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# Method Validation for the Determination of Ciprofloxacin in *Lucilia sericata* Larvae via Capillary Electrophoresis Combined With Mass Spectrometry

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**Keywords:** capillary electrophoresis | ciprofloxacin | *Lucilia sericata* | mass spectrometry | optimization

## ABSTRACT

Substances derived from insects can serve therapeutic functions due to their diverse biological properties. This article focuses on the species *Lucilia sericata* and the benefits of larval therapy in patients who, due to hospitalization, have developed pressure ulcers and other difficult-to-heal wounds. Larval therapy, also known as maggot debridement therapy, employs sterile fly larvae to treat chronic, non-healing wounds by enzymatically degrading necrotic tissue and decreasing bacterial colonization. The larvae are applied to the wound for a period of 48–72 h, during which they effectively clean the wound and stimulate tissue regeneration. This therapeutic approach is particularly efficacious for recalcitrant wounds, such as diabetic foot ulcers and pressure sores, which have not responded to conventional treatments. Larvae may also constitute an alternative material in entomotoxicological studies to detect substances ingested at not only toxic but also therapeutic doses. The present work describes a method for assaying ciprofloxacin in *L. sericata* larvae using capillary electrophoresis coupled to mass spectrometry. The developed method features high sensitivity with a limit of quantification of 100 ± 0.018 ng/mL, as well as accuracy and precision estimated within 87%–103% and 1%–4%, respectively. An application of a simple and fast precipitation of proteins procedure for sample cleaning resulted in a highly satisfactory recovery of the analyte (90%–104%). The method was linear in a range of 100–1000 ng/mL with a determination coefficient higher than 0.9973. The method was used to determine ciprofloxacin in larval homogenate after antibiotic administration to the patient at a dose of 500 mg twice daily per os during application of the larvae dressing. Ciprofloxacin was shown to distribute from the patient's circulation to the larvae at a concentration of 150 ng/mL (750 ng/g).

## 1 | Introduction

The use of insects in medicine is a practice that dates back to antiquity. For centuries, people have used representatives of different species from almost every taxonomic group for medicinal purposes and valued the health properties of their derived products. The medicinal properties of the larvae are still used in some cultures today [1–3].

**Abbreviations:** BGE, background electrolyte; CC, calibration curve; CIP, ciprofloxacin; DAD, diode array detector; ESI, electrospray ionization; LE, leading electrolyte; LOD, limit of detection; LOQ, limit of quantification; QC, quality control; TE, terminating electrolyte; tITP, transient isotachophoresis.

Modern research studies on natural medicine and increased interest in alternative treatments have led to a return to these ancient practices (e.g., larval therapy) [2]. The maggots, most commonly from the species *Lucilia sericata*, are grown under special laboratory conditions and then applied to the wound. After a few days, they remove necrotic lesions, and healthy tissue begins to regenerate. This occurs due to the ability of the larvae to actively clean the wound by secreting proteolytic enzymes, which are responsible for liquefying the necrotic lesion. Consequently, the liquid mass becomes a food source for the maggots [4–6]. An important aspect of wound healing is also maintaining proper asepsis. The larval secretions contain substances such as allantoin [7] that eliminate bacteria settled in the wound, as well as antibacterial peptides [8]. When the wound is clean, free of necrosis and bacteria, it is stimulated to heal through the release of growth factors, which lead to the proliferation of dermis cells and the epithelium. Studies have shown that substances present in larval secretions induce morphological changes that stimulate fibroblast migration and accelerate the reconstruction of the extracellular matrix, which promotes wound healing [9].

When a dressing prepared from larvae is applied to a patient's wound, the drugs taken by the patient can penetrate from the patient's circulation to the larvae [10]. It is therefore believed that the ability to determine exogenous substances in larvae leads to new diagnostic and research perspectives.

Another important point is the use of insects in the area of forensic toxicology. Forensic entomology, which is part of forensic medicine, uses knowledge of insects to determine the time and place of a crime [11]. In addition, necrophagic insects are tested for xenobiotics that may be ingested by larvae together with soft tissues, providing information on a potential cause of death [12].

This article focuses on the application of larvae as an alternative material in therapeutic drug monitoring, emphasizing the importance of this material in both the determination of drug intake at therapeutic doses and confirmation of drug overdoses. The study aimed to optimize and validate the method for determination of ciprofloxacin (CIP) in *L. sericata* larvae using capillary electrophoresis (CE) coupled to mass spectrometry (MS). The method was applied for determination of CIP in *L. sericata* larvae applied as a dressing for a decubitus wound in a patient receiving antibiotic therapy.

Ciprofloxacin is a broad-spectrum fluoroquinolone antibiotic used to treat various bacterial infections, including urinary tract, respiratory, gastrointestinal and skin infections. It is particularly effective against Gram-negative bacteria. In the case of this antibiotic routine, therapeutic drug monitoring is not performed but could prove necessary in critically ill patients, those with severe renal impairment or those being treated for multidrug-resistant infections [13].

## 2 | Materials and Methods

### 2.1 | Reagents and Materials

Ciprofloxacin (HPLC grade, ≥98% purity), the internal standard (IS), 2-(4-methyl-1-piperazinyl)-4-phenylquinazoline, HPLC grade water, acetonitrile, methanol, ammonium acetate, sodium hydroxide, ammonium hydroxide and formic acid were purchased from Sigma-Aldrich (Steinheim, Germany). Live fly larvae used as a control matrix were purchased from Biollab (Kędzierzyn-Koźle, Poland). Live fly larvae were also sourced from the dressing material (Biomantis, Krakow, Poland), which was applied to the patient's wound for 24 h during CIP therapy. Larvae were kept in physiological saline (pH = 7.4) at −20°C. Larvae extracted from the dressing has been classified as medical waste under category 18 01 04 according to the Waste Act issued on 14 December 2012.

### 2.2 | Instrumentation

#### 2.2.1 | Capillary Electrophoresis

Electrophoretic separations were performed using an Agilent 7100 CE system equipped with a diode array detector (DAD; Agilent Technologies, Waldbronn, Germany). ChemStation software and Lab Advisor/Instrument Utilities were used for equipment control, data collection and processing.

To optimize electrophoretic conditions, a fused silica capillary with an internal diameter of 50 μm and a total length ($L_{tot}$) of 70 cm, supplied by an Agilent Technologies, was used. Prior to the analyses, the capillary was flushed with 1 M NaOH for 20 min, followed by ultrapure water (10 min) and 40 mM ammonium acetate used as background electrolyte (BGE) for 20 min. Moreover, prior to each analytical run, the capillary was rinsed with a 0.1 M solution of sodium hydroxide (10 min) followed by ultrapure water (15 min) and then flushed with a BGE for 20 min. At the end of the analysis, the capillary was cleaned with methanol and deionized water for 4 min and left in an empty vial.

The BGE of 40 mM ammonium acetate, prepared by the appropriate dilution of 100 mM ammonium acetate with ultrapure water and brought to pH = 8.5 via the addition of ammonium hydroxide, was used. To ensure the adequate separation of the analytes in the capillary, a voltage of 27 kV was applied. CIP and IS solutions were loaded into the capillary at 30 mbar for 10 s. During the analysis, the room temperature was maintained at 22°C with the use of air conditioning.

#### 2.2.2 | Mass Spectrometry

CIP detection was performed using a system comprising CE apparatus, a single-quadrupole MS (1260 Infinity II Preparative LC/MSD System) with ionization induced by an electric field in combination with spraying (electrospray ionization, ESI). The sheath liquid was 1% formic acid in 50/50 (v/v) methanol/water, delivered by a pump equipped with a 1:100 splitter. The flow of heated dry nitrogen (200°C) as nebulizer gas was maintained at 10 L/min at 10 psi. ESI–MS was conducted in the positive ion mode, and the capillary voltage was set at −4 kV.

The analyses were performed in quadrupole scanning (SCAN) and selected ion monitoring (SIM) modes to obtain the appropriate mass spectrum for CIP and the IS. During the SCAN mode, the

monitored masses ranged from $m/z$ 100 to 1000, whereas in SIM mode, the following masses were selected: $m/z$ 331 (CIP mass), $m/z$ 332 (CIP mass after protonation) and $m/z$ 305 (IS mass).

## 2.3 | Sample Preparation

### 2.3.1 | Ciprofloxacin Solutions

Working CIP solutions at concentrations of 1000, 2500, 5000, 7500 and 10 000 ng/mL prepared via appropriate dilutions of the 1 mg/mL CIP stock solution in deionized water were used to generate the calibration curve (CC). Working CIP solutions at concentrations of 2000, 4000 and 8000 ng/mL, also prepared by appropriate dilutions of the CIP stock solution in deionized water, were used as the quality control (QC) samples to determine the precision, recovery and accuracy of the method.

### 2.3.2 | Matrix-matched Calibration and QC Samples

The CC of CIP in the larvae homogenate was prepared at concentrations of 100, 250, 500, 750 and 1000 ng/mL. Here, 90 µL of the larval homogenate has been pipetted into Eppendorf tubes, 10 µL of the respective CC working solutions were added and mixed thoroughly and 10 µL of the IS working solution at a concentration of 5 µg/mL was added, followed by incubation for 10 min at 4°C. To precipitate the proteins, 100 µL of acetonitrile was added, and the mixture was stirred for 20 min. The samples were then centrifuged (11 200 × $g$) for 10 min at 4°C, and 100 µL of supernatant was collected. The procedure was repeated twice, after which 100 µL of the supernatant was evaporated to dryness under nitrogen, and the dry residue was dissolved in 100 µL of deionized water. The sample was then centrifuged (11 200 × $g$) for 10 min at 4°C, and the sampled supernatant was subsequently analysed.

The QC samples of CIP in the larvae homogenate were prepared at the concentrations of 200, 400 and 800 ng/mL. After pipetting 90 µL of the larval homogenate to the Eppendorf tubes, 10 µL of the respective QC working solutions were added and mixed thoroughly, followed by the addition of 10 µL of the IS working solution at a concentration of 5 µg/mL. Subsequently, the sample preparation process was continued as described for the CC samples. The QC samples were used to determine the precision, accuracy, recovery and matrix effect (ME) of the method.

### 2.3.3 | Larvae Homogenate

The frozen larvae have been placed at room temperature until defrosted, collected with tweezers, weighed, mixed in deionized water in a 1:5 ratio and then ground with a tissue homogenizer.

## 2.4 | Method Validation

The method for the determination of CIP in the larval matrix was validated, including an assessment of the linearity, limit of detection (LOD), limit of quantification (LOQ), precision, accuracy, recovery and ME.

In this regard, to determine the linearity of the method, five concentration levels—100, 250, 500, 750 and 1000 ng/mL for CIP in the larval matrix—were used. The linearity was assessed based on a regression line and the coefficient of determination ($R^2$), with an acceptable value greater than 0.9973.

The lowest analyte concentration that can be reliably detected (LOD) was determined at a signal-to-noise ratio of 3:1. Similarly, the lowest analyte concentration that can be accurately quantified with acceptable precision and accuracy (LOQ) was evaluated at a signal-to-noise ratio of 10:1.

Three samples were prepared for each concentration of the QC samples (200, 400 and 800 ng/mL) and, thereafter, sequentially analysed to determine the precision, recovery and accuracy of the method and whether an analyte transfer effect was present.

The intraday accuracy (%A) and precision as relative standard deviation (%RSD) were determined by analysing three independent replicates of QC samples at each concentration level, performed within the same day across two separate analytical runs. Accuracy refers to the closeness of the measured values to the true value, expressed as a percentage, whereas precision reflects the consistency of the results, represented as the %RSD of the measurements.

The recovery of analytes [%RE] was assessed by comparison of the detector response for QC samples at three concentration levels before and after sample preparation in the larval matrix. The recovery was evaluated based on measurements of three independent replicates of QC samples per level.

The ME was calculated by comparing the responses (peak area) of CIP in the blank larval homogenate with the responses of CIP in the BGE solutions at the QC concentrations.

## 2.5 | Clinical Case Study

A 77-year-old male, weighing 70 kg, was hospitalized with severe respiratory failure. The patient was intubated, a tracheostomy tube was inserted and percutaneous endoscopic gastrostomy was performed for enteral feeding. The patient was discharged from the hospital after 4 months with a third-degree pressure ulcer. He remained under the constant care of his family and a health nurse. During his stay at home, a swab was taken from the pressure ulcer, and *Pseudomonas aeruginosa* was grown in the test medium.

Due to the infection, a targeted treatment based on the specific type of pathogen and its drug susceptibility was implemented. CIP was administered at a dose of 500 mg twice daily for 10 days. At the same time, according to the doctor's recommendations, dressings containing live cultures of sterile *L. sericata* larvae were applied (Biomantis, Krakow, Poland) to a pressure sore wound to eliminate necrotic lesions and eradicate bacteria. Larval therapy improved the cleaning of the pressure sore and shortened its healing period. In addition, together with antibiotic therapy, it allowed for faster elimination of the infection caused by *P. aeruginosa*.

**TABLE 1** | Conditions of the analysis after the method optimization.

| Parameters subject to optimization | | Analysis conditions |
|---|---|---|
| Capillary | Material | Unmodified silica |
| | Diameter Internal/External | 50 µm/375 µm |
| Temperature | Sample holder | 22°C |
| | Capillary | 23°C |
| Capillary conditioning | Eluent sequence | 0.1 M NaOH |
| | | Ultrapure water |
| | | 40 mM ammonium acetate, pH = 8.5 |
| | Total conditioning time | 50 min |
| Dosing | Duration | 10 s |
| | Pressure | 30 mbar |
| Separation | Voltage | 27 kV |
| | Time of analysis | 16 min |
| | Separation buffer | 40 mM ammonium acetate, pH = 8.5 |
| Mass spectrometer | Sheath liquid | Methanol/Water/ Formic acid (100/98/1) (v/v/v) |
| | Drying gas flow | 10 L/min |
| | Nebulizer pressure | 10 psi |
| | Drying gas temperature | 200°C |

## 3 | Results

### 3.1 | Method Optimization

The optimization of the CIP assay included the selection of appropriate analytical conditions, namely, the sample volume, capillary voltage, capillary conditioning method and quadrupole mode of operation. The final conditions of the analysis after the method optimization are presented in Table 1.

To determine the optimal migration times for CIP and the IS, the following voltages were used: 25, 27 and 30 kV. CIP migrated at a comparable rate relative to the applied voltage, whereas migration of IS showed a greater dependence on voltage. The migration time at 25 kV was increased; thus, to obtain the fastest possible analysis, the default voltage value of 27 kV was applied, as proposed by the equipment manufacturer. The application of higher voltage was waived due to a risk of Joule heat generation.

In CE, the pH of the buffer is of key importance, affecting the charge of the compounds and their migration and separation in the capillary. To ensure the adequate separation of analytes in the capillary, BGE buffer made of ammonium acetate at pH values ranging from 8 to 9 and concentrations ranging from 10 to 40 mM were used. On the basis of literature data [14, 15], the chemical characteristics of the substances, the p$K_a$ value of CIP, which are p$K_{_COOH}$ = 5.76 and p$K_{_NH3+}$ = 8.68 and our own experimental studies, the pH of BGE buffer was adjusted to 8.5 and the concentration of BGE buffer to 40 mM.

To obtain the appropriate peak sizes, a sample of CIP along with the IS was hydrodynamically loaded into the capillary at pressures of 20, 30 and 50 mbar, respectively. Because, at 50 mbar pressure, supersaturated peaks of CIP were obtained, thus the sample was loaded at 30 mbar for 10 s.

Another aspect of the work was selecting the mode of operation of the MS. The SIM mode is particularly useful when specific molecular weights are crucial for identification. However, in situations requiring a complete overview of the molecular weights in a sample—for example, when searching for unknown compounds—the SCAN mode may be more appropriate, although it may be less sensitive and precise in identifying specific mass. Finally, the SIM mode was selected due to the ampholyte nature of CIP. Changing the quadrupole operation to SIM mode led to a significant improvement in the baseline seen as noise reduction and lowering of its level. Non-specific peaks were also eliminated, and peaks corresponding to CIP and IS were obtained.

### 3.2 | Linearity, CC, LOD and LOQ

The method was linear from 100 to 1000 ng/mL. The CC for CIP in the larval matrix was characterized by a high coefficient of determination ($R^2$ > 0.9973) with the LOQ equal to 100 ± 0.018 ng/mL.

### 3.3 | Precision, Accuracy, Recovery and ME

The precision, accuracy, recovery and ME of the method were evaluated at three different concentrations of QC samples analysed in triplicate. Table 2 presents the results of the analyses.

Considering the influence of the larval matrix, the precision was 1%–4%, the recovery fell in the range of 90%–104% and the accuracy of the method was within the ranges of 87%–103%. To determine whether the larval matrix may affect the determination of the drug, the impact of the matrix has been studied. The ME was irrelevant for the assay (ME = −6.37%–3.95%). The results of the method validation confirm that the developed method meets the criteria and can be used routinely to determine the concentration of CIP in the larval matrix.

### 3.4 | Application of the Method

The validated method was used to determine the CIP concentration in larvae applied as a dressing to a decubitus wound. Figure 1 depicts example electropherograms in SIM mode of $m/z$ 331 and $m/z$ 332 for CIP in larval matrix (A), aqueous solutions (B) and sample of larvae homogenate living on the patient's wound (C), respectively.

**TABLE 2** | Accuracy, precision and recovery of the method for ciprofloxacin (CIP) determination in larval homogenate.

| CIP concentration [ng/mL] | Series | Repetition | Accuracy [%A] | Precision [%RSD] | Recovery [%RE] |
|---|---|---|---|---|---|
| 200 | 1 | 1 | 103 | 1 | 101 |
| | | 2 | 99 | | 97 |
| | | 3 | 101 | | 99 |
| | 2 | 1 | 98 | 1 | 96 |
| | | 2 | 100 | | 98 |
| | | 3 | 95 | | 92 |
| 400 | 1 | 1 | 99 | 1 | 101 |
| | | 2 | 95 | | 96 |
| | | 3 | 96 | | 97 |
| | 2 | 1 | 101 | 3 | 102 |
| | | 2 | 94 | | 96 |
| | | 3 | 102 | | 104 |
| 800 | 1 | 1 | 96 | 4 | 99 |
| | | 2 | 87 | | 90 |
| | | 3 | 94 | | 97 |
| | 2 | 1 | 90 | 2 | 93 |
| | | 2 | 93 | | 97 |
| | | 3 | 95 | | 99 |

Abbreviation: RSD, relative standard deviation.

Different migration times were evident for CIP in the larval matrix (10.7 min, Figure 1A) compared to aqueous solutions (9.1 min, Figure 1B). Considering the collected information, CIP was determined in the *L. sericata* larvae applied to a patient's wound as a dressing (Figure 1C). The peak, which migrates over approximately 10.7 min, corresponds to the drug. On the basis of the CC, the concentration of the antibiotic has been determined at 150 ng/mL (750 ng/g).

## 4 | Discussion

In optimizing the CIP assay conditions using CE/MS, the voltage selection, buffer pH, capillary conditioning method and dosed sample volume were considered. The mode of operation of the quadrupole and the method of collecting the masses of the compounds were also tested. The SIM mode increased the resolution of the method, reduced the occurrence of noise and stabilized the baseline, facilitating the reading of the results. CIP is an amphoteric drug and can, therefore, both donate and accept a proton under the appropriate conditions. The optimal selected weights were $m/z$ 331 and $m/z$ 332, respectively.

The parameter directly affecting the speed of the analysis is the voltage. The higher the voltage, the faster CIP migration, which speeds up the analysis. However, one should note that an increased voltage implies a risk of producing Joule heat, which may lead to undesirable effects. Therefore, a voltage of 27 kV was applied, despite obtaining a faster migration time for the drug at higher voltages.

The CC for CIP in the larval matrix was characterized by a high coefficient of determination ($R^2 > 0.9976$). Considering the influence of the larval matrix, the precision was 1%–4%, the repeatability fell in the range of 1%–5%, and the accuracy and recovery of the method were within the ranges of 87%–103% and 92%–104%, respectively. The above results confirm that the developed method is suitable for CIP determination in complex samples of the larval matrix.

The larval matrix delayed CIP migration by approximately 2 min relative to the aqueous drug solutions. The observed changes in the rate of migration were likely due to the formation of hydrogen bonds between CIP and the matrix components. The resulting complexes delayed the analyte's migration and were subsequently dissociated at a later stage of the analysis in the mass detector under the influence of ESI.

On the basis of the conclusions obtained, CIP was determined in the larval matrix. The tested drug migrated over a period of approximately 10.7 min, and its concentration in larvae homogenate was 150 ng/mL (750 ng/g). The presence of CIP in the larval sample originates from the antibiotic distribution from the patient circulation. CIP was administered to patient at a dose of 500 mg twice daily during the larval therapy, and according to the literature data corresponding to CIP plasma concentration of 1.5–2.9 μg/mL [16].

To date, no studies have been conducted to determine the concentration of CIP in larvae using any other available analytical technique. However, due to CIP wide application in human and

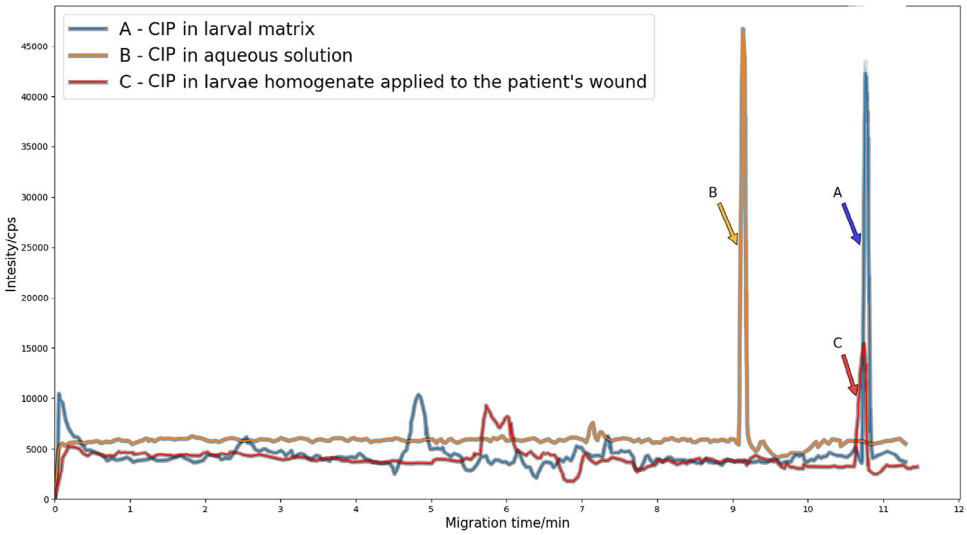


**FIGURE 1** | Example electropherograms for CIP at the concentration of 750 ng/mL added to the larval matrix (A), aqueous solution of CIP at the concentration of 750 ng/mL (B) and the larvae homogenate sample which were living on the patient's wound (C) where the concentration of a five-fold diluted sample was 150 ng/mL (750 ng/g). Separation conditions: ammonium acetate (40 mM, pH = 8.5), applied voltage: 27 kV, temperature: 23°C, injection pressure: 30 mbar, injection time: 10 s, MS detection, capillary: 70 cm × 50 μm. CIP, ciprofloxacin.

veterinary medicine, CIP is often quantified in various biota samples using a range of chromatographic techniques, including HPLC–UV [17, 18], HPLC–DAD [19, 20] and LC–MS/MS [21–23]. Concentration of CIP in the environment results from intensive use of this antibiotic both in humans and animals, which leads to its penetration into waters, soils and other ecosystems.

LC–MS/MS is a predominant analytical technique employed for the determination of CIP. This is exemplified by a study conducted in Northern Poland, where LC–MS/MS was used to detect the antibiotic in water and fish tissue samples from nearby rivers [21]. The authors point out that the concentration range for tissue samples was narrower than for water samples, which was probably due to the greater complexity of tissue matrix compared to water samples. These observations indicate that the analysis of complex samples presents an analytical challenge across multiple dimensions, as was evident in these studies. Comparing CE and LC, both methods demonstrate equivalent performance in terms of high sensitivity, precision and ability to accurately quantify trace levels of antibiotics. Regarding the detection method for CIP, this research employed a single quadrupole, which also presents certain limitations. In the investigation of antibiotic detection in urine and wipe samples for environmental and biological monitoring, HPLC–UV, LC–MS and LC–MS/MS are employed, and their results are comparable [24]. The authors recommend the use of MS/MS for the analysis of complex matrices, such as urine, due to their intricate nature and low expected concentrations. Although UV and single-MS detection methods are suitable for control measurements of wipe samples, LC–MS/MS is deemed essential for accurate and precise analysis in biological monitoring.

CIP is a broad-spectrum fluoroquinolone antibiotic, well absorbed orally with a bioavailability of 70%–80%, reaching peak plasma concentrations within 1–2 h. The drug is widely distributed in body tissues and fluids, binding to plasma proteins at a rate of 20%–40%. The total volume of distribution is 2–3 L/kg. CIP undergoes limited liver metabolism and is primarily excreted through the kidneys, with 40%–50% excreted in the urine in unchanged form. Elimination half-life of CIP is 4–6 h in individuals with normal renal function but longer in patients with renal impairment, requiring dosage adjustments. These properties make CIP effective in treating various bacterial infections [25]. CIP is characterized by a high capacity for distribution and penetration in tissues, which is associated with comparable or sometimes higher concentrations of the drug in tissues than in serum [26, 27]. Therefore, in our opinion, larvae can be used as an alternative material in entomotoxicological analyses to confirm both drug overdoses [10, 28] and drug intake at therapeutic doses.

The potential for combining larval therapy with antibiotic treatment lies in the synergistic effects of both methods. Larval therapy excels in mechanically debriding necrotic tissue and reducing bacterial load through the secretion of antimicrobial substances, thereby creating a cleaner wound bed that enhances the efficacy of systemic antibiotics. Additionally, this combination could potentially reduce the required dosage of antibiotics, thereby minimizing the risk of antibiotic resistance and adverse effects. Although currently rare in clinical practice, integrating these strategies could offer a more comprehensive approach to managing complex, chronic wounds, improving healing outcomes, and reducing treatment times.


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### Conflicts of Interest

The authors declare no conflicts of interest.

### Data Availability Statement

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

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