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Research Article

DEVELOPMENT AND VALIDATION OF AN HPLC-MS/MS METHOD FOR SIMULTANEOUS DETERMINATION OF IVERMECTIN, FEBANTEL, PRAZIQUANTEL, PYRANTEL PAMOATE AND RELATED COMPOUNDS IN FIXED DOSE COMBINATION FOR VETERINARY USE

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ABSTRACT

High performance liquid chromatography with tandem mass spectrometry (HPLC-MS/MS) method for simultaneous quantification of ivermectin, febantel, praziquantel and pyrantel pamoate in addition to the qualitative determination of febantel-related compound C and praziquantel-related compounds A, B and C in tablets was developed and validated. Chromatographic separations were achieved using isocratic elution (200 µL/min) on a C8 column (50 x 2.1 mm i.d) coupled with a C8 (10 x 2.1 mm i.d) guard column maintained at 20 °C. The mobile phase consisted in water/acetonitrile (15:85 v/v*)* containing 0.1% formic acid and 3 mmol/L ammonium formate. The injection volume used was 20 µL. The validation parameters indicated that the method has high sensitivity and selectivity with excellent linearity ($r \geq 0.99$). The observed range of recovery was 93.26% to 101.36% with a RSD of <5% for intraday and interday precision. Sample analyses demonstrated a lack of standardization in the amounts of these drugs, which may contribute to their toxicity or a reduction in efficacy, depending of the sample used. The proposed HPLC-MS/MS method is presented as an alternative option for the quality control of pharmaceutical preparations containing these drugs.

Keywords: Febantel / HPLC-MS/MS / Ivermectin / Praziquantel / Pyrantel pamoate / Validation.

INTRODUCTION

Helminthiases are parasitic diseases commonly found in pets and cause significant morbidity and mortality in dogs and cats. These infections are promoted mainly for nematodes, cestodes and trematodes and have public health significance because parasitic diseases are also transmissible to humans[1,2].

Anthelmintic drugs have been widely used on pets to treat or prevent parasitic diseases. In some cases, combinations of these drugs are required to enlarge the spectrum of action, and in most cases, associations of ivermectin, febantel, praziquantel and pyrantel pamoate are commonly used to improve the efficacy of treatment against helminthiasis. Administered orally in pets, associations of febantel, praziquantel and pyrantel pamoate showed high efficacy against *Giardia sp*[3] and *Ancylostoma ceylanicum*[4]. A synergistic effect of the combination of pyrantel pamoate and fenbendazole (febantel metabolite) has also been shown to be effective against *Toxocara canis*[5], while ivermectin and pyrantel pamoate have demonstrated high effectiveness against *Ancylostoma caninum*[6] and *Ancylostoma braziliense*[7].

Despite the large margin of safety, the administration of some anthelmintics can promote toxic effects. For example, ivermectin shows high toxicity in dogs at very low dosages levels (0.1-0.2 mg/kg)[2]. Considering that the toxic and therapeutic effects of anthelmintics are dose dependent, rigorous quality control becomes necessary, particularly in pharmaceutical preparations in which effectiveness and safety should be guaranteed.

Many analytical methods have been described in literature for the determination of ivermectin, febantel, praziquantel and pyrantel pamoate using various detection techniques, such as potentiometric titration[8], voltammetry[9,10], spectrophotometry[11-13] and high-performance liquid chromatography (HPLC) with a diode array detector[8,14-31]. However, none of these techniques simultaneously monitor these drugs or present adequate sensibility or selectivity. The use of HPLC coupled to tandem mass spectrometry (HPLC-MS/MS) has permitted the detection and quantification of low concentrations of these drugs in various matrices[32-44]. Despite this technique's potential for high selectivity and sensibility, none of the HPLC-MS/MS methods were applied for the simultaneous determination of ivermectin, febantel, praziquantel, pyrantel pamoate and related compounds of febantel and praziquantel in tablets.

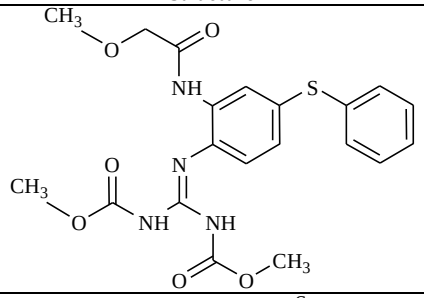
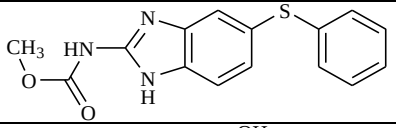
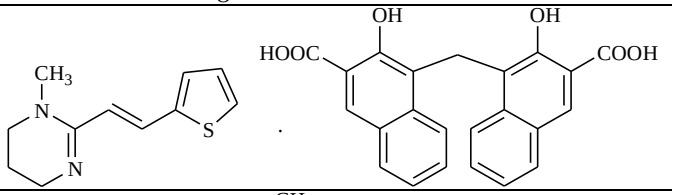
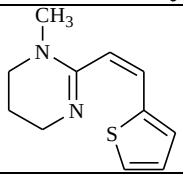
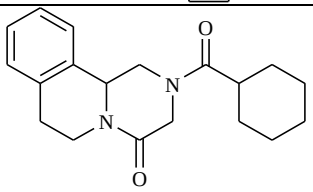
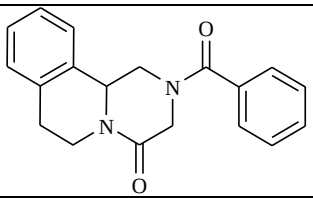
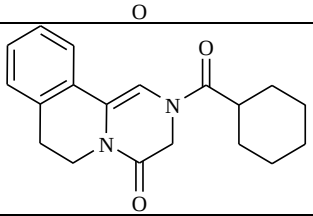
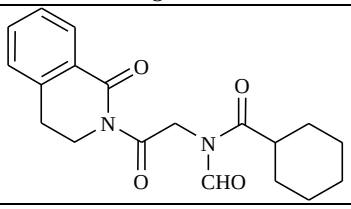
The present work deals with the development and validation of a fast, sensitive and selective HPLC-MS/MS method for the simultaneous quantification of ivermectin, febantel, praziquantel and pyrantel pamoate, and the qualitative determination of febantel-related compound C and praziquantel-related compounds A, B and C in tablets. The method described here is highly specific and sensitive and is capable of detecting the primary veterinary anthelmintics at low concentrations. Therefore, the proposed method provides the necessary framework for quality control of the main veterinary anthelmintics in all drug-processing stages.

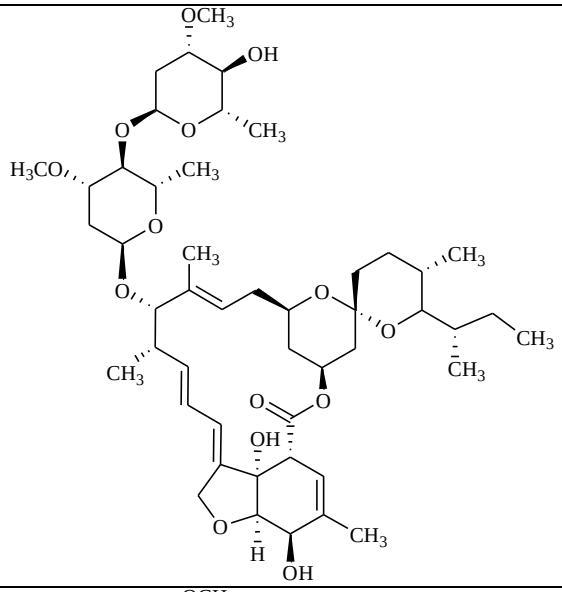
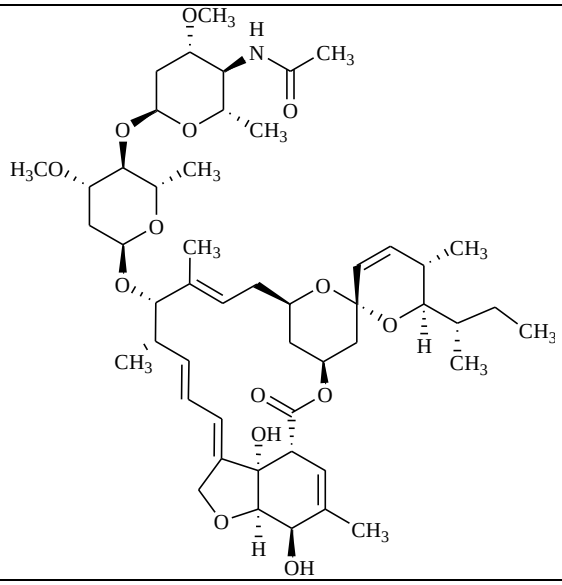
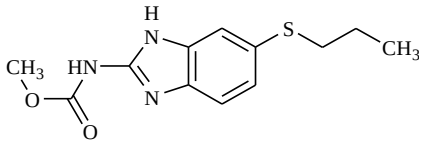
MATERIAL AND METHODS

Standards, reagents, chemicals and samples

Standards of febantel (99.6%), praziquantel (99.7%), pyrantel pamoate (96.7%) and febantel-related compound C (99.5%) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Pyrantel-related compound A (98.0%), praziquantel-related compound A (99.0%), praziquantel-related compound B (98.0%), praziquantel-related compound C (100.0%), albendazole (99.6%) and ivermectin (90.6%) were purchased from United States Pharmacopoeia (Rockville, MD, USA). Eprinomectin (100.0%) was obtained from Dr. Ehrenstorfer (Augsburg, Bavaria, Germany). The structures of all chemicals are shown in Table 1.

TABLE 1: COMPOUNDS AND RESPECTIVE CHEMICAL STRUCTURE.

Compound	Structure
Febantel Dimethyl N,N′-[[[2-[(methoxyacetyl)amino]-4-(phenylsulfanyl)phenyl]imino]methylene]dicarbamate	
Febantel related compound C Methyl [5-(phenylsulfanyl)-1H-benzimidazol-2-yl]carbamate	
Pyrantel pamoate 1-Methyl-2-[(E)-2-(thiophen-2-yl)ethenyl]-1,4,5,6-tetrahydropyrimidine hydrogen 4,4′-methylenebis(3-hydroxynaphthalene-2-carboxylate)	
Pyrantel related compound A 1-methyl-2-[(Z)-2-(thiophen-2-yl)ethenyl]-1,4,5,6-tetrahydropyrimidine	
Praziquantel (11bRS)-2-(Cyclohexylcarbonyl)-1,2,3,6,7,11b-hexahydro-4Hpyrazino[2,1-a]isoquinolin-4-one	
Praziquantel related compound A (11bRS)-2-benzoyl-1,2,3,6,7,11b-hexahydro-4H-pyrazino[2,1-a]isoquinolin-4-one	
Praziquantel related compound B 2-(cyclohexylcarbonyl)-2,3,6,7-tetrahydro-4H-pyrazino[2,1-a]isoquinolin-4-one	
Praziquantel related compound C N-formyl-N-[2-oxo-2-(1-oxo-3,4-dihydroisoquinolin-2(1H)-yl)ethyl]cyclohexanecarboxamide	

Ivermectin (B1a) 5-O-demethyl-22,23-dihydroavermectin A1a	
Eprinomectin (B1a) (4''R)-4''-(acetylamino)-5-O-demethyl-4''-deoxyavermectin A1a	
Albendazole Methyl [5-(propylsulphanyl)-1H-benzimidazol-2-yl]carbamate	

Acetonitrile and methanol (HPLC grade) were obtained from Tedia (Fairfield, CA, USA). Ammonium acetate, formic acid (88%), glacial acetic acid and hexane were obtained from J. T. Baker (Phillipsburg, NJ, USA). Ammonium formate (≥97%) was obtained from Acros Organics (Fair Lawn, NJ, USA). Acetone was obtained from Vetec (Rio de Janeiro, RJ, Brazil). Ammonium hydroxide was obtained from F. Maia (São Paulo, SP, Brazil). Ultrapure water was obtained using a Milli-Q purification system from Millipore (Milford, MA, USA). Three fixed dose combinations containing 0.06 mg of ivermectin, 150.0 mg of febantel, 50.0 mg of praziquantel and 144.0 mg of pyrantel pamoate were purchased from local markets (Curitiba, PR, Brazil).

Standard solutions

Stock solutions of ivermectin (IVE), febantel (FEB), praziquantel (PRA), pyrantel pamoate (PYR), pyrantel-related compound A (PYRA), praziquantel-related compound A (PRAA), praziquantel-related compound B (PRAB), praziquantel-related compound C (PRAC) and the internal standard (IS) eprinomectin (EPR) were prepared separately at a concentration of 1 mg/mL. Febantel-related compound C (FEBC) and the internal standard (IS) albendazole (ALB) were prepared separately at a concentration of 0.2 mg/mL. All stock solutions were prepared in acetonitrile/methanol (50:50 v/v) and stored at 4 °C. Working standard solutions were freshly prepared as needed for each experiment by direct dilution of the stock standard solutions in water/acetonitrile (15:85 v/v*)* containing 0.1% formic acid and 3 mmol/L ammonium formate. All working standard solutions were filtered through a polyvinylidene fluoride (PVDF) syringe filter (11 mm, 0.45 mm, Millipore Millex, Billerica, MA, USA) prior to injection.

Sample preparation

Twenty tablets of each fixed dose combinations were powdered and an aliquot of the powder equivalent of 25 mg of febantel, 24 mg of pyrantel pamoate, 8.33 mg of praziquantel and 0.01 mg of ivermectin was accurately weighed and transferred to a 50 mL volumetric flask. The samples were spiked with the internal standard solution to obtain a final concentration of 200 µg/mL of

ALB (IS) and 0.1 µg/mL of EPR (IS). A 25 mL aliquot of acetonitrile/methanol (50:50 v/v) was added to the flask and samples were subjected to mechanical stirring (30 rpm) and sonication for 10 min. The volume was completed with the same solvent and the samples were filtered through a quantitative filter paper (0.28 µm pore size, J Prolab, São José dos Pinhais, PR, Brazil). To determine febantel, praziquantel, pyrantel pamoate, febantel-related compound C and praziquantel-related compounds A, B and C, the filtrate was diluted 1:10000 v/v in water/acetonitrile (15:85 v/v*)* containing 0.1% formic acid and 3 mmol/L of ammonium formate. For the determination of ivermectin, the filtrate was diluted 1:10 v/v in acetonitrile/water (35:65 v/v*)* and a 1 mL aliquot of this solution was transferred to an Oasis HLB cartridge (Waters, Eschborn, Hesse, Germany), previously conditioned with 1 mL of hexane, 1 mL of acetone, 1 mL of acetonitrile, 1 mL of ultrapure water and 1 mL of acetonitrile/water (35:65 v/v). After sample loading, the sorbent was washed with 20 mL of acetonitrile/water (35:65 v/v). Ivermectin was eluted with 1 mL of acetonitrile and the eluate was evaporated in a sample concentrator until dry (40 °C, CentriVap Labconco, Kansas City, MO, USA). Finally, the sample was redissolved by vortex with 200 µL of water/acetonitrile (15:85 v/v*)* containing 0.1% formic acid and 3 mmol/L of ammonium formate. All samples were prepared under low light exposure and filtered through a PVDF syringe filter prior to injection.

HPLC-MS/MS instrumentation and conditions

HPLC-MS/MS analyses were performed on an Agilent 1200 HPLC system (Agilent Technologies, Santa Clara, CA, USA) that consisted of a G1312B binary pump, G1379B degasser and G1316B column oven. These apparatuses were connected with a CTC Sample Manager (Model 2777, Waters, Milford, MA, USA). The analytical separations were achieved on an XBridge-C8 (50 x 2.1 mm, 5 µm, Waters) column coupled with an XBridge C8 (10 x 2.1 mm, 5 µm, Waters) guard column maintained at 20 °C. The mobile phase consisted of water/acetonitrile (15:85 v/v*)* containing 0.1% formic acid and 3 mmol/L of ammonium formate. The isocratic flow rate was 200 µL/min and the injection volume was 20 µL. The needle was washed with 2 mL of acetonitrile/methanol (50:50 v/v) between injections. The mass spectrometer coupled to the HPLC system was a triple quadrupole API 3200 from Applied Biosystems MDS Sciex Instruments (Foster City, CA, USA) equipped with a syringe pump (Harvard Apparatus, Holliston, MA, USA) and an electrospray (ESI) ion source. The ESI source was operated in the positive ion mode and data acquisition was performed with the MS Workstation by Analyst 1.4 software (MDS Sciex, Concord, Ontario, Canada). The ion source parameters were the following: CUR 10 psi; GS1 45 psi; GS2 40 psi; CAD 4 psi; ion spray voltage (ISV) 5000 V; and temperature 450 ºC. Quantification was performed in multiple reaction monitoring (MRM) mode, maintaining a dwell time of 150 ms. The ion transitions and the individual compound parameters including the declustering potential (DP), entrance potential (EP), collision cell entrance potential (CEP), collision energy (CE) and cell exit potential (CXP) are shown in Table 2. The high purity nitrogen and zero grade air that were used as the CUR, GS1, GS2 and CAD gases were produced using a high-purity nitrogen generator from PEAK Scientific Instruments (Chicago, IL, USA).

TABLE 2 - SUMMARY OF THE MONITORED IONS AND THE MS/MS OPERATING CONDITIONS FOR THE ANALYTES AND IS

Compound	Identity	Precursor ion (*m/z*)	Fragment ions (*m/z*)	DP[a] (V)	EP[b] (V)	CEP[c] (V)	CE[d] (eV)	CXP[e] (V)
IVE	$[M+NH_4]^+$	892.3	307.2	41	8.5	34	35	6
			145.1	41	8.5	34	45	4
FEB	$[M+H]^+$	447.0	383.2	26	8	20	21	34
			415.1	26	8	20	19	32
PYR	$[M+H]^+$	207.2	150.2	46	5	14	37	4
			136.2	46	5	14	39	4
PRA	$[M+H]^+$	313.3	203.3	36	9.5	16	37	4
			174.3	31	7	15	15	4
FEBC	$[M+H]^+$	300.1	268.2	46	8	16	25	6
			159.1	46	8	16	47	4
PRAA	$[M+H]^+$	307.1	105.0	36	9	16	71	4
			77.1	41	10	14	21	4
PRAB	$[M+H]^+$	311.2	201.2	41	10	18	33	4
			173.1	26	7	15	33	4
PRAC	$[M+H]^+$	343.2	233.1	31	7	16	23	4
			148.1	36	9	16	23	4
ALB (IS)	$[M+H]^+$	266.1	234.2	41	7.5	16	41	4
			191.0	50	10	13	50	4
EPR (IS)	$[M+H]^+$	914.2	186.2	31	7	34	33	4
			154.2	31	7	34	49	4

a) DP, declustering potencial.b) EP, entrance potencial.c) CEP, collision cell entrance potencial.d) CE, collision energy. e) CXP, cell exit potential.

Selectivity and matrix effect

To confirm the selectivity, the method of addition of standard was applied. The method of addition of standard consists of the comparison of one analytical curve obtained with standard solutions, with another analytical curve obtained with sample spiked with analytes. In the proposed method, working standard solutions were prepared in triplicate at concentration levels of 12, 15 and 18 ng/mL for PRA; 80, 100 and 120 ng/mL for IVE; 40, 50 and 60 ng/mL for FEB, PYR, FEBC, PRAA, PRAB and PRAC; 20 ng/mL for ALB (IS) and 50 ng/mL for EPR (IS). The data was treated by linear regression and an analytical curve was obtained. Then, twenty tablets of each fixed dose combinations were powdered and an aliquot of the powder equivalent of 25 mg of febantel, 24 mg of pyrantel pamoate, 8.33 mg of praziquantel and 0.01 mg of ivermectin was accurately weighed and transferred to a 50 mL volumetric flask. A 25 mL aliquot of acetonitrile/methanol (50:50 v/v) was added to the flask and samples were subjected to mechanical stirring (30 rpm) and sonication for 10 min. The volume was completed with the same solvent and the samples were filtered through a quantitative filter paper. The filtrate was diluted (1:20000 v/v in mobile phase) and spiked with the analytes and ISs at the same concentration levels of the analytical curve obtained with standard solutions. The spiked samples were injected in triplicate into the HPLC-MS/MS and the same mathematical treatment was used to obtain the analytical curve. The slopes obtained from both analytical curves were then compared.

The matrix effect was evaluated by comparing the mean peak areas of the analytes obtained with spiked samples with those obtained with standard solutions. The spiked samples and standard solutions were prepared as described in selectivity study. The results of the mean peak areas comparison were expressed in percentage. Variations from 95 to 105% indicate the absence of matrix effect.

Limits of detection and quantification

The limits of detection (LOD) and quantitation (LOQ) were estimated from the signal-to-noise ratio. To conduct this study, a triplicate of working standard solution was prepared with 500 ng/mL of each compound. These solutions were diluted in mobile phase and injected until the smallest detectable peaks were obtained. The LOD was estimated at a signal-to-noise ratio of 3:1, and the LOQ was estimated as the peak at a signal-to-noise ratio of at least 10:1, until the acceptable accuracy and precision were reached (i.e., 10% relative standard deviation - RSD).

Linearity

Calibration curves were performed in triplicate at six varying concentration levels. To conduct this study, working standard solutions were prepared in mobile phase to obtain a concentration range of 6-30 ng/mL for PRA, 40-200 ng/mL for IVE and 20-100 ng/mL for FEB and PYR. Eprinomectin was used as an IS for ivermectin at a concentration of 50 ng/mL and albendazole was used for other analytes at a concentration of 20 ng/mL. For each compound, a calibration curve was generated to confirm the linear relationship between the analyte peak areas/IS peak areas and the analyte concentration/IS concentration. The slope, intercept and regression coefficient (r) were calculated as regression parameters by weighted (1/x) linear regression.

Precision and accuracy

The precision of the method was evaluated using the measurements of the repeatability (intraday) and intermediate precision (interday). To conduct this study, working standard solutions were prepared at concentration levels of 12, 15 and 18 ng/mL for PRA; 80, 100 and 120 ng/mL for IVE; 40, 50 and 60 ng/mL for FEB and PYR; 20 ng/mL for ALB (IS) and 50 ng/mL for EPR (IS). To investigate the repeatability, the same analyst completed three replicate injections of the working standard solutions in a short period of time. The intermediate precision was determined using freshly prepared solutions by a second analyst after two consecutive days. The results are expressed as the RSD, and statistical analysis (Student's t-test) was used to compare measurements obtained from the analytes in each assay.

The accuracy was measured in triplicate through a recovery assay. To perform this assay, one sample was prepared as described in "Sample preparation", and the filtrate was diluted (1:50000 v/v) in mobile phase and analyzed. The same sample was spiked with standard solutions at the same concentration levels of the precision assay. The amount of analyte recovered was calculated by subtracting the values of the analytes found in the spiked samples from those obtained in samples without fortification. The accuracy was expressed as a percentage of the amount recovered compared with the standard concentrations.

Stability assay

Studies were performed to evaluate the stability of the analyte solutions under normal working conditions. The stability was evaluated after a period of 1, 7 and 30 days stored under ambient temperature (21-23 °C, amber bottle); 1, 7 and 30 days stored under refrigeration (4 °C, amber bottle) and 24 h stored in the Sample Manager (20 °C, amber vial).

The analyses were performed using six replicate injections of the concentration of 15 ng/mL of PRA; 20 ng/mL of ALB (IS); 30 ng/mL of PRAA, PRAB and PRAC; 50 ng/mL of FEB, PYR, FEBC and EPR (IS) and 100 ng/mL of IVE. A statistical analysis (Student's t-test) was performed to compare the peak areas obtained from freshly prepared standard solutions with those obtained after each period of storage.

RESULTS AND DISCUSSION

Method development

HPLC-MS/MS methods have been previously reported in literature to determine ivermectin, febantel, praziquantel and pyrantel pamoate using various mobile phase compositions. As organic modifier the use of acetonitrile have been the most common, being reported the use of several additives for ions generation, including formic acid[32,36,42,45], acetic acid[34,35], ammonium formate[38,40], ammonium acetate[46] and combinations of these additives[33,37,39,43,44,47]. Thus, to compose the mobile phase, a comparison of different additives was performed.

Overall, the generation of $[M+H]^+$ ions was observed for all compounds, except ivermectin, which generated the formation of an $[M+NH_4]^+$ adduct ion. Methanol was not evaluated as an organic modifier to the mobile phase composition because it contains traces of sodium that lead to the formation of sodium adduct ions. According to Durden[37], sodium adducts are more stable than ammonium adducts but require higher collision energies to generate fragment ions. As a consequence, a less reproducible method is expected, and thus, only low intense fragment ions and non-linear calibration curves can be generated.

Consequently, acetonitrile was our first choice for the organic solvent in all experiments. The signal intensity of each analyte was evaluated by the direct infusion of working standard solutions (300 ng/mL of EPR (IS), 25 ng/mL of PYR and PYRA and 100 ng/mL of ALB (IS), IVE, FEB, FEBC, PRA, PRAA, PRAB and PRAC) prepared in water/acetonitrile (15:85 v/v) and containing different concentrations and combinations of additives (acetic acid, formic acid, ammonium acetate and ammonium formate).

Adequate signal intensity was observed for all compounds when 1% acetic acid and 5 mmol/L of ammonium acetate was used. However, during sample infusion, a green color was observed, suggesting sample oxidation or degradation. Thus, only combinations of formic acid with ammonium acetate or ammonium formate were considered to compose the mobile phase. Among these combinations, the use of 1% formic acid and 3 mmol/L of ammonium formate promoted excellent signal intensity for most of compounds. However, avermectins are not stable under acidic conditions[43,47], and the proportion of formic acid was reduced to 0.1%. With this modification, the signal intensity of all compounds increased, except PYR, which experienced a 30% reduction in signal. However, this reduction was not sufficient to compromise the analysis, as an excellent ionization of this compound was achieved and a high content of this substance is expected in the samples. Therefore, the combination of 0.1% of formic acid and 3 mmol/L of ammonium formate was selected to compose the mobile phase and diluent solution.

All of the MS and MS/MS parameters (DP, EP, CEP, CE, and CXP) were optimized by automatic multiple reaction monitoring (MRM). The two most intense fragment signals for each compound were obtained, except for PRAB and PRAC, which had identical fragments (55.2 and 83.2 *m/z*). To differentiate these compounds, lower intensity fragment ions were chosen. Furthermore, the experiment showed that all fragment ions of PYR and PYRA are identical, since they are *trans* and *cis* isomers, respectively. As such, the determination of PYR was prioritized, given that the conversion of PYR to PYRA occurs when the solution is exposed to light[11].

The optimization of the source parameters (CUR, CAD, IS, GS1, GS2, and temperature) was accomplished through flow-injection analysis (FIA), which coupled the chromatograph with the mass spectrometer. The pump was operated at 200 µL/min using an isocratic system of water/acetonitrile (50:50 v/v*)* containing 0.1% formic acid and 3 mmol/L of ammonium formate.

To develop the HPLC-MS/MS method, several combinations of water and acetonitrile were tested for mobile phase composition. Formic acid (0.1%) and 3 mmol/L of ammonium formate were retained as additives. Different columns (XBridge-C18 100 x 2.1 mm, 5 µm and XBridge-C8 50 x 2.1 mm, 5 µm - Waters Corporation) were also evaluated, maintaining the flow rate of 200 µL/min.

The initial experiments were conducted using a C18 column maintained at 20 °C and a mobile phase that consisted of water/acetonitrile (50:50 v/v*)* containing 0.1% formic acid and 3 mmol/L ammonium formate, eluted in isocratic system. Under this condition, an extensive run time analysis was observed (Tr > 20 min

for IVE and EPR) and the proportion of organic modifier was increased to 85%. With this modification, the retention times of EPR and IVE significantly decreased (Tr: 3.1 and 6.3 min, respectively). For comparative purposes, a C8 column was tested under the same conditions of the C18 column and the retention time of EPR and IVE reduced to 1.56 and 2.47 min, respectively. Using a C8 column, the most sensitive and best peak shapes were also achieved for all compounds, and no significant environmental waste was produced because the short run time (3.5 min). The representative chromatograms of the method are shown in Fig. 1.

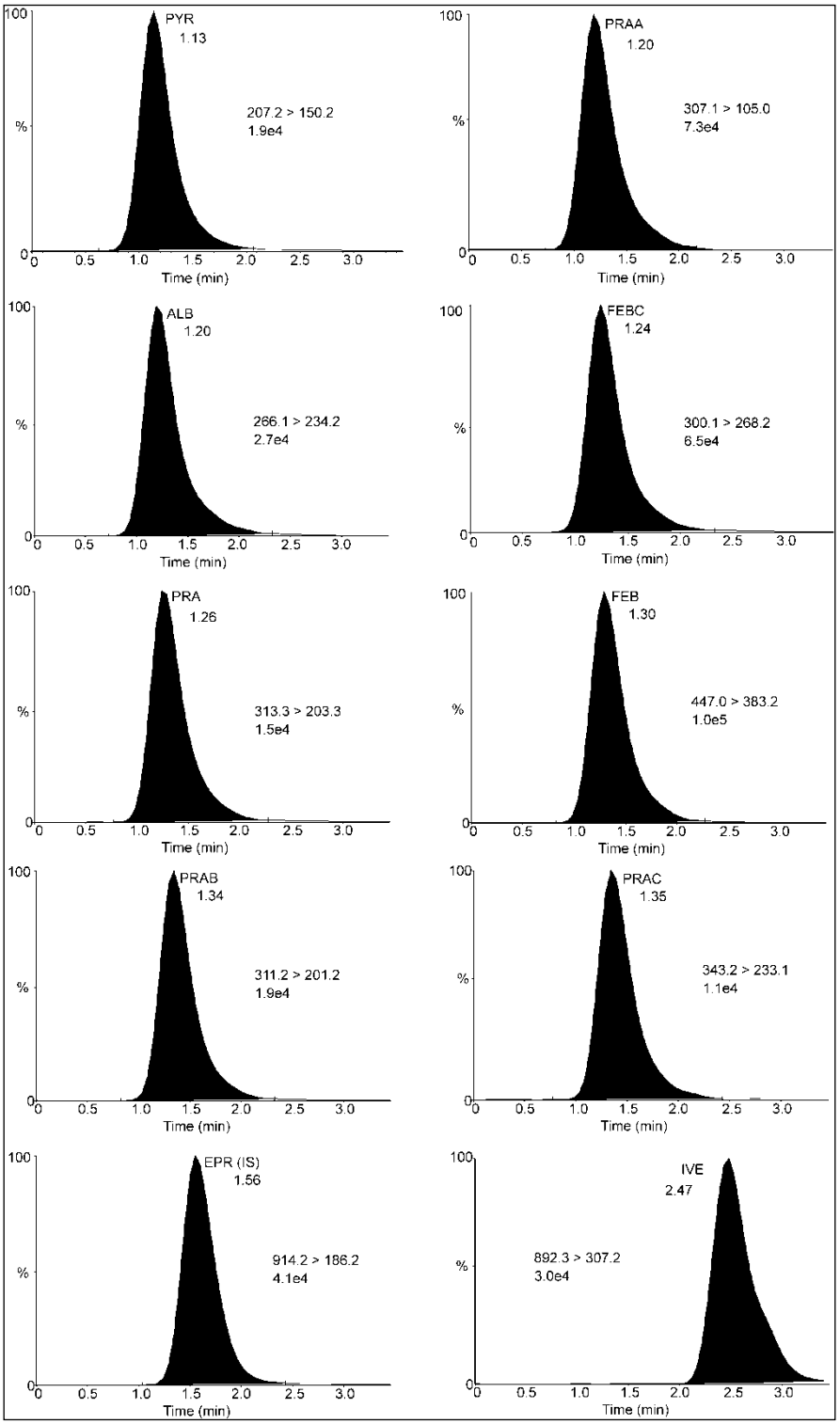


Figure 1: HPLC-MS/MS CHROMATOGRAMS OF PYR AT 25 NG/ML, PRAA AT 100 NG/ML, ALB (IS) AT 100 NG/ML, FEBC AT 100 NG/ML, PRA AT 100 NG/ML, FEB AT 100 NG/ML, PRAB AT 100 NG/ML, PRAC AT 100 NG/ML, EPR (IS) AT 300 NG/ML AND IVE AT 600 NG/ML.

After method development, sample preparation was the most critical step for analysis of anthelmintics. IVE has low ionization and is found in low concentrations in tablets. Thus, by making direct dilutions for the quantification of FEB, PRA and PYR, the IVE could not be detected. For that reason, the development of a selective method for ivermectin extraction was necessary.

Solid phase extraction (SPE) was the first choice for IVE extraction, because SPE has high selective sorbents adequate for trace analysis. C8, Oasis MCX and Oasis HLB cartridges were tested to obtain the maximum recovery of IVE, and among them, Oasis HLB was the most reproducible and effective system.

Primarily, the Oasis HLB cartridge was conditioned according to Krogh et al.[47], who demonstrated the high efficiency of Oasis HLB for recovery of IVE in water. However, using the same procedure, high amounts of FEB, PYR and PRA remained in the cartridge, and modifications in sample clean-up were necessary to avoid MS detector saturation. After sample loading, several combinations of acetonitrile and water were tested as wash solvent, and the best recovery of IVE (85.5%) with no interferences of FEB, PYR and PRA was achieved washing the sample with 20 mL of acetonitrile/water (35:65 v/v).

Selectivity and matrix effect

As demonstrated in Table 3, the slopes comparison from both curves (standard solutions and spiked samples) shows no significant variation for all compounds (RSD < 5%). Thus, no additional interferences were observed in the same retention time of the analytes of interest. Therefore, the selectivity of the developed method was found to be satisfactory. The present method also demonstrated no matrix effect with the results of the mean peak areas comparison (standard solutions and spiked samples) ranging from 95% to 105% (Table 4).

TABLE 3: COMPARATIVE SLOPE OF STANDARDS AND SPIKED SAMPLES IN DIFFERENT SAMPLES FOR SELECTIVITY STUDY

Compound	Sample 1			Sample 2			Sample 3		
	Slope			Slope			Slope		
	Standard	Spiked sample	RSD (%)	Standard	Spiked sample	RSD (%)	Standard	Spiked sample	RSD (%)
IVE	0.345	0.334	2.29	0.345	0.340	1.03	0.345	0.353	1.62
FEB	4.040	4.020	0.35	4.040	3.920	2.13	4.040	3.980	1.06
PYR	3.580	3.500	1.60	3.580	3.820	4.59	3.580	3.650	1.37
PRA	0.475	0.483	1.18	0.475	0.452	3.51	0.475	0.464	1.66
FEBC	4.310	4.600	4.60	4.310	4.070	4.05	4.310	4.200	1.83
PRAA	0.258	0.252	1.66	0.258	0.251	1.94	0.258	0.261	0.82
PRAB	0.471	0.498	3.94	0.471	0.457	2.13	0.471	0.474	0.45
PRAC	0.0606	0.0619	1.50	0.0606	0.0571	4.21	0.0606	0.0590	1.89

TABLE 4 : MATRIX EFFECT FOR ANALYTES AND INTERNAL STANDARDS IN DIFFERENT SAMPLES

Compound	Concentration (ng/mL)	Matrix effect (%)		
		Sample 1	Sample2	Sample 3
	40	100.5	96.5	97.0
FEB	50	103.7	96.7	96.3
	60	104.1	96.3	96.1
	40	104.4	96.7	96.2
PYR	50	104.2	98.7	98.2
	60	102.7	95.5	96.6
	12	103.2	96.9	99.2
PRA	15	102.5	96.5	96.9
	18	101.5	100.9	100.7
	80	101.2	102.6	101.2
IVE	100	102.4	102.4	100.2
	120	102.7	97.1	96.4
	40	99.0	97.7	98.5
FEBC	50	99.5	103.1	101.8
	60	100.8	96.0	98.7
	40	98.4	96.6	98.4
PRAA	50	99.4	97.5	99.2
	60	99.8	96.3	97.9
	40	98.9	98.0	98.4
PRAB	50	103.8	101.4	104.2
	60	103.2	96.3	99.9
	40	97.5	101.3	96.9
PRAC	50	95.7	98.9	99.0
	60	100.9	99.0	96.5
ALB[a)]	20	100.0	97.2	98.6
EPR[a)]	50	97.5	100.5	98.4

a) Internal standard

Limits of detection and quantification

The developed method showed high sensitivity, as demonstrated by the low LOD (signal-to-noise = 3), estimated at 0.05 ng/mL for FEB, FEBC and PRAA; 0.10 ng/mL for PYR, PRA and PRAB; and 0.50 ng/mL for PRAC and 1.0 ng/mL for IVE. The LOQ (signal-to-noise ≥ 10) was estimated to be 0.10 ng/mL for FEB, FEBC and PRAA; 0.25 ng/mL for PYR, PRA and PRAB; and 2.5 ng/mL for PRAC and IVE.

Linearity

The obtained calibration curves had correlation coefficients (r) higher than 0.99 for each analyte. The individual linear equations and correlation coefficients were as follows: IVE, $y = 0.105x + 0.0377$ ($r = 0.992$); FEB, $y = 0.79x + 0.0141$ ($r = 0.9989$); PRA, $y = 0.612x + 0.00131$ ($r = 0.9985$); and PYR, $y = 0.734x + 0.0726$ ($r = 0.9984$). Additionally, at all concentration levels, the analysis results of the analytes exhibited variations in precision and accuracy of <10%. These results guarantee a reliable response independent of the utilized concentrations. For impurities, linearity was not established, as the maximum limit allowed[8] is near the limit of quantification. Therefore, the impurities were evaluated qualitatively.

Precision and accuracy

Intra/interday precision and accuracy results are presented in Table 5. The results indicate that the method was precise with intraday

variation that ranged from 0.13 to 3.41% and interday variation that ranged from 0.44 to 4.40%. Furthermore, the *p*-value was greater than 0.05 for all compounds, indicating that the means compared are statistically equivalent. Satisfactory accuracy values were obtained for all compounds with a range of 93.3 to 101.4%.

TABLE 5: PRECISION AND ACCURACY RESULTS FOR FEB, PYR, PRA, IVE AND ISs ALB AND EPR

Compound	Standard concentration (ng/mL)	Precision				Accuracy
		Intraday		Interday		Main recovery (%) ± SD
		RSD (%)	(*p*-value)[a]	RSD (%)	(*p*-value)[a]	
FEB	40	0.34	0.76	2.58	0.07	95.5 ± 2.9
	50	0.64	0.44	1.48	0.16	99.7 ± 4.3
	60	0.24	0.84	1.25	0.19	97.0 ± 3.4
PYR	40	2.15	0.19	1.37	0.32	101.4 ± 2.4
	50	0.13	0.95	2.10	0.14	98.6 ± 4.6
	60	0.85	0.56	2.74	0.08	99.5 ± 4.6
PRA	12	2.72	0.20	2.06	0.24	100.7 ± 4.3
	15	2.93	0.12	0.44	0.74	101.2 ± 2.0
	18	0.38	0.86	3.50	0.09	96.9 ± 3.7
IVE	80	2.65	0.29	3.37	0.22	93.3 ± 1.0
	100	3.41	0.11	3.29	0.06	97.0 ± 3.5
	120	2.60	0.35	4.40	0.07	95.4 ± 5.0
ALB[b]	20	1.51	0.48	3.88	0.17	97.8 ± 3.4
EPR[b]	50	1.33	0.28	1.25	0.17	97.8 ± 1.1

a) Student's *t*-test, 95% confidence level. b) IS

Stability assay

The stability of the compounds and IS were investigated at one concentration level. The Table 6 summarizes the results of the differences between the peak areas obtained from freshly and stored standard solutions.

TABLE 6 - STABILITY OF STOCK AND WORKING STANDARD SOLUTIONS

Compound	Conc. (ng/mL)	Stability						
		Stock solutions						Working solution
		Stored at 4 ºC			Stored at ambient temperature			Stored at 20 ºC
		1 day (*p*-value)	7 days (*p*-value)	30 days (*p*-value)	1 day (*p*-value)	7 days (*p*-value)	30 days (*p*-value)	24 hours (*p*-value)
FEB	50	0.31	8.21^{-3}	3.72^{-13}	0,15	1.04^{-4}	1.95^{-14}	0.94
PYR	50	0.89	0.07	2.11^{-12}	0.11	7.41^{-5}	1.33^{-12}	0.21
PRA	15	0.34	0.10	9.99^{-13}	4.22^{-7}	2.13^{-6}	8.15^{-13}	0.08
IVE	100	0.48	0.37	4.72^{-9}	0.14	3.93^{-4}	5.30^{-11}	0.12
FEBC	50	0.38	0.01	1.32^{-13}	8.97^{-5}	1.21^{-5}	7.52^{-14}	0.17
PRAA	30	0.27	6.34^{-5}	2.30^{-12}	5.27^{-7}	2.95^{-8}	1.43^{-12}	0.74
PRAB	30	0.91	0.25	1.58^{-12}	0.66	0.63	4.06^{-14}	0.71
PRAC	30	0.78	3.36^{-12}	3.20^{-14}	8.80^{-7}	1.07^{-13}	1.80^{-14}	0.07
ALB[a]	20	0.47	0.08	8.43^{-14}	0.35	4.94^{-3}	2.81^{-14}	0.73
EPR[a]	50	0.24	0.17	1.29^{-9}	0.21	7.20^{-5}	2.92^{-10}	0.83

a) IS

Stock solutions of all analytes were stable after one day stored at 4 ºC (p>0.05). After seven days, the analytes stored at 4 ºC including PYR, PRA, IVE, PRAB, ALB (IS) and EPR (IS) remained stable (p>0.05). None of the stock solution were stable after 30 days of storage at 4 ºC (p<0.05).

When the stock solutions were stored at ambient temperature, FEB, PYR, IVE, PRAB, ALB (IS) and EPR (IS) were stable at one day (p>0.05). After 7 days, only PRAB was stable (p>0.05) and no stock solution was stable upon analysis after 30 days (p<0.05). The sample stability of all the working standard solutions was observed after a period of 24 hours (p>0.05) stored in a Sample Manager at 20 ºC.

Sample analysis

The developed method was successfully applied to quantitatively determine IVE, FEB, PRA and PYR in three commercial samples containing fixed dose of these drugs. Qualitative analysis was also performed to determine the main related compounds of FEB and PRA (FEBC, PRAA, PRAB and PRAC). Despite the high sensitivity achieved with the new method, none of the tested preparations presented related compounds. In contrast, all samples exhibited variable concentrations of anthelmintics with individual levels based on the percentage of the label claims, ranging from 65 to 152% of IVE, 98 to 116% for FEB, 92 to 107% for PRA and 98 to 114% for PYR (Table **7**). These results suggest a lack of standardization in the amounts of antiparasitic drugs contained in these products, contributing to their toxicity or efficacy reduction, depending on the sample.

TABLE 7 -CONTENTS OF IVE, FEB, PRA AND PYR OBTAINED IN COMMERCIAL SAMPLES BY THE DEVELOPED METHOD.

Compound	Sample 1		Sample 2		Sample 3	
	Found (mg) ± SD	%[a]	Found (mg) ± SD	%[a]	Found (mg) ± SD	%[a]
IVE	0.091 ± 0.003	151.67	0.045 ± 0.001	75.00	0.039 ± 0.001	65.00
FEB	174.6 ± 1.67	116.40	151.2 ± 6.75	100.80	147.3 ± 7.00	98.20
PRA	53.49 ± 1.13	106.98	46.59 ± 1.25	93.18	45.79 ± 0.35	91.58
PYR	163.8 ± 4.67	113.75	140.5 ± 4.49	97.57	143.0 ± 2.66	99.30

[a] Percentage of label claim

CONCLUSION

The new method was very efficient in the simultaneous determination of ivermectin, febantel, praziquantel and pyrantel pamoate in tablets, and achieved high sensitivity in the detection of the main related compounds of febantel and praziquantel. The present method was also found to be highly selective, linear, precise and accurate. Analyses of commercial samples demonstrated a lack of standardization in the amounts of these antiparasitics, mainly ivermectin, which has been described as a highly toxic substance. This result demonstrated the need for increased quality control of these medicines to guarantee the quality and safety of use of the tablets. The new method represents a significant improvement for the quality control of pharmaceutical preparations containing these drugs.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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