Postmortem sampling time effect on toxicity biomarkers in rats exposed to an acute lethal methomyl dose

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ABSTRACT

Regulations often are imposing long postmortem times before autopsy leading to certain toxicity-unrelated changes in biomarkers, which in turn may affect the reliability of toxicity evaluation during forensic investigations. Since methomyl pesticide shows significant toxicity and is frequently encountered in poisoning cases, the current study evaluated different parameters in methomyl intoxicated rats at three different postmortem intervals (Hour 0, Hour 3 and Hour 6). Eighteen adult Sprague Dawley rats were poisoned with methomyl to simulate actual methomyl poisoning cases. The time of death was assigned as Hour 0. The animals were divided into 3 groups (n = 6) to collect blood and tissue samples at the selected time points. Body weight, relative organ weight, protein concentration, methomyl concentration and acetylcholinesterase activity (AChE) were assessed in blood and different tissues (liver, spleen, kidney, brain, eye, and bone marrow) to evaluate the effect of postmortem sampling time. Outcomes revealed significant decreases in methomyl concentration in blood and bone marrow with advanced sampling time (P < 0.001). Similarly, there were significant reductions in AChE activity in the kidney (P < 0.01), while the enzyme activity significantly increased in brain samples (P < 0.05). Findings illustrated the importance of sampling time in toxicity studies because it could alter experimental results and impact consequent interpretations, as well as it may alter postmortem biomarkers in related forensic cases.

1. Introduction

Pesticide poisoning is commonly encountered in toxicity investigations due to its widespread use, easy access, and high toxicity in small quantities. The most frequent poisoning fatalities have been observed due to organophosphates and carbamates exposure [1]. Annual incidence rates of acute pesticide poisoning in agricultural workers in developed countries are around 18.2 per 100,000 among adults and 7.4 per million among children [1]. Methomyl, also called lannate, is a synthetic carbamate which is the third major group of pesticides used in agriculture [2], and a class of acetylcholinesterase (AChE)-inhibiting pesticides [3]. Although methomyl is a very toxic and banned substance, it is still used illegally by a minority of farmers. There are human fatalities due to additive or synergistic interactions between methomyl and other chemicals [3]. Methomyl is classified by the United States Environmental Protection Agency (US EPA) as highly toxic to humans via oral exposures [4]. Many fatal cases of accidental or suicidal exposure to lethal methomyl doses were within the range of 12–15 mg/kg [5].

The available diagnostic tests for evaluating methomyl toxicity are measuring AChE activity and measuring methomyl concentration in biological samples. Inhibition of AChE activity is utilized as a biomarker.
of methomyl poisoning [6]. The principal AChE members in the human body are AChE and plasma cholinesterase (ChE). AChE exists in the nervous system and the outer membrane of erythrocytes, while plasma ChE presents in plasma, liver, cerebrospinal fluid and glial cells [7]. AChE breakdowns the neurotransmitter acetylcholine, which, in turn, prevents the propagation of nerve impulses at the site of cholinergic transmission [7]. ChE is a circulating plasma glycoprotein that hydrolyzes naturally occurring esters [7]. Activity in plasma is generally more sensitive, but activity in erythrocytes correlates somewhat better with clinical signs and symptoms of pesticide toxicity [8]. Depression of ChE activity more than 50 % of reference values is associated with cholinergic signs; 90 % of depression of reference values is associated with severe poisoning cases [8]. Plasma ChE activity could be useful up to 20 h post methomyl exposure, while erythrocyte AChE activity offers an alternative diagnostic method for later analysis [9].

Measuring methomyl in biological samples is performed using liquid chromatography-mass spectrophotometry (LC-MS) and gas chromatography (GC). LC-MS techniques require less sample preparation (e.g., protein precipitation, extraction, dilution, filtration, and centrifugation) than GC techniques [10]. It is difficult to accurately determine methomyl quantities in biological tissues using GC analysis for the purpose of toxicological examinations, because methomyl is unstable and dissociates into its oxime in the GC column [7].

In this study, different parameters were selected to evaluate methomyl toxicity at three different postmortem sampling times in methomyl poisoned rats. The parameters were body weight, relative organ weight, protein concentration, AChE activity, and methomyl concentration in different biological samples (liver, spleen, kidney, brain, eye, bone marrow and blood) at 3-time points (Hour 0, Hour 3, and Hour 6). The findings highlight the effect of sampling time on postmortem toxicity findings and evaluations.

2. Materials and methods

2.1. Animal experimentation and simulation of lethal methomyl toxicity

The permission to use laboratory animals during all experiments was granted by the Animal Care Committee (ACUC) at Jordan University of Science and Technology [Approval No. 16/3/3/267]. A total of 18 healthy adult male Albino Sprague Dawley rats, 360–396 g body weight (bwt), were housed in the Laboratory Animals Research Unit at the University of Petra Pharmaceutical Center (UPPC), Amman, Jordan. The animals were examined by an assigned veterinarian and kept in cages supplied with a ventilation system that ensures isolation from external surroundings. Rats were left for one week for acclimatization at 25 °C, 55% humidity with a 12-hour-light/dark cycle. They had free access to standard rodent feed and water.

Considering that methomyl has high water solubility [3] and LD₅₀ of 17–24 mg/kg bwt [4,11], a dose of 20 mg/kg bwt was given orally to rats in 1 ml distilled water using a stainless-steel gavage needle (Harvard Apparatus, USA). In order to simulate methomyl lethal cases, rats were left to die without euthanasia of moribund animals, and this was considered as the experimental endpoint. Animals died within 10–12 min post methomyl exposure. The time of death was recorded per rat and set as “Hour 0”. Body weight, organs weight and samples were collected at three time points post death incidence (Hour 0, Hour 3, and Hour 6).

2.2. Samples collection and preparation

At each assigned sampling point, blood samples, body weight and tissue samples were collected from a group (n = 6). At Hour 0 and Hour 3, blood samples were collected via cardiac puncture using a 19–21 G needle. Later blood samples (i.e. Hour 6) were collected by 5-ml syringes without needles. Half of each blood sample was centrifuged in heparinized tubes at 3000× g for 5 min to harvest plasma for methomyl quantification, while the remaining half was collected in red cap tubes to harvest clotted blood for measuring protein content and AChE activity. Plasma and blood samples were divided into two aliquots per sample and stored at − 80 °C for later analysis.

Liver, spleen, kidneys, brain, eyes, and femoral bone marrow were collected, and their relative weight was calculated (i.e., organ weight/ final body weight × 100). Then two subsamples from these organs were kept at −80 °C for later homogenization. A homogenization solution was prepared by adding 0.03 % Triton X-100–0.5 L of phosphate buffer solution. 5 ml of the solution was added per 1 g tissue sample for homogenization using a homogenizer (Wiggenhauser, Germany). Processed samples were used for measurements of protein content and AChE activity. For methomyl analysis, 9 ml distilled water was added per 1 g tissue sample.

2.3. Chemicals and reagents

All solvents and reagents were analytical or HPLC grade. Methomyl PESTANAL®, formic acid, Triton X-100, and dichloromethane were purchased from Sigma-Aldrich (USA). Carisoprodol (internal standard) were purchased from Cayman Chemical Company (USA). Acetonitrile Ultragradient grade was from Carlo Erba Reagents (Spain). Methanol was supplied by Fisher Scientific (UK). Phosphate-Buffered Saline (PBS) was obtained from Capricorn Scientific GmbH (Germany). Deionized water was prepared using Milli-Q purification system (Germany).

2.4. Samples preparation for methomyl analysis

Samples were prepared by adding 100 µl plasma or 100 mg tissue homogenate to deionized water in a 1:10 ratio. An internal standard (IS) solution was prepared by adding carisoprodol in methanol to form a concentration of 4 µg/ml. Following addition of 100 µl of the IS solution, samples were extracted with 3 ml of dichloromethane and 200 µl acetonitrile. The mixture was mixed on a vortex for 2 min, then centrifuged at 3500 rpm for 4 min. The organic layer was transferred into conical glass tubes and evaporated to dryness under a nitrogen stream at 40 °C. Residues were stored at −25 °C for later reconstitution (≤ 24 h). Upon analysis, the residues from each sample were dissolved in 200 µl of acetonitrile: 0.1 % formic acid in deionized water (50:50, v/v). Of this preparation, 20 µl were used for later methomyl quantification.

2.5. Standard solutions and calibration

Methomyl stock solution (1 mg/ml) was prepared by dissolving 10 mg methomyl in 10 ml methanol. A methomyl working standard solution (51.5 µg/ml) was then prepared by diluting the stock solution in a mixture of methanol: deionized water (50:50, v/v). The internal standard carisoprodol stock solution (1 mg/ml) was prepared by dissolving 1 mg of carisoprodol in 1 ml methanol. Carisoprodol working stock solution (4 µg/ml) was prepared by diluting 0.2 ml carisoprodol stock solution in 50 ml of acetonitrile.

The methomyl standard curve was constructed using thirteen calibration points: 5150, 2575, 1287.5, 643.75, 321.88, 160.94, 80.47, 40.23, 20.12, 10.06, 5.03, 2.51, and 1.26 ng/ml. A standard curve (R² = 0.99) was used to calculate methomyl concentrations in samples.

The methomyl quality control samples were prepared at 1.26 (low), 80.47 (mid), and 5150 (high) ng/ml. Between and within batch analysis of quality control samples (n = 6) was performed to determine accuracy and precision. Between and within batch accuracy at the low concentration was 9.5 % and 10.7 %, respectively. While precision was 3.8 % and 5.5 %, respectively. Recovery was determined using aqueous solutions of methomyl (80.47 ng/ml), tissue homogenate and blood spiked to the same concentrations. Methomyl recovery from the aqueous solutions obtained according to the extraction method was about 81 %, from spiked tissue homogenate was 47–62 %, and from blood standards was about 58 % (mean of three extractions in each case). Limit of
temperature of 35°C. The chromatographic separation occurred in a 5 µm particle size Atlantis dC18 column, 100 Å, 4.6 mm × 100 mm (Atlantic Water, Taiwan) at a temperature of 35 °C. Samples were eluted with a mobile phase consisting of acetonitrile: 0.1 % formic acid (50:50, v:v) at a flow rate of 1 ml/min. The chromatography run time was 10 min per sample, and all samples were analyzed in triplicates.

Electrospray ionization (ESI) with a turbo ion spray on the positive-ion mode was used for the MS detection. The spray needle was set at a potential of 5500 V. The heated capillary was at 600 °C with a declustering potential of 31 V. The drying and nebulizing gas was nitrogen with a flow rate of 10 L/min. Two alternating scans were performed at mass-to-charge ratio (m/z) 261 and m/z 163 corresponding to protonated molecular ions [M+H]+ of carisoprodol and methomyl, respectively. Multiple reaction monitoring (MRM) for the transitions of m/z 261 → 97 and m/z 163 → 88 were used to quantify IS (Fig. 1) and methomyl (Fig. 2), respectively.

2.7. Protein estimation in blood and tissue samples

Protein was measured by a previously modified Bradford (1976) method to fit microassay protein quantification; bovine serum albumin was the standard [12,13]. The protein standard was diluted 1:1, 1:10, 1:100, and 1:1000 using 0.1 M PBS (pH 7.4) to construct the standard curve. The Bradford reagent was prepared by dissolving 100 mg Coomassie G250 in 50 ml of 95 % ethanol, then the solution was mixed with 100 ml of 85 % phosphoric acid to make one litre with distilled water. Finally, the reagent was filtered through No. 1 Whatman filter paper. Samples were added as 20 µl per well of a 96-well microplate, then serially diluted by 20 µl PBS, followed by 200 µl of Bradford reagent per well. Afterwards, the microplate was incubated for 15 min at room temperature. The absorbencies were measured at 595 nm using a microplate reader (Thermo Scientific, USA). Samples were measured in duplicates.

2.8. Measurement of AChE activity

Measuring AChE activity in blood and tissue samples was done using colorimetric AChE assay kit (Ab138871, Abcam, USA), and according to the manufacturer’s instructions. A trial run was performed using the previously prepared homogenates of tissue samples (liver, spleen, kidneys, brain, eyes, and femoral bone marrow). The homogenates were serially diluted with 0.03 % Triton X-100 in distilled water to achieve the optimum dilution for each organ. The dilution factor was 80 for liver, spleen, eyes, and bone marrow; was 40 for kidneys; and was 320 for brain samples. Blood samples were diluted 1:10 in 0.03 % Triton X-100.

The required kit solutions as well as the standard curve were prepared per instructions of the protocol booklets. 50 µl from each sample were loaded per well. Samples were tested in duplicates after 30 min of incubation. Absorbencies were measured at a wavelength of 410 nm in a kinetic mode using a microplate reader (ELx800, BioTek, USA). Readings were measured every 5 min for 60 min; the best reading was at 30 min. The minimum detection was as low as 0.1 mU/well of AChE. The enzyme activity was expressed as the amount of AChE per 1 milligram of protein [14].

2.9. Statistical analysis

Results were expressed as Mean ± standard deviation (M ± SD). Data were analyzed using Analysis of Variance (ANOVA) in GraphPad Prism 8, followed by Tukey’s contrast analysis to determine any significant differences between and within groups. The level of significance was set to the value P < 0.05.

3. Results

3.1. Body weight at different sampling time

The M ± SD body weights of methomyl poisoned rats are plotted against sampling time at Hour 0, 3, and 6 in Fig. 3. The values did not show significant changes between the three sampling times (P > 0.05).
3.2. Relative organs weight

The average relative weights of liver, spleen, right kidney, brain, right eye, and right femoral bone marrow collected from the poisoned rats are shown in Fig. 4. All tissue samples showed no significant weight alterations ($P > 0.05$) at the different sampling time.

3.3. Protein concentration in blood and tissue

The $\bar{M} \pm S.D$ protein concentrations in the tissue of liver, spleen, kidney, brain, eye, bone marrow, and blood are represented in Fig. 5. The values had no significant changes with advancing time of sampling ($P > 0.05$).

3.4. Methomyl concentration in blood and tissue

The $\bar{M} \pm S.D$ methomyl concentrations in the collected liver, spleen, kidney, brain, eye, bone marrow, and blood are listed in Fig. 6. Only bone marrow and blood had significant changes with increasing sampling time. In the bone marrow, there were significant decreases of methomyl concentration at Hour 3 ($P < 0.01$) and Hour 6 ($P < 0.001$) compared to Hour 0. In Blood, methomyl concentration significantly decreased at Hour 3 ($P < 0.01$) and Hour 6 ($P < 0.001$) compared to Hour 0. In addition, methomyl in blood showed a significant decrease at Hour 6 compared to Hour 3 ($P < 0.001$).

3.5. AChE activity level in blood and tissue

The $\bar{M} \pm S.D$ activity of AChE in blood and the collected tissues in control and poisoned rats are illustrated at the selected time points (i.e., Hour 0, 3, and 6) in Fig. 7. Only brain and kidney samples had significant changes in the activity of AChE at Hour 3 and Hour 6 relative to Hour 0. There were significant decreases in the enzyme activity of the kidney samples at Hour 3 and Hour 6 compared to Hour 0 ($P < 0.01$). On the contrary, brain samples showed significant increases at Hour 6 compared to Hour 0 ($P < 0.05$). Other samples recorded no significant changes in the enzyme activity at any timepoint of sampling.

4. Discussion

With increasing sampling time, putrefaction and postmortem changes of markers in autopsy samples are critical to evaluate toxicity cases. Therefore, sampling time is important for exclusion and inclusion of findings in toxicity cases. This study monitored changes of body weight, relative organ weight, protein content, methomyl concentration, and AChE activity in blood and different body tissues at three postmortem sampling points (Hour 0, Hour 3 and Hour 6). The outcomes highlighted the impact of sampling time on methomyl toxicity biomarkers and consequent evaluation of related poisoning cases.

The current study recorded no significant changes in body weight and relative organs weight at the selected sampling timepoints. Similarly, body weight remained unchanged in a study on euthanized non-fasted male and female Sprague-Dawley rats with carbon dioxide; the animals were weighed and were necropsied at 5, 10 and 25 min after respirations ceased [15]. The same study found that the liver weight increased with increasing postmortem interval due to sinusoidal congestion by plasma influx. The different sampling times and causes of death could explain the different findings in our study, and justify insignificant changes of the relative organs weight at the selected timepoints.

Since proteins could be utilized as a biomarker of toxicity [16], this is the first study to include measuring protein in blood and different body...
tissues in the assessment of lethal methomyl toxicity at early postmortem intervals. Regardless the sample type, protein content did not change significantly at the three sampling times. These results are in harmony with those of a systematic study on euthanized rats that were kept at 23°C to monitor changes in the brain proteins and serum albumin at several postmortem intervals within 72 h. The study recorded most alterations at about 48 h postmortem [17]. Another study demonstrated an extensive stability of the brain RNA in rat and human samples for up to 48 h postmortem [18]. An additional analysis of rat tissues found significant effects of postmortem interval on different
metabolites during 72 h. The researchers concluded that postmortem sampling must be done at timepoints less than 24 h to yield replicable results for metabolomics analyses on brain tissues [19]. Together, the presented findings herein would suggest that the increase in the brain AChE activity could have been due to the stability of brain samples relative to other samples. Up to date, there has been no study that completely matched our study conditions. However, within the current experimental settings, this parameter did not significantly change during 6 h postmortem. Studies for longer durations may produce notable changes.

Methomyl concentration did not significantly change in our study, except in the blood and the bone marrow samples. Both types of samples recorded significantly decreasing methomyl concentrations with increasing sampling time. Regarding this point, there are variable findings and explanations in previous studies. Possible explanations for the decreasing levels in blood could have been due to methomyl decomposition by serum albumin and hemoglobin, or due to methomyl binding ability to other blood proteins (e.g., esterase) leading to the insufficient recovery of free methomyl [20]. It is plausible that these explanations also apply to the bone marrow, since it is a highly vascularized organ, and functions as the primary blood-forming organ [21]. Although other samples had no significant changes in the methomyl concentrations, generally they showed decreasing methomyl levels with increasing postmortem intervals. Passive drug release from drug reservoirs such as the liver, the lungs and the myocardium may occur immediately and later after death through cell autolysis [22]. Moreover, the type of tissue parenchyma plays a vital role in the speed of autolysis and the significant release of toxins. Thus, longer postmortem durations could produce significant alterations in methomyl concentrations.

Both of the spleen and the kidney samples recorded methomyl levels below the detection limits at Hour 0 and Hour 6. Similarly, previous reports recorded undetectable levels in a suicidal case who had ingested 2.25 g methomyl. At 6 h post-ingestion, methomyl levels were 1.61 ppm in blood and 10.91 ppm in urine, while at 15 h the levels were 0.04 ppm in blood and 0.25 ppm in urine. Interestingly, at 22 h, levels were not detectable in both types of samples [23]. Uneven sedimentation and clotting followed by lysis of postmortem blood could have caused these findings. In fact, clots entrapped red blood cells, which may be entrapped within tissue parenchyma, and hence, sampling these clots could influence measurements of any substance in postmortem samples [22].

Significant reductions in the AChE activity were measured only in the kidney samples. A similar significant inhibition in the enzyme activity was observed in female rats following administration of 1/10 LD50 and 1/20 LD50 of methomyl for 28 days [24]. Another study reported a significant reduction in the kidney AChE activity after orally gavaging of 24 albino rats with 0.2, 2.5 and 5 mg/kg bwt doses of methomyl for 12 weeks [25]. These significant findings in the kidney were not only observed in rats but also were found in methomyl poisoned mice. In a time-dependent manner, the mice developed oxidative stress, renal damage, and other histopathological changes [26]. Low activities of some antioxidant enzymes in the kidneys could result from damaging impact of oxidative stress on the tissue structure, the enzymatic structure and function, and on the related gene expression [26].

Interestingly, the brain showed increasing AChE activity 6 h postmortem compared to Hour 0. Most literature concerning AChE activity is controversial. It contained a variety of AChE activity that increased, decreased and even unchanged at different postmortem sampling times [27–29]. It was observed that oxidative damage in the heart started earlier than in the kidneys; different antioxidant activities attributed to the different damage onset in both organs [30]. Likewise, it is possible that variable enzymatic activities of different tissues caused variable AChE findings in our study. Future research is recommended to investigate if other organs can record significant changes during longer postmortem intervals.

Generally, factors such as passive diffusion, functional loss of tissues and advanced autolytic processes could have influenced the current study. In addition, the controlled experimental conditions (i.e., temperature, feeding contents, animals’ age, and methomyl dose) are among the possible limitations of this study. Changes in these conditions, such as postmortem interval could alter the presented findings. However, monitoring toxicity biomarkers at different sampling intervals has broadened the spectrum of forensic analysis possibilities. Based on the possible potentialities, future studies are recommended for screening new biomarkers at different postmortem intervals, which encourages using of these biomarkers in the evaluation of lethal methomyl poisoning cases.

5. Conclusion

This is the first study of sampling time effect on postmortem toxicity biomarkers in methomyl poisoned rats. There were no significant changes in the body weight, relative organ weight, and protein content. Blood and bone marrow recorded significant decreases in methomyl concentration of samples that were collected at Hour 6 postmortem compared to immediately collected samples. Likewise, AChE activity in the kidney decreased significantly at late sampling times. In parallel, the
enzyme activity significantly increased in the brain at Hour 6 in proportion to immediate sampling time. The outcomes demonstrated that some biomarkers could change drastically in susceptible tissues at different postmortem sampling times. In addition, the present study highlighted the importance of sampling time for exclusion and inclusion of findings on methomyl toxicity biomarkers and consequent evaluation of related poisoning cases.

Ethics approval and consent to participate

The permission to use laboratory animals in the current experiments was granted by the Animal Care Committee (ACUC) at Jordan University of Science and Technology [Approval No. 16/3/3/267].

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CRediT authorship contribution statement

Mohammad I Ahmad: Methodology; Visualization; Roles/Writing - original draft; Writing - review & editing; Data curation; Formal analysis; Investigation; Methodology; Supervision; Administration; Resources; Software; Supervision; Validation; Visualization; Roles/Writing - original draft; Writing - review & editing.

S.D. Nusair: Conceptualization; Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; Project administration; Resources; Software; Supervision; Validation; Visualization; Roles/Writing - original draft; Writing - review & editing; Mohammad Ananbeh: Formal analysis; Investigation; Methodology; Visualization; Roles/Writing - original draft; Writing - review & editing; Aref Yazed: Data curation; Formal analysis; Methodology; Supervision; Visualization; Roles/Writing - original draft; Writing - review & editing; Mohammad I Ahmad: Data curation; Investigation; Methodology; Resources; Software; Supervision; Visualization; Roles/Writing - original draft; Writing - review & editing; Nidal A Qinna: Investigation; Methodology; Visualization; Roles/Writing - original draft; Writing - review & editing.

Consent for publication

Not applicable.

Author contributions

All researches contributed to the research.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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