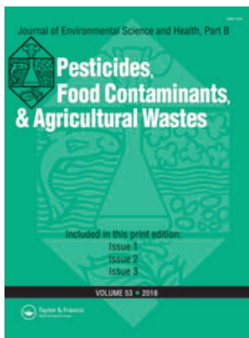

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Reference values for acetyl and butyrylcholinesterases in cattle under actual management conditions, hepatic and renal function by application of chlorpyrifos

Daniela M. Ferré[a,b], Valeria R. Lentini[a], R. Raquel Romano[a], Hector R. Ludueña[a], Paola J. Jotallán[a], and Nora B. M. Gorla[a,b]

[a]Laboratorio de Genética, Ambiente y Reproducción, Universidad Juan Agustín Maza, Guaymallén, Mendoza, Argentina; [b]Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina

ABSTRACT
Chlorpyrifos is an anticholinesterase organophosphate insecticide widely used in Argentina in the production of food derived from animal, fruit and horticultural origin and is reported as a residue within these products. Local reference values for acetyl and butyrylcholinesterase were determined in Aberdeen Angus bovine and cross bred cattle ($n = 25$), a requirement to be able to evaluate toxicity of commercial organophosphate and carbamate formulations. The activity of cholinesterase enzymes presented an overall mean of 2,183.00 ± 485.6 IU L^{-1} for erythrocyte acetylcholinesterase and 203.1 ± 42.06 IU L^{-1} for plasma butyrylcholinesterase, which are used as reference values for meat steers within a system of intensive production in a semi-arid region. The toxic potential of chlorpyrifos in steers of the same breeds ($n = 12$) was assessed applying chlorpyrifos 15.00% Tipertox® in a single therapeutic dose of 7.50 mg kg^{-1} by topical route. Prior to application and then on day 1 and day 21 post-application, both blood cholinesterases, serum chlorpyrifos concentration by ultra-high resolution liquid chromatography with mass detector, analysis of blood counts, total proteins, liver enzymes, urea and creatinine were evaluated. The mean plasma concentration of chlorpyrifos was 27.90 ug L^{-1} at 24 h. The findings indicate that the therapeutic treatment of castrated male bovines treated with chlorpyrifos, applied by pour-on according to the manufacturer's instructions, does not cause changes in the variables evaluated.

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Chlorpyrifos; cattle; chlolinesterases; UHPLC; hematology; renal and liver function

Introduction

The insecticide chlorpyrifos (CPF) (O, O-diethyl-O- [3, 5-trichloro-2-pyridinyl] phosphorothioate) is an organophosphate (OP) widely used in food production.[1] Its presence as food residues has been reported worldwide.[2,3] In Argentina a variety of concentrations and therapeutic presentations are commercialized for use in cattle, chlorpyrifos at 3.3, 5, 10, 12.5 and 15% as liquid for external antiparasitic through pour-on, at 6% as paste, 5% as a spray and CPF 24% liquid concentrate for bathing. It is also combined with other insecticides: CPF 50% + cypermethrin (CYP) 20% and CPF 41.6% + CYP 16% for bathing (http://www.motivar.com.ar/2015/12/listado-aprobado-de -garrapaticidas). At local and regional level our research group reported a 4.2% use of CPF as an external parasiticide in beef cattle production and a 6.2% use in vegetable and fruit production in the same semi-arid region. Additionally, the presence of chlorpyrifos has been confirmed in soils, surface water and groundwater in 3 different areas within the country.[4–6] These results from environmental biomonitoring give evidence that CPF is widely used in livestock and agricultural activities.

The main mechanism of action of CPF is the irreversible inhibition of cholinesterase enzymes (ChEs) in the nervous and blood systems. This results in the presentation of muscarinic and nicotinic effects, which are characteristics of OP and carbamates (CB) intoxication.[7] Vertebrates have two blood ChEs, based on their substrate specificities and inhibitors, acetylcholinesterase which is classified as true or erythrocyte cholinesterase (AChE, EC 3.1.1.7) and butyrylcholinesterase, pseudocholinesterase or plasma cholinesterase (BChE, EC 3.1.1.8). AChE is mainly present in erythrocytes, typically hydrolyzes acetylthiocholine (ATC) and is inhibited by excessive substrate. BChE is mainly present in plasma and generally hydrolyzes butyrylthiocholine at a higher rate than ATC but unlike AChE is not inhibited by high concentrations of substrate.[8]

In cattle, in contrast to what occurs in humans, 90% of enzyme activity is represented by AChE in erythrocytes and has very low plasma BChE values.[9,10] The correct use of the enzymes AChE and BChE as biomarkers of exposure to anticholinesterase compounds requires the procurement of the reference values of the species and population being studied. This is because the ability of the different blood enzymes to hydrolyze the different substrates varies depending on the animal species. In humans, the activity of these enzymes presents not only inter-species variations but also geographic, ethnic and altitudinal variations due to dietary effects, mainly temperature and inter-individual variations due to genetic factors.[11] The above mentioned variability of ChEs enzymes according to species and climatic and environmental conditions indicates that it is necessary to know the reference values in each production

CONTACT Nora B. M. Gorla noragorla@gmail.com; danitasol@hotmail.com CONICET, Universidad Juan Agustín Maza, Acceso Este, lateral Sur 2245, (5519) Guaymallén, Mendoza, Argentina.

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group prior to the application of CPF to be able to provide kinetic studies, new formulations, mixtures and concentrations.

Intoxications of cattle by chlorpyrifos are diagnosed according to the symptoms they present. However, signs are evident when the depletion of ChE activity in blood is greater than 50%.[12,13] When the signs of OP intoxication are not yet observable, the toxic potential can be characterized by the use of biomarkers of exposure and effect. These are the ChE activity,[14,15] blood determination of the active principle[16] and analysis of the effect of biomarkers as determination of hematological parameters and total proteins (TP) in blood circulation.[9] United States Environmental Protection Agency (USEPA) has concluded that ChE inhibition represents the most sensitive effect of CPF in animals and humans.[1] CPF also produces oxidative stress due to the generation of reactive oxygen species, mainly causing lipid peroxidation and thus injury to cell membranes, influencing the synthesis of proteins and affecting macromolecules such as DNA. Another biomarker of exposure is the fragility of erythrocyte membranes that can produce anemia and the degenerative changes in hemoglobin, both biomarkers of oxidative damage.[17,18]

The objectives of this study were twofold: (1) To establish reference values for acetyl and butyrylcholinesterase activity in cattle within an intensive production system of feed lot in a semi-arid region and (2) To evaluate the effect of a therapeutic exposure to chlorpyrifos on the activity of blood cholinesterases, hematological and biochemical parameters indicative of hepatic and renal function, and to analyze a possible association between these and plasma concentrations of the insecticide.

Materials and methods

Validation of the methods of determining acetyl and butyrylcholinesterase activity

Twenty mL of heparinized blood was extracted from a castrated crossbred male bovine and then fractionated to obtain whole blood and plasma to be able to measure AChE and BChE activity, respectively. AChE activity was determined according to the kinetic method of Ellman et al., 1961 modified by Villaamil Lepori et al., 2002 on erythrocytes previously washed with physiological solution and lysed with bidistilled water at a 1:25 dilution;[19,20] Chromogen 5,5-dithiobis-2-nitrobenzoic acid (DTNB Aldrich®) (0.26 mmol L^{-1}) in IFI® phosphate buffer solution at pH 7.7; and acetyl thiocholine iodide solution (CAS No. 1866-15-5 Sigma®). The reading was performed at 405 nm on a Varian Cary UV visible spectrophotometer at 30°C using the Cine soft kinetics program at 0, 30, 60, and 90 s.

The activity of BChE was determined in plasma stored for 48 h at 4–8°C according to Ellman et al.,[19] using the commercial kit GT LAB Cholinesterase in phosphate buffer solution (50 mmol L^{-1} pH 7.7), butyrylthiocholine (7 mmol L^{-1}) (CAS No. 141-75-3) and the chromogen DTNB (0.25 mmol L^{-1}). The spectrophotometric conditions used were the same as for AChE. All determinations were performed in duplicate in the four dilutions tested (6.25, 12.5, 25 and 50%) and by septuplets in the plasma and erythrocyte undiluted samples. The percentage of coefficient of variation (CV) was obtained. For the BChE method, the samples were refrigerated (8°C) for an additional 24 h and the activities were then determined again at under the same conditions. The percentage of CV of this inter-day determination was then calculated.

Cattle testing to determine ChE activity in castrated male bovines in order to obtain regional reference values

The AChE and BChE activities were determined in 25 Aberdeen Angus beef cattle and crossbred steers of between 15 to 18 mth of age. The animals were part of an intensive system of fattening, located at 621 masl in Lavalle (32°43′00″S 68°35′00″W) and were fed on a diet of balanced feed and were supplemented with maize, alfalfa and regional vegetables (potatoes, pumpkin, carrot) and water *ad libitum*. The blood extractions and determinations were performed in 3 groups of animals in different seasonal months: October ($n = 5$ animals), December ($n = 12$) and March ($n = 8$) with mean monthly temperatures of 17.79 ± 3, 09, 25.10 ± 3.85 and 21.61 ± 2.04°C respectively, and with a relative humidity of 52.03 ± 10.34, 40.06 ± 13.99 and 54.74 ± 14.34%.

Cattle testing to evaluate the toxic potential of chlorpyrifos

Twelve Aberdeen Angus and crossbred steers of between 15 and 18 mth of age with good sanitary conditions were used. They were part of an intensive system of fattening and were located at 715 masl in Maipú (Mendoza), 32°58′00″S 68°46′00″W, which had an average temperature of 19.41 ± 2.71°C and relative humidity of 55.83 ± 10.41. The animals were randomly divided into two groups of 6 animals each, a Control Group (CG) with animals weighing 246.80 ± 17.10 kg (media ± standard deviation) and an Exposed Group (EG) with weights of 253.00 ± 26.14 kg. All animals were fed with alfalfa, maize, regional vegetables (potato, sweet potato, and carrot) and balanced feed and had access to drinking water *ad libitum*. The EG animals were given CPF 15.00% Tipertox® at a single therapeutic dose of 7.50 mg kg^{-1} (13 mL per animal) by topical application in pour-on form applied on the back of the animals. Mineral oil was applied to the CG animals in equal volume. Prior to the application of CPF and mineral oil, blood samples were taken from the jugular vein, with heparin, with ethylenediaminetetraacetic acid anticoagulant (EDTA) and without anticoagulant and taken again 1 d and then 21 d after application. The following parameters were then determined; hematocrit (HCT), hemoglobin concentration (Hb), red blood cell (RBC) count, white blood cell (WBC) count, TP, albumin (A), globulins (G), A/G ratio, blood urea and creatinine, transaminase enzyme activities: aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), AChE, BChE. The presence/absence of CPF in the plasma of the animals of both groups were also determined.

The design of the trial was approved by the Institutional Committee for the Care of Laboratory Animals, Experimentation and Teaching (CICUALID) of the University Juan Agustín Maza. The health status of the animals was monitored regularly during the trial period.

Determination of hematology parameters, hepatic and renal function

HCT values were obtained in whole blood without anticoagulant (micro-hematocrit method, Biocap® capillaries of 80 μL). Hb (HCT/3) was estimated according to Dacie and Lewis (2011). WBC and RBC count was performed in blood samples with EDTA in Neubauer's chamber with isotonic sodium chloride solution and Turk's solution respectively.[21] Blood smears were fixed with methanol and stained with Biopur diagnostics® to determine differential WBC count and erythrocytes morphology.[21] The urea values were determined by spectrophotometry (Kit Urea, Berthelot method) and creatinine (Kit creatinine, kinetic method) in plasma as parameters of renal function. TP (Biuret colorimetric method) and albumin (colorimetric method) by the Total Proteins and Albumin Kit; the activity of the enzymes: AST (GOT/ AST Kit, modified UV-IFCC method), ALT (GPT/ALT Kit, modified UV-IFCC method) and ALP (alkaline phosphatase Kit, kinetic method DGKC/SSCC) in serum were quantified as parameters of hepatic functionality. All kits utilized were GT-LAB®.

Determination of chlorpyrifos in plasma by chromatography

The identification and quantification of CPF was performed in plasma extracted at 24 h post CPF application and stored at −8°C for 30 d. A liquid/liquid extraction was performed according to Ageda et al. (2006) modified with 1 mL of plasma, 1 mL of Baker® acetonitrile and then manually shaken for 1 min. It was centrifuged at 1,500 rpm for 10 min and the upper phase was then extracted. The deproteinized supernatant was filtered through cellulose acetate with 0.2 μm pore (Minisart®).[22]

Quantification of chlorpyrifos was performed using ultra-high resolution liquid chromatography with mass detector (UHPLC ms-ms), using a Thermo Scientific Ultimate 3000-TSQ Quantum™ Access Max Triple Quadrupole Mass Spectrometer (Waltham, Mass., USA) chromatograph. The chromatograph was equipped with an Acquity Beh C18 column 2.1 × 100 mm 1.7 μm and an Acquity Beh guard column 2.1 × 5 mm equal pore.[23] The mass detector conditions were as follows: electrospray ion source, spray voltage 4,000 V, vaporizer temperature 300°C, capillary temperature 270°C, sheath gas pressure 20 units, auxiliary gas pressure 10 units, collision gas argon 1.5 m Torr, cycle time 1 s, scan mode multiple reaction monitoring. Thermo TSQ Tune Master Software was used for the identification and quantification of chlorpyrifos. The detection limit was determined as the concentration of the analyte that produced a signal-to-noise ratio of 3.

Statistical analysis

The statistical program GraphPad Prism 6.0 was used. A Linear regression test (Pearson's test) was applied between the ChE activities (AChE and BChE) and the different dilutions of the blood sample. In the studies to establish regional reference values, the Kolmogorov–Smirnov test was applied to verify whether the overall AChE and BChE values followed a normal distribution. An analysis of variance (ANOVA) was then performed with Bonferroni posttest to evaluate if there were significant differences in the two ChE activities among the three groups of animals studied in different months. A Student's test was applied to observe the statistical differences between the global values of AChE and BChE. In the trial to evaluate the toxic potential of CPF, it was determined whether each one of the haematological and biochemical parameters studied followed a normal distribution (Kolmogorov Smirnov) and an ANOVA analysis was performed with Tukey's multiple comparisons between the analyzed variables of the CG and EG in the three sampling times. It was assessed whether there were significant differences between the AChE and BChE activities obtained as reference values and those obtained in the control group of the test to evaluate the toxic potential of CPF. It was then evaluated whether there was a correlation between AChE-BChE activities and plasma CPF concentration in the EG animals, between AChE-BChE activities and hematological parameters, and in AChE-BChE activities between the EG and CG in each of the sampling times used through the Pearson test.

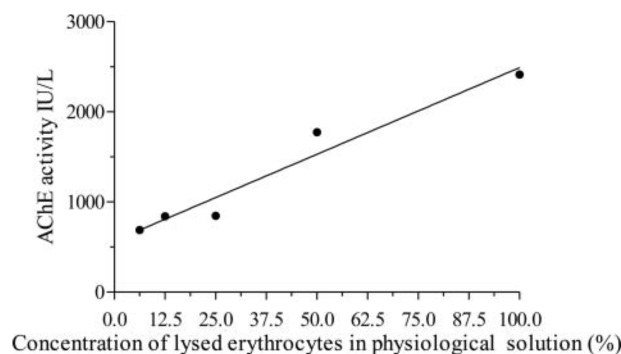

Figure 1. Linearity of the Acetylcholinesterase (IU L^{-1}) in bovine erythrocytes. The determinations were performed at 30°C. $r^2 = 0.9522$. AChE: Acetylcholinesterase.

Results

The linearity of the AChE and BChE blood activities are presented in Figures 1 and 2. The range of dilutions tested from an initial sample of erythrocytes or plasma showed a dilution-enzyme activity for AChE $r^2 = 0.9522$ (Fig. 1) and

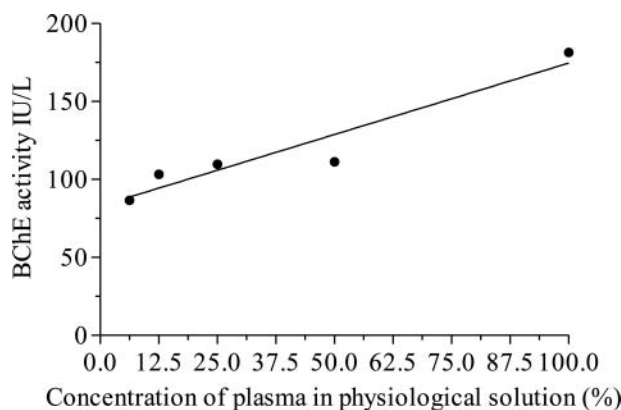

Figure 2. Linearity of the Butyrylcholinesterase (IU L^{-1}) in bovine plasma. The determinations were performed at 30°C. $r^2 = 0.9157$. BChE: Butyrylcholinesterase.

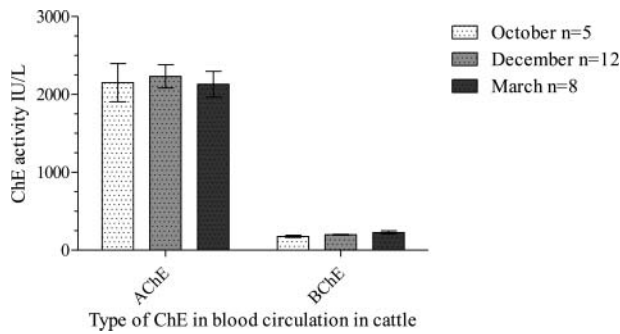

Figure 3. Values of Cholinesterases activity (Means ± SD) in cattle blood of an intensive system of fattening in a semi-arid region, summer season, Argentina. Activities determined in red blood cells (AChE) and plasma (BChE) at 30°C. IU: International Units, ChE: Cholinesterases, AChE: Acetylcholinesterase, BChE: Butyrylcholinesterase.

for BChE $r^2 = 0.9157$ (Fig. 2). The average activities (mean ± SD) of an initial sample analyzed by seven duplicates were 2,414.00 ± 258.1 IU L^{-1} for AChE with CV = 10.69% and 174.40 ± 9.97 IU L^{-1} for BChE with CV = 7.89%. In the evaluation of the stability of BChE activity against an additional cooling of the sample for 24 h, the mean BChE activity was 85.56 ± 1.52 IU L^{-1} for the 1:16 dilution; 98.95 ± 6.15 IU L^{-1} for the 1: 8 dilution; 103.80 ± 8.28 IU L^{-1} for the 1: 4 dilution; 116.00 ± 6.64 IU L^{-1} for the 1: 2 dilution and 174.40 ± 9.97 IU L^{-1} for the undiluted sample with a CV = 5.48%. No significant differences were observed with the BChE activities in the samples without additional refrigeration, the inter-day CV was <8.00%.

In order to establish the regional reference values for AChE and BChE activities in beef cattle the results obtained are shown in Figure 3. The average monthly temperatures and extreme values of the months of October, December and March were: 17.79 ± 3.09°C (11.53 ± 2.72–24.73 ± 4.23); 25.10 ± 3.85°C (17.83 ± 3.46–32.86 ± 4.36) and 21.61 ± 2.04°C (16.14 ± 2.92–27.77 ± 3.27), respectively. Mean relative humidities were 52.03 ± 10.34%; 40.06 ± 13.99% and 54.74 ± 14.34% (National Weather Service). The activities of the enzymes ChE did not present statistically significant differences between the groups and the different months and presented an overall mean of 2,183.00 ± 485.6 IU L^{-1} for erythrocyte AChE and 203.1 ± 42.06 IU L^{-1} for plasma BChE. In the global sample studied, the AChE and BChE activities passed the normality test (alpha = 0.05).

Plasma concentrations of CPF in animals administered with the parasiticide and in the respective controls, ChEs values, hematology, hepatic and renal function are presented in Table 1. The presence of CPF was not detected in the EG animals at 0 h or at 21 d post application. The CPF was quantified by UHPLC-ms ms with a precursor ion 349.9 m/z, collision energy 32 eV and ion product 1 = 96.9 m/z; and collision energy 20 eV and product ion 2 = 197.9 m/z. The limit of detection was 25 ng mL^{-1}. In the control group the absence of CPF in plasma was confirmed in the three sampling times.

Table 1. Biomarkers of exposure and effect in castrated male bovines treated with chlorpyrifos at a therapeutic dose (7.5 mg Kg^{-1}).

	Control Group (*n* = 6) 246.8 ± 17.10 Kg			*Exposed Group CPF 7,5 mg kg^{-1}* (*n* = 6) 253.00 ± 26.14 Kg		
	0 h	*1 d*	*21 d*	*0 h*	*1 d after treatment*	*21 d after treatment*
CPF in plasma (μg L^{-1})	0.00	0.00	0.00	0.00	27.90 ± 11.47	0.00
Cholinesterases						
AChE (IU L^{-1}) *n* = 3	2423.33 ± 97.12	2393.33 ± 41.63	2303.33 ± 137.96	2746.66 ± 557.88	2696.66 ± 682.52	2776.66 ± 446.35
BChE (IU L^{-1}) *n* = 3	234.66 ± 50.00	206.33 ± 13.57	222.66 ± 23.69	196.00 ± 26.23	200.00 ± 3.00	202.33 ± 17.21
Hematology						
HCT (%)	38.16 ± 1.83	38.00 ± 1.54	36.83 ± 2.13	35.33 ± 2.16	35.66 ± 1.21	36.33 ± 2.06
Hemoglobin (g dL^{-1})	12.70 ± 0.63	12.63 ± 0.54	12.25 ± 0.69	11.73 ± 0.72	11.85 ± 0.41	12.08 ± 0.69
RBC (cells μL^{-1})	6.23×10^6 ± 1.30[a]	4.71×10^6 ± 1.16	4.70×10^6 ± 0.60	5.69×10^6 ± 0.05	5.02×10^6 ± 0.54	5.27×10^6 ± 0.82
WBC (cells μL^{-1})	9841.66 ± 1668.65	7891.66 ± 731.72	8683.33 ± 959.51	9433.33 ± 765.28	9191.66 ± 1180.85	8558.33 ± 1779.44
Segmented neutrophils (cells μL^{-1})	4861.00 ± 1300.73[b,c]	3400.83 ± 364.30[b]	3535.16 ± 390.03	3952.16 ± 343.72	3626.50 ± 1034.78	3339.16 ± 627.05[c]
Neutrophils in band (cells μL^{-1})	80.16 ± 74.00	154.83 ± 65.14	274.00 ± 168.42	143.33 ± 84.24	128.16 ± 104.54	211.00 ± 140.14
Lymphocytes (cells μL^{-1})	4146.00 ± 600.18	3567.00 ± 543.82	4166.50 ± 761.42	4566.16 ± 699.74	4589.33 ± 1173.42	4136.33 ± 1413.57
Monocytes (cells μL^{-1})	303.66 ± 228.75[d]	620.33 ± 138.16	645.33 ± 106.46	513.66 ± 198.74	593.00 ± 195.09	691.66 ± 276.85[d]
Eosinophils (cells μL^{-1})	300.16 ± 123.09	148.66 ± 87.79	132.66 ± 104.30	258.00 ± 129.66	254.50 ± 88.59	180.10 ± 75.00
Basophils (cells μL^{-1})	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Renal function						
Urea (mg dl^{-1})	18.80 ± 2.70	18.80 ± 1.70	14.20 ± 3.30	17.30 ± 1.00	17.30 ± 1.60	17.70 ± 4.2
Creatinine (mg dl^{-1})	1.03 ± 0.24	0.92 ± 0.12	1.06 ± 0.21	0.91 ± 0.07	1.00 ± 0.15	1.05 ± 0.35
Liver function						
Total proteins (g dl^{-1})	6.32 ± 0.36	6.09 ± 0.43	6.10 ± 0.37	6.22 ± 0.33	5.95 ± 0.37	5.96 ± 0.50
Albumin (g dl^{-1})	4.31 ± 0.43	4.48 ± 0.38	4.48 ± 0.25	3.81 ± 0.48	4.38 ± 0.47	4.25 ± 0.66
Globulins (g dl^{-1})	2.00 ± 0.17	1.60 ± 0.38	1.62 ± 0.40	2.40 ± 0.70	1.60 ± 0.68	1.71 ± 0.83
Relation A/G	2.14 ± 0.39	2.92 ± 0.75	2.99 ± 1.13	1.79 ± 0.88	3.5 ± 2.24	2.84 ± 2.60
AST (IU L^{-1})	94.83 ± 6.17	106.33 ± 26.84	81.83 ± 34.35	102.50 ± 21.94	111.83 ± 26.09	77.16 ± 49.20
ALT (IU L^{-1})	35.16 ± 4.83	35.66 ± 3.01	27.00 ± 7.82	32.83 ± 11.68	38.50 ± 7.99	26.00 ± 5.76
ALP(IU L^{-1})	429.33 ± 71.64	425.00 ± 104.40	456.16 ± 115.37	446.66 ± 67.90	423.66 ± 42.90	450.83 ± 100.66

All values are Means ± standard deviation, CPF: chlorpyrifos, AChE: acetylcholinesterase, BChE: butyrylcholinesterase, HCT: hematocrit, RBC: red blood cell count, WBC: white blood cell count, A: Albumin, G: Globulins, AST: aspartate aminotransferase, ALT: alanine aminotransferase, ALP: alkaline phosphatase, IU: International Units. Significantly different; [a]in control group between 0 h and 21 d *P* = 0.0494; [b]in control group between 0 h and 1 d *P* = 0.0284; [c]between control and exposed groups at 21 d *P* = 0.0203; [d]between control and exposed groups at 21 d *P* = 0.00225.

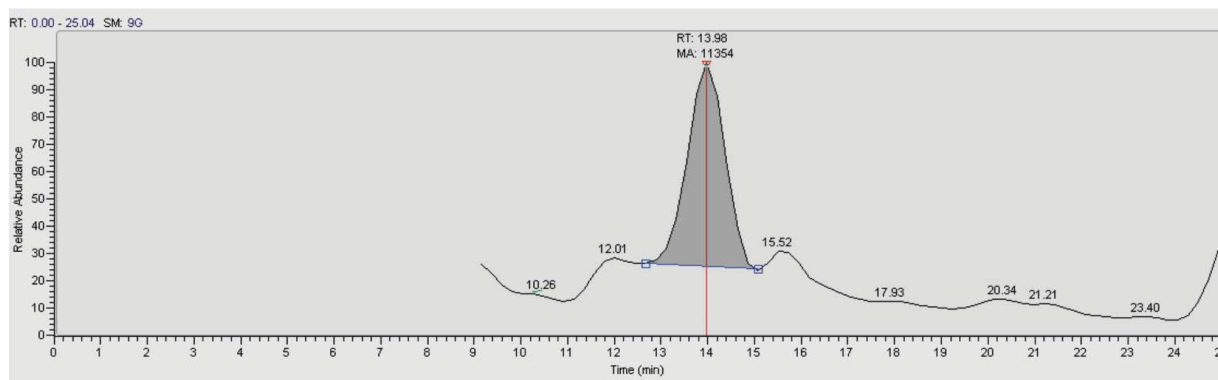

Figure 4. Representative UHPLIC-ms/ms chromatogram of chlorpyrifos (10 ng/mL) sample, transition 1 = 350 →96.90, transition 2 = 197.90 →96.90.

A chromatogram representative of the detection of CPF is presented (Fig. 4).

Positive direct correlation between AChE activity and the RBC count of CG animals at time 0 h (r^2 value = 0.9996) was evidenced. There were no statistically significant differences in ChE activities between CG and EG animals. There was no correlation between ChE activities and CPF concentrations in the exposed animals (r^2 value 0.0010 for AChE and 0.301 for BChE).

Discussion

The values of ChE activities in bovine blood in the 5 concentrations tested, including the undiluted concentration, showed the linearity of the AChE and BChE method. It is important to have regional reference values in the species of domestic animals where anticholinesterase parasiticides OP and CB are used, even more so when they are intimately related to human health through possible residues in food. Seasonal sampling months had a variation of 28.4°C in extreme temperatures and 43.01% in minimum and maximum environmental humidity values, which did not seem to influence the ChE activities obtained, which constitute reference values for meat steers of the region. Tests on animals exposed to OP should include the measurement of blood ChE activities either as a diagnosis in the event of accidental or intentional poisoning of animals or simply to follow the course and intensity of the response to the OP when it has been administered for therapeutic or research purposes. The AChe and BChe are produced by bone marrow and liver respectively and possess different tissue locations. The first is located in the membrane of neurons and erythrocytes and the second in plasma, liver, smooth muscle, erythrocyte membrane, intestine, pancreas, cardiac muscle and nerve tissue. Together they are both inhibited by OP and CB insecticides.[24] A decreased level of ChEs activities in tissues of animal origin is a strong indication that there has been some type of exposure to an inhibitory agent of this enzyme.

In the test to evaluate the toxic potential of CPF, the baseline mean ChEs values in pre-test (0 h), 2,585.00 ± 399.5 for AChE and 215.3 ± 41.52 for BChE had no statistically significant differences from the reference regional values (Fig. 3), $P = 0.07$ for AChE and $P = 0.52$ for BChE. In our study we did not obtain statistically significant differences in the EG either before or after the exposure. Picco et al.[10] used 10% chlorpyrifos at a dose 33% higher than that used in the present investigation, also in steers. In this study, the authors did not obtain ACh and BCH depression at 24 h but at 21 d with 50.77% and 30.92% inhibition, respectively. The basal values of ChE obtained by the authors in the province of Santa Fe were higher than the ranges obtained in the present investigation in the province of Mendoza. These differences may be related mainly to variations in geographic, environmental and genetic factors. Geographically, Santa Fe is located at 25 masl, which is approximately 432 m less than Mendoza which is close to the Argentinean Andes mountain range. Altitude is a non-physiological parameter affecting the erythrocyte count, which is the source of AChE in blood. Consistent with risk assessments for other OPs, USEPA has used a 10% inhibition response of erythrocyte ChE as the cutoff value for predicting acute and chronic exposure for chlorpyrifos.[1] In a herd of Holstein and Guernsey dairy cows, Arrieta et al.[25] found that there was virtually no BChE activity in erythrocytes or plasma, and erythrocyte AChE activity showed a mean activity of 0.89 ± 0.038 umoles min^{-1} mL^{-1}. The mean values of AChE in steers in the present study were 2,585 ± 399.5 IU L^{-1}, more than double the 896 IU L^{-1} equivalent obtained by Arrieta et al.[25] but lower than those obtained by Picco et al.[10] in Holstein steers (10,639.1 ± 1,631.36 IU L^{-1}) of lower body weight (175.57 ± 12.50 kg).

The variability of ChEs enzymes has been reported and can be found in available literature. Biological variability between individual and environmental factors can be confounding factors.[26] A number of environmental variables have been suggested as factors that may interfere with the exact interpretation of AChE activity. In invertebrates, temperature emerges as the most important factor, controlling levels of AChE activity and inhibition. However, using control samples which have had similar environmental temperatures, this source of variability can be eliminated.[26] Additionally, the analytical procedures and the temperatures at which the enzymes are measured also influence the results.[24] This information is generally poorly described so it is difficult to compare the results.[27] According to some authors, erythrocyte ChE accounts for 80–90% of total blood ChE activity.[28] In humans, inhibition of AChE generally is considered a better marker of toxicity, whereas inhibition of

BChE is a more sensitive marker of exposure because it is most effectively inhibited by most OP/CBs including chlorpyrifos, diazinon, and Malathion.[29] In this case study the erythrocyte ChE represented 92.31% of the total activity, therefore, in future toxicity studies by OP in steers we suggest the sole use of AChe as a biomarker of exposure. A greater understanding of the effects of OP on cattle is important because cattle could be exposed to anticholinesterase parasiticides repeatedly in a production period and also be exposed to potential contamination by OP and CB in surface water, pasture or air and could also be associated with fumigations such as the CPF used both in cattle production and in the production of fruits and vegetables within the region.

At 24 h post topical application of CPF in therapeutic doses in steers, plasma concentrations were on average 27.90 $\pm$ 11.47 $\mu g\ L^{-1}$, almost double that reported by other authors in a pharmacokinetic study also in steers.[30] The authors reported that after a CPF 10% dose, extremely low plasma concentrations ($<15\ \mu g\ L^{-1}$) were obtained and that they exhibited non-consistent temporal patterns during the 50 d of the study. The most common use of CPF is through pour on application, it begins on the skin, one of the biological barriers more impermeable to water that represents 10% of the weight in bovines, then continues with the dissolution of the active principle, liberation from the formulation towards the outermost layer of the skin, diffusion within the stratum corneum and then towards the dermis and finally diffusion towards the blood capillaries. Chlorpyrifos is characterized by a high liposolubility which facilitates its distribution on the cutaneous surface, the latter acting as a deposit zone from which the drug is slowly released into the systemic circulation. CPF absorption can be influenced by the blood perfusion and hydration status of the animal.[31] Skin temperature also affects the blood flow within the skin so the rate of drug absorption can have seasonal modifications. In the establishment where the study was conducted there was no shade for the animals and as a consequence the intense sunlight could have reduced CPF availability as the CPF is temperature sensitive but stable to UV light.[13]

None of the animals showed clinical or biochemical adverse effects due to CPF. In Argentina, other researchers used this compound at a concentration of 18% and a recommended dose of 18 mg kg^{-1} and although the manufacturer's recommendations were respected, there were cases of intoxication with a morbidity rate of 100% and mortality of 47%, which affected bulls exclusively, which suggests an association of effect by hormonal status of the animals.[32] In our study steers were used and they showed no toxicity effects, and are therefore an appropriate category for the use of this insecticide.

The HCT, RBC count and Hb values are within the ranges reported as normal according to Teare.[33] These parameters were not modified by exposure to 7.5 mg kg^{-1} CPF at 24 h or 21 d after topical application. In future studies, we propose the evaluation of the effects of a single therapeutic dose of CPF in membranes of blood cells through the measurement of the activity of enzymes indicative of the generation of reactive oxygen. The morphology of the erythrocytes of steers studied was normal (data not shown). The percentages of HCT were higher than those reported by Sidoti[34] for calves, heifers, cows and bulls of Aberdeen Angus breed and mixed cattle from two localities located between 800 and 1,880 masl with HCT 26.55 $\pm$ 2.34% and 29.20 $\pm$ 3.01%, respectively and in the same semi-arid region of this study but were fed from natural pastures. In contrast, HCT percentages are lower than the 43.20 $\pm$ 2.60% reported for other steers Cebu British crosses from Corrientes, a city located at 56 masl;[35] but were similar to those reported for Holstein females of the main dairy region of Argentina (130 m) during spring (HCT 36.57 $\pm$ 2.64).[36] The percentage values of HCT obtained are within the ranges obtained in a group of Holstein steers of 305.00 $\pm$ 28.00 kg (HCT 35.00 $\pm$ 21.00%) and 320.00 $\pm$ 28.00 kg (HCT 36.00 $\pm$ 4.00%) in different summer months with temperatures between 16 to 23°C and 25 to 30°C respectively and at an altitude of 1,500 m from Colorado, USA. These same steers continued to increase their HCT to 41.00 $\pm$ 3.00% as their body weights increased to 407.00 $\pm$ 33.00 kg, together with a decrease in ambient temperature to −7 to 10°C.[37]

Other authors have reported that HCT, RBC count and Hb were increased in calves when they switched to dry food.[38] Meat cattle have higher RBC count than milk cattle, so when indicating reference values it is necessary not only to indicate age, but also the other variables that influence the hematological parameters such as gender, gestation time in females, calving, lactation, diet, body condition, reproductive status, recent activity prior to sampling, degree of hydration, environmental temperature, altitude, therapeutic and prophylactic management. These variables can modulate biological rhythms in domestic animals, such as body temperature, that is modulated by physical activity and metabolic level, which in turn is synchronized with the hours of light exposure and ambient temperature.[39] All of the above justifies the need to establish regional reference values for hematological parameters. At the methodological level, the diameter of erythrocytes of ruminants is lower compared to that of humans, so that cellular counts performed with autoanalyzers may provide false low results.[40] In our study the counts were performed manually. The values of Hb presented are slightly higher than those reported by Sidoti[34] 10.25 $\pm$ 0.99 and 10.39 $\pm$ 1.48 g dl^{-1} for two bovine groups of different categories in areas located at a greater masl and at the end of the winter season and were in that study determined by spectrophotometry whereas those reported in our study were estimated from HCT and were determined in summer, the time of year when the values are higher than in the winter season according to Wilhem.[41]

During a 4-wk period Celik and Suzek[42] reported the presentation of leukocytosis after oral administration of OP methylparation to rats, assigning that effect to the stress conditions of the animals showing an immune system responding to the exposure of the insecticide with an increased mobilization of leukocytes.[42] There are no recent works similar to those produced in cattle. The values presented in the studied steers are within normal parameters according to Teare[33] and no differences were observed after exposure to CPF.

Plasma urea and creatinine values, if studied together over time, are indicators of renal function.[43] Those presented in this study are below those previously reported for cattle by Sidoti,[34] and even blood urea values were slightly lower than those reported as normal according to Teare,[33] which could

be due to decreased hepatic urea synthesis, dehydration or decreased protein catabolism.[43] Pardío et al.,[9] demonstrated that blood urea and TP levels increased in steers exposed to OP cumafos applied topically every 14 and 21 d over a 150-d period.[9,43] In our study there was no increase in TP after a single dose of CPF. HCT values, TP values and increased A/G ratio could reflect a mild dehydration in the animals under study, although clinical signs of dehydration were not detected. In addition, this mild dehydration state could influence the blood perfusion and hydration status of the skin, which reduces the absorption of CPF.[31]

Signs of liver toxicity with mild diffuse granular degeneration were observed in mice given daily oral doses of 60 mg kg^{-1} body weight (LD_{50}/80th) CPF for 2 wk which progressed to vacuolar degeneration of hepatic tissue when exposure continued up to 8 wk, in addition to renal toxicity with degeneration and necrosis of tubular epithelium and glomerular congestion.[44] Similar investigations are not available in cattle. In our study, there were no differences between urea and creatinine values measured before and after the application of CPF to a single topically applied dose of 7.5 mg kg^{-1} body weight. Liver and kidney histopathological changes demonstrated by Sharma in mice were accompanied by increased ALT and AST values and depressed AChE as the time of exposure to CPF increased.[44] The administration of sublethal doses of OP methylparation (oral) during a subacute time period to rats caused an increase in the serum enzymes AST, ALT and ALP, indicative of hepatotoxicity.[42] In our study the enzymatic activities of AST and ALT did not undergo significant modifications so it can be assumed that there was no cell injury of the hepatocytes, effects on the permeability of the plasma membranes, nor hepatic cellular necrosis since the ALP values did not increase either. It is extremely important to know the physiological values to be able to distinguish the pathological conditions.[45] The adaptation of cattle to different environmental factors, including nutrition, is accompanied by intense morphological, functional and biochemical changes.[46,47]

The regional reference values presented were obtained in a beef cattle population in a semi-arid region and can be used to improve the diagnosis and treatment of intoxications caused by the preventive or therapeutic use of organophosphate or carbamate parasiticides in this type of production. The usual therapeutic treatment of steers with chlorpyrifos in topical form does not cause immediate detectable changes in hematological parameters, hepatic and renal function, nor in the enzymatic activities acetyl and butyrylcholinesterase.

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