Validation of an Eiectrometeric Method for Cholinesterase Measurement in the Plasma of Ducks

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Abstract:
This research is designed to describe and validate electrometric method for measuring cholinesterase activity (ChE) in the plasma of 30-60 days old ducks. A suitable (ChE) assay mixture contain 0.2 ml of plasma , 3ml of distilled water , 3ml pH 8.1 buffer and 0.12ml of 7.5% acetylcholine iodide as the substrate .The one step incubation was for 30min. at 37C˚ was followed . Acetylcholine chloride was also a suitable substrate for the described procedure .The estimated level of true (ChE) activity in the plasma after inhibition of pseudo (ChE) by quinidine was 27%. The organophosphate insecticide parathion (0.5-8 µM) and carbamate insecticide aldicarb (5-40µM) significantly inhibited plasma (ChE) activites *in vitro*. Oral administration of parathion (2 and 4 mg/kg) and aldicarb (100 and 200 mg/kg) ducks induced a characteristic in toxication signs of cholinergic over stimulation and these sign were associated with significant decreases in plasma (ChE) activites .The results suggested that the present electrometric method for measuring of (ChE) activities in ducks is simple , accurate and efficient in monitoring the enzyme inhibition caused by organophosphate and carbamate insecticides.

Introduction:
Determination of plasma (ChE) is widely used to diagnose or monitor exposure to (ChE) inhibitors such as organophosphate or carbamate insecticides (1).One of the principle methods for measuring blood (ChE) activities exposure is the electrometric method which based on production of acetic acid which inturn decreases the pH of reaction mixture (12 , 14).The original electrometric method of Miche(12) is most commonly used in man. However the method is not directly applicable to samples from other animal species (14).This is because inherent variation in blood (ChE) activities between different animals species (12) and the special need for different buffer composition and substrate (2).

One of the most recent modifications of the electrometric method is that of Michel(12),which was introduced for rapid measurement of erythrocyte and plasma (ChE) activities in sheep .The method was not commonly applied on a limited scale in pigeon(1).It is characterize by simplycity and one step short incubation time (30min)(14).

Because of the limited application of the modified electrometric method in cockerels (12) and as part of the validation of these methods for use in duck, the present study was under-taken to examine the application of the technique in measuring plasma (ChE) activities in ducks. The accuracy, reproducibility and specifications of the method were consider in the present study .It is well known that avian species are widely used in anti (ChE) studies (12), an the introduction of these procedure in ducks would possibly be advantageous because of it's simplicity and accuracy .Parathion and aldicarb were used in this study because they are widely used organophosphate and carbamate insecticides respectively(7,8).

Materials and Methods:
Domestic ducks of both sexes 30-60 days old, were used .They are obtained from local licensed hatcheries, and housed under controlled environmental conditions with food and water available ad libitum. The ducks were euthanized by decapition and blood samples were collected using heparinized test tubes (2). Plasma was seperated from erythrocytes by centrifugation at 3000rpm (centurion, U.K) for 15min. All samples kept on ice until used for ChE determination within 1h.

Electrometric procedure for measurement plasma ChE activity used to measure plasma ChE activities of the ducks. The reaction mixture in a 10ml beaker contained 3ml distilled water, 0.2ml plasma and 3ml buffer, pH 8.1 .The pH of the mixture (pH_1) was measured with a glass electrode using a pH meter (Phillips, U.K), then 0.12ml of 7.5% aqueous solution of acetyl choline iodide (BDH,U.K.) was added to the mixture which was incubated at 37C for 30 min. At the end of the incubation period, the pH of reaction mixture (pH_2) was measured .The enzyme activity was calculated as follows: Plasma ChE activity ($\Delta pH/30min$) = (pH_1-pH_2) -ΔpH of blank the blank was with out plasma .The pH 8.1 buffer consisted of 1.237g sodium barbital (BDH) , 0.63g potassium dihydrogen phosphate (E-Merck.Dermstadt, Germany) and 35.07g sodium chloride (BDH)1 Liter of distilled water (9).

The experiment described below were performed on plasma samples to standardize the present electrometric method in ducks and to domenstrate it's precision , reproducibility , validity and efficiency in measuring enzyme inhibition as well as other specifications .The main emphasis was on plasma ChE because it is the most frequently monitored parameter in cases of anti ChE poisoning or exposure(4,8,11).

Substrate:

Plasma samples were collected from 5 ducks. Each sample was divided in to 2 portions .For the first portion 7.5% aqueous solution of acetyl choline iodide was used as the substrate , and for the second portion 7.94% of aqueous as solution of acetylcholine chloride was used as the substrate for the determination of the ChE activities .The two substrates were equimolar in concentration(0.275µM).

True cholinesterase activity in the plasma:

Plasma sample of 5 ducks were individually divided into 2 portions .The first portion was used for measuring the ChE activity as described befor .To the reaction mixture of the second portion, 40 ml of 0.1% of quinidine sulfate (sigma) was added, and incubated for 10 min at 37C˚.Quinidine specifically inhibits pseudo ChE activity in the plasma (6) .Following the 10min. incubation

period for inhibiting pseudo ChE activity (12) the remaining true ChE activity was measured as before.

In vitro ChE inhibition by parathion and aldicarb:

The method of inhibitor ChE incubation was used to measure the *in vitro* inhibition of plasma ChE activity (1) by parathion (Goldben , VAPCO, Jordan) and aldicarb (Goldben , VAPCO, Jordan).Parathion was added to the reaction mixture (N=5/concentrations) to obtained final concentration of (base line control) , 0.5 , 1 , 2 , 4 and 8 µM .The concentration of aldicarb in the reaction mixture (N=5/concentrations)5,10,20 and 40 µM .The reaction mixture contained the insecticides were incubated at 37C for 10min. There after the residual ChE activity in the mixture was measured as before (1).

The% inhibition of the enzyme was calculated as follows:

$$\%\text{inhibition of ChE} = \frac{\text{ChE activity (control)} - \text{ChE activity (with insecticide)}}{\text{ChE activity (control)}} \times 100$$

***In vivo* ChE inhibition by parathion and aldicarb:**

Ducks (5/group) were treated orally by a gavage needle with parathion at 0 (distilled water control), 2 and 4 mg/kg body weight or with aldicarb at 0(distilled water control) , 100 and 200 mg/kg body weight .

Distilled water was used to prepare the required insecticides concentration and the volume of administration was 4ml/kg. The ducks were monitored for the appearance of signs of poisoning characteristic of cholinergic over stimulation (9, 17) .Two hours after treatment , the ducks were decapitated to obtain the blood for ChE determination .

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 2 groups .The level of significant was at $P<0.05$ .Other statistical calculation used in the present study are found (13,15).

Results:

Substrate:

There was no significant difference in the plasma ChE activity when acetylcholine iodide and acetylcholine chloride were used as substrates (Table 1).

Table 1: Plasma ChE activity (Δ PH/30min) using two substrates (0.275µM)

Measurement	Acetylcholine Iodide	Acetylcholine chloride
Mean	0.82	0.79
SD	0.034	0.027
SE	0.006	0.011
Coefficient of variation	4.1	3.4

N=5 ducks /substrate group.

True Cholinesterase:

Using quinidine sulfate to inhibit pseudo ChE activity in the plasma, the percentage of true ChE activity was 27% (Table2).

Table(2) Estimation of true ChE activity (Δ PH/30min) in the plasma of ducks

Variable	Mean ± SE	% activity
Total cholinesterase	0.70±0.020	100
True cholinesterase*	0.51±0.014	27
Pseudo cholinesterase	0.19±0.013	73

N=5 ducks /group.

*Quinidine sulfate was used to inhibit pseudo ChE activity.

***In vitro* ChE inhibition:**

The insecticides parathion and aldicarb significantly and in a concentration dependent manner inhibited plasma ChE activities *in vitro* (table 3).

Table 3: *In vitro* inhibition of ducks plasma ChE by parathion and aldicarb

Inhibitor concentration(µM)	Plasma ChE	
	(Δ PH/30min)	% inhibition
Parathion		
0(control)	0.70±0.015	0
0.5	0.64±0.011*	8.5
1	0.55±0.013*	21
2	0.38±0.019*	45
4	0.18±0.020*	74
8	0.09±0.019*	87
Aldicarb		
0(control)	0.75±0.01	0
5	0.61±0.018*	18
10	0.58±0.020*	22
20	0.44±0.015*	34
40	0.35±0.011	53

N=5 ducks /conceatration group.

*significantly different from the respective control(0 concentration)$P<0.05$.

***In vivo* ChE inhibition:**

Oral dosing of parathion and aldicarb in ducks induced signs of toxicosis manifest salivation, lacrimation , ataxia , frequent defecation , tremors and difficulty in supporting the body weight.Parathion at 2 and 4 mg/kg , orally significantly and dose dependently inhibited plasma (60 and 86%) ChE activities in comparison with respective control value (Table 4) .Aldicarb at dose 100 and 200 mg/kg, orally significantly and dose dependently inhibited plasma (71 and 86%) ChE activities when compared with respective control values (Table 4).

Table (4): Plasma ChE inhibition in ducks dosed orally with parathion and aldicarb

Treatment mg/kg	Plasma ChE	
	(Δ PH/30min)	% inhibition
Parathion		
0(control)	0.76±0.011	0
2	0.30±0.021*	60
4	0.10±0.033*	86
Aldicarb		
0(control)	0.69±0.031	0
100	0.20±0.011*	71
200	0.09±0.013*	86

N=5 ducks /group.

*significantly different from the respective control $P<0.05$.

The individual values, mean , SE , SD and related statistics of plasma ChE activities of 13 ducks are presented in (table 5). The mean plasma ChE activity (ΔPH/30min) was 0.731 within a 95% confidence interval 0.70-0.73.The coefficient of variation of the electrometric method in measuring plasma ChE activity of the ducks was 6.3%.

Table 5: References of plasma ChE activity in ducks

Sample No.	ΔpH/30min
1	0.71
2	0.73
3	0.70
4	0.73
5	0.75
6	0.81
7	0.67
8	0.66
9	0.74
10	0.78
11	0.80
12	0.69
13	0.74
Mean	0.731
S.E	0.0128
S.D	0.0463
C.V	6.3
Range	0.15
95% confidence interval	0.70-0.73

N= 13 ducks

Discussion:

Measurement of blood (ChE) activity is a noninvasive diagnostic tool for monitoring anti (ChE) poisoning or exposure in man and animals (10). However the currently available method suffer from a wide range of variability and difficulties in reproducibility (18) ,what complicates the matter is that the Michel method is not directly applicable to animal ChE which differs considerably from that of the human(8,10).

The person method was previously applied once in a preliminary study on pigeons using acetylcholine chloride as the substrate (1). However, the method needed to be validated for further use in birds. The present study is the first extensive attempt to standardize and validate the present procedure in ducks.The use acetylcholine iodide and acetylcholine chloride as substrates produced almost identical results indicating the suitability of both substrates for measuring ChE by the electrometric method described here (Table 1). Other substrates such as acetylthiocholine and propionyl or butyryl choline ester can also be used as ChE substrates (9). However, they need to be tested for the present procedure.

Quinidine specifically inhibits pseudo ChE activity in the plasma (8) , thus permitting the estimation true ChE in the sample. In the present study the estimated true ChE activity in the plasma of the ducks was found to be 27% of the total ChE activity (Table2). This finding correlates with those (10-13%) reported by others in avian species (1) . It is also known that true ChE in the plasma of ducks is higher at hatching and gradually decreases with pseudo ChE activity increases (3,5).

In vitro inhibition of plasma ChE by parathion and aldicarb is agreement with the reported anti ChE effect of these insecticides (9) .This preliminary experiment suggested. The sensitivity of the described method for detecting ChE Inhibition caused by organophosphate or carbamates insecticides. However , further ChE inhibition should not be excluded from this *in vitro* system during the 30min incubation time(Table 3). In addition the original electrometric method can not be recommended for detection of ChE inhibition induced by carbamates (9, 14) carbamaylated ChE is unstable in the reaction mixture of the electrometric method of Michel because of considerable sample dilution and long incubation time (60min) (14).

Therefore further ***in*** *vivo* experiment is warranted using the present procedure. Dosing of ducks with parathion and aldicarb inducing signs of toxicosis characteristic of cholinergic over stimulation .The described electrometric method detected significant ChE inhibition especially plasma sample of the ducks (Table 4).

Cholinesterase activity is the most frequently examined parameter in birds exposed to anti ChE insecticides (9). It is also known that 20-30% decrease in ChE activity indicates exposure to ChE inhibitors and as the enzyme inhibition increases, signs of poisoning ChE inhibitors overtly in the animal (17) .A similar trend has been reported earlier in rats(19) and in birds (1) , intoxicated with organophosphate .Based on the *in vitro* and *in vivo* experiments of the present study. We conclude that ChE inhibition by insecticides parathion and aldicarb , and further supports a previous report in which the method was able to detect plasma and erythrocyte ChE inhibition by methomyl in rats (19) .However further studies are needed on the application of the present electrometric method for detection other avian species such as wild birds.

With regards to precision of the assay, the described electrometric method, produced acceptable low coefficient of variation in the plasma (6.3%) (Table 5).This result documents within laboratory precision of the assay (2) and agrees with the reported precision of the method in sheep (17). In spite of the expected limitation of comparisons between laboratories (2), the described method needs such a comparison. In an attempt to establish preliminary references range values for ChE activity of the ducks, the described electrometric method presents collectively for the first time ChE activity in the plasma.

In conclusion, the described electrometric method was precise and efficient in measuring ChE activity in the plasma of the ducks. The method, because of it's simplicity, efficiency and rapidity, could be an additional useful technique for monitoring exposure to ChE inhibitors in birds.

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تقييم الطريقة الكهرومترية لقياس نشاط خميرة الكولين استراز في بلازما دم البط

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الملخص:

صمم هذا البحث لتقييم الطريقة الكهرومترية وتقييسها وبيان شرعيتها لقياس نشاط خميرة الكولين استراز في بلازما دم البطة والتي تبلغ من العمر (٣٠-٦٠) يوماً. أحتوى مزيج التفاعل الخاص بهذه الخميرة على ٠,٢مل من البلازما و٣مل الماء المقطر و٣مل من المحلول الدارى بأها ٨,١ و ٠,١٢مل من المادة الحليلة يوديد الاستيل كولين٧,٥% وكانت مدة الحضن ٣٠ دقيقة بدرجة حرارة ٣٧مْ . وجد بانه من الممكن استعمال كلوريد الاستيل كولين ايضا كمادة حليلة في هذه الطريقة . كانت نسبة نشاط خميرة الكولين استراز الحقيقية في بلازما الدم بعد تثبيط الخميرة الكاذبة بالكوندين ٢٧% . ثبط المبيد الحشري الفسفوري العضوي الباراثيون(٠,٥-٨ مايكرومولار) والمبيد الحشري الكاربامايتي الالديكارب (٥-٤٠مايكرومولار) من نشاط خميرة الكولين استراز في البلازما في الزجاج في حين ادى اعطاء جرعات مسممة من الباراثيون(٢ و ٤ ملغم/كغم) والالديكارب (١٠٠ و ٢٠٠ ملغم/كغم) في البطة الى حدوث علامات التسمم الكوليني الفعلي مع تثبيط معنوي لنشاط خميرة الكولين استراز في البلازما ، تشير هذه النتائج الى ان الطريقة الكهرومترية الموصوفة تمتاز بالدقة والسهولة والكفاءة لقياس نشاط خميرة الكولين استراز وكذلك في الكشف عن التثبيط الحاصل في نشاط الخميرة الكولين استراز بعد تعرض البطة للمبيدين الباراثيون والالديكارب.