International Journal of Pharmaceutical Science Invention
ISSN (Online): 2319 – 6718, ISSN (Print): 2319 – 670X
www.ijpsi.org Volume 5 Issue 4 ǁ July 2016 ǁ PP. 47-51

Evaluation of LdhIsozymes Following the Treatment of Methyl Parathion in the Fish, *Labeo Rohita*

*S. N. C. Ray and *[1]R. C. Sinha
**Centre for Environment and Nature Conservation, Department of Zoology, Patna University, Patna-800005, India*
[1]Chief Executive, Centre for Environment and Nature Conservation, Department of Zoology, Patna University, Patna- 800005, India

ABSTRACT
Lactate dehydrogenase converts lactate into pyruvate and has a very important role in carbohydrate metabolism. LDH activity depends on its isozymes and their activities change under pathological conditions. The increase in LDH activity suggests the increased anaerobic conditionsunder the influence of methylparathion to meet the energy demand when the aerobic oxidation is lowered. Following the treatment of methyl parathion there was a differential percentage in increase or decrease of different isozymes in the fish Labeo rohita. There were three distinct bands during the 96h of treatment almost parallel to LDH5 band of human serum suggesting liver damage. This was also confirmed by histopathological studies.
Keywords*: Lactate dehydrogenase, isozymes, methyl parathion, Labeo rohita.*

I. Introduction

Methyl parathion, an organophosphate pesticide, extensively used in agriculture is known to be responsible for a number of physiological and biochemical disturbances to the non-target organisms. It is known to be a neurotoxic and hepatotoxic pesticide. It has been reported that some of the serum enzymes are useful in diagnosing liver diseases [1, 2]. Cellular enzymes in the extracellular space, although of no further metabolic function in this space are still of benefit because they serve as indicators suggestive of disturbances of cellular integrity induced by foreign interferences. Lactatedehydrogenase (LDH) is a cytoplasmic enzyme present in essentially all major organ systems. The extracellular appearance of LDH is used to detect cell damage or cell death [3]. Due to its extraordinarily interspersed distribution in the body, serum LDH is abnormal in many disorders [4]. It is released into peripheral space after cell rupture or cell death caused by e.g. excess heat or cold, starvation, exposure to pesticides or toxins, therefore the total serum LDH is highly sensitive but non-specific test.

In order to optimize the diagnostic value, LDH isozymes can be measured [5]. Lactate dehydrogenase (EC 3.1.1.27) is one of the chief enzymes of carbohydrate metabolism which catalyzes the oxidation of lactate and reduction of pyruvate anaerobic glycolysis. It is a dimeric molecule consisting of two separate loci which code for A and B sub-units of this enzyme. The A and B subunits indiscriminately associate and form five tetrameric isozymes (A4, A3B1, A2B2, A3, B1 and B4) [6]. Isozymes are multiple forms of single enzyme which have different isoelectric points and therefore be separated by electrophoresis. Electrophoretic separation of LDH were done extensively on different tissues of various animals from which it revealed that the enzyme exhibited multimolecular forms and functions [7].

Classically, five LDH isozymes are present in the serum, of which the electrophoretically slowest moving band (LDH5) corresponding to the liver isozymes. The appearance of LDH5 isozyme in the serum indicates hepatocellular damage. Since the LDH isozymes patterns are different in various organs and tissues, its serum analysis may be useful in establishing the cell injury.

LDH in fish has always been the subject of much attention. But not much information is available with the changes in the expression of LDH on the toxicity of methyl parathion. The aim of the present paper is to evaluate the expression of LDH isozymes in the serum of fish, *Labeo rohita* after exposure to sub- lethal concentration of methyl parathion for 96h using a fully automatic clinicalelectrophoresis and densitometer.

II. Materials And Methods

Ethical Statement: Presently, we do not have any Ethical Committee in our University. But however, we have followed the ethical norms, which are being followed elsewhere which is evident in the Materials & Methods Section.

Maintenance of animals:*Labeo rohita*, a common carp was obtained from the local hatchery. Fishes were acclimated to laboratory conditions for about 5-7 days. They were kept in aquarium tank (250 L) and water was

constantly aerated by a static system. During the acclimation period, they were given artificial (commercial) feed composed of ground shrimps available in the local market to avoid the possible effects of starvation. The feeding and maintenance of the fishes and physico-chemical characteristics of the aquaria water were measured (Tab. 1). Short-term test of acute toxicity over a period of 96h were performed on the fishes following the renewal of bioassay. The fishes were exposed intra-coelomatically with $1/3^{rd}$ of LC_{50} value of the pesticide methyl parathion. After 24, 48, 72 and 96 h of exposure fishes were processed for further investigations.

Determination of LC_{50}: The experiments were repeated several times and only arithmetic mean of the experiments at each concentration was taken to express the results. LC_{50} values were determined by EPA Probit analysis program [8]. The LC_{50} of methyl parathion for the fish *Labeo rohita* was 16.8 ppm.

Blood collection:The fishes were taken out of the aquarium water individually through fish net with a minimum possible disturbance. After preliminary investigations, the blood samples were collected from caudal fin as described by many authors. In the present study, the blood collection from caudal fin had to be abandoned because there was an unusual elevation in Lactate dehydrogenase (LDH) and Creatine phosphokinase (CPK) activities which were recorded due to leakage from the surrounding muscle tissues. Thus, cardiac sampling was the only suitable method available as an alternative to obtain blood under the present study. After the blood collection, the serum were separated and processed for enzymatic assays.

Histological procedures: Sample of fish from both control and experimental groups were excised, rinsed in physiological saline fixed in Bouin's fluid and thereafter processed for microtomy. The tissues were dehydrated in ethyl alcohol with series of ascending concentrations, embedded in paraffin and sectioned, which were subsequently stained with haemotoxylin and eosin. The slides were examined under low power (4X) objective and high power (10X) objective with binocular microscope. Photomicrographs were taken at high power (100X) zoom lens digital camera with software DigiPro 5.0 attached in the microscopeLabomed, model- LX400 and downloaded into a computer.

*Separation of LDH isozymes by Fully Automatic Clinical Electrophoretic Unit:*Fully automatic clinical electrophoretic unit is a system software controlled electrophoretic separation having a voltage, current and run time control, operated by an inbuilt programme present in the driver software of the electrophoresis. Fully Automatic Clinical Electriphoresis Model- Interlab- Pretty was used for the present study having software namely 'Interlab'. The system constituted of an automatic dispensing system for samples, a Teflon coated Peltier Plate for placing previously casted agarose gel over sterile plastic plate having bar- code over it and valve- compressor system for pouring appropriate amount of staining, destaining and washing solutions over the gel plate after completion of the migration. About 30µl of previously separated non-hemolyzed serum were placed in the sample space and loaded over the gel plate by the automatic dispensing system to achieve single straight line of application. The temperature for migration was set at 29^0C, time for migration was 5 minutes, voltage for migration was 400V and after migration secondary treatment of the gel was needed which was done in another chamber with an incubation period of 30 minutes at 45^0C after addition of staining solution. The staining solution contained pyruvate as substrate and NitroblueTetrazolium (NBT), NBT was reduced to formazan by NADH, also present in the staining solution. After the treatment with the staining solution, which stained the LDH isozyme bands, fixation of the band was achieved in the above mentioned external chamber. After staining and fixing the bands, the gel plate was re-placed into the electrophoretic chamber for repeated destaining with destaining solution comprising of acetic acid of strength 5% (v/v). Then washing was done with a surfactant solution for a number of times previously fed in the driver software. After completion of these steps, scanning of the gel plate was done in the densitometer with adriver software 'Scanlab' provided by the manufacturer.

III. Results And Discussion

Table-I. Physico-chemical characteristics of aquaria water

Sl no.	Parameter	Value
1.	Temperature	$(24\pm2)^0$C
2.	pH	7.1± 0.2 at 24^0C
3.	Dissolved Oxygen	8.5 ±0.5 mg/L
4.	Total Hardness	23.4± 3.4mg $CaCO_3$/L
5.	Conductivity	<10 µs/cm

Table-II. Name of LDH isozyme bands with their percentages in the electrophorogram from cathode to anode

Name of the LDH isozyme band (from Cathode to Anode)	Control	24h	48h	72h	96h
A	55.6%	65.6%	52.1%	45.7%	41.8%
A'	---	---	---	---	---
B	31.3%	34.4%	47.9%	54.3%	42.2%
B'	13.1%	---	---	---	16.0%

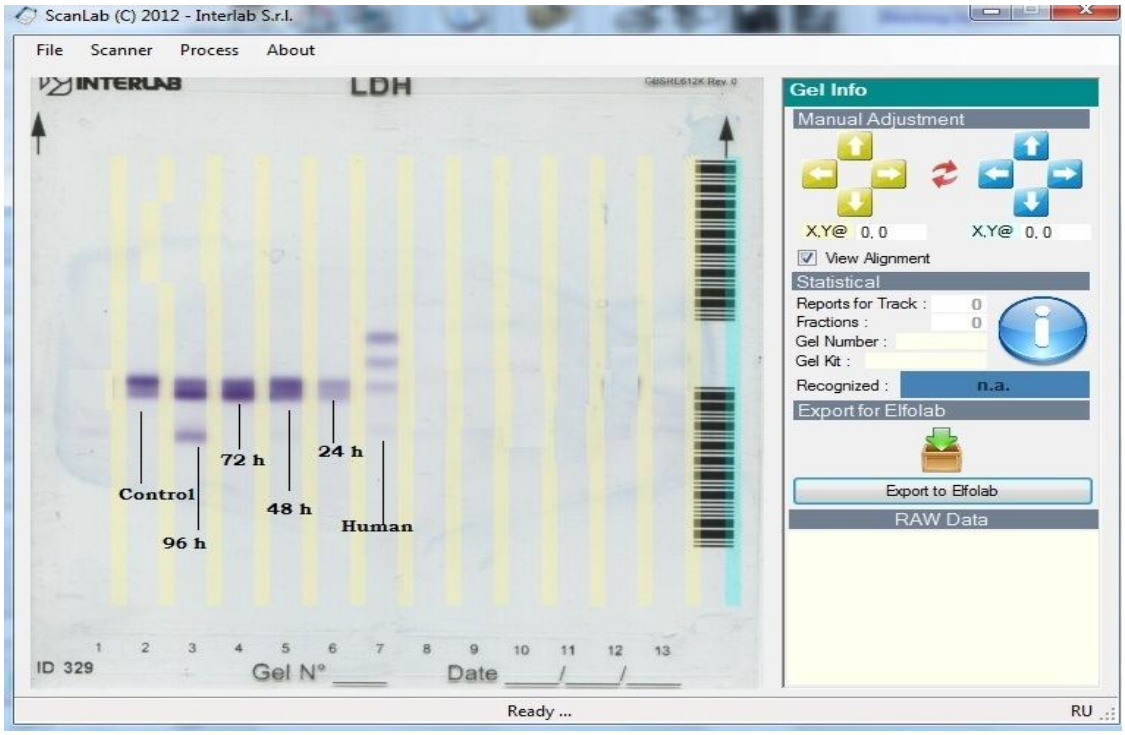

Figure 1. Agarose Gel Plate with positioning of the LDH isozyme bands of human and fish*Labeo rohita* (Control and treated 24h, 48h, 72h and 96h)

List of Abbreviations: HC= Hepatic Cord; CV= Central Vein; S= Sinusoid; BP= Bile Plug; SP= Sinusoidal Space; FN= Fragmented Nucleus

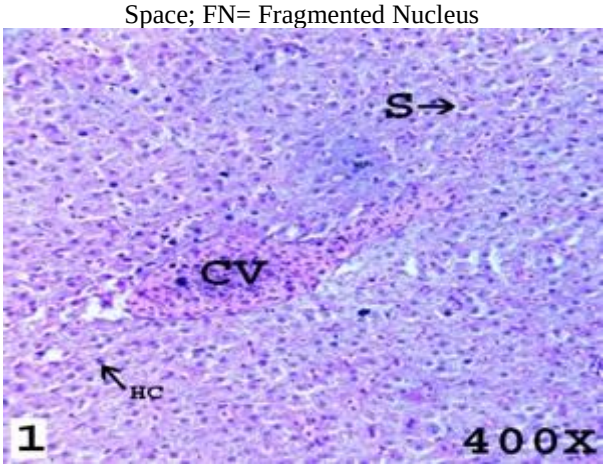

Plate 1: Control liver of *L. Rohita*

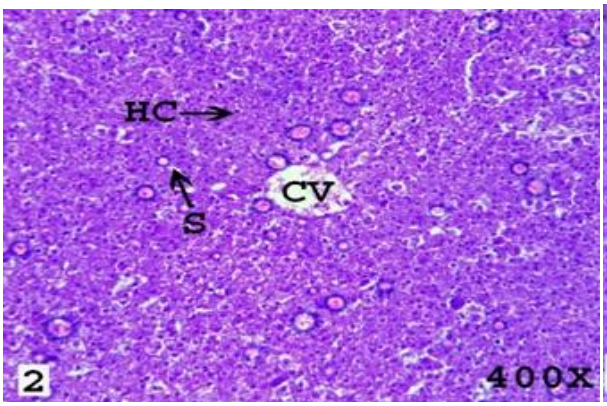

Plate 2: 24h Methyl parathion treated liver of *L. Rohita*

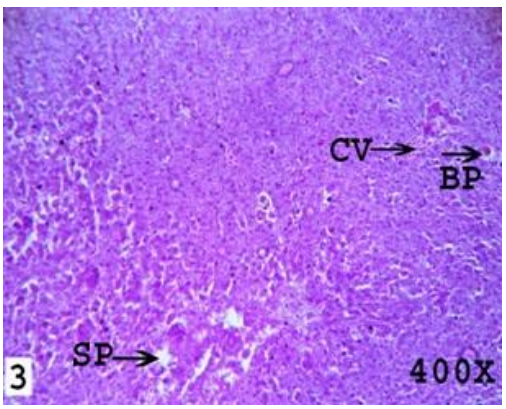

Plate 3: 48h Methyl parathion treated liver of *L. Rohita*

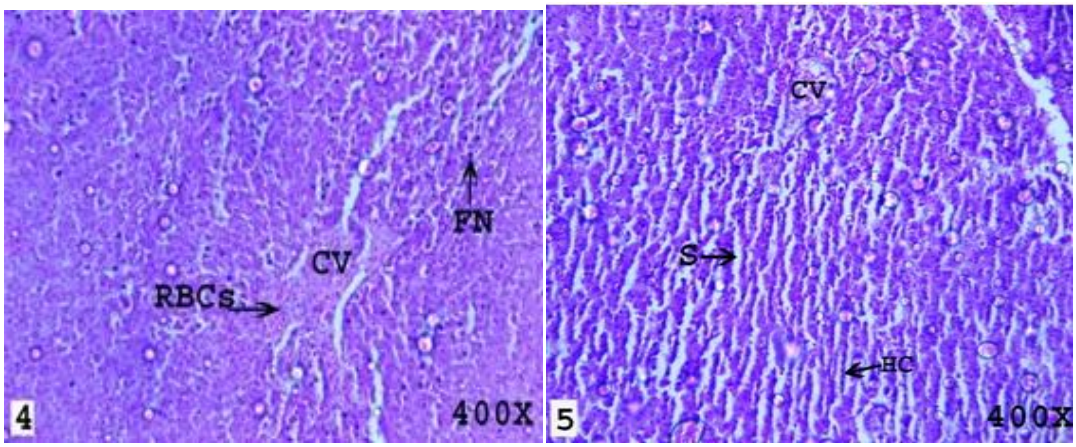

Plate 4: 72h Methyl parathion treated liver of *L. Rohita*

Plate 5: 96h Methyl parathion treated liver of *L. Rohita*

Figure 2: Plate numbers showing the Liver histopathology of the fish *Labeo rohita*

It is well known that LDH is present in the cells of almost every body tissue and in the case of damage it is released into the blood [9, 10]. Analyses have revealed LDH to be suitable biomarker since the results obtained after expositions of animals to different toxicants were found to be significant. It has already been mentioned earlier that LDH is a highly sensitive but not specific biomarker. Earlier, the authors have reported that LDH in the serum of the fish *Cirrhinus mrigala* increased significantly following the treatment of sub-lethal dose of methyl parathion [11]. Thus**,** in the present investigation, supplementary information is being reported by electrophoretic separation and quantification of LDH isozymes in the fish *Labeo rohita* as a function of methyl parathion. The LDH isozymeelectrophorogram have shown that there are four bands from cathode to anode. The bands of electrophorogram of LDH isozymes are designated as LDH A, LDH A', LDH B and LDH B'. In the control fish, LDH A is 55.6%, LDH A' is absent whereas LDH B is 31.3% and LDH B' is 13.1% (Table-2). But following the treatment of methyl parathion LDH A increased to 65.6% during 24h and thereafter decreased to 52.1%, 45.7% and 41.8% during 48h, 72h and 96h respectively. Whereas, LDH B increased to 34.4% in 24h and further increased to 47.9% and 54.3% in 48h and 72h respectively but decreased to 42.2% during 96h. LDH B' was absent during 24, 48 and 72h but present during 96h.

The differential percentage of increase or decrease of different isozymes in the treated fish during different periods may be due to the differential mode of action of methyl parathion in different tissues. Secondly, gene duplication as well as post- translational processing has been proposed as important factors in modulating tissue specific enzymes [12]. LDH A' is missing in the control fish as well as in the treated fish. Similarly, B' is absent following the treatment of methyl parathion during 24h, 48h, 72h but present in 96h (Table-2). The increase/ decrease/ absence of some of the isozymes could be interpreted as a result of certain mutational events that would have occurred in the regulatory genes which could leadto inhibition, alteration or constitutive gene expression. This is the reason some bands of isozyme became more intense or faint (Fig. 1). The differences of the distribution of the different LDH isozymesin the serum reflect differences in their metabolic activity.

It is interesting to observe that three distinct bands appear in the 96h (Fig. 1, Lane- 3) the third cathodic band, the slowest moving band which is almost parallel to LDH5 of human suggesting liver damage. Histological examination of the tissues could serve as complementary evidence [13] to enzyme studies towards revealing any distortion/damage to the normal structure of the tissues [14, 15, 16]. In the present study we have observed the histological changes in the liver of the fish, *Labeo rohita* as a function of methyl parathion.The histological changes in the animal tissues provide a rapid, cost effective method to detect effect of toxicants.

The liver of the control fish comprise of a continuous mass of large hexagonal hepatic cells containing more or less spherical nucleus. They are located among sinusoids and forming cord like structures known as hepatic cell cord. In fish these structures are generally obscure. Bile canaliculus is centrally located in each cord. There is no clear cut division of hepatic cells with lobules. These cells contain granular cytoplasm with distinct nuclei either exocentric or slightly centrally placed. Hepatic cells have many vital functions other than secretion of bile. They play an important role in protein, lipid and carbohydrate metabolism. Detoxification is another important function (Plate 1).

After the treatment of $1/3^{rd}$ of LC_{50} of methyl parathion, following changes were observed in 24h, 48h, 72h and 96h.

In 24h, hepatic cords were disorganized; bile pigment appeared; some hepatic cell nucleus divided; central vein became clear; interstitial space was filled with hazy liquid, very few RBCs were in between the hepatic cells; hepatic cells became dense in appearance (Plate- 2).

In 48h, the spaces between the hepatic cords were comparatively more; hepatic cord became disorganized; central vein became disorganized with more RBCs (Plate-3).
In 72h, central vein ruptured; lack of rounded structure of hepatic nuclei; hepatic cell membrane was found to be splitting leading to necrotic changes; formation of sinusoidal space was more nuclear disintegration was very clear and found scattered in the sinusoid space; appearance of bile pigment(Plate-4).
In 96h, sinusoidal space was more while the hepatic cells joined each other; nucleus was rounded; central vein had less RBCs (Plate-5).
The fish liver histology is an indicator of chemical toxicity and very useful method to study the effects of exposure of aquatic animals to toxicants present in the aquatic system [17]. The liver is the primary organ for detoxification of the xenobiotics. As mentioned above, the histopathological findings highly corroborates with the biochemical results i.e. during 72h to 96h the hepatic cell membrane ruptures with the release of isozymes especially LDH5 (Fig.- 1, lane-3) clearly suggesting that the LDH5 isozyme is the indicative of liver damage. Thus, it is concluded that histopathological changes coupled with biochemical changes could serve as valuable biomarkers for methyl parathion exposed fish.

Acknowledgement

The authors are grateful to DBT, Govt. of India for financial assistance for the project (Sanction Letter no. BT/PR/4762/BCE/8/903/2012; Dated: 11/3/2013). The authors also thank the Head of the Department of Zoology for extending all logistic support.

References

[1]. Reichling, J J and Kalpan, M M (1988). Clinical use of serum enzymes in liver diseases, *Dig. Dis. Sci.*, 33: 1601-1614.
[2]. Schmidt, E and Schmidt, F W (1990). Progress in the enzyme diagnosis in the liver diseases: Reality or illusion? *Clin. Biochem*, 23: 375-382.
[3]. Mess, D W and Henderson, A R (1986). Enzymes, In: Burtis, C A and Ashwood E R, eds. Textbook of clinical chemistry. Philadelphia, Saunders Co. Pp. 735-896.
[4]. Bansal, S K and Kaw, J L (1981). Lactate dehydrogenase isoenzymes in macrophages and serum during the development of pulmonary silicosis in the rat, *Toxicol. Letters*, 7: 279-283.
[5]. Glick, J H (1969). Serum lactate dehydrogenase isoenzyme and total lactate dehydrogenase values in health and disease and clinical evaluation of these tests by means of discriminant analysis. *Am. J. Clin. Pathol.*, 52: 320-328.
[6]. Fujio, Y and Kaneko, S (1980). Differentiation of lactate dehydrogenase isoenzymes in fish, *Thpoku J. Agri. Res.*, 31: 61-73.
[7]. Markert, C and Mollar, F (1959). Multiple forms of enzymes in tissues, ontogenic and species specific pattern, *Proc. Nat. Acad. Sci.*, 45: 753-763.
[8]. Finney D J (1971) Probitanalyss 3rd Edition Cambridge University Press London, pp. 330.
[9]. Ribeiro, S, Guilhermino, L., Sousa, J P and Soares, A M V M (1999).Novel bioassay based on acetylcholinesterase and lactate dehydrogenase activities to evaluate the toxicity of chemicals to soil isopods, *Ecootoxicol. Environ. Saf.*, 44: 287-293.
[10]. Wu, R S. S. and Lam, P K S (1997). Glucose-6-phosphate dehydrogenase and lactate dehydrogenase in the green lipped mussel (*Pernnaviridis*). Possible biomarker for hypoxia in the marine environment, *Water Resear.*, 31: 2797-2801.
[11]. Ray, S.N.C. and Sinha, R.C. (2014). Effects of methyl parathion on the carbohydrate metabolism of the fish, *Cirrhinus mrigala*. *J. Material Sci. &Engg. B*. (Davidson Publication, USA) 12: 366-371.
[12]. Laud, P R and Campbell, J W (1994). Genetic basis for tissue isozyme of glutamine synthetase in elasmobranches, *J. Molecul. Evol.*, 39: 93-106.
[13]. Shelby, M D, Newhold, R R, Tully, D B, Chae, K and Davis, V I (1996). Assessing environmental chemicals for estrogenecity using a combination of *in- vitro* and *in- vivo* assays, *Environ. Health Perspect.*, 104: 1296-1304.
[14]. Singh, NN and Srivastava, A K (1992). Biochemical changes in the freshwater Indian catfish *Hetropneustesfessilis* following exposure of sublethal concentration of aldrin, *J. Environ. Biol.*, 22: 21-43.
[15]. Singh, N N and Singh V V (2007). Endosulfan induced changes in the fresh water female catfish, *Hetropneustesfessili*s, *J. Environ. Biol.*, 28: 605-616.
[16]. Tripathi, G and Verma, P (2004). Endosulfan mediated biochemical changes in the fresh water fish, *Clariusbetrachus*, *Biomed. Environ. Sci.*, 17: 47-50.
[17]. Fernandez- Vega C, Sancho E, Ferrando MD and Andrew E. Thiobencarb induced changes in acetylcholinesterase activity of the fish *Anguilaanguila*. PesticiBiochemPhysiol 2002, 72: 55-63.