Determination of Deltamethrin in Mice Plasma and Immune Organs by Simple Reversed-Phase HPLC

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Summary. Deltamethrin, a well-known type 2 synthetic pyrethroid insecticide, is a widespread environmental toxicant. It has potential to accumulate in body fluids and tissues due to its lipophilic characteristics. The immune system is among the most sensitive targets regarding toxicity of environmental pollutants. Various methods are available in the literature to analyze deltamethrin (DLM) concentration in plasma and tissues, but regarding the immune organs, only one gas chromatography–tandem mass spectrometry (GC–MS/MS) method (on spleen tissues) has been reported. In the present investigation, a rapid and sensitive high-performance liquid chromatography (HPLC) method has been developed and validated to determine DLM concentration in plasma, thymus, and spleen using zaleplone as an internal standard. Liquid chromatography (LC) separation is performed on an Agilent Zorbax® C8 column (250 mm × 4.6 mm, i.d., 5 μm) with isocratic elution using a mobile phase consisting of acetonitrile–5 mM KH₂PO₄ (70:30, v/v) at a flow rate of 1 mL min⁻¹. The lower limit of quantification (LLOQ) for DLM is 10 ng mL⁻¹ (plasma, thymus, and spleen). The method has been validated in terms of establishing linearity, specificity, sensitivity, recovery, accuracy, and precision (intra- and inter-day) and stabilities study. This validated method was successfully applied to a pharmacokinetic and tissue distribution study of DLM in mice.

Key Words: deltamethrin, plasma, thymus, spleen, HPLC

Introduction

Pyrethroid insecticides are widely used in households to control pests, in agriculture to protect crops, and in public health to control diseases [1, 2]. Based on production of syndromes, pyrethroids are classified into two subclasses, type 1 and type 2. Type 1 pyrethroids are characterized by induction of whole body tremor, whereas type 2 pyrethroids produce choreoathetosis accompanied by salivation [3, 4]. They exert neurotoxicity by prolong-
ing the opening of sodium channels [5]. This prolonged opening of sodium channels results in persistent depolarization, leading to repetitive firing which causes seizures, paralysis, and death in the experimental animals [6].

\[ \text{C}_{22}\text{H}_{19}\text{Br}_{2}\text{NO}_{3} \]

Fig. 1. Chemical structure of deltamethrin

Deltamethrin, a \( \alpha \)-cyano type 2 synthetic pyrethroid ((S)-\( \alpha \)-cyano-3-phenoxybenzyl-(1R,cis)-2,2-dimethyl-3-(2,2-dibromvinyl)-cyclopropanecarboxylate), as shown in Fig. 1, was first synthesized in 1974 and has been widely used in controlling insect pests of medical and agricultural importance [2, 7]. The major advantages with deltamethrin (DLM) are their photostability, high potency, and low toxicity to birds and mammals [8, 9]. Deltamethrin is registered for use on various crops, including cotton, corn, cereals, soybeans, and vegetables, for pests such as mites, ants, weevils, and beetles, and the control of parasites on animals [10]. Presently it is also used in the control of malaria and other vector borne diseases [2, 11]. Due to this widespread use, pyrethroid contamination has become a potential problem. In the literature, various studies have shown that DLM exposure leads to immunotoxicity. Recently, we also reported DLM induced apoptosis in murine thymocytes [12]. The degree and duration of toxicity are largely dependent upon the concentration and time of duration of toxicant in the target tissue.

Chromatography is a common technique for determination of pyrethroids concentration. In the literature, variety of approaches like biological [13, 14], immunological [15, 16], and chemical assay [17] have been developed to quantify pyrethroids in biological samples. Recently, various studies have demonstrated the use of gas chromatography–mass spectrometry (GC–MS) and GC–MS/MS for the determination of deltamethrin in human urine and blood [18]. The GC methods are very sensitive, but they involve large sample sizes (3–15 mL) and extensive sample preparation (liquid–liquid extraction [LLE] followed by solid-phase extraction [SPE]) and have long run times (60–70 min). Another additional concern for the use of
gas chromatography for the determination of deltamethrin has been reported by Valverde et al. [19]. This report shows that the pyrethroid pesticide tralomethrin is thermally degraded to deltamethrin in the injection port of a gas chromatograph leading to concerns about the ability of GC methods to accurately quantitate deltamethrin in the presence of tralomethrin.

High-performance liquid chromatography (HPLC) is preferred with various detection systems including ultraviolet (UV) [20–23], diode array detection (DAD) [23–26], MS [27, 28] and tandem mass spectrometry (MS²) [29], and fluorescence detection (FLD) [30]. Generally, LC-MS techniques are considered superior to all the above-mentioned detection techniques, in terms of specificity and sensitivity. However, the high cost involved in the acquisition of LC-MS instrumentation does not allow its application in several analytical laboratories. Thus, there is need of a HPLC method which is less costly but sensitive enough and selective for analysis of pesticide residues in biological matrix.

HPLC method has been included in chemical assay and used to determine concentrations of DLM in milk, food, environmental specimens, blood, and various tissues [31–35], but all these methods are time-consuming. Immune system is very sensitive system regarding toxicity of environmental toxicants.

Thus, the main objective of the present study is to develop and validate more rapid and sensitive HPLC technique with photodiode array detector (PDA) to quantify DLM in plasma, thymus, and spleen of mice. The validation of method has been carried out according to Food and Drug Administration (FDA) guidelines for bioanalytical method validation [36].

**Materials and Methods**

**Chemicals**

DLM (purity >99.70%) was purchased from Sigma-Aldrich, USA. Zaleplone as internal standard (IS, purity >99.70%, Fig. 2) was obtained from Centaur Pharmaceuticals Pvt. Ltd. (Mumbai, India). HPLC grade methanol, acetonitrile, ethanol, dichloromethane, and KH₂PO₄ were purchased from Merck Ltd. (Mumbai, India). Ultra-pure water was obtained from a Milli-Q water purification system (Millipore, Milford, MA, USA).
Chromatographic Conditions

The analysis was conducted with a Knauer HPLC (Berlin, Germany) system consisting of a binary smartline 1100 pump, manual injector port having capacity of 20 μL, online degasser, and a PDA detector. The analyte and IS were eluted on an Agilent C8 (250 mm × 4.6 mm, i.d., 5 μm) column using acetonitrile–5 mM KH₂PO₄ (70:30, v/v) as mobile phase. The flow rate was set at 1.0 mL min⁻¹, and the total retention time was 10 min. The analyte and IS were monitored at 230 nm.

Preparation of Standards and Quality Control Samples

The standard stock solution (1 mg mL⁻¹) of DLM and IS was prepared in acetonitrile and was used to prepare working standard solutions. Working standard solutions of required concentration range of DLM for calibration and controls were prepared by serial dilution in mobile phase. The calibration standards were prepared with concentrations at 20, 50, 200, 400, 800, 1200, 1600, and 2000 ng mL⁻¹ for plasma, thymus, and spleen. Quality control (QC) samples at low (LQC), medium (MQC), and high (HQC) levels were prepared at the concentrations of 100, 500, and 1500 ng mL⁻¹. A 500 ng mL⁻¹ IS working solution was prepared by diluting its stock solution with diluent. All solutions described above were kept stored under refrigeration at 4 °C.
Extraction of DLM from Plasma, Spleen, and Thymus

A simple liquid–liquid extraction was used for the extraction of DLM from mice plasma, spleen, and thymus tissues. Aliquots of 50 μL (plasma) or 20 μL (spleen/thymus sample) were spiked with 10 μL of IS (500 ng mL⁻¹) and treated with 1.5 mL mixture containing diethyl ether and dichloromethane (50:50, v/v). After centrifugation at 4000g for 5 min, the upper organic layer was separated, transferred to a separate test tube, and evaporated to dryness at 40 °C under a gentle stream of nitrogen. The dried residue was reconstituted with 150 μL of mobile phase and vortex-mixed for 2 min, and 20 μL sample was injected into HPLC system.

Method Validation

The developed method was validated for sensitivity, selectivity, linearity, accuracy, precision (intra-day and inter-day), recovery, matrix effect, and stability, according to the FDA guidelines for bioanalytical method validation [38]. The selectivity was investigated by analyzing processed blank plasma or spleen/thymus tissues from six individual mice to test for interference at the retention time of all analytes and the IS. Matrix effects for analytes were evaluated by comparing the peak areas of analyte in extracted samples of blank plasma or spleen/thymus tissues from six different drug-free mice spiked with known concentrations (at QC levels) with the corresponding peak areas obtained by direct injection of standard solutions [37]. Matrix effects for the IS were also investigated. Recoveries of DLM and IS were determined by comparing the peak area of extracted analyte standard with the peak area of non-extracted standard. Linearity, accuracy, and precision were assessed by analyzing three validation batches. Each batch included a set of calibration standards and six replicates of QC (LQC, MQC, and HQC) levels of plasma and was processed on three separate days. The linearity of each calibration curve was assessed by plotting the peak area ratio (y) of the analytes’ peak areas to the IS peak versus the corresponding concentration (x) of the analytes in the freshly prepared plasma and spleen/thymus sample. The calibration curves were fitted with a weighted one/concentration (1/x) least-squares regression to determine the linearity. Accuracy was expressed as the ratio of the mean calculated concentration against the spiked concentration for each standard multiplied by 100%, and the precision was evaluated by percent relative standard deviation (% RSD). The bench-top, freeze-thaw, and 90 days storage condition (~80 °C) stability
of DLM and IS in spiked plasma samples was investigated. The stability of the analytes and IS in stock solution was also evaluated.

**Application to Pharmacokinetic Study**

Male balb/c mice (5–6 weeks old; 25–30 g) were used for all pharmacokinetic studies. Six young and healthy rodents were obtained from the Laboratory Animal Center, Department of Pharmaceutical Sciences and Technology, Mesra, Ranchi and housed in an environmentally controlled room (temperature: 20 ± 2 °C, humidity: 60 ± 5 °C, 12 h dark/light cycle) for at least 7 days before the experiment. All animal studies described were approved by the institutional animal ethical committee prior to the study. The rodents were given a commercial mice chow and water ad libitum. After a single dose by oral administration of 25 mg kg\(^{-1}\) of DLM to healthy mice (\(n = 6\)), a serial sacrifice sampling protocol was followed for the collection of blood and tissues. Blood samples were collected in heparinized tubes from the inferior vena cava of mice at 0, 0.15, 0.25, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 12, and 24 h post dose. Plasma samples were obtained by collecting the supernatant by centrifuging blood samples at 5000 rpm for 5 min at 4 °C. For collection of thymus and spleen tissue samples, animals were sacrificed by cervical dislocation under anesthesia and tissue samples (spleen/thymus) were collected at 0.15, 0.5, 1, 8, 12, and 24 h after administration. Tissue samples were quickly rinsed with physiological saline solution to remove the blood. After cleaning, samples were blotted with filter paper weighed and then homogenized in two-fold by weight physiological saline solution for all tissue samples using a homogenizer. All the abovementioned samples (plasma or tissues) were stored in −80 °C until further analysis.

**Results and Discussion**

**Optimization of Method**

Different solvent systems such as methanol–water and acetonitrile–water mixtures in different compositions, pumped at different flow rates (in the range of 0.8–1.2 mL min\(^{-1}\)) having variable pH range (3.0–5.0), and at different concentration of buffer (in the range of 5–20 mM) were evaluated. Best results were obtained with acetonitrile–10 mM KH\(_2\)PO\(_4\) mixture in the ratio of 70:30, \(v/v\) (pH adjusted to 3.0 with orthophosphoric acid), at a flow rate
of 1 mL min\(^{-1}\). The effect of column on the analysis of DLM and IS was also evaluated, various columns (C8, C18, phenyl etc.) were tried to get best results in terms of peak shape, retention time, and resolution. Finally, an Agilent Zorbax\textsuperscript{®} C8 (250 mm \times 4.6 mm, i.d., 5 μm) column was chosen to meet the above-mentioned requirements. Several organic solvents (methanol, acetonitrile, mixture of methanol–acetonitrile with water) were tried for the preparation of stock solutions of DLM. Acetonitrile was selected due to greater solubility and stability of analyte and IS in it. Various organic solvents including hexane, diethyl ether, ethyl acetate, and dichloromethane were investigated for the extraction of DLM and IS from plasma, spleen, and thymus samples. Mixture of diethyl ether and dichloromethane (50:50, \(v/v\)) provided the best recoveries with minimum endogenous interferences and was chosen as the extraction solvent.

**Method Validation**

**Linearity and sensitivity**

The response was found linear over a concentration range of 20.0–2000 ng mL\(^{-1}\) in mouse plasma, spleen, and thymus tissues. The calibration curve was created by plotting peak-area ratios of DLM to IS versus its concentrations in mice plasma or spleen/thymus tissues. Weighted \((1/x)\) factor with a regression equation was used to determine the slope, intercept, and correlation coefficient. All standard curves were linear \((r^2 \geq 0.9980)\). The developed method was very sensitive and has an LLOQ value of 10 ng mL\(^{-1}\) for plasma, spleen, and thymus tissue samples.

**Selectivity**

Assay selectivity was performed by analyzing drug-free plasma, spleen, and thymus tissues from six individual mice and evaluation of the peaks that interfered with DLM and the IS. Representative chromatograms were obtained from blank plasma, spleen, and thymus tissues and plasma or spleen/thymus tissues spiked with DLM (at LLOQ level) and the IS (at 500 ng mL\(^{-1}\)) as shown in Fig. 3. No interfering peak was observed in the samples at the retention time of either the analyte or IS. The retention time of DLM and IS was found to be 3.4 min and 4.3 min, respectively.
Fig. 3. Representative chromatograms for deltamethrin and internal standard in mice: (A1) blank plasma, (A2) blank plasma spiked with internal standard (at 500 ng mL$^{-1}$) and deltamethrin at LLOQ, (B1) blank spleen tissue homogenate, (B2) blank spleen tissue homogenate spiked with internal standard (at 500 ng mL$^{-1}$) and deltamethrin at LLOQ, (C1) blank thymus tissue homogenate, (C2) blank thymus tissue homogenate spiked with internal standard (at 500 ng mL$^{-1}$) and deltamethrin at LLOQ, and (D) 0.5 h plasma sample showing deltamethrin peak obtained following 25 mg kg$^{-1}$ oral dose of deltamethrin to mice.
The matrix effects and the extraction recovery were calculated by analyzing six replicates (plasma samples) or three replicates (spleen and thymus samples) at low, medium, and highest concentrations. Matrix effects for plasma or spleen/thymus were in the range of 77–93% for DLM and 78–91% for IS. Absolute recoveries were in the range of 76–93% for DLM and 80–86% for IS. All extraction recoveries and matrix effect values were within limits, suggesting good recoveries and no apparent matrix effect on the developed method (Table I).

Table I. Results of recovery and matrix effect of DLM and IS in mice plasma (n = 6)

| Biological matrix | Nominal conc. (ng mL⁻¹) | Recovery | RSD (%) | Matrix effect (%) | RSD (%) |
|-------------------|-------------------------|----------|---------|------------------|---------|
|                   |                         |          |         |                  |         |
| Plasma            | 100                     | 76.25    | 8.80    | 93.10            | 9.95    |
|                   | 500                     | 75.50    | 9.98    | 91.20            | 8.18    |
|                   | 1500                    | 80.60    | 9.30    | 90.15            | 6.10    |
|                   | 500 (IS)                | 80.50    | 5.15    | 90.90            | 4.06    |
| Spleen            | 100                     | 80.00    | 7.50    | 80.10            | 9.12    |
|                   | 500                     | 82.13    | 8.02    | 80.80            | 7.89    |
|                   | 1500                    | 90.15    | 8.20    | 79.82            | 5.13    |
|                   | 500 (IS)                | 85.12    | 4.49    | 79.50            | 3.95    |
| Thymus            | 100                     | 88.10    | 6.95    | 80.11            | 6.84    |
|                   | 500                     | 89.98    | 9.22    | 77.20            | 9.00    |
|                   | 1500                    | 92.15    | 9.10    | 78.16            | 6.10    |
|                   | 500 (IS)                | 86.10    | 4.48    | 78.22            | 4.99    |

**Precision and accuracy**

Precision and accuracy were determined at low, medium, and high-quality control samples (five replicates, n = 5) of plasma or spleen/thymus tissues (three replicates, n = 3). The precision (RSD < 10%) and accuracy (<15%) of the developed method were determined by analyzing the analytes at three QC (LQC, MQC, and LQC) levels for DLM. The results inferred that the developed method was accurate and precise (Table II).
Table II. Intra- and inter-day precision and accuracy in determination of DLM and IS in mice plasma, spleen, and thymus ($n = 5$ or $n = 3$)

| Biological matrix | Nominal conc. (ng mL$^{-1}$) | Mean conc. found (ng mL$^{-1}$) ± SD | Accuracy$^a$ (%) | Precision$^b$ (%) |
|-------------------|-------------------------------|-------------------------------------|-----------------|-----------------|
| **Plasma**        |                               |                                     |                 |                 |
| **Intra-day ($n = 5$)** | 100  | 80 ± 0.05                       | 80.00           | 8.50            |
|                   | 500  | 410 ± 0.20                      | 82.02           | 5.57            |
|                   | 1500 | 1208 ± 0.95                     | 80.50           | 3.66            |
| **Inter-day ($n = 5$)** | 100  | 82 ± 0.05                       | 82.00           | 8.80            |
|                   | 500  | 407 ± 0.50                      | 81.50           | 4.96            |
|                   | 1500 | 1282.5 ± 1.0                    | 85.50           | 3.50            |
| **Spleen**        |                               |                                     |                 |                 |
| **Intra-day ($n = 3$)** | 100  | 83 ± 0.05                       | 83.05           | 7.50            |
|                   | 500  | 416 ± 0.20                      | 83.20           | 5.10            |
|                   | 1500 | 1350 ± 1.15                     | 90.01           | 2.89            |
| **Inter-day ($n = 3$)** | 100  | 82 ± 0.50                       | 82.50           | 6.50            |
|                   | 500  | 420 ± 0.60                      | 84.60           | 4.99            |
|                   | 1500 | 1344 ± 1.15                     | 89.60           | 3.66            |
| **Thymus**        |                               |                                     |                 |                 |
| **Intra-day ($n = 3$)** | 100  | 84 ± 0.50                       | 80.50           | 6.30            |
|                   | 500  | 421 ± 0.20                      | 84.24           | 4.95            |
|                   | 1500 | 1351 ± 1.15                     | 90.14           | 3.66            |
| **Inter-day ($n = 3$)** | 100  | 84 ± 1.50                       | 85.50           | 6.82            |
|                   | 500  | 418 ± 0.20                      | 83.64           | 4.89            |
|                   | 1500 | 1350 ± 1.15                     | 90.01           | 3.12            |

$^a$Expressed as [(mean observed concentrations/nominal concentrations) × 100].

$^b$Expressed as relative standard deviation (RSD).

**Stability**

The stability was investigated for three storage conditions. The stability of plasma, spleen, and thymus samples was investigated for bench-top, three freeze–thaw cycles, and long term at −80 °C for 90 days. Stability of all the stock solutions was also investigated. All samples were found to be stable at the abovementioned stability conditions (Table III).
Table III. Stability of DLM after storage under indicated condition (mean % ± S.D., n = 3)

| Biological matrix | Nominal conc. (ng mL⁻¹) | Bench-top stability at 24 °C | Freeze-thaw stability (3 cycles) | Long term stability at 80 °C (90 days) |
|-------------------|-------------------------|----------------------------|---------------------------------|--------------------------------------|
|                   | Conc. found (ng mL⁻¹)± SD | CV (%)                     | Conc. found (ng mL⁻¹)± SD | CV(%)                             |
|                   |                         |                            | Conc. found (ng mL⁻¹)± SD | CV (%)                             |
|                   |                         |                            | Conc. found (ng mL⁻¹)± SD | CV (%)                             |
|                   |                         |                            | Conc. found (ng mL⁻¹)± SD | CV (%)                             |
| Plasma            | 100                     | 81 ± 0.01                  | 8.81                           | 81 ± 0.20                          | 8.69                                | 79 ± 0.50                          | 8.10                              |
|                   | 500                     | 415 ± 0.30                 | 5.30                           | 412 ± 0.53                         | 5.90                                | 400 ± 0.15                         | 5.85                              |
|                   | 1500                    | 1210 ± 0.10                | 2.01                           | 1355 ± 1.19                        | 2.18                                | 1200 ± 0.23                        | 2.80                              |
| Spleen            | 100                     | 82 ± 0.50                  | 6.92                           | 80 ± 0.14                          | 6.81                                | 79 ± 0.05                          | 6.10                              |
|                   | 500                     | 415 ± 0.20                 | 4.49                           | 418 ± 0.20                         | 4.50                                | 400 ± 0.20                         | 3.46                              |
|                   | 1500                    | 1344 ± 1.15                | 1.19                           | 1350 ± 1.85                        | 1.20                                | 1315 ± 1.15                        | 1.13                              |
| Thymus            | 100                     | 82 ± 0.10                  | 5.91                           | 83 ± 0.05                          | 5.82                                | 78 ± 0.06                          | 6.99                              |
|                   | 500                     | 410 ± 0.15                 | 4.35                           | 417 ± 0.30                         | 4.48                                | 399 ± 0.65                         | 5.59                              |
|                   | 1500                    | 1329 ± 1.13                | 1.18                           | 1360 ± 1.15                        | 1.32                                | 1338 ± 1.50                        | 1.85                              |

CV (%), expressed as relative standard deviation (RSD).

Pharmacokinetic and Tissue Distribution

Since a good understanding of pharmacokinetic information is necessary to explain a variety of events related to drug efficacy and toxicity, a simple and sensitive corresponding analytical method of DLM in biological samples is undoubtedly required. The present method has been applied successfully to the pharmacokinetic studies and tissue distribution (spleens and thymus) of DLM after an oral administration (25 mg kg⁻¹) in mice as shown in Fig. 4. The main pharmacokinetic parameters assessed using the non-compartmental method (WinNonlin software version 6.3; Pharsight Corporation, Mountain View, CA, USA) are summarized in Table IV. These results have shown that DLM absorbed rapidly and reached to systemic circulation in a very short time after oral administration in mice. DLM concentration in spleen was found to be more followed by thymus.
Fig. 4. Concentration–time profile of deltamethrin in mice plasma (A), spleen (B), and thymus (C) following oral dosing of 25 mg kg\(^{-1}\) of deltamethrin to mice

Table IV. Pharmacokinetic parameters of DLM in plasma, spleen, and thymus

| Parameters            | Plasma (h × ng mL\(^{-1}\)) | Spleen (h × ng mL\(^{-1}\)) | Thymus (h × ng mL\(^{-1}\)) |
|-----------------------|------------------------------|-------------------------------|-----------------------------|
| AUC (0–48 h)          | 2640                         | 742                           | 494.50                      |
| AUC\(_{\text{inf}}\)  | 2693.36                      | 859.01                        | 753.19                      |
| C\(_{\text{max}}\)    | 800.10                       | 60                            | 50                          |
| T\(_{\text{max}}\)    | 1.0                          | 1.0                           | 1.0                         |
| T1/2(\(z\))          | 3.69                        | 8.11                          | 17.93                       |
| MRT (0–48 h)          | 4.30                         | 18.02                         | 21.09                       |

Conclusion

Although the acute toxicity of DLM is well characterized, there is a lack of published simple and reliable analytical methods to support pharmacokinetic studies of this compound. The developed method has various advantages as compared to previously published methods, such as inexpensive, simple extraction procedure from small volume of plasma or tissues, shorter run time, and full validation (which support pharmacokinetic studies of deltamethrin). Moreover, the endogenous matrix substances (plasma or tis-
sues) did not interfere with the elution time of analyte and internal standard, which showed the high specificity of the method. Previously published methods based on HPLC have several disadvantages such as longer run time (in some methods, the run time was more than 25 min), high level interferences from the biological matrix with the analyte(s) and IS, less sensitivity, low accuracy and precision, and low recoveries as compared to the developed method [35, 38]. Even though LC–MS/MS or GC–MS/MS methods are more sensitive, required less volume of sample injection, and have short run time as compared to the developed method [18], still, the developed analytical method is sensitive enough to study the pharmacokinetic and toxicokinetic parameters of deltamethrin. Thus, this analytical method can be utilized in toxicokinetic investigations of deltamethrin.

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