Analytical and Bio-Analytical Method Development and Validation of Dichlorvos Pesticide Using RP-HPLC Method

Vaishnav L C L, Chandan R S*, Maruthi R, Gurupadayya B M
Department of Pharmaceutical Chemistry, JSS College of Pharmacy Mysuru, JSS Academy of Higher Education and Research, Sri Shivarathreeshwara Nagarag, Mysuru-570015, Karnataka, India

Article History:
Received on: 04 Nov 2019
Revised on: 05 Dec 2019
Accepted on: 11 Dec 2019

Keywords:
PDA detector, RP-HPLC method, Dichlorvos, organophosphate

ABSTRACT
Organophosphorus compounds were synthesised in the 1800s. Later they are used as insecticides in the late 1930s and early 1940s. The German scientist Gerhard Schrader is known for the creation of the basic chemical structure of anticholinesterase organophosphate compounds and development of the first commercialised Organophosphorous insecticide. Such chemicals are anticholinesterase insecticide commonly used in agriculture and horticulture. To a lesser extent, they are used for domestic use. Due to the absence of bio persistence in organophosphates, most of the western countries opted to substitute organochlorines with organophosphates. Organophosphate pesticides are commonly used around the world, and contamination by these compounds is a serious public health concern in developing countries. Toxicokinetics and toxicodynamics of OP poisoning not only differ with path or level of exposure. But also the agent's chemical composition. Organophosphates are a group of pesticide that was developed in the 1940s in Germany and soon became an effective defence against agricultural pests. Dichlorvos which is a commonly used group of pesticide is a broad-spectrum organophosphate compound having insecticidal activity. Dichlorvos is a cholinesterase inhibitor exhibiting stomach, contact and systemic mode of action. Therefore, an accurate, fast, cost-effective and straightforward RP-HPLC technique for detecting Dichlorvos was developed. The RP-HPLC method is established by using ACN and Millipore water 50:50 v/v as mobile phase, the Flow rate is maintained at 1.5mL/minute. Detection of Dichlorvos was performed by using a PDA detector at 200nm. By this RP-HPLC procedure, RT of Dichlorvos was identified at 2.9 min.

INTRODUCTION
Organophosphorus compounds are synthesised in the 1800s, and later they are used as insecticides in the late 1930s and early 1940s (Costa, 2006). The German scientist Gerhard Schrader is known for the creation of the basic chemical structure of anticholinesterase organophosphate compounds and development of the first commercialised Organophosphorous insecticide (Costa, 2006). Such chemicals are anticholinesterase insecticide commonly used in agriculture and horticulture (Kwong, 2002). To a lesser extent, they are used for domestic use. Due to the absence of bio persistence in organophosphates, most of the
western countries opted to substitute organochlorines with organophosphates (Rusyniak and Nañagas, 2004). Organophosphate pesticides are commonly used around the world, and contamination by these compounds is a serious public health concern in developing countries (Sukirtha et al., 2013). Toxicokinetics and toxicodynamics of OP poisoning not only differ with path or level of exposure. But also, the agent’s chemical composition (Kwong, 2002).

The toxicity mechanism of organophosphates is by suppression of acetylcholinesterase, which results in building up of acetylcholine neurotransmitter and the continues activation of acetylcholine receptors (Jones et al., 1992; Blair et al., 1976; Inoue et al., 2007). The recommended treatment comprises of reactivating blocked acetylcholinesterase with an oxime antidote and suppressing acetylcholine’s action on the receptor with atropine (Costa, 2006; Rusyniak and Nañagas, 2004). A patient who received an appropriate diagnosis recover from acute toxicity. Dichlorvos is the active component of many insecticidal formulations (Jones et al., 1992; Blair et al., 1976). The toxicity of Dichlorvos was reported by FAO/WHO at a joint meeting on pesticide residues (1965, 1967, 1968 and 1970) and a permissible daily intake of 0.004 mg/kg was suggested (Blair et al., 1976). High concentration exposure over the short-term also up to 50 days of exposure on monkeys and rat to 0.1-0.5mg. The only effect seen was a depression of cholinesterase. Dichlorvos is a cholinesterase inhibitor exhibiting stomach, contact and systemic mode of action (Sharma et al., 1990). Dichlorvos is a broad-spectrum organophosphate compound having insecticidal activity. In the early days, most organophosphorous insecticides were also dangerous to mammals, including humans, i.e. they were not selectively toxic to insects. Chromatographic techniques are commonly used for the chemical isolation, detection and quantification of many pesticides. The HPLC, GC or TLC methods were used to determine Dichlorvos, which are having some advantages and disadvantages (Cho, 1997; Parrilla et al., 1994). To increase food production, pesticide applications have become vital. India is the world’s fifth pesticide consumer. The proposed RP-HPLC method for the detection of Dichlorvos has a short retention time compared to other HPLC methods. Dichlorvos chemical structure is shown in Figure 1.

MATERIALS AND METHODS

Instrumentation and Chromatographic conditions

For the current study, high- pressure liquid chromatography (HPLC) LC-20AD with PDA detector is used. The separation was attained by using a Phenomenex Luna C18 column (250 mm X 4.60 mm 5µ). The run time was set to 10min. Acetonitrile (ACN) and Millipore water (50:50 v/v) at a flow rate of 1.5 ml/min are used as the mobile phase. The temperature of the column was set at 40°C. The wavelength of detection was set at 200 nm. PHENEX PTFE0.02µm syringe sensor is used for filtration purposes.

Figure 1: Chemical structure of Dichlorvos

Figure 2: Uv spectrum

Chemicals and reagents

Dichlorvos standard was procured from Sigma Aldrich, Bengaluru. Action-3, which is 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. HPLC grade ACN and Millipore water is used as mobile phase. HPLC grade ACN was used as the diluent for preparation of the solutions.
### Table 1: Optimized Chromatographic conditions

| Column                        | Phenomenex luna C18 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                                         |

### Table 2: System suitability results

| Parameters         | Acceptance criteria | Results  |
|-------------------|---------------------|----------|
| Tailing factor    | NMT 2.0             | 1.270    |
| Theoretical plates| NLT 2000.0          | 8652.304 |

### Table 3: Concentration and peak area for calibration curve

| Concentration | Area   |
|---------------|--------|
| 10            | 549627 |
| 20            | 937318 |
| 30            | 1271496|
| 40            | 1670206|
| 50            | 2147712|

### Table 4: Method precision intraday studies

| Concentration | Peak area | Concentration | Peak area | Concentration | Peak area |
|---------------|-----------|---------------|-----------|---------------|-----------|
| 10            | 545384    | 30            | 1237318   | 50            | 2170206   |
| 10            | 546254    | 30            | 1229528   | 50            | 2194037   |
| 10            | 541658    | 30            | 1241167   | 50            | 2138502   |
| 10            | 536916    | 30            | 1206536   | 50            | 2097184   |
| 10            | 534522    | 30            | 1283667   | 50            | 2198853   |
| 10            | 544521    | 30            | 1231128   | 50            | 2095633   |
| average       | 541542.5  | 1238224       | 2163143   |
| STD deviation | 4407.041  | 23110.79      | 42044.54  |
| %RSD          | 0.813794  | 1.866447      | 1.956407  |

Acceptance criteria: The RSD calculated on 8 determinations must be ≤ 2.0%

### Table 5: Method precision interday studies

| Concentration | Peak area | Concentration | Peak area | Concentration | Peak area |
|---------------|-----------|---------------|-----------|---------------|-----------|
| 10            | 535624    | 30            | 1218965   | 50            | 2193468   |
| 10            | 546985    | 30            | 1204563   | 50            | 2185524   |
| 10            | 546685    | 30            | 1208265   | 50            | 2099835   |
| 10            | 539863    | 30            | 1218167   | 50            | 2122558   |
| 10            | 548467    | 30            | 1205837   | 50            | 2192538   |
| 10            | 546997    | 30            | 1218462   | 50            | 2184935   |
| average       | 544103.5  | 1212377       | 2163143   |
| Std deviation  | 4695.094  | 6254.223      | 37448.89  |
| %Rsd          | 0.862904  | 0.515865      | 1.731226  |

Acceptance criteria: The RSD calculated must be ≤ 2.0%.
### Table 6: Accuracy

| Level of recovery | Amount of formulation | Amount of Pure drug | The total amount of drug | Peak area | Difference | % Recovery | Mean |
|-------------------|-----------------------|---------------------|--------------------------|-----------|------------|------------|------|
| 50                | 20                    | 10                  | 30                       | 36085012  | 35535385   | 98.47686   |      |
| 50                | 20                    | 10                  | 30                       | 36084635  | 35535008   | 98.47684   |      |
| 50                | 20                    | 10                  | 30                       | 36081465  | 35531838   | 98.47671   |      |
| 100               | 20                    | 20                  | 40                       | 78090259  | 77152941   | 98.7997    |      |
| 100               | 20                    | 20                  | 40                       | 71885632  | 70948314   | 98.6961    |      |
| 100               | 20                    | 20                  | 40                       | 71852784  | 70915466   | 98.6955    |      |
| 150               | 20                    | 30                  | 50                       | 82284526  | 81013030   | 98.45461   |      |
| 150               | 20                    | 30                  | 50                       | 82276485  | 81004989   | 98.45461   |      |
| 150               | 20                    | 30                  | 50                       | 82276352  | 81004856   | 98.4546    |      |

Acceptance criteria: Mean % recovery and individual at each level should be between 102.0% and 98.0%

% Recovery = (Amount of drug recovered/ Amount of drug added) * 100

### Table 7: LOD and LOQ

| Average of SD | Average of slope |
|---------------|------------------|
| 101937        | 12302            |
| 102339        | 12301            |
| 102659        | 12377            |

LOD = 0.046 μg/ml

LOQ = 0.139 μg/ml

### Table 8: Robustness

| Parameters                               | Change in units | Acceptance criteria | Results |
|------------------------------------------|-----------------|---------------------|---------|
| Wavelength                               | 205 ± 3         | %RSD ≤ 2            | 1.329   |
| Flow rate                                | 1ml/min ± 0.1   | %RSD ≤ 2            | 1.256   |
| Column temperature                       | 40°C ± 5°C      | %RSD ≤ 2            | 0.546   |
| Mobile phase ratio                       | Acetonitrile: orthophosphoric acid 0.1% 50:50 (v/v) ± 2 | %RSD ≤ 2 | 0.217 |

### Table 9: Ruggedness

| Concentration | Trial 1 | Trial 2 | Mean | SD  | %RSD |
|---------------|---------|---------|------|-----|------|
| by changing the analyst 0 | 0       | 0       | 0    | 0   | 0    |
| 10            | 542356  | 5354867 | 2948612 | 3402959 | 0.925648 |
| 20            | 931234  | 931025  | 931129.5 | 147.7853 | 0.765426 |
| 30            | 1215482 | 12256718 | 6736100 | 7807333 | 0.896253 |
| 40            | 1610250 | 16203652 | 8906951 | 10319094 | 0.925625 |

By changing the instrument 0 | 0 | 0 | 0 | 0 | 0 |
| 10            | 539524  | 545264  | 542394 | 4058.793 | 0.748311 |
| 20            | 935246  | 945698  | 940472 | 7390.68 | 0.785848 |
| 30            | 12854691 | 1226524 | 7040608 | 8222356 | 0.862546 |
| 40            | 1621534 | 1660214 | 1640874 | 27350.89 | 0.965246 |
### Table 10: Calibration data of Dichlorvos

| Sl.no | Conc. (µg/ml) | Peak area |
|-------|---------------|-----------|
| 1     | 0             | 0         |
| 2     | 10            | 312932    |
| 3     | 20            | 615335    |
| 4     | 30            | 907831    |
| 5     | 40            | 1095066   |
| 6     | 50            | 1513468   |

### Table 11: Results showing precision for Dichlorvos (within run)

| Concentration | Mean peak area | Mean concentration | SD    | %CV    |
|---------------|----------------|--------------------|-------|--------|
| 10 (LLOQ)     | 305015.4       | 9.9026             | 0.8183| 8.2643 |
| 20 (LQC)      | 585185.0       | 19.962             | 0.8725| 4.3708 |
| 30 (MQC)      | 861875.4       | 29.897             | 1.0632| 3.5563 |
| 40 (HQC)      | 1136209.2      | 39.748             | 1.9101| 4.8055 |

### Table 12: Results showing precision for Dichlorvos (between run)

| Concentration | Mean peak area | Mean concentration | SD    | %CV    |
|---------------|----------------|--------------------|-------|--------|
| 10 (LLOQ)     | 307135.8       | 9.97877            | 0.7763| 7.7795 |
| 20 (LQC)      | 581728.6       | 19.8809            | 0.8446| 4.2483 |
| 30 (MQC)      | 862136.0       | 29.9069            | 1.0649| 3.5609 |
| 40 (HQC)      | 1134797.2      | 39.6973            | 1.8912| 4.7640 |

### Table 13: Results showing recovery for Dichlorvos

| Standards | Concentration | Analytical Peak Area | Bioanalytical Peak area | % Recovery |
|-----------|---------------|----------------------|-------------------------|------------|
| LLOQ      | 10            | 549627               | 312932                  | 56.93533978|
| LQC       | 20            | 937318               | 615335                  | 65.64847789|
| MQC       | 30            | 1271496              | 907831                  | 71.39865167|
| HQC       | 40            | 1670206              | 1095066                 | 65.56472675|
| UQC       | 50            | 2147712              | 1513468                 | 70.46885243|

### Table 14: Result showing stability for Dichlorvos

| Stability | Standards | Concentration µg/mL | Mean recovered concentration µg/mL | SD       | %CV     |
|-----------|-----------|---------------------|-----------------------------------|----------|---------|
| Bench-top| LQC       | 20                  | 19.9169                           | 0.5020   | 2.5205  |
|           | HQC       | 40                  | 39.2690                           | 0.7317   | 1.8633  |
| Freeze and thaw | LQC | 20                  | 19.9370                           | 0.2522   | 1.2650  |
|           | HQC       | 40                  | 39.9585                           | 0.7294   | 1.8347  |
| Long term stability | LQC | 20                  | 19.9120                           | 0.2621   | 1.3166  |
|           | HQC       | 40                  | 38.3938                           | 0.0700   | 0.1827  |
Figure 3: Blank chromatogram

Figure 4: Standard chromatogram of Dichlorvos at 100µg/ml concentration showing RT at 2.9 min

Figure 5: Sample chromatogram of Dichlorvos at 20µg/ml concentration showing RT at 2.9 min

Figure 6: Chromatogram of blank serum

Figure 7: Standard chromatogram of Dichlorvos

Figure 8: Sample chromatogram of Dichlorvos

Figure 9: Calibration curve for Dichlorvos

Figure 10: Linearity graph of Dichlorvos

Analytical method development

Selection of wavelength

The λ max of Dichlorvos was determined by using UV-visible spectrophotometer 1800. Uv spectrum for Dichlorvos is shown in Figure 2.

Mobile phase selection and preparation

Dichlorvos being less polar, different mobile phase combinations of various ratios were tried for the selection of mobile phase. The standard Dichlorvos drug was injected with various combination of
mobile phase at different ratios and flow rate for the peak optimisation. The procedure was continued until obtaining a sharp peak. The sharp peak was obtained at 50:50 (v/v) of ACN and Millipore water.

**Preparation of standard stock solution**

The standard stock of Dichlorvos was prepared by dissolving 10mg of the standard drug in 10ml of HPLC grade acetonitrile to obtain 1mg/ml concentration. From the stock solution, the standard stock solutions of 10, 20, 30, 40, and 50µg/ml were prepared. All dilutions were made up by using HPLC grade acetonitrile.

**Sample preparation**

0.131ml of marketed formulation (Action-3) containing 76% of Dichlorvos was diluted to 100ml by using HPLC grade acetonitrile to form 1mg/ml solution. From the above sample solution, pipette out 0.2ml and make-up to 10ml by using HPLC grade acetonitrile to get 20µg/ml solution. The above resolution was passed through 0.20µm syringe filter and injected to RP-HPLC.

**Optimization of the method for Dichlorvos**

Study of the effect of various parameters in developing method was carried out. Initially, the solubility of Dichlorvos in multiple solvents was tested. Then a suitable column for separation is selected for the proposed method. To achieve a proper separation of eluted compounds in HPLC, the chromatographic conditions were optimised. Initially, different diluent was tested to elute the drug.

Flow rate and mobile phase choice are determined based on peak parameters like tailing factor or asymmetry, run time, resolution. Acetonitrile and Millipore water in ratio 50:50 (v/v) was used as mobile phase at a flow rate of 1.5ml/min. The blank chromatogram was shown in Figure 3.

The standard and sample chromatogram of Dichlorvos at 2.9 min were shown in Figure 4 and Figure 5 respectively. Chromatographic conditions used for the method is shown in Table 1.

**RESULTS AND DISCUSSION**

The precise, quick and easy RP-HPLC technique for the identification of Dichlorvos has been developed. The proposed technique was evaluated following the ICH Q2(R1) guidelines. The RT of Dichlorvos was found to be 2.9min from the chromatogram. And the coefficient of correlation was found to be 0.995 for analytical and 0.9934 for bio-analytical method. The quantification limit (LOQ) was found to be 0.139µg/ml, and the detection limit (LOD) was found to be 0.461µg/ml. The inter-day and intra-day precision value (RS D percentage) were identified to be less than two.

**Analytical method validation**

**System suitability**

The standard stock of Dichlorvos was injected six times for testing system suitability parameters, The results were shown in Table 2.

**Linearity**

It is the ability to obtain experimental results equal to the analyte content in the specimen. The calibration curve was attained by using five different concentrations in triplicate 10, 20, 30, 40, and 50µg/ml and the linearity was established by applying linearity expression y=mx+c, and the slope was calculated. The calibration curve for Dichlorvos is shown in Figure 9. The concentration and peak area was established in Table 3.

**Precision**

The repeatability of the method was validated by using different concentration of the drug 10, 30 and
50 μg/ml. The above solutions were prepared from the stock solution and used to inject in interday and intraday for the evaluation of precision. The concentrations were prepared at three different times in a day for intraday studies. The results for accuracy was shown in Table 4 and Table 5.

**Accuracy**

It is the closeness of the obtained value to the true value of the sample, to check the accuracy of the method, the formulation was spiked with 50%, 100% and 150% of Dichlorvos standard drug. The results were analysed to find the % recovery of the Dichlorvos. The result for accuracy was given in Table 6.

**Limit of detection and limit of quantification**

The LOD and LOQ for the HPLC method were determined by using a calibration standard. LOD can be calculated as per the ICH guidelines by using the formula $LOD = 3.3 \times N / S$, $N$ is the standard deviation and $S$ is the slope. LOQ can be calculated by the formula $LOQ = 10 \times N / S$ where $N$ is the standard deviation and $S$ is the slope. The results for LOD and LOQ was shown in Table 7.

**Robustness**

A method can stay unchanged when small differences in parameters are applied. The robustness of the suggested technique was verified by increasing and decreasing the wavelength, flow rate, column temperature and 30 μg/mL concentration were injected. The result of the robustness was shown in Table 8.

**Ruggedness**

An experimental procedure’s ruggedness is its ability to remain unaffected by minor or intentional changes in system parameters. The ruggedness of the proposed procedure is validated by changing analyst and instrument. The result was shown in Table 9.

**Bioanalytical method validation**

**Calibration curve**

It consisted of a matrix sample processed without analyte and matrix sample with calibration standards. It is showing good linearity over the range of 10 to 50 μg/ml with a coefficient of correlation 0.9934. The calibration curve for Dichlorvos is shown in Figure 10. The concentration and peak area was shown in Table 10.

**Specificity/selectivity**

An analytical technique can differentiate and quantify the analyte in the presence of other components in the sample. For selectivity blank plasma of two different lots were taken and analysed. Selectivity was assessed by comparing the extracted blank plasma response with extracted LLOQ. At the RT of the drug, no significant interference from the blank plasma was observed.

**Sensitivity**

This parameter was evaluated by injecting six different aliquots of extracted LLOQ concentration. Percentage deviation from the nominal concentration and percentage CV were calculated. The developed method was found to be sensitive to %CV.

**Accuracy and precision**

Within run and between run accuracy were performed by five replicates of LLOQ, LQC, MQC and HQC. Between run, accuracy was assessed by analysing sample on different days. The accuracy and precision for all batches at LLOQ, LQC, MQC and HQC levels were calculated. Mean percentage nominal concentration and CV for all the batches were found to be within the acceptance limit. The results for precision is shown in Table 11 and Table 12.

**Recovery**

After spiking the extracted QC samples were analysed and percentage recovery at each level was calculated by comparing the peak area of low, medium and high QC levels. Mean recovery across all the QC levels is found to be 66.0%. Results for recovery is shown in Table 13.

**Stock solution stability**

Both main stock and spiking stock of Dichlorvos was found to be stable at 2-10°C for 20 days (long term) and 8 hours at room temperature.

**Bench top stability**

Low and high QC was prepared and kept at the benchtop at room temperature for a minimum of 4 hrs (stability samples). Then analysed the response is compared with the freshly prepared calibration standard responses. Mean percentage change was calculated

**Freeze and thaw stability**

The samples were exposed to three freeze-thaw cycles. The peak area response is then compared with standard calibration responses. Mean percentage change was calculated and verified against acceptance criteria.

**Long term stability**

The stability of the sample is evaluated by keeping it for an extended period in freeze state and extracted then analysed. The response is compared with a
fresh calibration standard response. Results for stability is shown in Table 14.

CONCLUSION

The developed RP-HPLC approach has been validated in terms of device suitability, linearity, precision, accuracy, LOD and LOQ, robustness and ruggedness in compliance with the ICH guidelines. It was inferred from the above finding that the system developed was reliable, accurate and unique for the detection of Dichlorvos.

ACKNOWLEDGEMENT

We would like to thank the Principal, JSS College of Pharmacy, Mysuru and JSS Academy of Higher Education & Research, Mysuru for providing the facilities in the successful completion of the research work.

Conflict of Interest

The authors declare that they have no conflict of interest for this study.

Funding Support

The authors declare that they have no funding support for this study.

REFERENCES

Blair, D., Dix, K. M., Hunt, P. F., Thorpe, E., Stevenson, D. E., Walker, A. I. T. 1976. Dichlorvos ? a 2-year inhalation carcinogenesis study in rats. *Archives of Toxicology, 35*(4):281–294.

Cho, Y. 1997. Nancy Matsuoka and Akira Kamiya Determination of organophosphorus pesticide in biological sample of acute poisoning by HPLC with diode-array detector. *chem pharm. Bull, 45*(4):737–740.

Costa, L. G. 2006. Current issues in organophosphate toxicology. *Clin Chim Acta, 366*(1-2):1–13.

Inoue, S., Saito, T., Mase, H., Suzuki, Y., Takazawa, K., Yamamoto, I., Inokuchi, S. 2007. Rapid simultaneous determination for organophosphorus pesticides in human serum by LC–MS. *Journal of Pharmaceutical and Biomedical Analysis, 44*(1):258–264.

Jones, M. W., Sommerville, C., Wootten, R. 1992. Reduced sensitivity of the salmon louse, Lepeophtheirus salmonis, to the organophosphate dichlorvos. *Journal of Fish Diseases, 15*(2):197–202.

Kwong, T. C. 2002. Organophosphate Pesticides: Biochemistry and Clinical Toxicology. *Therapeutic Drug Monitoring, 24*(1):144–149.

Parrilla, P., Vidal, J. L. M., Galera, M. M., Frenich, A. G. 1994. Simple and rapid screening procedure for pesticides in water using SPE and HPLC/DAD detection.

Rusyniak, D., Nañagas, K. 2004. Organophosphate Poisoning. *Seminars in Neurology, 24*(02):197–204.

Sharma, V. K., Jadhav, R. K., Rao, G. J., Saraf, A. K., Chandra, H. 1990. High performance liquid chromatographic method for the analysis of organophosphorus and carbamate pesticides. *Forensic Science International, 48*(1):21–25.

Sukirtha, T. H., Usharani, M. V., Jones, O. 2013. Original Research Article Gas Chromatography-Mass Spectrometry Determination of Organophosphate Pesticide Residues in water of the irrigation canals in the North Zone. *Sci Total Environ [Internet], 2*(321–330).