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Research Article

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Determination of chlorpyrifos residues in water and liver tissue of zebrafish *(Danio rerio)* by high performance liquid chromatography (HPLC) with UV detection

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ABSTRACT

Chlorpyrifos is one of the most toxic pollutants for aquatic ecosystem. The present study investigated the extraction of chlorpyrifos in water and liver tissue of zebrafish (Danio rerio). Water was collected at 0, 24, 48, 72 and 96hrs after the addition of toxicant and the amount of chlorpyrifos present in the water and liver tissue. In the concentration level range of 200 µg/L for the extraction of chlorpyrifos from water and liver tissue. Amount of chlorpyrifos in water and liver tissue analyzed by HPLC to know the amount of residue left out in the water and zebrafish liver after addition of 200 µg/L of chlorpyrifos initially. The recoveries were the decrease of concentration chlorpyrifos in water and increase in liver with increase in time of exposure is an indication of the accumulation of the toxicant in the organism through uptake. This could be hazardous as it could make its way into the food chain.

Key words: Chlorpyrifos, Residue analysis, Zebrafish, Liver, Water, HPLC.

INTRODUCTION

To meet the demands of the growing population, it is necessary to increase the food production. The introduction of high yielding varieties has necessitated the farmers to use fertilizers and pesticides for the control of insect's pesticides. However, improper use of pesticides has resulted in resistance in pest populations, contamination of soil, water and the environment and pesticide residues in the food products [1].

Fish representing as bio-indicators of environmental contamination and may play an important role in the evaluation of the potential risk of pollution in aquatic environments, since they may directly expose to chemicals caused by agricultural output through runoff or indirectly by food chain of the ecosystem, this may reflect the biological influences of environmental contamination in water [2]. The zebrafish (*Danio rerio*) is a small equatorial freshwater fish that has a great tolerance for a wide range of breeding circumstances has been employed as an experimental species in the intensive studies of many other scientists, from the 1980s. Particularly in recent years, numerous studies have shown the identified benefits of hiring of zebrafish in environmental toxicological studies [3].

At present, India is the largest producer of pesticides in Asia and ranks twelfth in the world in the use of pesticides with an annual production of 81647 MT [4]. The Indian pesticide industry is dominated by insecticides, whereas

globally, herbicides and fungicides are the key segments [5]. Andhra Pradesh is the first highest pesticide consuming state in India [6].

In recent studies, a residue of chlorpyrifos was most frequently detected pesticide in aquatic systems, food products, etc., and thereby raising public concern on safety. Residues above the maximum amount (0.05 mg kg^{-1}) was detected in cauliflower in places where chlorpyrifos was sprayed at the recommended dose [7], 0.02 µg/kg in spinach and rice [8] in Okra and eggplant in different countries [9] and in green vegetables sold in Tokyo market [10].

Different concentrations of chlorpyrifos in water and sediment samples across the world were also detected. It was found in water (0.3 ug/L) collected from Horqueta stream, in sediment (30.3 µg/kg), in the runoff water (0.28 µg/L) and in the suspended particles (63 and 225.8 µg/kg) in Brown stream of Argentina [11], in water (24.5 to 303.8ppb) and sediment (0.9 to 303.8ppb) samples collected from New Damietta drainage canal in Egypt [12], in water (up to 72pg L^{-1}) of the coastal lagoon system of Laguna de Terminos, Campeche, Mexico [13], in surface water (0.007 µg/L) and in ground water (0.016 µg/L) in southern coast watershed of Caspian Sea, Iran [14], in El-Embaby drain (9.38ng g^{-1}), Egypt [15], in Lake Naivasha (between 8.8 and 26.6 µg L^{-1}), Kenya [16] and in paddy field water samples (0.06 ± 0.001 µg L) in Bangladesh [17], in water (0.01-1.31 mg/kg) and soil (0.01-0.81 mg/kg) in the central agricultural areas of Thailand [18].

In India also chlorpyrifos residues were detected in water samples (0.003-0.006 µL/L) collected from Kaithal and Pant Nagar areas [19], at measurable levels in breast milk from nursing mothers [20], made tea samples exceeding the MRL levels in 16% and 20% of the Dooars and Hill regions of West Bengal respectively [21] and in tissues of fish (88.6 µg/g) collected from Kolleru Lake in Andhra Pradesh, the state where the present study was carried out [22]. The extent of residues of chlorpyrifos found in different parts of India and the world in different systems has lead to the present study.

India is an agricultural country. Its 80% population is dependent on agricultures. To achieve economic benefit and to make sufficient supply of food to a vast population, it becomes necessary to increase the yield of crops. Sometimes they might cause economic losses. The use of pesticides contaminated the food stuffs, thus instead of supporting health these becomes a great health hazard. Considering the Indian context, population has been growing at an annual growth rate of 1.2% in India and exceeded 1.271 billion people in the 2015 itself. As demand for food is increasing, so provide the quality of food in India should be geared up in order to meet increasing demand for food.

The wide use of pesticides for agricultural performance represents thousands of molecules with an enormous variety of physicochemical properties that are hazardous to living organisms. It is due to contamination of a number of aquatic ecosystems, including sediments, water and biota. The present study was planned to estimate the level of chlorpyrifos residues in water and liver tissue of zebrafish and their biomagnifications at Indian region.

EXPERIMENTAL SECTION

Maintenance of zebrafish

Zebrafish (*Danio rerio*) are maintained in our department aquarium facility for more than two generations with continuous breeding under defined conditions as described by Westerfield [23]. Fifty fish were housed in each 100L glass aquaria with continuous aeration at a temperature of 27 ± 1°C and 13:11 hr light:dark photoperiod. Fish were fed twice a day with alternating diet of freshly hatched brine shrimp (sanders brine shrimp co, Utah) and dry flake food (tetra brand).

Preparation of stock solution

Technical grade chlorpyrifos (99%) was obtained from the Nagarjuna Agri Chem Limited, Hyderabad. A stock solution was prepared by dissolving 50 mg chlorpyrifos in 5ml acetone. This was stored at 4°C and from this daily requirements are taken and added to respective fish tanks.

Experimental design

Mature adult zebrafish from our aquarium stock were kept in 20L glass aquaria with continuous aeration for four days. Later 200 µg/L of chlorpyrifos was added. Simultaneously control fish were maintained separately. Liver was collected after 24h, 48h, 72h and 96hrs from fish for conducting the experiments. Water was analyzed for the concentration of chlorpyrifos at 0h, 24h, 48h, 72h and 96hrs after addition of 200 µg/L of toxicant.

Residue analysis

Water analysis

Water samples were collected at 0h, 24h, 48h, 72h and 96hrs after the addition of toxicant in 500 ml amber glass bottles. The bottles were prewashed with a non-phosphate detergent and then rinsed with distilled water and methanol. The amount of chlorpyrifos residues was analyzed using High performance Liquid Chromatography (HPLC) following the method described by Rao et al., [24] with some modifications.

Tissue analysis

The experimental fish were rinsed with distilled water prior to the dissections to avoid the external pesticide residue. Fish were dissected and liver tissue was collected. Liver tissue was subjected to lyophilisation using liquid nitrogen. Chlorpyrifos was extracted from the tissue samples by homogenization in a Potter-Elvejhem glass-teflon homogenizer using HPLC grade petroleum ether and anhydrous sodium sulphate (residue analysis grade). The resultant extracts were centrifuged to remove all the cell debris and unwanted materials. The extracts were passed through an anhydrous sodium sulphate column to remove traces of water in the samples. Further, the extracts were again passed through the Florisil column for cleanup of the sample. The resultant extracts were evaporated to dryness under reduced pressure in a rotary evaporator at 40°C. Dry extract was dissolved in 1 ml of acetonitrile for HPLC analysis. Quantification of chlorpyrifos was carried out by means of external standard method using peak areas of individual samples. The efficiency of the extraction procedure was 95%, with a relative standard deviation of 9% at 1 ng/g wet wt. (n = 6). The limit of detection of the method was 0.11 ng/g wet wt. of tissue.

Statistical analysis

Data were statistically analyzed using the DMR (Duncan's Multiple Range) test. The value $P<0.05$ was used as the criterion for statistical significance [25]. All data are expressed as mean ± SD (n=6).

RESULTS

Chlorpyrifos residue levels in water and liver tissue

The presence of chlorpyrifos in water after 0, 24, 48, 72 and 96 h was measured by HPLC. The amount of chlorpyrifos added to water was 200µg/L initially. The results showed that chlorpyrifos degraded quite quickly and the residue of chlorpyrifos detection was shown in Table 1 and Figure 2. The presence of chlorpyrifos in liver after 24, 48, 72 96 h was measured by HPLC. Concentration of chlorpyrifos recorded in liver was shown in Table 1 and Figure 2.

Table 1: Residue of Chlorpyrifos in water and liver tissue as determined by High performance liquid chromatography (HPLC)

Amount added	Concentration of Chlorpyrifos (µg/L)								
	0 h	24 h		48 h		72 h		96 h	
	Water	Water	Liver	Water	Liver	Water	Liver	Water	Liver
200 µg/L	79.3 ± 3.05	61.3 ± 5.13	4.2 ± 0.42	36.6 ± 3.21	19.6 ± 0.14	29.5 ± 2.41	48.1 ± 6.92	18 ± 2.64	63 ± 5.65

Note: Values are mean (n=3) ± SD.

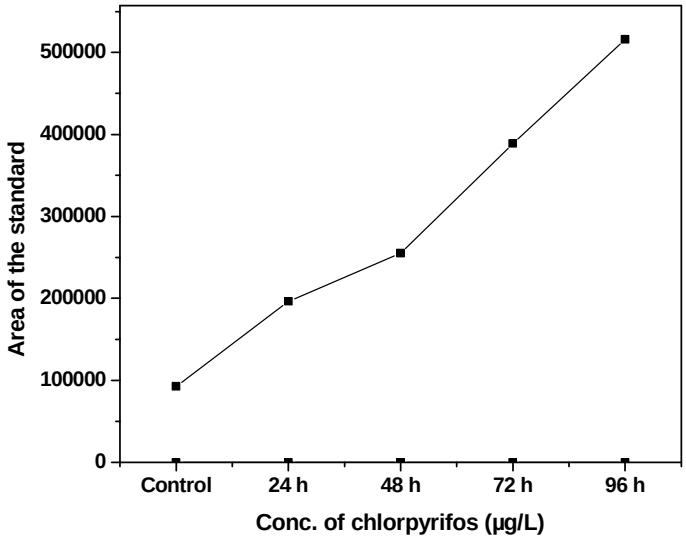

Figure 1. Standard graph: Concentration of Chlorpyrifos vs Area, 20µl of Chlorpyrifos dissolved in acetonitrile was injected

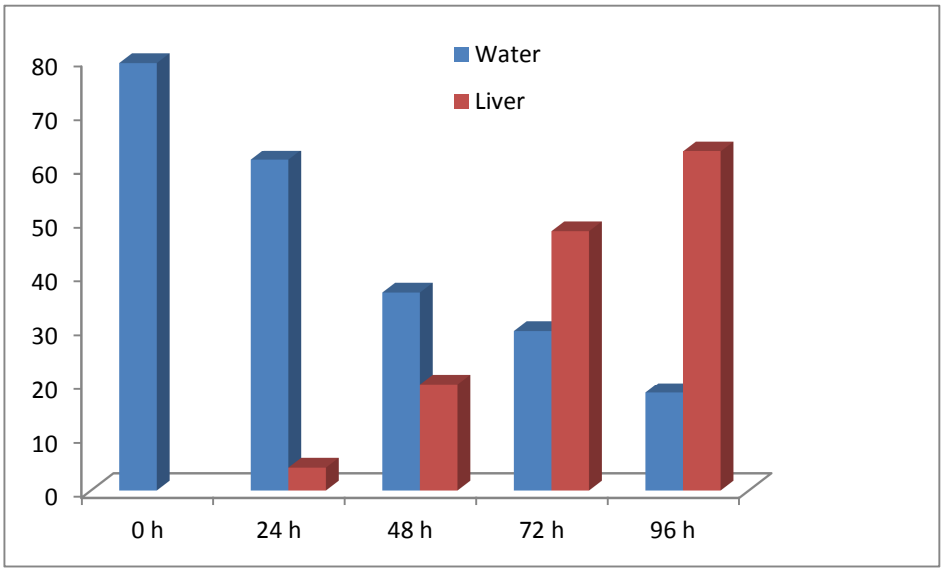

Figure 2. Residue of Chlorpyrifos in water and liver tissue after 0h, 24h, 48h, 72h and 96hrs. 200µg/L of Chlorpyrifos was added initially

DISCUSSION

From earlier research it is understood that chlorpyrifos was stated to be producing oxidative stress resulting in the accumulation of lipid peroxidation products in different tissues of rats [26, 27] but we cannot conclusively say that similar oxidative stress is caused in fish also. Chlorpyrifos residue in water refers to its concentration that has remained in the water after it has been added to water. Concentration of chlorpyrifos remained is analyzed to correlate the relationship between the concentration added and the concentration remained. The presence of chlorpyrifos residue in water after 0, 24, 48, 72 and 96 h the amount of chlorpyrifos added to water was 200 µg/L initially and was shown in Figure 2. A quick degradation of the toxicant was noticed. It was shown by Saad et al., [28] that organophosphorus compounds are quickly degradable in the aquatic environment.

Laboratory studies on the fate of chlorpyrifos in pure water indicate that hydrolysis and photolysis occur at moderate rates under neutral conditions with half-lives of about a month at a neutral pH and 25°C [29]. The major degradation pathway of chlorpyrifos begins with cleavage of the phosphorus ester bond to yield 3,5,6-trichloro-2-pyridinal (TCP) and this is degraded by microbial activity and photolysis to carbon dioxide and organic matter [30]. Studies by Karen et al., [31] indicate some aquatic macrophytes can absorb chlorpyrifos and help remove it from the aqueous environment. As a result the residue of chlorpyrifos in the water of Nanjing and Guangxi was undetectable after 21 days [32]. Adsorption by biomass with maximum occurring within 3 h, Sorption to dried leaves and different waxes influencing photodegradation of chlorpyrifos was reported earlier [33, 34, 35]. Also, aerobic bacteria tend to transform chlorpyrifos by hydrolysis to produce diethylthiphosphoric acid (DEPT) and 3,5,6-trichloro-2-pyridinol (TCP) [36]. Several studies have demonstrated that different biological systems like straw, peat can effectively retain and degrade pesticides, including chlorpyrifos [37, 38, 39]. Though there are no dried leaves, waxes are macrophytes in the zebrafish aquarium, planktons, faecal and any other biological matter present could be absorbing chlorpyrifos adding to the quick degradable property of chlorpyrifos. This could be the reason for less amount of residues even among the 24 h and thereafter.

CONCLUSION

Chlorpyrifos residue in water refers to its concentration that has remained in the water after it has been added to water and the residue in liver refers to concentration that has accumulated in liver tissue as a result of uptake of water by fish. Analysis of the concentration of chlorpyrifos remained/accumulated is an effort to correlate the relationship between the concentration added and the concentration remained/accumulated. Decrease of concentration of chlorpyrifos in water and increase in liver with increase in time of exposure is an indication of the accumulation of the toxicant in the organism through uptake. This could be hazardous as it could make its way into the food chain. A constant monitoring program should be introduced by the Government of India to provide a hazard free environment to the aquatic biota and to ensure safe and healthy supply of fish for human consumption.

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