Recent Research in Science and Technology 2013, 5(4): 15-19
ISSN: 2076-5061
Available Online: http://recent-science.com/

Development of an enzymatic method for quantification of dichlorvos from environmental samples

Uma Maheswara Rao Ganguru[1] and Yalavarthy Prameela Devi[2]

[1]Center for Biotechnology, Institute of Science and Technology, Jawaharlal Nehru Technological University, Hyderabad 500085, India.
[2]Environmental Biology Laboratory, Department of Zoology, Kakatiya University, Warangal 506009, India.

Abstract
A simple, low cost and sensitive novel enzymatic tablet method was developed for the quantification of an organophosphorus pesticide, dichlorvos (2,2-dimethyl dichlorovinyl phosphate) at microgram level. The method involves in quantification of dichlorvos is based on inhibition of the enzyme, Succinate Dehydrogenase (SDH) (EC.No. 1.3.5.1). The substrate, sodium succine specifically binds to the enzyme (SDH) and develops color in the presence of chromogenic reagent. This chromogenic reagent contains INT (2-(4- Ido-phenyl)-3-(4-nitrophenyl)-5 phenyl tetrazolium chloride) and PMS (N-methyl phenazonium methosulphate). Lyophilized egg albumin powder was used as a source of SDH enzyme. Two tablets, one containing the enzyme and the other containing a mixture of substrate and chromogenic agent were prepared. Optimization of the tablet method was done by standardizing the conditions for colorimetric analysis: optimum pH (7), optimum temperature (80ºC), enzyme concentration (lyophilized egg albumin powder) (10mg/tablet) and optimum reaction incubation time (30 min). Percentage of enzyme inhibition and concentration of dichlorvos at a range of 0-65 µg was plotted, which was observed to follow the Beer Lambert's Law. A color chart was prepared for quantification of dichlorvos based on the formation of formazan in the reaction. If the concentration of environmental samples is within the given range, the samples can be quantified by comparing with the color chart. The method is successfully applied for quantification of dichlorvos from environmental samples.

Keywords: Dichlorvos, SDH, quantification, environment.

INTRODUCTION

Dichlorvos, known as DDVP, is an organophosphate insecticide, commercially manufactured since 1961 and used in different parts of the world as a contact and stomach poison against insects (Gallo and Lawryk, 1999). This insecticide is generally used in control of agricultural insects, internal and external parasites of farm animals and also insects in houses, buildings and aircraft environment (Sekizawa, 1989). It is also used for controlling planktonic invertebrates and parasites during culture of aquatic species (Brandal and Egidius, 1979). The poisonous effect of dichlorvos is shown directly by inhibiting the enzyme, acetyl cholinesterase (Sekizawa, 1989). It is soluble in fat and hence can easily penetrate the biological membranes (Hofer, 1981). The symptoms of dichlorvos exposure in humans are: increase in salivary secretion, sweating, wetness of eyes, muscle contractions, heart rhythm change, headache, weakness, and finally coma (ATSDR, 1997).

The main methods for detection and determination of dichlorvos are thin layer chromatography (TLC), gas chromatography (GC) and esterase based enzymatic methods (Sofia and Nikos, 2005; Sofia et al. 2005; Zheng et al. 2006; Songhui et al. 2012a). It is commonly analyzed by GC, because they have low ultraviolet absorbance (El-Refai and Giuffrida, 1965). Songhui et al. (2012b) recently introduced a HPLC based method for analysis of dichlorvos from environmental samples. But monitoring the environmental samples by these methods are cumbersome, time consuming and expensive. Hence, in the present study, we introduced an alternate method for quantification of dichlorvos at microgram levels.

Received: Feb 10, 2013; Revised: March 18, 2013; Accepted: April 25, 2013.

*Corresponding Author
Uma Maheswara Rao Ganguru
Center for Biotechnology, Institute of Science and Technology,
Jawaharlal Nehru Technological University, Hyderabad 500085, India.

Email: maheswaraos@gmail.com

MATERIALS AND METHODS

Materials

Dichlorvos (2,2-dimethyl dichlorovinyl phosphate) (98.7% pure) technical grade sample was procured from Hyderabad chemicals, Balanagar, Hyderabad. The standard solution was prepared as stock in distilled water at 10mg/ml (w/v) concentration. Lyophilized emulsion of egg albumin was employed as an enzyme source. Sodium succinate, INT (2-(4- Ido-phenyl)-3-(4-nitrophenyl)-5 phenyl tetrazolium chloride), PMS (N-methyl phenazonium methosulphate), lactose (β-D-galactopyranosyl-(1→4)-D-glucose), water insoluble starch, magnesium stearate (Magnesium octadecanoate) and SDS (Sodium dodecyl sulfate) were supplied by HIMEDIA. Solvents - chloroform, ethyl acetate, propanone (acetone) and hexane required for quantification and identification of dichlorvos were procured from MERCK chemicals.

Enzyme

Egg albumin was extracted from fresh eggs and 20% (v/v)

emulsion was prepared in double distilled water. This emulsion was lyophilized and the powder was used as a source of Succinate dehydrogenase (SDH) (EC.No. 1.3.5.1) enzyme.

Quantification of dichlorvos
Preparation of tablets

Two tablets (tablet-A and tablet-B) were prepared for quantification of dichlorvos. Tablet A was prepared with lyophilized egg albumin powder, the composition of which is given in table 1. Tablet B was prepared with sodium succinate, INT and PMS mixture, which acts as a substrate (sodium succinate) as well as chromogenic reagent (INT and PMS) (table 2). The above compositions for tablet A and B were separately homogenized and prepared tablets by direct compression method.

Table 1. Composition of Tablet A

Component	Concentration (mg/ tablet)
Enzyme powder	10
Lactose	335
Starch (in soluble)	50
Magnesium stearate	5
Total:	**400 mg**

Table 2. Composition of Tablet B

Component	Concentration (mg/ tablet)
INT	8
PMS	3
Sodium succinate	4
Lactose	330
Starch (in soluble)	50
Magnesium stearate	5
Total:	**400 mg**

Optimization of tablet method

The tablet method was optimized by the colorimetric method. The tablets, A and B were dissolved separately in 20ml and 10ml of distilled water respectively. One ml of the reaction mixture (pre dissolved tablet A - 0.4ml, tablet B - 0.2ml and distilled water - 0.4ml) was incubated at various conditions to optimize the assay i.e. pH (4-9), temperature 10-100˚C, time 0-60 minutes and enzyme concentration 0-20mg/per tablet. The enzyme reaction was stopped by the addition of 2ml of 1% SDS and the optical density (OD) was measured at 495nm using a colorimeter. A graph was made for each parameter based on the amount of formazan (pink color) formed.

Table 3. Composition of reaction mixture

Tablet - A (Enzyme)	0.4 ml
Dichlorvos standard	0.2 ml
Incubation for 10 minutes at 37°C	
Tablet – B (Substrate)	0.2 ml
Distilled water	0.2 ml
Total:	**1 ml**

Preparation of standard graph and color chart for quantification of dichlorvos

Dichlorvos standard solutions were prepared in various concentrations to assess the inhibition of SDH activity. As shown in table 3, reaction mixture was prepared and incubated at 80ºC for about 30 min. The reaction was terminated by using 1% SDS. The OD was measured at 495nm with the help of a colorimeter. The control was carried out with 0.2ml of distilled water instead of dichlorvos standard solution. The amount of formazan produced was calculated from formazan standard graph. A standard graph was made with various concentrations of dichlorvos and percent SDH inhibition. A standard color chart was also prepared based on visual color appearance.

Quantification of dichlorvos from environmental samples

The present work was undertaken to evaluate the applicability of the developed enzymatic method for quantification of dichlorvos from environmental samples. Environmental samples were collected from the industrial and agricultural areas of Balanagar and Jeedimetla, Hyderabad. All the samples were collected in fresh plastic bags, which were analyzed within 4 hours of collection.
The water samples were filtered using whatman no. 40 filter paper. The dichlorvos present in the samples (1ltr) was extracted into hexane and further concentrated using rota vapour at 37°C. After complete removal of hexane, it was dissolved in 1 ml of distilled water. Soil samples (50gm) were ground and extracted with 100ml of water. This water was separated from soil by filtration and concentrated by the above procedure. After hexane evaporation, the residue was dissolved in 1 ml of distilled water for evaluating the content of dichlorvos. If necessary, extraction and concentration procedures were repeated. The samples were analyzed and compared by the developed enzymatic method as well as by the GC-MS (EPA method 622).

Calculation for percent SDH inhibition (I%)

Percent SDH inhibition was calculated by the below formula which is based on optical density at 495nm.

$$\text{Percent inhibition} = \frac{C - E}{C} \times 100$$

C = Optical Density of control
E = Optical Density of sample

RESULTS AND DISCUSSION

The principle involved here is the biochemical reaction between dichlorvos and SDH enzyme. SDH, a member of citric acid cycle, catalyses the oxidation of succinate to fumarate (Michele et al. 2004). SDH activity can be determined by the reduction of tetrazolium salts to deeply colored formazan in the presence of substrate, sodium succinate (Defendi and Pearson, 1995; Glick and Nayyar, 1956; Kun and Ahood, 1949). In the presence of dichlorvos, this enzyme reaction is inhibited. This kind of inhibitory nature was made use for determination of dichlorvos. Same principle was already employed in detection, separation and identification of dichlorvos (Uma and Prameela, 2011).

Optimum assay conditions

As shown fig. 1, the optimum assay conditions are pH 7, temperature 80°C, incubation time 30 min and enzyme concentrations 10mg/tablet. Generally, the concentration of substrate influences enzyme inhibition. Enzyme inhibition increases with increase in substrate concentration (Kok et al., 2002). In case of

SDH assay, it was reported that sodium succinate is not the rate limiting factor (Chandra Mouli, 1999). Hence, in the present study, a stable concentration of the substrate was used. Whereas, the maximum enzyme activity was observed at a concentration of 10mg/tablet. After that, enhanced concentration of enzyme did not result in significant increase of activity. In the present study tablet A was prepared with 10mg of lyophilized egg albumin powder and it is sufficient for analyzing 50 samples. The enzyme sensitivity was found to increase in the presence of inhibitor at lower concentrations of enzyme (Sofia and Nikos, 2005; Sofia et al. 2005; Shan et al. 2004; Mohammadi et al. 2005).

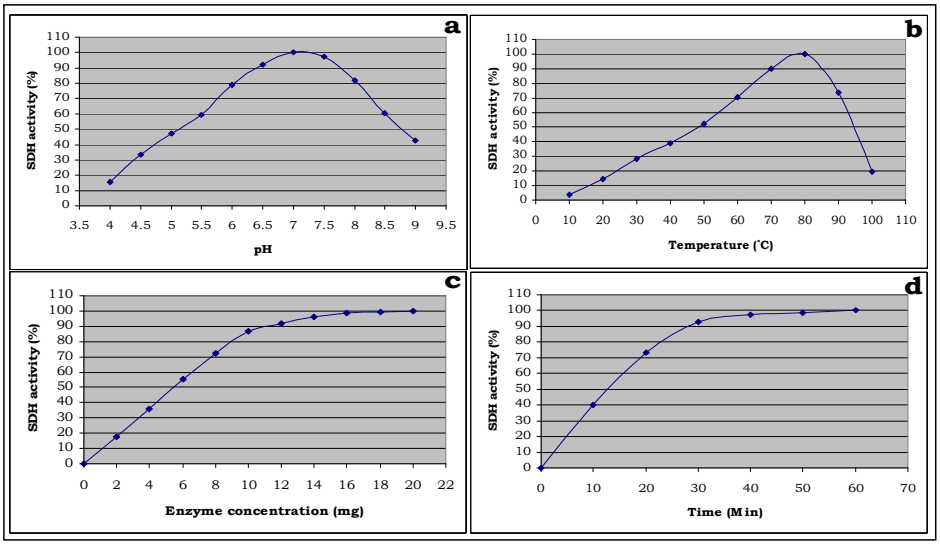

Fig 1. Standardization profile for the tablet method of egg albumin SDH. Standardization of (a) pH; (b) temperature; (c) enzyme concentration per tablet and (d) maximum reaction time.

Detection and determination of inhibitors such as pesticides and heavy metals are generally performed in aqueous solutions. Some of the compounds exhibit low solubility in water and may be highly soluble in organic solvents. Some enzymes are strongly inhibited when experiments are conducted in organic solvents based on their nature and concentration (Amine et al. 2005). The pesticides, such as; dichlorvos, diazinon and fenthion detection was reported in the presence of ethanol using an immobilized acetyl cholinesterase enzyme based screen-printed biosensor (Andreescu et al. 2002). Dichlorvos is water soluble, so the present experiment was conducted in aqueous solution.

Standard graph and color chart

The developed method was successfully applied for the quantification of dichlorvos. The standard graph was prepared to analyze the samples in the range of 0-65 µg based on the % SDH inhibition. As shown in Fig. 2, the percent enzyme inhibition has shown an increasing trend with increased concentration of dichlorvos. The Limit of detection (LOD) is based on the concentration of inhibitor, where the interval does not overlap that of zero concentration of the inhibitor standard (Aziz et al. 2006). However, the detection limit also depends on the incubation time of the enzyme with inhibitor (Kuswandi, 2003). Ciucu. et al. conducted an experiment with incubation time of 30 min for the detection of paraoxan with 10mg detection limit (Ciucu et al. 2003). The residual enzymatic activity after incubation with inhibitor was studied using different incubation times (5, 15, 30 min) in the presence of AChE and ChO bienzymatic system (Kok et al. 2002). Usually, the materials used as support matrices may also inhibit the enzyme activity. But here, the support materials used for the preparation of tablet A and B were lactose, starch and magnesium stearate which have been proved to have no effect on the enzyme SDH (Chandra Mouli, 1999).

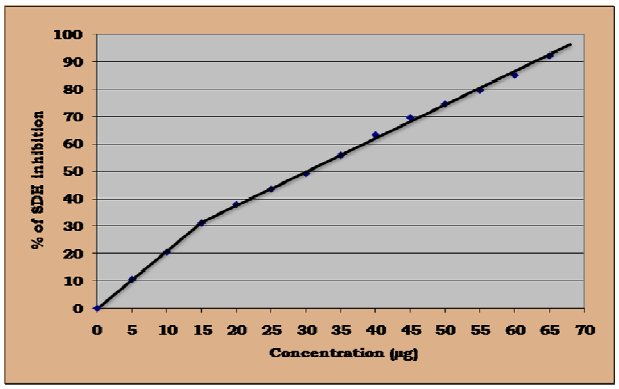

Fig 2. Calibration curve showing SDH inhibition and concentration of dichlorvos.

A color chart was developed (fig. 3), which shows the gradation of color with increased inhibition at various concentrations of dichlorvos. This chart helps to quantify the samples by color comparison. This is useful, if the concentration of dichlorvos in the sample is between 0-65µg. It is based on the gradation of pink color formazan due to the reaction with various concentrations of dichlorvos, i.e. 0-65µg. The pink color obtained by formazan is inversely proportional to the concentration of dichlorvos (fig. 3). In case of higher dichlorvos levels i.e., beyond the quantification range, samples can be diluted accordingly for bringing them to the within the range for further analysis. BOROSIL reaction test tube (12x75mm) was employed to prepare the color chart. This information has to be noted, because the transmission of color intensity varies with size and types of the glass tube and hence advised to use the same.

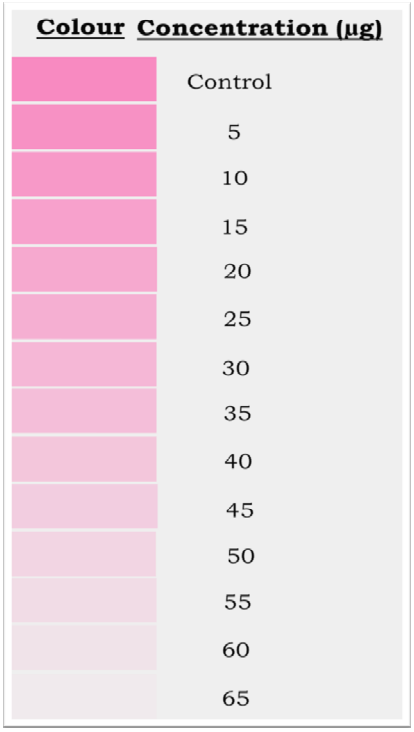

Fig 3. Standard color chart for quantification of dichlorvos by tablet method.

Analysis of dichlorvos from environmental samples

The developed tablet method was successfully employed for testing amount of dichlorvos in environmental samples. The extracted environmental samples were analyzed by the developed enzymatic method and also by standard instrumental method GC-MS to know the accuracy of the developed tablet method in quantification of dichlorvos. Dichlorvos concentration was found to be well outside the quantification limit in all water samples, by both the methods. The concentration of dichlorvos in Jeedimetla pesticide industrial area soil sample was found to 0.022 ppm by GC-MS and 0.02ppm by developed enzymatic method. However, there was no dichlorvos found in all other soil samples by both the methods (table 4).

Table 4. Monitoring of environmental samples for presence of dichlorvos.

S.No.	Sample	Quantification by tablet method (ppm)	Quantification by GC/MS (ppm)
Water Samples			
1.	Agricultural waste water	- ve	- ve
2.	Balanagar pesticide industrial waste water	- ve	- ve
3.	Jeedimetla pesticide industrial waste water	- ve	- ve
Soil Samples			
1.	Agricultural land soil	- ve	- ve
2.	Balanagar pesticide industrial area soil	- ve	- ve
3.	Jeedimetla pesticide industrial area soil	0.02 ± 0.01	0.022 ± 0.003

± S.D of mean of 3 observations.

The method that is developed in the present study is quite useful as it can also be used in the field. As, the SDH enzyme is capable of inhibiting a number of pollutants, it is advised to know the Rf value of dichlorvos for confirmation by TLC or Paper Chromatography, which has been developed earlier by the same authors (Uma and Prameela, 2008).

CONCLUSION

The tablet method developed in the present study is simple, sensitive and useful for timely monitoring of dichlorvos from environmental samples where the other methods are time consuming, expensive and unsuitable for field use.

ACKNOWLEDGEMENTS

The authors acknowledge UGC, INDIA for the financial support offered to this project and selecting one of the authors, Prof. Yalavarthy Prameela Devi for UGC Research Award 2006. The authors thank JNTUH authorities for providing laboratory facilities in undertaking this research work.

REFERENCES

[1] Gallo M.A. and Lawryk N.J. 1999. Handbook of pesticide toxicology, Academic. Press. New York.

[2] Sekizawa J. 1989. Environmental health criteria 79 for dichlorvos, International programme on chemical safety.

[3] Brandal P.O. and Egidius E. 1979. Treatment of salmon lice (lepeophtheirus salmonis kroyer, 1838) with neguvon sdescription of method and equipment. *J. Aquaculture.* 18:183-188.

[4] Hofer W. 1981. Chemistry of metrifonate and dichlorvos. *J. Acta. Pharmacol. Toxicol.* 49:7-14.

[5] ATSDR. 1997. Toxicological profile for dichlorvos, U.S. department of health and human services. Atlanta. GA.

[6] Sofia S. and Nikos A.C. 2005. Lowering the detection limit of the acetyl cholinesterase biosensor using a nanoporous carbon matrix. *J. Analytica. Chemical. Acta.* 530:199-204.

[7] Sofia S., Dider F. and Nikos A.C. 2005. Genetically engineered acetyl cholinesterase based biosensors for attomolar detection of DDVP. Biosens. *Bioelectron.* 20:2347-2352.

[8] Zheng Y.H., Hua T.C., Sun D., Xiao J.F. and Wang F. 2006. Detection of DDVP residue by flow injection calorimetric biosensor based on immobilized chicken liver esterase. *J. food eng.* 74:24-29.

[9] Songhui W., Bingren X., Yilong S. and Qianqian T. 2012a. Direct determination of dichlorvos in water by partial least square-discriminant analysis. Environ. Chem. Let. DOI: 10.1007/s10311-012-0363-5(available online).

[10] El-Refai A.R. and Giuffrida L. 1965. Separation and micro quantitative determination of dipterex and DDVP by gas-liquid chromatography. *J. Assoc. Off. Anal. Chem.* 48:374-379.

[11] Songhui W. Bingren X and Qianqian T. 2012b. Trace determination of dichlorvos in environmental samples by room temperature ionic liquid-based dispersive liquid-phase microextraction combined with HPLC. J. Chroma. Sci. DOI: 10.1093/chromsci/bms058 (available online).

[12] Michele S., Paola C.B., Ilaria D.M., Tiziana L. and Claudio F. 2004. The deletion of the succinate dehydrogenase gene KlSDH1 in Kluyveromyces lactis does not lead to respiratory deficiency. J. Eukaryotic. Cell. 3(3):589-597.

[13] Defendi V. and Pearson B. 1995. Quantitative estimation of succinic dehydrogenase activity in a single microscopic tissue section. *J. Histochem. Cytochem.* 3:61-69.

[14] Glick D. and Nayyar S.N. 1956. Studies in histochemistry, XLII. Further studies on the determination of succinic dehydrogenase in microgram amounts of tissue and distribution of the activity in the bovine adrenal. *J. Histochem. Cytochem.* 4(4):389-396.

[15] Kun E. and Ahood L.G. 1949. Colorimetric estimation of succinic dehydrogenase by triphenyltetrazolium chloride. *Science.* 109:144-146.

[16] Uma Maheswara Rao G. and Prameela Devi. Y 2011. Development of enzymatic method for environmental monitoring of monocrotophos. *RRST.* 3(4):58-65.

[17] Kok F.N., Bozoglu F. and Hasirci V. 2002. Construction of an acethylcholinesterase-choline oxidase biosensor for aldicarb determination. Biosens. *Bioelectron.* 17:531-539.

[18] Chandra M.G.V. 1999. Development of tablet method for the determination of heavy metals from environmental samples. Ph.D. diss. Kakatiya University.

[19] Shan D., Mousty C. and Cosnier S. 2004. Subnanomolar cyanide detection at polyphenol oxidase/clay biosensors. *Anal. Chem.* 76:178-183.

[20] Mohammadi H., Amine A., Cosnier S. and Mousty C. 2005. Mercury enzyme inhibition assays with an amperometric sucrose biosensor based on a trienzymatic–clay matrix. *Anal. Chem. Acta.* 543:143-149.

[21] Amine A., Mohammadi H., Arduini F., Ricci F., Moscone D. and Palleschi G. 2004. Extraction of enzyme inhibitors using a mixture of organic solvent and aqueous solution and their detection with electrochemical biosensors. Eighth world congress on biosensors. Granada in Spain.

[22] Andreescu S., Noguer T., Magearu V. and Marty J. L. 2002. Screenprinted electrode based on AChE for the detection of pesticides in presence of organic solvents. *Talanta.* 57:169-176.

[23] Aziz A., Hasna M., Ilhame B. and Giuseppe P. 2006. Enzyme inhibition-based biosensors for food safety and environmental monitoring. *Biosens and Bioelec.* 21:1405-1423.

[24] Kuswandi B. 2003. Simple optical fibre biosensor based on immobilized enzyme for monitoring of trace heavy metal ions. *Anal. Bioanal. Chem.* 376:1104-1110.

[25] Ciucu A., Negulescu C. and Baldwin R.P. 2003. Detection of pesticides using an amperometric biosensor based on ferophthalocyanine chemically modified carbon paste electrode and immobilized bienzymatic system. *Biosens. Bioelectron*. 18:303-310.

[26] Uma Maheswra Rao G. and Prameela Devi Y. 2008. Detection, separation and identification of dichlorvos (DDVP) by enzymatic method. *The Bioscan*. 3(4):773-376