Comparative measurement of serum Acetyl Cholinesterase Enzyme using three different methods

Akram Jamshhidzade [1], Hossein Nicknahad[1], Mohammadi-Bardbori A[1], Talati M[1]*

ABSTRACT

Introduction: IAChE (acetylcholinesterase) is one of the plasma's enzymes that plays a crucial role in nervous signal transduction in synapses and is responsible for the hydrolysis of Ach.

Methods: We investigated three methods for the determination of AChE in human blood serum, using three different assays. Michel methods are based on the measurement of hydrogen ion produced in processes. Ellman methods are of colorimetric type that is based on produced choline and its reaction with coloriogenic reagents. The other way to determine the enzyme activity is related to a decrease in the substrate level during hydrolysis process. De la Huerge is the method in which decrease in the Ach (acetylcholine) level is determined after the completion of reaction and than enzyme activity is calculated.

Results: No significant difference was found among the results of the three methods. According to our results, Michel method has the least variation in the results and Ellman has shown the highest variation. On the other hand, Ellman needs the shortest time for each test and de la Huerga requires a longer time, so in the cases we need to report the results in a short time, Ellman is the best choice.

Conclusion: These results suggest that the three methods are reliable and are comparable for determining of AChE in human blood serum. It is concluded that Michel method may be preferable to Ellman and de la Huerga methods because of its simplicity, low cost, highest precision, accuracy, sensitivity and objectivity. In addition, this method takes little time to be performed.

Key words: Cholinestrase, De La Huerge method, Ellman method, Michel method

[1] Department of Pharmacology Faculty of Pharmacy, Shiraz University of Medical Sciences, Shiraz, Iran
*Corresponding author:Akram Jamshidzadeh, Associate professor, Department of Pharmacology and Toxicology Faculty of Pharmacy, Shiraz University of Medical Sciences, Shiraz, Iran
Tel: #98 711 2424126, Fax: #98 711 2426070
E-Mail: ajamshid@sums.ac.ir

INTRODUCTION

The chemical substance that plays an essential role in nervous signal transduction in synapses is acetylcholine (ACh). Acetylchol inesterase (AChE) is responsible for the hydrolysis of Ach which transforms it into choline and acetate (1). Measurement of AChE has a variety of applications in the diagnosis and treatment of a large number of diseases (2). Determination of this enzyme activity level is used in the diagnosis of organophosphate (3) and carbamate toxicity (4) and also chemical agents active on CNS (5-6). AChE activity level changes in many diseases, such as hepatic disorder (7), Alzheimer disease (8), Myasthenia gravis (9-10), acute infection (11), diabetes (2), some carcinomas (12) and some physiologic conditions such as pregnancy, infancy and in the elderly (13).

During the past decade, use of analytical methods such as HPLC, GC and other analytical methods to evaluate the measurement of compounds in toxicological research has been developed. These methods take a long time to be done and are not cost effective. Automated methods do not eliminate laborious sample preparation steps. To overcome these problems, a great variety of workable colorimetric assays for determination of AChE has been developed. The widely used ones include Michel method (14), Ellman method (15-16), and de la Huerge method (16) which mainly focus on the measurement of hydrogen ion produced in process (14), on the produced choline and on its reaction with coloriogenic reagents respectively (15). Determination of enzyme activity is related to the decrease in substrate level during hydrolysis process (16).

In this study, we compared three different methods for the determination of AChE in human blood serum: Michel method, Ellman method and de la Huerge method.

MATERIALS AND METHODS

Butyriltiocholineiodide and DTNB were purchased from the Randox Chemical Co (England) and Ferric choloror from Kimiya mavad (Iran). Diazinon was obtained from Kimiya kavosh (Iran) and plasma from the Iran transfusion organization (Iran, Fars).

All other chemicals were obtained from the Merck Chemical Co. (Germany).

Measurement Of Ache Activity Based On Michel Method

Michel method was done as follow (14): In order to determine the AChE activity, experimental test tubes were divided into 4 groups of 9 samples and each test was performed for 10 times. Group 1 was considered as control, group 2 received plasma + buffer, group 3 received buffer+ Diazinon(0.6%), and group 4 considered as toxic received plasma + buffer + Diazinon(0.6%). All the samples were incubated *in vitro* for 1 hr at 25 °C. For a typical assay condition, the reaction mixture in 10-ml beaker contained 3 ml distilled water, 0.2 ml plasma and 3 ml barbital-phosphate buffer with pH 8.1. The pH of the mixture (pH1) was measured with a glass electrode using a pH meter. Then 0.1 ml of aqueous solution of acetylthiocholine iodide (7.5%) was added to the reaction mixture which was incubated at 37 °c for 20 minutes. At the end of the incubation period, and the pH of the reaction mixture (pH2) was measured. The enzyme activity was calculated as follows: ChE activity (ΔpH/20 minutes) = (pH1 – pH2) - Δ pH of blank

The pH 8.1 buffer consisted of 1.237 g sodium barbital (BDH), 0.63 g potassium dihydrogen phosphate and 35.07 g sodium chloride (BDH)/1 L of distilled water.

Measurement Of Ache Activity Based On Ellman Method

AChE activity was studied using DTNB Kit. The assay of human serum cholinesterase with the Ellman reaction involves reaction of 5, 5-dithio-bis (2-nitrobenzoic acid) (DTNB) with thiocholine liberated from its esters by enzymatic hydrolysis. The yellow 5-thio-2-nitro-benzoate (TNB) is formed that is detected by colorimetry substrates for the assay. The absorbance at 412 nm was measured immediately (16).

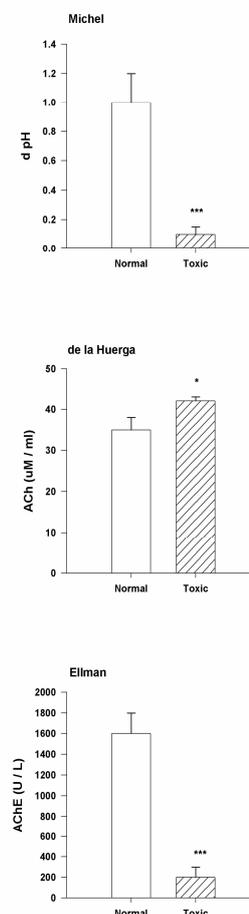## Measurement Of Ache Activity Based On De La Huerga Method

AChE activity was determined using the method developed by Dietz (16). In order to determine the AChE activity based on de la Huerga method, experimental test tubes were divided into 3 groups of 9 samples and each test was performed for 10 times. Tube1 received 2 ml of solution containing pH 8.1 barbital-phosphate buffer and 0.03 M KCl, , 0.44M $MgCl_2$ at a ratio of 8:1+0.4 ml of plasma. Tube 2 received 2 ml of solution containing pH 8.1 barbital-phosphate buffer, 0.03 M Kcl , 0.44M $MgCl_2$ +0.4ml of plasma+ 50uM of Ach solution. Tube 3 received 2 ml of solution containing pH 8.1 barbital-phosphate buffer, 0.03 M KCl , 0.44M $MgCl_2$ +0.4ml of plasma+ 50uM of Ach solution +0.1 ml of Diazinon(0.6%). All the tubes were incubated at 37 °C for 1 hr and received 2 ml of hydroxylamine solution containing hydroxylamine chloride 14%W/V and NaOH solution 14% W/V at a ratio of 1:1. Then 0.7 ml of 0.5 M HCl was added to 0.5 ml of the above mixture. Ten ml of ferric choloror 1%W/v was added to 0.5 ml of this solution and was centrifuged at 2000 rpm for 5 min. The absorbance at 412 nm was measured.

Statistical Analysis: Statistical differences were determined by the analysis of variance (ANOVA) followed by the Tukey-Kramer multiple comparison test on the Instat package. Differences were regarded as significant at p<0.05.

RESULTS

Simultaneous *in vitro* treatment of Diazinon (0.6%) with plasma significantly changed the AChE activity, compared to the controls (p<0.001, Fig 1 A, B and C1).

*(p<0.05) significantly different when compared with the control.
***(p<0.001) significantly different when compared with the control. Plasma samples obtained from 9 subjects of either sex were pooled separately. Each test was carried out 10 times.*

As shown in Table 1, no significant difference was found among the percentage of changes in the results of the three methods. These results suggest that the three methods are reliable and are comparable for determining the cholinesterase (ChE) in human blood serum (Table 1, p>0.05).

Table1. Percentages of variation, cost and time for each test (min) are shown. Plasma samples obtained from 9 subjects of either sex were pooled separately. Each test was performed 10 times. ®= Rial

Methods	% Variation	Cost for each test®	Time for each test(min)
Michel	2±1.5	264.93	100
Ellman	4±2	6202.5	25
De la Huerga	5±4	867.95	140

A significant difference was found among the time, variation and cost for each test, accuracy, and precision for the three different methods; i.e., Michel, Ellman and de la Huerga (Table. 2)

Table2. Time, variation and cost for each test, accuracy, and precision for the three different methods; Michel, Ellman and de la Huerga are compared. Plasma samples obtained from 9 subjects of either sex were pooled separately. Each test was performed 10 times. ↑ High; ↕ Moderate; Low↓

Methods	Time for each test	Variation	Cost for each test	Accuracy	Precision
Michel	↕	↓	↓	↑	↑
Ellman	↓	↑	↑	↓	↓
De la Huerga	↑	↕	↕	↕	↕

DISCUSSION

One of the common methods used to measure blood ChE activity is the electrometric method of Michel which is based on the hydrolysis of acetylcholine and production of acetic acid which in turn decreases the pH of the reaction medium (17). This consists essentially of a measurement of the rate of the fall of pH in weakly buffered solutions, upon the accumulation of acid due to the enzymic hydrolysis of an appropriate choline ester. Since the only apparatus required is a pH meter, a constant temperature bath and suitable test-tubes, and the only observations needed are a limited number of pH determinations; the method seemed particularly suitable for the performance of large number of estimations. The principle of the electrometric method is that the fall in pH should be directly proportional to the time over a suitably wide range of pH. However, the shortcomings of the Michel's electrometric method are relatively low in sensitivity, sample size, long incubation period and low throughput (18). In addition, the original electrometric method is not preferred for the detection of ChE inhibition induced by carbamates. 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 >60 minutes) (19).

The Ellman method use for cholinesterase determination is a spectrophotometric method which entails the use of 5, 5'-dithiobis-(2-nitrobenzoic) acid (DTNB) as a chromogen and recording the level of cholinesterase activity as the change in absorbance at 412 nm. Although this procedure commonly poses no problem, an exception arises when analyzing tissues rich in hemoglobin, because hemoglobin also optimally absorbs light at 400-430 nm. The use of 6, 6'-dithiodinicotinic acid (DTNA) might be a solution because like DTNB, it is also a chromogen for sulfhydryl groups, but with an optimal absorption wavelength of 340 nm (ie removed from the maximum hemoglobin absorbance range). Moreover, because the assay is read at 340 nm instead of 412 nm, the DTNA assay is markedly more sensitive in determining cholinesterase activity in hemoglobin-rich tissues. Since the advantages of the DTNA method far outweigh the disadvantages, it should be regarded as a sensitive and convenient procedure for determining cholinesterase activity, especially in hemoglobin-rich tissues (20).

According to thes result, Michel method has the least variation in the results and Ellman has shown the highest variation. On the other hand, Ellman needs the shortest time for each test and de la Huerga needs a longer time, so in the cases we need to report

the result in a short time, Ellman conserve as the method of choice (Table2). In conclusion, the Michel method might be preferable to Ellman and de la Huerga methods because of its simplicity, low cost, high precision, accuracy, sensitivity and objectivity. In addition, this method takes less time to be performed.

REFERENCES

1. Koelle GB. Pharmacology of organophosphates. Journal of applied toxicology. 1994;14:105-109.
2. Tiets. Fundamental of clinical chemistry (5 edition):C. Burtis A, Ashwood ERA 2001:385-388.
3. Queen MJMC. Clinical and analytical considerations in the utilization of cholinesterase measurements. Clinica chimica acta. 1995;237:91-105.
4. Ducatman AM and Moyer TP. The role in the clinical laboratory in the evaluation of polygenated polycyclic toxins: DDT, PCB, diabenzodioxins and diabenzofurans, Chlordecone (Kepone), and hexachlorophene. In: Therapeutic drug monitoring continuing education and Quality control program. Washington, DC: American association for clinical chemistry, Jan 1983.
5. Layish I, Krivoy A, Rotman E, Finkelstin A, Tashmaz Z, Yehezkelli Y. Pharmacollogic prophylaxis against never agent poisoning. The Israel Medical Association journal. 2005;7:182-187.
6. Bajgar J. Organophosphates/ nerve agent poisoning: mechanism of action, diagnosis prophylaxis and treatment. Advances in clinical chemistry. 2004;38:151-216.
7. Schmidt E, Schmidt FW, Lautz U. Biochemical aspects of transplantation. Clin Chem. An overiew. New Yourk-London: plenum Press. 1989: 345-353.
8. Giacobini E. Cholinesterase inhibitors: new roles and therapeutic alternatives. Pharmacological research. 2004;50:433-446.
9. Milin J, Kothar DO. Myasthenia Gravis. The Journal of the American Osteopathic Association. 2004;104:377-384.
10. Haveson RF et al. Myasthenia Gravis Association Information Pack. Volume 1. 2nd edition. March: 2005:1-23.
11. Cynthia CC, Barbara, JB. Laboratory test and diagnostic procedures. 2nd edition 1997:852-853.
12. Soreq H, Lapidot-Lifson Y, Zakut H. A role of cholinesterase in tumor genesis. Cancer Cells. 1991;3:511-516.
13. Depeyter A, Willis WO, Liebhaber M. Cholinesterase activity in pregnant women and newborns. Clinical toxicology. 1994;32:638-696.
14. Stahr HM. Anyone for testing water and algae--for free. Veterinary and human toxicology. 1991;33(1):69.
15. Ellman G, Courtney D, Andres V, Featherstone R. A new rapid colorimetric determination of acetylcholinesterase activity. Biochemical pharmacology. 1961; 7:88–95.
16. Dietz AA, Rubinstein HN, Lubrano T. Colorimetric determination of serum cholinesterase and its generic variants by the propionylthiocholine-dithio-bis-(nitrobenzoic acid) procedure. Clinical chemistry. 1973;19:1309–13.
17. Michel HO. An electrometric method for the determination of red blood cell and plasma cholinesterase activity. The Journal of laboratory and clinical medicine. 1949; 34:1564-1568.
18. Wilson BW. Clinical enzymology. In: Loeb WF, Quimby FW (eds.). The clinical chemistry of laboratory animals. Philadelphia, PA: Taylor and Francis. 1999:399-454.
19. Nostrandt AC, Duncan JA, Padilla S. A modified spectrophotometric method appropriate for measuring cholinesterase activity in tissue from carbaryl-treated animals. Fundamental and applied toxicology. 1993;21:196-203.
20. Willig S, Hunter DL, Dass PD, Padilla S. Validation of the use of 6, 6'-ithiodinicotinic acid as a chromogen in the Ellman method for cholinesterase determinations. Veterinary and human toxicology. 1996;