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Validation and Applicability of an HPLC Method for Analysis of Eprinomectin in Responsive Systems for *Pour-on* Administration in Cattle

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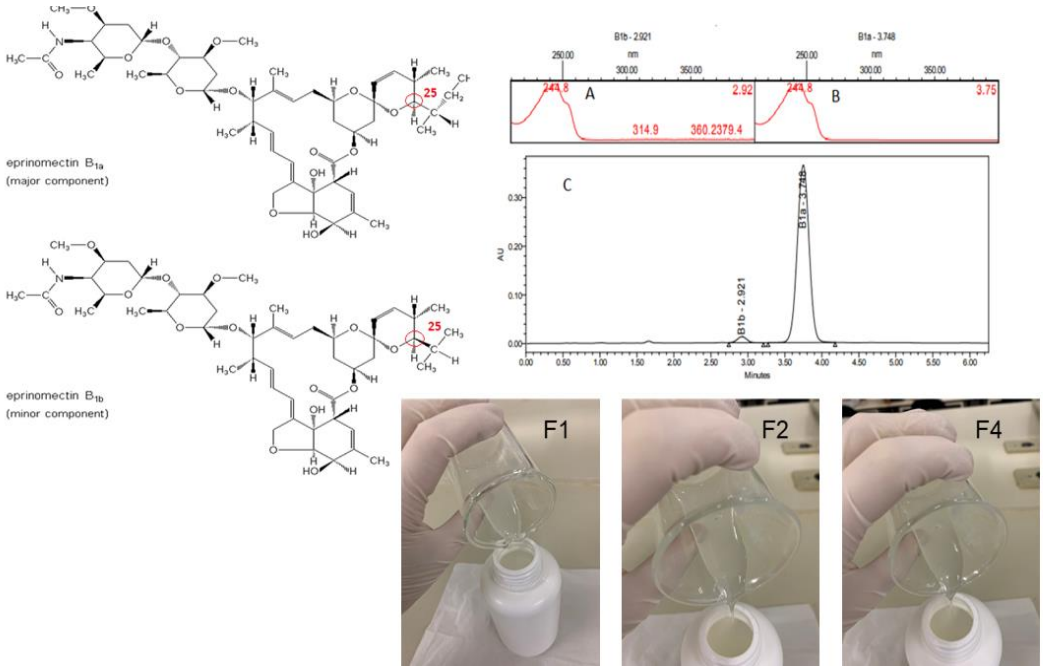
HIGHLIGHTS

- An HPLC method for eprinomectin (EPR) analysis was developed and validated.
- Five formulations containing poloxamer 407, carbomer 974P, isopropanol and EPR were prepared.
- The $T_{sol/gel}$ of formulations F1, F2 and F4 were compatible for use on the animal skin.
- F1, F2 and F4 displayed good recovery for EPR content and trapping efficiency.

Abstract: This study investigated the development, validation and applicability of a high-pressure liquid chromatographic (HPLC) method for analysis of eprinomectin (EPR) in thermoresponsive bioadhesive systems aiming the future *pour-on* administration in animals. For the determination of the both two types of EPR (B1a and B1b), an HPLC method was developed and validated according to the international standardized guidelines. A factorial design 2^2 plus central point was utilized for the system design, rendering five formulations containing poloxamer 407, carbomer 974P, isopropanol and EPR. The formulations were evaluated as visual characteristics and sol-gel transition temperature ($T_{sol/gel}$). In addition, the applicability of HPLC method was evaluated by analysis of the selected formulations, analyzing the drug content and trapping efficiency. The HPLC method was selective, linear, homoscedasticity, precise, accurate and robust. Formulations F3 and F5 presented instability; however, F1, F2 and F4 were considered suitable. $T_{sol/gel}$ of F1, F2 and F4 were compatible for use on the animal skin, and they displayed good recovery for EPR content and trapping efficiency. The proposed HPLC method for eprinomectin analysis was valid and could determine the eprinomectin content in systems F1, F2 and F4. Moreover, the methodology showed to be of importance for EPR analysis in thermoresponsive systems, displaying applicability.

Keywords: technology; eprinomectin; drug delivery; liquid chromatography; bioadhesive.

GRAPHICAL ABSTRACT



INTRODUCTION

Due to population growth, a major challenge is to meet the demand for food and the nutritional needs of human beings. The Food and Agriculture Organization of the United Nations (FAO) predicts the need to feed 8 billion people by the year 2030, combined with an expected increase in per-capita consumption. Meat is one of the foods that provide high nutritional quality in terms of balanced nutrients, and consumption is expected to increase in developed countries at an annual rate of 2.1% [1, 2]. The control of the parasitic infections plays an important role in the performance of animal production to meet the growing demand for protein worldwide. The control of parasites in cattle is an important factor in livestock, since parasites cause great economic losses due to the drop in productivity and transmission of pathogens, which can cause death in animals [1, 3].

Avermectins constitute a group of macrocyclic lactones that began to be used as anthelmintics from the 1980s with efficiency and safety. They are obtained from the fermentation of microorganisms present in the soil called *Streptomyces avermectilus* and *Streptomyces cyanogriseus* [4]. Anthelmintic macrocyclic lactones have a high degree of lipophilicity, are transported by lipoproteins in the lymph and plasma and are stored in adipose tissue, which vary between drugs due to differences in their chemical structures. Avermectins are amphiphilic compounds that typically possess both hydrophilic (affinity for water, polar) and lipophilic (affinity for fat) properties. The anthelmintic macrocyclic lactones ring and benzofuran cycles impart high degrees of lipophilicity. They have high octanol/water partition coefficients (log P), which testify to their property to solubilize in organic solvent like octanol or in lipid membranes rather than in aqueous solutions. Log P ranges from 4.4 to 5.6. [5,6]. Avermectins are classified into semi-synthetic and biosynthetic. Thus, eight different structures were isolated and divided into four main components (A1a, A2a, B1a and B2a) and four secondary components (A1b, A2b, B1b and B2b). In addition to their anthelmintic activity, these compounds are potent ectoparasiticides [7, 8].

Currently, eight avermectins are licensed as veterinary drugs and insecticides, namely abamectin, ivermectin, doramectin, emamectin benzoate, selamectin, moxidectin, milbemycin oxime and eprinomectim [9]. They have complex ring structures (Figure 1), with a 16-membered macrocyclic ring, containing a spiroketal group, a benzofuran ring and disaccharide functionality [8]. In this group, eprinomectin (EPR) is a substance that stands out for not accumulating in the tissues, making the withdrawal period unnecessary for the consumption of meat and milk and, therefore, can be recommended for lactating cows [10]. Among avermectins, EPR is the only one licensed for the treatment of parasitic infections in lactating cows, due to the zero-day withdrawal period for milk [9, 11].

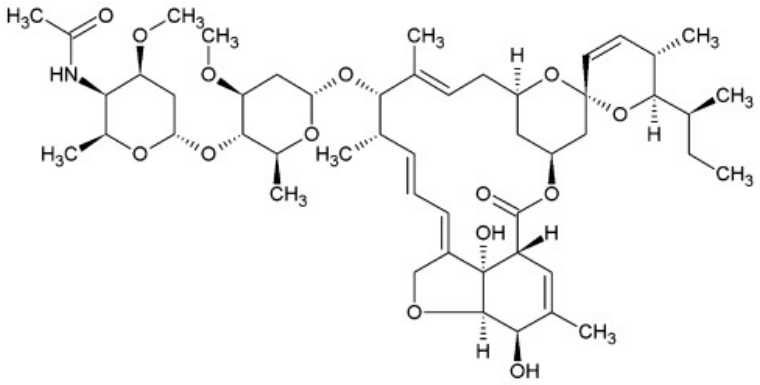


Figure 1. General chemical structure of eprinomectin.

EPR is a semi-synthetic lactone of the avermectin family, registered as a broad-spectrum endectocide, constituted by the mixture of two homologues, eprinomectin B1a (≥ 90%) and eprinomectin B1b (≤ 10%), which differ by a methylene group at position C25 [12, 13]. This drug acts on glutamate-dependent chloride channels, which are common in nematodes, insects and ticks, thus paralyzing the pharynx and somatic muscles, binding to these receptors, produces an increase in the permeability of chloride ions, which leads to membrane hyperpolarization cell, thereby opening chloride channels controlled by gamma-amino butyric acid (GABA). Increased flow of chloride ions at nerve synapses in worms, and in the neuromuscular system in arthropods, results in paralysis and death. In vertebrates, it stimulates the release of gamma-amino butyric acid (GABA) in neurons, but as they are usually in the brain and therefore, protected by a blood-brain barrier, the drug is safe for mammals [14].

In the past, to overcome the shortage of zero withdrawal anthelmintic drugs in milk, the off-label use of EPR was adopted by some dairy sheep breeders and only recently has the EPR been registered for use in dairy sheep. Today, it represents a promising anthelmintic drug with easy and well-being-friendly administration (topical) and zero withdrawal periods in milk [15]. The anthelmintic efficacy of the EPR topical administration has reported in sheep [12], goats [16] and cattle [17].

Thermoresponsive and bioadhesive polymeric systems can improve drug availability, increasing the contact time between the system and the skin and still controlling drug release [18]. The use of bioadhesive polymers, such as carbomers, is of great value due to their physicochemical characteristics, such as viscoelastic properties, presence of groups that make hydrogen bonds, degree of hydration, pH, charge, length, mass and chain conformation [18, 19]. Carbopol 974P® (C974P) and Carbopol 934P® (C934P) are examples of carbomers widely used in the pharmaceutical industry and are distinguished by the degree of crosslinking and manufacturing conditions [20].

One of the most widely used thermoresponsive polymer types is those of poloxamers, xyloglucan and poly(N-isopropylacrylamide). Poloxamers, also known by the trade names Pluronic®, Synperonic® and Tetronic®, have displayed many pharmaceutical applications, as well as an excellent compatibility with other compounds, studies show their high capacity to solubilize drugs, good drug release and the absence of toxicity in the mucous membranes, thus being recognized as a safe polymeric material for pharmaceutical application [21, 22]. The blends of poloxamer/Carbopol® were extensively studied and presented sol-gel transition temperatures around 30 ºC [20-25].

Numerous studies have been carried out using this thermoresponsive mucoadhesive system, reporting that they are suitable systems for the release of various drugs, both hydrophilic and hydrophobic [20, 23, 24]. In this context, bioadhesive thermoresponsive platforms composed of poloxamer 407 (P407) and C974P or C934P for topical administration of EPR have been proposed.

The United States Pharmacopeia (USP) monograph for the EPR uses two independent HPLC methods [26]. An HPLC method is used for the eprinomectin detection of all related substance peaks except for 8a-oxo-B1a. The second HPLC method is used exclusively to quantify the 8a-oxo-B1a peak [27]. Some studies reported the validation of different methodologies for quantification of EPR by HPLC [28 - 30]. However, no study reported the application of the method for quantification of the active in a polymeric system (containing Poloxamer P407, Carbopol C974P and isopropanol) for veterinary use. Most studies are applied to detection in milk [27,28]. Therefore, the aim of this work was to develop and validate a high-performance liquid chromatographic (HPLC) method for analysis of EPR from the polymeric systems. The HPLC methodology was investigated as validation, suitability and applicability. The results were evaluated and discussed focused on the developed methodology is able to provide reliable data.

MATERIAL AND METHODS

Materials

Eprinomectin (EPR; pharmaceutical grade) was obtained from Hebei Veyong (Shijiazhuang, Hebei, China) and analytical standard of EPR (purity 97.54%; B1b = 3.78% + B1a = 93.76%) was purchased from Sigma-Aldrich (Saint Louis, MO, USA). Acetonitrile and methanol (LC grade) were purchased from J. T. Baker (Mexico City, Mexico). Isopropanol (analytical grade) and triethanolamine (TEA) were purchased from Neon (Suzano, SP, Brazil). Carbopol 974P® (C974P) was kindly donated by Lubrizol (Sao Paulo, SP, Brazil). Poloxamer 407 (P407) was purchased from BASF Corporation (Geismar, LA, USA). A Milli-Q apparatus (Millipore, Bedford, USA) was utilized to obtain ultra-pure water (conductivity of 18 mΩ), which was utilized in all experiments.

HPLC analysis of eprinomectin (EPR)

Instrumentation and chromatographic conditions

The chromatographic analyses were performed using an HPLC system [27-30] model Alliance 2696 (Waters®, Wexford, Ireland), equipped with a C18 reversed phase column (4.6 x 75mm, 3.5µm) (Symmetry Waters®, Wexford, Ireland), a photodiode array detector (Waters®, Wexford, Ireland) and the Empower 3® software (Waters®, Milford, MA, USA). The study was performed using isocratic elution of mobile phase comprised of acetonitrile:methanol:water (47:33:20, v/v/v), flow of 1.5 mL/min, and at the temperature of 30 ºC. The injection volume was 15 µL and detection wavelength was 245 nm.

Preparation of standard and working solutions

For validation of analytical methodology, three stock solutions (SPM1, SPM2 and SPM3) were prepared by dissolving the EPR standard in the mobile phase to a concentration of 1 mg/mL (B1a + B1b).

Validation of the analytical method

The parameters evaluated during the validation of HPLC analytical method were determined according to the guidelines established by the International Conference on Harmonization (ICH Q2-R1 and Q2-R2) [31,32], evaluating the selectivity, specificity, linearity, limit of quantification, limit of detection, precision, accuracy and robustness.

Selectivity

The selectivity of the method was evaluated by comparing the chromatograms generated after injection of the diluent (mobile phase) and the EPR standard solution, respecting the test concentration of 300 µg/mL (B1a + B1b). Samples of the polymeric systems without the drug were also diluted in the mobile phase and analyzed. The objective of the test was to verify a possible elution of some interference in the same retention times of the chromatographic peaks of the B1b and B1a components of EPR.

Linearity and homoscedasticity

For the linearity evaluation, 21 samples were prepared by diluting the three independent standard solutions (SPM1, SPM2 and SPM3), at concentration levels of 70% (210 µg/mL), 80% (240 µg/mL), 90% (270 µg/mL), 100% (300 µg/mL), 110% (330 µg/mL), 120% (360 µg/mL) and 130% (390 µg/mL) of the theoretical concentration stipulated for the test (300 µg/mL of B1a + B1b). All solutions were prepared and analyzed in triplicate, and the individual concentrations of B1b and B1a were determined considering the levels provided in the EPR standard certificate (Table 1).

Table 1. Concentrations of standard solutions (S1, S2 and S3) used for the linearity evaluation

Conc.	S1 (µg/mL)			S2 (µg/mL)			S3 (µg/mL)		
level	B1b	B1a	B1b+B1a	B1b	B1a	B1b+B1a	B1b	B1a	B1b+B1a
70%	7.970	197.684	205.653	8.002	198.471	206.473	8.144	202.015	210.160
80%	9.108	225.924	235.032	9.145	226.824	235.969	9.308	230.875	240.182
90%	10.247	254.165	264.411	10.288	255.177	265.465	10.471	259.734	270.205
100%	11.385	282.405	293.790	11.431	283.530	294.961	11.635	288.593	300.228
110%	12.524	310.646	323.170	12.574	311.883	324.457	12.798	317.453	330.251
120%	13.662	338.886	352.549	13.717	340.236	353.953	13.962	346.312	360.274
130%	14.801	367.127	381.928	14.860	368.589	383.449	15.125	375.171	390.297

The analytical curves were obtained for linearity evaluation, referring to the components B1b and B1a of EPR, relating the concentrations of the standard solutions with the respective peak`s area obtained. The results were also evaluated by verifying the possible presence of discrepant points (outliers) by the Grubbs method for outlier diagnostic [33,34].

Analysis of variance (ANOVA) was utilized to evaluate the significance of linear regression and the analysis of lack of fit. The dispersion of the regression residues was evaluated, and thus the homoscedasticity of the data. The homogeneity of variances was assessed using the Cochran test at a significance level of 5%. The Cochran value (C_{calc}) was determined and compared with the Cochran tabulated value (C_{tab}) [34]. C_{calc} was calculated by Equation 1 and the homoscedasticity was confirmed if $C_{cal} \leq C_{tab}$:

$$C_{calc}c = \frac{s^2major}{\sum s^2} \quad (1)$$

Where s^2major is the largest variance and $\sum s^2$ is the sum of the variances.

Sensitivity

The limits of detection (LD) and quantification (LQ) were determined from the data obtained from the calibration curves, and were determined according to the equations 2 and 3 [31,32].

$$LD = \frac{3.3 \text{ x } \sigma}{s} \quad (2)$$

$$LQ = \frac{10 \text{ x } \sigma}{s} \quad (3)$$

Where σ is the standard deviation and s is the slope of the equation.

Precision

The precision of the methodology was investigated through the test of repeatability and intermediate precision [31,32]. For repeatability, a standard sample was prepared and analyzed in six replicate samples at a concentration of 300 µg/mL of eprinomectin (B1b + B1a), and the coefficient of variation of the concentrations obtained was calculated. For intermediate precision, a sample was prepared and analyzed in six replicate samples, on a different day than the repeatability test, and the concentrations obtained were compared with those obtained in the repeatability test, and the coefficient of variation among them was calculated. The results were evaluated by ANOVA statistical analysis.

Accuracy

The accuracy of an analytical method is obtained by the degree of agreement between the individual results of the method under study in relation to a value accepted as true. The accuracy must be expressed by the percentage recovery ratio of the analyte of known concentration added to the sample or by the ratio between the mean concentration, determined experimentally, and the corresponding theoretical concentration [31,32]. The accuracy of the method was evaluated based on the analysis of five concentrations of EPR 75% (225 µg/mL), 80% (240 µg/mL), 100% (300 µg/mL), 120% (360 µg/mL) and 125% (375 µg/mL) of the theoretical test concentration of 300 µg/mL, with three replicates each. Accuracy was expressed by the ratio between the experimentally determined mean concentration and the corresponding theoretical concentration according to the equation 4.

$$Recovery\ (\%) = \frac{Experimental\ concentration}{Theoretical\ concentration}\ X\ 100 \quad (4)$$

Robustness

The robustness of the method was evaluated by analyzing a sample of EPR standard, of 300 µg/mL (B1b + B1a), under the following conditions [31,32]: flow rate of 1.48 mL/min and 1.52 mL/min; column temperature of 28 °C and 32 °C; wavelength (λ) of 243 nm and 247 nm. The ANOVA analysis was performed to evaluate the results.

Applicability of the method

For the development of the polymeric platforms, a full factorial 2^2 design, with central point, was employed to evaluate the influence of P407 (15, 16.25 or 17.5%, w/w) (X_1) and C974P (0.2, 0.25 or 0.3%, w/w) (X_2) on physicochemical characteristics of systems. Moreover, the systems were also composed of isopropanol (15%, w/w) and EPR (0.5%, w/w) (Table 2). The tested concentrations were randomized, chosen according to previous studies carried out with the research group [17]. For the polymeric systems preparation, EPR was dispersed in isopropanol, and then P407 and an amount of ultra-purified water were added. C974P was dispersed in ultra-purified water and stirred until the total dispersion, which was added to the previous mixture. After complete homogenization, the pH was adjusted (pH = 7) using triethanolamine. The formulations were stored in hermetically sealed containers for 24 h prior to the analysis.

Table 2. Matrix of full factorial 2^2 design for the polymeric systems containing Poloxamer P407 (%, w/w) (*X*1), Carbopol C974P (%, w/w) (*X*2) and Isopropanol (15%, w/w) as fixed concentration, for the low and high levels of each variable and a central point

Independent variables (%, w/w)	**Low**	**Central**	**High**
	(-1)	**(0)**	**(+1)**
***X₁* = P407**	15	16.25	17.5
***X₂* = C974P**	0.2	0.25	0.30
Formulations	***X₁***		***X₂***
F1	-1		-1
F2	-1		1
F3	1		-1
F4	1		1
F5 [C]	0		0

The sol-gel transition temperature ($T_{sol/gel}$) analysis of each formulation was carried out using 20 g of sample at low temperature (5 ± 2 °C), which were placed in a 50-mL glass beaker with a magnetic bar and a magnetic stirrer with heating (IKA Works Inc., Wilmington, NC, USA) was used. The formulations were gradually heated under slow agitation, and the temperature observed with the aid of a thermometer. The $T_{sol/gel}$ was determined when the magnetic bar stopped rotating. Samples were heated to 60 ± 2 °C and analyzed in triplicate [22,23,25,35].

For determination of the EPR content in each system, an amount of formulation was added in a 10-mL volumetric flask, and the volume was made up with mobile phase, obtaining a solution with 300 µg/mL of EPR. The solution was filtered, and the determination of EPR was conducted using the chromatographic conditions previously described, and determined using the valid analytical curve. The analysis was performed in at least six replicate samples. The trapping efficiency (TE) was calculated according to Equation 5:

$$TE\ (\%) = \frac{Experimental\ EPR\ content}{Theoretical\ EPR\ content} x\ 100 \quad (5)$$

Statistical analysis

The responses obtained in the different tests were statistically compared using Analysis of Variance (ANOVA). In all cases of ANOVA, *post-hoc* comparisons of individual group means were performed using Tukey's Honestly Significant Difference test ($p < 0.05$). Excel software (Microsoft, Redmond, WA, USA) were used through.

RESULTS AND DISCUSSION

Validation of HPLC method

The selectivity of the method was evaluated and the comparison of the chromatograms, obtained from the standard solution of EPR (Figure 2), demonstrated that there was no elution of any possible interfering agent at the same time of retention of components B1b (2.92 min) and B1a (3.75 min) of EPR. It was possible

to verify that there was no elution of any other component in the same retention times of the B1b and B1a chromatographic peaks of eprinomectin, thus proving the selectivity of the method.

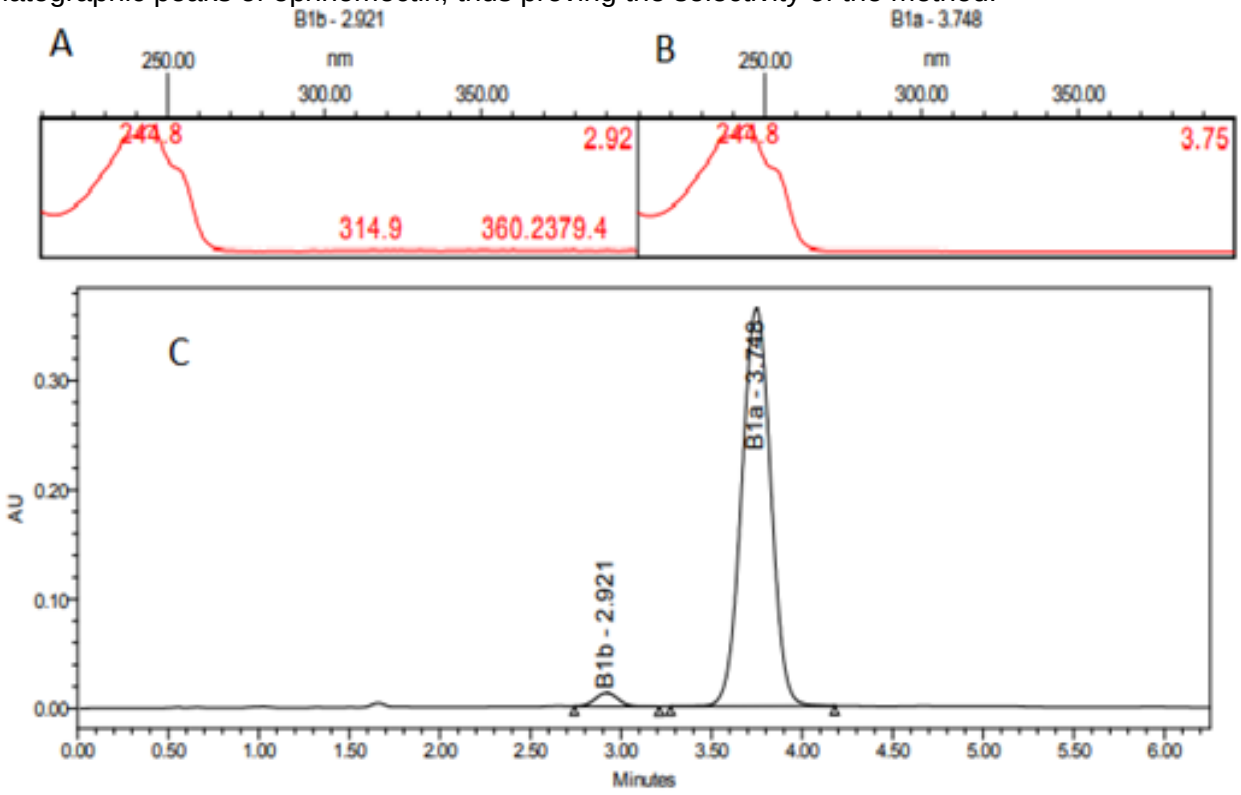


Figure 2. High-performance liquid chromatographic analysis, at λ = 245 nm, showing the specificity of the method for eprinomectin (EPR) at concentration of 300 µg/mL (B1b + B1a): (A) B1b peak scan spectrum; (B) B1a peak scan spectrum; (C) The chromatogram showing the EPR peaks B1b (2.921 min) and B1a (3.748 min).

The capacity of the method to obtain results directly proportional to analyte sample concentration is called linearity [31,32]. For the linearity, the interval between the upper and lower concentrations (range) has been determined as from 70% to 120% of 300 µg/mL. The results of the linearity of the proposed method test were according, and proving that the method was linear, guaranteeing the reliability of the area-concentration correlation. The calibration curves obtained through the Empower 3® Software showed a good correlation, for eprinomectin B1b the linear equation obtained was y = 8546.5 x – 5138, with determination coefficient (r^2) of 0.9940. For eprinomectin B1a, the r^2 was also 0.9940 and the linear equation was y = 20460 x – 93218. The statistical data of the regression found by the ANOVA test, where it was possible to observe that there is a very strong correlation between the variables.

In order to verify that the regression of the equation was statistically significant, tests were carried out to verify the adjustment of the linear model with analysis of the lack of fit and significance of the regression. If $F_{value} \geq F_{tab}$, the regression slope is not null at the selected confidence level. Therefore, there is an indication that the regression is significant. Table 3 shows the ANOVA results for linearity of the method, including the significance of linear regression and lack of fit analyses, for determination of EPR B1a and B1b.

The homoscedasticity was evaluated by the Grubbs test, and the results were satisfactory because the calculated G values were lower than the tabulated G value for n=9 (2.11), and none of the values obtained was discrepant to be. Homoscedasticity was also confirmed by the Cochran test where the value of C_{cal} (0.1970) ≤ C_{tab} (0.3384) [34].

The range of the analytical procedure was obtained from the linearity test, by determining the interval in which the method remained linear, supported by the results of sensitivity, precision and accuracy [34]. The work interval made it possible to detect the components of eprinomectin (B1b and B1a) within the limits of interest, and the detector response remained linear at concentrations from 1.231 µg/mL to 15.125 µg/mL for B1b and 1.157 µg/mL to 375.171 µg/mL for B1a. This range is supported by accuracy and precision tests.

Table 3. ANOVA results for linearity of the method for eprinomectin (EPR; B1a and B1b) analysis: significance of regression and lack of fit analysis

Parameter	DOF	SQ	MS	F_{value}	F_{tab}
			EPR B1b		
Regression	1.00	15970084781.57	15970084781.57	6322.72	4.08
Residual	40.00	101033037.65	2525825.94		
Lack of fit	5.00	1664915.70	332983.14	0.16	2.49
Pure error	35.00	118958718.70	2124262.83		
			EPR B1a		
Regression	1.00	5.29724E+13	5.29724E+13	6651.27	4.09
Residual	39.00	3.10606E+11	7964247767.00		
Lack of fit	5.00	10761812931.13	2152362586.23	0.30	2.49
Pure error	34.00	396744486195.54	7084722967.78		

DOF = Degree of freedom; SQ = Sum of squares; MS = Mean square.

The sensitivity of the method developed has been evaluated and the limit of detection (LD) and limit of quantification (LQ) were determined. The lowest amount of EPR that can be detected (LD) for B1b and B1a were 0.4061 µg/mL and 0.3817 µg/mL, respectively. Moreover, the LQ (the lowest amount of EPR that can be determined) was 1.2307 µg/mL and 1.1568 µg/mL for B1b and B1a, respectively.

The precision parameter was evaluated in terms of repeatability and intermediate precision. In this regard, the methodology proved to be reproducible and accurate, with results of less than 5% variation, analyzed in six replications on the same day (reproducibility), and with the comparison of the sample analyzed on different days by different analysts (intermediate precision). In the repeatability test performed, the coefficients of variation were less than 5%, both for B1b and B1a. The intermediate precision evaluation was also evaluated, and the results showed relative standard deviations were less than 5%, either for B1b and B1a.

The accuracy of a method is defined by the agreement between the results of an assay and the reference value accepted as true. Accuracy implies a combination of random and systematic error components, when applied to a series of different test results [32-34]. The method proved to be accurate (Table 4) for the quantification of B1b and B1a, with a recovery rate of less than 102%. For B1b, the recovery was 100.50% ± 0.99. The mean recovery found for B1a in the accuracy test was 100.21% ± 0.40.

Table 4. Recovery results for method accuracy analysis for eprinomectin (EPR; B1a and B1b)

B1b concentration (µg/mL)	Concentration (µg/mL)		Recovery (%)
	Experimental	Theoretical	
8.00	8.61 ± 0.10	8.70 ± 0.10	101.02 ± 0.33
9.00	9.19 ± 0.11	9.35 ± 0.09	101.75 ± 0.26
11.00	11.48 ± 0.13	11.51 ± 0.14	100.24 ± 0.75
13.00	13.78 ± 0.16	13.83 ± 0.06	100.36 ± 0.69
14.00	14.35 ± 0.17	14.23 ± 0.22	99.10 ± 0.48
B1a concentration (µg/mL)			
215	215.47 ± 1.84	213,63 ± 2.47	100.86 ± 0.49
225	228.28 ± 1.41	227,87 ± 2.64	100.19 ± 1.20
280	285.33 ± 2.84	284,84 ± 3.30	100.17 ± 0.69
340	341.83 ± 2.45	341,81 ± 3.96	100.01 ± 0.91
360	355.39 ± 8.4	356,05 ± 4.12	99.80 ± 1.20

The robustness of an analytical method is the method's ability of the method to support small variations of some parameters [32-34]. Thus, some chromatographic parameters were varied, such as mobile phase

flow, oven temperature, and variation of wavelength for detection. Table 5 displays the results when small changes in oven temperature, with the sample processed at temperatures of 28 °C and 32 °C. The flow was varied (1.48 and 1.52 mL/min) as well. For the robustness test, the sample was also processed with the wavelengths of 243 nm and 247 nm in addition to the 245 nm as described in the method, the results were satisfactory, proving the robustness of the method in the tested wavelength range.

The method proved to be robust with a coefficient of variation of less than 5% in the face of small variations [32-34].

Table 5. Determination of eprinomectin B1b and B1a concentrations relative standard between flow (1.48 and 1.52 mL/min), variations of wavelengths (λ = 243, 247 nm), oven temperature (28 °C and 32 °C) for robustness evaluation

Variation	Concentration (µg/mL)		RSD (%)	
	B1b	B1a	B1b	B1a
Flow (mL/min)				
1.48	11.77 ± 0.08	296,60 ± 1,29	2.01	0.56
1.52	11.54 ± 0.10	291,90 ± 3,60	1.67	0.89
Wavelength (λ; nm)				
243	11.74 ± 0.11	283.70 ± 0,82	0.63	1.82
247	11.93 ± 0.05	288.53 ± 1,71	1.07	1.02
Oven temperature (ºC)				
28	11.81 ± 0.06	291.21 ± 1.22	1.58	0.47
32	11.73 ± 0.11	291.56 ± 0.98	1.55	0.45

RSD = Relative standard deviation

Applicability of the HPLC method

Five polymeric systems containing EPR were prepared and microscopically evaluated just after the preparation and after 72 h. The visual analysis aimed to evaluate possible instability or precipitation. Moreover, the formulations were also evaluated as $T_{sol/gel}$ (Table 6).

Table 6. Tsol/gel transitional temperature, visual analysis (precipitation and crystallization) and eprinomectin content evaluation from polymeric formulations containing eprinomectin

Formulations	$T_{sol/gel}$ (ºC)	Crystallization	Eprinomectin content (g/100g)
F1	36.0 ± 1.0	absence	0.4981 ± 0.0002
F2	38.0 ± 1.0	absence	0.4985 ± 0.0017
F3	19.0 ± 1.0	presence	-
F4	32.0 ± 1.0	absence	0.4967 ± 0.0003
F5	45.0 ± 1.0	presence	-

Formulations F3 and F5 showed precipitation of the drug, indicating they are not suitable for further analysis. One of the major challenges of this work, in addition to validating an adequate method for the quantification of eprinomectin, was to prepare a stable polymeric system, since EPR is not easily solubilized, and it can precipitate or crystallize, leaving the system unstable. The polymeric systems F1, F2 and F4 displayed to be promising systems, and they could be utilized to deliver EPR.

The F3 and F5 formulations displayed very discrepant and statistically different values from the other formulations. As the purpose of the formulation is to use pour on, poured directly onto the animal's skin, the ideal gelation temperature would be close to body temperature (34-37 ºC). And in this way, the F3 ($T_{sol/gel}$ result of 19.0 ± 1.0 ºC) and F5 (Tsol/gel result of 45.0 ± 1.0 ºC) formulations are not as expected and were discarded from the study.

The EPR content was also evaluated in each polymeric formulation, and displayed similar results for F1, F2 and F4 ($p > 0.05$). The EPR content was 0.4981, 0.4985 and 0.4967 (%, w/w) for formulations F1, F2 and F3, respectively. Moreover, analyzing the drug content and EPR trapping efficiency (TE) of systems, it was possible to observe that the formulations displayed good TE results, showing their ability for entrapment of EPR in the polymeric structure (Table 6). The TE displayed 99.62 %, 99.70 and 99.34 for F1, F2 and F4,

respectively. Some liposoluble drugs require a polymeric system to become dispersed in water or to be incorporated into the formulation. In this sense, systems composed of a mixture of poloxamer 407 and carbomers can be used. These preparations were conceived as bioadhesive thermoresponsive platforms for drug delivery and demonstrated the ability to achieve close contact with the mucosa during a long residence time [18-25]. Some studies have shown that the polymeric systems containing poloxamer and Carbopol are very favorable platforms for both soluble [24,25] and insoluble drug incorporation (being trapped in the core of the micelles) [36]. These results indicate the HPLC could analyze the EPR content in the different formulations and considering the complex sample, composed of different polymers and isopropanol.

CONCLUSION

A methodology for analysis of EPR by HPLC was performed displayed to be valid. This methodology displayed well resolved peaks, with good selectivity. The linearity of the method was evaluated for both B1b and B1a, being considered linear and without lack of fit. The homoscedasticity was evaluated by Cochran, also confirmed by Grubbs method and showed no outliers. The method showed to be accurate and precise. Moreover, small changes of mobile phase flow, column oven temperature, and wavelength did not significant changed the results, indicating the robustness of methodology for these analyzed parameters. Moreover, this method displayed to be useful to analyze EPR in the designed thermoresponsive bioadhesive systems, which displayed high trapping efficiency. Therefore, this HPLC methodology showed to be valid, versatile and applicable for EPR analysis in the complex polymeric matrices, such as formulations F1, F2 and F4. Future studies using these systems should be conducted for the optimization of a final formulation for pour-on administration of EPR.

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