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Electrometric Determination of Erythrocyte, Plasma and Whole Blood Cholinesterase Activities in Sheep, Goats and Cattle and Their *in vitro* Inhibition by Anticholinesterase Insecticides

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Abstract: Determination of blood cholinesterase activity in animals is used for diagnosis and monitoring poisoning induced by organophosphate and carbamate insecticides. An electrometric method was applied in one step single incubation period (30 min) for measurement of normal reference range values of erythrocyte, plasma and whole blood cholinesterase activities in sheep, goats and cattle, of both sexes. The reaction mixture contained 3 mL distilled water, 3 mL barbital-phosphate buffer (pH 8.1), 0.2 mL erythrocytes, plasma or the whole blood and 0.1 mL acetylcholine iodide (7.5%) as a substrate. The mixture was incubated at 37°C for 30 min in the three animal species. This step avoided variations in enzyme activity across animal species due to differences in the incubation periods found in previous studies. The pH of the reaction mixture was determined by a pH meter before and after the incubation. The initial pH was measured before the substrate addition. The unit of enzyme activity was expressed as Δ pH/30 min. The mean normal reference range values of erythrocytes, plasma and whole blood cholinesterase activities (Δ pH/30 min) in males of the three animal species were as follows, respectively: Sheep (0.306, 0.133 and 0.249), goats (0.366, 0.135 and 0.234) and cattle (0.469, 0.135 and 0.374) whereas those of the females were as follows, respectively: Sheep (0.436, 0.121 and 0.257), goats (0.338, 0.175 and 0.252) and cattle (0.645, 0.137 and 0.450). The method of inhibitor-cholinesterase incubation was used to measure the *in vitro* inhibition of plasma, erythrocyte and whole blood cholinesterase activities by the organophosphate insecticides chlorpyrifos and methidathion and by the carbamate insecticide carbaryl. Chlorpyrifos in concentrations of 0.5 and 1 μM inhibited plasma, erythrocyte and whole blood cholinesterase activities by 5-30% in sheep, 10-55% in goats and 5-61% in cattle. Methidathion in concentrations of 0.5 and 1 μM inhibited plasma, erythrocyte and whole blood cholinesterase activities by 3-70% in sheep, 8-53% in goats and 6-65% in cattle. Carbaryl in concentrations of 4 and 8 μM inhibited them by 8-54% in sheep, 8-53% in goats and 14-74% in cattle. The study establishes for the first time by using the described electrometric method in a unified way across the three ruminant species sheep, goats and cattle, normal reference range values of erythrocytes, plasma and whole blood cholinesterase activities. The results also suggest that the described electrometric method could be efficiently used for detecting cholinesterase inhibition by organophosphate and carbamate insecticides. Further, the experimental protocol of *in vitro* cholinesterase inhibition is of value in preliminary toxicological examinations of anticholinesterase pesticides.

Key words: Cholinesterase, electrometric method, ruminant, organophosphate, carbamate, insecticide

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INTRODUCTION

Determination of blood cholinesterase activity in animals is used for diagnosis and monitoring poisoning induced by organophosphate and carbamate insecticides (Halbrook *et al*., 1992; Wilson, 1998; Wilson *et al*., 1998; Pardio *et al*., 2001). Various electrometric methods are available for the determination of blood cholinesterase activity in man and animals (Wills, 1972; Mohammad and St. Omer, 1982; Imerman, 1993; Munro *et al*., 1999; Wilson, 1999). All electrometric methods are based on the hydrolysis of the substrate (acetylcholine) in the enzymatic reaction mixture and production of acetic acid which in turn decreases the pH of the reaction mixture (Mohammad and St. Omer, 1982; Imerman, 1993; Munro *et al*., 1999; Wilson, 1999). The unit of cholinesterase activity is expressed as Δ pH/incubation time, e.g., 30 min (Mohammad and St. Omer, 1982; Wilson, 1999). The original electrometric method of Michel is most commonly used in man (Wilson, 1999). However, the method cannot be applied in animals because of the inherent variations in blood or tissue cholinesterase activities of different animal species that require different buffers, reaction temperatures, incubation times and sample volumes (Wills, 1972; Mohammad and St. Omer, 1982; Wilson, 1999; Al-Qarawi and Ali, 2003; Mohammad *et al*., 2005). In addition, the original electrometric method is not recommended for detection of cholinesterase inhibition caused by carbamate insecticides since carbamylated enzyme undergoes reactivation in the reaction mixture of the electrometric method of Michel because of considerable sample dilution and long incubation time (totally>60 min) (Osweiler *et al*., 1985; Nostrandt *et al*., 1993).

The modified electrometric method of Mohammad *et al*. (1997) was introduced for rapid measurement of erythrocyte and plasma cholinesterase activities in sheep and other ruminants (Al-Jobory and Mohammad, 2005; Mohammad *et al*., 2005; 2006a, b). The method has also been used in non ruminants such as mice (Ahmed and Mohammad, 2005), rats (Mohammad *et al*., 2002), chickens (Mohammad and Al-Baggou, 2005), wild birds (Alias and Mohammad, 2005) as well as man (Ahmed and Mohammad, 2005). The method is simple, reproducible, accurate and cheap and can be used to detect cholinesterase inhibition induced by organophosphate and carbamate insecticides in animals (Mohammad *et al*., 2002; Ahmed and Mohammad, 2005; Mohammad and Al-Baggou, 2005; Mohammad *et al*., 2006b). Recent studies from our laboratory reported plasma and erythrocyte cholinesterase activities in sheep, goats and cattle using the presently described electrometric method but with different incubation times for the three animals species (Al-Jobory and Mohammad, 2005; Mohammad *et al*., 2005; 2006a). These incubation times were 20, 30 and 40 min for the cattle, sheep and goats, respectively (Mohammad *et al*., 2005). A single incubation time of thirty minutes is suitable for the three ruminants as blood cholinesterase activity lies in the linear portion of the enzyme activity vs. time curves (Al-Jobory and Mohammad, 2005; Mohammad *et al*., 2005).

Organophosphate or carbamate insecticides are widely used in ruminants against ectoparasites. Measuring blood cholinesterase activities of ruminants may be used as a biomarker of environmental contamination with pesticides (Munro *et al*., 1991; Halbrook *et al*., 1992). The technique of *in vitro* ChE inhibition by organophosphates and carbamates has various toxicological implications and can also be used to assess the potential toxicity of anti cholinesterase compounds (Iyaniwura, 1990; Khan *et al*., 1990; Karanth and Pope, 2003; Al-Jobory and Mohammad, 2005; Mohammad *et al*., 2006a).

The purpose of the present study was to establish normal reference range values of blood (erythrocytes, plasma and whole blood) cholinesterase activities in sheep, goats and cattle using the described electrometric method with only a single 30 min incubation period. Using one-step 30 min incubation period allows enzyme activities in ruminants to be compared easily and in a meaningful manner as they all share low plasma and relatively high erythrocyte cholinesterase activities (Mohammad and St. Omer, 1982; Osweiler *et al*., 1985; Mohammad *et al*., 2005).

MATERIALS AND METHODS

Venous blood samples of 1-2 years old domestic mixed breeds of sheep, goats and cattle of both sexes were obtained at the slaughterhouse and from local herds in Mosul region in Iraq. The animals were apparently healthy and not exposed to any insecticide for at least two weeks before blood sampling. Blood samples were collected using heparinized test tubes (Coles, 1986). Plasma was separated from erythrocytes by centrifugation at 3000 rpm (Centurion, U.K.) for 15 min.

The modified electrometric method of Mohammad *et al*. (1997) was used to measure blood cholinesterase activities of the sheep, goat and cattle. The reaction mixture in a 10 mL glass container contained 3.0 mL distilled water, 0.2 mL erythrocytes, plasma or whole blood and 3.0 mL barbital-phosphate buffer solution (pH = 8.1). The pH of the mixture (pH 1) was measured with a glass electrode using a pH meter (Hanna Instruments, Romania). This step was followed by the addition of 0.1 mL of 7.5% aqueous solution of acetylcholine iodide (BDH, U.K) to the mixture, which was then incubated at 37°C for 30 min. At the end of the incubation period, the pH of the reaction mixture (pH 2) was measured. The enzyme activity was calculated as follows:

Cholinesterase Activity (Δ pH/30 min) = (pH1-pH 2) - Δ pH of Blank

The blank was without the blood sample. The pH 8.1-buffer solution consisted of 1.24 g sodium barbital (BDH, U.K.), 0.63 g potassium dihydrogen phosphate (E-Merck, Germany) and 35.07 g sodium chloride (BDH) dissolved in one liter of distilled water (Mohammad *et al*., 1997).

Plasma, erythrocyte or whole blood samples of 4-5 animals from each species regardless of the sex were pooled for cholinesterase inhibition experiments. The method of inhibitor- cholinesterase incubation was used to measure the *in vitro* inhibition of plasma, erythrocyte and whole blood cholinesterase activities (Mohammad *et al*., 1997; Karanth and Pope, 2003; Mohammad *et al*., 2005) by chlorpyrifos (40%, VAPCO, Jordan) and methidathion (50%, Agricultural Chemicals Manufacturing Enterprise, Jordan) as well as by carbaryl (85%, Sociedad Anonima De Agroquimicos, Spain). Chlorpyrifos and methidathion are organophosphate insecticides, whereas carbaryl is a carbamate one. The insecticides were prepared in distilled water and individually added in a volume of 0.1 mL to the reaction mixtures of the plasma, erythrocytes or whole blood. The final concentrations of chlopyrifos and methidathion in the reaction mixtures were 0.5 and 1 μM, whereas those of carbaryl were 4 and 8 μM. Control reaction mixtures did not contain any insecticide and used for measurement of base-line cholinesterase values. The reaction mixtures were incubated at 37°C for 10 min (Mohammad *et al*., 1997; Mohammad *et al*., 2005). Thereafter, the residual cholinesterase activity in the mixtures was measured as before.

The mean, standard deviation, standard error, range and 95% confidence interval of erythrocyte, plasma and whole blood cholinesterase activities were determined (Petrie and Watson, 1989). One way analysis of variance was used to determine the statistical difference between plasma, erythrocyte and whole blood cholinesterase activities within each animal species followed by the least significant difference test (Petrie and Watson, 1989). The level of significance was at $p<0.05$. The % of enzyme inhibition was calculated as follows:

% cholinesterase inhibition = [cholinesterase activity (without insecticide) - cholinesterase activity (with insecticide)/cholinesterase activity (without insecticide)] X 100

RESULTS

Table 1-3 show the normal reference range values, 95% confidence interval and related statistics for erythrocyte, plasma and whole blood cholinesterase activities of the sheep, goats and cattle, respectively. The mean normal reference range values of erythrocytes, plasma and whole blood

Table 1: Normal reference range values of blood cholinesterase activities (Δ pH/30 min) in sheep

	Erythrocyte		Plasma		Whole blood	
Parameters	Male	Female	Male	Female	Male	Female
n	100	98	100	97	100	98
Mean	0.306	0.436*	0.133†	0.121†	0.249†[a]	0.257†[a]
SD	0.114	0.093	0.073	0.053	0.105	0.069
SE	0.011	0.009	0.007	0.005	0.011	0.007
Minimum	0.10	0.24	0.02	0.02	0.09	0.12
Maximum	0.57	0.66	0.38	0.22	0.51	0.46
Range	0.47	0.42	0.36	0.20	0.42	0.24
95% CI	0.28, 0.33	0.42, 0.45	0.12, 0.15	0.11, 0.13	0.23, 0.27	0.24, 0.27

n: Number of animals; SD: Standard deviation; SE: Standard error; CI: Confidence interval. * Significantly different from the corresponding male value, $p<0.05$. † Significantly different from the corresponding erythrocyte cholinesterase value, $p<0.05$. [a] Significantly different from the corresponding plasma cholinesterase value, $p<0.05$

Table 2: Normal reference range values of blood cholinesterase activities (Δ pH/30 min) in goats

	Erythrocyte		Plasma		Whole blood	
Parameters	Male	Female	Male	Female	Male	Female
n	59	53	59	53	59	53
Mean	0.366	0.338*	0.135†	0.175*†	0.234†[a]	0.252†[a]
SD	0.084	0.093	0.072	0.072	0.058	0.071
SE	0.010	0.011	0.009	0.010	0.008	0.010
Minimum	0.20	0.19	0.02	0.01	0.12	0.12
Maximum	0.56	0.47	0.35	0.39	0.34	0.50
Range	0.36	0.28	0.33	0.38	0.22	0.48
95% CI	0.35, 0.39	0.32, 0.36	0.12, 0.15	0.16, 0.19	0.22, 0.25	0.23, 0.27

n: Number of animals; SD: Standard deviation; SE: Standard error; CI: Confidence interval. * Significantly different from the corresponding male value, $p<0.05$. † Significantly different from the corresponding erythrocyte cholinesterase value, $p<0.05$. [a] Significantly different from the corresponding plasma cholinesterase value, $p<0.05$

Table 3: Normal reference range values of blood cholinesterase activities (Δ pH/30 min) in cattle

	Erythrocyte		Plasma		Whole blood	
Parameters	Male	Female	Male	Female	Male	Female
n	103	43	103	43	103	43
Mean	0.469	0.645*	0.135†	0.137†	0.374†[a]	0.450*†[a]
SD	0.160	0.092	0.076	0.071	0.142	0.075
SE	0.016	0.014	0.007	0.011	0.014	0.011
Minimum	0.18	0.43	0.02	0.02	0.13	0.25
Maximum	0.88	0.90	0.33	0.32	0.81	0.64
Range	0.70	0.47	0.31	0.30	0.68	0.39
95% CI	0.44, 0.50	0.62, 0.67	0.12, 0.15	0.12, 0.16	0.35, 0.40	0.43, 0.47

n: Number of animals; SD: Standard deviation; SE: Standard error; CI: Confidence interval. * Significantly different from the corresponding male value, $p<0.05$. † Significantly different from the corresponding erythrocyte cholinesterase value, $p<0.05$. [a] Significantly different from the corresponding plasma cholinesterase value, $p<0.05$

cholinesterase activities (Δ pH/30 min) in males of the three animal species were as follows, respectively: sheep (0.306, 0.133 and 0.249), goats (0.366, 0.135 and 0.234) and cattle (0.469, 0.135 and 0.374), whereas those of the females were as follows, respectively: sheep (0.436, 0.121 and 0.257), goats (0.338, 0.175 and 0.252) and cattle (0.645, 0.137 and 0.450).

Erythrocyte cholinesterase activity of the females was significantly ($p<0.05$) higher than that of the males in sheep (Table 1) and cattle (Table 3) but lower than that of the males in goats (Table 2). Plasma cholinesterase activity of the females was significantly higher than that of the males in goats (Table 3). Plasma and whole blood cholinesterase activities of the males and females within each animal species were significantly lower than those of the erythrocytes, with the lowest enzyme activity seen in the plasma component of the blood (Table 1-3). The combined frequency distributions of male and female blood cholinesterase activities of the three ruminants are shown in Fig. 1-3.

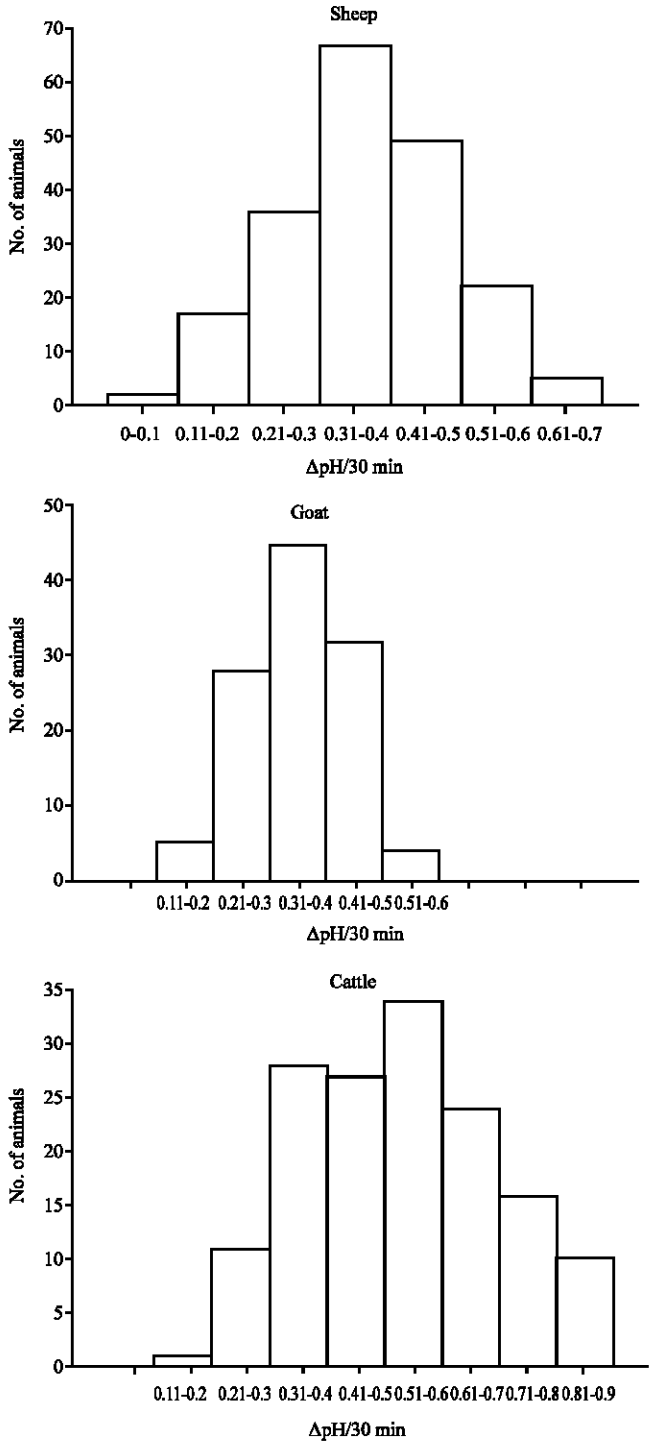

Fig. 1: Frequency distribution of erythrocyte cholinesterase activities in male and female sheep, goats and cattle

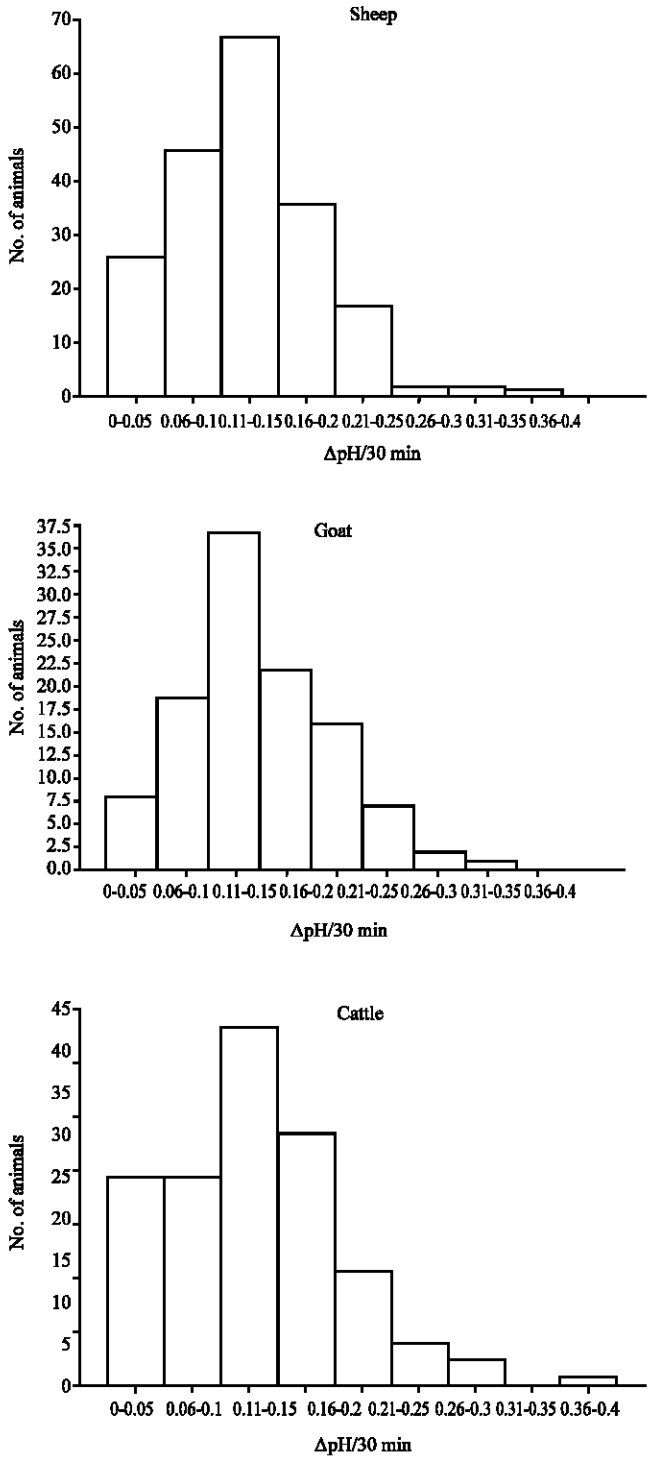

Fig. 2: Frequency distribution of plasma cholinesterase activities in male and female sheep, goats and cattle

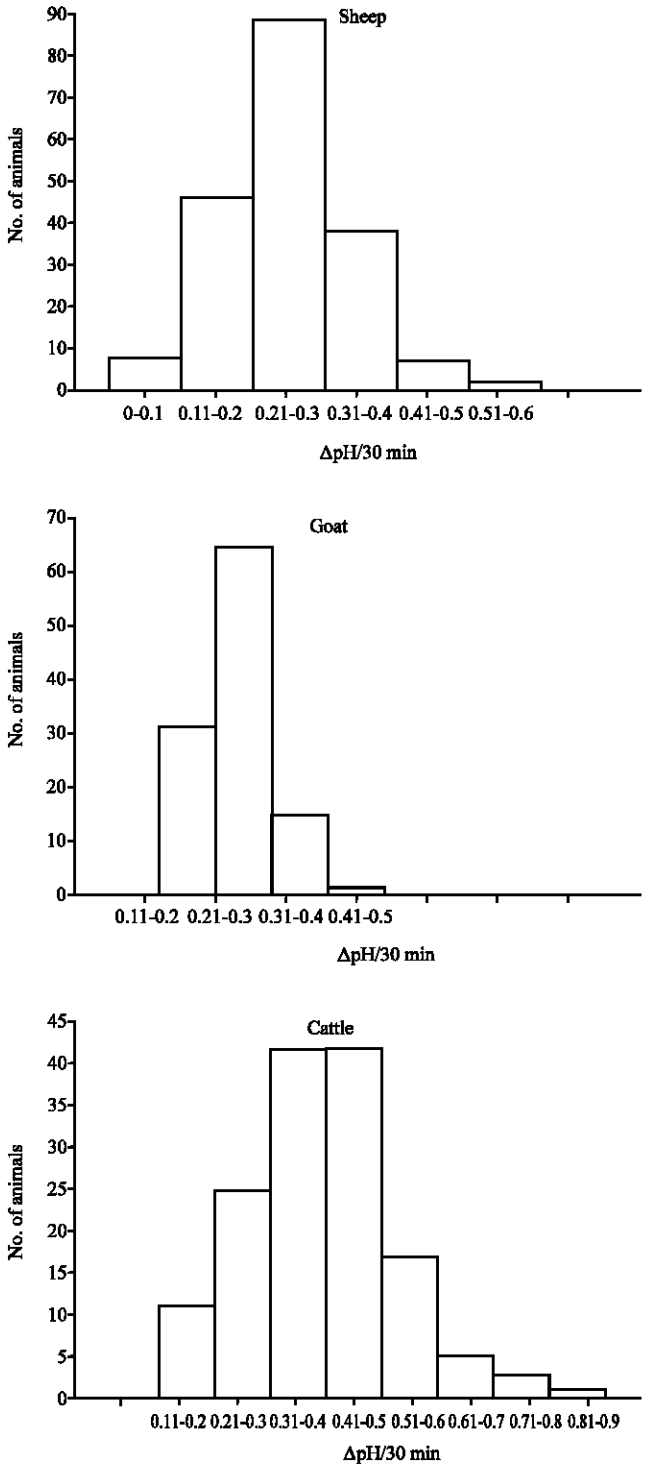

Fig. 3: Frequency distribution of whole blood cholinesterase activities in male and female sheep, goats and cattle

Table 4-6 show *in vitro* inhibition of plasma, erythrocyte and whole blood cholinesterase activities in sheep, goats and cattle, respectively by the insecticides chlorpyrifos, methidathion and carbaryl. Chlorpyrifos in concentrations of 0.5 and 1 μM inhibited plasma, erythrocyte and whole blood cholinesterase activities by 5-30% in sheep, 10-55% in goats and 5-61% in cattle. Methidathion in concentrations of 0.5 and 1 ìM inhibited plasma, erythrocyte and whole blood cholinesterase activities by 3-70% in sheep, 8-53% in goats and 6-65% in cattle, whereas carbaryl in concentrations of 4 and 8 μM inhibited them by 8-54% in sheep, 8-53% in goats and 14-74% in cattle.

Table 4: *In vitro* inhibition of sheep cholinesterase activities in the plasma, erythrocytes and whole blood by chlorpyrifos, methidathion and carbaryl

Inhibitor concentration (μM)	Plasma		Erythrocytes		Whole blood	
	Δ pH/30 min	% inhibition	Δ pH/30 min	% inhibition	Δ pH/30 min	% inhibition
No inhibitor (distilled water-control)						
	0.10±0.005		0.61±0.056		0.37±0.024	
Chlorpyrifos						
0.5	0.09±0.013	10	0.53±0.032	13	0.35±0.015	5
1.0	0.07±0.010	30	0.51±0.013	16	0.30±0.030	19
Methidathion						
0.5	0.06±0.015	40	0.52±0.036	15	0.36±0.013	3
1.0	0.03±0.008	70	0.48±0.027	21	0.33±0.022	11
Carbaryl						
4	0.09±0.015	10	0.40±0.027	34	0.34±0.027	8
8	0.08±0.008	20	0.28±0.077	54	0.22±0.016	41

Cholinesterase activity values are mean±SE, n = 4/each concentration

Table 5: *In vitro* inhibition of goat cholinesterase activities in the plasma, erythrocytes and whole blood by chlorpyrifos, methidathion and carbaryl

Inhibitor concentration (μM)	Plasma		Erythrocytes		Whole blood	
	Δ pH/30 min	% inhibition	Δ pH/30 min	% inhibition	Δ pH/30 min	% inhibition
No inhibitor (distilled water-control)						
	0.13±0.018		0.51±0.015		0.40±0.024	
Chlorpyrifos						
0.5	0.10±0.011	23	0.46±0.025	10	0.24±0.023	40
1.0	0.09±0.016	31	0.44±0.014	14	0.18±0.025	55
Methidathion						
0.5	0.12±0.016	8	0.41±0.011	20	0.26±0.011	35
1.0	0.07±0.011	46	0.38±0.009	25	0.19±0.032	53
Carbaryl						
4	0.10±0.019	23	0.40±0.013	22	0.30±0.020	25
8	0.07±0.012	46	0.37±0.029	27	0.21±0.006	48

Cholinesterase activity values are mean±SE, n = 4/each concentration

Table 6: *In vitro* inhibition of cattle cholinesterase activities in the plasma, erythrocytes and whole blood by chlorpyrifos, methidathion and carbaryl

Inhibitor concentration (μM)	Plasma		Erythrocytes		Whole blood	
	Δ pH/30 min	% inhibition	Δ pH/30 min	% inhibition	Δ pH/30 min	% inhibition
No inhibitor (distilled water-control)						
	0.31±0.043		0.70±0.019		0.59±0.019	
Chlorpyrifos						
0.5	0.17±0.008	45	0.63±0.021	10	0.56±0.044	5
1.0	0.12±0.015	61	0.57±0.068	19	0.44±0.085	25
Methidathion						
0.5	0.16±0.030	48	0.66±0.025	6	0.55±0.028	7
1.0	0.11±0.017	65	0.61±0.010	13	0.46±0.029	22
Carbaryl						
4	0.11±0.031	65	0.48±0.023	31	0.51±0.018	14
8	0.08±0.009	74	0.39±0.033	44	0.37±0.039	37

Cholinesterase activity values are mean±SE, n = 4/each concentration

DISCUSSION

Normal reference range values of erythrocyte, plasma and whole blood cholinesterase activities of male and female sheep, goats and cattle are collectively reported in the present study for the first time using the described electrometric method in a unified way with a single 30 min incubation period. These values could be reference points for future studies involving diagnosis of organophosphate or carbamate poisoning in ruminants or monitoring exposure of such animals to anticholinesterase pesticides. Measurement of blood cholinesterase activities in animals is a non-invasive method for monitoring exposure to organophosphate and carbamate insecticides (Mohammad *et al*., 1982; Halbrook *et al*., 1992; Wilson, 1998; Pardio *et al*., 2001). However, it should be stressed that these cholinesterase values reported in the present study, though of healthy animals, are in no way substitutes for control values that should be obtained from the same locality in which poisoning might occur (Osweiler *et al*., 1985; Halbrook *et al*., 1992; Wilson, 1998; Pardio *et al*., 2001).

Plasma cholinesterase values were significantly lower than those of the erythrocytes and whole blood within each animal species. The relatively low cholinesterase activity in the whole blood compared with that of the erythrocytes is thus attributable to the diluting effect of the plasma. Lower plasma cholinesterase activity in ruminants has been reported by others using electrometric or colorimetric methods (Silvestri, 1977; Mohammad and St. Omer, 1982; Al-Qrawi and Ali, 2003; Mohammad *et al*., 2005). This finding is in contrast to those reported in man and non ruminant animal species such as rodents and horses as they have high plasma cholinesterase activity (Wills, 1972; Wilson, 1999; Ahmed and Mohammad, 2005). However, as found in the present study, sex differences in blood cholinesterase activities should not be excluded (Wills, 1972; Wilson, 1999). The differences in blood cholinesterase activities among animal species as well as between sexes could be considered normal physiological differences (Wills, 1972; Wilson, 1999) and they might form the basis of the differential and blood-fraction dependent variations in sensitivity to organophosphate and carbamate insecticides (Wills, 1972; Osweiler, 1985; Wilson, 1999).

The present electrometric method described for measurement of blood cholinesterase activities in the three ruminants depended mainly on the modifications introduced earlier in sheep (Mohammad *et al*., 1997) and then expanded to include other ruminants, but with different incubation periods for the enzymatic reaction mixture (Mohammad *et al*., 2005; 2006a, b). The present study unified this difference by introducing only a single incubation period of 30 min, thus allowing a direct comparison of blood fraction cholinesterase activities across the three animal species. The 30 min one step incubation time and the 0.2 mL sample volume appeared to be suitable for the assay conditions in the three animal species to measure the enzyme activity without interference with the buffering capacity in the reaction mixture. The results of the present study further add to and expand, by reporting whole blood cholinesterase activities, previous studies in ruminants that advocated the described electrometric for measurement of blood cholinesterase activities (Mohammad *et al*., 1997; Mohammad *et al*., 2005; 2006a, b; Al-Jobory and Mohammad, 2005). The method has been also applied successfully for the determination of blood or tissue cholinesterase activities in other animal species exposed to anticholinesterase insecticides (Ahmed and Mohammad, 2005; Mohammad *et al*., 2002; Mohammad and Al-Baggou, 2005).

The *in vitro* inhibition of plasma, erythrocyte and whole blood cholinesterase activities by the organophosphate insecticides chlorpyrifos and methidathion and by the carbamate insecticide carbaryl in the three animal species are in agreement with the reported anticholinesterase effects of these insecticides in various animal species including the ruminants (Abdelsalam, 1987; Iyaniwura, 1990; Khan *et al*., 1990; Long *et al*., 2003; Wilson, 1998; Karanth and Pope, 2003; Al-Jobory and Mohammad, 2005; Mohammad *et al*., 2006a). The present findings suggest the sensitivity of the described method in detecting cholinesterase inhibition caused by organophosphate or carbamate

insecticides. *In vitro* cholinesterase inhibition is a useful technique for detecting the potential anticholinesterase activity of chemicals (Iyaniwura, 1990; Khan *et al*., 1990; Karanth and Pope, 2003; Al-Jobory and Mohammad, 2005; Mohammad *et al*., 2006a).

In conclusion, the present study reports normal range values of erythrocyte, plasma and whole blood cholinesterase activities in sheep, goats and cattle as described by a simple and efficient electrometric method. The described electrometric method could be efficiently used for detecting cholinesterase inhibition in ruminants exposed to anticholinesterase insecticides and further point out the value of *in vitro* blood cholinesterase inhibition in preliminary toxicological examinations of insecticides with potential therapeutic applications in ruminants.

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