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# DETERMINATION OF FIPRONIL IN BOVINE PLASMA BY SOLID-PHASE EXTRACTION AND LIQUID CHROMATOGRAPHY WITH ULTRAVIOLET DETECTION

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A fast and efficient method has been developed and validated for the determination of fipronil in bovine plasma. Samples were subjected to solid-phase extraction (SPE) followed by reversed phase liquid chromatography (LC) separation, using acetonitrile/water (60:40 v/v) as the mobile phase with a flow rate of 1.0 mL/min and ultraviolet (UV) detection at 210 nm. Ethiprole was used as the internal standard (IS). The method was found to be linear over the range 5-500 ng/mL (r = 0.999). The limit of quantitation (LOQ) was validated at 5 ng/mL. The method was successfully applied to monitor plasma concentrations following subcutaneous administration of fipronil in cattle.

Keywords: fipronil; bovine plasma; LC-UV.

## INTRODUCTION

Fipronil is a broad-spectrum phenylpyrazole recommended for agricultural, phytosanitary and veterinary uses.[1] Its mechanism of action involves neurotransmitter γ-aminobutyric acid (GABA) block[2] via chloride channels resulting in insect death.[3] GABA plays an important role in neural transmission of both vertebrates and invertebrates, however fipronil presents some selectivity since its binding with GABA receptor is weaker in vertebrates.[4]

Currently, fipronil is commercially available only in topical forms for tick control in cattle. The indiscriminate use of pesticides leads to the emergence of resistant tick active molecules. This resistance drives the search for new compounds with other therapeutic targets and novel mechanisms of action. However, the limited size of the market along with animal health problems related to the resistance and adverse effects of acaricide compounds reduce the number of new molecules introduced. Possible innovations include the development of new pharmaceutical forms that promote a broader spectrum of action, ease of use, and safety for the environment and the applicator.[1]

The therapeutic action of drugs is dependent on their effective concentration at the site of action for a given period of time. Once a drug concentration at the site of action is in equilibrium with the same concentration in the bloodstream, for most drugs, the measurement of drug concentration in plasma becomes its measurement at its site of action. The drug availability from the dosage form plays a critical role in a drug's clinical efficacy. Therefore, the drug's plasma profile from dosage form studies is crucial in assessing the performance of new formulations. Fipronil plasma profile studies through subcutaneous administration enable the characterization of pharmacokinetic parameters and provides the basis for studies related to dose adjustment. Correlated with efficacy tests, plasma profile studies can support the development of new formulations containing fipronil to control ticks.

For plasma profile analysis, it is necessary to develop an analytical method for quantification of fipronil in plasma. Due to its extensive use as a pesticide in agriculture, a large number of studies on the determination of fipronil in water, soil and food samples is available. Fipronil has been detected in soil and water samples by gas chromatography with mass spectrometric detection (GC-MS),[5,6] LC-UV[7] and GC with electron-capture detection (ECD).[8] Its determination has also been widely described in honeybees, honey and pollen samples by GC with nitrogen phosphorous detection (NPD),[9] LC with tandem mass spectrometry detection (MS/MS),[10] GC-ECD/MS[11-13] and LC-UV.[14] Methodology to determine fipronil in milk samples by GC-MS/MS[15] and animal tissues by GC-ECD[16] has also been reported. Nevertheless, there are few studies that describe determination of fipronil in plasma samples using GC-MS/MS,[17] LC-UV/MS,[18] and GC-MS.[19] Concerning sample preparation, liquid-liquid extraction (LLE)[6,7] and SPE[10,12,15-18] are generally used, however solid-phase micro extraction (SPME),[5] gel permeation chromatography (GPC),[8] Quick, Easy, Cheap, Effective, Rugged, and Safe (QuEChERS)[14] and matrix solid-phase dispersion (MSPD)[9,11,13,19] have also been used.

In analytical methods, the customary procedure is the IS method, where a compound that is not part of the sample (IS) is added at known and constant concentrations to the sample before performing the analysis. In bioavailability studies, IS use is recommended when chromatographic methods are used.[20] Ethiprole is a phenylpyrazole pesticide that differs structurally from fipronil by a group $–C_2H_5$ rather than a $–CF_3$ on sulfinyl function[21] (Figure 1). Because of this structural similarity, it has been used as an IS in methods developed for fipronil determination in animal plasma.[18]

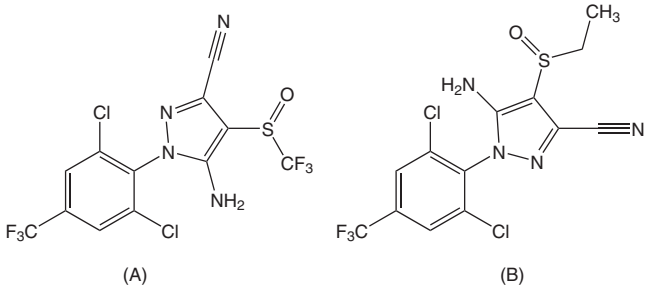


***Figure 1.*** *Chemical structure of (A) fipronil and (B) ethiprole (IS)*

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In order to assess whether the method produces reliable results, so that it can be applied in routine analysis, it is necessary to perform the validation of the developed method. Validation is the evaluation of an analytical process's ability to produce consistent results with satisfactory precision and accuracy. The parameters usually involved in bioanalytical method validation are stability, selectivity, linearity, limit of quantitation, accuracy and precision.[20,22]

The aim of the present study was to develop and validate a fast and efficient analytical method for fipronil determination in bovine plasma using SPE and LC-UV. The method was applied to a bovine plasma sample collected after subcutaneous administration of fipronil.

## EXPERIMENTAL

### Chemicals and reagents

HPLC grade solvent methanol and acetonitrile were purchased from Tedia (Ohio, USA). HPLC grade water was obtained using a Gehaka ultra purified system (Gehaka, São Paulo, Brazil). Fipronil (97.5%) and Ethiprole (98.2%) were purchased from Sigma-Aldrich Co. (Lower Saxony, Germany).

### Instrumentation

The chromatographic separation was performed using a Symmetry C18 column (5 µm, 4.6 × 250 mm) (Waters, Massachusetts, USA) preceded by a Kromasil C18 (5 µm, 4.6 × 10 mm) (Tedia Brazil, Rio de Janeiro, Brazil), guard column of matching chemistry, both maintained at 25 °C. The LC-UV system consisted of a Dionex Ultimate 3000 system separation module coupled with a Dionex Ultimate 3000 UV-Vis detector (Dionex, California, USA). The mobile phase consisted of acetonitrile:water (60:40, v/v) with a flow rate of 1.0 mL/min. The UV wavelength was set at 210 nm and the injection volume was 10 µL. The LC-UV system was controlled by Chromeleon 6.8 software (Dionex, California, USA).

### Preparation of standard solutions

Fipronil (1 mg/mL) and Ethiprole (IS) (0.5 mg/mL) stock solutions, and the respective working standard solutions, were prepared in acetonitrile. Fipronil working standards at concentrations of 50, 100, 500, 1000, 2500 and 5000 ng/mL were prepared for calibration standards and used to spike plasma. Ethiprole working standard was prepared at the concentration of 2500 ng/mL.

### Preparation of calibration standards and quality control (QC) samples in plasma

Blood was collected in heparin tubes by jugular venipuncture of cattle. Plasma was obtained by centrifugation at 756 x g for 10 min at 4 °C. Plasma samples used for spiking and blank studies were taken from experimental animals that were found to be free of pesticides. A set of calibration standards (5-500 ng/mL) in plasma was constructed by spiking plasma with fipronil working standard solution to give final concentrations of 5, 10, 50, 100, 250, and 500 ng/mL. The QC samples at the concentrations of 5 ng/mL (LOQ), 100 ng/mL (middle QC) and 500 ng/mL (high QC) were prepared separately in a similar manner. Ethiprole was added to all plasma samples at a concentration of 250 ng/mL. Plasma spiked with fipronil and ethiprole (IS) was stored at -20 °C until analysis.

### Sample extraction

Plasma samples were subjected to the SPE clean-up using Oasis® HLB cartridges (Waters, Massachusetts, USA). The solid-phase extraction was carried out manually using a Supelco SPE vacuum manifold (Pennsylvania, USA). The cartridges were conditioned with methanol (2 mL) and water (2 mL) following which the plasma (1 mL) was loaded, washed with water (1 mL) and then eluted with 1 mL of methanol. The eluate was evaporated to dryness at 40 °C and reconstituted in 100 µL of acetonitrile. Solvent evaporation was performed using a Tecnal TE-0197 Evaporator workstation (Tecnal, São Paulo, Brazil). A volume of 10 µL was injected for LC-UV.

### Validation procedures

All validation experiments were performed according to ANVISA guidelines.[20]

*Selectivity*

The selectivity of the developed method was determined by analyzing blank plasma samples from 6 different sources (4 different individuals, 1 lipemic and 1 hemolysate).

*Limit of quantitation*

The limit of quantitation was determined according to a signal-to-noise ratio of 5 on spiked sample chromatograms.

*Linearity*

The linearity of the method was assessed using 2 calibration curves analyzed on 2 consecutive days. The peak area ratios (response) against the respective analyte concentration were used to assess the relationship between response and concentration. The calibration curves were fitted by least squares linear regression to calculate slopes, intercept and correlation (r).

*Precision and accuracy*

The precision and accuracy of the assay was determined by the analysis of 5 replicate sets of each of the three concentrations (5, 100, 500 ng/mL) of the QC samples on two separate occasions. The precision of the methods was expressed as the relative standard deviation (% RSD). Accuracy was calculated by comparing the measured concentration with the nominal (true) concentration as the mean recovery percent (%).

### Stock solution stability

The stock solution stability for both fipronil and the IS was performed at room temperature (25 °C) after a period of 6 h to 10 days by comparing with those of freshly prepared stock solution.

### Plasma sample stability

Post-preparative stability, i.e. the stability during the residence time in the auto sampler, was performed by assaying QC samples at low and high concentrations at 12 h after extraction. Short-term stability was evaluated by assaying QC samples at low and high concentrations after 24 h at room temperature. Freezing/thawing cycle stability was evaluated by assaying QC samples at low, medium and high concentrations after three freeze and thaw cycles. Long-term stability was checked by assaying QC samples at low and high concentrations after storage at -20 °C for 1, 2 and 3 months. Stability was expressed as percentage of mean recoveries (n = 3) from the nominal concentration.

### Characterization of bovine internal exposure

A dose of 1 mg/kg body weight (b.w.) of fipronil solution in glycerol formal/propylene glycol was administrated by single subcutaneous injection to 4 parasite-free male zebu calves (250 ± 5 kg). Blood was collected in heparin tubes by jugular venipuncture of cattle before and at 1, 2, 4, 8, 10, 24, 48 and 72 h after administration. Plasma was obtained by centrifugation at 756 x *g* for 10 min at 4 °C and stored at -20 °C until analysis. Sample extraction was carried out as described previously.

Animal procedures were conducted in accordance with accepted standards for good clinical practice from The European Agency for the Evaluation of Medicinal Products.[23]

## RESULTS AND DISCUSSION

### Method development

The use of UV has the advantage of its low price and maintenance costs, being an economical alternative detector. The wavelength used in this study (210 nm) differs from other methods described for determination of fipronil by UV (280 nm).[7,18] With a greater absorption at 210 nm we obtain a gain in sensitivity and therefore decrease the LOQ at least 10 fold. The LOQ values found were below those previously described for fipronil in plasma samples by LC-UV[18] and also by GC-MS,[19] although did not reach the values demonstrated by GC-MS/MS.[17] The method still has advantages over the isocratic elution mode in as far as its simplicity compared with the gradient elution mode described earlier.[18] A decrease in run time and mobile phase flow rate led to a reduction in analysis time and solvent used. The extraction and clean-up of plasma samples in a single process renders the SPE method a simpler and faster alternative compared with techniques that combine LL and SPE.[10-12,15,16] In addition, this ensures a clean matrix without matrix effects, guaranteeing the selectivity of the method which has been confirmed as advantageous for use in matrices such as plasma.[17,18]

### Method validation

The method was fully validated according to bioanalytical method recommendations described in ANVISA guidelines in terms of selectivity, linearity, precision, accuracy and stability.[20] All parameters tested also fulfilled the acceptance criteria of the FDA guidelines.[22]

*Selectivity*

The selectivity of the method was established by the analysis of blank, standard solution and plasma spiked at the LOQ (5 ng/mL) and IS (250 ng/mL) as shown in Figure 2. No significant interference or matrix effect was observed at the retention times of fipronil and IS in spiked plasma samples.

*Limit of quantitation*

The quantitation limit (LOQ) attained by the procedure was 5 ng/mL according to a signal-to-noise ratio of 5 on spiked sample chromatograms.

*Linearity*

Calibration plots of fipronil/IS peak area ratios versus the nominal concentration of fipronil in plasma were constructed and a linear regression applied to the data. A procedure to assess linearity by the ordinary least squares method was used.[24] Linear response was observed over the range 5 to 500 ng/mL with a mean r = 0.9993 ± 0.0003 (n=2) (Table 1). The mean slope and intercept values from the calibration curves were 0.331 and 0.022, respectively. All the values obtained were well within the guidelines published by the ANVISA.[20]

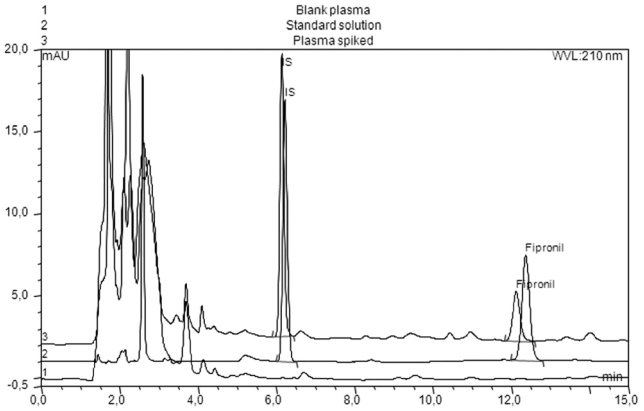


***Figure 2.*** *Chromatograms of (1) blank plasma (2) standard solution at the LOQ (5 ng/mL) and IS (250 ng/mL); (3) plasma spiked at the LOQ (5 ng/mL) and IS (250 ng/mL)*

**Table 1.** The intra and inter-day linearity, precision and accuracy for fipronil in bovine plasma

| | Fortified levels (ng/mL) | Parameters | Intra-day | | Inter-day |
|---|---|---|---|---|---|
| | | | Day 1 | Day 2 | |
| Linearity | 5 - 500 | a | 0.307 | 0.321 | 0.331 |
| | | b | 0.020 | 0.024 | 0.022 |
| | | r | 0.999 | 0.999 | 0.999 |
| Accuracy (%) | 5 | n=5 | 113.8 | 110.3 | 112.1 |
| | 100 | n=5 | 89.2 | 85.3 | 85.9 |
| | 500 | n=5 | 100.8 | 99.1 | 99.9 |
| Precision (RSD %) | 5 | n=5 | 7.2 | 12.2 | 9.9 |
| | 100 | n=5 | 2.8 | 2.7 | 4.4 |
| | 500 | n=5 | 1.3 | 1.5 | 1.6 |

*Precision and accuracy*

Intra-day and inter-day precision and accuracy were established by analyzing samples (n = 5) at three different concentrations 5, 100 and 500 ng/mL, on 2 separate days. Inter and intra-day data for accuracy and precision in spiked plasma are given in Table 1. The inter-day precision for the QCs 5, 100 and 500 ng/mL was between 1.6 and 9.9% while the accuracy was well within ± 15%. All the values obtained were well within the guidelines published by the ANVISA.[20] Thus, the method exhibited good accuracy and precision and proved suitable for application in pharmacokinetic studies.

### Standard solution stability

Fipronil and IS stock solutions were stable in acetonitrile at room temperature for a period of 10 days.

### Plasma sample stability

Evaluation of biological sample stability is important to guarantee safe application of the method in sample analysis. Stability tests assess sample stability at room temperature (short-term), of samples already processed, after undergoing the extraction process (post-processing), in case of freezing and thawing (freeze-thaw cycle) and freezing

**Table 2.** Stability of fipronil in bovine plasma under various storage conditions

| Fortified levels (ng/mL) | Accuracy (% recovery) | | | | |
|---|---|---|---|---|---|
| | Inicial | Post-preparative | Short-term | Freezing/thawing cycle | Long-term (3 months) |
| 5 | 96.7 | 100.4 | 112.7 | 109.6 | 86.6 |
| 100 | - | - | - | 102.0 | - |
| 500 | 107.2 | 106.8 | 104.2 | 108.7 | 103.5 |

samples (long-term). Table 2 shows the stability of fipronil in bovine plasma kept under various conditions. Percentage mean recoveries (n = 3) from the nominal concentration ranged from 85 to 115% for all concentrations under all conditions tested. Frozen samples remained stable for 3 months.

### Applicability of the method for pharmacokinetic studies

This method was developed for the measurement of fipronil concentrations in bovine plasma dedicated to pharmacokinetic studies. To evaluate the effectiveness of this method, fipronil was subcutaneously administered to four calves at a dose of 1 mg/kg b.w. No difficulties were found during the analysis. All the values obtained from calf sample analysis were within the calibration range. As shown in Figure 3, fipronil concentrations ranged from 17.3 ± 8.7 to 256.3 ± 91.7 ng/mL.

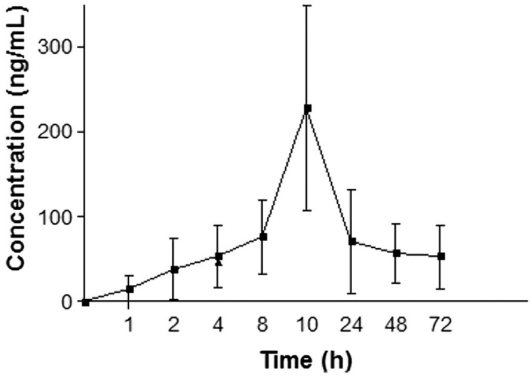


***Figure 3.*** *Mean plasma concentration of fipronil versus time after administration to four male cattle, at dose of 1 mg/kg b.w. Fipronil concentrations were assayed at 1, 2, 4, 8, 10, 24, 48 and 72 h after administration*

## CONCLUSION

Fipronil were determined by liquid chromatography with ultraviolet detection, providing a simple and rapid procedure for the determination of this compound in bovine plasma samples with good reproducibility and low detection limits. The proposed procedure, SPE, allows performing of the extraction and clean-up of plasma samples in a single process, thereby requiring only a low volume of organic solvents. The good sensitivity, resolution and short analysis time (15 min) coupled with the simplicity of the procedure should make this method a useful tool for the routine analysis of the examined compound. Finally, the present method has been co-validated to analyze fipronil in canine plasma (not shown) and can be easily adapted to more general animal plasma samples rendering it an effective investigative tool for routine analysis of plasma profile in the development of new pharmaceutical forms for fipronil administration.

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