Original Article

นิพนธ์ต้นฉบับ

Stability of methomyl in blood under experimental condition

ความคงตัวของเมทโธมิลในเลือดภายใต้สภาวะทดลอง

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Abstract

Objective: To study the effect of storage time on the analysis of blood samples for methomyl concentration and to examine the stability of methomyl concentration in blood in different temperatures.

Materials and Methods: Blood samples from autopsy and the expired blood from blood bank were prepared for the testing of stability. Methomyl was spiked into both sources of blood to make a final concentration of 5 µg/ml, and then the samples were kept at 2 different conditions, 4˚C and room temperature (RT), for 6 months. The samples were extracted with chloroform : isopropanol (9:1) at each of the observation periods (0, 3, 7, 14, 28, 90 and 180 days) and then were analyzed with HPLC.

Results: The results showed that methomyl concentration at both 4˚C and RT conditions decreased drastically within the first 3 days, 69.35% and 41.59% decreasing from the beginning concentration at 4˚C and RT, respectively in the autopsy blood, and 68.15% and 51.04% decreasing at 4oC and RT, respectively in the expired blood; therefore, they were stable throughout the rest of storage time. Methomyl concentration of the autopsy blood samples stored at 4˚C significantly decreased lesser than those stored at RT only in the day 3 and 7, but there was no significant difference in its decrement in those of the expired blood. In addition, there was no significant difference in the change of methomyl concentrations in both of the autopsy and expired blood between both different temperature storages.

Conclusion: Methomyl concentration in blood is stable whether the samples are stored at 4˚C or RT and still be that for 6 months. The concentration may decrease from the initial concentration only in the first 3 days. Moreover, methomyl concentration from blood sample stored at 4˚C decrease lesser than that stored at RT in the first period of time.

Keywords: Methomyl blood concentration, Storage stability, Forensic toxicology

บทคัดย่อ

วัตถุประสงค์: เพื่อศึกษาผลของระยะเวลาที่ใช้ในการเก็บรักษาที่มีต่อการวิเคราะห์ และเพื่อศึกษาความคงตัวของเมทโธมิลที่เก็บรักษาไว้ที่สองสภาวะที่ต่างกันคือ 4 องศาและอุณหภูมิห้อง

วัสดุและวิธีการศึกษา: ในการศึกษาครั้งนี้ได้นำเลือดจากผู้เสียชีวิตและเลือดหมดอายุจากธนาคารเลือดมาเติมเมทโธมิลลงไปให้มีความเข้มข้น 5 µg/ml และเก็บรักษาไว้ที่ 4 องศาเซลเซียส (˚C) และที่อุณหภูมิห้องเป็นเวลาถึง 6 เดือน การตรวจวิเคราะห์จะทำการสกัดด้วย chloroform : Isopropanol ในอัตราส่วน 9:1 ที่ระยะเวลาต่างๆกัน คือ 0, 3, 7, 14, 28, 90 และ 180 วัน ก่อนจะนำไปตรวจวิเคราะห์ด้วยเครื่องไฮเพอร์ฟอร์แมนซ์ลิควิดโครมาโตกราฟี (HPLC)

ผลการศึกษา: พบว่าที่ทั้งสองอุณหภูมิ ในช่วง 3 วันแรกมีการลดลงของระดับความเข้มข้นเมทโธมิลอย่างรวดเร็วและมีนัยสำคัญทางสถิติเมื่อเปรียบเทียบกับความเข้มข้นเริ่มต้น คือลดลงไป 69.35% และ 41.59% ในเลือดจากผู้เสียชีวิตที่ 4˚C และอุณหภูมิห้องตามลำดับและลดลงไป 68.15% และ 51.04% ในเลือดหมดอายุจากธนาคารเลือดที่ 4˚C และอุณหภูมิห้องตามลำดับ หลังจากนั้นความเข้มข้นเมทโธมิลจะคงตัวไปจนตลอดระยะเวลาทดลอง ความเข้มข้นของเมทโธมิลที่เก็บไว้ที่อุณหภูมิ 4˚C ในเลือดจากผู้เสียชีวิตมีการลดลงที่น้อยกว่าตัวอย่างที่เก็บที่อุณหภูมิห้องอย่างมีนัยสำคัญในวันที่ 3 และ 7 แต่ไม่พบว่ามีความแตกต่างอย่างมีนัยสำคัญในเลือดหมดอายุจากธนาคารเลือด ยิ่งไปกว่านั้นการลดลงของความเข้มข้นเมทโธมิลที่เก็บไว้ทั้งสองอุณหภูมิก็ไม่มีการเปลี่ยนแปลงที่แตกต่างกันอย่างมีนัยสำคัญ นอกจากนี้การเปรียบเทียบผลระหว่างเลือดจากผู้เสียชีวิตและเลือดหมดอายุจากธนาคารเลือดพบว่า การลดลงของความเข้มข้นเมทโธมิลที่เก็บไว้ทั้งสองอุณหภูมิไม่มีความแตกต่างกันอย่างมีนัยสำคัญทางสถิติ

สรุป: ระดับเมทโธมิลในตัวอย่างเลือดที่เก็บรักษาไว้ที่ 4˚C หรือที่อุณหภูมิห้องเป็นเวลา 6 เดือนจะคงที่ แต่จะลดลงอย่างมีนัยสำคัญทางสถิติเมื่อเปรียบเทียบกับความเข้มข้นเริ่มต้นภายหลัง 3 วันแรกเท่านั้น และการเก็บรักษาตัวอย่างไว้ที่ 4˚C จะช่วยชะลอการลดลงของเมทโธมิลในช่วงแรกได้ดีกว่าการเก็บไว้ที่อุณหภูมิห้อง

คำสำคัญ: เมโธมิล, ความเข้มข้นในเลือด, ความคงตัวในการเก็บรักษา, นิติพิษวิทยา

Introduction

Nowadays, the rate of committing suicide in Thailand is higher. Hanging is the most popular way to commit suicide following by toxic substance ingestion. Most of the substances are agriculture-used. The most one is methomyl, or widely known as Lannate® or Nudrin® [1]. Methomyl (methyl-N-[(methylcarbanomoyl)oxy]-thioacetimidate) is a carbamate insecticide frequently used worldwide whose toxicity is over-stimulation of parasympathetic activity, as organophosphate does, by reversibly binding and inhibiting acetylcholinesterase, and results in excess accumulation of acetylcholine at

muscarinic and nicotinic receptors [2]. This mechanism clinically presents with slowing of heart rate, constriction of pupil, bronchoconstriction, increasing in secretion (known as the four classical symptoms, salivation, lacrimation, urination, and defecation), seizures and even death due to paralysis of respiratory muscles [3, 4]. Methomyl is highly toxic while administrating via oral and ocular routes or via inhalation, but it has a low toxicity via dermal exposure. It is absorbed from gastrointestinal tract and rapidly metabolized to weak compounds through hepatic hydrolysis and other pathways, and finally excreted in urine [2, 5].

Several types of body fluid are obtainable from routine autopsy procedure such as pericardial fluid, blood, bile, urine, cerebrospinal fluid and vitreous humor. In drug analysis, including methomyl, fluid samples such as blood are much easier to handle in the process than solid tissue samples. In the death due to poisoning, blood is a direct evidence to prove a cause of the death by detecting and determining drug concentration. Most forensic laboratories preserve specimens for at least 6 months or a period of time which fulfils requirement of the law so a study of its stability is necessary to discover whether drug concentrations are different between each period of time. Previous studies explained that postmortem change in blood is able to affect concentration of drugs and poisons; thus, specimens should be taken shortly after death where it is hardly possible. To understand the effect of postmortem change on the stability of blood concentration of methomyl, the expired donor blood from blood bank was a choice for using as a control in order to compare with the autopsy blood. Both groups of specimen are simultaneously tested to examine the difference of methomyl concentration in blood among specific periods of time to observe its stability.

Materials and Methods

This study was divided into 3 consecutive steps as follows:

1. Optimal extraction and analysis

The optimal condition for extraction of methomyl from blood was taken after a 2-ml. spiked sample (in the final concentration of 5µg/ml of methomyl) was diluted to a half fold with normal saline (1:1). After vortex for 1 min, to homogenize, the mixture was transferred to a separating funnel, then 10 ml. of chloroform : 2-propanol (9:1 v/v) was added and shaken for 10 min. The under-layer was removed by filtering with Whatman® filter paper N0. 1PS and then it was dried and re-dissolved with 2 ml. of acetonitrile : water (60:40 v/v). Finally, it was filtered and analyzed with HPLC.

2. Method validation

2.1 Selectivity

We used the suitable standard reagents, methomyl, aldecarb, carbofuran and carbaryl, each of which has a retention time at 3.314, 4.005, 4.575 and 6.144 min., respectively; thus, each component must be well separated and easily distinguishable as the resolution factor (R) >1 for each pair of the peaks.

2.2 Accuracy

Preparation of 1 mg/ml of methomyl was spiked into blood to make 5 µg/ml concentration and the analysis results revealed their concentrations ranging between 1.758-2.037 µg/ml. The accuracy was calculated into the percentage of recovery which was 35.16-40.74%.

2.3 Precision

The precision in this study was determined by Horwitz ratio. Observed relative standard deviation (RSD) was calculated from mean and standard deviation of 10 extractions of methomyl-spiked blood and the result was 5.894, whereas, expected RSD calculated from Horwitz equation was 5.582. So the HORRAT or Horwitz ratio was 1.06 that was an acceptable value.

2.4 Linearity range

Five concentration levels of methomyl-spiked blood samples which were 1, 5, 10, 15 and 20 µg/ml, were prepared for plotting the calibration curve. The correlation coefficient (r^2) was 0.9901.

2.5 LOD/LOQ

Five concentration levels of methomyl-spiked blood samples, which were 1, 5, 10, 15 and 20 µg/ml, were extracted and analyzed. LOD and LOQ were calculated by three and ten times, respectively, from Y-intercept point of the plotted graph, which was plotted between mean, in X axis, and standard deviation, in Y axis. The LOD and LOQ of methomyl concentration in blood were 0.4242 and 1.414 µg/ml, respectively.

3. Stability testing

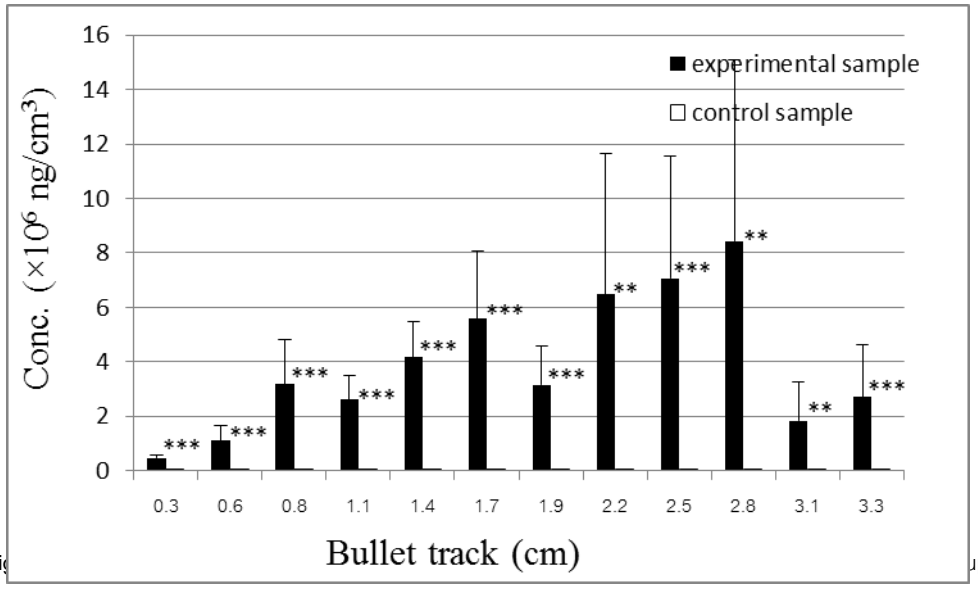
Methomyl-free samples from both autopsy and expired donor blood were spiked with 1mg/ml standard-preparation methomyl to make a final concentration of 5 µg/ml and stored at 2 different temperatures, 4˚C and room temperature (RT). Optimal extraction and analysis method was done at specific points-of-time (0, 3, 7, 14, 28, 90 and 180 days), which made 14 groups of data, and statistical comparisons among mean concentration of each group were calculated for paired t-test, between autopsy and expired blood at both storage temperatures, and repeated ANOVA for analysis between

each specific point-of-time with the initial concentration in both storage conditions. These statistical analysis were programmed by SPSS V.11 .

Results

1. The stability of methomyl concentration in autopsy blood stored at 4˚C and room temperature (RT)

Spiked autopsy blood stored at 4˚C and room temperature was extracted at specific time periods: 0, 3, 7, 14, 28, 90 and 180 days and then were analyzed with HPLC. The results were shown in the graph below (Fig. 1). Methomyl concentration in blood decreased rapidly in the first 3 days when it was compared with the beginning concentration in both 4˚C and RT conditions (p-value≤0.01), after that it was constant throughout storage time (p-value≤0.01).



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*Significant difference between 4°C and RT at each storage time (p-value <0.05). * (p-value <0.01).

2. The stability of methomyl concentration in expired blood from blood bank stored at 4˚C and room temperature (RT)

Spiked expired blood stored at 4˚C and room temperature was extracted at specific time periods: 0, 3, 7, 14, 28, 90 and 180 days and then were analyzed with HPLC. The results were shown in the graph below (Fig. 2). The decrement of methomyl concentration in expired blood was the same pattern as those found in the autopsy blood. It rapidly decreased in the first 3 days in comparison with the beginning concentration in both 4˚C and RT conditions (p-value≤0.01), and then it was constant throughout the rest of storage time (p-value≤0.01).

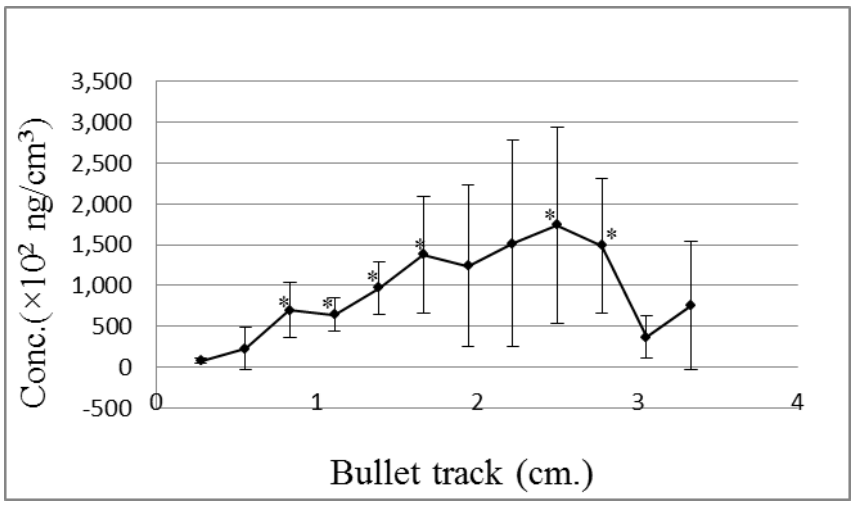

Fig. 2. The stability of methomyl concentration in expired blood from blood bank stored at 4°C and RT. (n=6 for each group)

*Significant difference between 4°C and RT at each storage time (p-value <0.05). ** (p-value <0.01).

3. Comparison between autopsy blood and expired blood

Both groups of autopsy and expired blood were compared at each storage condition and plotted as shown in the graph below (Fig. 3 & 4). Statistical calculation revealed that, at 4˚C condition, there was significant difference at the day 90 (p-valued≤0.01); at RT condition, there was significant difference at the day 7 and 90 (p-valued ≤0.05).

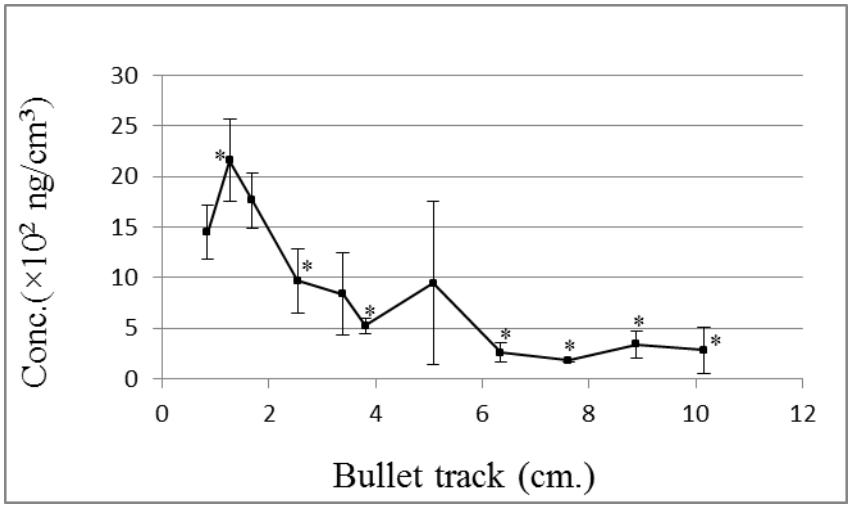

Fig. 3. The stability of methomyl concentration in autopsy and expired blood stored at 4°C. (n=6 for each group)

*Significant difference between autopsy blood and expired blood at each storage time (p-value <0.05). ** (p-value <0.01).

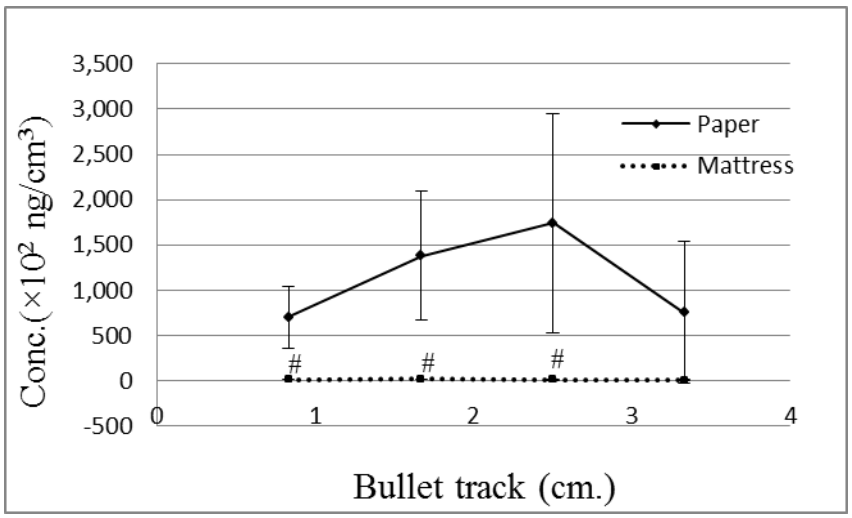

Fig. 4. The stability of methomyl concentration in autopsy blood and expired blood stored at room temperature. (n=6 for each group)

*Significantly different between autopsy blood and expired blood of each storage time (p-value <0.05) ** (p-value <0.01).

Discussion

LLE (liquid-liquid extraction) was chosen to perform in this study because it is favorably used in most laboratories and takes a low cost. Several methods were brought to try for seeking the best way of methomyl extraction from blood, including protein precipitation with zinc sulfate to remove the contaminating matrix, and non-precipitated diluted samples. The results showed that diluted sample without protein precipitation was the most satisfying for its recovery yield, so this procedure was chosen. In the optimizing method trial, its product was an emulsion which interfered with the analytic process. Therefore, mixture of chloroform and isopropanol was applied to solve this problem. The optimizing proportion found from our trail was a ratio of 9 : 1 for chloroform : isopropanal produced the least emulsion while the recovery product of methomyl was acceptable.

In the part of stability testing, standard methomyl was spiked into postmortem and expired packed red cell pool at the concentration of 5 µg/ml, and then all samples were stored at 2 different conditions (4˚C and room temperature). The extracted solution was performed by HPLC at each specific observation periods which was at 0th, 3rd, 7th, 14th, 28th, 90th and 180th days. The results showed that storage time was a significant factor which influenced methomyl concentration in both types of blood, while the different preserved condition was a factor which significantly affected on methomyl concentration only in the first 3 days, but not for the rest of time. These results were similar to the study of Ito et al. [6] who reported in the rapid decrement of methomyl concentration in blood after storage at room temperature. According to the dramatic decrement of methomyl concentration within the first 3 days, this might be affected by metabolism which occurred rapidly. Due to its chemistry, a

sulfur containing compound, this is susceptible to a variety of enzymatic biotransformation [7, 8] including carboxylesterase in blood 4. Carboxylesterase enzyme is known as a detoxifying enzyme of many drugs including carbamates and organophosphates[9-11]. This process results in rapid metabolization of methomyl into its metabolites, acetamide and acetonitrile, which are evaporable. The reaction may effect the change of methomyl concentration in blood in this study which directly detected methomyl, a parent compound. After 3 days, the results showed that methomyl concentration was constant throughout the rest of storage time. This might be affected by decomposition after death. The first stage of decomposition is in a first few days. At this stage, cell autolysis begins as green or purple discoloration on the body appears due to blood decomposition [12]. Autolysis is spontaneous self-destruction of cells which occurs when they cannot obtain oxygen because of a dysfunction of blood circulation [13] and the cells finally break down, and it also degrades enzymes. This might be the reason why methomyl concentration in our study was constant after the first three days. In addition, aeration can increase the rate of methomyl degradation [14] but in this study methomyl-spiked blood was an aliquot, containing 3 ml. in each tube, which was enough for one extraction; thus, the factor of oxidation or aeration from re-opening of the tube might not result in degradation of methomyl.

In both of the different storage conditions, 4˚C and room temperature, methomyl concentration decreased significantly from the initial concentration in the whole storage time, but in the first 7 days, the decrement of that at 4˚C were less than that at room temperature. This might be a result from the effect of temperature on enzyme activity, in the other words; an increment of temperature causes an increment in the activity. This result was similar to that of Ramajiti et al. [15] who reported about the decrement of Baygon® , a compound in the same group as methomyl. They found that degradation of the compound at 4˚C and at room temperatures decreased rapidly in the whole storage time (60 days) but it was stable at -20˚C and -80˚C. This could be summarized that carbamate compound concentration in blood less decreased when they were stored at 4˚C than at room temperature.

For a comparison between autopsy and expired blood, the aim of this part was to focus on whether postmortem factors might affect methomyl degradation. The time of death is an uncontrollable factor as it varies from one case to another at the time of autopsy. More decomposition it is, more degradation may be, on the other hand, donated blood is much fresher and is assumed as the least degradation of methomyl. Surprisingly, the results showed that methomyl concentration in both types of blood degraded in the same pattern. Expired blood showed degradation of methomyl slightly lesser than in autopsy blood from our whole observation period without significant statistical difference. According to the aforementioned, the factors affecting degradation of methomyl in blood were from

both enzymatic metabolism and degradation of methomyl. From this study, although the stage of decomposition was not equal, the degradation showed no significant change. Moreover, it seemed that enzymatic metabolism of methomyl in blood might play a role in changing concentration more potentially than degradation of methomyl.

Conclusion

The results indicated that the effect of storage time influenced methomyl concentration in blood. The decrement was found in both autopsy and expired blood throughout the observation period (6 months) in comparison with the initial concentration, especially in the first 3 days, and it occurred in both 4˚C and room temperature conditions. The decrement in the first 7 days at 4˚C was slightly lesser than at room temperature with statistical significance only in autopsy blood, not in expired blood. It is suggested that detection of methomyl concentration in blood can be performed in samples stored at 4˚C or RT for up to 6 months without significant difference, and the decrement of methomyl concentration is slower while keeping blood sample at 4˚C than at room temperature only in the first period. Methomyl concentration from both storage temperatures decreased in the same pattern but there was no significantly different. These illustrated that enzymatic metabolism of methomyl in blood might affect either samples from autopsy or fresh blood. Decrement of methomyl concentration was probably caused by enzymatic metabolism more potentially than blood decomposition.

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