmol/L)	V_{mapp} (mIU/mg protein)
0.001	1.023 ± 0.174^{b}	242.374 ± 8.012^{b}	0.440 ± 0.072^{a}	198.893 ± 4.613^{b}	0.642 ± 0.130^{b}	219.66 ± 7.179^{b}	0.497 ± 0.104^{ab}	205.584 ± 5.586^{b}	0.475 ± 0.052^{a}	225.974 ± 6.207^{b}	0.564 ± 0.129^{ab}	223.114 ± 7.252^{b}
0.010	0.964 ± 0.106^{b}	254.282 ± 5.284^{b}	0.494 ± 0.090^{a}	208.317 ± 5.147^{b}	-	-	0.429 ± 0.092^{ab}	199.394 ± 4.588^{b}	0.221 ± 0.161^{a}	210.687 ± 10.142^{b}	-	-
0.100	0.714 ± 0.121^{a}	224.005 ± 6.275^{a}	0.226 ± 0.107^{a}	198.949 ± 7.282^{b}	-	-	0.474 ± 0.069^{ab}	199.131 ± 3.829^{b}	0.605 ± 0.117^{a}	212.462 ± 7.265^{b}	0.706 ± 0.148^{ab}	131.415 ± 4.477^{c}
1.000	-	-	0.187 ± 0.121^{a}	106.905 ± 4.656^{c}	0.506 ± 0.123^{ab}	144.686 ± 4.631^{d}	0.497 ± 0.093^{ab}	178.530 ± 4.558^{c}	0.410 ± 0.081^{a}	182.720 ± 4.449^{c}	1.640 ± 0.577^{b}	109.380 ± 8.790^{d}
10.000	-	-	0.197 ± 0.324^{a}	32.749 ± 3.769^{d}	1.126 ± 0.105^{d}	75.425 ± 1.620^{e}	0.579 ± 0.052^{b}	134.952 ± 7.979^{d}	-	-	2.601 ± 0.890^{b}	71.761 ± 6.767^{e}
Possible classification	Competitive-like		Non-competitive		Mixed		Mixed		Non-competitive		Mixed	

K_{mapp}: Michaelis-Menten constant in presence of inhibitors; V_{mapp}: Maximum rate of substrate hydrolysis in presence of inhibitors; Lowercase letters in column indicate significant differences ($P < 0.05$) using ANOVA and Tukey's test.

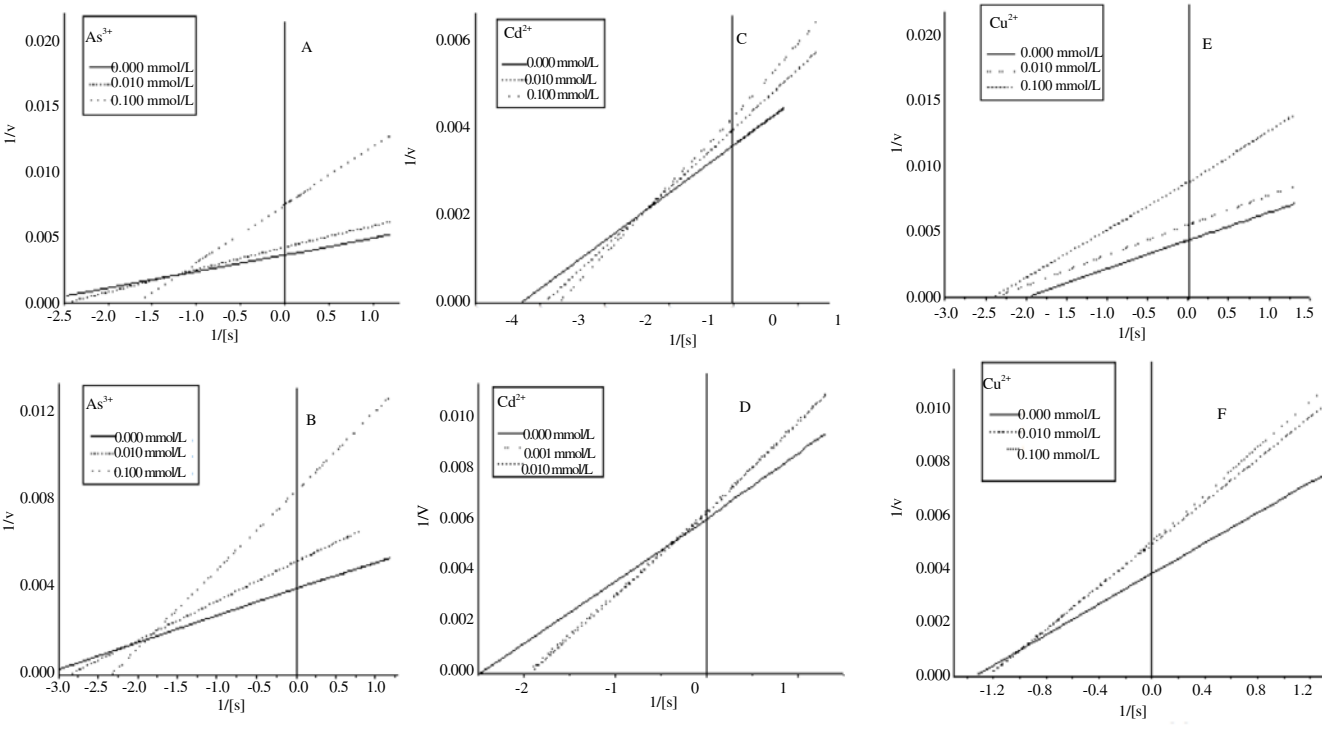

Figure 2. Double reciprocal regression plots of brain AChE activity from *R. canadum* (A, C and E plots) and *O. niloticus* (B, D and F plots) exposed to several concentrations of inhibitory ions (As^{3+}, Cd^{2+} and Cu^{2+}).

Table 4

Kinetic parameters of AChE from *O. niloticus* concerning several concentrations of five inhibitory ions using hyperbola model.

Concentration (mmol/L)	Hg^{2+}		Cu^{2+}		Zn^{2+}		Cd^{2+}		As^{3+}	
	K_m (mmol/L)	V_{max} (mIU/mg protein)	K_m (mmol/L)	V_{max} (mIU/mg protein)	K_m (mmol/L)	V_{max} (mIU/mg protein)	K_m (mmol/L)	V_{max} (mIU/mg protein)	K_m (mmol/L)	V_{max} (mIU/mg protein)
0.000	0.856 ± 0.093[a]	194.497 ± 3.784[a]	0.477 ± 0.153[a]	224.490 ± 9.559[a]	0.617 ± 0.086[a]	196.170 ± 4.170[a]	0.786 ± 0.115[a]	210.869 ± 7.447[a]	0.092 ± 0.077[a]	220.897 ± 7.179[a]
Concentration (mmol/L)	K_{mapp} (mmol/L)	V_{mapp} (mIU/mg protein)	K_{mapp} (mmol/L)	V_{mapp} (mIU/mg protein)	K_{mapp} (mmol/L)	V_{mapp} (mIU/mg protein)	K_{mapp} (mmol/L)	V_{mapp} (mIU/mg protein)	K_{mapp} (mmol/L)	V_{mapp} (mIU/mg protein)
0.001	0.781 ± 0.090[a]	188.803 ± 3.906[a]	0.736 ± 0.128[a]	192.806 ± 5.575[b]	0.649 ± 0.097[a]	199.061 ± 4.853[a]	0.952 ± 0.202[a]	196.964 ± 10.168[ab]	0.481 ± 0.076[ab]	196.529 ± 3.968[b]
0.010	0.812 ± 0.163[a]	190.900 ± 6.977[a]	-	-	0.683 ± 0.113[a]	195.810 ± 5.427[a]	0.798 ± 0.159[a]	186.694 ± 6.725[b]	0.482 ± 0.107[ab]	191.680 ± 5.455[b]
0.100	3.905 ± 0.692[b]	192.573 ± 10.353[a]	0.679 ± 0.101[a]	161.086 ± 4.037[c]	0.952 ± 0.248[a]	191.231 ± 9.397[a]	0.686 ± 0.161[a]	178.677 ± 6.725[b]	0.591 ± 0.142[b]	127.205 ± 4.090[c]
1.000	-	-	0.424 ± 0.072[a]	109.233 ± 3.011[d]	0.978 ± 0.286[a]	164.343 ± 9.492[b]	-	-	1.443 ± 0.268[c]	97.529 ± 4.145[d]
10.000	-	-	0.411 ± 0.216[a]	22.615 ± 1.385[e]	1.541 ± 0.442[b]	91.084 ± 5.831[c]	0.918 ± 0.153[a]	153.044 ± 5.003[c]	-	-
Possible classification	Competitive-like		Non-competitive		Mixed		Non-competitive		Mixed	

K_{mapp}: Michaelis-Menten constant in presence of inhibitors; V_{mapp}: Maximum rate of substrate hydrolysis in presence of inhibitors; Lowercase letters in column indicate significant differences ($P < 0.05$) using ANOVA and Tukey's test.

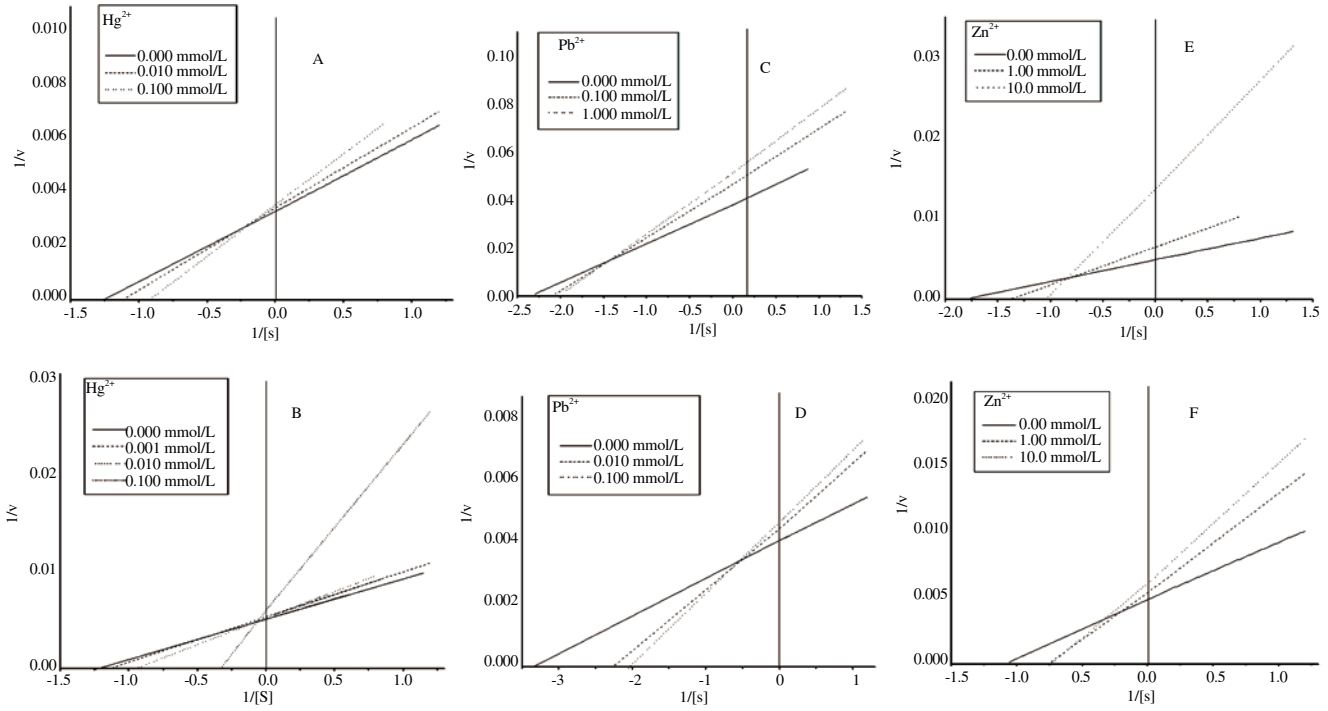

Figure 3. Double reciprocal regression plots of brain AChE activity from *R. canadum* (A, C and E plots) and *O. niloticus* (B, D and F plots) exposed to several concentrations of inhibitory ions (Hg^{2+}, Pb^{2+} and Zn^{2+}).

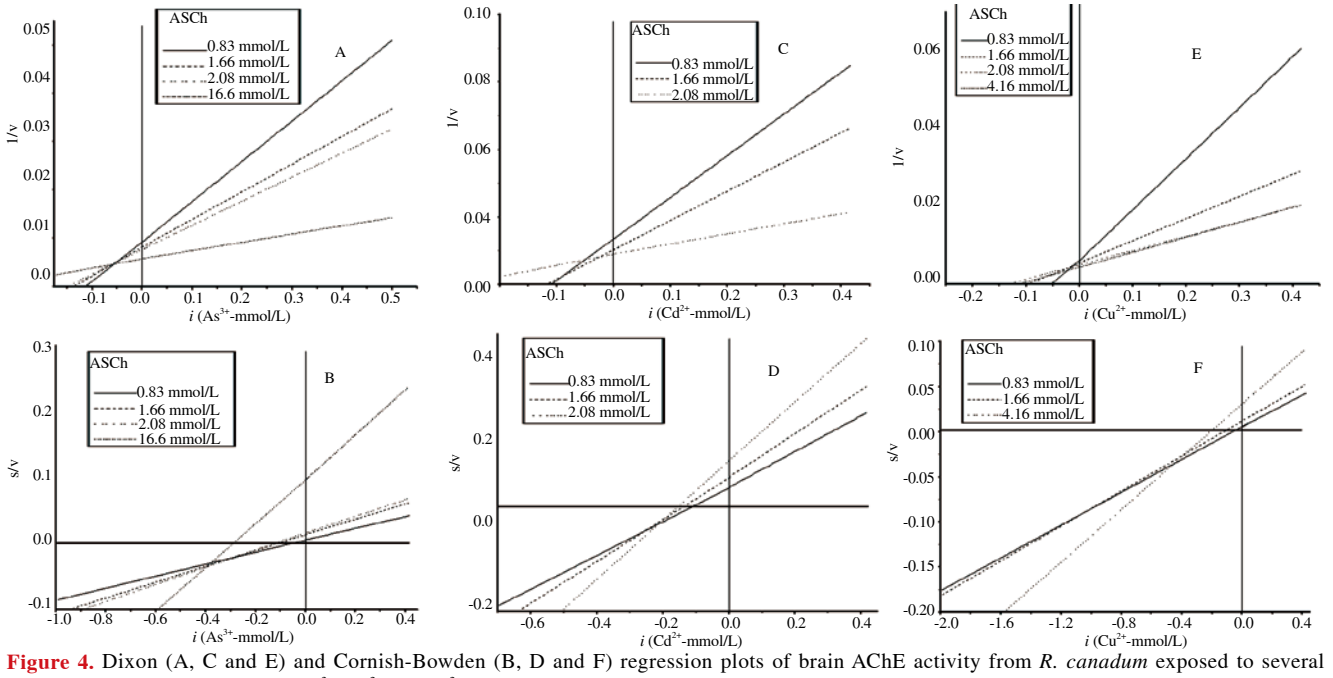

Figure 4. Dixon (A, C and E) and Cornish-Bowden (B, D and F) regression plots of brain AChE activity from *R. canadum* exposed to several concentrations of inhibitory ions (As^{3+}, Cd^{2+} and Cu^{2+}).
ASCh: Acetylthiocholine iodide.

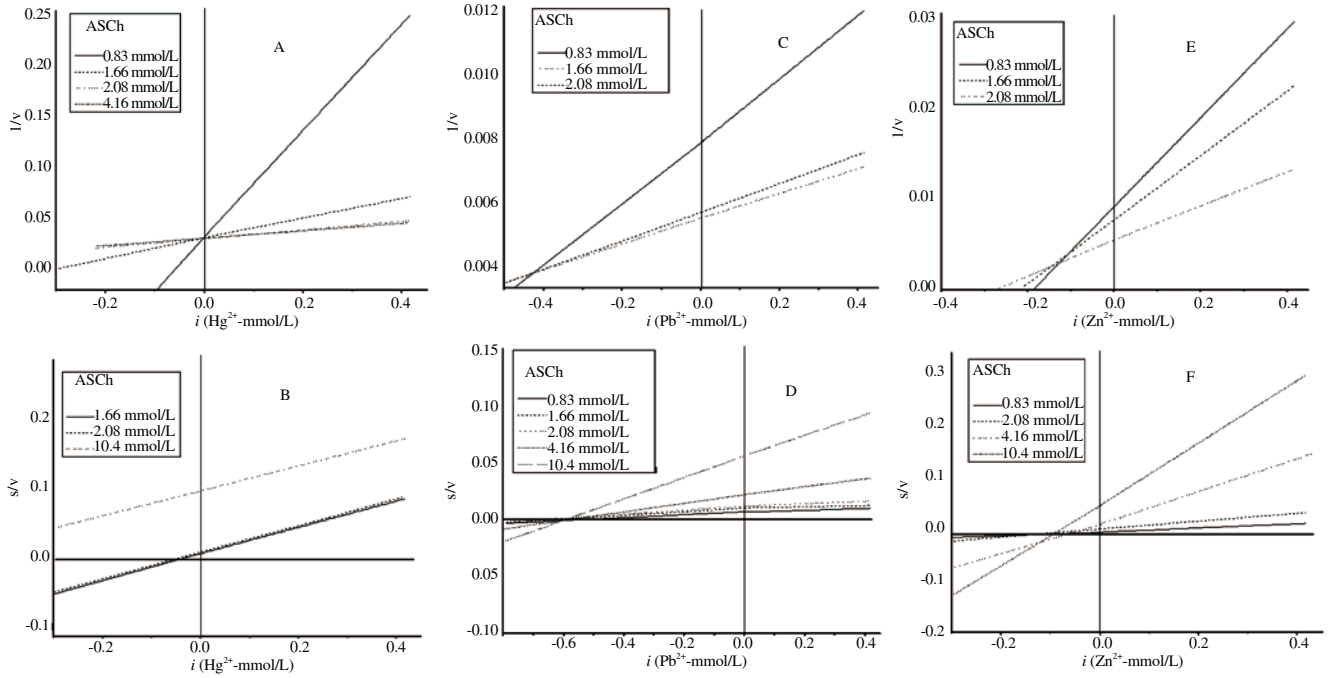

Figure 5. Dixon (A, C and E) and Cornish-Bowden (B, D and F) regression plots of brain AChE activity from *R. canadum* exposed to several concentrations of inhibitory ions (Hg^{2+}, Pb^{2+} and Zn^{2+}).
ASCh: Acetylthiocholine iodide.

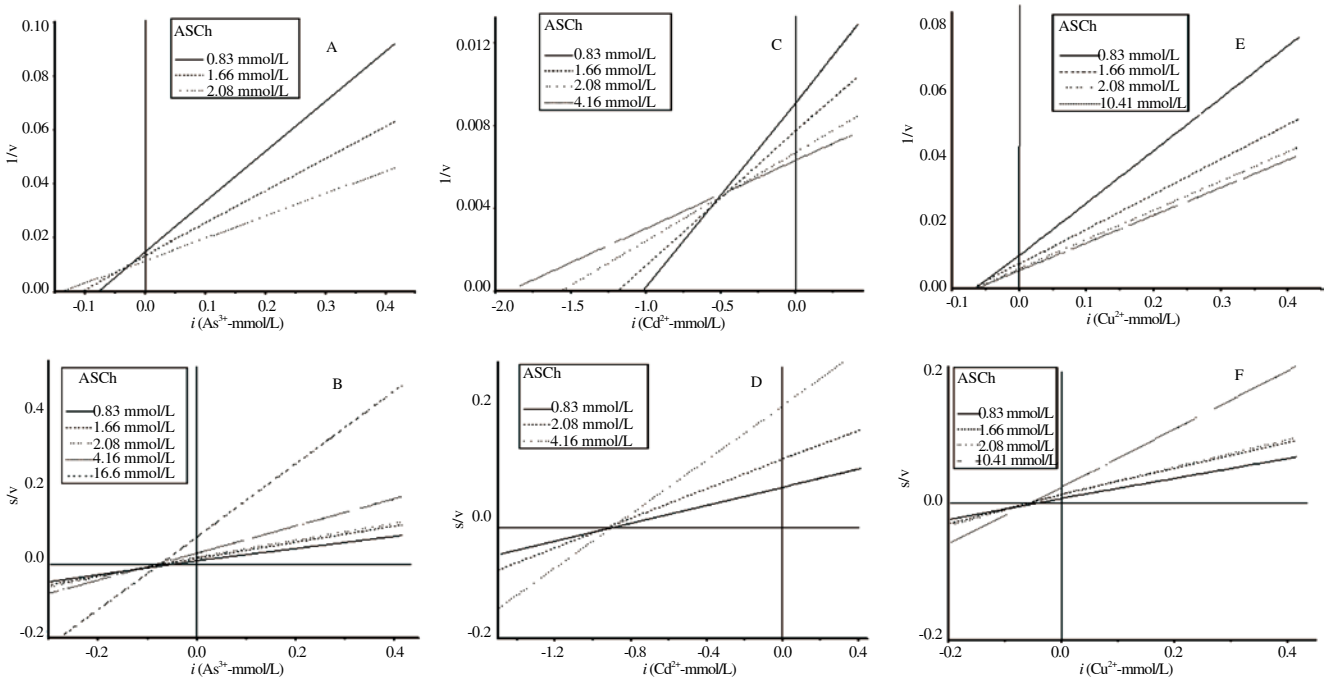

Figure 6. Dixon (A, C and E) and Cornish-Bowden (B, D and F) regression plots of brain AChE activity from *O. niloticus* exposed to several concentrations of inhibitory ions (As^{3+}, Cd^{2+} and Cu^{2+}).
ASCh: Acetylthiocholine iodide.

Table 5
k_i^* and $k'_i{}^{**}$ (μmol/L) of ions on brain AChE activity from *O. niloticus* and *R. canadum*.

Species	As^{3+}		Cd^{2+}		Cu^{2+}		Hg^{2+}		Pb^{2+}		Zn^{3+}	
	k_i	k'_i	k_i	k'_i	k_i	k'_i	k_i	k'_i	k_i	k'_i	k_i	k'_i
O. niloticus	32.25	116.0[b]	482.4	920.5	16.5	58.5[c]	4.14	-	105.3	554.7	167.42	124.5[c]
		77.1				96.5						187.9
R. canadum	50.00	276.3[b]	37.8[a]	210.4	63.1	133.5[c]	3.29	-	426.1	612.0	120.20	117.5[c]
		379.0	93.6			211.0						148.1

*k_i: the dissociation constant of the enzyme-inhibitor complex estimated by Dixon plots (1953); [a]: Substrate concentration from 2.08 mmol/L; k'_i: The dissociation constant of the enzyme-inhibitor-substre complex estimated by Cornish-Bowden plots (1974); [b]: Substrate concentration from 16.60 mmol/L; [c]: Substrate concentration from 4.16 mmol/L.

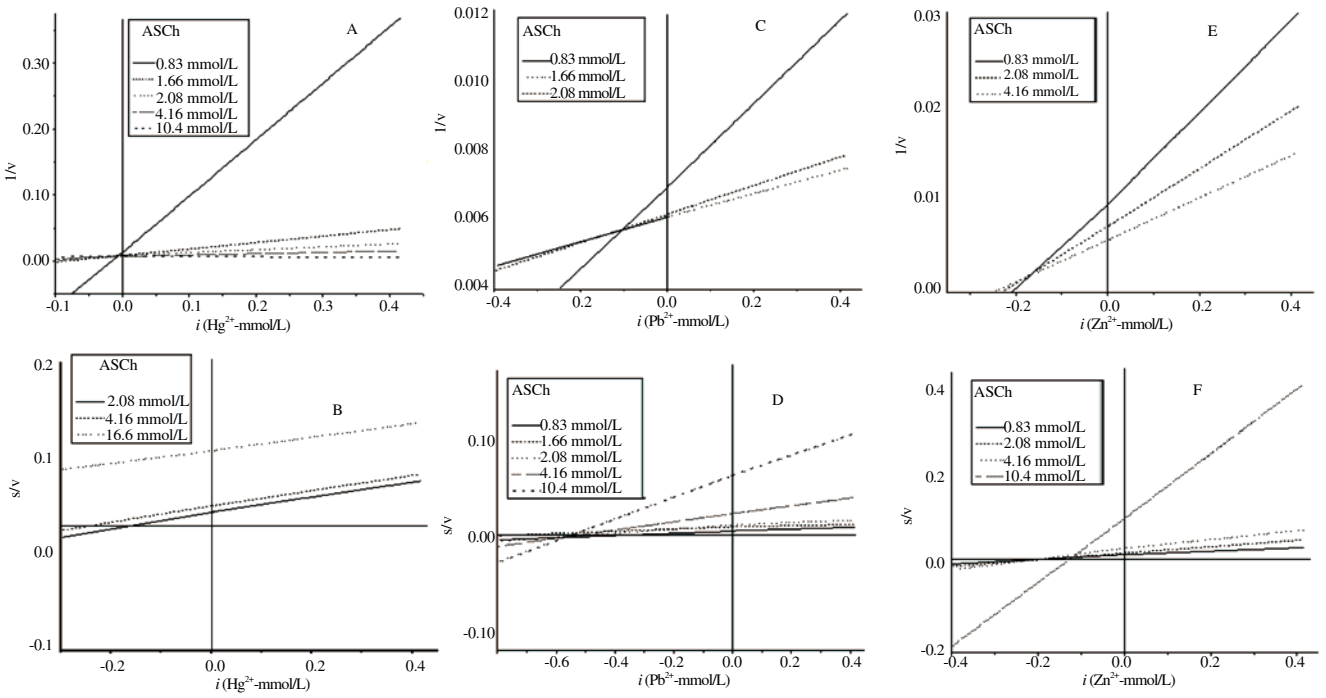

Figure 7. Dixon (A, C and E) and Cornish-Bowden (B, D and F) regression plots of brain AChE activity from *O. niloticus* exposed to several concentrations of inhibitory ions (Hg^{2+}, Pb^{2+} and Zn^{2+}).
ASCh: Acetylthiocholine iodide.

the k_i values from Dixon plots and the enzyme-substrate-inhibitor complex (k'_i) from Cornish-Bowden plots for the inhibitory ions. The values for k'_i were higher than ki in all situations (excepting competitive inhibition in which k'i does not exist) in both species.

4. Discussion

Some studies pointed to the influence of ions on the AChE activity by binding to peripheral sites promoting conformational modifications or changing the hydration state of the active center which alters the rate of substrate hydrolysis by the enzyme[12,19,20]. Hughes and Bennett[12] working with *E. electricus* AChE reported three classes of metal ion effects on AChE activity; activation by Ca^{2+}, Mg^{2+} and Al^{3+}; inactivation by Cu^{2+}, Na^{+}, Pb^{2+} and Zn^{2+} and a non-specific effect of Li^{+} whereas Tomlinson *et al.*[13] using the same species divided the effect of ions into two groups: the first performs an activating action comprising Ca^{2+}, Mg^{2+}, Mn^{2+} and Na^{+}; the second one exerts inhibitory effects and is formed by Cd^{2+}, Cu^{2+}, Hg^{2+}, Ni^{2+}, Pb^{2+} and Zn^{2+}. Our results differ from these groups only in relation to Al^{3+} and Li^{+}.

The main peripheral anionic site in AChE is described as a region near the rim of the gorge where the active center is located[21]. Nevertheless, there are binding sites for positively charged activators and inactivators far from the active site of the enzyme which are different for organic and inorganic molecules[13]. Roufogalis and Quist[22] called α-site the anionic sub-site of the active center (choline binding site). According to them, the β-site is located bordering the gorge while the γ-site is the one far from the active site. Rosenberry[23] named the same sites, respectively as C, P1 and P2. In addition, this author reported the sites P3 and P4 which are binding sites for inorganic cations and hydrophobic organic cations as well as P2 and, according to Roufogalis and Wickson[24], these sites can cause allosteric disturbances in enzymatic activity.

The β-site (or P1) is known to bind metal ion of the Tomlinson's first group such as Ca^{2+}, Mg^{2+}, Mn^{2+}, polar cations and is considered an accelerator site whose occupancy can even stabilize the activated conformation of the enzyme[13]. Furthermore, Mg^{2+} binds to α and β-sites and can act as competitive inhibitor at low substrate concentrations or low affinity substrates ("poor" substrates). At high substrate concentrations, it probably is displaced from α-site to only occupy the β-site, therefore causing activation[22]. In the present work, only *R. canadum* AChE was positively affected by the activator ions Mn^{2+}, Mg^{2+} and Ca^{2+} (this last in concentrations above 1 mmol/L) whereas *E. electricus* AChE was inhibited by Mn^{2+} at 0.01 mmol/L. This occurred possibly by the fact that, according to Tomlinson *et al.*[13] working with *E. electricus*, activation is not well demonstrable in high ionic strength conditions as in the present work and *R. canadum* is a sea water species which, in other words, means that their enzymes may have evolved under conditions of higher salt fluctuations compared to freshwater species and could mirror activation effects even under high ionic strength conditions. These findings may seem contrasting with the influence of Ca^{2+} and Mg^{2+} on *E. electricus* AChE activity in other studies. Tomlinson *et al.*[20] and Hughes and Bennet[12] found activations of 60% and 40%, respectively by Ca^{2+}, while Tomlinson *et al.*[20] reported an increase of about 60% in their work with Mg^{2+}. However, the activation reported for Ca^{2+} and Mg^{2+} by these authors occurred at high substrate concentrations and low ionic strength buffers. In relation to Mn^{2+}, the results here corroborate other works[25,26].

Al^{3+} didn't appear among the groups of ions reported by Tomlinson *et al.*[13] and the results in literature are contradictory. In some works, Al^{3+} inhibited AChE from bovine brain and from electric organ of *E. electricus* and this inhibition occurred in an ionic strength-dependent manner[27,28]. These studies advocated the existence of an interaction between this ion and the residue Glu in the active site of the enzyme. However, such residue can only provide a weak interaction, confirmed by the findings for the active site of butyrylcholinesterase (BChE) (which also present this

residue in its catalytic triad) by Sarkarati *et al*.[29]. Here, the ionic strength conditions are different and Al^{3+} did not affect AChE from any species under study.

The activator ions (Ca^{2+}, Mg^{2+} and Mn^{2+}) plus Al^{3+} and K^{+} presented no complexation or interaction with protein or colorimetric reagents capable of increase absorbance in the assay causing false positives when using the enzyme as biomarker. Besides binding to the anionic sites of AChE, these activator ions can interact with "hard bases" side chains such as carboxylate groups in the sample preparation[13]. However, this effect did not appear to be important in our experimental conditions.

Cu^{2+}, $Zn^{2+}$and Cd^{2+} are part of the second group defined by Tomlinson and co-workers[13] and are known as strong inhibitors of AChE. The action of ligands on P2, P3 and P4 sites allows allosteric disturbances at the catalytic site inhibiting enzymatic activity[23]. The inhibitions exerted by Cu^{2+} and Zn^{2+} found in literature are varied: Tomlinson *et al*.[20] which observed inhibition of 100% by Cu^{2+} and Zn^{2+} in the activity of *E. electricus* AChE at 1 mmol/L and the value reported by Hughes and Bennett[12] who found 20% inhibition promoted by Cu^{2+} for the same species and ion concentration. Nemcsók *et al*.[30] observed an inhibition of 69% at 0.36 mmol/L of Cu^{2+} and no effect for Zn^{2+} whereas Bocquené *et al*.[31] reported an inhibition of 100% in two marine species (*Scomber scomber* and *Pleuronectes platessa*) under Cu^{2+} exposure at 1 mmol/L. These last authors found for the same species, respectively, 57.4% and 70% at 1 mmol/L for Zn^{2+}. In the present study, *E. electricus* AChE was the most sensitive enzyme for Cu^{2+}, Zn^{2+} and Cd^{2+} presenting IC_{50} of 0.05, 0.01 and 1.0 mmol/L, respectively. Silva *et al*.[6] reported IC_{50} values for Cu^{2+}, Zn^{2+} and Cd^{2+} of 2.10, 2.57 and 6.14 mmol/L, respectively, using *Cichla ocellaris* AChE. Olson and Christensen[19] observed IC_{50} values by Cu^{2+}, Zn^{2+} and Cd^{2+} of 0.16, 10.0 and 0.57 mmol/L, respectively, on the activity of AChE from *Pimephales promelas* (*P. promelas*).

Ba^{2+} and Fe^{2+} induced, under our experimental conditions, a similar pattern of inhibition. Only *E. electricus* AChE presented IC_{50} for these ions. Unlike the other species under study, Fe^{2+} inhibited significantly *E. electricus* enzyme at concentrations such as 0.1 mmol/L and Ba^{2+} did it at 0.01 mmol/L. Ba^{2+} and Fe^{2+} only exerted inhibitory effects on *C. macropomum*, *A. gigas* and *R. canadum* at 1 mmol/L or higher concentrations. Similarly, Li^{+} only inhibited significantly *E. electricus* enzyme (IC_{50} of 0.38 mmol/L) and seems to compete for the same site with Pb^{2+} although they present different valences, as evidenced by the study of Hughes and Bennett[12] which reported a relief of Pb^{2+} inhibition effects by the presence of Li^{+}. These authors described a non-defined effect of Li^{+} alone and in fact for *A. gigas*, *C. macropomum*, *O. niloticus* and *R. canadum* no positive or negative effect was observed.

The chelating ion $EDTA^{2-}$ only inhibited *R. canadum* and *E. electricus* AChE presenting for the latter an IC_{50} value of 21.25 mmol/L. The enzymes from the other species in study were significantly inhibited only above 50 mmol/L by this ion. Such results (except for *E. electricus*) are in accordance with Tomlinson *et al*.[13], and enable this chelating agent to protect against divalent metallic interferences when using AChE from these species as biomarker for anticholinesterase agents analyzes.

According to Olson and Christensen[19], the ion As^{3+} (from AsO^{2-}) exhibits much more significant inhibitory activity, as compared to As^{5+}. These authors reported a 50% inhibition at 0.03 mmol/L using *P. promelas*. Here, we used As^{3+}, which induced values of IC_{50} in concentrations lower than 1 mmol/L for *C. macropomum*, *R. canadum*, *E. electricus* and *O. niloticus*. Another report about exposure to arsenic in S. scomber and P. platessa describes 33 and 31% of inhibition, respectively at 1 mmol/L[31]. Recently, Silva *et al*.[6] reported an IC_{50} of 0.1 mmol/L by this ion on C. ocellaris AChE.

Hg^{2+} ion completely inactivated AChE from *C. macropomum*, *R. canadum*, *E. electricus* and *O. Niloticus*, when these enzymes were exposed to 1 mmol/L or lower concentrations. The AChE activity from *A. gigas* was the most resistant . These values are not too discrepant from those reported by Olson and Christensen[19], who found 50% inhibition at 1.6 mmol/L for *P. promelas*. Gill *et al*.[32], using AChE from *Puntius conchonius*, observed 67% of inhibition at 0.001 mmol/L. Here, Pb^{2+} was able to inhibit the enzymes from *A. gigas*, *R. canadum* and induced an IC_{50} of 0.01 mmol/L on the activity of *E. electricus* AChE. Hughes and Bennett[12] observed an inhibition of about 100% with *E. electricus* at 1mmol/L, while Olson and Christensen[19] reported 50% inhibition at 7.1 mmol/L for *P. promelas* AChE.

Hg^{2+} and Pb^{2+} belongs to the Tomlinson's second group of ions and according to Valle and Ulmer[33], inhibit a large number of enzymes by strongly interacting with their functional sulfhydryl groups. AChE was in the past included among such enzymes although no free sensitive sulfhydryl group are present in its structure except the one described in the *Torpedo californica* electric organ. It was noted that most of these enzymes present such groups in form of disulfide bonds (*e.g*.: *E. electricus* AChE) or only one in a position (not conserved) buried or accessible through the solution but not always capable to react with thiol agents[10,34,35]. Investigations in the binding sites of Hg^{2+} to human BChE, observed no mercury bound to sulfhydryl groups in crystal structure and the only free accessible cysteine was persulfured (Cys-S-SH) and not easily susceptible to reduction[10]. Moreover, arsenic was not listed in the second group of Tomlinson but was also regarded as a free -SH ligand. According to Mounter and Whittaker[36], As-S link is readily hydrolyzed in alkaline solutions. However, in the present work the enzymes remained inhibited by As^{3+} in basic conditions evidencing the binding of this metal with sites other than free sulfhydryl groups. In other words, the classical explanation about the action of inhibitory ions on free sulfhydryl groups of enzymes is not sufficient for the inhibition of cholinesterases activity[36,37].

Tomlinson *et al*.[13], working with AChE from *E. electricus* reported that Hg^{2+} and Pb^{2+} complex with the product of Ellman method, thiocholine (TCh), interfering in the assay. Nevertheless, in the same work it was found that Hg^{2+} strongly inhibited the enzyme when using p-nitrophenyl acetate as substrate. Additionally, they demonstrated that this ion decreased the rate of carbamoylation of the enzyme active site by M7C (7-(dimethylcarbamoyloxy)-N-methylquinoline iodide), which proves the tight binding of Hg^{2+} to the peripheral sites of AChE and their interference on the active site. The same was observed in the work of Frasco *et al*.[9] in which was reported the binding of TCh and thiobis-nitrobenzoate ion (TNB) with not only Hg^{2+}, but also Cd^{2+}, Cu^{2+} and Zn^{2+} interfering in Ellman's method. They also proposed another substrate, o-nitrophenyl acetate. However, as occurs with p-nitrophenyl acetate, this substrate is not specific for AChE being hydrolyzed by other esterases and requiring higher concentrations for the assays. Here, these problems with TNB and TCh were minimized due to the separated incubation of enzyme plus ion and the blanks with total cholinesterase inhibitor neostigmine bromide. DTNB and acetylthiocholine were only added immediately before the readings.

Hg^{2+} and Pb^{2+} precipitates were not observed during the assays.

Here, according to inhibition kinetic analyzes using hyperbola model with *R. canadum* and *O. niloticus*, Cu^{2+} behaved as non-competitive inhibitor and Zn^{2+} as mixed-type. Double reciprocal plot analyzes also showed Zn^{2+} as mixed-type inhibitor for both species whereas showed Cu^{2+} as non-competitive (with *R. canadum*) or mixed (with *O. niloticus*) inhibitor. Dixon and Cornish-Bowden plots compared analyzes can provide disambiguation on kinetic behaviour[16] and, in the present work, suggest that these two ions act as mixed-type inhibitors. In addition, Cd^{2+} behaved as non-competitive inhibitor towards AChE from *R. canadum* and *O. niloticus* when analyzing with hyperbola model. Nevertheless, all the other three graphic approaches such as double reciprocal, Dixon and Cornish-Bowden pointed this ion as a mixed-type inhibitor. These results for Cu^{2+} and Cd^{2+} are corroborated by Sarkarati *et al*.[29] using human serum BChE, while Hughes and Bennett[12] regarded Cu^{2+} and Zn^{2+} as non-competitive inhibitor exposing AChE from *E. electricus* to these metals.

Kinetic analysis of the inhibitory behaviour of As^{3+} and Pb^{2+} on *R. canadum* and *O. niloticus* brain AChE pointed to mixed-type inhibitors in all models used (hyperbola, double reciprocal, Dixon and Cornish-Bowden). For the ions classified as mixed or non-competitive inhibitors in this study (As^{3+}, Cd^{2+}, Cu^{2+}, Pb^{2+} and Zn^{2+}), the values of k'i were higher than that of ki which means that the enzyme-substrate-inhibitor complex is the limiting step in the rate of substrate hydrolysis confirming the type of their inhibitory behavior.

In the present work, Hg^{2+} ion seems to present a competitive-like inhibitory action using all the models excepting double reciprocal plots which showed a mixed-type behavior. However, Frasco *et al*.[10] reported that no Hg^{2+} ion was attached to the anionic sub-site (choline binding site or ammonium binding site) of the AChE active center in the three-dimensional structure of the enzyme and therefore could not be a competitive inhibitor. Nevertheless, the effects of Hg^{2+} binding could present competitive-like consequences. The same authors state that the first and main binding site of Hg^{2+} to AChE is located at the omega loop (cysteine loop or W-loop) behind the choline binding site of the active center. These two regions are mutually responsive to ligand-dependent conformational changes and it was proposed by other authors that occupancy of peripheral site induces movements in the loop which in turn modify the orientation of the key tryptophan residue present in the choline binding site of the active center[23,38-40]. It suggested that conformational alterations from the binding of Hg^{2+} to the loop and the other three peripheral mercury-binding sites reported by Frasco *et al*.[10] could allosterically be transmitted to the choline binding locus of the active center interfering in substrate binding and, inversely, the substrate binding to peripheral (substrate inhibition site) and active sites, in increasing concentrations, could hinder Hg^{2+} binding to AChE loop since the position of residues in this region undergo a modification becoming more exposed to the solution after peripheral site occupancy[39,40]. The ion would be displaced from the loop and not from the active center therefore mimicking the behavior of a competitive inhibitor. This is corroborated by our results of Cornish-Bowden analysis of Hg^{2+} action that presented no k'i value. It means that no enzyme-substrate-inhibitor complex was perceptible as in the case of competitive inhibition. Further studies are necessary to directly confirm such behavior.

These results suggest that AChE from the species under study could be useful as biomarker of Hg^{2+} ion, according to the type of effluent discharged in a given area. For the other ions, in most of cases they have little potential to interfere on the enzyme activity in samples not associated with mine and industrial effluents.

R. canadum AChE was responsive to activator ions (Ca^{2+}, Mg^{2+} and Mn^{2+}) in the high ionic strength conditions of the present work. In addition, the commercial enzyme from *E. electricus* was strongly influenced by the majority of the ions analyzed (unlike the other enzymes proposed in this paper) which is an undesirable feature for a biomarker of anticholinesterasic agents. In contrast with *E. electricus* enzyme, AChE from *A. gigas* was the most resistant to the ions. Nevertheless, EDTA can be used to protect the enzyme activity against divalent metallic cations since only exerted interfering effects on *R. canadum* and *E. electricus* enzyme at 1 mmol/L.

Inhibition kinetic analyzes in the present experimental conditions classified As^{3+}, Cd^{2+}, Cu^{2+}, Pb^{2+} and Zn^{2+} as mixed or non-competitive inhibitors of *R. canadum* and *O. niloticus* brain AChE.

The most reactive ion was Hg^{2+}, which strongly inhibited the AChE from the five species. This ion presented competitive-like features of inhibitory behavior, even without binding to the active center of the enzyme (as demonstrated in other studies) probably due to its interaction with regions responsive to peripheral site occupancy. Further studies are required to elucidate such competitive mimicking effect.

Ions and heavy metals may arise as probable contaminants in samples from different sources, and can cause false positives or negatives in the analyses of pesticides or other anticholinesterasic agents. On the other hand, analyzing the inhibition produced by these substances along with other methods, it is possible to use the enzyme also as a biomarker for the presence of some heavy metals, according to the waste composition from a given area.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgment

The authors would like to dedicate this study to Dr. Patrícia Fernandes de Castro (in memoriam) for her invaluable help and to thank Financiadora de Estudos e Projetos (FINEP/RECARCINE), Petróleo do Brasil S/A (PETROBRAS), Secretaria Especial de Aqüicultura e Pesca (SEAP/PR), Conselho Nacional de Pesquisa e Desenvolvimento Científico (CNPq) and Fundação de Apoio à Ciência e Tecnologia do Estado de Pernambuco (FACEPE) for financial support (Grant numbers: IBPG-1301-2.08/08 FACEPE, IBPG-0523-2.08/11 FACEPE, BFP-0036-2.08/13 FACEPE and BFP-0111-2.08/13). Our gratitude also goes to Universidade Federal Rural de Pernambuco and Aqualider, for providing fish juvenile specimens.

References

[1] Campanha HM, Carvalho F, Schlosser PM. Active and peripheral anionic sites of acetylcholinesterase have differential modulation effects on cell proliferation, adhesion and neuritogenesis in the NG108-15 cell line. *Toxicol Lett* 2014; **230**: 122-31.

[2] Karczmar AG. Cholinesterases (ChEs) and the cholinergic system in ontogenesis and phylogenesis, and non-classical roles of cholinesterases - a review. *Chem Biol Interact* 2010; **187**: 34-43.

[3] Yldirim NC, Yldirim N, Danabas D, Danabas S. Use of acetylcholinesterase, glutathione S-transferase and cytochrome P450 1A1 in *Capoeta umbla* as biomarkers for monitoring of pollution in

Uzuncayir Dam Lake (Tunceli, Turkey). *Environ Toxicol Pharmacol* 2014; **37**: 1169-76.

[4] Sogorb MA, González-González I, Pamies D, Vilanova E. An alternative *in vitro* method for detecting neuropathic compounds based on acetylcholinesterase inhibition and on inhibition and aging of neuropathy target esterase (NTE). *Toxicol In Vitro* 2010; **24**: 942-52.

[5] Valdés-Ramírez G, Fournier D, Ramírez-Silva MT, Marty JL. Sensitive amperometric biosensor for dichlorovos quantification: application to detection of residues on apple skin. *Talanta* 2008; **74**: 741-6.

[6] Silva KC, Assis CR, Oliveira VM, Carvalho LB Jr, Bezerra RS. Kinetic and physicochemical properties of brain acetylcholinesterase from the peacock bass (*Cichla ocellaris*) and *in vitro* effect of pesticides and metal ions. *Aquat Toxicol* 2013; **126**: 191-7.

[7] Rodríguez-Fuentes G, Armstrong J, Schlenk D. Characterization of muscle cholinesterases from two demersal flatfish collected near a municipal wastewater outfall in Southern California. *Ecotoxicol Environ Saf* 2008; **69**: 466-71.

[8] Monserrat JM, Geracitano LAG, Bianchini A. Current and future perspectives using biomarkers to assess pollution in aquatic ecosystems. *Comments Toxicol* 2003; **9**: 255-69.

[9] Frasco MF, Fournier D, Carvalho F, Guilhermino L. Do metals inhibit acetylcholinesterase (AChE)? Implementation of assay conditions for the use of AChE activity as a biomarker of metal toxicity. *Biomarkers* 2005; **10**: 360-75.

[10] Frasco MF, Colletier JP, Weik M, Carvalho F, Guilhermino L, Stojan J, et al. Mechanisms of cholinesterase inhibition by inorganic mercury. *FEBS J* 2007; **274**: 1849-61.

[11] Reddy GR, Basha MR, Devi CB, Suresh A, Baker JL, Shafeek A, et al. Lead induced effects on acetylcholinesterase activity in cerebellum and hippocampus of developing rat. *Int J Dev Neurosci* 2003; **21**: 347-52.

[12] Hughes RJ, Bennett J. Effect of metal ions on the activity of acetylcholinesterase. *Biochem Soc Trans* 1985; **13**: 219-20.

[13] Tomlinson G, Mutus B, McLennan I. Activation and inactivation of acetylcholinesterase by metal ions. *Can J Biochem* 1981; **59**: 728-35.

[14] Assis CR, Linhares AG, Oliveira VM, França RC, Carvalho EV, Bezerra RS, et al. Comparative effect of pesticides on brain acetylcholinesterase in tropical fish. *Sci Total Environ* 2012; **441**: 141-50.

[15] Sedmak JJ, Grossberg SE. A rapid, sensitive and versatile assay for protein using Coomassie brilliant blue G250. *Anal Biochem* 1977; **79**: 544-52.

[16] Cornish-Bowden A. A simple graphical method for determining the inhibition constants of mixed, uncompetitive and non-competitive inhibitors. *Biochem J* 1974; **137**: 143-4.

[17] Dixon M. The determination of enzyme inhibitor constants. *Biochem J* 1953; **55**: 170-1.

[18] Cheng Y, Prusoff WH. Relationship between the inhibition constant (Ki) and the concentration of inhibitor which causes 50 per cent inhibition (I_{50}) of an enzymatic reaction. *Biochem Pharmacol* 1973; **22**(23): 3099-108.

[19] Olson DL, Christensen GM. Effects of water pollutants and other chemicals on fish acetylcholinesterase (*in vitro*). *Environ Res* 1980; **21**: 327-35.

[20] Tomlinson G, Mutus B, McLennan I. Modulation of acetylcholinesterase activity by peripheral site ligands. *Mol Pharmacol* 1980; **18**: 33-9.

[21] Eastman J, Wilson EJ, Cerveñansky C, Rosenberry TL. Fasciculin 2 binds to the peripheral site on acetylcholinesterase and inhibits substrate hydrolysis by slowing a step involving proton transfer during enzyme acylation. *J Biol Chem* 1995; **270**: 19694-701.

[22] Roufogalis BD, Quist EE. Relative binding sites of pharmacologically active ligands on bovine erythrocyte acetylcholinesterase. *Mol Pharmacol* 1972; **8**: 41-9.

[23] Rosenberry TL. Acetylcholinesterase. *Adv Enzymol Relat Areas Mol Biol* 1975; **43**: 103-218.

[24] Roufogalis BD, Wickson VM. Acetylcholinesterase - specific inactivation of allosteric effects by a water soluble carbodiimide. *J Biol Chem* 1973; **248**: 2254-6.

[25] Martinez H, Bonilla E. Water intake and brain choline-acetyltransferase and acetylcholinesterase activities in manganese treated rats. *Neurobehav Toxicol Teratol* 1981; **3**: 277-80.

[26] Santos D, Milatovic D, Andrade V, Batoreu MC, Aschner M, Marreilha dos Santos AP. The inhibitory effect of manganese on acetylcholinesterase activity enhances oxidative stress and neuroinflammation in the rat brain. *Toxicology* 2012; **292**: 90-8.

[27] Moraes MS, Leite SR. Inhibition of bovine brain acetylcholinesterase by aluminum. *Braz J Med Biol Res* 1994; **27**: 2635-8.

[28] Sharp TR, Rosenberry TL. Ionic strength dependence of the inhibition of acetylcholinesterase activity by Al^{3+}. *Biophys Chem* 1985; **21**: 261-4.

[29] Sarkarati B, Çoku ra AN, Tezcan EF. Inhibition kinetics of human serum butyrylcholinesterase by Cd^{2+}, Zn^{2+} and Al^{3+}: comparison of the effects of metal ions on cholinesterases. *Comp Biochem Physiol C Pharmacol Toxicol Endocrinol* 1999; **122**: 181-90.

[30] Nemcsók J, Németh Á, Buzás ZS, Boross L. Effects of copper, zinc and paraquat on acetylcholinesterase activity in carp (*Cyprinus carpio* L.). *Aquat Toxicol* 1984; **5**: 23-31.

[31] Bocquené G, Galgani F, Truquet P. Characterization and assay conditions for use of AChE activity from several marine species in pollution monitoring. *Mar Environ Res* 1990; **30**: 75-89.

[32] Gill TS, Tewari H, Pande J. Use of the fish enzyme system in monitoring water quality: effects of mercury on tissue enzymes. *Comp Biochem Physiol C* 1990; **97**: 287-92.

[33] Valle BL, Ulmer DD. Biochemical effects of mercury, cadmium and lead. *Ann Rev Biochem* 1972; **41**: 91-128.

[34] MacPhee-Quigley K, Vedvick TS, Taylor P, Taylor SS. Profile of the disulfide bonds in acetylcholinesterase. *J Biol Chem* 1986; **261**: 13565-70.

[35] Steinberg N, Roth E, Silman I. Torpedo acetylcholinesterase is inactivated by thiol reagents. *Biochem Int* 1990; **21**: 1043-50.

[36] Mounter LA, Whittaker VP. The effect of thiol and other group-specific reagents on erythrocyte and plasma cholinesterases. *Biochem J* 1953; **53**: 167-73.

[37] Ali N, Hoque MA, Haque A, Salam KA, Karim MR, Rahman A, et al. Association between arsenic exposure and plasma cholinesterase activity: a population based study in Bangladesh. *Environ Health* 2010; **9**: 36-44.

[38] Barak D, Ordentlich A, Bromberg A, Kronman C, Marcus D, Lazar A, et al. Allosteric modulation of acetylcholinesterase activity by peripheral ligands involves a conformational transition of the anionic subsite. *Biochemistry* 1995; **34**: 15444-52.

[39] Simon S, Le Goff A, Frobert Y, Grassi J, Massoulie J. The binding sites of inhibitory monoclonal antibodies on acetylcholinesterase. Identification of a novel regulatory site at the putative "back door". *J Biol Chem* 1999; **274**: 27740-6.

[40] Shi J, Boyd AE, Radic Z, Taylor P. Reversibly bound and covalently attached ligands induce conformational changes in the omega loop, Cys69-Cys96, of mouse acetylcholinesterase. *J Biol Chem* 2001; **276**: 42196-204.