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EVALUATION OF CLINICAL UTILITY OF A SIMPLE AND RAPID RP- HPLC METHOD FOR THE DETERMINATION OF DICHLORVOS POISONING IN HUMAN SERUM OF POISONED PATIENTS

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ABSTRACT

Organophosphates (OPs) are a class of pesticides used worldwide and also a common poison for agrochemical poisoning. As Dichlorvos is a common OP compound used in agriculture, it in turn is a commonly implicated OP compound. Yet there was a lack of availability of effective analytical method for its determination in a tertiary care hospital in Mysuru on an urgent basis. Hence, a simple and quick reversed phase high performance liquid chromatography (RP- HPLC) method was developed using Acetonitrile (ACN) and Millipore water 50:50 v/v as mobile phase. From this method, Dichlorvos was identified at a shorter retention time (RT) of 2.9 min when compared to other HPLC methods. The main objective of this study was to evaluate the clinical utility of the proposed RP- HPLC method for the determination of Dichlorvos poisoning in human serum of poisoned patients. The method was successfully applied to determine Dichlorvos poisoning in samples of 48 poisoned patients treated at JSS Medical College Hospital, Mysuru. Out of 48 samples analyzed, 21 and 27 samples showed the presence and absence of Dichlorvos respectively. Outcomes of the analysis were also sub categorized based on the information collected by clinician with respect to the Dichlorvos poisoning, the types of exposures of poison and the types of clinician's queries. In short, the proposed RP-HPLC method for determination of Dichlorvos is effective and reliable for diagnosis and prognosis of Dichlorvos poisoning in a clinical setting.

INTRODUCTION

Organophosphates (OPs) are one of the major classes of pesticides used worldwide (Costa, 2018). These chemical compounds are also a common poison for intentional, occupational and accidental poisoning which results in significant morbidity and mortality in poisoned patients (Balali-Mood and Saber, 2012; Banday *et al.*, 2015). During OP poisoning, acetyl cholinesterase (AChE) which is an enzyme responsible for hydrolyzing and Inactivating the neurotransmitter acetyl choline (ACh) is inhibited, thereby leading to excessive accumulation of Ach at the neuronal synapses and the neuromuscular junction. This in turn causes overstimulation of cholinergic receptors resulting in clinical features like excessive sweating, salivation, bronchospasm, bronchorrhea, bradycardia, hypotension and reduced consciousness (Hundekari *et al.*, 2013; Eddleston, 2019). In addition, butyryl

cholinesterase (BuChE) and neuropathy target, esterase, are also inhibited; but clinical significance of these interactions are uncertain. According to World Health Organization (WHO), 3 million cases of pesticide (mainly OP compounds) poisoning occur every year, resulting in an excess of 250,000 deaths (Narang *et al.*, 2015). In India, a developing country in South Asia, agrochemicals are easily available as it is an agriculture based country and therefore, responsible for higher number of pesticide poisoning incidents in the rural parts (Jesslin *et al.*, 2010; Maheswari *et al.*, 2016). As Mysuru is a South Indian district in the state of Karnataka where agriculture is the major occupation in rural areas, both retrospective and prospective studies were conducted in the poison information center (PIC) established by Department of Clinical Pharmacy, JSS College of Pharmacy, located at JSS Hospital, Mysuru to assess the pattern of poisoning cases admitted to the hospital. From these studies, it was found that the commonly used agents for poisoning of all poisoned cases admitted to JSS Hospital (a tertiary care teaching hospital) were pesticides. Amongst which, OP class of pesticides were the most implicated pesticides (Jesslin *et al.*, 2010; Prashar and Ramesh, 2018; Churi *et al.*, 2012). As Dichlorvos is a common OP compound used in agriculture, it in turn is a commonly implicated OP compound (Peshin *et al.*, 2014; Narang *et al.*, 2015). Despite the above fact, only a few studies have been done to perform quantitative analysis of Dichlorvos poisoning on an urgent basis in the poisoned patients. In order to overcome this limitation, a simple, rapid and effective analytical method was developed and validated by the analysis department of JSS College of Pharmacy, Mysuru using reversed phase high performance liquid chromatography (RP- HPLC). Therefore, the purpose of this study was to evaluate the clinical utility of the newly developed RP-HPLC method in detecting and quantifying Dichlorvos poisoning in human serum of poisoned patients at JSS Hospital, Mysuru, so that methodological support for diagnosis, therapy and prognostic evaluation of Dichlorvos poisoning can be provided at the earliest.

MATERIALS AND METHODS:

Chemicals and reagents: Dichlorvos standard was procured from Sigma Aldrich, Bengaluru. Action-3, a Dichlorvos marketed formulation manufactured by Jayakrishna pesticides private limited, was procured from a local market. All chemicals used were analytical grade purchased from Merck pharmaceuticals. High pressure liquid chromatography (HPLC) grade Acetonitrile (ACN) and Millipore water were used as mobile phase. HPLC grade ACN was used as the diluent for the preparation of the solutions.

Instrumentation and Chromatographic conditions:

BD red topped vacutainer (BD- 367812 Hemogard) blood collection tubes and a centrifuge were used to collect blood samples from the poisoned patients and to separate serum from blood respectively. A high pressure liquid chromatography LC-20AD with photometric diode array (PDA) detector were used to perform analysis and the separation was attained by using Phenomenex luna C18 column (250 mm X 4.60 mm 5µ). The run time was set to 10min. ACN and Millipore water (50:50 v / v) at a flow rate of 1.5 ml / min were used as mobile phase. Temperature of the column was set at 40°C. The wavelength of detection was set at 200 nm. PHENEX PTFE 0.02µm syringe sensor was used for filtration purposes. Chromatographic conditions used for the method is shown in Table 1.

Steps involved in analytical method development:

1) Preparation of standard stock:

Accurately weigh 10mg of pure Dichlorvos into 10ml volumetric flask, dissolve and makeup the volume by using HPLC grade ACN to get 1mg/ml concentration. From the above solution, prepare 75, 150, 225, 300 and 375µg/ml so that after diluting it with serum and ACN, the final concentration will be 10, 20, 30, 40 and 50µg/ml respectively. All dilutions were made up by using HPLC grade ACN.

2) Sample preparation:

0.131ml of marketed formulation (Action-3) containing 76% of Dichlorvos was diluted to 100ml by using HPLC grade ACN to form 1mg/ml solution. From the above sample solution, pipette out 0.2ml and makeup to 10ml

by using HPLC grade ACN to get 20µg/ml solution. The above solution was passed through 0.20µm syringe filter and injected to RP-HPLC.

3) Optimized extraction procedure:

In this procedure, ACN acts as a protein precipitating agent. To the Eppendorf tube, add 100µl of human serum and 100µl of drug and vortex the above mixture in a vortex meter for 20 seconds. To the mixture, add ACN and make-up the volume to 1.5ml; then centrifuge it at 9500 RPM for 10 minutes at 4˚c. The supernatant was filtered through 0.20µm syringe filter and injected to RP-HPLC. Moreover, the proposed technique was evaluated in accordance with the USFDA May 2018 guidelines. The blank chromatogram is shown in [Figure 1]. The standard and the sample chromatogram of Dichlorvos at 2.9 min are shown in [Figure 2 and 3] respectively.

Application of the proposed method to determine Dichlorvos poisoning in clinical setting:

On admission, patients with OP poisoning were diagnosed on the basis of a medical history from the patient and/or relatives, containers brought to hospital, records in patient-transfer forms, characteristic smell in the breath and clinical features typical of OP poisoning. Dichlorvos concentrations in serum samples from 48 poisoned patients (both confirmed and suspected OP poisoned patients) treated at JSS Hospital, Mysuru, were determined by RP-HPLC proposed procedure. For performing the analysis, blood samples of OP poisoned patients were collected in suitable vacutainer and were centrifuged to separate serum from the blood. By using the RP- HPLC proposed method, quantitative analysis of Dichlorvos poisoning in human serum of poisoned patients were carried out and the results were communicated to the clinicians.

RESULTS AND DISCUSSION:

The objective of the study was to determine the clinical utility of the newly developed RP-HPLC method in detecting and quantifying Dichlorvos poisoning in human serum of poisoned patients at JSS Hospital, Mysuru. A total of 48 samples were examined to determine the reliability and accuracy of the proposed RP-HPLC method to evaluate Dichlorvos poisoning. When findings of toxicological analysis are communicated, this is often followed very quickly by the question of ‘‘how much’’, that is to say, the issue of quantification. Therefore, the quantitative analysis of the samples were carried out to identify the presence of Dichlorvos poison in poisoned patients where there was a lack of details available on the consumed product and if the clinicians are not able to identify the specific poison. The results that were obtained using the proposed RP- HPLC method was divided into two based on the following criteria:

1) If the RT is found to be 2.9 min, then the presence of Dichlorvos in human serum is confirmed i.e. a positive result
2) If the RT was not found to be 2.9 min, then it shows the absence of Dichlorvos in human serum i.e. a negative result

Out of 48 samples requested for the analysis, a total of 21 samples were confirmed with positive results and the remaining 27 samples were found to be negative as shown in Figure 4. The chromatogram of poisoned patients with a positive result and a negative result is shown in [Figure 5 and 6] respectively.

A) From the information collected by clinician with respect to the Dichlorvos poisoning:

In the absence of analysis of samples collected from the poisoned patients, the treatment is initiated mainly based on the information collected by the clinician with respect to the Dichlorvos poisoning like the medical history from the patient and/or relatives, the clinical symptoms indicative of OP poisoning and so on. But, if the information collected goes wrong, then the proper treatment may not be provided to the patient leading even to the death of the patient. Therefore, the samples of poisoned patients those were taken to carry out the analysis were divided into two categories based on the information collected by the clinician. They were:

1) Samples from confirmed OP poisoned patients
2) Samples from suspected OP poisoned patients

Among 48 samples analyzed, 30 samples were from confirmed OP poisoned patients and 18 samples were from suspected OP poisoned patients.

1) Based on the details provided on the containers (which were suspected to contain the substance responsible for poisoning) brought to hospital along with the patient, records in forms of patient transfer and from patient's medical history, the samples obtained from the hospital titled as the samples of confirmed OP poisoned patients were sub divided into two categories:

1.1) Samples of Dichlorvos poisoned patients (the analytical tests were performed to confirm that the poisoning was due to Dichlorvos whereby to confirm that the information collected was correct; if the analytical results showed negative results, then the information collected with respect to the presence of Dichlorvos poisoning was not correct.)

1.2) Samples of suspected Dichlorvos poisoned patients

Out of 30 samples taken from confirmed OP poisoned patients, 17 samples were confirmed with positive results and 13 samples were showing negative results. A total of 17 samples were identified as Dichlorvos poison depending on any of the above mentioned information sources. As the information collected may not be always correct, to confirm and/or to quantify it, analysis of those 17 samples were performed. Out of which, 13 samples were confirmed with positive results whereas 4 samples showed negative results suggesting that the information collected with respect to the presence of Dichlorvos poisoning in 4 patients were not correct. Among the 13 samples which were identified as suspected Dichlorvos poison, 4 samples showed positive results whereas 9 showed negative results.

2) Based on the patient's medical history, characteristic smell in the breath and clinical symptoms associated with OP poisoning, the samples obtained from the hospital titled as suspected OP poisoned patients were of 18 in number of which 2 samples were confirmed with Dichlorvos poison and the remaining 16 samples were showing negative results. The number of samples analyzed from confirmed and suspected OP poisoned patients using the proposed RP- HPLC method is depicted in Table 2. It was observed that, though the samples of unknown poisoning is analyzed using the proposed RP- HPLC method, if the RT was found to be 2.9 min, then the samples were confirmed with Dichlorvos poison.

B) From the types of exposures of poison:

Based on the type of poison exposure, the samples of poisoned patients which were taken to carry out the analysis were divided into three categories:

1) Oral exposure
2) Inhalation exposure
3) Dermal exposure

Among the 48 samples, the quantitative analysis of 33 samples after oral exposure, 11 samples after inhalation exposure and 4 samples after dermal exposure was carried out. 17 out of 33 samples after oral exposure, 3 out of 11 samples after inhalation exposure and 1 out of 4 samples after dermal exposure were confirmed with positive results as shown in Table 3.

It was observed that, whatever be the type of poison exposure, the quantitative analysis of Dichlorvos poisoning was carried out using the proposed PR- HPLC method and the results were communicated to the clinicians as early as possible.

C) From the types of clinician's queries:

The analysis of Dichlorvos poisoning in the human serum of poisoned patients were carried out to answer 3 main queries of the clinicians at the hospital. They were:

Query 1) Query for the detection of Dichlorvos poison in the poisoned patients

Query 2) Query for the confirmation of Dichlorvos poison in the poisoned patients

Query 3) Query for knowing the quantity of Dichlorvos poison in human serum

As shown in Figure 7, Among the 48 samples analyzed, 14,6, 28 samples were analyzed for answering query 1, query 2 and query 3 respectively.

Table 1: Optimized Chromatographic Conditions

Column	Phenomenex luna C_{18} column (250 mm X 4.60 mm 5µ)
Wavelength	200nm
Flow rate	1.5ml/min
Detector	PDA
Injection volume	10µl
Mobile phase	ACN and Millipore water 50:50 (v/v)
Retention time	2.9 min

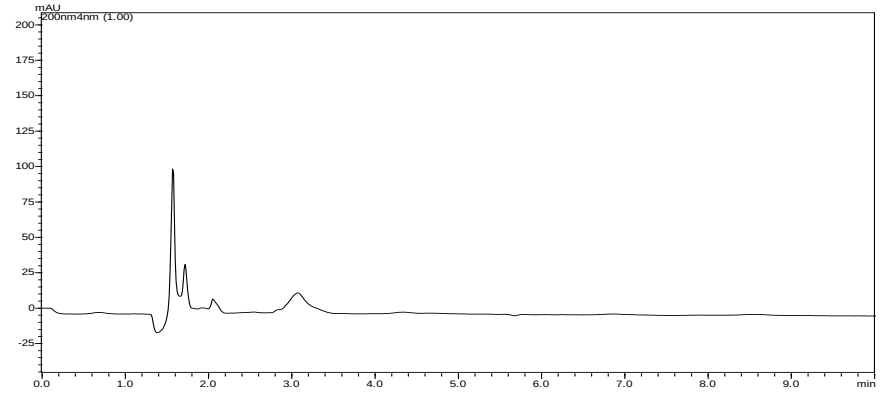

Figure 1: Chromatogram of blank serum

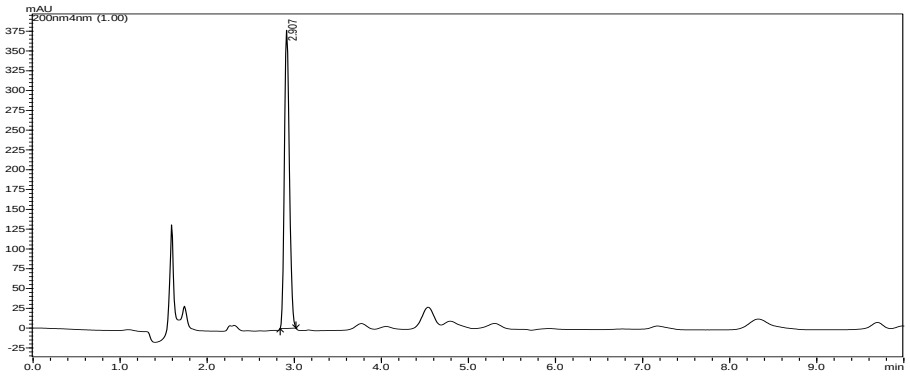

Figure 2: Standard chromatogram of Dichlorvos

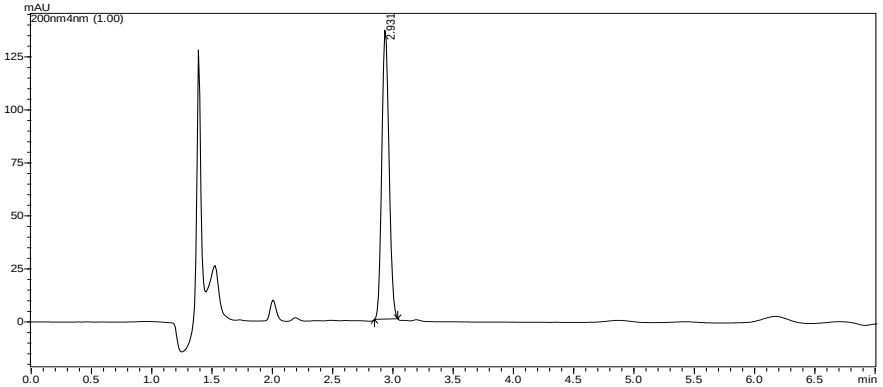

Figure 3: Sample chromatogram of Dichlorvos

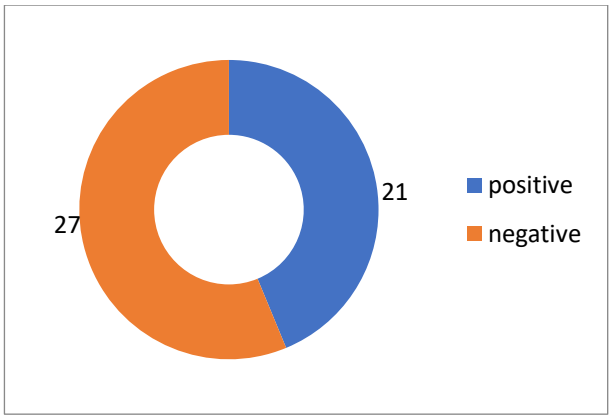

Figure 4: Number of positive and negative results

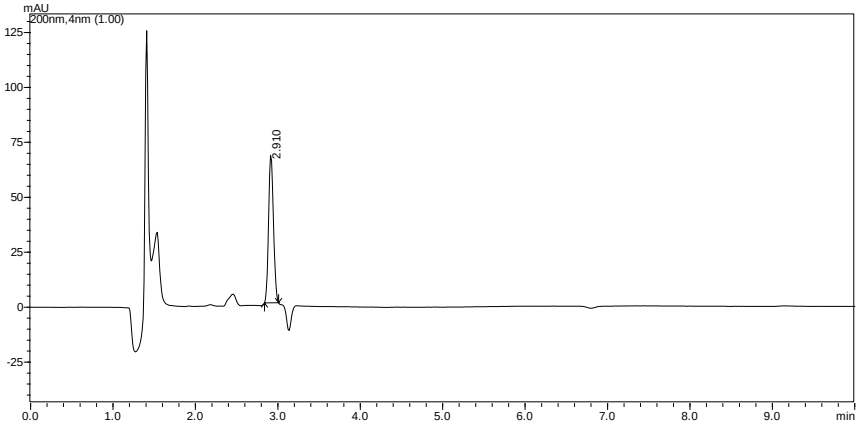

Figure 5: Chromatogram of poisoned patients showing positive result

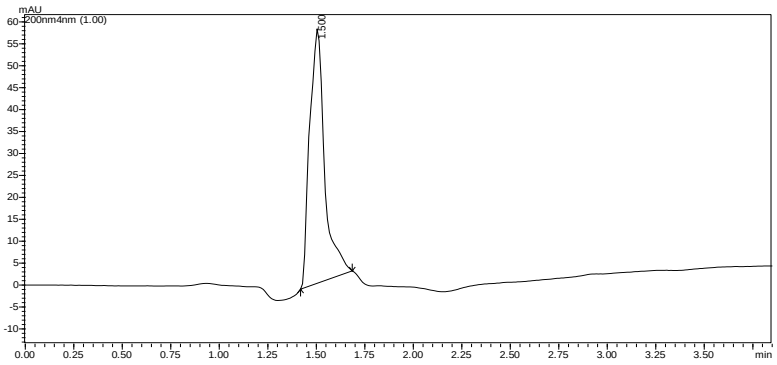

Figure 6: Chromatogram of poisoned patients showing negative result

Table 2: Number of Samples Analyzed Based on Confirmed and Suspected OP Poisoning

Total No. of samples analyzed (n= 48)					
No. of samples from confirmed OP poisoned patients (n = 30)				No. of samples from suspected OP poisoned patients (n = 18)	
No. of samples of Dichlorvos poisoned patients (n= 17)		No. of samples of suspected Dichlorvos poisoned patients (n= 13)			
No. of positive results (n= 13)	No. of negative results (n= 4)	No. of positive results (n = 4)	No. of negative results (n = 9)	No. of positive results (n= 2)	No. of negative results (n= 16)

Table 3: Number of Samples Analyzed Based on Types of Exposures

Total No. of samples analyzed (n= 48)					
No. of oral exposure (n = 33)		No. of inhalation exposure (n =11)		No. of dermal exposure (n = 4)	
No. of positive results (n=17)	No. of negative results (n=16)	No. of positive results (n=3)	No. of negative results (n=8)	No. of positive results (n=1)	No. of negative results (n=3)

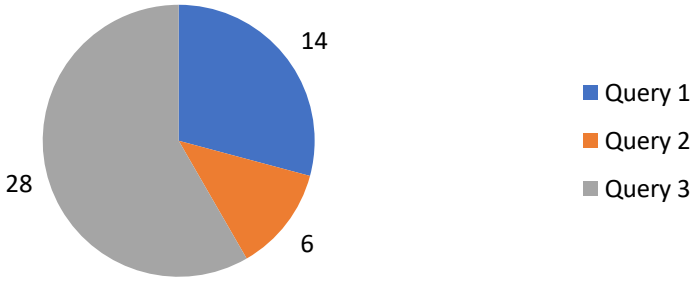

Figure 7: Number of samples analyzed based on the clinician's queries

It was observed that, all the type of clinician's queries were able to be answered at the earliest as the proposed RP-HPLC method for detection of Dichlorvos is having short retention time compared to other HPLC methods. Therefore, this analytical method was suitable and valuable for diagnosis and prognosis of Dichlorvos poisoning and could be used as an effective tool for carrying the quantitative analysis of Dichlorvos poisoning in the human serum of poisoned patients.

CONCLUSION:

OP poisoning is one of the major health concerns in developing countries. As Dichlorvos is a commonly implicated OP compound for poisoning, an accurate, fast, simple and cost-effective RP-HPLC technique was developed for its determination in human serum of poisoned patients. In order to evaluate the clinical utility of proposed RP – HPLC method, serum samples of poisoned patients were collected and analyzed. From this study, it was concluded that the newly developed RP-HPLC method for detecting and quantifying the Dichlorvos poisoning in human serum of poisoned patients is reliable, accurate and can be performed rapidly, easily, and economically. Hence, this method is a helpful tool for assisting the clinician in the detection, management and prognosis of Dichlorvos poisoning.

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