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Detection of Herbicide Clomazone in Freshwater Teleost by Using HPLC Method

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Abstract: Clomazone is widely used in cotton, soybean, tobacco and rice fields to control broadleaf weeds. The present study shows the accumulation of the herbicide clomazone in the tissues of the non-target organism, i.e. the fish *Channa punctatus*. After determining the LC_{50} of clomazone, different dosages for treating the fish with the herbicide were calculated. The fish were treated with three different concentrations, i.e. treated with high (0.5 ppm), mild (0.25 ppm) and low (0.08 ppm) doses of the herbicide. The fish were exposed to the three concentrations for 28 days and were sacrificed after 7, 14, 21 and 28 days. The herbicide clomazone was detected and quantified in gills, kidneys and liver extracted from fish by HPLC using the mobile phase (methanol:water) at a flow rate of 1 ml/min measured at 220 nm. This study gave an RSD of less than 1%, reflecting the accuracy of the method. The standard curve was linear in the range of 0.1 to 0.006 ppm. The detection and determination limits were 0.004 and 0.015 ppm, respectively. The study showed that gill accumulation of clomazone occurs after each fixed interval and at each dose. In contrast, kidney and liver only showed dose independent accumulation after prolonged exposure.

Keywords: Clomazone, *Channa punctatus*, Gills, Liver, Kidney, HPLC

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Introduction

Several herbicides and pesticides are being used by farmers to increase crop yield. These pesticides contaminate the groundwater and nearby water bodies, affecting the non-target organisms. One such herbicide is clomazone. Herbicide clomazone (Fig. 1) is a member of the chemical class isoxazolidinone. The clomazone is volatile and has high water solubility. This herbicide prevents carotenoid synthesis by acting as an inhibitor. It is resistant to a broad range of pH values and weakly sorptive to soil (Murussi *et al*., 2015). Due to the herbicide's practical weed control ability, it is frequently used in fields. However, it does threaten the ecology and fauna (Norsworthy *et al*., 2019; Song *et al*., 2020; Hussan *et al*., 2020; Panasenko *et al*., 2022). The clomazone employed

in the agricultural fields may also affect the Nitrogen-cycle, increase fungal abundance, and reduce the bacterial number (Du *et al*., 2018). When zebrafish was exposed to clomazone, it affected the development at early stages (Stevanovic *et al*., 2017). It also exerts toxic effects in fish by producing reactive oxygen species (Menezes *et al*., 2013).

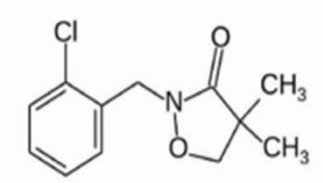

Fig. 1: Molecular geometry of clomazone.

Clomazone concentrations in various matrices have been measured using multiple analytical techniques. Various researchers have found clomazone residues in rice-field water, soyabean farms, soil, and nearby water bodies using HPLC with DAD or UV (Hu *et al*., 2011; Farajzadeh *et al*., 2014; Liao *et al*., 2014; Pang and Hu, 2020). A reliable analytical technique for isolating, identifying, and quantifying both novel and well-known compounds and clarifying their chemical and structural properties is LC-MS (liquid chromatography-mass spectrometry).

For the identification of herbicides and pesticides, a quick and reliable method is HPLC-UV (Al-Rimawi, 2014). The aim of the present study was to validate and develop method for determining clomazone in the tissues of fish. The HPLC technique was employed after extracting tissue samples from the fish to determine the concentration of clomazone accumulated in the fish's tissues (gills, liver, and kidney). Furthermore, a UV detector was used for the detection of the herbicide.

Materials and Methods

Chemicals:

Hubei Ruichi Technology Development Co. Ltd, Hong Kong, China, supplied analytical standards for clomazone (purity-95%).

Experimental design:

The fish were acclimatized for two weeks prior to the treatment. The LC_{50} was calculated. The fish were treated with three doses of the herbicide, i.e., a high dose of 0.5 ppm, a mild dose of 0.25 ppm, and a low dose of 0.08 ppm for 7, 14, 21, and 28 days respectively. After regular exposure to each dose, fish were sacrificed, and tissues (gills, kidneys, and liver) from the fish were collected and analysed.

Apparatus:

HPLC system (Agilent 1200 series) equipped with pump (G-1311A) and UV-visible absorbance detector (G1365B), C18 column of 250 mm in length, 4.6 mm in diameter and 5µm particle size, autosampler (G1367B), and column oven (G1316A). The mobile phase consisted of methanol: water (60:40) and 0.1% H_3PO_4 at a flow rate of 1 ml/min at room temperature with an injection volume of 10 µl. Analytes were measured at 220 nm. The software used was Agilent chemstation. Peaks obtained in the chromatogram were compared with the standard solutions' retention time and absorption spectra of the samples, and identification was made.

Standard Solution Preparation:

Four different standard solutions of clomazone were prepared. Each solution designed was injected into the HPLC, and a detector recorded peaks in the chromatogram. The standard solutions prepared were of concentrations 0.1 ppm, 0.05 ppm, 0.025 ppm, and 0.006 ppm.

Validation Method:

The parameters of the confirmed method were composed of linearity (LR), recoveries (detection of herbicide), the LOD (limit of detection), the LOQ (limit of quantification), precision, and selectivity.

$$LOD = \frac{3 * SDy}{m}$$

$$LOQ = \frac{10 * SDy}{m}$$

where, SDy= standard deviation of the y-intercepts; m= slope from the standard curve.

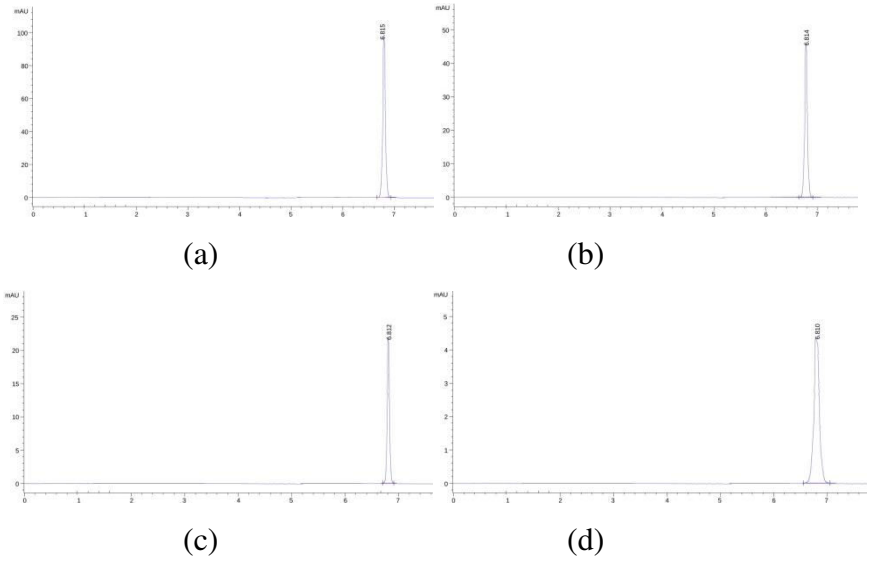

Fig. 2: Chromatograms obtained at different concentration of standard solutions (a) 0.10 ppm showing retention time (min) 6.815, (b) 0.05 ppm showing retention time (min) 6.814, (c) 0.025 ppm showing retention time (min) 6.812 and (d) 0.006 ppm showing retention time (min) 6.810.

Interday and intraday investigations were done to confirm the method's precision. By evaluating the response of clomazone five times within a day and five distinct days, respectively, were calculated. RSD (%) was used to describe the outcomes.

Results and Discussion

Several trials were conducted in order to optimize the mobile phase. Finally, methanol:water (60:40) was chosen as the optimal mobile phase since it displayed extremely well resolved analyte peaks.

Retention time of standard solutions of clomazone was recorded at 6.815, 6.814, 6.812, and 6.810, yielding a perfect peak resolution with distinct baseline separation as shown in Figure 2.

The measurement of clomazone revealed that there was no interday and intraday variation, and that the RSD values were always less than 1% throughout the analysis.

The method's linearity was examined using the linear regression analysis of the standard solution. The respective calibration curve's coefficient of correlation (R^2) was used to assess the linearity (Kim *et al.*, 2022). Linearity was calculated at different standard solutions of clomazone, where peak areas were plotted against the concentration of clomazone over the range of 0.01- 0.006 ppm. The R^2 was estimated to be 0.9981 (Fig. 3).

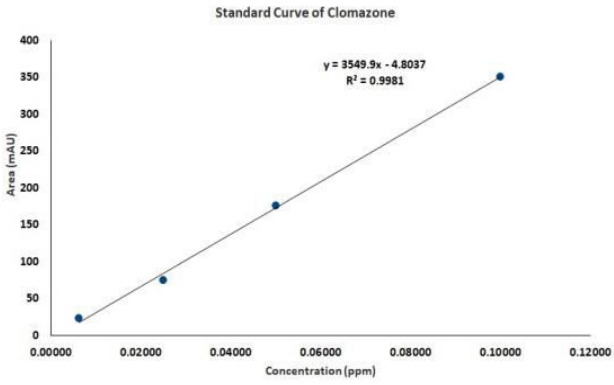

Fig. 3: Standard curve of standard solutions of the clomazone.

Selectivity was determined by analyzing the chromatograms and no interfering peaks were obtained in the chromatogram of the prepared standard solutions.

The detection and quantification of clomazone at low concentrations is made possible by its low LOD and LOQ (Table 1).

Table 1: Validation parameters of clomazone

Linearity range (ppm)	0.01-0.006
Regression equation	Y = 3549.9x-4.8037
Correlation coefficient (R^2)	0.9981
Intra-day precision (%RSD)	0.615
Inter-day precision (%RSD)	0.027
Specificity	Specific
LOD (ppm)	0.004
LOQ (ppm)	0.015

Table 2: The concentration of clomazone accumulated in the different tissues of fish *Channa punctatus* upon exposure to three clomazone concentrations for 7, 14, 21, and 28 days.

		Clomazone accumulated at different doses (in ppm)		
Sample	Days	High dose (0.5 ppm)	Mild dose (0.25 ppm)	Low dose (0.08 ppm)
GILLS	7	0.00315	0.00256	0.0021
	14	0.00902	0.00676	0.0045
	21	0.01315	0.00607	0.0037
	28	0.01834	0.01294	0.0091
KIDNEY	7	-	-	-
	14	0.01660	0.01202	0.0090
	21	0.01754	0.01496	0.0128
	28	0.01398	0.01283	0.0092
LIVER	7	-	-	-
	14	-	-	-
	21	0.01853	0.00995	0.0085
	28	0.02459	0.01646	0.0149

Treatment with High Dose (*0.5 ppm*):

The amount of clomazone accumulated in various tissues after being administered to fish at a concentration of 0.5 ppm was calculated. The concentration in gills were found to be 0.003 ppm after treatment with clomazone for seven days, 0.009 ppm after 14 days of treatment, 0.013 ppm after 21 days of treatment, and 0.018 ppm after 28 days of treatment (Table 2; Fig. 4). The concentration of accumulation was determined by the peak obtained in the chromatogram. No accumulation was detected in the kidney after the

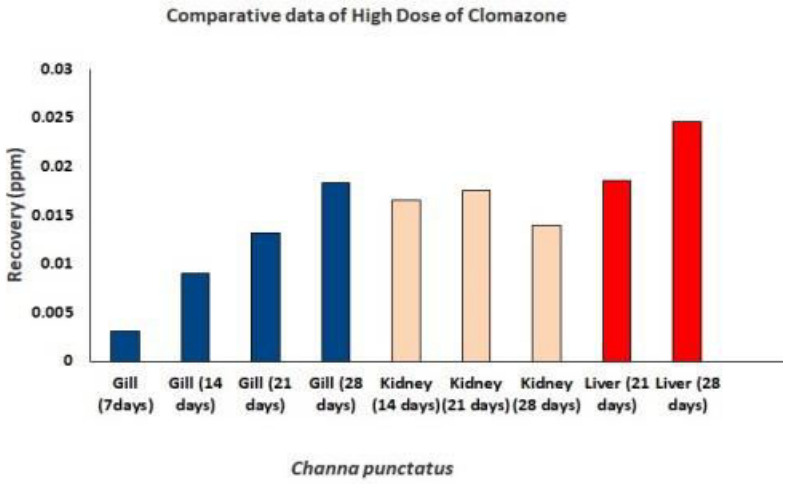

Fig. 4: Concentrations of clomazone accumulated in tissues of *Channa punctatus* after exposing it to high dose.

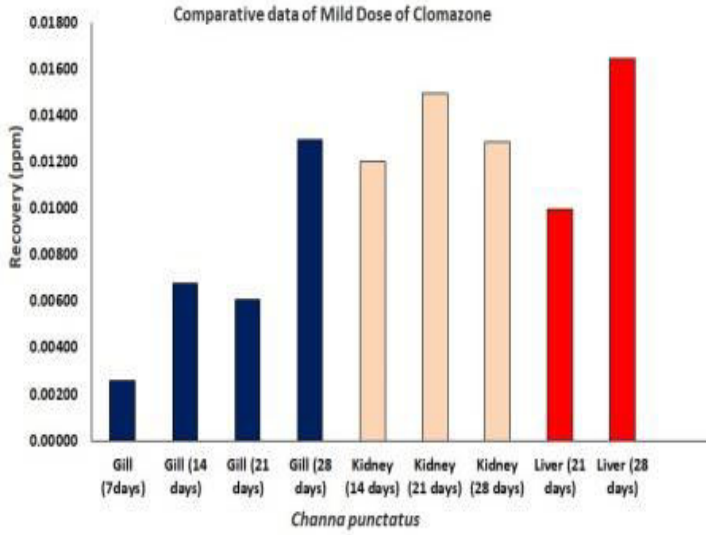

Fig. 5: Concentrations of clomazone accumulated in tissues of *Channa punctatus* after exposing it to mild dose.

7th day of administration; however, after 14, 21 and 28 days it did accumulate clomazone, with concentrations of 0.016, 0.017 ppm and 0.013, respectively. No accumulation of the clomazone was calculated in the liver after 7 and 14 days since no peaks were obtained in the chromatogram, but after 21 and 28 days, it was evident from the peak obtained in the chromatogram that clomazone had accumulated to concentrations of 0.018 and 0.024 ppm, respectively (Table 2; Fig. 4).

Treatment with Mild Dose (*0.25 ppm*):

After 7, 14, 21, and 28 days, *Channa punctatus* was sacrificed, and tissues were collected for the HPLC analysis. Accumulation of clomazone in gills was calculated to be 0.002 (after seven days), 0.006 (after 14 days), 0.006 (after 21 days), and 0.012 ppm (after 28 days) (Table 2; Fig. 5). After seven days, there was no accumulation in the kidney, but after 14, 21 and 28 days of treatment, clomazone was accumulated, with concentrations of 0.012, 0.014 ppm and 0.0128 respectively. After 7 and 14 days of exposure to 0.25 ppm concentration of clomazone, there was no accumulation in the liver. However, after 21 and 28 days, the liver showed clomazone accumulation; the accumulated concentrations were calculated to be 0.009 and 0.016 ppm, respectively (Table 2; Fig. 5).

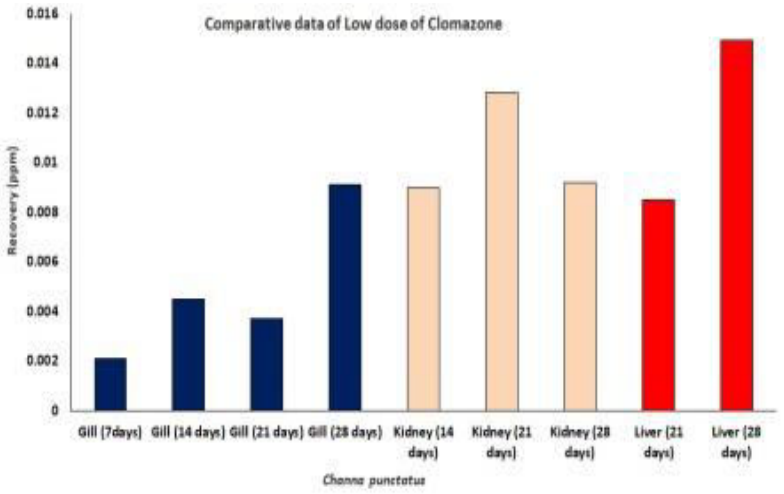

Fig. 6: Concentrations of clomazone accumulated in tissues of *Channa punctatus* after exposing it to low dose.

Treatment with Low Dose (*0.08 ppm*):

In gills, the concentration of clomazone accumulated was calculated to be 0.002, 0.004, 0.003, and 0.009 ppm after 7, 14, 21, and 28 days of exposure, respectively (Table 2; Fig. 6).. After seven days of exposure, no accumulation was obtained in the kidney. However, herbicide clomazone was accumulated in the kidney after 14, 21 and 28 days, and the concentration was calculated to be 0.009, 0.012 and 0.0092 ppm, respectivelt (Table 2; Fig. 6). In the liver, no peaks were obtained in the chromatogram upon exposure for 7 and 14 days. Therefore, no accumulation was calculated in the liver after 7 and 14 days. However, the liver showed accumulation of the herbicide after 21 and 28 days. Therefore, the concentration of accumulated clomazone in the liver was calculated to be 0.008 and 0.014 ppm (Table 2; Fig. 6).

The fish were treated with a high dose, i.e. 0.5 ppm concentration of clomazone, for 28 days. After 28 days, the fish were kept in clean water free from any herbicide for another 28 days to assess the recovery from the accumulated clomazone in the fish tissues. All the gills, liver, and kidney tissues showed 0% accumulation after transferring into the clean water after treatment. Therefore, the fish tissues fully recovered from the clomazone toxicity when kept in clean water for 28 days.

With the aid of HPLC, the quantification was monitored for 28 days at various intervals. HPLC analysis is a method that widely used in identification of different chemicals and substances in biological samples (Zhou *et al*., 2022). Studies show that the concentration of clomazone present in the soil solution is dependent on the amount of carbon and water in the soil (Lee *et al*., 2004). According to the findings of Cumming *et al*. (2002) clomazone is a fairly persistent herbicide that can be identified on an average for up to 30 days. Clomazone was also the most frequently found herbicide in irrigation water in other studies (Quayle, 2003). The characteristic of this herbicide results in the maintenance of high concentration of clomazone in the rice field enhancing the possibility of environmental pollution (Zanella *et al*., 2011). Zanella *et al.* (2000) reported that the mobile phase consisting of methanol and water, and the wavelength (220 nm) used for the detection of clomazone in water samples is valid and adequate. Therefore, the method development for detection of clomazone in fish is valid. The correlation coefficient (R^2) of the standard curve is valid if the value is higher than 0.98 (Oyedeji *et al*., 2021). Thus, as the R^2 for individual analytes was achieved to be >0.998, the proposed method is linear and acceptable. Furthermore, due to the absence of significant interference peaks at the retention times of the clomazone the proposed

method was proven to have selectivity. The LOD and LOQ in this investigation were 0.004 and 0.015 ppm, respectively.

Conclusion

This research aimed to develop a simple method for detecting and quantifying herbicide clomazone in the tissues of fish *Channa punctatus*. The proposed method is applicable in reliably measuring the extent of clomazone in fish tissues and may allow a quick and convenient way to explore the various herbicides and pesticide accumulation in non-target organisms. The study highlighted the bioaccumulation of clomazone. The LOD and LOQ were 0.004 and 0.015, respectively. As the days of exposure increased, the herbicide concentration in the gills also increased significantly. In kidney and liver, accumulation of clomazone was recorded when exposed for longer period of time. In the future a study on oxidative stress can be done for analysing the effect of clomazone in *Channa punctatus*.

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