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RESEARCH ARTICLE

SIMULTANEOUS QUANTITATIVE ANALYSIS OF TWO ANTHELMINTIC DRUGS IN A VETERINARY DOSAGE FORM

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ABSTRACT

In this research article, a new chromatographic method ultra-performance liquid chromatography (UPLC) method was developed for the quantification of ivermectin (IMT)-preziquantel (PRZQ) in a two-component mixture and veterinary dosage form mixture. The separation of the studied veterinary drugs (IMT and PRZQ) was carried out on Waters Acquity ® BEH C18 column (50mm x 2.1 mm i.d., 1.7 m) using a mobile phase consisting of water-acetonitrile-methanol (10:70:20, v/v). In the chromatographic analysis, flow rate was 0.38 mL/min, and column temperature was maintained at 42 0C. The chromatographic detection of IMT and PRZQ was made at the wavelength of 220.0 and 245.0 nm, respectively. The developed UPLC method was validated and applied to real samples consisting of IMT and PRZQ. Recovery assay results were found to be 100.4 % for PRZQ and 101.8 % for IMT. The presented UPLC method is introduced as an alternative choice for the quality control of pharmaceutical preparations containing these drugs. For the quality control of pharmaceutical preparations containing these drugs, the presented UPLC method is introduced as an alternative experimental analysis choice.

Keywords: Ultra performance liquid chromatography, Ivermectin, praziquantel, Anthelmintic veterinary dosage form

1. INTRODUCTION

Parasites cause failure to show normal performance and productivity in animals, sensitivity to diseases in animals, weakening as a result of insufficient use of food, enlargement of postpartum spaces, deterioration of hair cover, diarrhea, and death in more severe cases. Anthelmintic drugs are used to treat parasites in animals. These drugs include ivermectin (IMT) and praziquantel (PRZQ).

Ivermectin, one of the drugs that disrupt neuromuscular coordination, is effective in parasites by mimicking neurotransmitters or changing their effects. Eventually, the parasite becomes paralyzed. Spastic or loose paralysis in parasites causes them to be expelled by normal peristaltic movements of the intestines [1]. Praziquantel, an isoquinoline compound, is effective in neurotransmission. The parasite increases cell membrane tension, causing depolarization and contraction. It facilitates the passage of glucose through the parasite skin [2, 3].

A literature survey showed that the simultaneously quantification of ivermectin and preziquantel in their binary mixture and their combinations with other active substances was reported, including ultraviolet spectroscopy [4], diffuse reflectance spectroscopy [4] high-performance liquid chromatography [5-9], high-performance thin layer chromatography [10] and high-performance liquid chromatography with tandem mass spectrometry [11].

In the comparison of the methods, the newly developed UPLC method has shorter retention time than that of traditional HPLC method in [12].

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UPLC or HPLC method is useful technique for the separation and analysis of analytes in complex mixtures. In previous studies, it was observed that the traditional HPLC was used to the simultaneous quantitative analysis of the related veterinary drugs. However, the literature method has time consuming with long runtime and the studies for the analysis of analytes. Therefore, the improved of new UPLC method is a reasonable to get short runtime with low cost. In this study, a new UPLC method with Photodiode Array (PDA) detection was developed for the quantitative resolution of a two component mixture or a combined veterinary dosage forms containing the studied veterinary active compounds. The validity of the developed UPLC approach was performed by analyzing validation samples, synthetic mixtures, intra-day and inter-day samples, and standard addition samples. IMT and PRZQ in the veterinary tablets were determined by using the newly improved UPLC method.

2. MATERIAL AND METHODS

2.1. Standard, Chemical and Samples

Standards of ivermectin (99.6 %), and praziquantel (99.7 %) were get from Sigma-Aldrich. Acetonitrile and methanol (UPLC grade) were obtained from Merck. Ultrapure water was acquired using a Milli-Q purification system from Millipore (Milford, MA, USA). The commercial formulation, Dicromec tablet consisting of PRZQ and IMT was obtained from a pharmacy, Ankara, Turkey.

2.2. Preparation of Solutions

Stock solution of 10 mg/100 mL of IMT and PRZQ were individually prepared in methanol. A series of the calibration solutions consisting of 16 solutions at 8 different concentrations in the range of 2.0-44.0 µg/mL for IMT and 20.0-160.0 µg/mL for PRZQ was prepared by using the stock solutions.

Validation test set containing 17 different concentrations compounds in the working range of 2.0-44.0 µg/mL for IMT and 20.0-160.0 µg/mL for PRZQ was prepared.

Standard addition samples consisting of an appropriate amount of veterinary formulation at a constant concentration; IMT and PRZQ at four different concentration levels 5.0 µg/mL, 10.0µg/mL, 20.0 µg/mL, and 30.0 µg/mL for IMT and 10.0 µg/mL, 20.0 µg/mL, 30.0 µg/mL, and 40.0 µg/mL for PRZQ were prepared. Three different concentrations of IMT containing IMT and PRZQ in the working ranges between 2.0-44.0 µg/mL for IMT (at three different concentration levels 2.0 µg/mL, 26.0 µg/mL and 44.0 for µg/mL) and 10.0-80.0 µg/mL for PRZQ (at three different concentration levels 10.0 µg/mL, 60.0 µg/mL and 80.0 µg/mL) intra-day and inter-day solutions were prepared, respectively.

2.3. Preparation of Veterinary Tablet Sample

10 tablets, each containing 10.0 mg IMT and 250.0 mg PRZQ, was weighted and finely powdered in a mortar. An amount of this powder equivalent to the average mass of half tablet of DICROMEC® (Anatolia Medicine & Chemical Industry Co., Konya,Turkey) was weighted, dissolved in the solvent mixture by sonication, than diluted to 50 mL. The stock mixture prepared in the balloon was mixed with a magnetic stirrer for 35 minutes and filtered through a membrane filter (Sartorius Minisart = 0.20 µm). After 4 mL of this stock solution was taken and added into a 10 mL flask, the volume was made up the mark with methanol.

The solution was filtered by a syringe filter (Acrodisc, Pall Industries). This procedure was repeated 10 times.

2.4. Experimental Conditions of UPLC

Waters Acquity Series UPLC System (Waters Corporation 34 Maple Street Milford, MA 01757 USA) (with PDA system) was used for the chromatographic quantification of PRQ and IMT. The system was operated up by Empower UPLC Software program.
The analytical separation was achieved on an Waters Acquity ® BEH C18 column (50mm x 2.1 mm i.d., 1.7 m) maintained at 42 0C. The mobile phase consisted of water-acetonitrile-methanol (10:70:20, v/v). The isocratic flow rate was 0.38 mL/min and the injection volume was 1 µL. The needle was washed with 2 µL of acetonitrile/methanol (50:50 v/v) between injections.

3. RESULTS AND DISCUSSION

Many mobile phase systems containing various organic solvents, such as water, methanol and acetonitrile, at different amount of solvents were tried to find the optimum conditions to get desirable elution of IMT and PRZQ. By using the Waters Acquity ® BEH C18 column (50mm x 2.1 mm i.d., 1.7 m), a mobile phase consisting of water-acetonitrile-methanol (10:70:20, v/v), with isocratic flow rate of 0.38 mL/min and column temperature of 42 0C, was found appropriate for adequate elution of IMT and PRZQ in samples. The injection volume for the sample was 1 µL for the chromatographic procedure. As stated in Figure 1 A and B, the optimum chromatographic detection for IMT and PRZQ was carried out at 220.0 nm and 245.0 nm, respectively. The concentration set consisting of IMT and PRZQ in the working ranges between of 2.0-44.0 µg/mL and 20.0-160.0 mg/mL were gotten by using the stock solutions of the related drugs. The UPLC chromatograms for the calibration samples of IMT and PRZQ were registrated according to optimized chromatographic conditions, as shown in Figure 1 A and B, respectively). A analogue chromatographic treatment was used to the standard addition samples and commercial veterinary samples. From Figures 1 A and B, the retention time of IMT and PRZQ were found as 0.482 minutes, 0.720 minutes, respectively. Calibration curves for IMT and PRZQ were calculated using linear regression analysis between concentration and peak-area ratio 220.0 nm and 245.0 nm, respectively. The statistical results for the calculations were listed in Table 1. IMT and PRZQ in the veterinary tablets were determined via the calibration curves.

Table 1. Results of linear regression analysis

	PRZQ	IMT
m	5744	6023.5
n	18448.4	288.6
r	0.9992	0.9997
SD(m)	95.4	58.8
SD(n)	482	157.6
SD(r)	6185.8	2287.1
LOD	2.52	0.78
LOQ	8.39	2.62

m = The slope of the linear regression equation
n = The cutoff point of the linear regression equation
r = Correlation coefficient of linear regression equation
SD (m) = Standard deviation of slope
SD (n) = Standard deviation of the cutoff point
SD(r) = Standard deviation of the correlation coefficient
LOD = Limit of detection (µg/mL)
LOQ = Limit of quantification (µg/mL)

The UPLC peak results of the concentration sample set involving of were depicted in Figure 1 A and B. From these UPLC chromatograms, the elution times of IMT and PRZQ were observed as 0.482 min and 0.720 min, respectively.

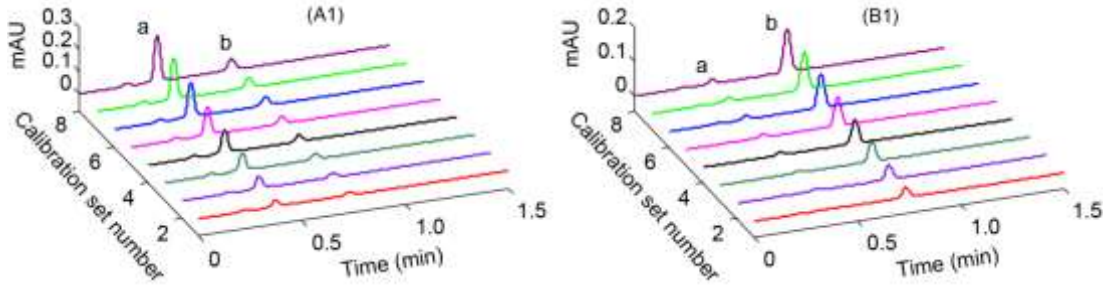

Figure 1. Chromatograms of calibration set were obtained by applying the chromatographic condition to the calibration samples (CS number) for separated compounds IMT (A1, 220.0 nm (a) and PRZQ (B1, 245.0 nm) (b).

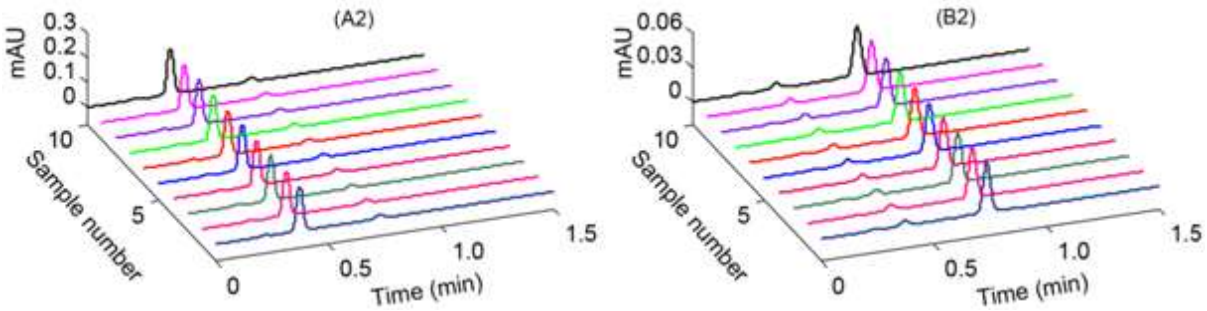

Figure 2. Chromatograms of Dicromec commercial veterinary samples A2 (220 nm) and B2 (245 nm) for IMT and PRZQ active compounds, respectively.

This study showed that the proposed and validated UPLC technique gave us a good separation of the analytes with short analysis time of 1.5 min. The results showed that, the newly improved UPLC technique was very promising to accomplish the rapid and dependable analysis of the veterinary tablet preparation containing IMT and PRZQ.

3.1. UPLC Method Validation

As explained in the section Standard solutions, a concentration set of the mixture solutions consisting of IMT and PRZQ was prepared then, under optimal chromatographic conditions, the UPLC results of the concentration set were plotted by PDA detection at the wavelength, 245.0 nm for IMT and 220.0 nm for PRZQ. Calibration graphs for IMT and PRZQ with high correlation coefficients were obtained. Their statistical data consisting of concentration ranges, slope, intercept and correlation coefficients of the linear regression equations, limit of detection (LOD) and limit of quantification (LOQ) were presented in Table 1. For both IMT and PRZQ, suitable linearity's having advanced correlation coefficients were detected (see Table 2.). The LOD and LOQ values of IMT and PRZQ were given in Table 1.

An objective set consisting of 17 different mixture samples of IMT and PRZQ in the different mixture compositions was analyzed by the newly developed UPLC method. Recovery results and relative standard deviations were calculated and shown in Table 2. As it was seen in from Table 2, recovery

values were 101.6% for IMT and 99.7% for PRZQ, and relative standard deviation values were 1.87% for IMT and 1.36% for PRZQ. It was deduced that the newly improved UPLC technique has a good accuracy and precision for the UPLC analysis of IMT and PRZQ.

Table 2. Analysis results were obtained from the optimized and validated UPLC method of IMT and PRZQ synthetic mixture solutions.

Added (µg/µL)		Found (µg/µL)		Recovery (%)		
Mix no.	PRZQ	IMT	PRZQ	IMT	PRZQ	IMT
1	10	3	9.8	2.9	97.6	95.4
2	20	3	20.4	3.0	101.8	101.4
3	30	3	29.8	3.1	99.4	102.0
4	40	3	40.1	3.0	100.4	101.1
5	50	3	49.4	3.1	98.8	103.3
6	60	3	61.4	3.1	102.3	102.6
7	70	3	70.5	3.1	100.8	101.9
8	80	3	81.0	3.0	101.3	101.6
9	75	2	74.8	2.0	99.8	102.4
10	75	8	75.2	8.2	100.2	102.1
11	75	14	74.2	14.3	99.0	101.9
12	75	20	73.6	21.0	98.2	104.9
13	75	26	74.0	26.4	98.7	101.4
14	75	32	74.5	32.4	99.4	101.2
15	75	38	75.4	38.2	100.5	100.6
16	75	44	73.6	44.8	98.1	101.8
17	75	3	73.9	3.0	98.6	101.5
				Mean	99.7	101.6
				SD	1.36	1.87
				RSD	1.36	1.84

SD = Standart deviation
RSD = Relative standard deviation

Selectivity/ specificity for the UPLC technique was checked according to presence or absence of the excipient's effect on the analysis of IMT and PRZQ from veterinary tablet dosage form. As explained in the section 2.2, the standard solutions were prepared by adding of the stock solutions of IMT (at four different amounts; 5.0, 10.0, 20.0, and 30.0 µg/mL) and PRZQ (at four different amounts; 10.0, 20.0, 30.0, and 40.0 µg/mL) to the sample solution of veterinary tablet dosage form. From the Table 4, it was observed that there is no interference of the veterinary tablet's excipient on the determination of analytes.

Accuracy and precision testing for the newly improved UPLC method were performed with the analysis of the intra-day and inter-day samples. The samples containing IMT (at three different concentrations: 2.0, 26.0, and 44.0 µg/mL) and PRZQ (at three different concentrations: 10.0, 60.0, and 80.0 µg/mL) were analyzed. Intra-day and inter-day experiments were carried out at different three times and at different three days with three replications, respectively. Experiment for each concentration level was repeated three times. Mean recoveries; relative standard deviations and relative standard errors were calculated and shown in Table 3. These analyses indicated that the newly improved UPLC method is suitable to analyze the related compounds in samples with required precision and accuracy.

Table 3. Analysis results of intra-day and inter-day samples

	Added (µg/µL)		Found (µg/µL)	
	PRQ	IMT	PRZQ	IMT
Inter-day	10	2	10.39	2.03
	60	26	60.48	26.68
	80	44	82.98	46.13
Intra-day	10	2	9.93	2.00
	60	26	60.21	26.15
	80	44	81.14	46.09
			Recovery (%)	
			PRZQ	IMT
Inter-day			103.9	101.6
			100.8	102.6
			103.7	104.8
Intra-day			99.3	100.0
			100.4	100.6
			101.4	104.8
			RSD (%)	
			PRZQ	IMT
Inter-day			0.82	2.61
			0.49	1.19
			0.22	0.16
Intra-day			1.05	4.36
			0.98	0.67
			1.08	1.58
			RSE (%)	
			PRZQ	IMT
Inter-day			3.85	1.58
			0.80	2.61
			3.72	4.84
Intra-day			-0.65	0.00
			0.35	0.56
			1.43	4.76

RSD = Relative standard deviation
RSE = Relative standard error
n= 3 (for all concentration level)

Table 4. Analysis results of standard addition sapmles

	Added		Found (µg/mL)	
	PRZQ	IMT	PRZQ	IMT
Formulation	10	5	9.69	5.06
Formulation	20	10	19.62	9.98
Formulation	30	20	29.20	20.20
Formulation	40	30	40.40	30.65
			Recovery (%)	
			PRZQ	IMT
			96.9	101.3
			98.1	99.8
			97.3	101.0
			101.0	102.2
			RSD (%)	
			PRZQ	IMT
			0.47	0.10
			0.46	0.43
			0.47	0.54
			1.09	0.69

RSD = Relative standard deviation

3.2 UPLC Method Application to Veterinary Tablet

After the method validation step, the newly developed UPLC technique was employed to simultaneously quantify IMT and PRZQ in commercial veterinary formulation. The UPLC chromatograms of these industrial veterinary samples were plotted under separation conditions. The peak areas of IMT and PRZQ obtained at the retention times, 0.482 min and 0.720 min were computed and replaced into the calculations of the calibration curves to determine the related drugs in veterinary tablet dosage form, respectively (see Figure 2.). Assay results obtained from veterinary tablet dosage form samples were shown in Table 5. As it was seen in the experimental results listed in Table 5, the improved UPLC technique is very appropriate for the quantitative analysis of IMT and PRZQ in the studied veterinary tablet dosage form.

Table 5. Quantitative analysis results of IMT and PRZQ in commercial veterinary preparation (Label claim: PRQ 250.0 mg/mL and IMT 10.0 mg/mL).

Exp no.	PRZQ µg/µL	IMT µg/µL
1	245.43	9.87
2	246.10	10.02
3	246.91	9.59
4	246.93	9.70
5	248.98	9.38
6	242.94	9.56
7	244.58	9.70
8	240.77	9.64
9	246.24	10.23
10	255.27	9.76
Mean	246.4	9.7
SD	3.86	0.24
RSD	1.57	2.50

SD = Standard deviation
RSD =Relative standard deviation

4. CONCLUSION

In our work, a new UPLC technique was improved for the simultaneous analysis of the substance of IMT and PRZQ in industrial veterinary tablet preparation with short runtime and few experiments. In the development of selective, precise, accurate and reliable UPLC technique a equilibrium between high-quality separation and short analysis time was considered. Thus, the analysis by the improved UPLC method were accomplished within a short runtime of 1.5 min with a good separation of the IMT and PRZQ peaks in a UPLC chromatogram. In evaluated with HPLC (12), the newly applied UPLC is more economical with suitable chromatographic separation of IMT and PRZQ in an industrial tablet formulation. It was concluded that the proposed UPLC method was a very useful and promising for the simultaneous quantification and routine analysis of the active compounds, IMT and PRZQ in commercial veterinary tablets.

CONFLICT OF INTEREST

The authors stated that there are no conflicts of interest regarding the publication of this article.

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