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Chromatographic Quantification of Ivermectin and Pranziquantel in the Tablets using Stability Indicating RP-HPLC Method

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Abstract

Background:

A new stability indicating RP-HPLC based assay method was developed to quantify ivermectin and praziquantel simultaneously and applied effectively to tablets.

Methods:

The simultaneous assay of ivermectin and praziquantel by RP-HPLC was done using an YMC C18 (250 mm × 4.6 mm, 5 µm) column with a mobile phase mixture of 0.1M disodium hydrogen phosphate (pH 4.5) and acetonitrile (55:45, v/v) using a isocratic flow rate of 1.0 ml/min and measured at 242 nm using photodiode array detector. All parameters were validated following the ICH guiding principles. The method was applied to quantify ivermectin and praziquantel simultaneously in tablets.

Results:

The retention values of ivermectin and praziquantel were 3.465 min and 4.468 min, respectively. The method's linearity was found to be 1-3 µg/ml (ivermectin) and 25-75 µg/ml (praziquantel). The limit of detection was 0.010 µg/ml (ivermectin) and 0.046 µg/ml (praziquantel); limit of quantification was 0.033 µg/ml (ivermectin) and 0.155 µg/ml (praziquantel). The percent relative standard deviation of ivermectin and praziquantel was <1.0%. The percent assay was 99.51% and 99.20% for ivermectin and praziquantel, respectively. In tablets, the percent recovery of ivermectin and praziquantel was 99.60% and 99.38% with a percent relative standard deviation value of 0.353% and 0.106%, respectively. Stability indicating capability of the method was demonstrated through the stress degradation studies.

Conclusion:

The developed method was proved to be selective, precise and accurate for the quality control of ivermectin and praziquantel in tablets.

Key words: Ivermectin, Praziquantel, Antihelmintic agent, Stress degradation, RP-HPLC, Analysis

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Introduction:

Ivermectin is a broad spectrum anthelmintic and antiparasitic agent that works against different parasites[1]. Ivermectin is obtained from avermectins. Avermectins is an anti-parasitic agent obtained as a product of fermentation from *Streptomyces avermitilis*[2]. Ivermectin is used for treating onchocerciasis, strongyloidiasis, enterobiasis, ascariasis, filariasis, trichuriasis and scabies in humans[3]. Also, ivermectin is widely used in domestic animals to give treatment against endoparasites and ectoparasites[4]. Recently, ivermectin is being studied as an antiviral agent against the viruses that causes chikungunya and yellow fever[5]. Ivermectin is chemically known as (2aE,4E,8E)-(5′S,6S, 6′R,7S, 11R,13R, 15S,17aR, 20R, 20aR,20bS)-6′-(S)-sec-butyll-3′,4′,5′,6,6′,7,10,11,14,15,17a,20,20a,20b-tetradeacahydro-20,20b-dihydroxy-5′,6,8,19-tetra methyl-17-oxospiro[11,15-methano-2H,13H,17H-furo[4,3,2-pq][2,6]-benzodioxacycloocta decin-13,2′-[2H]pyran]-7-yl-2,6-dideoxy-4-O-(2,6-dideoxy-3-O-methyl-α-l-arabino-hexo pyranosyl)-3-O-methyl-α-l-arabino-hexopyreanoside (Figure 1).

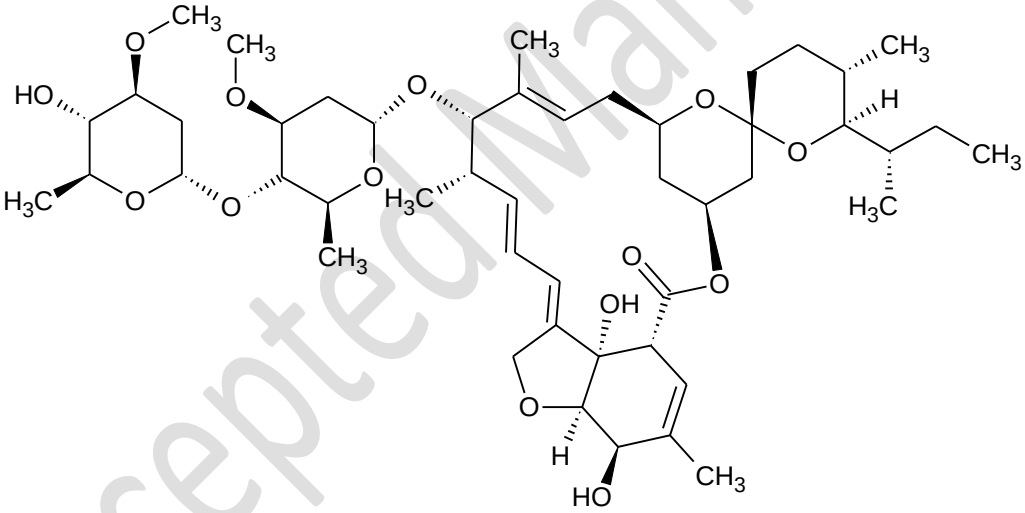

Figure 1. Ivermectin chemical structure

Praziquantel is a broad spectrum antihelmintic agent that works against several cestodes and trematodes[6]. In humans, praziquantel is employed to treat the infections in blood and liver caused by worms. Praziquantel is used in therapy of liver flukes, schistosomiasis and cysticercosis. Praziquantel acts through killing and paralyzing the parasites[7,8]. This makes them to let loose their grip on blood vessel walls. Then the body eliminates parasites naturally. In dogs, horses and cats, praziquantel is prescribed to treat tapeworm infections, pancreas fluke and

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blood flukes[9-11]. Praziquantel is chemically known as (*RS*)-2-(Cyclohexylcarbonyl)-1,2,3,6,7,11b-hexahydro-4*H*-pyrazino[2,1-*a*]isoquinolin-4-one (Figure 2).

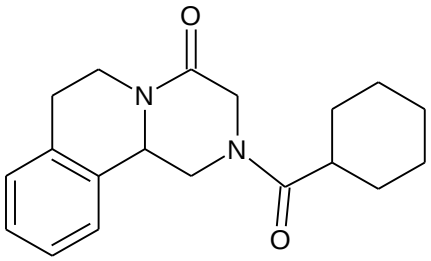

Figure 2. Praziquantel chemical structure

Ivermectin and praziquantel combination is available in paste form (Equimax paste with strength 14.03% praziquantel and 1.87% ivermectin) and tablet dosage form (Ipraz tablet with strength 2 mg ivermectin and 50 mg praziquantel)[12,13]. Equimax is used as dewormer in horses mares, foals, breeding stallions and ponies. It is used to treat and control bots and tapeworms[14,15]. Ipraz tablets are used as dewormer in dogs. These tablets are prescribed to treat and control flat worms and round worms in dogs[13].

The literature described quite a few analytical methods for the estimation of ivermectin and praziquantel in combination with other drugs. These methods are based on techniques, such as ultraviolet spectroscopy[16], diffuse reflectance spectroscopy[16], high performance liquid chromatography[17-21], high performance thin layer chromatography[22] and high performance liquid chromatography with tandem mass spectrometry[23].

To the best of our knowledge, till now only one HPLC method for the simultaneous quantification of ivermectin and praziquantel in Equimax paste is reported[24]. Quantification of ivermectin and praziquantel simultaneously in tablets using a stability indicating RP-HPLC method is not reported. Therefore, a stability indicating RP-HPLC method for quantifying ivermectin and praziquantel simultaneously in tablets has been proposed and validated based on ICH requirements.

Experimental

HPLC instrumentation

The HPLC system consists of a Waters alliance HPLC module quaternary pump, a rheodyne sample injection valve with a 10 µl sample loop, a YMC C18 (5 µm particle size, 4.6

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mm by 250 mm) analytical column, and a Waters photodiode array detector. Acquisition and interpretation of chromatographic data was performed with Empower2 version software.

Chemicals, solvents and suppliers

HPLC grade acetonitrile used was procured from Merck India Ltd, Mumbai, India. Remaining other chemicals used in this study was analytical regent grade and obtained from SD. Fine Chemicals Ltd., Mumbai, India. The chemicals used include disodium hydrogen phosphate, sodium hydroxide, hydrochloride, hydrogen peroxide and orthophosphoric acid. Milli-Q water used was obtained from Millipore system.

Reference standards of ivermectin and praziquantel and their tablet dosage form

Reference standards of ivermectin and praziquantel were procured from Rainbow Pharma Training Labs, Hyderabad, India. Ipraz tablets with strength 2 mg ivermectin and 50 mg praziquantel (Virbac animal health, Mumbai, India) were purchased from a local pharmacy market.

Chromatographic conditions for assay

So as to produce reproducible and reliable chromatographic results, following conditions were used: 0.1 M Na_2HPO_4: acetonitrile (55:45 *v/v*) as mobile phase, 1 ml/min isocratic flow rate, 25 ^oC column temperature, 242 nm detection wavelength and 10 µl injection volume.

Ivermectin and praziquantel stock solution

Ivermectin and praziquantel stock solution was prepared by dissolving 2 mg of ivermectin and 50 mg of praziquantel in 100 ml of mobile phase. Final concentration of stock solution is 20 µg/ml ivermectin and 500 µg/ml praziquantel.

Solutions for calibration curve and validation

Five solutions with following concentrations were prepared for calibration curves of ivermectin and praziquantel by diluting stock solution appropriately with the mobile phase:

Solution I: 1 µg/ml ivermectin and 25 µg/ml praziquantel

Solution II: 1.5 µg/ml ivermectin and 37.5 µg/ml praziquantel

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Solution III: 2 µg/ml ivermectin and 50 µg/ml praziquantel

Solution IV: 2.5 µg/ml ivermectin and 62.5 µg/ml praziquantel

Solution V: 3 µg/ml ivermectin and 75 µg/ml praziquantel

Working solution for validation studies was prepared through diluting stock solution with mobile phase to achieve a final concentration of 2 µg/ml ivermectin and 50 µg/ml praziquantel.

Tablet solution preparation

Ten tablets were powdered. Exactly weighed tablet powder equal to 2 mg of ivermectin and 50 mg of praziquantel was dissolved in 100 ml of mobile phase (concentration - 20 µg/ml ivermectin and 500 µg/ml praziquantel). The solution was homogenized thoroughly using sonication for 20 min. The obtained solution was diluted aptly with the mobile phase to get a final concentration of 2 µg/ml ivermectin and 50 µg/ml praziquantel for analysis following filtration via a 0.45 µm membrane filter.

Calibration graphs of ivermectin and praziquantel

Calibration graphs of ivermectin and praziquantel were prepared as per the guidelines of ICH. Five calibration solutions in the concentration range 1-3 µg/ml of ivermectin and 25-75 µg/ml of praziquantel were prepared. 10 µl volume of each calibration solution was injected into the system under the assay conditions explained above. The peak areas of ivermectin and praziquantel were determined at 242 nm. Calibration graphs were plotted with peak area against concentration. The evaluation of linearity was made with linear regression analysis.

Assay of ivermectin and praziquantel in marketed tablet

Tablet solution with a concentration of 2 µg/ml ivermectin and 50 µg/ml praziquantel was prepared and analyzed using described assay conditions through triplicate injections into the system. The peak areas of ivermectin and praziquantel were determined. The content of ivermectin and praziquantel in tablets was estimated using the corresponding linear regression or corresponding calibration curve.

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Stress degradation of ivermectin and praziquantel

Guidelines Q1A (R2) was followed to study the stress degradation[25]. Ivermectin and praziquantel tablet stock solution (20 µg/ml ivermectin and 500 µg/ml praziquantel) was used for degradation to establish an indication for the method's stability indicating property and specificity.

For acid, base, water and hydrogen peroxide induced degradation, 10 ml of 0.1N HCl, 10 ml of 0.1N NaOH, 10 ml of distilled water and 10 ml of 30% hydrogen peroxide, respectively were added separately into 10 ml of ivermectin and praziquantel tablet stock solution at room temperature and the mixtures were sonicated for 30 min. After specified period of degradation, the mixture was diluted to 100 ml with mobile phase for analysis. For photo and thermal degradation, 10 ml of ivermectin and praziquantel tablet stock solution was exposed to sunlight for 24 h and 105^{o}C for 30 min in hot air oven, respectively. Following degradation, the degraded solutions were diluted to 100 ml with mobile phase for analysis. Before injecting the degraded solutions into system, they are filtered through 0.45 µm membrane filter.

Results and discussion

Assay method development

Various stationary phases (Waters C18, Xterra C18 and YMC C18) were used to check the chromatogram with acceptable peak shape, tailing factor and plate count for reproducibility at 25 ^{o}C. Various trials have been performed by using 0.1% orthophosphoric acid/methanol, 0.1M sodium dihydrogen phosphate/methanol, 0.1M disodium hydrogen phosphate/acetonitrile combinations in different ratios, different pH ranges and different flow rates. The trails were done to optimize the mobile phase composition, pH and flow rate for the separation of ivermectin and praziquantel peaks with acceptable resolution and with good peak shape. Based on the observations and conclusions obtained from the good enough number of chromatographic trials, a particular set of chromatographic conditions were optimized and found to be suitable for simultaneous estimation of ivermectin and praziquantel. The optimized chromatographic conditions are as follows:

- Column (stationary phase) : YMC C18 (250 mm x 4.6 mm, 5 µm particle size)
- Mobile phase composition and ratio: 0.1M disodium hydrogen phosphate and acetonitrile combination in ratio 55:45 (*v/v*)

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- Mobile phase flow rate : 1.0 ml/min

The chromatogram of ivermectin and praziquantel obtained after method optimization was given in Figure 3.

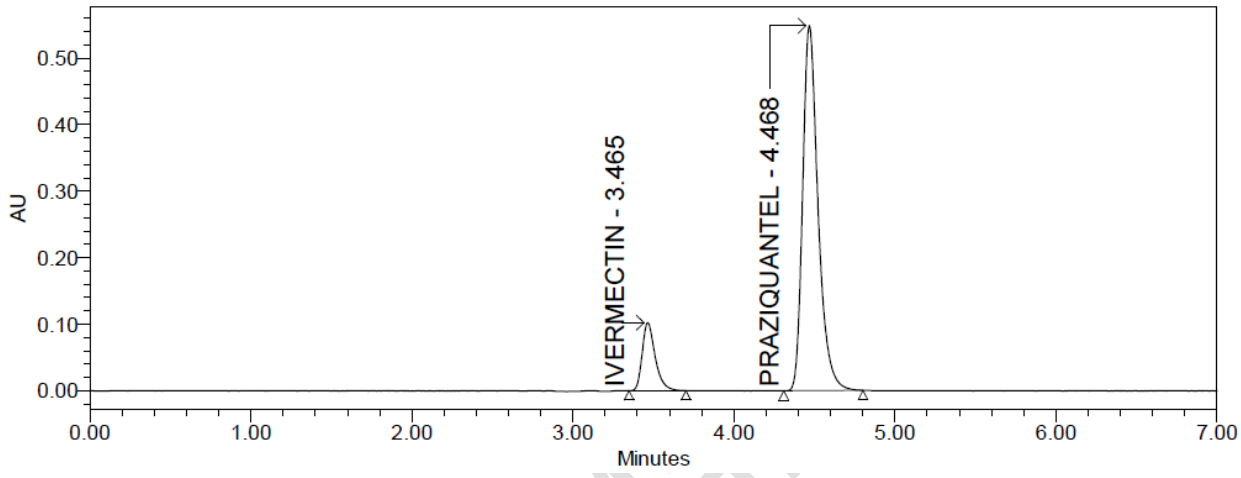

Figure 3. Chromatogram of ivermectin and praziquantel obtained with optimized chromatographic conditions

Method validation

The optimized method was validated as per ICH Q2 (R1) guidelines[26]. The validation parameters evaluated are given below.

System suitability

Test parameters were assessed by injecting 10 µl of ivermectin and praziquantel working solution 6 times, with a concentration of 2 µg/ml ivermectin and 50 µg/ml praziquantel. As per the chromatograms obtained, parameters such as injection precision for working solution (relative standard deviation of peak areas and retention times of ivermectin and praziquantel), theoretical plates for working solution, resolution for working solution and tailing factor for working solution were determined, and stated in Table 1. The values are within the acceptable limit. Hence the system is suitable for the assay of ivermectin and praziquantel simultaneously.

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Table 1. Test for system suitability parameters

Test parameters	Ivermectin		Praziquantel		Acceptable limit
	Mean of five values	% RSD	Mean of five values	% RSD	
Peak area (mAU)	572623	0.053	3649863	0.083	RSD ≤2
Retention time (Rt)	3.467	0.024	4.471	0.029	RSD ≤2
Theoretical plates (N)	9214	0.888	10985	0.549	> 2000
Tailing factor (T)	1.364	0.402	1.350	0.524	≤2
Resolution (Rs)	-	-	6.174	0.185	≥2

Linearity

The assay method reported, gave good quality linear detectable response in the range of 1 to 3 µg/ml (ivermectin) and 25 to 75 µg/ml (praziquantel) with coefficient of regression (R^2) of 0.9998 (ivermectin) and 0.9999 (praziquantel) with well resolved ivermectin and praziquantel peaks. The regression equation (PA = mC + I, where 'PA' is peak area, 'm' is slope, 'C' is concentration of drug and 'I' is intercept) is:

PA = 28621 C + 33.50 ---- ivermectin

PA = 73050 C + 63.92 ---- praziquantel

These values indicated good correlation between peak area and drug concentration.

Limit of detection and limit of quantification

The limits of detection (LOD) and quantification (LOQ) were determined as signal to noise ratio of 3:1 and 10:1, respectively for ivermectin and praziquantel. LOD value for ivermectin and praziquantel was calculated as 0.010 µg/ml and 0.046 µg/ml, and LOQ value for ivermectin and praziquantel was 0.033 µg/ml and 0.155 µg/ml, respectively. The values of LOD and LOQ for ivermectin and praziquantel obtained proved adequate sensitivity of the assay method.

Selectivity

The developed assay method was examined for selectivity to ensure there was no interference from the components of solvent system used and common tablet excipients. The

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mobile phase (blank), placebo (blank), working solution (2 µg/ml ivermectin and 50 µg/ml praziquantel) and tablet solution (2 µg/ml ivermectin and 50 µg/ml praziquantel) were injected. The respective chromatograms obtained were compared (Figure 4). No interfering peaks were observed in the mobile phase (blank) and placebo (blank) chromatograms at retention times of ivermectin and praziquantel. The results proved the selectivity of the assay method.

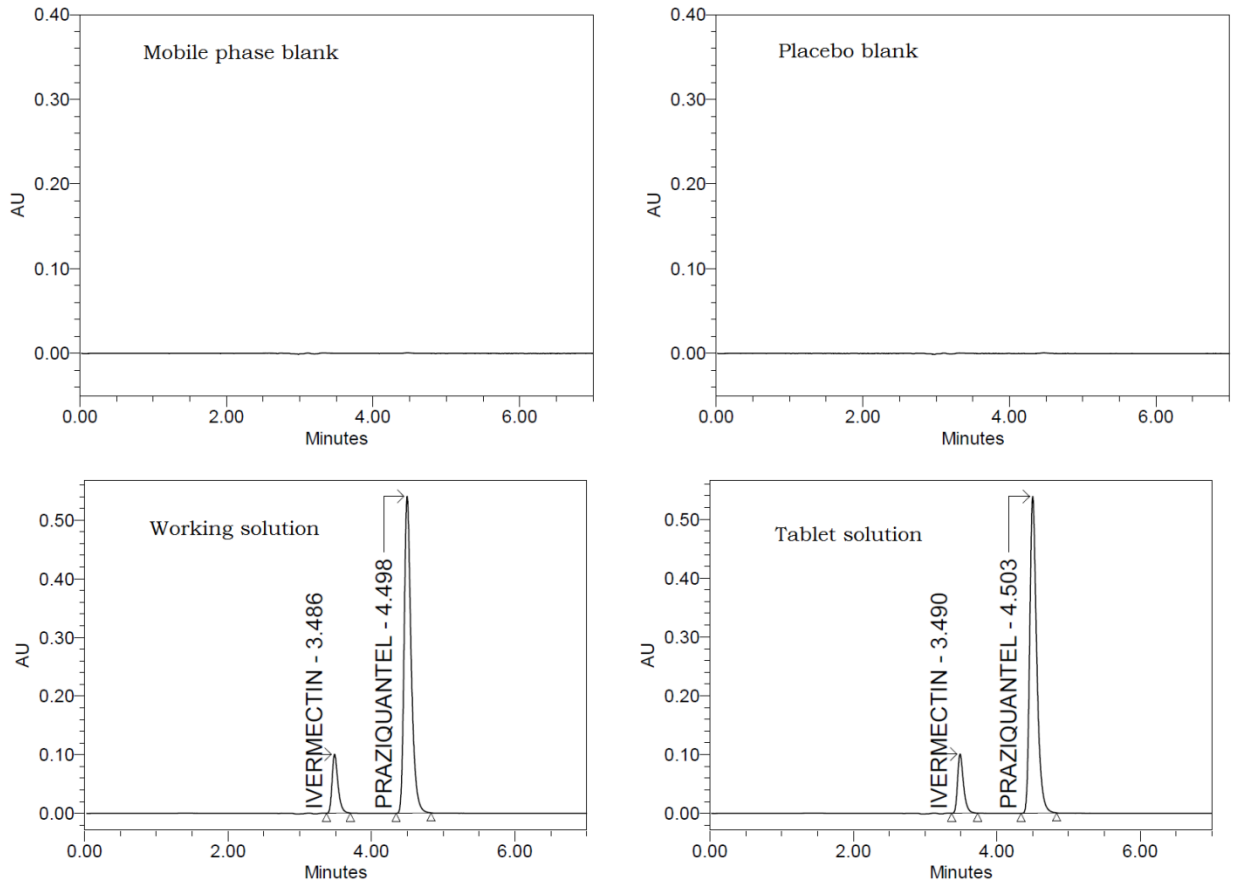

Figure 4. Selectivity testing chromatograms

Precision and accuracy

Both precision and accuracy of the assay method were evaluated. Precision and accuracy tests were done using working solution (2 µg/ml ivermectin and 50 µg/ml praziquantel) and executed in six replicates. Precision and accuracy are expressed by the value of percent relative standard deviation and percent assay, respectively. Precision is stated well if percent relative standard deviation is not greater than 2%. The percent relative standard deviation is 0.078% and 0.079% for ivermectin and 0.244% and 0.241% for praziquantel. The accuracy is

considered good, as percent assay in the range of 98.90-99.62%. The average of percent assay is 99.51% and 99.20% for ivermectin and praziquantel, respectively. The precision and accuracy data of ivermectin and praziquantel are shown in Table 2.

Table 2. Precision and accuracy data of ivermectin and praziquantel

Sample	Ivermectin (2 µg/ml)			Praziquantel (50 µg/ml)		
	Precision	Accuracy		Precision	Accuracy	
	Peak area (mAU)	Concentration assayed (µg/ml)	Percent assay (%)	Peak area (mAU)	Concentration assayed (µg/ml)	Percent assay (%)
A	572304	1.991	99.54	3652886	49.740	99.48
B	572170	1.990	99.52	3646385	49.650	99.30
C	571858	1.989	99.46	3632158	49.460	98.92
D	572594	1.992	99.60	3631456	49.450	98.90
E	572352	1.991	99.56	3645014	49.630	99.26
F	571340	1.988	99.38	3648468	49.680	99.36
Mean	**572103**	**-**	**99.51**	**3642727**	**-**	**99.20**
%RSD	**0.078**	**-**	**0.079**	**0.244**	**-**	**0.241**

Recovery:

To confirm the recovery of the assay method, the results of assay of ivermectin and praziquantel were evaluated as the percentage of recovery of known concentration of ivermectin and praziquantel spiked to the preanalyzed tablet sample. Each sample was injected three times and recovery was assessed in the range of 50%, 100% and 150% for ivermectin and praziquantel. The results are depicted in Table 3. High recovery pointed out that the assay method has a good degree of selectivity and accuracy.

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Table 3. Recovery data of ivermectin and praziquantel

Spiked level (%)	Concentration of drug (mg)			Percent assay (%)	RSD (%)
	In tablet	Spiked	Recovered		
Ivermectin					
50	2	1	2.989	99.63	0.153
100	2	2	3.984	99.60	0.087
150	2	3	4.978	99.56	0.039
Praziquantel					
50	50	25	74.610	99.48	0.070
100	50	50	99.273	99.27	0.095
150	50	75	124.244	99.40	0.074

Robustness

Robustness test was done by making changes in assay conditions. These changes include: flow rate (±0.2 ml/min), ratio of acetonitrile in mobile phase (±5%), column temperature (± 2 ^{o}C), pH of mobile phase (±0.2 units) and detection wavelength (± 2 nm). During robustness testing, the system suitability parameter values were determined. The values in all changed conditions are within the acceptable limit (Table 4). Hence the method is robust.

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Table 4. Robustness data of ivermectin and praziquantel

Parameter	Ivermectin (2 µg/ml)			Praziquantel (50 µg/ml)		
	T	N	Rs	T	N	Rs
Flow rate 0.9 ml/min	1.35	8191	-	1.33	9913	5.85
Flow rate 1.1 ml/min	1.36	8674	-	1.34	10429	5.99
Temperature 23 ºC	1.39	9613	-	1.37	10971	6.26
Temperature 27 ºC	1.41	9756	-	1.40	11272	6.32
Ratio of acetonitrile 40%	1.35	8191	-	1.33	9913	5.85
Ratio of acetonitrile 50%	1.39	9613	-	1.37	10971	6.26
pH of mobile phase 4.3 units	1.36	9165	-	1.35	10896	6.17
pH of mobile phase 4.7 units	1.36	9206	-	1.35	10999	6.17
Detection wavelength 240 nm	1.31	9335	-	1.34	10992	6.20
Detection wavelength 244 nm	1.36	9182	-	1.36	10904	6.17

T = Tailing factor; N = Theoretical plates; Rs = Resolution

Specificity and stress degradation study

Stability indicating ability and specificity of the assay method were demonstrated through the stress degradation studies. The degradation tests employed hydrolysis (acid, base and water), oxidation (hydrogen peroxide), dry heat and direct sun light. The percentage recovery, percentage degradation, purity angle and purity threshold values of ivermectin and praziquantel were calculated. The results obtained from degradation tests have been depicted in Table 5. Degradation was observed in all the stress conditions applied. The developed method was able to separate the peaks of degradants from the peaks of ivermectin and praziquantel, indicating that the method has suitable specificity and stability indicating ability (Figure 5).

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Peak purity of ivermectin and praziquantel peaks was evaluated by photodiode array detector. The less value of purity angle than purity threshold indicated an excellent degree of purity of ivermectin and praziquantel peaks.

Table 5. Summary of degradation of ivermectin and praziquantel under applied stress conditions

Tablet solution treated with	Recovered (%)	Degraded (%)	Purity Angle	Purity Threshold
Ivermectin (2 µg/ml)				
0.1 N HCl	83.43	16.57	0.361	0.476
0.1 N NaOH	87.81	12.19	0.345	0.587
30 % H_2O_2	89.03	10.97	0.242	0.493
Sunlight	84.05	15.95	0.423	0.723
105 ^oC	86.05	13.95	0.390	0.517
Distilled H_2O	94.21	5.79	0.244	0.385
Praziquanel (50 µg/ml)				
0.1 N HCl	84.27	15.73	0.275	0.462
0.1 N NaOH	87.04	12.96	0.283	0.464
30 % H_2O_2	87.3	12.70	0.181	0.363
Sunlight	83.28	16.72	0.281	0.465
105 ^oC	87.64	12.36	0.191	0.363
Distilled H_2O	95.7	4.3	0.190	0.262

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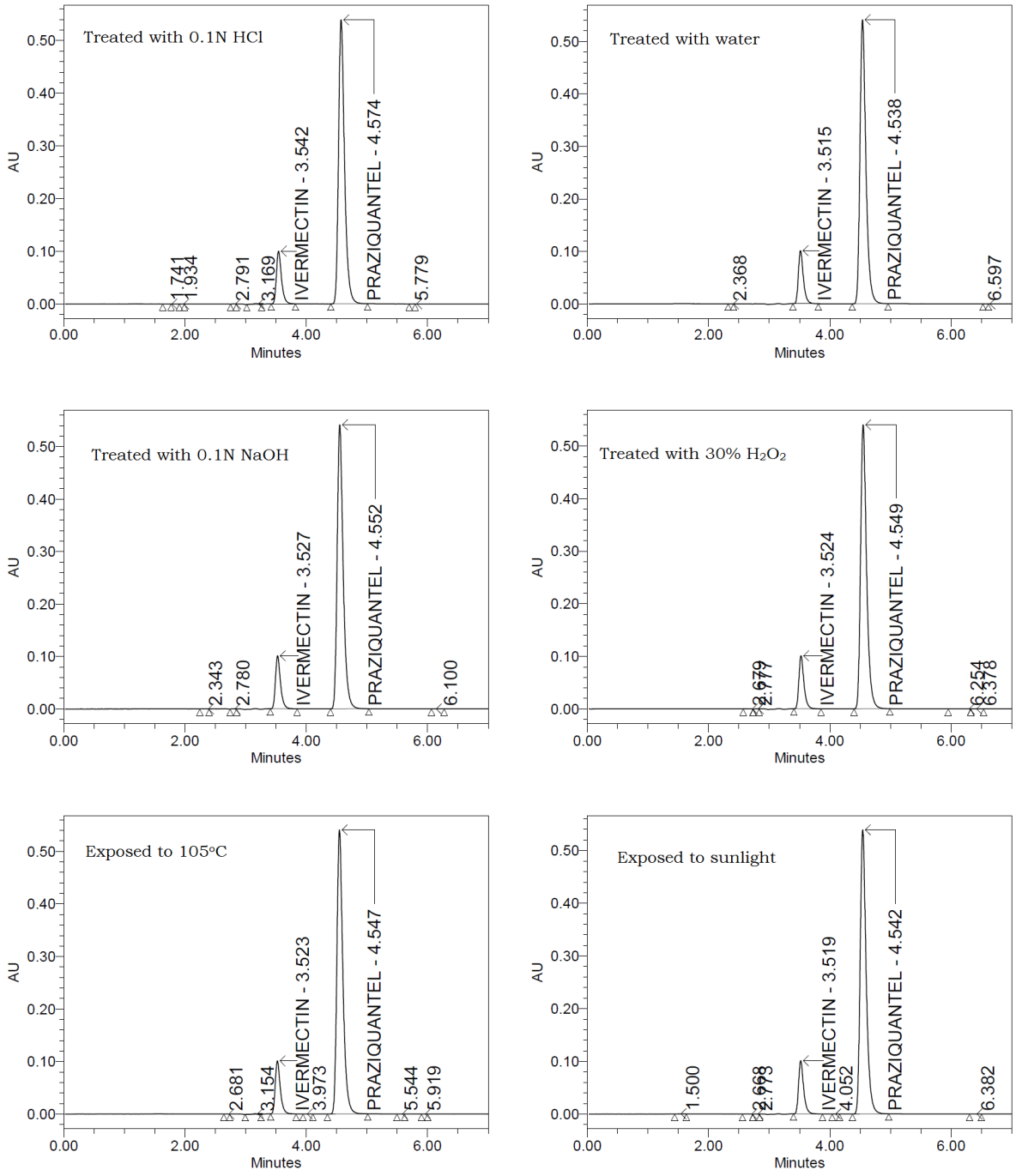

Figure 5. Chromatogram obtained from degradation studies of ivermectin and praziquantel

Application of assay method to tablet formulation

Applicability of the assay method developed was demonstrated by determining ivermectin and praziquantel content in marketed tablet dosage form. The recovery of ivermectin and praziquantel were determined in triplicates. The determined results were satisfactory with excellent recovery of the labeled amount (Table 6).

Table 6. Assay of ivermectin and praziquantel in tablet

Drug	Concentration of drug (mg)		Recovered	RSD
	In tablet	Determined*	(%)	(%)
Ivermectin	2	1.992	99.60	0.353
Praziquantel	50	49.652	99.38	0.106

* Average of three determined values

Conclusion

A stability indicating RP-HPLC based assay method was developed and validated for the assay of ivermectin and praziquantel in tablet and bulk forms. Linearity and remaining other validation parameters were good enough in the concentration ranges of 1–3 μg/ml and 25–75 μg/ml for ivermectin and praziquantel, respectively. The results of stress degradation divulged the specificity and stability indicating property of the method. The method was applied to tablet form and demonstrated to be accurate and precise for the assay of ivermectin and praziquantel simultaneously and its applicability for the quality control of ivermectin and praziquantel.

Ethical issues:

Not applicable.

Conflicts of interest:

No conflicts of interest exist in this investigation.

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