Malays. Appl. Biol. (2015) 44(1): 133–138

PARAQUAT DICHLORIDE DETECTION FROM FORENSIC BLOWFLY SAMPLES

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ABSTRACT

Accidental ingestion of paraquat dichloride contributed for a high number of pesticide-related deaths. It is a major suicide agent in many developing countries because of its easy accessibility. Based on the escalated death events related to misuse of paraquat, a study was conducted to observe its effect on decomposition process of New Zealand white rabbits, hence, biological and entomological samples were obtained for analysis. Entomological samples especially blowflies which are the main processor of dead remains applied in forensic investigations to obtain time and cause of death. In this research, high performance liquid chromatography (HPLC) was introduced in toxicological analysis to determine the presence of paraquat dichloride from biological specimens, and exploring the potential of blowfly samples as an alternate sample when biological specimen had decomposed. Paraquat dichloride was extracted using C_{18} solid phase extraction (SPE) to retain the interferences. Mobile phases used were acidified water (pH 3) and acetonitrile with ratio of 85:15. Limit of detection (LOD) and limit of quantitation (LOQ) were 0.03 mg/L and 0.09 mg/L, respectively with a linear curve (R^2 = 0.9982). From the analysis, concentration of paraquat dichloride found out to be the highest in liver as well as in the third instar of blowfly larvae. The isolation and analytical method proposed in this present study were able to be applied in detection of paraquat dichloride in post-mortem specimens. In conclusion, blowfly samples were suitable to be applied in forensic toxicological analysis to replace decomposed biological samples.

Key words: paraquat dichloride, entomotoxicology, HPLC, blowflies, *Chrysomya rufifacies*

INTRODUCTION

Forensic entomotoxicology is a field that has advanced in recent years to provide clues on intoxication-related death whenever the biological specimens are unavailable due to decomposition happened on the remains. The time of death can be determined by estimating the minimum post-mortem interval (PMI) using age of the oldest blowflies larvae obtained from dead remains. The content of the larvae provides clues to the possible cause of death using toxicological analysis (Yi *et al.,* 2013). Larvae feed on tissues of an individual who has consumed drugs or poison ante mortem, will ingest the substance, as well as its metabolites (Campobasso *et al.,* 2004). Bourel *et al.* (2001) demonstrated that morphine had accumulated in puparial cases of blowfly *Lucilia sericata*. Rapid absorption of drugs present in human tissues through intestinal epithelium of the larvae was demonstrated along with subsequent deposition of drugs in an area lying between the endocuticle and exocuticle. This information is pivotal to assists in determining minimum PMI estimates (Abd El-bar and Sawaby, 2011). Toxicological analysis was applied to the forensically-important blowflies in order to identify drugs and toxins present on intoxicated tissues, and the effects caused by such substance on arthropods development. *Chrysomya megacephala* and *Chrysomya rufifacies* were among the most species reported to colonize dead remain in Malaysia (Omar *et al.,* 2002) and they were available in a large number of decomposition sites (Gosselin *et al.,* 2011).

Unintentional death cases usually occurred from overdose usage of certain toxic agents such as paraquat. World Health Organization (WHO) had

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categorised paraquat as Class II pesticides which are moderately toxic. Paraquat is extremely toxic to humans and animals. In the third world country, paraquat is one of the herbicide that is used as a method of suicide because of its easy accessibility (Dinham, 1996). In Malaysia, between the year of 1986 – 1996, 700 poisoning cases are due to paraquat. About 73% are suicide cases while the remainder are occupational and accicdental exposures (Majid, 1997).

This study concerns on the transfer of poison or drugs to necrophagous arthropods which is the essence of forensic entomotoxicology (Yi *et al.,* 2013). The purposes of this study were to quantitate the concentration of paraquat dichloride levels in entomological and biological samples by high performance liquid chromatography (HPLC) hence their effect onto the development of blowfly *Chrysomya rufifacies* using a simple, fully validated and reliable method.

MATERIALS AND METHODS

Six adult New Zealand white rabbits were supplied by Aman Semesta Sdn. Bhd. Malaysia. Paraquat dichloride hydrate ($C_{12}H_{14}C_{l2}N_2.xH_2O$) was purchased from Dr. Ehrenstorfer GmbH, Germany. Acetonitrile HPLC Grade, phosphoric acid (H_3PO_4) 85% and methanol (CH_3OH) HPLC Grade were purchased from Merck KGaA, Germany. Ultrapure water was obtained from Thermo Scientific with 18.2 MΩ-cm pores. HPLC C_{18} column was obtained from Agilent, USA; with the dimension of 4.6 x 250 mm x 5 µm. Solid phase extraction (SPE) 200 mg silica-based octadecyl sorbent cartridge was purchased from Waters, USA.

Administration of paraquat dichloride

Six adult New Zealand white rabbits weighing from 1.5 kg to 2.0 kg were used in this experiment. The control and test group rabbits were eradicated by asphyxiation using carbon dioxide and lethal dose of paraquat (LD_{50}=100 mg/kg), respectively. No anesthetization was done on the test group prior to exposure to paraquat, to avoid any contamination. After sacrificing, biological samples were obtained from control and test groups from a small incision on its abdomen and entomological samples were obtained from the carcass as the carcass decomposition progressing. This study was approved by UiTM Animal Ethic Committee.

Extraction of paraquat dichloride from biological and entomological samples

All samples were homogenised in phosphate buffer (pH 8.0) and centrifuged for 10 minutes. Supernatant was used in the following sample preparation steps. Paraquat is highly ionic compound; therefore, the use of plastic or polypropylene apparatus is pivotal. This method was adapted from Almeida and Yonamine (2007) with slight modification.

SPE C_{18} cartridges were preconditioned by using 10 mL methanol and 5 mL of phosphate buffer (pH 8.0). Then, the sample was added into the column and the eluate was used in analysis as the sorbent traps the interferences. The eluate were allowed to evaporate using a gentle stream of nitrogen and reconstituted with phosphate buffer (pH 8.0). A total of 20 µL of the analyte was injected into HPLC. This method adapted from Rumiza *et al.* (2008) with some modification.

Chromatographic separation of paraquat dichloride

Separation was achieved using Agilent Zorbax Eclipse XDB-C18 (4.6 x 250 mm x 5 µm) with mobile phases of acidified water and acetonitrile (85:15). The mobile phase flow rate was set at 1 mL/min with 254 nm of diode array detector (DAD). Overall analysis took about 4.0 minutes.

Method validation for paraquat dichloride

Paraquat dichloride standard was diluted using methanol to five dilutions ranging between 1.0 mg/L to 50 mg/L in order to construct a calibration curve. Limit of detection (LOD), limit of quantification (LOQ), precision, percentage of relative standard deviation (%RSD) were determined from the analysis.

RESULTS AND DISCUSSION

The method was validated using control entomological and biological samples. The selectivity of the method was determined by analysing control samples in identifying the interferences as well as the absence of paraquat dichloride. The background noise was also determined in this process. Paraquat dichloride calibration curve was constructed using five points where the range spans from 1.0 mg/L to 50 mg/L. The area response of paraquat dichloride was plotted against the concentrations that generate a representative regression analysis of y = 114.3x with R^2 of 0.998. The precision was done by analysing five aliquots of 10 mg/L standard paraquat dichloride. Table 1 and Fig. 1 summarize the method validation and the standard calibration curve respectively.

In extracting paraquat dichloride, C_{18} sorbent was used to separate the analyte and the interferences. Paraquat dichloride is a polar compound, hence it did not retained in the sorbent

as C_{18} is a non-polar sorbent. Analyte was collected after sample had run through the sorbent. An example of interference from entomological and biological samples is fatty acids which are non-polar compound. Hence, they did retain on the C_{18} sorbent which extraction achieved. Using this method, the extraction time was reduced with the recovery of 67.6%.

Entomological samples of *Chrysomya rufifacies* were used for chemical analysis in detecting paraquat dichloride. They were the first, second and third instar of larvae, pupa, puparial cases and adult fly. These blowfly samples grew on the rabbit carcasses and consume the flesh of the carcasses as their food substrate. Chromatograms of paraquat analysis are shown in Fig. 2. Paraquat dichloride was eluted out at 1.9 min ± 0.023 min. Except for adult, *C. rufifacies* samples and biological samples from the carcasses were positive for paraquat dichloride. Concentration of paraquat in entomological and biological samples was calculated and summarised in Table 2.

Based on the previous research, paraquat dichloride were able to be separated using high performance liquid chromatography with vltraviolet detector (HPLC – UV) mobile phase contained methanol, acetonitrile as well as ion – pairing agents (Ito *et al.,* 1993, Sellero *et al.,* 1996, Hara *et al.,* 2007). Zou *et al.* (2011) use the combination of 0.1 M phosphoric acid at pH 3.0 and acetonitrile as the mobile phases in analysing paraquat dichloride. The ratio was 85% phosphoric acid and 15% acetonitrile. It was found that we were able to maximise the detection of paraquat dichloride from entomological, as well as biological samples using technique done by Zou *et al.* (2011) with slight modification. The acidified water at pH 3.0 and acetonitrile were used as the mobile phases with the ratio of 85% acidified water and 15% acetonitrile. Paraquat dichloride is a polar compound that has the affinity towards water rather than acetonitrile. As the separation was done by using a C_{18} column, the analyte eluted by water rather than retained on the stationary phase. This explains the elution of paraquat dichloride that happened as early as 1.9 min ± 0.023 min. Based on the control chromatograms obtained, the specificity of the method was good as the analyte was absent in the control samples. This shows that the method was suitable to analyse the presence of paraquat from the entomological and biological samples without any endogenous species that have the same retention time as the analyte.

When larvae fed on a tissue that was intoxicated with some kind of drugs or poison, there were two processes that will occur in their system. The two processes are bioaccumulation or excretion of the drugs as well as its metabolites (Carvalho *et al.,*

Table 1. Validation of paraquat di chloride analysis using high performance liquid chromatography (HPLC)

Parameter	Value
Linearity	1.0 – 50 mg/L
R^2	0.9982
LOD	0.03 mg/L
LOQ	0.1 mg/L
Recovery	67.6%
Precision (%RSD)	3.75%

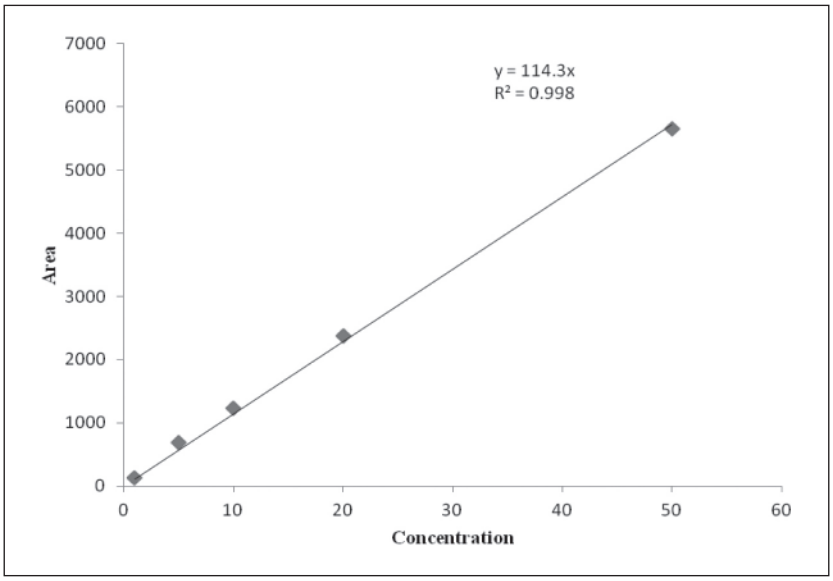

Fig. 1. Calibration curve of paraquat dichloride standard

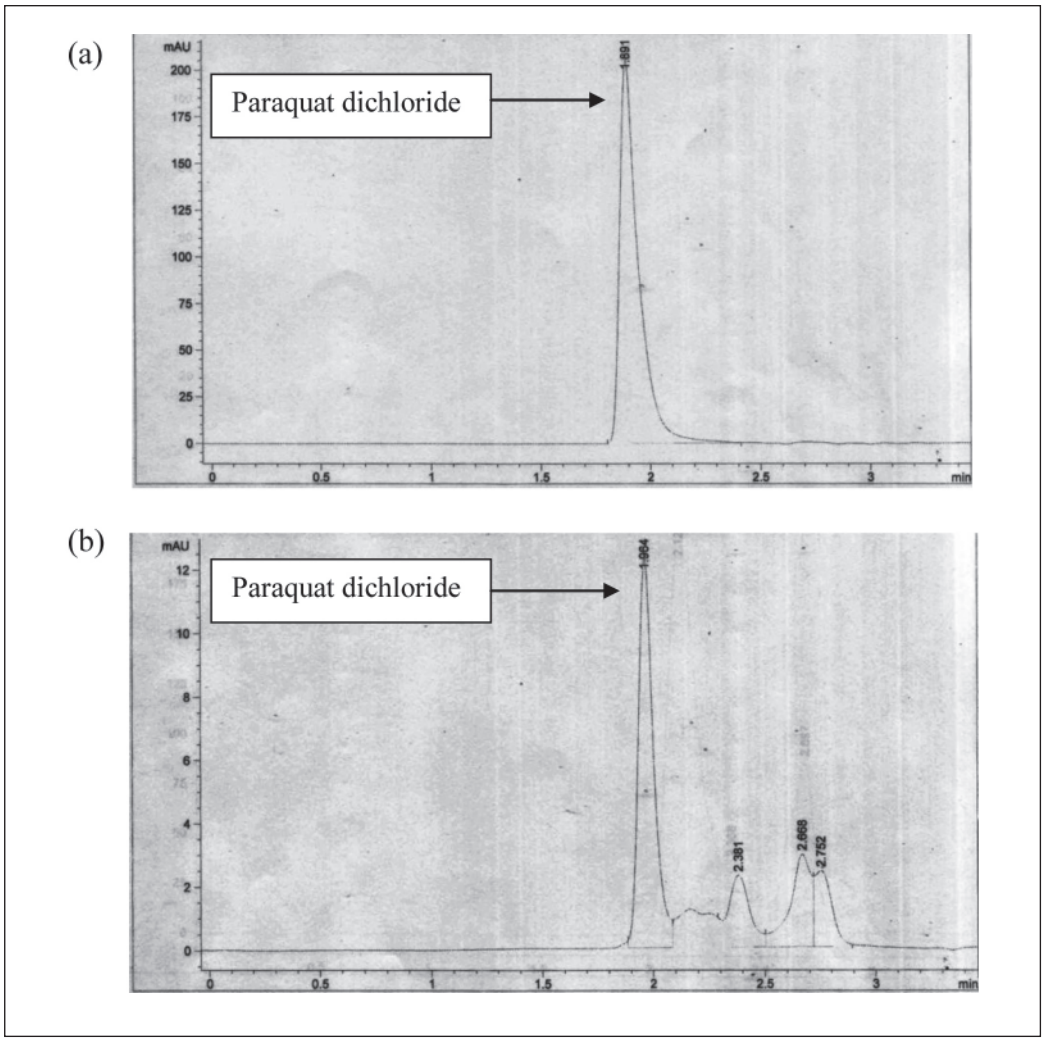

Fig. 2. Chromatogram obtained from analysis of (a) standard paraquat 10 mg/L (b) larvae of second instar

Table 2. Concentration of paraquat dichloride (mg/L) in entomological (blowflies *C. rufifacies*) and biological samples of rabbit carcasses

Samples	Concentration (mg/L)
Second instar	0.35 (± 0.04)
Third instar	0.57 (± 0.01)
Pupa	0.47 (± 0.02)
Puparial cases	0.16 (± 0.01)
Adult	Not detected
Liver	0.88 (± 0.06)
Lungs	0.61 (± 0.06)
Urine	Not detected
Blood	Not detected

2001). In this study, we found that bioaccumulation occurred, revealed from detection of the substance in every stage of development; except on adult. Thus, paraquat dichloride may have a significant impact onto the developmental rate of the larvae since it was accumulated in the *C. rufifacies* larvae. Rashid *et al.* (2013) conducted a study using ketum extract on *C. rufifacies* blowflies and found that the concentration of ketum extract was higher in feeding phase compared to the post-feeding phase of the blowflies. The rate of accumulation was higher than the rate of excretion during feeding phase where it consumes the intoxicated carcasses and stores it in its gut as it continues to grow. Ketum extract had an impact onto the development of *C. rufifacies* where it accelerates the growth of larvae. Thus if affect the estimation of PMI.

The concentration of paraquat dichloride was highest in the liver sample compared to the other samples as portrayed in Table 2. Generally, paraquat dichloride accumulates in the liver as well as the lungs. The concentration obtained from these samples could not be correlated with the concentration of paraquat dichloride administrated at the beginning of the sampling stages as there were no verified equations due to the metabolic processes by the rabbit as well as the larvae itself. Introna *et al.* (2001), suggested that if a toxin can be detected in a larvae sample, the rate of absorption exceeds the rate of elimination. The concentration of paraquat dichloride detected was increased from second instar to the third instar and decrease as soon as it reaches puparial stages. Third instars of *C. rufifacies* have the highest concentration of paraquat dichloride due to the active feeding activity if compared to other species. The amount of paraquat dichloride accumulated in the third instar was more than the second instar might be due to the rate of absorption exceeds the rate of elimination. As it begins to pupate, it migrates from the carcasses; hence the consumption of intoxicated carcasses had been halted. This explains the concentration of paraquat in pupa samples was lower than third instar samples. No paraquat dichloride was detected in adult sample; however paraquat dichloride exhibited an impact onto the appearance of adult emergence. Some of adult blowflies emerge with no wings. This shows that the content of toxic substance had cause a severe malfunction in the blowflies' physiology. Paraquat dichloride that had accumulated in the larvae and pupae system might have been metabolised into other metabolites, hence excreted from the adult. This explained the absence of paraquat dichloride from adult sample.

CONCLUSION

Paraquat dichloride had been detected in all of the entomological and biological samples with the highest concentration in the third instar larvae and liver of the carcasses. This suggested that entomological samples of *Chrysomya rufifacies* can be used as an alternative specimen in paraquat-intoxicated cases. A detailed summary on the correlation of each drugs or poison with vast species of forensically-important arthropods should be documented to aid in estimating PMI.

ACKNOWLEDGEMENTS

We would like to thank Universiti Teknologi Mara (UiTM) for supporting this study and providing the research facilities. We would also like to thank UiTM staff for their help and support throughout this study.

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