RESEARCH ARTICLE

A Rapid High Performance Liquid Chromatography Method for Simultaneous Quantification of Praziquantel, Ivermectin and Abamectin from Veterinary Formulations: Development, Validation and Application

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ABSTRACT

A simple, sensitive, accurate and robust analytical method was developed for separation and quantification of Praziquantel, Ivermectin and Abamectin from pharmaceutical preparations. The chromatographic separation was achieved on a C8 column (Waters Luna C8, 250×4.6 mm, 5 µm) with a short runtime of 11 minutes. The mobile phase used was 65 parts of acetonitrile mixed with 20 parts of methanol and 15 parts distilled water. The flow rate was set to 1.5 ml/min while column oven was maintained at 30 ºC. The UV detector was set at 244 nm. The method was validated according to the regulatory guidelines with respect to specificity, precision, accuracy, linearity and robustness. The working concentration for the assay method was 750 µg/ml for Praziquantel, 100 µg/ml for Ivermectin and 60 µg/ml Abamectin. Working range was selected based on the label claim of the oral paste formulations and keeping the final concentration of Praziquantel constant for the two formulations containing Ivermectin and Abamectin. Linearity range for the assay method was proven in the range of 375 to 1125 µg/ml for Praziquantel, 50 to 150 µg/ml Ivermectin and 40-80 µg/ml for Abamectin. The accuracy was between 98.0 to 102.0%. The precision was assessed by calculating %CV of six different assay preparations in intra-day. Intermediate precision was assessed as inter-day precision by calculating cumulative %CV of total twelve assay preparations. The cumulative %CV of twelve assay results was less than 2.0% for Praziquantel, Ivermectin and Abamectin. The method was found to be specific, finally, the method was demonstrated to be robust, resistant to small variations of chromatographic variables for oral paste formulations containing Praziquantel, Ivermectin and Abamectin.

INTRODUCTION

Drug resistance development is on the rise due to the rampant and uncontrolled use of antibiotics and other drugs used in the treatment of infections. Combination drug treatments and therapies are being developed for many drugs that were originally used as standalone therapies for a faster and better control of the infections.

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For the analytical chemist this presents with a new set of challenges. Many times the components of this combination therapy have significantly differing physical (solubility, melting point etc) and chemical (pKa, UV absorption maxima, stability in solvent etc.) properties. In addition to this, there are the excipients and preservatives which also need to be separated from the analytes of interest for the quantification of the active drugs in the formulation. If separate methods are employed

for the determination of the active ingredients then efficiency of the QC lab is affected as more time and efforts are needed from the chemist and less output is delivered in terms of number of samples analysed. Efforts are now being put in developing a common analytical method for multicomponent formulation analysis.

Praziquantel (PZ) is a heterocyclic prazino-isoquinoline derivative with a broad spectrum of activity against several trematodes (Fasciola, Schistosoma) and cestodes. Praziquantel is believed to act by interference with tegument calcium transport, resulting in paralysis of the parasitic worms with subsequent loss of adherence to tissue, degradation and expulsion. Praziquantel is available commercially as oral treatments in the form of tablets and paste formulation alone or in combination with other anthelmintic agents for veterinary use.

Ivermectin (IV) is a macrocyclic lactone and semisynthetic derivative of avermectin which is produced by Streptomyces avermitilis. Ivermectin has potent activity against several parasites and arthropods. It believed to act by interference with a glutamate gated chloride channel, which interferes with the parasite's neural and neuromuscular transmission. It has a broad spectrum of activity against several nematodes cestodes and trematodes. Ivermectin has particularly potent activity against onchocerciasis (river blindness) and lymphatic filariasis, which are important endemic diseases in Africa and South America. It is used alone or in combination with other anthelmintic agents and is available in various oral dosage forms.

Abamectin (AB) is a mixture of avermectins containing more than 80% avermectin B1a and less than 20% avermectin B1b and is obtained directly from fermentation extracts of Streptomyces avermitilis. These two components, B1a and B1b have very similar biological and toxicological properties. It is a systemic & contact broad-spectrum ectoparasiticide and endoparasiticide. Abamectin is used in combination with other anthelmintic agents for the treatment of cattle and Horses.

An available literature contains only sparsestudies related to the determination of Abamectin, Ivermectin or praziquantel in drugs. The analysis was carried out by colorimetric[1], voltamperometric[2], spectrophotometric[3] and chromatographic[4-8] methods. In a single study, a quantification of abamectin with praziquantel in veterinary oral paste by HPLC in isocratic system was described[8]. Other publications deal with the determination of a single active substance or its metabolites in a biological material ñ fruits and food[9-12], milk[13-15], meat and animal tissues[16-20], plasma[21-28], and environment[29-31]

There is a method reported for the simultaneous quantification of Praziquantel and Ivermectin and Praziquantel and Abamectin in public domain [32]. The method utilizes a reverse phase method using Supelcosil ABZ+ column and gradient elution using mixtures of water and acetonitrile and pure acetonitrile. Mobile phase rate was maintained at 1.2 mins and the detector was maintained at 245 nm. Even though the chromatographic conditions are identical for both the methods, the analysis was conducted separately for formulations containing Abamectin and Ivermectin as the retention time of the components of Ivermectin and Abamectin are having same retention time.

The method uses a complex composition of water and acetonitrile along with the gradient mixing to effect the separation. The total analysis time is about 30 mins, which is significantly high considering a quantification for a binary mixture. The peak of last eluting component if the mixture Ivermectin B_{1a} is eluting at around 25 mins. The authors could have tried for a shorter column to reduce the analysis time. Since the author have not even injected all the three components together in the same analytical run, there is significant scope of reducing the run time. Under the scenario, it would be much preferred if a single standard containing all the components could be injected so as to reduce the multiple injections and save analysis time as well as cost.

There is hence a need for developing an analytical method better suited for quantification of the three drugs for routine quality control

analysis. The current research involves development and validation of a new HPLC-UV method to quantify the drugs from marketed veterinary formulations as per the ICH Q2 (R1) guidelines[33].

MATERIAL AND METHODS

Chemical and Reagents

The working standards of Praziquantel, Ivermectin and Abamectin were provided by Cipla Ltd., India. HPLC grade acetonitrile and methanol was used from J.T. Baker Inc, USA. The Millex-GV 0.22 µm PVDF hydrophilic filters were procured from Millipore (India) Pvt. Ltd, Bangalore, India. Purified water was generated from TKA water purification system.

Preparation of Solutions

Two separate stock solutions each of Praziquantel (PZ), Ivermectin (IV) and Abamectin (AB) were prepared for the calibration curve and precision and accuracy experiment for the method validation exercise. The concentrations of the stock solutions were 3000 µg/ ml, 1000 µg/ ml and 600 µg/ ml for PZ, IV and AB respectively.

The stock solutions were prepared in Methanol and stored at 2-8°C. The stock concentrations were prepared by dissolving required quantity of each standard in 50 ml of mobile phase. Subsequent dilutions of the stock solutions were prepared from stock solutions by dilution with mobile phase. The working standard solutions thus prepared were used to prepare the solutions used in the validation experiment.

A seven-point standard curve was prepared. The calibration curve concentrations were 375, 450, 600, 750, 900, 1050 and 1125 µg/ml for PZ 50, 60, 80, 100, 120, 140 and 150 µg/ml for IV and 20, 24, 32, 40, 48, 56 and 60 µg/ml for AB.

Sample Preparation Procedure

For Equimax paste: The Equimax oral paste samples of about 1.1 gm were weighed and transferred to a clean dried 200 ml volumetric flask. Care was taken so that the paste sample does not stick to the neck of the flask. 70 ml of mobile phase was added to the flasks and sonicated for 60 seconds to disperse the formulation in the mobile phase. The flasks after sonication were allowed to come to the room temperature and then diluted to volume with mobile phase. The samples were further filtered with syringe filters into HPLC vials for analysis.

For Promectin Plus paste: The Promectin plus oral paste samples of about 1.6 gm were weighed and transferred to a clean dried 100 ml volumetric flask. Care was taken so that the paste sample does not stick to the neck of the flask. 30 ml of mobile phase was added to the flasks and sonicated for 60 seconds to disperse the formulation in the mobile phase. The flasks after sonication were allowed to come to the room temperature and then diluted to volume with mobile phase. The samples were further filtered with syringe filters into HPLC vials for analysis.

HPLC Method Parameters

The analysis was performed on liquid chromatography system (Shimadzu UFLC prominence, USA) composed of Binary pump, Thermostated auto sampler, column oven and diode array detector with Shimadzu Lab solutions software. The column Luna C8 (250 X 4.6 mm, 5 µm) from Waters Corp., USA was used. Column oven temperature was maintained at 30 °C. The UV detection was performed at 244 nm. The injection volume was 20 µL and injector compartment was maintained at 30 °C. The mobile phase was a 65:20:15 v/v mixture of distilled Acetonitrile, methanol and distilled water respectively. Mobile phase flow rate of 1.5 ml/minute was used throughout the run.
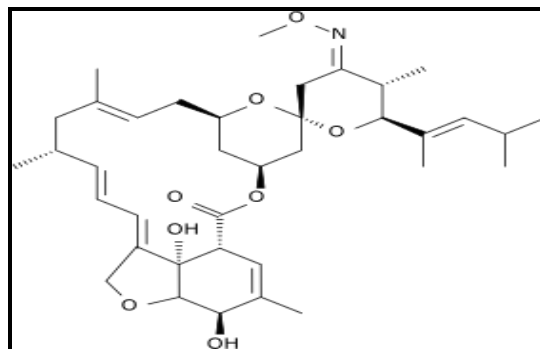

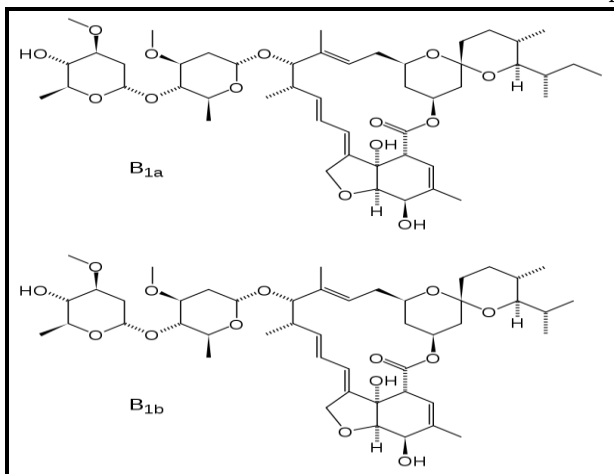Figure 1: Chemical structure of Praziquantel
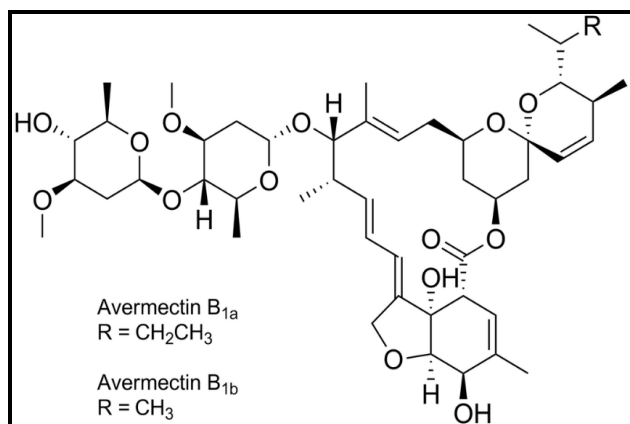
Figure 2: Chemical structure of Ivermectin

Figure 3: Chemical structure of Abamectin

Method Validation

The analytical method for quantification of PZ, IV and AB from cream formulation has been validated for selectivity, linearity, precision, accuracy, solution stability, ruggedness and robustness following appropriate recommendations of the ICH Q2 (R1) regulatory guidelines recommendations[33].

RESULTS AND DISCUSSION

Specificity

Specificity was performed by chromatographing the individual working level solutions of Praziquantel, Ivermectin and Abamectin. PZ, IV and AB solutions of 750 ppm, 100 ppm and 40 ppm were injected in the chromatographic system. No interfering peak of excipients or blank was observed at the retention time of the analytes. The theoretical plates, tailing factor observed for peaks of PZ, IV and AB are 4320 &

1.25; 5967 & 1.16 and 7523&1.15 respectively. The resolution between the peaks of PZ and IV was 13.13 and that between IV and AB was 10.78. Hence the peaks were well resolved from each other. Representative chromatograms of Praziquantel, Ivermectin and Abamectina represented in Figure 4, Figure5 and Figure 6 respectively. The mix standard chromatogram is presented in Figure 7 while sample solution chromatograms for Equimax paste and Promectin plus paste are presented in Figure 8 and Figure 9 respectively.
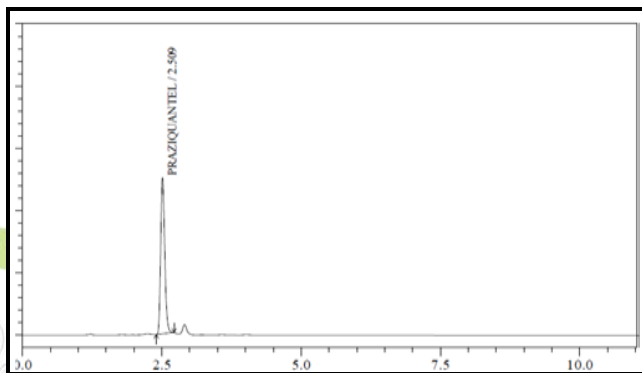

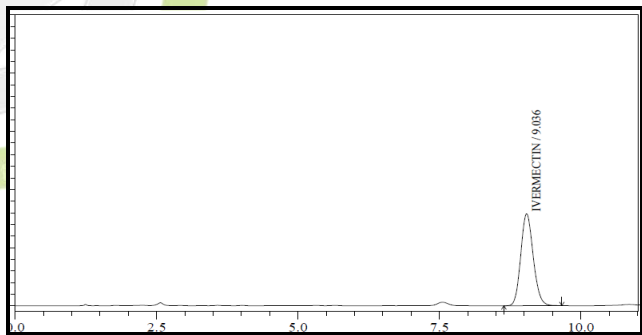Figure 4: Representative Chromatogram of Praziquantel

Figure 5: Representative Chromatogram of Ivermectin
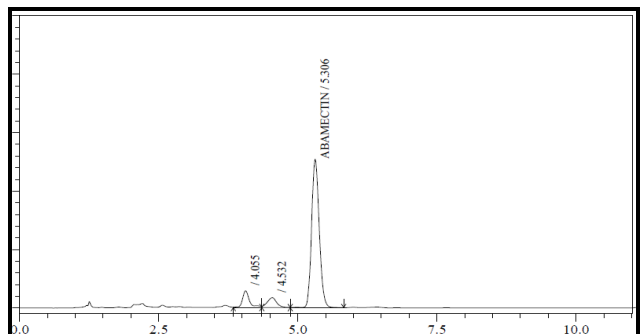

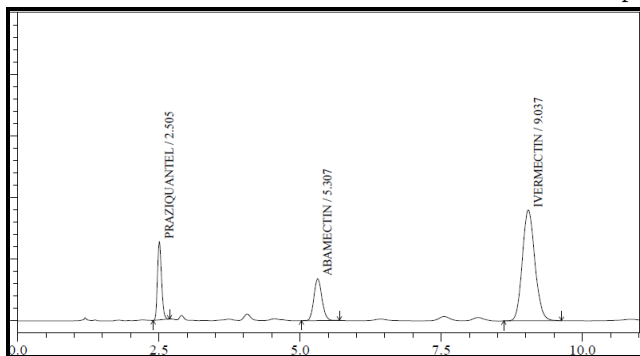Figure 6: Representative Chromatogram of Abamectin

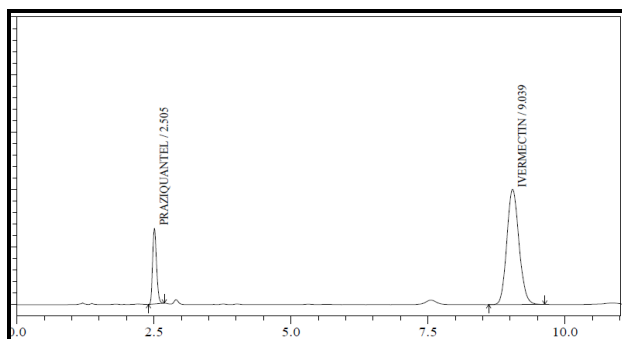Figure 7: Representative Chromatogram mixed standard

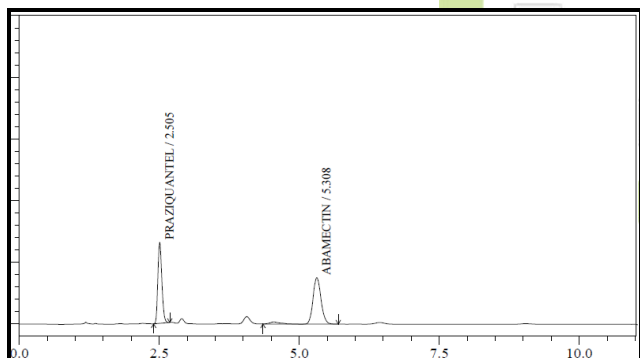Figure 8: Representative Chromatogram of sample (Equimax paste)

Figure 9: Representative Chromatogram of sample (Promectin Plus paste)

Precision

System Precision

System suitability was evaluated by injecting six replicates of the mix standard preparation in the chromatographic system. The relative standard deviation (RSD) of the area response and the retention time were evaluated. The RSD values for area response was found to be 0.75, 0.21 and 0.89 for PZ, IV and AB respectively. The RSD

values for retention time was found to be 0.34, 0.49 and 0.10 for PZ, IV and AB respectively.

Method Precision

Method precision was evaluated by injecting six preparations each of the two marketed formulations. The RSD for the back calculated % label claim of the active components was evaluated. The RSD values for the assay of PZ, IV and AB was found to be 0.74, 0.38 and 0.32respectively.

Linearity and Range

The response against concentration relationship was evaluated using a seven point calibration curve. Standard stock solution equivalent 3000 µg/ml of PZ and 1000 µg/ml of IV and 600 µg/ml of AB were prepared respectively. Series of dilutions were made from this Standard stock solution to achieve a linearity range of 50% to 150% of WL. The concentrations were 375, 450, 600, 750, 900, 1050 and 1125 µg/ml for PZ 50, 60, 80, 100, 120, 140 and 150 µg/ml for IV and 20, 24, 32, 40, 48, 56 and 60 µg/ml for AB. Calibration curves for concentration versus peak area were plotted for each compound and the obtained data were subjected to regression analysis with equation $y = mx + c$. The regression coefficient value was observed to be 1.0 for PZ and IV, and 0.9997 for AB. The linearity plot for PZ, IV and AB are shown in Figure10, Figure 11 and Figure 12 respectively.
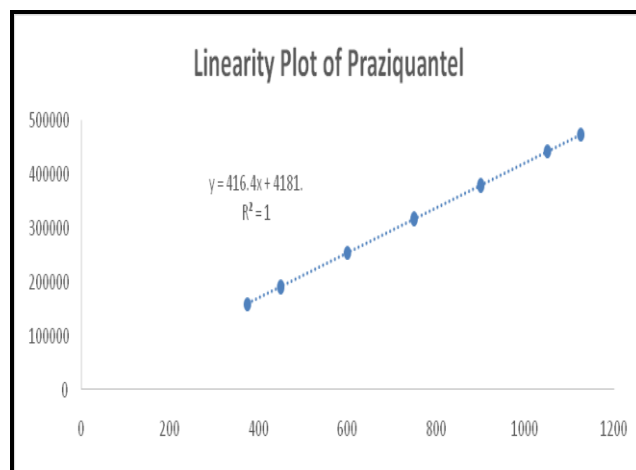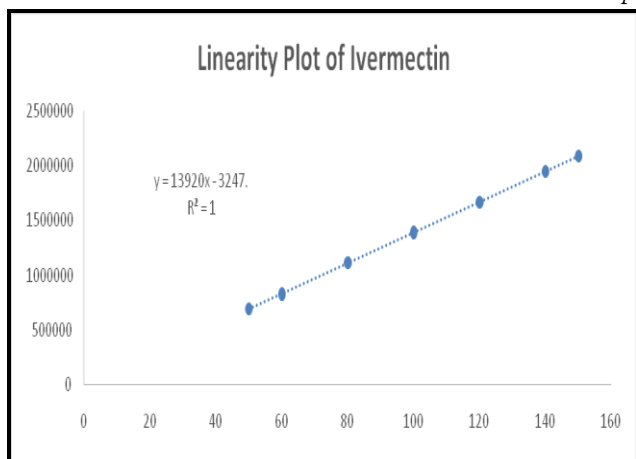

Figure 10: Linearity plot for Praziquantel
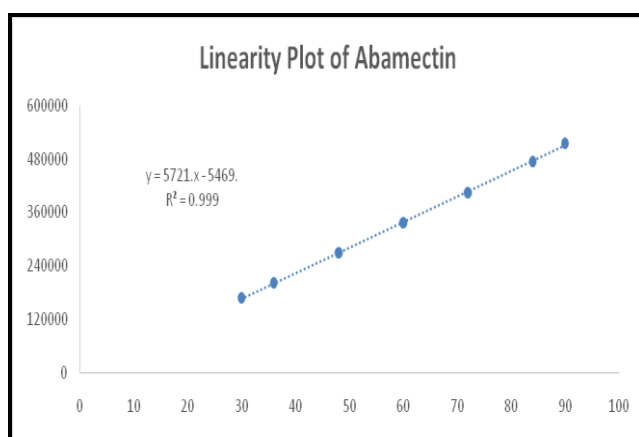
Figure 11: Linearity plot for Ivermectin

Figure 12: Linearity plot for Abamectin

Accuracy

Accuracy of the method was checked recovery method i.e. by spiking the WS solution in formulation sample and checking the recovery. Since the formulations under investigation were procured from the market, the placebo was not available; hence the recovery was conducted by adding the standard of each component in the cream formulation and then checking the recovery for added standard.

The Accuracy of the method was evaluated at three levels i.e. 50, 100 and 150 % of the working concentration in triplicate. The back calculated content of each of the three replicates at an individual level were evaluated. The accuracy was found to be consistent for PZ, IV and AB across the three concentration levels. The accuracy for PZ was found to be 99.63% to 100.77% and for IV was found to be 99.69% to 100.47% for IV for the Equimax paste. The

accuracy by recovery method was 99.77% to 100.44% for PZ and 99.89% to 100.22% for IV for the Promectin Plus paste.

The global mean accuracy for PZ and IV was 99.98% and 100.14% respectively for Equimax paste. The global mean accuracy for PZ and IV was 100.01%, 100.25% and 99.98% respectively for Promectin Plus paste.

Ruggedness (Intermediate Precision)

For ruggedness experiment the sample preparation and analysis was performed by another analyst using the same method of analysis. Six replicates of each of the marketed formulation were prepared and chromatographed on the next day of the method precision experiment. Intermediate assay method precision was evaluated by injecting six preparations each of the two marketed formulations. The RSD for the back calculated % label claim of the active components was evaluated. The RSD values for the assay of PZ, IV and AB was found to be 0.51%, 1.40 and 0.29% respectively. The cumulative RSD values for the 12 samples for assay was found to be 0.57, 0.31 and 0.75 for PZ, AB and IV respectively.

Solution Stability

The sample solutions prepared for the assay method precision experiment were re-injected after intervals of 12, 24, 36 and 48 hrs after initial injections. The stability of the analytes in the sample solution was evaluated by comparing the back calculated assay values for PZ, IV and AB. The analytes were found to be stable in the sample solution for at least 48 hrs. The stability was found to be 99.83%, 99.35% and 99.37% for PZ, IV and AB respectively.

Robustness

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage. Statistical analysis showed no significant difference between results obtained employing the analytical conditions established for the method

and those obtained in the experiments in which variations of some parameters were introduced. Thus, the method was shown to be robust for changes in mobile phase such mobile phase flow rate (+0.2 ml/min and -0.2 ml/min), column oven (25 ℃ and 35 ℃).

Application of Method to Marketed Formulations

The assay of PZ, IV and AB was performed on commercial marketed samples of the veterinary oral paste formulation. Purchased samples of Equimax paste and Promectin Plus paste were analysed using the analytical method. The assay results were 99.14% and 99.36% for PZ and IV in Equimax paste and 99.64% and 100.39% for PZ and AB in Promectin Plus paste. Assay testing performed on different days showed similar results.

CONCLUSION

The HPLC-UV assay method has been developed and validated for quantification of PZ, IV and AB for oral paste formulations. The validation data demonstrate good precision and accuracy of the method. The method was robust and did not encounter any variation with minor changes in the method parameters. This method was appliedfor the analysis of marketed formulations and was found to provide consistent and accurate results. This method significantly improves upon the drawbacks of the previously reported methods. It has also been observed that placebo interference is drastically reduced in the present method compared to the reported methods.

The method significantly improves on the analysis time required compared to the available method. The analysis time has been cut down from 25 minutes to 11 minutes; which is an improvement of 60%. This will enable the analysis to be completed faster. For the analysis of Praziquantel-Abamectin combination, the analysis time can be even further reduced to 7 minutes.

This assay method for simultaneous quantification of PZ, IV and AB will be beneficial for the routine and Quality control analysis of oral paste formulations containing these active ingredients, by saving the time and efforts of the analyst.

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