การศึกษาความคงตัวของสารคาร์บาเมตในเลือดภายใต้สภาวะทดลอง

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บทคัดย่อ

ยาฆ่าแมลงกลุ่มคาร์บาเมตเป็นสารพิษที่พบบ่อยในผู้ป่วยที่ได้รับสารพิษในประเทศไทย แต่ข้อมูลความคงตัวของสารกลุ่มคาร์บาเมตยังมีน้อย ดังนั้นการศึกษานี้มุ่งศึกษาความคงตัวของสารกลุ่มคาร์บาเมต 3 ชนิด ได้แก่ คาร์บาริล คาร์โบฟูราน และโพรพอกเซอร์ ที่อุณหภูมิแตกต่างกัน ทำการศึกษาโดยเติมสารคาร์บาริล คาร์โบฟูราน และโพรพอกเซอร์ในเลือดที่ความเข้มข้น 3 ระดับ (ต่ำ, กลาง, สูง) จากนั้นแบ่งเลือดดังกล่าวออกเป็นกลุ่มๆ และเก็บไว้ที่อุณหภูมิห้อง และ 4 องศาเซลเซียส โดยวิเคราะห์หาสารทั้งสามชนิดในเลือดที่วันเริ่มต้นก่อน จากนั้นวิเคราะห์เลือดที่เก็บไว้ที่อุณหภูมิห้องที่วันที่ 1, 2, 3, 7, 14 และ 30 และวิเคราะห์เลือดที่เก็บไว้ที่ 4 องศาเซลเซียสที่วันที่ 3, 7, 14, 30, 60, 90 และ 180 วิเคราะห์ข้อมูลด้วยสถิติเชิงพรรณาและการทดสอบอะโนวา ผลพบว่าสารทั้งสามชนิดที่เก็บในอุณหภูมิห้องลดลงอย่างมีนัยสำคัญ โดยเฉพาะอย่างยิ่งน้อยกว่า 15% เทียบกับค่าตั้งต้นตั้งแต่วันที่ 7 ถึง 30 ($p<0.001$) และสารทั้งสามชนิดที่เก็บใน 4 องศาเซลเซียสลดลงอย่างมีนัยสำคัญ โดยเฉพาะอย่างยิ่งน้อยกว่า 15% เทียบกับค่าตั้งต้นตั้งแต่วันที่ 7 ถึง 180 ($p<0.001$) คาร์โบฟูรานมีอัตราลดลงเร็วที่สุดตามด้วยโพรพอกเซอร์ และคาร์บาริล โดยสรุปคาร์บาริล, คาร์โบฟูราน และโพรพอกเซอร์มีความไม่คงตัวที่อุณหภูมิห้อง และ 4 องศาเซลเซียส คาร์โบฟูรานมีความไม่คงตัวที่สุดตามด้วยโพรพอกเซอร์ และคาร์บาริล ดังนั้นจึงแนะนำว่าตัวอย่างเลือดจากผู้ที่สงสัยว่าได้รับสารพิษกลุ่มคาร์บาเมตควรเก็บตัวอย่างและตรวจวิเคราะห์โดยเร็ว เพราะสารพิษกลุ่มคาร์บาเมตทั้งสามชนิดนี้มีความไม่คงตัวทั้งที่อุณหภูมิห้อง และ 4 องศาเซลเซียส

คำสำคัญ: คาร์บาเมต ความคงตัว เลือด อุณหภูมิ

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The Stability of Carbamates in Blood Samples under Experimental Conditions

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Abstract

Carbamate insecticides are the most prevalent compounds involved in cases of poisoned patients in Thailand. However, the stability data of carbamate insecticides are relatively limited in literatures. Thus, this study aimed to investigate the stability of three common carbamate insecticides including carbaryl, carbofuran and propoxur under different temperature conditions. Pooled blood samples were spiked with carbaryl, carbofuran and propoxur at three concentration levels (low, medium and high). These three compounds were analyzed for initial concentrations, after which they were divided into aliquots and stored both at room temperature (25°C) and in a refrigerator (4°C). Next, all samples were analyzed for these three compounds on the 1st, 2nd, 3rd, 7th, 14th and 30th day at room temperature and on the 3rd, 7th, 14th, 30th, 60th, 90th and 180th day at 4°C. Descriptive statistics and one-way ANOVA were performed to investigate the changing trend of these three compounds. The changing rates in these three analyte concentrations at room temperature were significantly decreased, particularly less than 15% from the initial concentrations on the 7th day to the 30th day ($p<0.001$). At 4°C, all three analytes decreased significantly, particularly less than 15% from initial the concentrations on the 7th day to the 180th day ($p<0.01$). Carbofuran decreased at the fastest rate, followed by propoxur and carbaryl, respectively. In conclusion, carbaryl, carbofuran and propoxur were significantly unstable at both room temperature and 4°C. Carbofuran was the most unstable compound, followed by propoxur and carbaryl, respectively. It was suggested that blood samples from suspected carbamate poisoning cases should be collected and analyzed as soon as possible because these three ordinary carbamate insecticides were not stable both at room temperature and 4°C.

Keywords: Carbamates, Stability, Blood, Temperature

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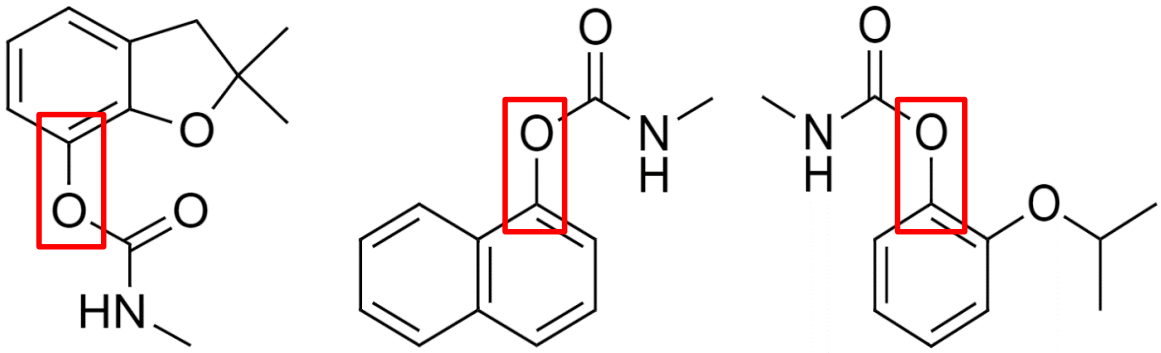
Introduction

Carbamate insecticides are one of the most common insecticides used in Thailand. According to a previous study, carbamate insecticides were the most prevalent compounds detected in Thai patients suspected of poisoning[1]. Among several types of carbamate insecticides, methomyl, carbofuran and carbaryl were commonly involved in cases of poisoned patients in Thailand[2]. The diagnosis of carbamate poisoning can be performed using cholinesterase activities[3], and the detection of carbamate insecticides in blood, urine and gastric content[4]. However, the detection of carbamate insecticides in the blood may be problematic because several carbamate insecticides are relatively unstable and can be degraded into their metabolite forms[5]. Thus, there may be some limitations in using the detection of carbamate insecticides in blood to diagnose carbamate poisoning. According to a previous study, when some animals including goats, chicken and monkeys were exposed to methomyl, methomyl oxime and acetonitrile that were conjugated with sulfate and cysteine were detected as metabolites of methomyl in these animals[6]. This study showed that methomyl could be rapidly degraded by esterase enzymes into metabolites in animal bodies[6]. In addition, a previous study stated that the detection of some types of carbamate insecticides such as methomyl in cadavers who were dead from methomyl poisoning could produce negative results or low concentrations of methomyl at below the toxic level[7]. The proposed hypotheses of this phenomenon were the activities of blood esterase enzymes or the effect of bacterial activities in blood[6]. Consequently, data concerning the stability of carbamate insecticides should be a priority for interpretation, particularly in case of suspected poisoning. However, the stability data of carbamate insecticides in biological samples remains limited.

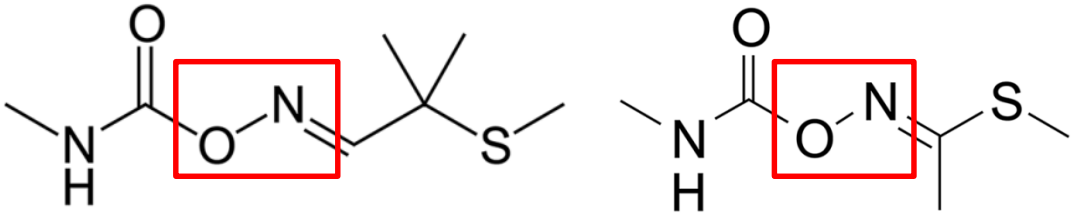
Ramagiri S et al. studied the stability of propoxur in blood and urine using spiked samples obtained from living volunteers[8]. They found that propoxur in the blood samples decreased to approximately 20% and 5% of initial concentrations on the 28th day and 60th day at room temperature, respectively[8]. When samples were stored at 4°C, propoxur in blood samples decreased to approximately 80% and 58% of initial concentrations on the 28th day and 60th day, respectively[8]. These data showed that propoxur was unstable in spiked blood samples both at room temperature and 4°C. The chemical structures of carbamate insecticides can be divided into two groups including ordinary carbamates and oxime carbamates. Carbofuran, carbaryl, and propoxur are examples of ordinary

carbamates as shown in Figure 1 whereas methomyl and aldicarb are classified as oxime carbamates as shown in Figure 2. The main difference between these two types of carbamate insecticides was the connection of ester bond of N-methyl carbamic acid. Ordinary carbamates had ester bonds of carbamic acid with phenol groups (O-phenol) whereas oxime carbamates had ester bonds with oximes (O-N=). Carbofuran and carbaryl are two types of carbamate insecticides which are commonly found in Thai patients suspected of poisoning and belong to ordinary carbamates. Data concerning stability of these two carbamates should be investigated and compared with the stability data of propoxur that belongs to the same class. Thus, this study aimed to investigate the stability of ordinary carbamates including carbofuran, carbaryl and propoxur to obtain stability data. These stability data will be useful for the interpretation of these carbamate concentrations in blood samples in cases of suspected poisoning.



Carbofuran Carbaryl Propoxur

Figure 1. Chemical structures of carbofuran, carbaryl and propoxur



Aldicarb Methomyl

Figure 2. Chemical structures of methomyl and aldicarb

Materials and Methods

Chemicals and reagents

Carbaryl and carbofuran at concentrations of 1 mg/mL were purchased from AccuStandard, Inc. Propoxur and carbofuran-d_3 at concentrations of 1 mg/mL and 100 µg/mL were from LGC Standards Ltd. Acetonitrile and methanol LC/MS grade were obtained from Duksan Pure Chemicals Co., Ltd. Ammonium acetate and formic acid were purchased from Merck Ltd. Potassium phosphate monobasic and potassium phosphate dibasic were purchased from Daejung Chemicals & Metals Co., Ltd. All standards and chemicals were imported from all manufacturers stated above by U&V Holding (Thailand) Co., Ltd. Deionized water (dH_2O) was produced from a Merck Millipore Direct-Q® 3 UV-R Water Purification System.

Instrumentation

The analysis of carbaryl, carbofuran and propoxur in blood was performed using an electrospray ionization liquid chromatography Quadrupole Time-of-Flight (QTOF) mass spectrometry (ESI-LC-QTOF-MS). A Thermo Scientific Dionex Ultimate 3000 Model high performance liquid chromatography (HPLC) was coupled with a Maxis Impact Bruker Daltonics QTOF-MS instrument. Chromatographic separation was conducted using a Phenomenex Luna C18 column (10 cm x 2 mm x 3 µm, 100 A) equipped with a guard column (4 cm x 2 mm x 3 µm, 100 A). The mobile phase was composed of 0.1% formic acid and 5 mM ammonium acetate in dH2O (A) and Acetonitrile (B). The analysis was performed using gradient elution with a flow rate of 0.3 mL/min. The gradient program started with a mobile phase of 75%A:25%B, increased mobile phase B to 50% in 6 minutes, then increased mobile phase B to 90% in 1 minute and holding 10%A:90%B for 4 minutes, then back to 75% A:25% B within 0.1 minutes; this condition was continued for 4 minutes. The total run time was 15 minutes. The injection volume was 5 µL and the column temperature was set at 40°C.

Mass spectrometry was operated in positive ESI mode. ESI parameters were set as capillary voltage 2900 V, Nebulizer gas 2.0 bar, drying gas 8.0 L/min and drying temperature 180°C. Broad band collision induced dissociation (bbCID) mode was performed with a mass range of 50-1500 m/z. Collision energy for MS and MS/MS (bbCID) modes were set at 4.0 and 35.0 eV, respectively. Multiple reaction monitoring (MRM) transitions for carbaryl, carbofuran and propoxur were shown in Table 1 (a quantitation ion was defined as an underscored ion). The retention times for carbaryl, carbofuran and propoxur were 6.7,

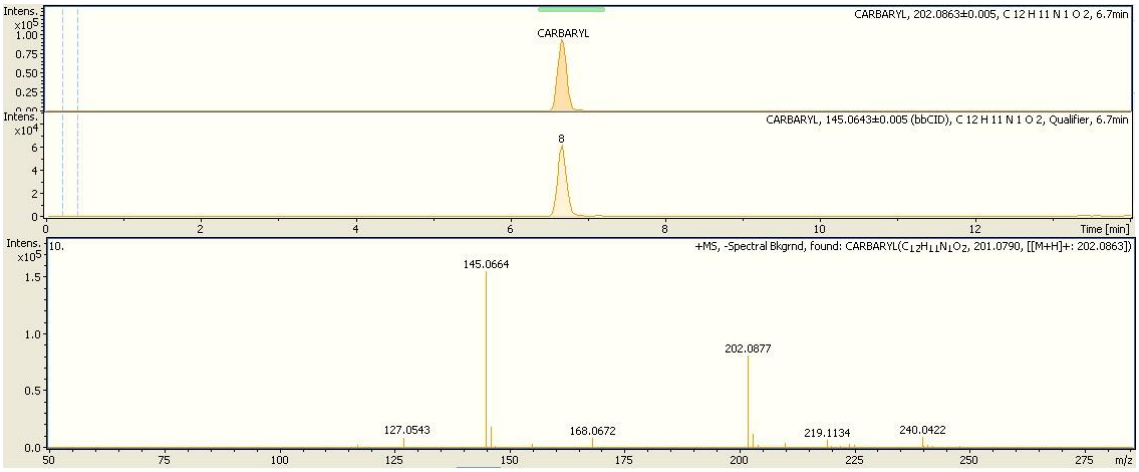
6.2 and 6.1 minutes, respectively. Chromatograms and MS spectra of these three carbamate insecticides were shown in Figure 3.

Table 1. MRM transitions for carbaryl, carbofuran and propoxur

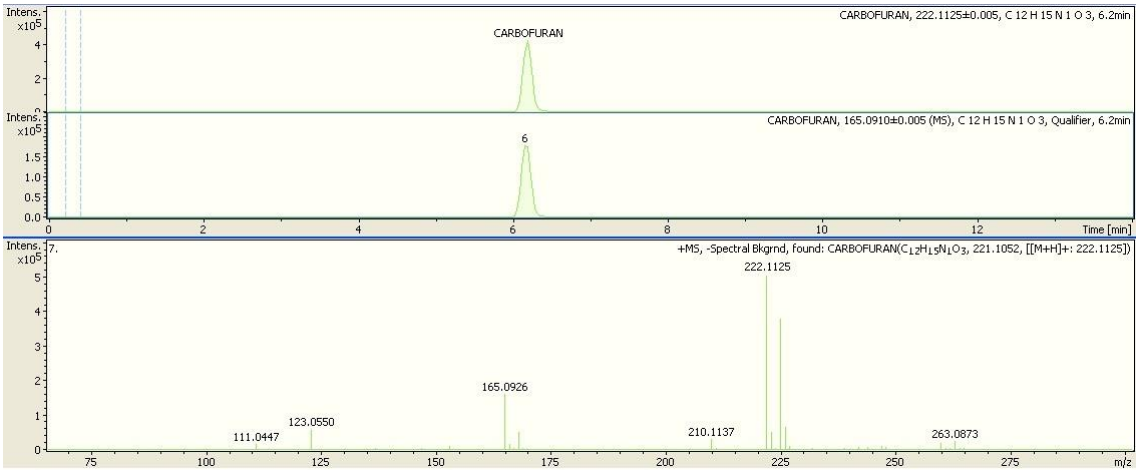
Compound	Transition 1	Transition 2
Carbaryl	202.1>145.1	202.1>127.1
Carbofuran	222.1>165.1	222.1>123.1
Propoxur	210.1>111.0	210.1>168.1

Preparation of stability samples and storage conditions

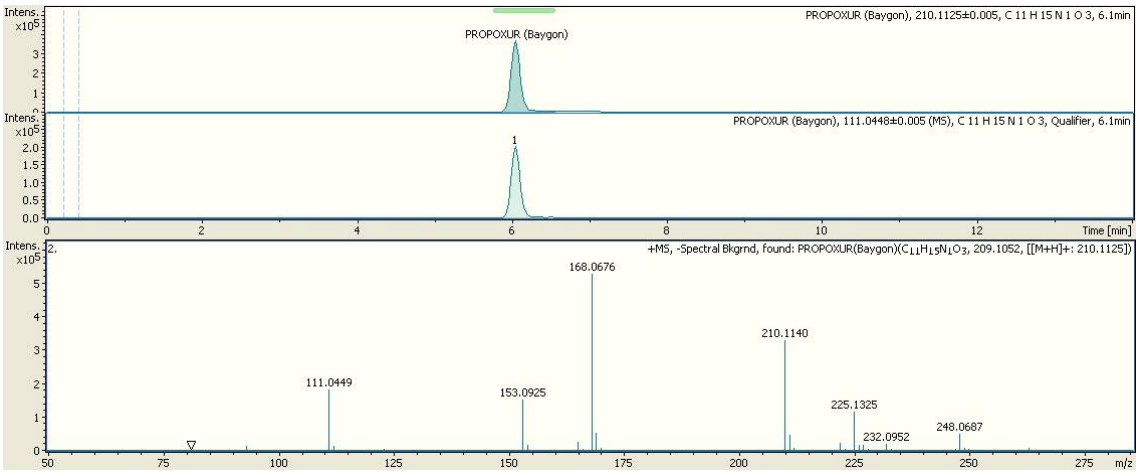
Expired whole blood was obtained from the Department of Transfusion Medicine, Siriraj Hospital, Mahidol University. Three pools of whole blood were prepared for spiking with carbaryl, carbofuran and propoxur. Each pool of blood consisted of three groups including low, medium and high concentrations. For carbaryl and carbofuran, low, medium and high concentration groups were spiked to achieve concentrations of 60, 150 and 400 ng/mL. For propoxur, low, medium and high concentration groups were spiked to achieve concentrations of 150, 450 and 750 ng/mL. All spiked blood samples were analyzed for carbaryl, carbofuran and propoxur in triplicate at the initial period (day_0). Then, all spiked blood samples for each compound were divided into 39 aliquots. 18 aliquots were stored at room temperature (RT, 25°C) and 21 aliquots were stored in a refrigerator at 4°C. For room temperature, each aliquot for each concentration was analyzed on the 1^{st}, 2^{nd}, 3^{rd}, 7^{th}, 14^{th} and 30^{th} day. For 4°C, each aliquot for each concentration was analyzed on the 3^{rd}, 7^{th}, 14^{th}, 30^{th}, 60^{th}, 90^{th}, and 180^{th} day. Each aliquot was analyzed in triplicate. Mean and standard deviation (SD) were calculated for each concentration for all three analytes. Percent changes from initial concentrations for all three analytes were calculated by subtracting each replicate from each date to the same order of the other replicate on the initial day and divided by the concentration on the initial day. Then, all figures obtained from calculation were transformed into percent. Thus, three percent changes for each concentration for each analyte were obtained and mean and SD for percent changes were calculated from these three percent changes. Then, all data were submitted for statistical analysis.



Carbaryl



Carbofuran



Propoxur

Figure 3. Chromatograms and MS spectra of carbaryl, carbofuran and propoxur

Sample preparation

After 1 mL of blood sample was pipetted into a test tube, 1.5 mL of cold acetonitrile was added. The blood sample was vortexed and centrifuged at 4000 rpm/min. The supernatant part was taken and 2 mL of 0.1M phosphate buffer pH6.0 was added to the supernatant. Then, the sample was extracted by solid phase extraction (SPE) using SPE cartridge Oasis HLB® 60 mg 3mL. The SPE cartridge was conditioned with methanol and dH_2O. Subsequently, the sample was loaded into the SPE cartridge followed by washing with 10% methanol in dH_2O. Finally, the elution step was achieved with methanol. The eluent solution was evaporated under a nitrogen stream at 40°C. The sample was then re-constituted with 75%:25% mobile phase A:B and injected into LC-QTOF-MS.

Method validation

As this analytical method was an in-house method that was developed in the laboratory, this in-house method was validated to ensure that analytical results were reliable. Method validation was conducted following the Standard Practices for Method Validation in Forensic Toxicology issued by the Scientific Working Group for Forensic Toxicology (SWGTOX) 2013[9]. Method validation was conducted using expired whole blood from the Department of Transfusion Medicine, Siriraj Hospital, Mahidol University. Selectivity and interference studies were performed to achieve complete separation between carbaryl/carbofuran/propoxur and baseline noise. In addition, other carbamate compounds including methomyl, aldicarb and carbosulfan were tested to ensure that they did not interfere with carbaryl/ carbofuran/propoxur analysis. The limit of detection (LOD) and lower limit of quantitation (LLOQ) were evaluated by spiking decreasing concentrations of carbaryl, carbofuran and propoxur into blood samples. LOD and LLOQ were determined by the lowest concentrations that generated a signal-to-noise (S/N) ratio greater than 3 and 10 times, respectively. LOD for carbaryl, carbofuran and propoxur were 5, 5 and 25 ng/mL, whereas LLOQ for carbaryl, carbofuran and propoxur were 10, 10, and 50 ng/mL, respectively.

The matrix effect (ionization suppression/enhancement experiment) was assessed because the method was involved in using LC-QTOF/MS. Average peak areas for three concentrations (low, medium and high concentrations) of carbaryl, carbofuran and propoxur in standards prepared in the mobile phase were compared with standards spiked in extracted blank blood samples. Three concentration levels for carbaryl and carbofuran were 30, 150 and 400 ng/mL, whereas 150, 450 and 750 ng/mL were employed for propoxur. The matrix effect

for all analytes should not exceed ±25%. The matrix effects for the three concentrations of carbaryl, carbofuran and propoxur in this study were -8.67-6.67%, -5.65-16.94% and -1.40-6.27%, respectively.

The linearity ranges for carbaryl and carbofuran were assessed using six calibrators at 10, 20, 50, 100, 200 and 500 ng/mL whereas the linearity range for propoxur was evaluated using six calibrators at 50, 100, 200, 400, 800 and 1000 ng/mL. All calibrators were prepared as five replicates and run on five separate days. Calibration curves for carbaryl, carbofuran and propoxur were generated by Bruker Daltonics Compass for OTOF Series 1.7 Software®. Criteria of r2 ≥0.99, and accuracy of each calibrator within ±15% and %coefficient of variation (%CV) ≤15% should be achieved for these two analytes. The accuracy and %CV of carbaryl calibrators were -13.0-13.4% and ≤10.1%. The accuracy and %CV of carbofuran calibrators were -11.8-12.5% and ≤12.6%. The accuracy and %CV of propoxur calibrators were -13.4-13.9% and ≤13.1%.

Accuracy and precision were evaluated by the injection of five replicates of spiked blood samples at low, medium and high concentrations on five separate days using similar concentrations in the matrix effect study. Accuracy for each concentration should be within ±15%, and precision that was evaluated by %CV should also be ≤15%. The accuracy of the three concentrations of carbaryl was -10.1-4.7%, and %CV was ≤7.1%. The accuracy of the three concentrations of carbofuran was -13.8-10.9%, and %CV was ≤9.8%. The accuracy of the three concentrations of propoxur was -11.5-13.0%, and %CV was ≤8.7%.

Statistical analysis

Statistical analysis was performed using SPSS for Windows® version 25. Descriptive statistics including mean and standard deviation (SD) were analyzed for three concentration levels of all three compounds. One-way ANOVA with multiple comparisons was performed for comparisons of all three compounds on the initial day and over a period of 180 days. Multiple comparisons were performed using post-hoc test and Fisher's LSD method with $p<0.05$. Post-hoc test with Fisher's LSD method was employed in this study because SD in each concentration from each analyte over the period of 30 days at room temperature and 180 days at 4°C was relatively small-scale and closed to each other and could be comparable.

This study was approved and exempted by the Siriraj Institutional Review Board, Faculty of Medicine, Siriraj Hospital, Mahidol University (Document

No.78.071/EC 02654, on 16th September 2021).

Results

At room temperature, carbaryl, carbofuran and propoxur in spiked blood samples at low, medium and high concentrations decreased during the experimental period, as shown in Table 2. Mean concentrations of each analyte at each time were compared with the initial concentrations and expressed as percent change from the initial concentrations, which were shown in Table 3. The SWGTOX guidelines stated that the compound stability had a cut-off value of ±15% compared with initial concentrations[8]. According to Table 3, all three compounds were unstable at room temperature, particularly carbofuran. Carbofuran decreased to less than 15% from the initial concentrations on the 2nd day and rapidly decreased to complete disappearance on the 14th day. Carbaryl decreased to less than 15% from initial concentrations on the 1st day to the end of 30-day period and achieved a significant reduction ($p<0.05$) on the 1st day. All three concentrations of carbofuran decreased to less than 15% from the initial concentrations on the 2nd day, whereas all three concentrations of propoxur decreased to less than 15% from the initial concentrations on the 7th day. All three compounds produced a highly significant reduction for three concentrations on the 3rd day ($p<0.001$) and produced a reduction less than 15% from the initial concentrations on the 7th day ($p<0.001$).

When considering blood samples stored in a refrigerator (4°C), data of all three compounds in spiked blood samples at three concentration levels also decreased during the 180-day period, as shown in Table 4. Mean concentrations of these three compounds at each period were also compared with the initial concentrations and expressed as percent change from the initial concentrations, which were shown in Table 5. According to Table 5, all three compounds were also unstable at 4°C. Carbofuran still decreased at the most rapid rate. Carbofuran developed a significant reduction on the 3rd day ($p<0.001$) and completely disappeared for all three concentration levels on the 90th day. However, all of three concentration levels of carbofuran decreased to less than 15% on the 7th day ($p<0.001$). Carbaryl decreased to less than 15% from initial concentrations on the 3rd day to the end of 180-day period and it developed a significant reduction on the 3rd day ($p<0.001$). Propoxur decreased to less than 15% from initial concentrations and produced a significant reduction on the 7th day ($p<0.01$ for low concentration and $p<0.001$ for medium and high

concentrations). All three compounds at 4°C produced a highly significant decrease for three concentrations on the 7th day (p<0.01) and the 14th day (p<0.001), respectively.

Table 2. Carbaryl, carbofuran and propoxur concentrations in spiked blood samples at room temperature (RT)

Compound	Mean ± SD concentrations at each period (ng/mL)						
	Day_0	1st Day	2nd Day	3rd Day	7th Day	14th Day	30th Day
Carbaryl							
Low	61.92 ±4.26	44.37 ±4.36	39.78±3.50	31.82±2.83	27.43±2.13	18.55±2.21	10.75±5.42
Medium	151.31 ±5.18	116.07±6.93	92.51±4.01	80.65±4.86	72.12 ±7.85	55.83 ±8.73	19.11±8.67
High	374.33±10.40	275.43±12.67	255.07±11.12	192.98±8.57	200.93±8.65	205.40±5.64	118.39±6.30
Carbofuran							
Low	64.69 ±7.25	34.31 ±8.18	21.50 ±6.96	ND	ND	ND	ND
Medium	160.15 ±6.75	150.84 ±5.89	94.96 ±4.65	14.98 ±5.96	ND	ND	ND
High	393.84 ±4.67	394.43 ±5.41	328.46 ±4.51	190.20±5.39	58.78 ±6.53	ND	ND
Propoxur							
Low	162.66 ±8.31	160.85 ±6.97	137.51 ±5.38	77.97 ±7.31	27.78 ±8.21	ND	ND
Medium	461.56 ±5.46	454.17 ±6.67	455.18 ±8.79	317.86±7.24	212.45±9.55	63.28 ±8.17	ND
High	751.49±10.24	719.12±11.39	693.24 ±9.21	651.46±9.89	595.17±10.04	512.28±12.52	22.92±12.82

*ND = Not detected (<LOD of each compound)

When considering blood samples stored in a refrigerator (4°C), data of all three compounds in spiked blood samples at three concentration levels also decreased during the 180-day period, as shown in Table 4. Mean concentrations of these three compounds at each period were also compared with the initial concentrations and expressed as percent change from the initial concentrations, which were shown in Table 5. According to Table 5, all three compounds were also unstable at 4°C. Carbofuran still decreased at the most rapid rate. Carbofuran developed a significant reduction on the 3rd day (p<0.001) and completely disappeared for all three concentration levels on the 90th day. However, all of three concentration levels of carbofuran decreased to less than 15% on the 7th day (p<0.001). Carbaryl decreased to less than 15% from initial concentrations on the 3rd day to the end of 180-day period and it developed a significant reduction on the 3rd day (p<0.001). Propoxur decreased to less than 15% from initial concentrations and produced a significant reduction on the 7th day (p<0.01 for low concentration and p<0.001 for medium and high concentrations). All three compounds at 4°C produced a highly significant decrease for three concentrations on the 7th day (p<0.01) and the 14th day (p<0.001), respectively.

Table 3. Mean percent change from the initial concentrations and p-value of carbaryl, carbofuran and propoxur at room temperature (RT) over 30 days

Compound	Mean percent (%) change from the initial concentrations (Day_0) ± SD					
	(D1-D0)/D0	(D2-D0)/D0	(D3-D0)/D0	(D7-D0)/D0	(D14-D0)/D0	(D30-D0)/D0
Carbaryl						
Low	-28.35±6.15	-35.76±5.98	-48.61±5.84	-55.70±5.42	-70.04±4.69	-82.64 ±2.94
p-value	0.018*	0.006*	<0.001*	<0.001*	<0.001*	<0.001*
Medium	-23.29±6.65	-38.86±5.45	-46.70±5.95	-52.33±6.48	-63.10 ±5.30	-87.37±2.93
p-value	0.006*	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*
High	-26.42±3.08	-31.86±3.16	-48.45±2.65	-46.32±2.70	-45.13±2.02	-68.37±1.63
p-value	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*
Carbofuran						
Low	-46.96±5.82	-66.77±2.35	-100	-100	-100	-100
p-value	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*
Medium	-5.81±5.52	-40.71±4.40	-90.64±1.63	-100	-100	-100
p-value	0.148	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*
High	0.15±1.43	-16.60±2.92	-51.71±3.21	-85.07±1.69	-100	-100
p-value	0.997	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*
Propoxur						
Low	-1.12±4.05	-15.46±5.87	-52.07±3.13	-82.92±4.08	-100	-100
p-value	0.972	0.044*	<0.001*	<0.001*	<0.001*	<0.001*
Medium	-1.60±2.37	-1.38±3.27	-31.13±2.14	-53.97±1.76	-86.29±1.27	-100
p-value	0.495	0.643	<0.001*	<0.001*	<0.001*	<0.001*
High	-1.69±2.67	-5.23±2.70	-10.94±2.39	-18.64±1.60	-29.97±2.04	-96.87±0.97
p-value	0.029	0.002*	<0.001*	<0.001*	<0.001*	<0.001*

When percent changes of each concentration in each carbamate were compared with one another over the experimental periods using ANOVA with multiple comparisons with Fisher's LSD method, it was found that concentrations had an effect on the pattern of percent changes. For carbaryl stored at room temperature, low and medium concentrations were not significantly different for all percent changes from the initial concentrations over 30 days. However, comparison of percent changes from the initial concentrations between low and high concentrations and comparison between medium and high concentrations produced significant difference at percent change between the 14^{th} day and the initial day and percent change ($p<0.05$) between the 30^{th} day and the initial day ($p<0.01$). For carbofuran stored at room temperature, percent changes from the initial

concentrations at low concentration were significantly greater than medium and high concentrations for all comparisons ($p<0.01$) except for the dates that carbofuran was undetectable. In addition, percent changes from the initial concentrations at medium concentration were also significantly greater than high concentration for all comparisons ($p<0.05$ for comparison between the 1st day and the initial day and $p<0.01$ for the other comparisons) except for the dates that carbofuran was undetectable. For propoxur stored at room temperature, comparisons of percent changes between low and medium concentrations between the 1st day and the initial day and between the 2nd day and the initial day were not significantly different. However, comparisons of percent changes between low and medium concentrations from the 3rd day were highly significantly different ($p<0.001$) except for the 30th day when propoxur was undetectable. Comparisons between low and high concentrations and medium and high concentrations also produced the similar pattern of significant difference ($p<0.001$ from the 3rd day). All of these findings indicated that all of these three carbamates at high concentrations stored at room temperature had significantly lower reduction rate than low and medium concentrations.

Table 4. Carbaryl, carbofuran and propoxur concentrations in spiked blood samples at 4°C

Compound	**Mean ± SD concentrations at each period (ng/mL)**							
	Day_0	3rd Day	7th Day	14th Day	30th Day	60th Day	90th day	180th Day
Carbaryl								
Low	64.99±3.57	47.60 ±3.08	45.47±3.93	43.52±4.49	41.88±4.19	29.43±3.90	23.00±5.41	ND
Medium	153.05±4.89	127.36±5.89	119.61±3.81	116.57±4.40	117.62±5.82	101.07±5.89	77.22±4.74	14.65±6.93
High	416.35±8.40	338.42±9.85	335.35±9.17	326.60±9.61	288.70±7.98	275.27±9.35	276.80±8.06	54.23±9.92
Carbofuran								
Low	52.93±2.11	38.77±2.18	22.28±3.13	ND	ND	ND	ND	ND
Medium	153.75±5.73	136.62±4.58	120.80±4.18	49.50±5.43	ND	ND	ND	ND
High	402.44±7.96	330.99±8.27	315.78±5.21	258.73±6.58	130.11±6.50	16.82±7.23	ND	ND
Propoxur								
Low	151.23±6.56	137.61±4.23	123.26±4.88	74.68±6.82	29.46±7.16	ND	ND	ND
Medium	434.19±8.21	407.59±7.57	346.72±6.65	315.73±10.84	223.46±10.35	77.61±9.80	ND	ND
High	748.91±8.16	660.10±9.78	630.92±9.03	600.65±6.13	447.56±8.80	376.09±7.76	161.91±9.66	ND

*ND = Not detected (<LOD of each compound)

Table 5. Mean percent change from the initial concentrations and p-value of carbaryl, carbofuran and propoxur at 4°C over 180 days

Compound	Mean percent (%) change from the initial concentrations (Day_0) ± SD						
	(D3-D0)/D0	(D7-D0)/D0	(D14-D0)/D0	(D30-D0)/D0	(D60-D0)/D0	(D90-D0)/D0	(D180-D0/D0)
Carbaryl							
Low	-26.76 ±3.52	-30.04 ±5.59	-33.04 ±2.26	-35.56 ±2.75	-54.72 ±4.14	-64.61 ±1.61	-100
p-value	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*
Medium	-16.79 ±3.44	-21.85 ±3.98	-23.84 ±2.27	-23.15 ±2.61	-33.96 ±3.01	-49.55 ±4.03	-90.43 ±0.90
p-value	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*
High	-18.71 ±3.59	-19.45 ±4.40	-21.56 ±2.55	-30.66 ±2.54	-33.89 ±2.81	-33.52 ±2.44	-86.98 ±0.85
p-value	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*
Carbofuran							
Low	-26.75 ±3.21	-57.91 ±5.58	-100	-100	-100	-100	-100
p-value	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*
Medium	-11.14 ±3.41	-21.43 ±3.26	-67.81 ±3.57	-100	-100	-100	-100
p-value	0.001*	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*
High	-17.66 ±4.80	-21.45 ±2.68	-35.64 ±2.80	-67.63 ±2.21	-95.82 ±1.09	-100	-100
p-value	0.001*	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*
Propoxur							
Low	-9.01 ±5.05	-18.50 ±5.13	-50.62 ±2.57	-80.52 ±2.54	-100	-100	-100
p-value	0.038*	0.001*	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*
Medium	-6.13 ±2.68	-20.15 ±1.75	-27.28 ±2.43	-48.53 ±2.36	-82.13 ±1.31	-100	-100
p-value	0.005*	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*
High	-11.86 ±2.39	-15.76 ±2.16	-19.80 ±2.57	-40.68 ±2.39	-49.78 ±0.94	-78.38 ±0.89	-100
p-value	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*

For the experiment at 4°C, percent changes of carbaryl at low concentration were significantly greater than medium and high concentrations particularly from the 14th day ($p<0.01$). However, percent changes of carbaryl at medium concentration were significantly greater than high concentration only at the 90th day and the 180th day ($p<0.01$). For carbofuran stored at 4°C, percent changes of carbofuran at low concentration were significantly greater than medium and high concentrations from the 3rd day to the end of the period of 180-day ($p<0.001$) except for the comparison between low and medium concentrations from the 60th day and the comparison between low and high concentrations from the 90th day because carbofuran at all three concentrations were completely undetectable. However, percent changes of carbofuran at medium concentration were significantly greater than high concentration from the 14th day to the 60th day ($p<0.001$) except for the comparison from the 90th day due to undetectable concentrations of carbofuran.

For propoxur stored at 4°C, percent changes of propoxur at low concentration were significantly greater than medium and high concentrations from the 14th day ($p<0.001$) except for the comparison between low and medium concentration from the 90th day and the comparison between low and high concentration at the 180th day due to undetectable concentrations of propoxur. For the comparison between medium and high concentrations, percent changes of propoxur at medium concentration were significantly greater than high concentration with $p<0.05$ from the 14th day to the 30th day and with $p<0.01$ from the 60th day to the 180th day. These findings still represented that these three carbamates at high concentrations stored at 4°C showed significantly lower reduction rate than low and medium concentrations. However, carbaryl and propoxur at medium concentration showed slower reduction rate until the 60th day and the 7th day, respectively.

When percent changes of each concentration for each analyte were compared with each other to compare the reduction rate of different insecticides, these comparisons were shown in Table 6 for samples stored at room temperature and Table 7 for samples stored at 4°C, respectively. According to Table 6, the comparison of percent changes between carbaryl and carbofuran at low concentration produced significant difference from the 1st day ($p<0.05$). Carbofuran decreased at a significantly slower rate than carbaryl on the 1st day for medium concentration and from the 1st day to the 2nd day for high concentration. However, after these two periods, carbofuran at both medium and high concentrations decreased more significantly rapidly than carbaryl ($p<0.001$). For the comparison of percent changes between carbaryl and propoxur, carbaryl decreased more significantly rapidly than propoxur until the 2nd day for low concentration and until the 3rd day for medium concentration and they came to similar reduction rate on the 3rd day and the 7th day, respectively. However, propoxur decreased more significantly than carbaryl after periods stated above. For high concentration, carbaryl decreased more significantly than propoxur from the 1st day until the 14th day ($p<0.001$). However, propoxur decreased more significantly rapidly than carbaryl on the 30th day ($p<0.001$). For the comparisons of percent changes between carbofuran and propoxur, carbofuran decreased more significantly rapidly than propoxur from the 1st day at low concentration ($p<0.01$) except the dates when these two carbamates were undetectable. However, there was no significant difference between percent changes between carbofuran and propoxur at medium and high concentrations on the

1st day. The significant difference between carbofuran and propoxur at medium and high concentrations at room temperature occurred from the 2nd day until the end of experimental period ($p<0.01$).

Table 6. Comparison of percent changes from the initial concentrations between each carbamate insecticide at different concentrations and p-values at room temperature over 30 days

Difference between compounds at different concentrations	p-values from comparison of percent changes from the initial concentrations at defined dates between each compound					
	(D1-D0)/D0	(D2-D0)/D0	(D3-D0)/D0	(D7-D0)/D0	(D14-D0)/D0	(D30-D0)/D0
Low						
ΔCarbaryl-Carbofuran	0.046*	0.01*	<0.001*	<0.001*	<0.001*	<0.001*
ΔCarbaryl-Propoxur	<0.001*	0.04*	>0.05	0.005	<0.001*	<0.001*
ΔCarbofuran-Propoxur	0.002*	<0.001*	<0.001*	0.002*	>0.05	>0.05
Medium						
ΔCarbaryl-Carbofuran	0.009*	>0.05	0.001*	0.001*	0.003*	0.002*
ΔCarbaryl-Propoxur	0.003*	0.001*	0.03*	>0.05	0.02*	0.002*
ΔCarbofuran-Propoxur	>0.05	<0.001*	<0.001*	<0.001*	<0.001*	>0.05
High						
ΔCarbaryl-Carbofuran	<0.001*	0.002*	>0.05	<0.001*	0.001*	<0.001*
ΔCarbaryl-Propoxur	<0.001*	<0.001*	<0.001*	<0.001*	0.001*	<0.001*
ΔCarbofuran-Propoxur	>0.05	0.002*	<0.001*	<0.001*	<0.001*	0.002*

$\Delta compound_1$-$compound_2$ = comparison of percent changes from the initial concentrations between two compounds with shown statistical significance

According to Table 7, the difference between percent changes of carbaryl and carbofuran at low concentration at 4°C occurred at the 7th day ($p<0.01$) whereas percent changes at medium and high concentrations produced significant difference on the 14th day ($p<0.001$). When the comparison of percent changes between carbaryl and propoxur at low concentration stored at 4°C was considered, propoxur decreased more significantly slowly than carbaryl from the 1st day to the 2nd day ($p<0.05$) but propoxur decreased more significantly rapidly than carbaryl from the 14th day ($p<0.001$). For medium and high concentrations, the reduction rate of carbaryl and propoxur had no significant difference until the 14th day except for the 3rd day of medium concentration. However, propoxur at medium and high concentrations declined significantly compared with carbaryl from the 30th day ($p<0.001$). For the comparison of percent changes between carbofuran and propoxur at low concentration stored at 4°C, carbofuran decreased more significantly

rapidly than propoxur from the 3rd day ($p<0.01$) until it was undetectable. For medium and high concentration, carbofuran decreased more significantly rapidly than propoxur particularly from the 14th day ($p<0.001$) until it was undetectable. According to these results, it could be suggested that these three carbamates at low concentrations were more unstable than medium and high concentrations and carbofuran was the most unstable compound for all concentrations compared with the other two compounds both at room temperature and at 4°C.

Table 7. Comparison of percent changes from the initial concentrations between each carbamate insecticide at different concentrations and p-values at 4°C over 180 days

Difference between compounds at different concentrations	p-values from comparison of percent changes from the initial concentrations at defined dates between each compound						
	(D3-D0)/D0	(D7-D0)/D0	(D14-D0)/D0	(D30-D0)/D0	(D60-D0)/D0	(D90-D0)/D0	(D180-D0/D0)
Low							
ΔCarbaryl-Carbofuran	>0.05	0.003*	<0.001*	<0.001*	<0.001*	<0.001*	>0.05
ΔCarbaryl-Propoxur	0.005*	0.049*	<0.001*	<0.001*	<0.001*	<0.001*	>0.05
ΔCarbofuran-Propoxur	0.005*	0.001*	<0.001*	<0.001*	>0.05	>0.05	>0.05
Medium							
ΔCarbaryl-Carbofuran	>0.05	>0.05	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*
ΔCarbaryl-Propoxur	0.02	>0.05	>0.05	<0.001*	<0.001*	<0.001*	<0.001*
ΔCarbofuran-Propoxur	>0.05	>0.05	<0.001*	<0.001*	<0.001*	>0.05	>0.05
High							
ΔCarbaryl-Carbofuran	>0.05	>0.05	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*
ΔCarbaryl-Propoxur	>0.05	>0.05	>0.05	0.009*	<0.001*	<0.001*	<0.001*
ΔCarbofuran-Propoxur	>0.05	0.01*	<0.001*	<0.001*	<0.001*	<0.001*	>0.05

$\Delta compound_1$-$compound_2$ = comparison of percent changes from the initial concentrations between two compounds with shown statistical significance

When percent changes from the initial concentrations of these three compounds at room temperature and 4°C were plotted along the experimental period, they were shown in Figure 4A and 4B. According to Figure 4A and 4B, carbofuran decreased more rapidly during the experimental periods under both room temperature and 4°C conditions, followed by propoxur and carbaryl, respectively. Carbaryl decreased at the most rapid rate at the first interval but decreased at the slower rate compared with the other two compounds in the later period. In contrast, propoxur decreased at the slower rate at the first two periods but developed a rapid

reduction after the 2nd day of room temperature condition and the 7th day of 4°C condition. The decreasing trends of room temperature conditions for all three compounds at three concentration levels were similar to the decreasing trends of 4°C conditions. Carbaryl was the only compound that could be detected at the end of the 30-day period at room temperature and the 180-day period at 4°C.

Discussion

This study showed that all three carbamate compounds examined were not stable during the 30-day period at room temperature and the 180-day period at 4°C. When the same period was compared for each compound, room temperature developed more unstable conditions than 4°C for all three concentrations. The previous study showed that propoxur decreased to around 20% of initial concentrations on the 28th day[8]. This study showed that carbaryl decreased to 12.63-31.63% compared with initial concentrations at the 30th day whereas carbofuran completely disappeared and propoxur nearly completely disappeared at the 30th day. This finding showed the similar trend to the study of Ramagiri S et al[8]. At 4°C, Ramagiri S et al. indicated that propoxur decreased to 58% of initial concentrations on the 60th day[8]. This study showed that carbaryl decreased to 45.28-66.11% on the 60th day. However, carbofuran completely disappeared for low and medium concentrations and decreased to only approximately 4% on the 60th day whereas propoxur completely disappeared at the low concentration and decreased to 17.87% and 50.22% at the medium and high concentrations on the 60th day. Thus, propoxur stored at low temperature decreased at a slower rate than at room temperature and this finding was consistent with a previous study[8]. In addition, when the stability data for propoxur in this study were compared with a previous study, this study produced a decreasing trend similar to the previous study, although this study showed a more rapid decrease[8]. One hypothesis for the instability of these three compounds was attributed to their chemical structures. A previous study showed that the greater stability of carbamate was achieved when more N-H bond was substituted[10]. Thus, these three compounds were susceptible to chemical hydrolysis since they had no N-H bond substitution[10]. Furthermore, when whole blood was held overnight or more at room temperature, blood pH would be lower and lactate would

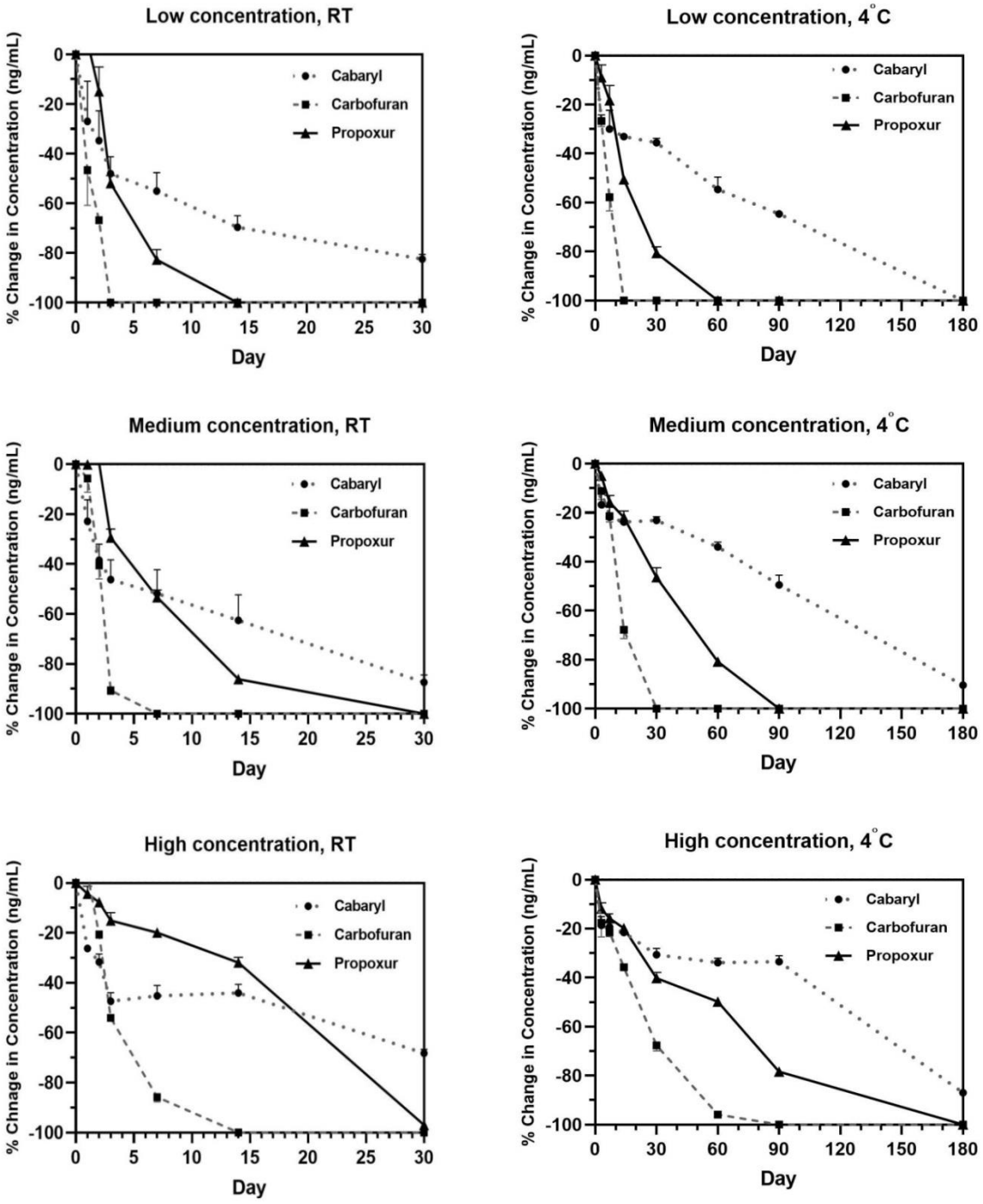


Figure 4. Percent change from the initial concentrations of carbaryl, carbofuran and propoxur at (A) room temperature and (B) 4°C

increase[11]. This pH change could be attributed to more instability of these three carbamate insecticides at room temperature than at 4 °C because pH change could have an effect on hydrolysis of compounds containing ester and amide bonds like these

three carbamate insecticides[8].In addition, carbofuran was the most unstable compound, followed by propoxur and carbaryl, respectively when these three compounds were compared with one another. Carbofuran decreased rapidly, even at 4°C, and was completely undetected for all three concentrations on the 90th day and 180th day, whereas propoxur was completely undetected for all three concentrations on the 180th day. Carbaryl is the only compound that could be detected on the 180th day for medium and high concentrations. The main difference between these three compounds was the different functional groups connected to ester bond sides. This should be studied further to elucidate the effect of the different functional groups connected to ester bond sides on the stability of carbamate insecticides. These findings indicated that carbaryl, carbofuran and propoxur were unstable in spiked blood samples at both room temperature and 4°C. The main difference of blood sample from living volunteers from the previous study and the expired whole blood in this study was the citrate anticoagulant used in the expired whole blood that obtained from blood donation unit. There was no available data about the effect of citrate on the stability of carbamates. However, the effect of citrate on the stability of these three carbamates may not be obviously significant because the stability pattern of propoxur in this study was similar to the previous study that used blood samples obtained from living volunteers. However, further study should be conducted to prove the effect of citrate and other types of anticoagulants on the stability of carbamates. According to our results, it could be suggested that blood samples containing these three carbamate insecticides should be stored at least at 4 °C and the analysis should be performed within 7 days after storage because these three carbamate insecticides would decrease significantly at the 7th day. In addition, due to instability of these three carbamate insecticides in blood samples at room temperature, it could be suggested that blood samples from suspected cases of carbamate poisoning should be collected as soon as possible particularly within one day. When blood samples were collected from suspected poisoned patients within one day and were analyzed within 7 days for blood samples stored at 4 °C, these three carbamate concentrations could be interpreted corresponding with clinical settings of suspected carbamate poisoning.

There were two main limitations in this study. First, there was no identification of degradation products from these three carbamate compounds. Thus, sites of bond breaking in these three compounds could not be determined, and instability sites could not be identified. Second, this study

was conducted for only ordinary carbamate insecticides. Oxime carbamate insecticides contained different bonding in their molecules. Thus, this stability pattern may not be applied to oxime carbamate insecticides. In addition, this study did not include other factors that could have an impact on the stability of carbamates in blood samples such as pH, the amount of some specific blood proteins and capacity of protein binding to carbamates. Blood pH may have an important role on the stability of carbamates because carbamates contained ester bonds in their molecules and acidic condition that occurred particularly in postmortem blood[12] could enhance ester hydrolysis. Furthermore, a previous study showed that methomyl was able to bind to some specific proteins such as albumin and some protein residues of hemoglobin and this protein binding may have an effect on the stability of methomyl[13]. Thus, further research should include the effect of pH and protein binding in the stability study of carbamates.

Conclusion

Carbaryl, carbofuran and propoxur were significantly unstable at both room temperature and 4°C. At 4°C, these three compounds at all concentration levels decreased to less than 15% from initial concentrations on the 7th day. Carbofuran was the most unstable compound, followed by propoxur and carbaryl, respectively. It was suggested that blood samples from suspected cases of carbamate insecticide poisoning should be collected and analyzed as soon as possible because these three ordinary carbamate insecticides were not stable in blood samples both at room temperature and 4°C.

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Conflict of interest

The authors declare that there is no conflict of interest.

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