BJPS

Brazilian Journal of
Pharmaceutical Sciences

http://dx.doi.org/10.1590/s2175-97902022e19587

Article

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

Flávia Viana Avelar Dutra[#], Leila Suleimara Teixeira[#], Bruna Carneiro Pires, Diego Hernando Ângulo Florez, Roseane Andrade Teixeira, Keyller Bastos Borges*

Departamento de Ciências Naturais, Universidade Federal de São João del-Rei, Campus Dom Bosco, São João del-Rei, Minas Gerais, Brazil

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.

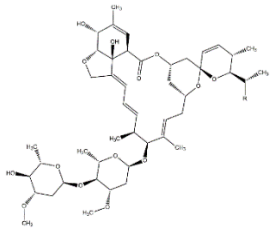
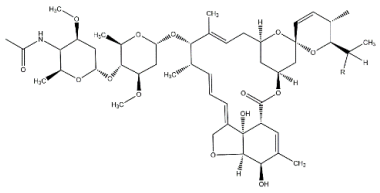
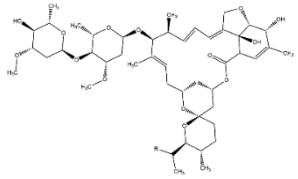
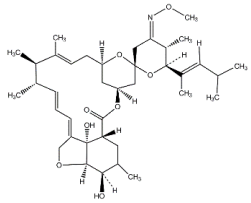
INTRODUCTION

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.**

***Correspondence:** K. B. Borges. Departamento de Ciências Naturais. Universidade Federal de São João del-Rei. Campus Dom Bosco. Praça Dom Helvécio 74, Fábricas. 36301–160, São João del-Rei, Minas Gerais, Brazil. E-mail: keyller@ufsj.edu.br. Orcid: https://orcid.org/0000-0003-1067-1919

TABLE I - Antiparasitics structures and properties (Chemicalize)

Antiparasitics		Properties
ABA	 ABA B1a: R = CH_2CH_3 ABA B1b: R = CH3	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	 EPR B1a: R = CH_2CH_3 EPR B1b: R = CH3	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	 IVM B1a: R = CH_2CH_3 IVM B1b: R = CH3	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	 (Chen, Hung, 2002)	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.

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).

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 Pharmacopeia 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).

$$RSD\% = \frac{S}{\bar{X}} \times 100 \quad \textbf{(1)}$$

in which S is the absolute standard deviation and 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,

$$F = \frac{QMR}{QMr} \quad \textbf{(2)}$$

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**:

$$LOD = 3.3 \times \frac{S}{a} \quad \textbf{(3)}$$

$$LOQ = 10 \times \frac{S}{a} \quad \textbf{(4)}$$

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).

$$RE\% = \frac{value_{obtained} - value_{real}}{value_{real}} \times 100 \qquad (5)$$

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: ± 2 °C; (ii) flow rate: ± 0.05 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® C18 column (150 mm × 4.6 mm, 5 µm) 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, 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.

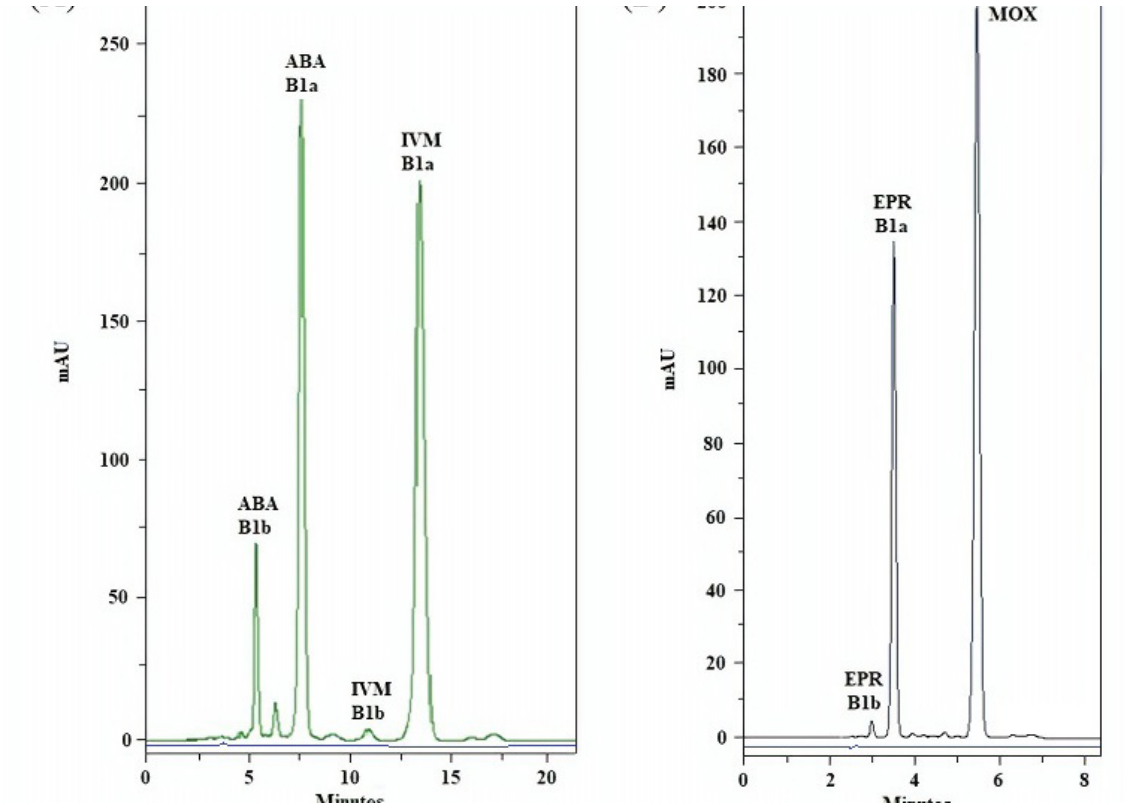


FIGURE 1 - Optimized chromatograms referring to analysis of **(a)** ABA B1b and ABA B1a, IVM B1b and IVM B1b; and **(b)** EPR B1b and EPR B1a, and MOX. Chromatographic conditions: mobile phase of acetonitrile: methanol: ultrapure water (53: 35: 12, v/v/v) and acetonitrile: ultrapure water (87: 13, v/v), 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 B1a and B1b, MOX, ABA B1b and B1a, and IVM B1b and B1b.

TABLE III - The linearity of the analytical curve

Analytes	Concentrations (mg mL^{-1})	Linear equation	Correlation coefficient (r)	*F*-values [a]	RSD%
EPR B1b	0.24, 0.26, 0.28, 0.30, 0.32, 0.34, and 0.36	$y = 8.69\times10^{5}\,x + 9.55\times10^{3}$	0.9962	3663.77	1.22
EPR B1a		$y = 3.48\times10^{7}\,x + 5.51\times10^{5}$	0.9972	4339.71	1.29
MOX		$y = 5.05\times10^{7}\,x - 3.06\times10^{5}$	0.9957	1929.22	1.64
ABA B1b	0.80, 0.87, 0.93, 1.00, 1.07, 1.13, and 1.20	$y = 8.53\times10^{6}\,x + 3.21\times10^{5}$	0.9965	3959.01	1.16
ABA B1a		$y = 3.62\times10^{7}\,x + 2.08\times10^{6}$	0.9929	1049.21	2.05
IVM B1b		$y = 1.58\times10^{6}\,x + 1.80\times10^{5}$	0.9943	2455.88	1.77
IVM B1a		$y = 4.80\times10^{7}\,x + 4.15\times10^{6}$	0.9948	2671.04	1.73

[a] *F*-values $\geq F_{critic}$ (4.20), significant linear regression.

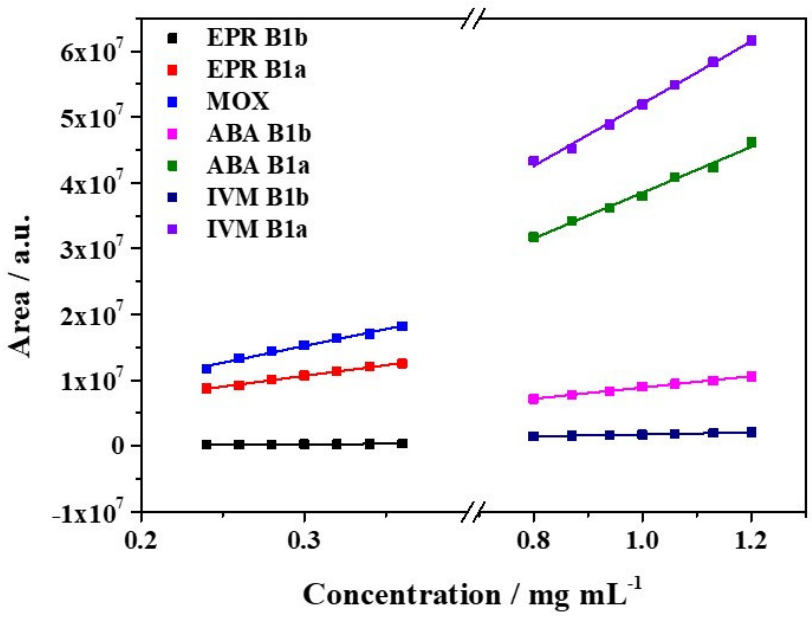


FIGURE 2 - Calibration curves for EPR B1a and B1b, MOX, ABA B1b and B1a, IVM B1b and B1b.

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 $F_{crictical}$ (tabulated), the $F_{crictical}$ 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

	EPR B1b	EPR B1a	MOX	ABA B1b	ABA B1a	IVM B1b	IVM B1a
LOD (mg mL^{-1})	0.003	0.012	0.010	0.021	0.026	0.034	0.037
LOQ (mg mL^{-1})	0.004	0.015	0.033	0.068	0.026	0.113	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 ±3 % (ICH, 2005; Silva *et al.*, 2017; Ribani *et al.*, 2004).

TABLE V - Medium values of the precision and accuracy for EPR B1b and B1a and MOX analysis

Analytes	EPR B1b			EPR B1a			MOX		
Nominal concentration (mg mL^{-1})	0.260	0.300	0.340	0.260	0.300	0.340	0.260	0.300	0.340
	Intraday (n = 6)[a]								
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)[b]								
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

[a] n = number of determinations; [b] n = number of days.

TABLE VI - Medium values of the precision and accuracy for ABA B1b and B1a, IVM B1b and B1a analysis

Analytes	ABA B1b			ABA B1a			IVM B1b			IVM B1a		
Nominal concentration (mg mL^{-1})	0.870	1.000	1.130	0.870	1.000	1.130	0.870	1.000	1.130	0.870	1.000	1.130
	Intraday (n = 6)[a]											
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)[b]											
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

[a] 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})	
		Conc.	%	Conc.	%	Conc.	%	Conc.	%	Conc.	%	Conc.	%	Conc.	%
Flow rate (mL min^{-1})	**1.15**	0.299	99.79	0.299	99.58	0.297	98.90	0.996	99.56	1.017	101.67	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	0.301	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	0.302	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})	
		Conc.	%	Conc.	%	Conc.	%	Conc.	%
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.

ACKNOWLEDGMENTS

The authors would like to thank the Brazilian agencies CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico), and FAPEMIG (Fundação de Amparo à Pesquisa do Estado de Minas Gerais) for financial support. Also, this study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code 001, and part of the project involving the Rede Mineira de Química (RQ-MG) supported by FAPEMIG (Project: REDE-113/10; Project: CEX-RED-0010-14).

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.

REFERENCES

ABIEC (Associação Brasileira das Indústrias Exportadoras de Carnes). Série histórica de carne bonvina. Accessed in 17 July 2020. Available from: http://abiec.com.br/exportacoes/

Albers-Schonberg G, Arison BH, Chabala JC, Douglas AW, Eskola P, Fisher H, et al. Avermectins. structure determination. J Am Chem Soc. 1981;103(14):4216-4221.

Alvinerie M, Sutra JF, Galtier P, Mage C. Pharmacokinetics of eprinomectin in plasma and milk following topical administration to lactating dairy cattle. Res Vet Sci. 1999;67(3):229-232.

Aragão NM, Veloso MCC, Andrade JB. Validação de métodos cromatográficos de análise - um experimento de fácil aplicação utilizando cromatografia líquida de alta eficiência (CLAE) e os princípios da "química verde" na determinação de metilxantinas em bebidas. Quim Nova. 2009;32(9):2476-2481.

Benz GW, Roncalli RA, Gross SJ. Use of Ivermectin in Cattle, Sheep, Goats, and Swine, in: WC Campbell, Ivermectin and Abamectin. Springer, New York. 1989;215-229.

Cassiano NM, Barreiro JC, Martins LRR, Oliveira RV, Cass QB. Validação em métodos cromatográficos para análises de pequenas moléculas em matrizes biológicas. Quim Nova. 2009;32(4):1021-1030.

ChEMBL. Abamectin. Accessed in 17 July 2020. Available from: https://www.ebi.ac.uk/chembl/compound_report_card/CHEMBL3215298/

Chemicalize. Available from: https://chemicalize.com/

Chen YC, Hung YP, Fleckenstein L. Liquid chromatographic assay of moxidectin in human plasma for application to pharmacokinetic study. J Pharm Biomed Anal. 2002;29(5):917-926.

Cruza BC, Lopes WDZ, Maciel WQ, Felippellia G, Fávero FC, Teixeira WFP, et al. Susceptibility of Rhipicephalus (Boophilus) microplus to ivermectin (200, 500 and 630 µg/kg) in field studies in Brazil. Vet Parasitol. 2015;207(3-4):309-317.

Danaher M, Howells LC, Crooks SRH, Cerkvenik-Flajs V, O'Keeffe M. Review of methodology for the determination of macrocyclic lactone residues in biological matrices. J Chromatogr B. 2006;844(2):175–203.

Dionisio AC, Rath S. Abamectin in soils: Analytical methods, kinetics, sorption and dissipation. Chemosphere. 2016;151:17-29.

DRUGBANK. Eprinomectin. Accessed in 17 July 2020. Available from: https://www.drugbank.ca/drugs/DB11431

Egerton JR, Ostlind DA, Blair LS, Eary CH, Suhayda D, Cifelli S, Riek RF, CampbelL WC. Avermectins, new family of potent anthelmintic agents: efficacy of the bla component. Antimicrob Agents Chemother. 1979;15(3):372-378.

Florez DHA, Teixeira RA, Silva RCS, Pires BC, Dutra FVA, Borges KB. Pipette-tip solid-phase extraction using polypyrrole as efficient adsorbent for extraction of avermectins and milbemycins in milk. Anal Bioanal Chem. 2018;410(14):3361-3374.

Frenich AG, Luiz MMA, Vidal J, González RR. Comparison of several extraction techniques for multiclass analysis of veterinary drugs in eggs using ultra-high pressure liquid chromatography-tandem mass spectrometry. Anal Chim Acta. 2010;661(2):150-160.

Giannetti L, Giorgi A, Necci F, Ferretti G, Buiarelli F, Neri B. Validation study on avermectine residues in foodstuffs. Anal Chim Acta. 2011;700(1-2):11-15.

Gomes LVC, Lopes WDZ, Cruz BC, Teixeira WF, Felippelli G, Maciel WG, et al. Acaricidal effects of fluazuron (2.5 mg/kg) and a combination of fluazuron (1.6 mg/kg) + ivermectin (0.63 mg/kg), administered at different routes, against *Rhipicephalus (Boophilus) microplus* parasitizing cattle. Exp Parasitol. 2015;153:22–28.

Hernández-Borges J, Ravelo-Pérez LM, Hernández-Suárez EM, Carnero A, Rodríguez-Delgado MA. Analysis of abamectin residues in avocados by high-performance liquid chromatography with fluorescence detection. J Chromatogr A. 2007;1165(1-2):52–57.

Hernando MD, Suàrez-Barcena JM, Bueno MJM, Garcia-Reyes JF, Fernández-Alba AR. Fast separation liquid chromatography–tandem mass spectrometry for the confirmation and quantitative analysis of avermectin residues in food. J Chromatogr A. 2007;1155(1):62-73.

Higa LOS, Garcia MV, Barros JC, Koller WW, Andreotti R. Evaluation *of Rhipicephalus* (*Boophilus*) *microplus* (Acari: Ixodidae) resistance to different acaricide formulations using amostras from Brazilian properties. Rev Bras Parasitol Vet. 2016;25(2):163-171.

ICH - International Conference on Harmonization. Validation of Analytical Procedures: Methodology, Technical Requirements for the Registration of Pharmaceuticals for Human Use. 2005. Acessed in 17 July 2020. Available from: https://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q2_R1/Step4/Q2_R1__Guideline.pdf.

Janer EC, Klafkec GM, Capurrob ML, Schumaker TTS. Cross-resistance between fipronil and lindane in Rhipicephalus (Boophilus) microplus. Vet Parasitol. 2015;210(1-2):77–83.

Krogh KA, Björklund E, Loeffler D, Fink G, Halling-Sørensen B, Ternes TA. Development of an analytical method to determine avermectins in water, sediments and soils using liquid chromatography–tandem mass spectrometry. J Chromatogr A. 2008;1211(1-2):60–69.

Milhome MAL, Sousa DOB, Lima FAF, Nascimento RF. Avaliação do potencial de contaminação de águas superficiais e subterrâneas por pesticidas aplicados na agricultura do Baixo Jaguaribe, CE. Eng Sanit Ambient. 2009;14(3):363-372.

Oliveira HL, Anacleto SS, Silva ATM, Pereira AC, Borges WS, Figueiredo EC, et al. Molecularly imprinted pipette-tip micro solid phase extraction for selective determination of fluoroquinolones in human urine using HPLC/DAD. J Chromatogr B. 2016;1033–1034:27-39.

Pollmeier M, Maiera S, Moriarty K, DeMontigny P. High-performance liquid chromatographic assay for the determination of a semisynthetic avermectin analog (eprinomectin) in bovine milk at parts per billion levels-method development and validation. J Chromatogr B. 2002;772(1)99–105.

Prichard R, Ménez C, Lespine A. Moxidectin and the avermectins: Consanguinity but not identity. Int J Parasitol Drugs Drug Resist. 2012;2:134–153.

Rabel SR, Stobaugh JF, Heinig R, Bostick JM. Improvements in detection sensitivity for the determination of ivermectin in plasma using chromatographic techniques and laser-induced fluorescence detection with automated derivatization. J Chromatogr. 1993;617(1):79-86.

Ribani M, Bottoli CBG, Collins CH, Jardim ICSF, Melo LFC. Validação em métodos cromatográficos e eletroforéticos. Quim Nova. 2004;27(5):771-780.

Silva ATM, Oliveira HL, Silva CF, Fonseca MC, Pereira TFD, Nascimento Jr CS, et al. Efficient molecularly imprinted polymer as a pipette-tip solid-phase sorbent for determination of carvedilol enantiomers in human urine. J Chromatogr B. 2017;1061–1062:399-410.

SINDAN (Sindicato Nacional da Indústria de Produtos para Saúde Animal). Representatividade por Classe terapêutica durante 2014-2018. Accessed in July 17, 2020. Available from: http://www.sindan.org.br/mercado-brasil-2018

Souza SVC, Junqueira G. A procedure to assess linearity by ordinary least squares method. Anal Chim Acta. 2005;552(1-2):25–35.

The United States Pharmacopeial Convention, Validation of Compendial Procedures, n. 1225, 2017. Accessed in July 2020. Available from: https://hmc.usp.org/sites/default/files/documents/HMC/GCs-Pdfs/c1225_1SUSP40.pdf.

Trapero DP, Sonseca-Yepes A, Moreira-Romero S, Hernández-Carrasquilla M. Determination of macrocyclic lactones in bovine liver using QuEChERS and HPLC with fluorescence detection. J Chromatogr B. 2016;1015–1016:166–172.

VICH GL 49: Studies to evaluate the metabolism and residue kinetics of veterinary drugs in food-producing animals: validation of analytical methods used in residue depletion studies. Available from: http://www.vichsec.org/guidelines/pharmaceuticals/pharma-safety/metabolism-and-residue-kinetics.html

Wang F, Chen J, Cheng H, Tang Z, Niu G, Pang Z, et al. Multi-residue method for the confirmation of four avermectin residues in food products of animal origin by ultra-performance liquid chromatography–tandem mass spectrometry. Food Addit Contam. 2008;28(5):627–663.

Wang X, Li P. Rapid screening of mycotoxins in liquid milk and milk powder by automated size-exclusion SPE-UPLC–MS/MS and quantification of matrix effects over the whole chromatographic run. Food Chem. 2015;173:897–904.

Received for publication on 26th September 2019
Accepted for publication on 16th June 2020