Original Article

นิพนธ์ต้นฉบับ

The Study in Postmortem Serum Cholinesterase

การศึกษาระดับเอนไซม์คอลีนเอสเทอเรสในเลือดภายหลังตาย

Peerayuht Phuangphung M.D.*, Wichai Wongchanapai M.D., LL.B., Ph.D.*, Somboon Thamtakerngkit M.D., LL.B.*

**Department of Forensic Medicine, Faculty of Medicine, Siriraj Hospital, Mahidol University, Bangkok, Thailand*

พีรยุทธ เฟื่องฟุ้ง พ.บ.†, วิชัย วงศ์ชนะภัย พ.บ., น.บ., ปร.ด.†, สมบูรณ์ ธรรมเถกิงกิจ พ.บ., น.บ.†

†ภาควิชานิติเวชศาสตร์ คณะแพทยศาสตร์ศิริราชพยาบาล มหาวิทยาลัยมหิดล กรุงเทพฯ ประเทศไทย

Abstract

Objective: To study the stability of the postmortem serum cholinesterase activities under the storage at 4°C and room temperature at the prospective time.

Materials and Methods: Postmortem blood samples were recruited from the 34 cadavers sent for autopsy at the Department of Forensic Medicine, Siriraj Hospital. Measurement of serum cholinesterase (ChE) activities was analyzed by delta pH method of Michel.

Results: The postmortem serum cholinesterase activities were not significantly different between gender. The initial value of postmortem serum cholinesterase activities ranged from 2369.46 mU/ml to 4879.68 mU/ml, with a mean value of 3584.55 ± 652.11 mU/ml (Mean ± SD). The enzyme activities in the first three days were not significantly different between serum samples stored at 4°C and room temperature. But the enzyme activities in serum samples from the 4^{th} day to the 6^{th} day stored at 4°C were significantly lower than those in serum samples stored at room temperature.

Conclusion: The analysis of postmortem serum cholinesterase activities should be performed within three days after the collection of specimen and the normal range of postmortem serum cholinesterase activities was not significantly different from that of ante-mortem enzyme activities.

Keywords: postmortem, serum cholinesterase, organophosphate, carbamate, insecticide poisoning

บทคัดย่อ

วัตถุประสงค์ เพื่อศึกษาความคงตัวของระดับเอนไซม์คอลีนเอสเทอเรสในเลือดภายหลังตายโดยเปรียบเทียบระหว่างเลือดที่เก็บที่อุณหภูมิ 4°C และอุณหภูมิห้อง

วิธีการศึกษา ศึกษาโดยการเก็บเลือดจำนวน 34 ตัวอย่างจากศพที่ถูกส่งมาตรวจที่ภาควิชานิติเวชศาสตร์ โรงพยาบาลศิริราช มาทำการวิเคราะห์ โดยใช้วิธีการของมิเชลในวิเคราะห์หาระดับเอนไซม์คอลีนเอสเทอเรสในเลือด

ผลการศึกษา ระดับเอนไซม์คอลีนเอสเทอเรสในเลือดภายหลังตายไม่มีความแตกต่างกันระหว่างเพศ โดยค่าตั้งต้นของระดับเอนไซม์คอลีนเอสเทอเรสในเลือดภายหลังตายอยู่ในช่วง 2369.46 mU/ml ถึง 4879.68 mU/ml โดยมีค่าเฉลี่ย ± ค่าเบี่ยงเบนมาตรฐานของระดับเอนไซม์คอลีนเอสเทอเรสในเลือดภายหลังตายเท่ากับ 3584.55 ± 652.11 mU/ml ระดับเอนไซม์คอลีนเอสเทอเรสในเลือดภายหลังตายจากเลือดที่เก็บที่อุณหภูมิ 4°C และอุณหภูมิห้องไม่มีความแตกต่างกันในช่วง 3 วันแรกที่เก็บ แต่ในช่วงวันที่ 4 ถึงวันที่ 6 ระดับเอนไซม์คอลีนเอสเทอเรสในเลือดภายหลังตายจากเลือดที่เก็บที่อุณหภูมิ 4°C จะมีค่าน้อยกว่าเลือดที่เก็บที่อุณหภูมิห้องอย่างมีนัยสำคัญทางสถิติ

สรุป การวิเคราะห์หาระดับเอนไซม์คอลีนเอสเทอเรสในเลือดภายหลังตายควรจะทำการวิเคราะห์ภายใน 3 วัน นับตั้งแต่วันที่ทำการเก็บเลือดตัวอย่าง และค่าปกติของระดับเอนไซม์คอลีนเอสเทอเรสในเลือดภายหลังตายไม่มีความแตกต่างจากค่าปกติของระดับระดับเอนไซม์คอลีนเอสเทอเรสในเลือดของผู้ที่มีชีวิต

คำสำคัญ : ระดับเอนไซม์คอลีนเอสเทอเรสในเลือดภายหลังตาย, ยาฆ่าแมลงกลุ่มออร์กาโนฟอสเฟต, ยาฆ่าแมลงกลุ่มคาร์บาเมท, พิษจากยาฆ่าแมลง

Introduction

Pesticide poisoning still seems to be the common cause of toxicological-related death. Among the pesticide group, insecticide is the most common chemical poisoning. Insecticide covers about 40-50% of all pesticide-related deaths in Thailand. Organophosphate and carbamate are commonly used in the world including Thailand and mainly responsible for insecticide-related deaths in Thailand.

Deaths due to insecticide poisoning can be suicidal, accidental or even homicidal in manner. The most common route of administration is oral ingestion even though the victims can be exposed to insecticide poisoning via dermal or respiratory route. In the crime scene that has the evidence about the insecticide poisoning such as the glass containing the blue-green liquid or the container of insecticide left some blue-green particles, the physician and the police who co-operate in the postmortem examination at the crime scene should suspect for insecticide poisoning. In the autopsy cases those are suspected for organophosphate or carbamate poisoning, the measurement of postmortem serum cholinesterase activity will be beneficial for the diagnosis of these insecticide poisonings combined with the investigation of gastric content.

The diagnosis of organophosphate or carbamate poisoning in the autopsy cases is based on the detection of organophosphate or carbamate from gastric content and the decrement of serum cholinesterase activity in the postmortem blood sample. The serum cholinesterase activity in the patients who died of organophosphate or carbamate poisoning will decrease because the mechanism of action of organophosphate or carbamate involves in acetylcholinesterase inhibition.

The properties of postmortem blood are different from blood obtained from living person in several aspects. Hemolysis is the principal difference that occurs in the postmortem blood. Because cholinesterase enzyme exists in two forms, true cholinesterase found in red blood cell, nerve endings, brain and lung tissues and pseudocholinesterase (plasma or serum cholinesterase) found in plasma, so theoretically if hemolysis occurs, the serum cholinesterase activities in the postmortem blood will be affected. Moreover, if the autopsy is performed in the weekend, the postmortem blood samples will be collected and stored in the refrigerator at 4°C for 2-3 days before the analysis of serum cholinesterase activities is done. Therefore the objective of this study is to evaluate whether the change of serum cholinesterase activities in the postmortem blood samples from the cadavers who were not dead from insecticide poisoning stored in 4°C is different from that stored in the room temperature at prospective time. It will be applied to the interpretation of the postmortem serum cholinesterase activity in the suspected cases and the normal range of postmortem serum cholinesterase activity may be derived from this study.

Materials and Methods

Postmortem blood samples were recruited from the 34 cadavers those were sent for autopsy at the Department of Forensic Medicine, Siriraj Hospital from January 2008-July 2008. Postmortem blood samples selection was based on the following eligible criteria:

1. Postmortem interval of all cadavers was less than 24 hours
2. The cadaver did not have the severe injuries
3. The cadavers did not die of chronic illness
4. The cadavers did not die of massive hemorrhage

All postmortem blood samples were collected from cardiac blood. After postmortem blood samples were collected, they were centrifuged at 1200g for 5 minutes and the serum was collected. Then the serum was divided into 2 groups:

1. The first group of serum was conducted to measure for postmortem serum cholinesterase activity at the 0, 2^{nd}, 4^{th}, 6^{th}, 8^{th}, 24^{th} hours and then at the 2^{nd}, 3^{rd}, 4^{th}, 5^{th}, 6^{th} day and finally at one month. The serum was kept for the 4°C along the process of analysis
2. The first group of serum was conducted to measure for postmortem serum cholinesterase activity at the 0, 2^{nd}, 4^{th}, 6^{th}, 8^{th}, 24^{th} hours and then at the 2^{nd}, 3^{rd}, 4^{th}, 5^{th}, 6^{th} day and finally at one month. The serum was kept for the room temperature along the process of analysis

Analytical Procedure[5, 10, 12, 13]

Measurement of serum cholinesterase (ChE) activity was analyzed by delta pH method of Michel following these steps:

1. Preparing the Buffer's solution for 2 tubes by adjusting the temperature of the Buffer's solution to 25°C before analysis and each tube contained 3 ml of the Buffer's solution (The Buffer's solution is 0.02% dithiobisnitrobenzoic acid (DTNB) in 0.1 M sodium dihydrogen phosphate buffer solution (pH 7.4))
2. The testing tube was added by 20µl of serum sample and 0.1 ml of 5% acetylthiocholine iodide solution and the other tube was the control tube that nothing was added.

3. Cholinesterase activity to catalyze acetylthiocholine (ATCh) hydrolysis was summarized in the reaction 1 and 2

$$\text{Acetylthiocholine} \xrightarrow{\text{ChE}} \text{Acetate + Thiocholine} \quad (1)$$

$$\text{Thiocholine + DTNB} \longrightarrow \text{5-thio-2-nitrobenzoic acid (yellow color)} \quad (2)$$

4. The reaction was performed in the UV-Visible Spectrophotometer. Cholinestease activity was measured spectrophotometricallly by following the formation of the hydrolysis product, yellow colored 5-thio-2-nitrobenzoic acid (TNB) compound, at the Absorbance of 405 nm for 30 seconds. The cholinesterase activity was calculated as:

   Cholinesterase activity (mUnits/ml) = Change in absorbance in 30 seconds × 23460

Statistical Analysis

- Descriptive Data was analyzed by Microsoft Excel for Windows version 2003
- Student t-test analysis was performed with SPSS software version 13 for comparisons of serum cholinesterase activities between the group of postmortem blood samples kept in 4°C and room temperature at the following consecutive times
- Repeated measured ANOVA analysis was performed with SPSS software version 13 for comparisons of each value of serum cholinesterase activities in the group of postmortem blood samples kept in 4°C and in the group of postmortem blood samples kept in room temperature respectively.

Results

There were 34 postmortem blood samples included in this study and these samples were recruited from 23 male cadavers (67.6%) and 11 female cadavers (32.4%). The age range of all cadavers was from 8 years to 60 years, with a mean age of 33.97 years. The mean age of male group was 32.65 years and the mean age of female group was 36.73 years. The initial value of postmortem serum cholinesterase activity ranged from 2369.46 to 4879.68, with a mean value of 3584.55. The mean value of postmortem serum cholinesterase activities of male group was 3562.86

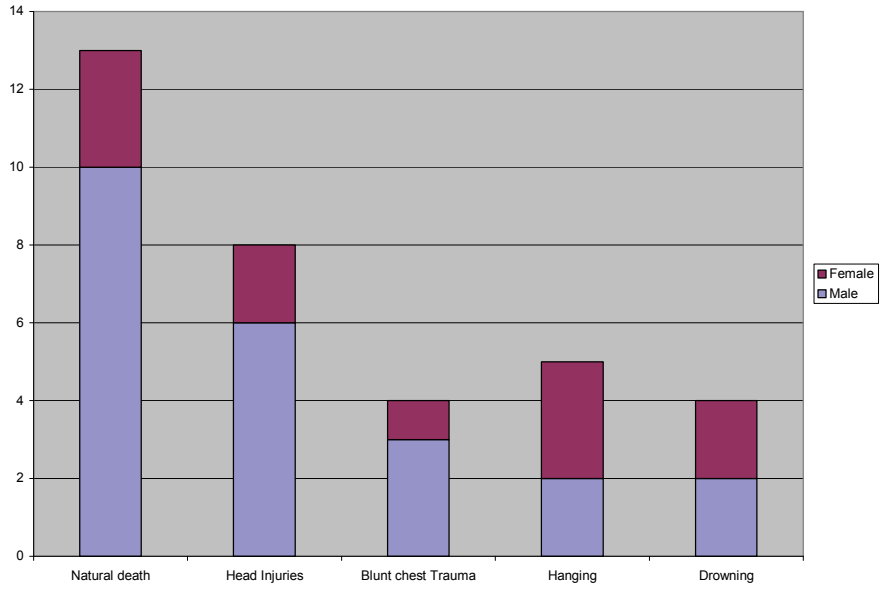
and the mean value of postmortem serum cholinesterase activities of female group was 3629.9. The initial values of postmortem serum cholinesterase activities were not significantly different between male and female. Summary of the demographic data was shown in Table 1 and summary of the initial values of postmortem serum cholinesterase activities characterized by gender was shown in Table 2. Summary of cause of death of the deceased was shown in Graph 1.

Case	N (%)	Mean Age (Range)
Male	23 (67.6%)	32.65 (16-59)
Female	11 (32.4%)	36.73 (8-60)
Total	34 (100%)	33.97 (8-60)

Table 1 Sex and age of the deceased

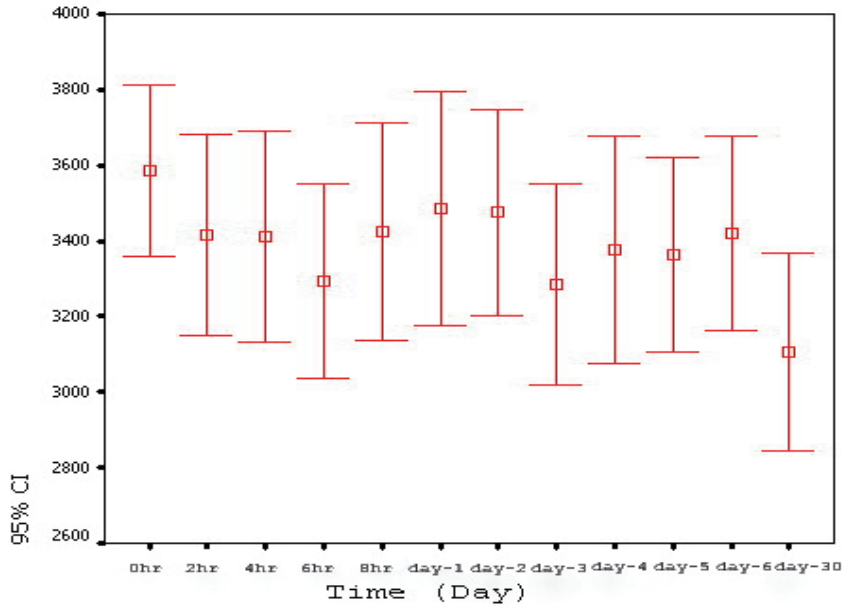
Case	N	ChE Activity			
		Mean ± SD	Maximal Value	Minimum Value	% CV
Male	23	3562.86 ± 646.45	4879.68	2369.46	18.14
Female	11	3629.90 ± 693.25	4785.84	2697.90	19.10
Total	34	3584.55 ± 652.11	4879.68	2369.46	18.19

Table 2 Postmortem serum cholinesterase activities in all of the deceased characterized by gender

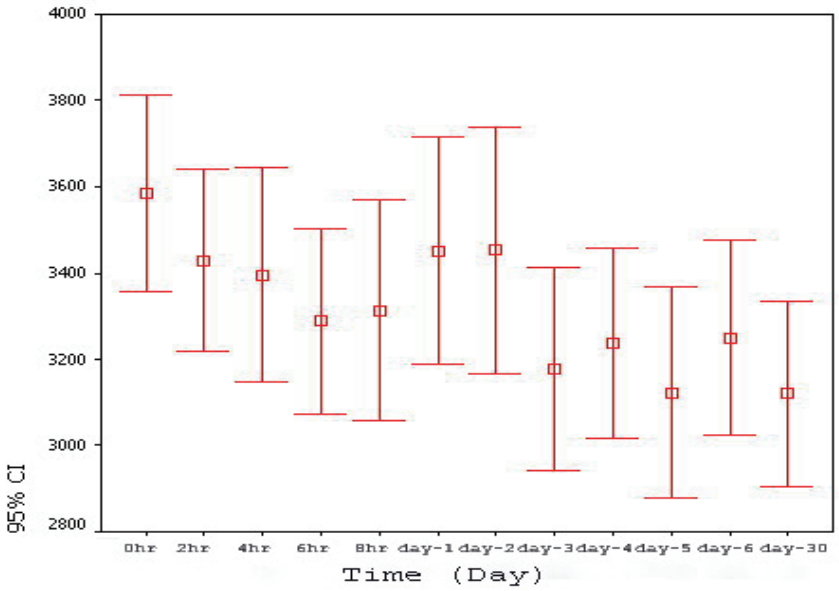


Graph 1 Cause of Death of deceased

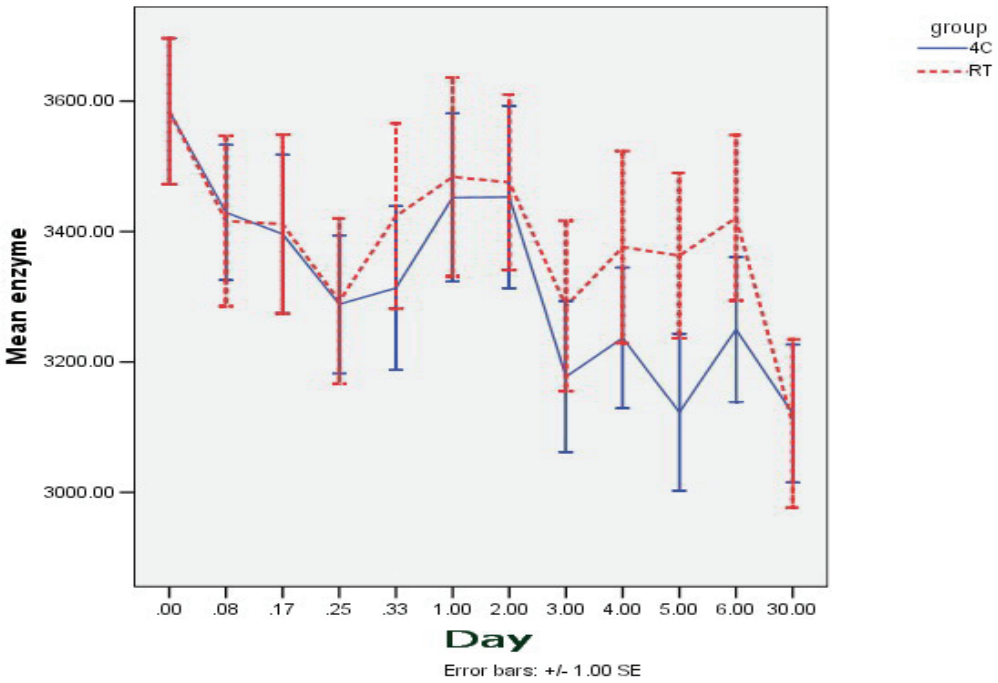
The change of postmortem serum cholinesterase activities of the group of postmortem blood samples kept in room temperature on the consecutive timeline was presented in Graph 2 and the change of postmortem serum cholinesterase activities of the group of postmortem blood samples kept in 4°C on the consecutive timeline was presented in Graph 3. Comparison of the changes of postmortem serum cholinesterase activities between these two groups of samples was shown in Graph 4. There were no statistically significant differences between the postmortem serum cholinesterase activities kept in 4°C and room temperature on the first three days whereas there were statistically significant differences between the postmortem serum cholinesterase activities kept in 4°C and room temperature on 4^{th}-6^{th} day ($p < 0.05$). When the enzyme activities were followed to the end of the month, there were no statistically significant differences between the postmortem serum cholinesterase activities of these two groups at the end of the month.



Graph 2 The change of postmortem serum cholinesterase activities of the group of postmortem blood samples stored in room temperature



Graph 3 The change of postmortem serum cholinesterase activities of the group of postmortem blood samples stored in 4°C



Graph 4 Comparison of the changes of postmortem serum cholinesterase activities between these two groups of postmortem blood samples

Statistically, there were no significant differences in the change of postmortem serum cholinesterase activities of the group of postmortem blood samples kept in 4°C and room temperature from the initial time to the end of the week. The values of enzyme activities revealed

significant differences in postmortem serum between the initial time and the end of the month ($p < 0.001$).

Discussion

Organophosphate and Carbamate are the commonly used insecticides in Thai agricultural areas. These insecticides are easily accessible because they are ubiquitously sold for the agricultural purpose. Therefore, the toxicities from these insecticides frequently occurred not only in the accidental cases but also in the many suicidal cases.

Mechanism of action of organophosphate and carbamate intoxication is that of cholinesterase inhibition. Therefore, the effects of these insecticides are cholinergic excess at the synaptic areas. Due to the cholinergic excess from the toxicities of organophosphate and carbamate poisoning, it produces classic signs and symptoms of "Cholinergic Toxidromes or DUMBELS" in the lived patients. But in the cases of postmortem examination at the crime scene are different from the situations of lived patients.[7, 9] The signs produced from "Cholinergic Toxidromes" such as miosis and hyper-secretion state are unreliable when the postmortem changes come over and the cadavers dead from these poisonings produce the non-specific findings both in external and internal examination. So the diagnosis of organophosphate or carbamate poisoning in the autopsy cases is only based on the detection of organophosphate or carbamate from gastric content and the decrement of serum cholinesterase activity in the postmortem blood sample.

Huizenga JR et al. reported that there was no change in catalytic activities of human serum cholinesterase from the initial time to about two months between serum samples stored at room temperature (20°C), at 4°C and at -20°C.[4] Klette KL et al. reported that postmortem blood samples kept at 25°C had no significant difference between the initial values of postmortem blood cholinesterase activities and the values of enzyme activities at the 7^{th} day. Klette KL et al. also found that the enzyme activities of the specimen had a mean value (range) of 4800 mU/ml (2000-7400 mU/ml) initially and 4500 mU/ml (700-7000 mU/ml) after one week.[6] But Klette KL et al. did not report about the enzyme activities of the postmortem blood kept at 4°C comparing with those kept at 25°C.

This study is aimed to determine the effect of temperature on stability of the postmortem serum cholinesterase activities when the measurement of serum cholinesterase activities is performed prospectively from initial day to one month. From this study postmortem serum cholinesterase activities of the postmortem blood samples kept at 4°C from the initial time to the 3rd day were not significantly different to that of the postmortem blood samples kept at room temperature. But from the 4th day to the 6th day there were statistically significant differences between the postmortem serum cholinesterase activities of these two groups. Graph 4 were shown that the mean values of postmortem serum cholinesterase activities in the group of 4°C were less than those of room temperature from the 4th day to the 6th day. It was anticipated that this result occurred due to the property of this enzyme in the postmortem state. The optimum pH for the hydrolysis of acetylcholine by this enzyme lies between 7.6 and 8.5 at 25°C and the optimum temperature for Serum Cholinesterase lies about 45°C.[1, 2] This was shown that the optimum conditions for cholinesterase enzyme are in the weak base and in the warm temperature. In fact, the postmortem blood was in the acidic state. Therefore serum cholinesterase in the postmortem blood was not in the favorable state to catalyze substrate. Moreover, the serum kept at 4°C was in the cool state, so theoretically the enzyme could not work well. In this experiment the effect of temperature obviously affected the enzyme activities from the 4th day to the 6th day, not at the first three days. When the postmortem serum cholinesterase activities of these two groups were followed to the end of the month, there were not statistically significant differences between the postmortem serum cholinesterase activities of these two groups. It may be concluded that temperature and pH have the equal effect to the enzyme activities when following the enzyme activities to the end of the month. Practically, because the postmortem serum cholinesterase activities in the first three days both kept at room temperature and at 4°C were not significantly different due to the fact that the temperature and pH had the little effect on the specimen, so this measurement procedure should be performed within three days after the collection of specimen.

From the general knowledge, the normal range of the ante-mortem plasma (or serum) cholinesterase activities was 1900-4000 mU/ml. From the previous studies, it was found that the normal range of the ante-mortem plasma cholinesterase activities was about 3000-10000 mU/ml in

adult and about 5000-12000 mU/ml in children[8, 3, 11]. There was no difference in the ante-mortem plasma cholinesterase activities between male and female groups. The enzyme activities from the previous studies seemed to be higher than that described in the general knowledge and the enzyme activities in the children groups seemed to be slightly higher than those in the adult groups.

When the postmortem cholinesterase activities were considered as shown in Table 3, they were found that at the initial day the postmortem cholinesterase activities were not different from the normal range of the ante-mortem serum cholinesterase activities described in the general knowledge but they appeared to be lower than that derived from the previous experiment as shown in Table 3. Before this study was conducted, it may be anticipated that the postmortem serum cholinesterase activities should be just higher than ante-mortem serum cholinesterase activities because the postmortem blood had the process of hemolysis. From the previous knowledge, there were two forms of the cholinesterase enzyme existing in the bodies, true (RBC) cholinesterase and serum cholinesterase. When the hemolysis began, some of true (RBC) cholinesterase should leak into the serum and the total serum cholinesterase activities should slightly increase. But in this experiment the postmortem serum cholinesterase activities were not different from the ante-mortem serum cholinesterase activities. It could be hypothesized that this phenomenon was due to the preference of this enzyme to the substrate. True (RBC) cholinesterase prefers to hydrolyze acetylcholine while serum cholinesterase prefers to hydrolyze butyrylcholine. In this experiment, acetylthiocholine (ATCh), not acetylcholine, was used as the substrate in the measurement procedure. Because the enzyme generally reacted to the substrate following the “lock and key” model, so true (RBC) cholinesterase leaked into the serum might not catalyze this substrate well. Therefore the postmortem serum cholinesterase activities measured in this study were not significantly changed from the normal range of ante-mortem serum cholinesterase activities.

Study	Specimen	N	Temp (°C)	Initial Day	1st Week
Klette et al. (1993)	Whole blood	53	25°C	4800 ± 1400 (2000-7400)	4500 ± 1400 (700-7000)
			4°C	N/A	N/A
Present study (2008)	Serum	34	RT	3584.55 ± 652.11 (2369.46-4879.68)	3421.05 ± 739.64 (2205.24-5677.32)
			4°C	3584.55 ± 652.11 (2369.46-4879.68)	3249.90 ± 647.66 (2369.46-5255.04)

Table 3 Summary of comparison of postmortem blood cholinesterase activities from the previous study to the present study

Finally, from the previous studies, there were no statistically significant changes in ante-mortem serum cholinesterase activities from the initial time to about two months between serum samples stored at room temperature (20°C), at 4°C and there were no statistically significant changes in postmortem whole blood cholinesterase activities from initial time to the first week in blood samples stored at 25°C. In this study, the results were somewhat different to the previous studies. In the first week, there were no statistically significant differences in the values of postmortem serum cholinesterase activities in both groups of these postmortem blood samples. This result was similar to the previous studies and it was shown that the enzyme was mostly stable within the first week. But when the enzyme activities were followed to the end of the month, their values were significantly different from that of initial time. It was suggested that the postmortem enzyme activities had the tendency to decline when their values were followed up to the end of the month. This may be due to the effect of environment of postmortem serum and the loss of normal structure and conformation of the enzyme when the one month elapsed. It could be concluded that the postmortem enzyme activities had the most reliable stability in the first week and the postmortem enzyme activities would decrease when the analysis was performed on the one month later.

Conclusion

From this study, it may be concluded that

1. The values of postmortem serum cholinesterase activities derived from serum stored at 4°C were not significantly different from that derived from serum stored at room temperature in the first three days. Whereas the values of postmortem serum cholinesterase activities derived from serum stored at 4°C were significantly different from that derived from serum stored at room temperature from the 4^{th} day to the 1^{st} week. So the values that came from the serum in the first three days were more reliable than the values that came from the serum after three days. Therefore, we should perform the analysis of postmortem serum cholinesterase activities within three days after the collection of specimen.
2. The mean value of the postmortem serum cholinesterase activities at the initial time was 3584.55 ± 652.11 mU/ml, with the range from 2369.46 mU/ml to 4879.68 mU/ml. The normal range of postmortem serum cholinesterase activities was not significantly different from the normal range of ante-mortem serum cholinesterase activities described in many literatures.
3. Postmortem serum cholinesterase activities had the most reliable stability when they were measured in the first week and their values would decrease when the measurement of the enzyme activities was performed on the one month later.

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