Development and validation of analytical methods by HPLC for quality control of avermectins and milbemycins in bulk samples

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This work aims to develop analytical methods using high-performance liquid chromatography with a diode array detector (HPLC-DAD) for analysis and quantification of avermectins (AVMs) and milbemycins (MBMs) in bulk samples. First, the methods were optimized and some parameters such as temperature, flow rate, injection volume and mobile phase with different proportions of solvents were evaluated. The best chromatographic conditions were obtained using the Phenomenex® C18 (150 × 4.60 mm, 5 μm) column at a temperature of 20 °C, flow rate of 1.2 mL min⁻¹, injection volume of 20 μL, and detection at 250 nm. Acetonitrile: ultrapure water (87: 13, v/v) was used as mobile phase for moxidectin and eprinomectin, and acetonitrile: methanol: ultrapure water (53: 35: 12, v/v/v) for abamectin and ivermectin. Under these conditions satisfactory results were obtained, with appropriate limits of detection and quantification, acceptable linearity, precision, accuracy, and robustness. These methods satisfy the need for analytical methods for the multi-determination of MBMs and the B1a and B1b forms of AVMs by HPLC-DAD, which can be considered simple, effective, innovative and should aid in the development of the field.

Keywords: Avermectins. Milbemycins. Quality control. Bulk samples.

INTRODUCTION

Several veterinary drugs have been widely used in animals for disease control, for increasing productivity, and supply of food of animal origin (Dionisio, Rath, 2016; Hernando et al., 2007). Within the wide variety of veterinary drugs can be found the avermectins (AVMs) and milbemycins (MBMs). AVMs are semisynthetic derivatives of macrocyclic lactones (MLs) (Krogh et al., 2008). AVMs are used for the treatment of infections caused by nematodes and arthropods and for the control of parasites specific to the livestock (Trapero et al., 2016; Benz, Roncalli, Gross, 1989).

AVMs are produced by the fermentation of the bacterium Streptomyces avermitilis, in which are generated eight different components, such as B1a, B1b, A1a, A2b, among others. These components differ according to some characteristics in their chemical structures, for example, the series B represents the hydroxyl group in the C5 position, the series “a” and “b” are homologous and have similar antiparasitic activities, but the different bioactivities are due the structural differences between the components of series “1” and “2”. In general, AVM B1 is more active and has greater antiparasitic activity than B2 when administered orally, but the commercial product has approximately 90 % AVM B1a and 10 % B1b (Egerton et al., 1979; Albers-Schonberg et al., 1981). This class includes abamectin (ABA), eprinomectin (EPR) and ivermectin (IVM). Moxidectin (MOX) belongs to the class of MBMs, another important MLs subfamily, which is produced by Streptomyces cyaneogriseus (Krogh et al., 2008). The structures and properties of these molecules are shown in Table I.
ABA consists of a mixture of ABA B1a (>90 %) and ABA B1b (<10 %) (Prichard, Ménez, Lespine, 2012). The discovery of IVM and its later commercialization led to considerable chemical and microbiological efforts to explore its general structure. IVM is the generic name given to commercialization of the mixture of two homologs of AVM, which contains not less than 80 % IVM B1a and not more than 20 % IVM B1b. IVM B1a differs from B1b by a single methylene group at the C-25 position. When ABA B1a has a secondary butyl substituent and IVM B1b an isopropyl substituent, EPR and MOX are obtained, which are semisynthetic compounds, derived from ABA and IVM, respectively (Milhome et al., 2009; Danaher et al., 2006; Hernández-Borges et al., 2007). In conclusion, the compounds of series A are methoxylated at the 5-position, unlike the B-series with a hydroxyl group not derived from the same 5-position. AVMs are characterized by the presence of a substituent of a glucose molecule at position 13 and a sec-butyl substituent at carbon 25 (Milhome et al., 2009; Danaher et al., 2006; Hernández-Borges et al., 2007; Florez et al., 2018).

### TABLE I - Antiparasitics structures and properties (Chemicalize)

| Antiparasitics | Properties |
|----------------|------------|
| **ABA**        | Molecular form: C_{48}H_{72}O_{14} (B1a)  
C_{47}H_{70}O_{14} (B1b)  
Molecular mass: 873.090 g mol^{-1} (B1a)  
859.063 g mol^{-1} (B1b)  
Solubility in water: 1.21 (ChEMBL)  
pKa: 12.47 |
| **EPR**        | Molecular form: C_{50}H_{75}NO_{14} (B1a)  
C_{49}H_{73}NO_{14} (B1b)  
Molecular mass: 914.143 g mol^{-1} (B1a)  
900.116 g mol^{-1} (B1b)  
Solubility in water: NA  
pKa: 12.42 (DRUGBANK) |
| **IVM**        | Molecular form: C_{48}H_{74}O_{14} (B1a)  
C_{47}H_{72}O_{14} (B1b)  
Molecular mass: 875.106 g mol^{-1} (B1a)  
861.079 g mol^{-1} (B1b)  
Solubility in water: 4.0 (Alvinerie, Galtier, Mage, 1999)  
pKa: 12.47 |
| **MOX**        | Molecular form: C_{37}H_{53}NO_{8}  
Molecular mass: 639.83 g mol^{-1}  
Solubility in water: 5.2 µg mL^{-1}  
pKa: 12.55 and 2.81 |

NA: not available.
Because of the lipophilic properties of the AVMs and the long-term permanence of their residues in the animals’ bodies, especially in milk, they promote the risk of intoxication for consumers. Therefore, their detection and monitoring have become extremely important (Wang, Li, 2015; VICH GL 49; Janer et al., 2015; Gomes et al., 2015; Pollmeier et al., 2002; Wang et al., 2008; Frenich et al., 2010). In Europe as well as in the United States, ABA and IVM are prohibited for the treatment of lactating animals. The EPR and MOX dairy cow maximum residue limits have been set at 20 µg Kg⁻¹ and 40 µg Kg⁻¹, respectively, as maximum permissible without affecting food safety or human health (Wang, Li, 2015). Different Codex Alimentarius Commissions based on evaluations of the Food and Agriculture Organization of the United Nations (FAO) and World Health Organization (WHO) established values of 5 µg kg⁻¹ for ABA, 10 µg kg⁻¹ for IVM, 20 µg kg⁻¹ for EPR, and values between 10 and 20 µg kg⁻¹ for MOX (Gomes et al., 2015; Pollmeier et al., 2002).

Brazil is among the world’s largest exporters of beef, with about 1.866.476 tons (2019) (ABIEC, 2020), thus antiparasitics became the most marketed therapeutic class in the country, with about 23-29 % (2014-2018) in relation to other classes of medicines (SINDAN, 2020). IVM, ABA, EPR and MOX are among the most common antiparasitic drugs administered to cattle, representing more than 44 % of the total veterinary antiparasitic compounds marketed in Brazil. This preference is due to the extended endectocidal activity of these compounds, causing the elimination rates to be slow in bovine animals (Dionisio, Rath, 2016; Wang et al., 2008; Frenich et al., 2010).

Because most of the AVMs used at relatively low doses have good stability and safety, there is a great deal of flexibility in their formulation. Medications are used in veterinary practice as endectocides (Giannetti et al., 2011) for small ruminants (e.g., Ivomec® Drench), injectable (e.g., Ivomec® Injection for cattle and pigs), including long-acting injectable formulations in some markets (e.g., Ivomec Gold®), pour-on (Ivomec® Pour-on), and long-acting boluses in some markets for cattle, and pastes for horses (e.g., Eqvalan®), which are all composed mostly of IVM and ABA (Cruza et al., 2015). There are also combinations between EPR and MOX with other pharmacologically active ingredients to broaden the action spectrum (Higa et al., 2016).

Different analytical methods have been employed for the analysis of pharmaceutical inputs, such as thin layer chromatography, liquid chromatography, and immunochemistry (Rabel et al., 1993). The instrumental analytical technique most used in the methodologies for the determination of AVMs and MBMs is high performance liquid chromatography (HPLC), taking advantage of the different chromatographic detectors of the chromatographic column that when in contact with the analytes present in the eluent, emit electrical signals that are recorded in the form of peaks. Through this record, qualitative and quantitative data can be obtained on the analytes present in the sample (Frenich et al., 2010).

Therefore, the aim of this work was to develop analytical methods using HPLC with a diode array detector (DAD) for analysis and quantification of ABA and IVM (B1a and B1b) and also EPR (B1a and B1b) and MOX in bulk samples. This study was realized because of the great importance of the development of effective and reliable analytical methods for the control of the quality of the pharmaceutical inputs or bulk samples, which do not contemplate the forms B1a and B1b.

**EXPERIMENTAL**

**Standards and solvents**

For the analysis of antiparasitics, ABA (97.60 %) and EPR (97.04 %) from Sigma-Aldrich (St. Louis, United States), MOX (95.78 %) from Zhejiang Hisun Pharmaceutical (Zhejiang, China), and IVM (89.83 %) from Hebei Veyong (Hebei Sheng, China) were used. Methanol and acetonitrile HPLC grade solvents were acquired from J.T.Baker (Mexico City, MX, Mexico). The water was distilled and purified using a Millipore Milli-Q Plus system (Bedford, MA, USA).

**Analytical instrumentation and separation conditions**

Chromatographic analyses were performed on an Agilent Technologies HPLC (Palo Alto, CA, USA) consisting of the quaternary pump model 1260 (G1311...
B), automatic injector model 1260 Hip ALS (G1367E), a column oven model 1290 TCC (G1316C), a thermostat model 1290 (G1330B) and a DAD model 1260 VL+ (G1315C). An Agilent Open LAB Chromatography Data System® was used to control the HPLC system and for data acquisition. All separations were performed on an analytical Phenomenex® Gemini C18 column (150 mm × 4.6 mm, 5 µm) at a temperature of 25 °C. The injection volume was 20 µL. A flow rate of 1.2 mL min⁻¹ was used and detection was performed at 250 nm. The mobile phase for EPR and MOX consisted of acetonitrile: ultrapure water (87: 13, v/v) and the mobile phase for ABA and IVM consisted of a mixture of acetonitrile: methanol: ultrapure water (53: 35: 12, v/v/v).

Preparation of solutions

Prior to the preparation of the working solutions, standard solutions of each analyte were prepared in methanol at a concentration of 1.5 mg mL⁻¹. For the validation of the method, seven solutions with different concentrations were prepared by diluting the stock solution with methanol in the appropriate concentrations for each drug: EPR and MOX: 0.24, 0.26, 0.28, 0.30, 0.32, 0.34 and 0.36 mg mL⁻¹; ABA and IVM: 0.80, 0.87, 0.93, 1.00, 1.07, 1.13 and 1.20 mg mL⁻¹.

Validation of methods

After the optimization of the chromatographic conditions, the validation of the two analytical methods (ABA/IVM and EPR/MOX) was performed following the recommendations established by the United States Pharmacopeia - USP guidelines (The United States Pharmacopeial Convention, 2017), and the International Conference on Harmonization (ICH, 2005). From the analyses of the solutions of different concentrations, the analytical curve was constructed and some parameters, such as linearity, limits of detection (LOD) and quantification (LOQ), precision, accuracy, and robustness were evaluated.

Linearity

Linearity can be determined by the analytical curve, which is constructed from different concentrations, and the responses of the instrument should be directly proportional to them. Linearity can be determined by the equation of the line \( y = ax + b \), the coefficient of determination \( R^2 \) or correlation \( r \) and relative standard deviation (RSD%) (Equation 1) being that these values determine the quality of the curve (Aragão, Veloso, Andrade, 2009; Oliveira et al., 2016).

\[
RSD\% = \frac{S}{\bar{x}} \times 100
\]  

in which \( S \) is the absolute standard deviation and \( \bar{x} \) is the mean of the results (The United States Pharmacopeial Convention, 2017).

In addition, the \( F \)-test was also performed to validate the linear regression, resulting from analysis of variance. Subsequently, the \( F \)-value was calculated by Equation 2 and compared with the \( F \)-value tabulated, with 5 % of significance,

\[
F = \frac{QMR}{QMr}
\]  

in which QMR is the quadratic mean of regression value and QMr is quadratic mean of residue (Souza, Junqueira, 2005; Cassiano et al., 2009).

Limit of detection and quantification

The LOD and LOQ were calculated according to the parameters of the analytical curve, according to Equations 3 and 4:

\[
LOD = 3.3 \times \frac{S}{a}
\]  

\[
LOQ = 10 \times \frac{S}{a}
\]
in which \( S \) is the standard deviation estimate and \( a \) is the angular coefficient of the analytical curve (Oliveira et al., 2016; Silva et al., 2017; Ribani et al., 2004).

**Precision**

Precision was assessed by the intermediate precision test which expresses within-laboratory variation on different days, and by repeatability for short periods of time using the same analyst with the same equipment (intraday). For this assessment, low, intermediate, and high three-level concentrations, 0.24, 0.28 and 0.32 for MOX and EPR and 0.87, 1.00 and 1.13 for ABA and IVM in six replicates \( (n = 6) \) were evaluated and the value was estimated by the calculation of the RSD\% (Equation 1). The determination of precision must obey the proximity between the results obtained from the analysis of the same sample, in the same laboratory, on the same day and on two different days (ICH, 2005; The United States Pharmacopeia Convention, 2017).

**Accuracy**

The agreement between the individual results of an assay and a reference value accepted as true represents the accuracy. Accuracy is always considered within certain limits, at a given level of confidence, that is, it is always associated with precision values. Accuracy can be calculated as the percentage of recovery by the assay of the known added amount of analyte in the sample, or as the difference between the mean and the accepted true value, together with confidence intervals. In this case, it was expressed in terms of the relative error (RE\%), as shown in Equation 5 (ICH, 2005).

\[
RE\% = \left(\frac{\text{value obtained} - \text{value real}}{\text{value real}}\right) \times 100
\]

**Robustness**

The robustness of the method was verified by investigating the effects caused by deliberate minor changes in experimental conditions in both methods, such as: (i) temperature of column: \( \pm 2 \text{ °C} \); (ii) flow rate: \( \pm 0.05 \text{ mL min}^{-1} \); (iii) three different Gemini C18 columns. All other conditions were kept constant. For each condition, a standard solution of 0.3 mg mL\(^{-1}\) for EPR/MOX and 1.00 mg mL\(^{-1}\) for ABA/IVM were prepared and injected into the chromatography system. The robustness of the method was evaluated from the % found of the peak area for each analyte after three consecutive injections in triplicate \( (n = 3) \).

**RESULTS AND DISCUSSION**

**Development of the chromatographic method**

To obtain a chromatographic analytical method, optimization of the MOX/EPR and ABA/IVM separation conditions were performed for both methods. The composition and proportion of the solvents used in the mobile phase, flow rate, temperature, and injection volumes were studied.

For the optimization, only one Phenomenex\textsuperscript{®} C18 column \( (150 \text{ mm} \times 4.6 \text{ mm}, 5 \mu\text{m}) \) was used. The first conditions were evaluated using the mobile phase composed of acetonitrile, methanol, and ultrapure water in different compositions, flow rate of 1.2 mL min\(^{-1}\), temperature of 25 °C, injection volume of 20 µL, and wavelength at 250 nm. However, it was not possible to obtain an efficient separation of the four pharmaceutical inputs simultaneously, as well as the forms B1a and B1b. The use of acetonitrile: methanol: ultrapure water \( (53: 35: 12, \text{v/v/v}) \) as mobile phase led to the separation of the IVM (B1a and B1b) and ABA (B1a and B1b), as shown in Figure 1a. For the separation of MOX and EPR (B1a and B1b), different proportions of the mobile phase composed of acetonitrile and ultrapure water were studied, in which the proportion of 87:13 (v/v) was the most efficient, as can be seen in Figure 1b. Table II shows the optimized chromatographic conditions.
FIGURE 1 - Optimized chromatograms referring to analysis of (a) ABA B1b and ABA Bla, IVM B1b and IVM B1b; and (b) EPR B1b and EPR B1a, and MOX. Chromatographic conditions: mobile phase of acetonitrile: methanol: ultrapure water (53: 35: 12, v/v/v) and acetonitrile: ultrapure water (87: 13, v/v), respectively, Phenomenex® Gemini C18 column (150 × 4.60 mm, 5 µm), temperature of 25 °C, injection volume of 20 µL, flow rate of 1.2 mL min⁻¹, and λ = 250 nm.

TABLE II - Optimized conditions for simultaneous analysis of drugs

| Parameters                  | EPR and MOX | ABA and IVM |
|-----------------------------|-------------|-------------|
| Mobile Phase                | acetonitrile: ultrapure water (87: 13, v/v) | acetonitrile: methanol: ultrapure water (53: 35: 12, v/v/v) |
| Column                      | Phenomenex® Gemini C18 (150 × 4.60 mm, 5 µm) |
| Temperature                 | 25 °C       |
| Injection volume            | 20.0 µL     |
| Mobile phase flow           | 1.2 mL min⁻¹ |
| Wavelength                  | 250 nm      |
| Elution mode                | Isocratic  |
Method validation

The validation of the analytical method was performed by the construction of the analytical curve and the parameter evaluations of linearity, LOD and LOQ, precision, accuracy, and robustness. For linearity evaluation, analytical curves were obtained in the concentrations presented in Table III, as well as the linear equations obtained, correlation coefficients, and RSD%. As can be seen, the values of r were between 0.9929 and 0.9972 showing the linearity of these analytical models which closely describe the concentration–response relationship (ICH, 2005; The United States Pharmacopeia Convention, 2017). Figure 2 shows the calibration curves for EPR Bla and Blb, MOX, ABA Blb and Bla, and IVM Blb and Blb.

### Table III - The linearity of the analytical curve

| Analytes | Concentrations (mg mL⁻¹) | Linear equation | Correlation coefficient (r) | F-values a | RSD% |
|----------|---------------------------|-----------------|-----------------------------|------------|------|
| EPR Blb  | 0.24, 0.26, 0.28, 0.30, 0.32, 0.34, and 0.36 | \( y = 8.69 \times 10^5 x + 9.55 \times 10^3 \) | 0.9962 | 3663.77 | 1.22 |
| EPR Bla  | 0.24, 0.26, 0.28, 0.30, 0.32, 0.34, and 0.36 | \( y = 3.48 \times 10^7 x + 5.51 \times 10^5 \) | 0.9972 | 4339.71 | 1.29 |
| MOX      | 0.80, 0.87, 0.93, 1.00, 1.07, 1.13, and 1.20 | \( y = 5.05 \times 10^7 x - 3.06 \times 10^5 \) | 0.9957 | 1929.22 | 1.64 |
| ABA Blb  | 0.80, 0.87, 0.93, 1.00, 1.07, 1.13, and 1.20 | \( y = 8.53 \times 10^6 x + 3.21 \times 10^5 \) | 0.9965 | 3959.01 | 1.16 |
| ABA Bla  | 0.80, 0.87, 0.93, 1.00, 1.07, 1.13, and 1.20 | \( y = 3.62 \times 10^7 x + 2.08 \times 10^6 \) | 0.9929 | 1049.21 | 2.05 |
| IVM Blb  | 0.80, 0.87, 0.93, 1.00, 1.07, 1.13, and 1.20 | \( y = 1.58 \times 10^6 x + 1.80 \times 10^5 \) | 0.9943 | 2455.88 | 1.77 |
| IVM Bla  | 0.80, 0.87, 0.93, 1.00, 1.07, 1.13, and 1.20 | \( y = 4.80 \times 10^7 x + 4.15 \times 10^6 \) | 0.9948 | 2671.04 | 1.73 |

*F-values ≥ F_{critic} (4.20), significant linear regression.*
The $F$-test of significance was performed to evaluate the regression of the analytical curve, and the values are described in Table III. Comparing the $F$-values with $F_{critical}$ (tabulated), the $F_{critical}$ value was 4.20, which corresponds to the ratio between the degree of freedom of the numerator (regression) and the degree of freedom of the denominator (residue), being the values equal to 1 and 28, respectively, at the confidence level of 5%. With all values of $F \geq F_{critical}$, there is an indication that the regression is significant, since it is accepted that the slope of the line is not zero and there is an indication of the relationship of linearity between $x$ and $y$, the larger the value of $F$ (Cassiano et al., 2009).

The LOD and LOQ were calculated by Equations 2 and 3 respectively and the values obtained are shown in Table IV. The LOD values ranged from 0.003 to 0.037 mg mL$^{-1}$ and LOQ values from 0.004 to 0.123 mg mL$^{-1}$.

**TABLE IV** - Limits of detection and quantification of drugs analyzed

| Drug   | LOD (mg mL$^{-1}$) | LOQ (mg mL$^{-1}$) |
|--------|--------------------|--------------------|
| EPR B1b | 0.003              | 0.004              |
| EPR B1a | 0.012              | 0.015              |
| MOX    | 0.010              | 0.033              |
| ABA B1b | 0.021              | 0.068              |
| ABA B1a | 0.026              | 0.026              |
| IVM B1b | 0.034              | 0.113              |
| IVM B1a | 0.037              | 0.123              |

Table V and VI show the medium values of intra and interday for the different analytes, in which the precision was evaluated by RSD% and accuracy by RE%. Intraday precision values ranged between 0.321–1.859, and interday between 0.152–2.315. The values of intraday and interday accuracy ranged from $-2.552$ to $1.302$ and $-2.713$ to $1.423$, respectively. These results were satisfactory, showing RSD% up to 3% and RE% up to $\pm 3\%$ (ICH, 2005; Silva et al., 2017; Ribani et al., 2004).
TABLE V - Medium values of the precision and accuracy for EPR Blb and Bla and MOX analysis

| Nominal concentration (mg mL⁻¹) | EPR Blb | EPR Bla | MOX |
|-------------------------------|---------|---------|-----|
| 0.260 | 0.300 | 0.340 | 0.260 | 0.300 | 0.340 | 0.260 | 0.300 | 0.340 |

**Intraday (n = 6)***

| Concentration analyzed (mg mL⁻¹) | 0.253 | 0.286 | 0.327 | 0.246 | 0.291 | 0.329 | 0.262 | 0.300 | 0.338 |
|---------------------------------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| Precision (RSD, %)              | 1.499 | 0.853 | 0.913 | 0.413 | 0.360 | 0.472 | 0.461 | 0.688 | 0.503 |
| Accuracy (RE, %)                | 0.667 | -1.290 | -0.161 | -2.037 | 0.373 | 0.228 | 1.060 | 0.103 | -0.439 |

**Interday (n = 2)***

| Concentration Analyzed (mg mL⁻¹) | 0.254 | 0.282 | 0.321 | 0.247 | 0.291 | 0.329 | 0.263 | 0.291 | 0.327 |
|---------------------------------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| Precision (RSD, %)              | 1.003 | 1.517 | 1.293 | 0.219 | 0.386 | 0.174 | 0.371 | 0.246 | 0.649 |
| Accuracy (RE, %)                | 1.145 | -2.713 | -2.247 | -1.635 | 0.375 | 0.365 | 0.958 | 0.629 | -0.281 |

* * n = number of determinations; b n = number of days.

TABLE VI - Medium values of the precision and accuracy for ABA Blb and Bla, IVM Blb and Bla analysis

| Nominal concentration (mg mL⁻¹) | ABA Blb | ABA Bla | IVM Blb | IVM Bla |
|-------------------------------|---------|---------|---------|---------|
| 0.870 | 1.000 | 1.130 | 0.870 | 1.000 | 1.130 | 0.870 | 1.000 | 1.130 |

**Intraday (n = 6)***

| Concentration analyzed (mg mL⁻¹) | 0.860 | 1.013 | 1.125 | 0.877 | 0.997 | 1.116 | 0.862 | 1.007 | 1.129 | 0.869 | 0.992 | 1.135 |
|---------------------------------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| Precision (RSD, %)              | 0.512 | 0.665 | 0.664 | 1.739 | 0.740 | 1.010 | 0.860 | 1.859 | 0.998 | 0.485 | 1.607 | 0.710 |
| Accuracy (RE, %)                | -1.225 | 1.302 | -0.461 | 0.845 | -0.266 | -1.228 | -0.880 | 0.799 | -0.007 | -0.165 | -0.822 | 0.458 |

**Interday (n = 2)***

| Concentration Analyzed (mg mL⁻¹) | 0.859 | 1.014 | 1.124 | 0.872 | 0.996 | 1.113 | 0.865 | 1.002 | 1.131 | 0.869 | 0.992 | 1.137 |
|---------------------------------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| Precision (RSD, %)              | 0.527 | 0.548 | 0.152 | 0.839 | 0.814 | 0.855 | 2.315 | 2.164 | 0.959 | 1.113 | 1.219 | 0.749 |
| Accuracy (RE, %)                | -1.242 | 1.423 | -0.531 | 0.194 | -0.377 | -1.462 | -0.564 | 0.164 | 0.114 | -0.161 | -0.827 | 0.616 |

* * n = number of determinations; b n = number of days.
The results of the robustness study are given in Table VII and VIII. During all variation conditions, the assay values were not affected and the data were in accordance with the actual values, showing that minor changes in experimental conditions did not significantly affect the determination of analytes, hence both developed HPLC methods were robust for the determination of EPR/MOX and ABA/IVM in bulk samples.

**TABLE VII - Chromatographic conditions and range investigated during robustness testing**

| Variable          | Range investigated | EPRI B1b (mg mL⁻¹) | EPRI B1a (mg mL⁻¹) | MOX (mg mL⁻¹) | ABA B1b (mg mL⁻¹) | ABA B1a (mg mL⁻¹) | IVM B1b (mg mL⁻¹) | IVM B1a (mg mL⁻¹) |
|-------------------|--------------------|--------------------|--------------------|--------------|------------------|------------------|------------------|------------------|
| Flow rate (mL min⁻¹) | 1.15              | 0.299             | 99.79             | 0.297        | 98.90            | 0.996            | 99.56            | 0.996            | 99.64            | 1.002            | 100.16           |
|                   | 1.20              | 0.296             | 98.58             | 0.295        | 98.18            | 0.302            | 100.52           | 0.995            | 99.52            | 0.995            | 99.45            | 0.992            | 99.21            | 0.981            | 98.12            |
|                   | 1.25              | 0.300             | 100.12            | 0.301        | 100.24           | 0.298            | 99.23            | 1.006            | 100.58           | 1.013            | 101.26           | 1.008            | 100.82           | 1.018            | 101.84           |
| Column            | Gemini C18 (1)     | 0.305             | 101.59            | 0.306        | 102.01           | 0.300            | 100.12           | 1.007            | 100.65           | 0.985            | 98.53            | 1.006            | 100.55           | 1.012            | 101.15           |
|                   | Gemini C18 (2)     | 0.298             | 99.32             | 0.296        | 98.64            | 0.299            | 99.76            | 0.993            | 99.32            | 0.991            | 99.12            | 0.995            | 99.54            | 1.002            | 100.22           |
|                   | Gemini C18 (3)     | 0.295             | 98.49             | 0.296        | 98.65            | 0.296            | 98.80            | 0.992            | 99.22            | 0.995            | 99.49            | 1.001            | 100.12           | 0.981            | 98.12            |
| Column temperature (°C) | 23                | 0.301             | 100.46            | 0.303        | 100.92           | 0.300            | 100.25           | 1.001            | 100.14           | 0.991            | 99.12            | 1.015            | 101.54           | 0.981            | 98.12            |
|                   | 25                | 0.305             | 101.56            | 0.303        | 101.14           | 0.303            | 100.64           | 0.994            | 99.36            | 1.003            | 100.26           | 1.002            | 100.16           | 0.992            | 99.22            |
|                   | 27                | 0.301             | 100.45            | 0.303        | 100.90           | 0.300            | 99.89            | 1.001            | 100.10           | 0.983            | 98.25            | 0.993            | 99.31            | 1.009            | 100.90           |

% found (mean): all analyses were performed in triplicates (n = 3).

**TABLE VIII - Chromatographic conditions and range investigated during robustness testing for ABA B1b, ABA B1a, IVM B1b and IVM B1a.**

| Variable          | Range investigated | ABA B1b (mg mL⁻¹) | ABA B1a (mg mL⁻¹) | IVM B1b (mg mL⁻¹) | IVM B1a (mg mL⁻¹) |
|-------------------|--------------------|------------------|------------------|------------------|------------------|
| Flow rate (mL min⁻¹) | 1.15              | 0.996            | 99.56            | 1.017            | 101.67           | 0.996            | 99.64            | 1.002            | 100.16           |
|                   | 1.20              | 0.995            | 99.52            | 0.995            | 99.45            | 0.992            | 99.21            | 0.981            | 98.12            |
|                   | 1.25              | 1.006            | 100.58           | 1.013            | 101.26           | 1.008            | 100.82           | 1.018            | 101.84           |
| Column            | Gemini C18 (1)     | 1.007            | 100.65           | 0.985            | 98.53            | 1.006            | 100.55           | 1.012            | 101.15           |
|                   | Gemini C18 (2)     | 0.993            | 99.32            | 0.991            | 99.12            | 0.995            | 99.54            | 1.002            | 100.22           |
|                   | Gemini C18 (3)     | 0.992            | 99.22            | 0.995            | 99.49            | 1.001            | 100.12           | 0.981            | 98.12            |
| Column temperature (°C) | 23                | 1.001            | 100.14           | 0.991            | 99.12            | 1.015            | 101.54           | 0.981            | 98.12            |
|                   | 25                | 0.994            | 99.36            | 1.003            | 100.26           | 1.002            | 100.16           | 0.992            | 99.22            |
|                   | 27                | 1.001            | 100.10           | 0.983            | 98.25            | 0.993            | 99.31            | 1.009            | 100.90           |

% found (mean): all analyses were performed in triplicates (n = 3).
CONCLUSIONS

Two simple and efficient chromatographic methods were developed for the determination of ABA/IVM and EPR/MOX antiparasitics that are widely used in veterinary treatment. The developed methods presented good linearity, precision, accuracy, robustness, limits of detection, and adequate quantification, which can be useful in analyses of different pharmaceutical veterinary formulations, after proper modifications in analytical methods or sample preparation procedures. In addition, the simultaneous resolutions of EPR B1a and B1b, MOX, ABA B1b and B1a, and IVM B1b and B1b can be used in other matrices such as biological fluids together with adequate techniques of sample preparation. These methods satisfy the need for analytical methods for the multi-determination of B1a and B1b forms of AVMs and MBMs by HPLC-DAD, and they were effective, innovative, and should aid in the development of the field.

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AUTHOR CONTRIBUTIONS

# These authors contributed equally.

CONFLICTS OF INTEREST

The authors declare no conflict of interest, particularly no financial or personal relationships with other people or organizations that could inappropriately influence this work.

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