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An experimental study of postmortem decomposition of methomyl in blood

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ABSTRACT

Methomyl (*S*-methyl-1-N-[(methylcarbamoyl)oxy]thioacetimidate) is a carbamate pesticide. It has been noted that in some cases of methomyl poisoning, methomyl is either not detected or detected only in low concentrations in the blood of the victims. However, in such cases, methomyl is detected at higher concentrations in the vitreous humor than in the blood. This indicates that methomyl in the blood is possibly decomposed after death. However, the reasons for this phenomenon have been unclear. We have previously reported that methomyl is decomposed to dimethyl disulfide (DMDS) in the livers and kidneys of pigs but not in their blood. In addition, in the field of forensic toxicology, it is known that some compounds are decomposed or produced by internal bacteria in biological samples after death. This indicates that there is a possibility that methomyl in blood may be decomposed by bacteria after death. The aim of this study was therefore to investigate whether methomyl in blood is decomposed by bacteria isolated from human stool. Our findings demonstrated that methomyl was decomposed in human stool homogenates, resulting in the generation of DMDS. In addition, it was observed that three bacterial species isolated from the stool homogenates, *Bacillus cereus*, *Pseudomonas aeruginosa*, and *Bacillus* sp., showed methomyl-decomposing activity. The results therefore indicated that one reason for the difficulty in detecting methomyl in postmortem blood from methomyl-poisoning victims is the decomposition of methomyl by internal bacteria such as *B. cereus*, *P. aeruginosa*, and *Bacillus* sp.

1. Introduction

Methomyl (*S*-methyl-1-N-[(methylcarbamoyl)oxy]thioacetimidate) is a carbamate pesticide. The mechanism underlying its toxicity is based on acetylcholinesterase inhibition. Methomyl caused approximately 60 fatalities annually from 2009 to 2013 in Japan, contributing to about 15% of all poisoning-related deaths caused by agricultural chemicals [1]. In studies on methomyl-poisoning deaths, methomyl was either not detected or detected only in low concentrations in the blood of the victims. Meanwhile, in some cases, methomyl was detected at quite a higher concentration in the vitreous humor than in the blood [2–5]. This indicated the possibility that methomyl decomposes in blood after death; however, the reasons for this phenomenon have remained unclear so far.

The fate of methomyl in the body has been studied in rats that were orally administered radiolabeled methomyl-^{14}C. In that study, ^{14}C-carbon dioxide and ^{14}C-acetonitrile were detected as the major metabolites of the radiolabeled methomyl-^{14}C [6]. In experiments in goats and hens, a sulfate conjugate of methomyl-oxime, thiocyanate ion, acetamide, acetic acid, N-acetyl-S-1-[(methylamino)carbonyloxyiminoethyl]cysteine, and an N-sulfate cysteine conjugate of acetonitrile {N-[1-(sulfoimino)ethyl]cysteine} were recovered as metabolites of methomyl in the urine and excrement of the animals [7,8].

In a previous study, we examined the cause of a specific odor that was emitted from autopsy specimens following methomyl-poisoning, which we believed was generated from sulfur compounds. Our study revealed that methomyl is decomposed into dimethyl disulfide (DMDS) in pig livers and kidneys [9]. However, when methomyl was incubated with fresh blood, we did not observe the production of DMDS or the decomposition of methomyl.

In the field of forensic toxicology, it is known that nitrobenzodiazepines are decomposed into 7-amino metabolites. In addition, ethanol is produced with time by bacteria in biological samples after death [10,11]. Therefore, we must carefully evaluate results of the analysis of compounds obtained from biological samples collected after death. We conducted this study to investigate the

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influence of bacteria, especially internal bacteria, on methomyl decomposition in the blood after death.

2. Materials and methods

2.1. Reagents and materials

All the chemicals (methomyl, methomyl-oxime, oxamyl, fenobucarb [BPMC], isoprocarb [MIPC], xylycarb [MPMC], metolcarb [MTMC], propoxur [PHC], macbal [XMC], toluene, barbital, and triazolam) used in the experiment were purchased from Wako Pure Chemical Industries, Ltd.(Osaka, Japan). Methomyl-d_3 (S-methyl-1-N-[(methylcarbamoyl)oxy] thioacetimidate(d_3)) and S-CD_3-methomyl (S-methyl(d_3)-1-N-[(methylcarbamoyl)oxy]thioacetimidate) were obtained from Hayashi Pure Chemical Ind., Ltd. (Osaka, Japan). Defibrinated horse blood was purchased from Nippon Bio-Test Laboratories Inc. (Tokyo, Japan). Nutrient agar medium was purchased from Eiken Chemical Co., Ltd. (Tokyo, Japan). *Bacillus cereus* (ATCC® 14579™), *Citrobacter freundii* (ATCC® 8090™), *Enterobacter faecalis* (ATCC® 43062™), *Escherichia coli* (ATCC® 8739™, ATCC® 25922™), *Klebsiella pneumoniae* (ATCC® 23357™), and *Pseudomonas aeruginosa* (ATCC® 27853™) were purchased from the American Type Culture Collection (Manassas, VA, USA). We used human stool samples provided voluntarily by six healthy adults. Identification of bacteria at the species level was done by SRL Inc. (Tokyo, Japan).

Working solutions of each compound were prepared as follows. Methomyl (10 mg) was dissolved in 1 mL of H_2O. Toluene (10 mg) was mixed with 10 mL of 10% Tween and then diluted 100 times with 10% Tween. Sodium barbital (111.8 mg) was dissolved in 10 mL of H_2O. Triazolam (10 mg) was dissolved in 10 mL of CH_3OH and diluted 10 times with CH_3OH. Methanol solutions of BPMC, MIPC, MPMC, MTMC, PHC, and XMC were prepared, each at a concentration of 100 μg/mL. Methanol solutions of methomyl-oxime and oxamyl were prepared, each at a concentration of 10 mg/mL, and used as the working solutions. Toluene was used as the internal standard (IS) for detecting DMDS. Sodium barbital was used as the IS for detecting methomyl, methomyl-oxime, and oxamyl, whereas triazolam was used as the IS for detecting BPMC, MIPC, MPMC, MTMC, PHC, and XMC.

2.2. Analyses of compounds

2.2.1. Gas chromatography-mass spectrometry (GC/MS) analysis

Volatile compounds were analyzed using a gas chromatograph (GC-2010; Shimadzu, Kyoto, Japan) equipped with a mass spectrometer (PARVUM2, Shimadzu). A DB-5MS fused-silica capillary column (30 m × 0.25 mm (ID); film thickness, 0.25 μm; Agilent, Santa Clara, CA, USA) was used for chromatographic separation. Helium (99.9999% purity) was used as the carrier gas at a flow rate of 1 mL/min. Injections were made in split mode (1:10). The temperatures of the injection port and the oven were set at 200 °C and 55 °C, respectively. The MS was operated in electron impact ionization mode (ionization voltage, 70 eV). The temperatures of the ion source and the interface were set at 200 °C and 250 °C, respectively. Mass spectral scans were carried out in the range of *m*/*z* 1–350. Select-ion monitoring mode was carried out in the following conditions: *m*/*z* 79 and 94 for DMDS and *m*/*z* 91 and 92 for toluene.

2.2.2. High-performance liquid chromatography (HPLC)

HPLC analyses were performed using a Shimadzu LC-10A system composed of an LC-10AS pump, an SPD-10A UV detector, and a CTO-6A column oven. The HPLC system was equipped with an AS-100 automated sample injector (Bio-Rad Laboratories, Tokyo, Japan) and a Chromatopac C-R6A chromatograph data processer (Shimadzu). A Nova-Pak® C_{18} stainless steel cartridge column (3.9 × 150 mm, 4 μm; Waters, Milford, MA, USA) was used for chromatographic separation. The oven temperature was set at 40 °C. The wavelengths for drug detection were set at 230 nm (for methomyl, oxamyl, and methomyl oxime) and 210 nm (for BPMC, MIPC, MPMC, MTMC, PHC, and XMC). The mobile phase used for analyzing the compounds was composed of acetonitrile and 0.05% HCOOH, which were used at a ratio of 4:96 (v/v) to analyze methomyl, oxamyl, and methomyl-oxime, and at a ratio of 3:7 (v/v) for BPMC, MIPC, MPMC, MTMC, PHC, and XMC. The flow rate of the mobile phase was set at 1 mL/min and the injection volume was 20 μL.

2.3. Experiments

2.3.1. Preparation of samples for DMDS analysis

2.3.1.1. Preparation of volatile compounds from methomyl-stool homogenate. Human stool samples were homogenized with saline (10 times the weight of the stool sample). The homogenate (100 μL), defibrinated horse blood (0.5 mL), saline (0.5 mL), methomyl working solution (10 μL), and toluene working solution (10 μL) were placed in a headspace vial. The vial was sealed tightly with Teflon-coated septum and incubated at 35 °C for 24 h in a drying chamber (ETTAS EO-450B; AS ONE Co., Osaka, Japan). A 100-μL aliquot of vapor in the vial was injected into the GC/MS system using a gas-tight syringe. The volume of the produced DMDS was determined from the DMDS peak area/toluene peak area. The same experiments were performed using methomyl-d_3 and S-CD_3-methomyl solutions instead of the methomyl solution.

It was observed that, after incubating blood samples spiked with DMDS, the peak area values of DMDS that were obtained were inconsistent because they decreased over time. Therefore, the construction of a calibration curve was extremely difficult. As a result, we were unable to accurately quantitate DMDS in the biological samples.

2.3.1.2. Bacterial culture from the human stool homogenates and methomyl decomposition. A 100-μL aliquot of the human stool homogenate sample, which had been suspended in saline at 100 times its weight, was cultured in agar medium at 35 °C for 24 h. Colonies were isolated after repeated successive cultures and the isolated bacteria were suspended in saline. The suspension was adjusted so that the turbidity of the suspension, which was diluted 100 times with saline, at 600 nm (OD_{600}) was 0.38–0.40.

Using the method described in Section 2.3.1.1 for the stool homogenate, 100 μL of the turbidity-adjusted suspension was placed in a vial and incubated. Then, a 100-μL aliquot of vapor in the vial was injected into the GC/MS system to detect DMDS. The isolated bacteria were identified at the species level by SRL Inc.

2.3.1.3. Decomposition of methomyl by the bacteria guaranteed by ATCC. A suspension of the bacteria purchased from ATCC: *B. cereus* (ATCC® 14579™), *C. freundii* (ATCC® 8090™), *E. faecalis* (ATCC® 43062™), *E. coli* (ATCC® 8739™, ATCC® 25922™), *K. pneumoniae* (ATCC® 23357™), and *P. aeruginosa* (ATCC® 27853™) was prepared as described in Section 2.3.1.2 and placed in a vial. After incubation, a 100-μL aliquot of vapor in the vial was injected into the GC/MS system.

2.3.1.4. Decompositions of methomyl-oxime, oxamyl, and phenyl carbamate by B. cereus or P. aeruginosa. A 100-μL aliquot of the phenyl carbamate (BPMC, MIPC, MPMC, MTMC, PHC, and XMC) working solution was placed in a vial. Next, methanol was evaporated using a stream of nitrogen gas, followed by the addition of 100 μL of the turbidity-adjusted bacteria suspension, 0.5 mL of