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Validation of an electrometric blood cholinesterase measurement in goats

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A modified electrometric method was described and validated for measurement of plasma and erythrocyte cholinesterase activities in 6~18 months old goats. The enzymatic reaction mixture contained 3 ml distilled water, 3 ml barbital-phosphate buffer (pH 8.1), 0.2 ml plasma or erythrocytes and 0.1 ml acetylthiocholine iodide (7.5%) as a substrate. The mixture was incubated at 37°C for 40 minutes. 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 enzyme activity was expressed as ΔpH/40 min. The coefficients of variation of the described method in measuring plasma and erythrocyte cholinesterase activities were 4 and 2%, respectively. Preliminary reference values (n = 14) of the mean cholinesterase activity (ΔpH/40 min) and 95% confidence interval in the plasma were 0.194 and 0.184~0.204, respectively, and those of the erythrocytes were 0.416 and 0.396~0.436, respectively. The pseudocholinesterase activity of the plasma cholinesterase was 63.5% as determined by quinidine sulfate inhibition. The organophosphorus insecticides dichlorvos and diazinon at 0.5~4 μM and the carbamate insecticide carbaryl at 5~20 μM in the reaction mixture significantly inhibited plasma (13.7~85.5%) and erythrocyte (16.4~71.9%) cholinesterases *in vitro* in a concentration-dependent manner. The results suggest that the described electrometric method is simple, precise and efficient in measuring blood cholinesterase activity in goats.

Key words: carbaryl, cholinesterase, diazinon, dichlorvos, goat, organophosphate

Introduction

Determination of erythrocyte and plasma cholinesterase (ChE) activity is used to monitor exposure to organophosphate (OP) or carbamate insecticides [7,19,20]. One of the principle methods for measuring blood ChE activity is the electrometric method which is based on production of acetic acid that decreases the pH of the reaction mixture [18,19]. The original electrometric method of Michel [10] is commonly used in man [7,19]. However, the method is not directly applicable to samples of different animal species [7,18,19]. This is because of the inherent variations in blood ChE activities between different animal species [1,4,5,13, 18,19] and the special need for different buffer compositions, reaction temperatures, incubation times and sample volumes [5,12,13,17,18].

Various modifications of the electrometric method are available for measuring blood ChE activity in animals [12,13,17,18,19]. These modifications include increasing sample volume, increasing or decreasing incubation time, increasing incubation temperature or using buffers of different compositions. A modified electrometric method was introduced for rapid measurement of erythrocyte and plasma ChE activities in sheep [12]. It is characterized by its simplicity and one-step short incubation time (30 min) [12]. The described electrometric method correlates well with the original electrometric method and with the spectrophotometric method [2,12]. The method is based on measurement of the decrease in pH of the enzymatic reaction mixture as a result of hydrolysis of the substrate acetylcholine iodide or acetylthiocholine iodide and the production of acetic acid [2,12]. However, the application of the method in other ruminant species needs validation before clinical application. The purpose of the present study was to examine the applicability of the technique in measuring blood (plasma and erythrocytes) ChE activities in goats taking into consideration the accuracy, reproducibility and specifications of the method.

Materials and Methods

Domestic goats (*Capra hircus*, 6~18 months old) were used in the study. All experiments complied with regulations addressing animal use, and proper attention has been given to ethical consideration towards the goats used in the present study. 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

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test tubes [6]. Plasma was separated from blood by centrifugation at 3,000 rpm (Centurion, UK) for 15 min.

Electrometric procedure for measurement of plasma and erythrocyte ChE activities

The modified electrometric method of Mohammad *et al*. [12] was used to measure blood ChE of the goats. For a typical assay, the reaction mixture in a 10 ml beaker contained 3 ml distilled water, 0.2 ml plasma or erythrocytes and 3 ml of pH 8.1 buffer solution. The pH of the mixture (pH1) was measured with a glass electrode using a pH meter (Phillips, UK), and then 0.1 ml of 7.5% aqueous solution of acetylthiocholine iodide (BDH, UK) was added to the mixture. The reaction mixture was incubated at 37°C for 40 min. At the end of the incubation period, the pH of the reaction mixture (pH2) was measured. The enzyme activity was calculated as follows:

ChE activity (ΔpH/40 min) = (pH1 – pH2) – ΔpH of blank

The blank was without plasma or erythrocytes. The pH 8.1 buffer solution consisted of 1.237 g sodium barbital (BDH, UK), 0.63 g potassium dihydrogen phosphate (Merck, Germany) and 35.07 g sodium chloride (BDH, UK) dissolved in one liter of distilled water [12].

Preliminary experiments using pooled plasma or erythrocyte samples indicated that an incubation time of 40 min after the addition of the substrate with a sample volume of 0.2 ml were suitable for measuring the ChE activity. The experiments described below were performed to standardize the present electrometric method in goats, and to demonstrate its precision, reproducibility, validity and efficiency in measuring enzyme inhibition, as well as other specifications.

Precision of the electrometric method

The coefficient of variation of the electrometric method was determined in pooled plasma and erythrocyte samples [16].

Preliminary reference ChE values

Blood samples were obtained from 14 goats to measure ChE activity in the plasma and erythrocytes.

True ChE activity in the plasma

Plasma samples of 6 goats were individually divided into two portions. The first portion was used for measuring the ChE activity as described before. To the reaction mixture of the second portion, 40 µl of 0.1% quinidine sulfate (Sigma, USA) was added, and incubated for 10 min at 37°C. Quinidine specifically inhibits pseudo ChE activity in the plasma [8]. Following the 10 min incubation period for inhibiting pseudo ChE activity [12], the remaining (true ChE) activity was measured as before. Pseudo ChE activity = ChE activity (without quinidine) – true ChE activity (with quinidine).

***In vitro* ChE inhibition by dichlorvos, diazinon and carbaryl**

The method of inhibitor-ChE incubation [12] was used to measure the *in vitro* inhibition of plasma and erythrocyte ChE activities by dichlorvos (Al-Tariq, Iraq), diazinon (Ciba Geigy, Swiss) and carbaryl (Sociedad Anonima de Agroquimicos, Spain). The insecticides were individually added to the reaction mixtures of the plasma or erythrocytes (n = 4/group), and the final concentrations obtained for each insecticide in the reaction mixture were as follows:

Dichlorvos and diazinon: 0 (base-line control), 0.5, 1, 2 and 4 µM.

Carbaryl: 0 (base-line control), 5, 10, and 20 µM.

The reaction mixtures containing the insecticides were incubated at 37°C for 10 min. Thereafter, the residual ChE activity in the mixture was measured as before. The percent of enzyme inhibition was calculated as follows:

% ChE inhibition = ChE activity (without insecticide) – ChE activity (with insecticide)/ChE activity (without insecticide) × 100

Statistics

When applicable the data were subjected to analysis of variance followed by the least significant difference test [16]. Student's *t*-test was used for the means of two groups [16]. The level of significance was at $p < 0.05$. Other statistical calculations used in the present study are found elsewhere [16].

Results

Precision of the electrometric method

The coefficients of variation of the described method in measuring plasma and erythrocyte cholinesterase activities of the goats were 4 and 2%, respectively (Table 1).

Table 1. Precision of the described electrometric method for measurement of cholinesterase (ChE) activity in the plasma and erythrocytes of goats

Sample	No. of replicates	Mean ChE activity (ΔpH/40 min)	Standard deviation	Coefficient of variation (%)
Plasma	12	0.202	0.008	4
Erythrocytes	10	0.368	0.007	2

Table 2. Preliminary reference cholinesterase activity (ΔpH/40 min) in the plasma and erythrocytes of goats

Sample	Plasma	Erythrocytes
Mean	0.194	0.416*
Standard error	0.005	0.010
Standard deviation	0.017	0.038
95% Confidence interval	0.184~0.204	0.396~0.436
Range	0.06	0.11

*Significantly different from plasma cholinesterase activity, $p < 0.05$.

Table 3. Estimation of true cholinesterase (ChE) activity (ΔpH/40 min) in the plasma of goats (n = 4)

Variable	Mean ± SE	% Activity
Total ChE	0.170 ± 0.016	100
True ChE*	0.062 ± 0.006	36.5
Pseudo ChE	0.108 ± 0.001	63.5

*Quinidine sulfate was used to inhibit pseudo ChE activity.

Preliminary reference ChE activity

Table 2 shows the normal ChE values, 95% confidence interval and related statistics for plasma and erythrocyte ChE activities of 14 goats. Preliminary reference values of the mean cholinesterase activity (ΔpH/40 min) and 95% confidence interval in the plasma were 0.194 and 0.184~0.204, respectively, and those of the erythrocytes were 0.416 and 0.396~0.436, respectively (Table 2). Erythrocyte ChE activity was significantly higher than that of the plasma.

True ChE and *in vitro* ChE inhibition

Using quinidine sulfate to inhibit pseudo ChE activity in the plasma, the percentage of true ChE activity was estimated to be 36.5% (Table 3). The insecticides dichlorvos, diazinon and carbaryl significantly and in a concentration-dependent manner inhibited plasma (13.7~85.5%) and erythrocyte (16.4~71.9%) ChE activities *in vitro* (Table 4).

Discussion

Measurement of blood ChE activity in animals is a non-invasive method for monitoring poisoning or exposure to OP and carbamate insecticides [7,18,19,20]. Methods available for measuring ChE activity have a wide range of variability and difficulties in reproducibility [7,9,18,19]. Further, the shortcomings of the original electrometric method are relative insensitivity, sample size and low throughput [19]. The matter is more complicated because of the fact that the Michel method [10] is not directly applicable to measure animal ChE which differs considerably from that of the human [4,13,18,19]. Therefore, several investigators advocated many modifications of the original electrometric method. These modifications included increasing the sample volume, increasing the reaction temperature, the use of different buffers and increasing or decreasing the incubation time [2,13,17,18,19].

The present electrometric method described for measurement of blood ChE activities in goats depended mainly on the

Table 4. *In vitro* inhibition of goat plasma and erythrocyte cholinesterases (ChE) by dichlorvos, diazinon and carbaryl (mean ± SE)

Inhibitor concentration (μM)	Plasma ChE		Erythrocyte ChE	
	ΔpH/40 min	% inhibition	ΔpH/40 min	% inhibition
		Dichlorvos		
0	0.193 ± 0.003	0	0.445 ± 0.03	0
0.5	0.150 ± 0.003*	22.3	0.418 ± 0.02	6.1
1.0	0.100 ± 0.010*	48.2	0.372 ± 0.02*	16.4
2.0	0.068 ± 0.006*	64.8	0.263 ± 0.02*	40.9
4.0	0.028 ± 0.004*	85.5	0.125 ± 0.02*	71.9
		Diazinon		
0	0.223 ± 0.008	0	0.510 ± 0.01	0
0.5	0.198 ± 0.005	11.2	0.453 ± 0.02	11.2
1.0	0.175 ± 0.005*	21.5	0.430 ± 0.02	15.7
2.0	0.150 ± 0.026*	32.7	0.382 ± 0.02*	25.1
4.0	0.133 ± 0.020*	40.4	0.360 ± 0.05*	29.4
		Carbaryl		
0	0.255 ± 0.012	0	0.590 ± 0.01	0
5	0.220 ± 0.013*	13.7	0.490 ± 0.02*	17.0
10	0.150 ± 0.010*	41.2	0.440 ± 0.01*	25.4
20	0.085 ± 0.006*	66.7	0.375 ± 0.03*	36.4

*Significantly different from the respective control (0 concentration), $p < 0.05$.

modifications previously reported in sheep [12] and it correlates well with the original method and the spectrophotometric method [2,12]. The method has been applied successfully for the determination of blood or tissue ChE activities in other animal species such as chickens [11], rats [3], mice [2] as well as in man [2]. However, the method has not been validated for use in goats. The present study is the first attempt to standardize and validate the present electrometric procedure in goats.

The 40 min one step incubation time and the 0.2 ml sample volume appeared to be suitable for the assay conditions to produce enzyme activity without interference with the buffering capacity in the reaction mixture. This is in agreement with our earlier finding in sheep [12]. The one step short incubation time of the described method would be useful in increasing the efficiency of the procedure for multiple samples when compared to more than 60 min of the original Michel method [10]. The method also decreases substantially handling of the reaction mixture as found in other electrometric methods [12,17,18,19].

With regards to precision of the assay, the described electrometric method produced acceptable low coefficient of variation in the plasma (4%) and erythrocytes (2%). This result documents within-laboratory precision of the assay [7] and agrees with the reported precision of the method in sheep [12]. In spite of the expected limitations of between-laboratories comparisons [7,9], the described method needs such a comparison. In an attempt to establish preliminary reference range values for plasma and erythrocyte ChE activities of the goats, the described electrometric method also presents for the first time blood ChE activity in this species.

Quinidine specifically inhibits pseudo ChE activity in the plasma [8,19], thus permitting the estimation of true ChE in the sample. In the present study, the estimated % of true ChE activity in the plasma of the goats was found to be 36.5% of the total ChE activity. This finding correlates with those reported in other animal species [18,19]. The overall ChE activities in the plasma and erythrocytes also correlate with normal values reported in goats by other methods [1,4,5,17].

In vitro inhibition of plasma and erythrocytes ChE by dichlorvos, diazinon and carbaryl is in agreement with the reported antiChE effects of these insecticides [1,2,11,12]. This particular experiment suggested the sensitivity of the described method for detecting ChE inhibition caused by OP or carbamates. However, further ChE inhibition should not be excluded from this *in vitro* system during the 40-min incubation time. In addition, the original electrometric method cannot be recommended for detection of ChE inhibition induced by carbamates [14,15]. Carbamylated ChE is unstable in the reaction mixture of the original electrometric method because of considerable sample dilution and long incubation time (>60 min) [14,15]. Further, the described electrometric method also detected ChE inhibition in the plasma and erythrocytes of goats dipped in 0.1% diazinon (unpublished data). Previous reports from our laboratory also indicated the efficiency of the method in detecting *in vivo* ChE inhibition induced by OP or carbamates in other animal species [2,3,11]. The described electrometric method in goats was also comparable to the original method from which it was derived in detecting low level of plasma ChE in sheep [12].

In conclusion, the described electrometric method was precise and efficient for rapid determination of ChE activity in the plasma and erythrocytes of goats, and it could be an additional useful technique for monitoring exposure to ChE inhibitors in goats.

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