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A Modified Method for Measurement of True Acetylcholinesterase Activity

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ABSTRACT

The measurement of true cholinesterase (ChE) activity is a useful tool for monitoring exposure to organophosphate and carbamate insecticides. Blood cholinesterase activity is measured colorimetrically or electrometrically. Recently, a simple and practical electrometric method has been described and validated for measuring blood cholinesterase activity in people and animals. The aim of the present report was to use the modified technique for measuring blood cholinesterase activity in organophosphorus and carbamate intoxicated patients compared to the original electrometric method. True (ChE) of healthy subject (N=35), patients suffering from organophosphorus (N=50) and carbamate poisoning (N=25) were measured by the modified and original electrometric method. The modified method involved the addition of 0.2 ml erythrocytes to three ml of redistilled water and 3 ml barbital-phosphate buffer (pH 8.1). The pH of the mixture was measured (pH1), and then 0.1 ml of 0.11 M acetylcholine chloride was added. The mixture was incubated at 37 ^{O}C for 20 minutes and the pH (pH2) was measured. Enzyme activity was expressed as ΔpH/20 min = pH1-pH2 − (ΔpH of the blank). The blank was without the blood sample. The determination of ChE activity (mean ± SE) of organophosphorus group by the modified procedure and original method recorded (0.337±0.097 VS .328 ±0.087 respectively). In carbamate group the enzyme activity measured by the modified procedure shows a mild significant decrease of 16% ($P<0.01$) than the original method. Using both assay methods, there were a significant positive correlation (Pearson's correlation coefficient, $r = 0.973$, $P < 0.0001$ and $r=0.974$, $P<0.001$ for organophosphorus and carbamate groups respectively). The coefficient of variation of the described electrometric method in measuring blood cholinesterase activity was <1. The results suggested that the present electrometric method for measurement of blood ChE activity in man is a simple, accurate, and efficient in monitoring the enzyme inhibition caused by organophosphate and carbamate poisoning.

Introduction

Poisoning with organophosphorus (OP) and carbamate compounds are a global public health problem [1,2]. They pose major environmental pollution problems and health hazards to people and animals [3–6]. These insecticides inhibit cholinesterase (ChE) activity in the nervous tissues and neuromuscular junctions, causing an accumulation of acetylcholine at the nerve endings which subsequently produces signs of toxicities characterized by nicotinic, muscarinic, and central nervous system effects [7–9].

Measurement of blood or tissue cholinesterase activity is an ideal biomarker for monitoring exposure to organophosphate and carbamate insecticides and for diagnosing poisoning induced by these pesticides [10-16]. Usually a 20-30% decrease in blood ChE activity indicates exposure to ChE inhibitors [16-17], and further enzyme inhibition is an indication of hazardous condition and poisoning [17-19]. Various colorimetric and electrometric methods exist for measurement of true ChE activity in the blood and other tissues such as the brain [11-13,20]. One of the common methods for measuring blood ChE activity is the electrometric method of Michel [21] which is based on the hydrolysis of acetylcholine and production of acetic acid which in turn decreases the pH of the reaction medium. However, the shortcomings of

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the Michel's electrometric method are relative insensitivity, sample size, long incubation period and low throughput [11-13]. In addition, the original electrometric method is not preferred for detection of ChE inhibition induced by carbamates [12,22,23]. Carbamylated ChE is unstable in the reaction mixture of the electrometric method of Michel because of considerable sample dilution and long pre-reaction and post-reaction incubation times (totally >90 minutes) [22,24].

Various modifications of the electrometric method have been described for measuring erythrocyte ChE activities in clinical and research applications [12,13,20,25,26]. These modifications include increasing or decreasing sample volume, incubation time and incubation temperature, or using buffers of different compositions. A simple modification of the electrometric method has been reported; it was originally modified from electrometric method described in sheep [25,26]. By omitting apparently unnecessary steps of saline washing of the erythrocytes and the one or two incubation periods of 10 minutes each before substrate addition, the modified method was advocated for its one-step short incubation time (20 minutes), the use of a single barbital-phosphate buffer, and the overall shorter assay time [19,20]. The modified method has been tested on spiked human and several animal species blood samples with organophosphorus and carbamate insecticides [26,27]. Further, the modified method (in contrast to the original electrometric method of Michel) can detect ChE inhibition caused by organophosphorus and carbamate insecticides [21,26]. Other than a single validation report, no attempt has been made to use the modified method for measuring ChE activity in people on a large scale [26].

Therefore, the purpose of our study was to apply the modified electrometric methods for measuring blood ChE activities in patients suffering from insecticide intoxication taking into consideration the accuracy, reproducibility and specifications of the method.

Materials and methods

Subjects: This study was conducted at the poison control center, Ain Shams University hospital (ASUH) from Mars 2015 to September 2016. 110 candidates participated in this work. They were classified into;

1) 50 patients: 28 males and 22 females, mean (±SD) age of 28 ±6 years diagnosed as organophosphorus insecticides intoxication (Organophosphorus Group).
2) 25 patients: 11 males and 14 females, mean (±SD) age of 24 ±5 years diagnosed as carbamate insecticides intoxication (Carbamate Group).
   All patients were selected from in-patient's department and intensive care unit.
3) Control group of male (n=18) and female (n=17) healthy volunteer, mean (±SD) age of 31 ±7 years, had no history of exposure to anti ChE insecticides for at least two months before blood sampling was included.

Ethical aspects: This study was approved by the Ethics Committee of Ain Shams University Hospital (ASUH).

Chemicals: Acetylthiocholine iodide, acetylcholine chloride and sodium barbital were supplied by the Sigma Chemical Company (Poole, UK). All other reagents and solvents used in this work were of analytical grade and were supplied by Fisher (Loughborough, UK).

Reagents: *Buffer I*: 20 mM sod. barbital, 4 mM potassium dihydrogen phosphate and 0.6 M KCL pH adjusted to 8.1 with HCL. *BufferII*: 6 mM sodium barbital, 4 mM potassium dihydrogen phosphate, and 600 mM sodium chloride, pH adjusted also to 8.1 with hydrochloric acid (0.1N). *SubstrateI*: 0.11 M acetylcholine chloride. *SubstrateII*: 27.5 mM acetylthiocholine iodide. *Saponin*: 0.001mM and Saline (0.9g %). All reagents used successfully for 3 months if kept refrigerated.

Blood samples: Venous blood (7 ml) was withdrawn and collected in heparinized vacutainers (28), the erythrocytes were separated by centrifugation at 3000 rpm (Jwan, France) and divided into two portions. The first portion was used for the modified method while the rest of the erythrocytes kept in graduated tube for measuring the ChE activity by Michel method.

Enzyme activity measurement (Modified Method):

Preliminary experiments using different erythrocyte sample volumes indicated that an incubation time of 20 min after the addition of the substrate with a sample volume of 0.2 ml were suitable for measuring the ChE activity as follow.

Three mL of redistilled water and 3 ml buffer I were added to 0.2 ml erythrocyte. The pH of the mixture (pH1) was measured just after the substrate I (0.1 ml) was added using a glass electrode connected to a pH meter (Hanna 213 microprocessor pH Meter, Romania) and the mixture incubated in a water bath at 37°C for 20 min, after which the pH was again measured (pH2).

Michel method (Original Method):

Cholinesterase activity was also determined by the Michel method [14]. In brief, the cells of 5 ml heparinized blood were mixed with two volumes of saline, then centrifuge at 3000 rpm for 15 min. The supernatant was discarded, and the previous step was repeated twice. The volume of the cells was noted, and the saline was discarded to the point where the remaining volume was double the volume of RBCs. Well mixed RBCs suspension (0.4 ml) was hemolyzed with 9.6 ml of saponin. Two milliliter of haemolyzate were added to 2 ml of buffer II. The pH of the mixture (pH1) was measured just after the substrate II (0.4ml) was added. The pH was again measured exactly after one hour (pH2).

Calculation: The activity of cholinesterase for both methods was calculated as follow:

Cholinesterase activity (Δ pH/20 min) = (pH1-pH2) - Δ pH of blank

The blank was without erythrocytes. The percentage of enzyme inhibition was calculated as the following formula:

$$\% \text{ ChE inhibition} = \frac{(\text{ChE activity of healthy subjects} - \text{ChE activity of patient}) \, 100}{\text{ChE activity of healthy subjects}}$$

Assay validation: The precision of the modified method for measuring the activity of true acetylcholinesterase was estimated by calculating the coefficient of variation for ten replicates using three pooled samples of all studied groups.

Statistical Analysis: Conventional statistical methods were used to calculate the means, coefficient of variance, and standard deviation. Pearson's correlation coefficient and the 2 sample t-tests were applied to test for any significant differences ($P<0.05$). All statistics were analyzed using the statistical package for the social sciences (SPSS) software (version 18; IBM Corporation, Armonk, New York, USA).

Results

The determination of true cholinesterase activity (mean ± SD) of organophosphorus group by the modified procedure and original method yielded numerically close values (0.337 ± 0.097 vs 0.328 ± 0.087, respectively) (**Table 1 and Fig. 1**). In carbamate group the enzyme activity measured by the modified procedure showed a mild significant decrease of 16% ($P < 0.01$) than the original method (**Table 2 and Fig.1**).

Using both assay methods there was a significant positive correlation (Pearson's correlation coefficient, $r = 0.973$, $P < 0.001$ and $r = 0.974$, $P < 0.001$ for organophosphorus and carbamate groups, respectively).

The coefficient of variation of the described electrometric method in measuring blood cholinesterase activities were 0.01, 0.29 and 0.3 for the healthy subject, organophosphorus and carbamate groups, respectively (**Table 3**).

Table 1: True cholinesterase inhibition % and activities in organophosphorus group measured by the original and modified methods.

	Original method		Modified Method	
	Inhibition %	**Activity**	**Inhibition %**	**Activity**
Mean	47.16	0.328	47	0.337
St D	12.5	0.087	13.2	0.097
% Change			0.33	-2.7
P<			N.S.	N.S.

Table 2: True cholinesterase inhibition % and activities in carbamate group measured by the modified and original methods.

	Original method		Modified Method	
	Inhibition %	**Activity**	**Inhibition %**	**Activity**
Mean	59.24	0.448	47.68	0.383
St D	13.1	0.112	14.5	0.122
% Change			19.4	15.9
P<			< 0.01	< 0.01

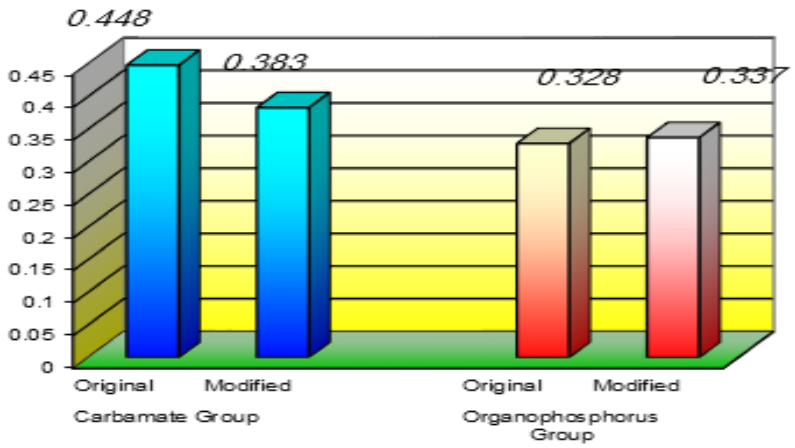

Fig. 1: The mean values of ChE activity determined by the original and modified methods in organophosphorus and carbamate groups

Table 3: Precision of the modified method for measuring the activity of true cholinesterase in all studied groups.

	No. of Pooled Sample	No. of Replicates	Mean	S.D	CV
Healthy Subject Group	3	10	0.7	0.01	0.01
Organophosphorus Group	4	10	0.343	0.107	0.29
Carbamate Group	5	10	0.361	0.11	0.3

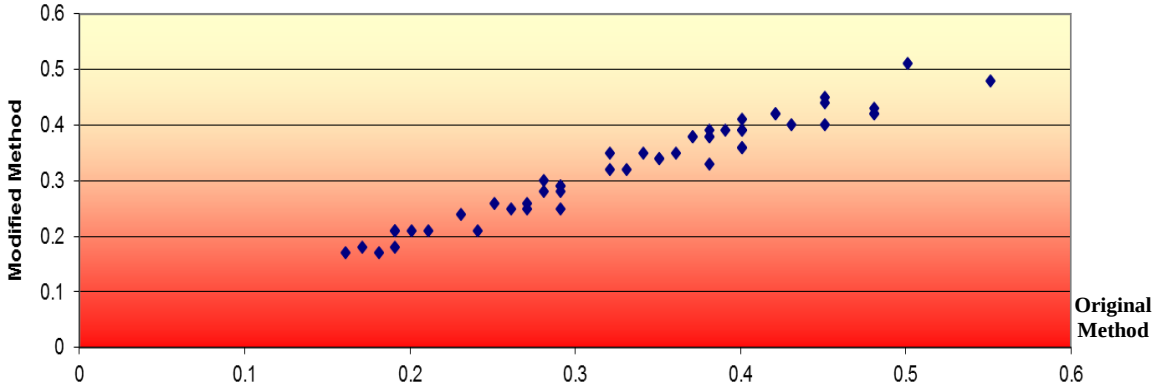

Fig. 2: Linear regression correlation of the individual ChE activity using original and modified methods of organophosphorus group (r = 0.973).

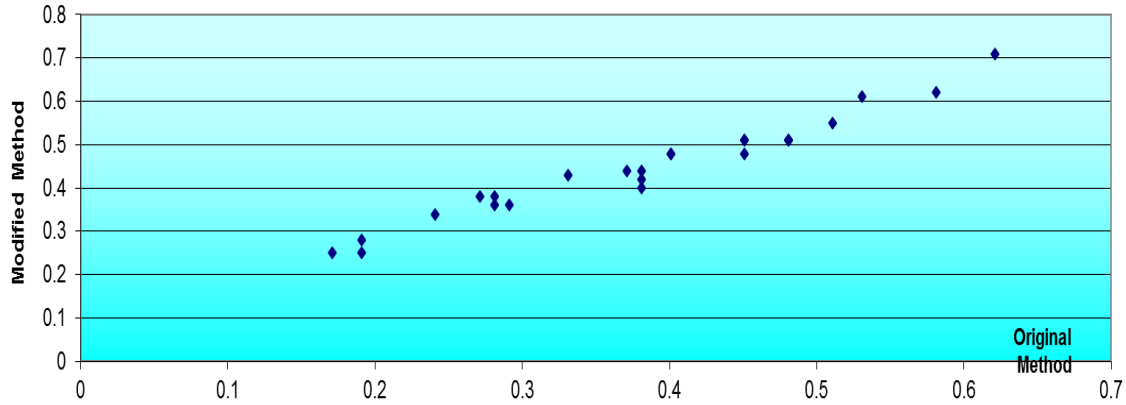

Fig. 3: Linear regression correlation of the individual ChE activity using original and modified methods of carbamate group (r = 0.973).

Discussion

The use of organophosphorus and carbamate pesticides in a large scale and the dangers associated with their application have resulted in cholinesterase (ChE) activities being used as biomarkers for evaluating both exposure to and the effect of these pesticides [7]. The original Michel's electrometric method [21] available for measuring ChE activity suffer from a wide range of variability and difficulties in reproducibility, relative insensitivity, sample size and low throughput [11-13].

Different modifications of this original electrometric method have been described for measuring erythrocyte ChE activities in clinical and research applications [11-13,20,25]. All these modified methods have been applied for the determination of blood or tissue ChE activities in several animal species such as mice [29], rats [30], rabbits [31], goats [32], chickens [33,34] and wild birds [35]. However, the method has not been validated for use in man. In vitro inhibition of spiked plasma, erythrocyte, and whole blood ChE activities by chlorpyrifos, methidation (organophosphates), and by carbaryl (a carbamate) applied also [10,23,26,36].

The present study is the first attempt to standardize and validate the determination of the of true ChE activity of apparently intoxicated patients with organophosphorus and carbamates insecticide as determined by a simple modified electrometric method. The ChE activities obtained by the original and our method in organophosphorus group show no significantly changes (**Table 1 and Fig 1**) which close to those reported in the literature [21,37,38]. Of the two methods a correlation of the individual ChE activities, a very highly significance

(r=0.973 and r=0.974) reported in organophosphorus and carbamate groups respectively. With regards to precision of the assay, the described electrometric method produced acceptable low coefficients of variation.

The main advantage of the described method over other electrometric methods is the one-step short incubation period (20 min) and the overall shorter assay time in comparison with the original method [21]. The described procedure shortens considerably the time by omitting the apparently unnecessary steps of saline-washing of the erythrocytes and the 2 incubation periods of 10 min each before substrate addition. Furthermore, the erythrocytes were not washed with saline since only a small difference (0.02 ΔpH) was found between ChE activities of washed and non-washed erythrocyte sample. The method also decreases substantially handling of the reaction mixture as found in other electrometric methods [12-14,20] and eliminates the problem of color interference found in the spectrophotometric methods [11-14] by using a glass electrode. Further, it is inexpensive, like the Michel's method, as it uses commonly available laboratory equipment and reagents.

Keeping in mind that the original electrometric method cannot be recommended for detection of ChE inhibition caused by carbamates [22-24]. The enzyme activity measured by the modified procedure in carbamate group recorded a mild significant decrease reached 16% in compared to the original method (**Table 2 and Fig. 1**). Our results are in accordance with many literatures [22-24,26], who reported that the carbamylated ChE is unstable in the reaction mixture of the electrometric method of Michel because of considerable sample dilution and long pre and post-reaction incubation times (totally > 60 minutes) [22-24]. Long incubation time may facilitate regeneration of insecticide inhibited enzymes [12,39], especially in cases of carbamate poisoning [22,23]. The results of current study suggest the possibility of using the present method in case of poisoning with carbamates and organophosphorus.

Conclusions

The described electrometric method was simple, precise, economic, rapid, and useful technique for monitoring exposure to ChE inhibitors.

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