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SIMULTANEOUS ESTIMATION OF PYRANTEL PAMOATE, PRAZIQUANTEL & FEBANTEL BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY USING DUAL WAVELENGTH

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ABSTRACT

A sensitive, fast, and stability-indicating gradient reverse-phase liquid chromatography method was developed for quantitative simultaneous estimation of Pyrantel pamoate, Praziquantel and Febantel using reverse phase high performance liquid chromatography by gradient elusion with dual wavelength at a specified time interval. All three ingredients were well separated with Phenominax Hypersil C18 (ODS) (4.6 X100mm) 3µm column. The various factors affecting different parameters during method development by HPLC was analyzed and standardized.

Key words: High Performance liquid chromatography, Reverse Phase, Pyrantel Pamoate, Praziquantel , Febantel

INTRODUCTION

Three active ingredients having different mode of action and spectra of activity namely Praziquantel Pyrantel pamoate and Febantel present in single dosage form as tablet containing 68.0 mg Praziquantel, 68.0 mg Pyrantel base as Pyrantel Pamoate and 340.2 mg Febantel was used for the simultaneous estimation. Praziquantel is active against cestodes (tapeworms). Pyrantel Pamoate is active against hookworms and ascarids. Pyrantel Pamoate acts on the cholinergic receptors of the nematode resulting in spastic paralysis i.e. Peristaltic action of the intestinal tract then eliminates the parasite. Febantel is active against nematode parasites including whipworms. Febantel is rapidly absorbed and metabolized in the animal [1, 2]. Pyrantel Pamoate is Yellow to tan solid, practically insoluble in water (Solubility of <0.1 g/100 mL at water 19 ºC) & methanol; soluble in dimethyl sulfoxide; slightly soluble in dimethyl formamide. Molecular Formula is $C_{34}H_{30}N_2O_6S$, Molecular weight: 594.68 [3]. Praziquantel is white crystalline powder very slightly soluble in water (water solubility 400 mg/L); freely soluble in alcohol & in chloroform. Molecular formula is $C_{19}H_{24}N_2O_2$, Molecular weight: 312.41 [3]. Febantel is white or almost white, crystalline powder practically insoluble in water (is soluble in 1 to 2 ppm in water at pH 5 to 9), soluble in acetone, slightly soluble in anhydrous ethanol. Molecular formula is $C_{20}H_{22}N_4O_6S$. Molecular weight: 446.48 [4].

The proposed method for separation of these three molecules is reverse phase method. Reversed phase HPLC (RP-HPLC) has a non-polar stationary phase and polar mobile phase. In Reverse phase liquid chromatography the separation of molecules is based upon their inter-action with a hydrophobic matrix which is largely based on their polarity. Molecules are bound to the hydrophobic matrix in an aqueous buffer (polar) and eluted from the matrix using a gradient of organic solvent (non-polar). One common stationary phase is silica. Silica beads (3-5 micron) have linear octadecane groups (C18) attached to the surface via co-valent bonds. These beads are usually porous in order to increase the surface area of the beads available for binding. The C18 groups are very hydrophobic (non-polar) and can bind quite polar molecules such as charged peptides in a highly polar solvent such as water. Silica has been surface-modified with RMe_2SiCl, where R is a straight chain alkyl group such as $C_{18}H_{37}$ or C_8H_{17}. Generally n-Octadecyl(RP-18),n-octyl (RP-8).ethyl (RP-2), phenyl,(CH_2)n-CN, $(CH_2)_n$-Diol. hydrophobic polymers can be used as stationary phase. In these stationary phases nonpolar molecules retention time is longer than the polar molecules. [5,6,7].

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METHOD

Fluconazole was supplied as gift sample from (Synergene Active Ingredients (p) ltd.), curcumin was procured from (molychem pvt Ltd.), ethanol from (Rankem lab, Mumbai), HPMC& carbapol, Peptone purchased from (Sd Fine Chem. Limited), Agar from (Lobachem), Sodium chloride, Glycerine, yeast extract & Beef extract were purchased from (Molychem pvt Ltd).

To develop the method for simultaneous estimation of Pyrantel Pamoate, Praziquantel and Febantel different experiments were carried out using different diluents. Different concentrations of drug were injected at different wavelengths for simultaneous estimation using different mobile phase to optimize the method. A few of these trials are presented in this article to understand the effect of different factors affecting in this method. The method development was started with solubility determination. As described earlier Pyrantel Pamoate, Praziquantel and Febantel are practically insoluble in water. So organic solvents in combination with phosphate buffer and surfactant were used to dissolve. For optimization of chromatographic conditions the method development was started using different columns like Symmetry C18 waters 150X4.6mm, 5µ; Kromasil 100-5C18,150X4.6 mm; Phenomenex Gemini C18 250 x 4.6 mm , 5 µ particle size) & Phenominax Hypersil C18 (ODS) 100X4.6 mm,3µm.All three ingredients were well separated with Phenominax Hypersil C18 (ODS) 100X4.6 mm, 3µm column.

Preparation of Mobile Phase

Buffer preparation: Accurately weighed 1.0 gm of 1-Heptane sulphonic acid sodium salt and dissolved in 950 ml of water, Added 1.0 ml of TEA and then adjusted to pH 7 with OPA and made up the volume up to 1000 ml with water. Filtered the buffer with 0.45 µ nylon filter.

Diluent Preparation:

Buffer preparation: Buffer solution was prepared by dissolving 3.12 gm of Sodium dihydrogen phosphate dihydrate in 1500 ml of water. 40 gm of Sodium lauryl sulfate was dissolved and sonicated for 30 minutes to dissolve completely. pH of the solution was adjusted up to 6.8 with dilute NaOH (50 % solution) and made the volume up to 2000 ml with water.

Diluent 1 was prepared by mixing above Buffer and Acetonitrile at the ratio of 20 : 80.

Diluent 2 was prepared by mixing Acetonitrile, Glacial acetic acid, water and Tri ethyl Amine at the ratio of 92.8 : 3 : 3: 1.2 .

Preparation of blank solution: Diluent 1was taken in 50ml volumetric flask by maintaining the temperature about 25° C . 10 ml of this diluent 1 was taken separately in to 50 ml volumetric flask and diluted up to the mark with diluent 2 and mixed. 2.5 ml from this preparation was taken in to 10ml volumetric flask and diluted with diluent 2 and mixed. This solution was taken as blank.

Preparation of placebo solution: Accurately weighed placebo powder equivalent to 51 mg Pyrantel Pamoate, 51mg Praziquantel and 255.15mg Febantel was taken into 50 ml volumetric flask. 25 ml diluent 1 was added and sonicated till it dissolved completely by maintaining the temperature at 25° C and made volume up to the mark with Diluent 1 and mixed. The solution was filtered with SY25TG filter and few ml of filtered solution was discarded. From the filtered solution second stock solution was prepared which was further diluted to obtain the final test solution.

Second stock preparation: 10 ml of above stock was taken separately in to 50 ml volumetric flask and diluted up to the mark with diluent 2 and mixed.

Final test solution preparation: 2.5 ml from above stock preparation was taken in to 10ml volumetric flask and diluted with diluent 2 and mixed.

Preparation of standard solution:

Pyrantel Pamoate stock preparation: Accurately weighed about 51 mg of Pyrantel Pamoate Working standard in 50ml volumetric flask. 25 ml diluent 1 was added and sonicated till it dissolved completely by maintaining the temperature at about 25° C and made volume up to the mark with diluent 1 and mixed.

Praziquantel stock preparation: Accurately weighed about 51 mg of Praziquantel working standard in 50ml volumetric flask . 25 ml diluent 1 was added and sonicated till it dissolved completely by maintaining the temperature at about 25° C and made volume up to the mark with diluent 1 and mixed.

Febantel stock preparation: Accurately weighed about 255.15 mg of Febantel Working standard in 50ml volumetric flask. 10 ml diluent 1 was added and sonicated till it dissolved completely by maintaining the temperature at about 25° C and made volume up to the mark with diluent 1 and mixed.

Standard stock preparation: 10 ml of Pyrantel stock preparation, 10 ml of Praziquantel stock preparation and 10 ml of Febantel stock preparation was taken separately in to 50 ml volumetric flask and diluted up to the mark with diluent 2 and mixed.

Working Standard Preparation: 2.5 ml from standard stock preparation was taken in to 10ml volumetric flask and diluted with diluent 2 and mixed.

Preparation of test solution:

Stock solution preparation: Twenty Tablets were weighed and average weight was determined. These tablets were triturated in a mortar pestle and transferred an accurately weighed tablet powder equivalent to 51 mg Pyrantel Pamoate, 51mg Praziquantel and 255.15 mg Febantel in to 50 ml volumetric flask. 25 ml diluent 1 was added and sonicated till it dissolved completely by maintaining the temperature at 25° C and made volume up to the mark with diluent 1 and mixed. The solution was filtered with SY25TG filter and few ml of filtered solution was discarded. From the filtered solution second stock solution was prepared which was further diluted to obtain the final test solution.

Second stock preparation: 10 ml of above test stock was taken separately in to 50 ml volumetric flask and diluted up to the mark with diluent 2 and mixed.

Final test solution preparation: 2.5 ml from standard stock preparation was taken in to 10ml volumetric flask and diluted with diluent 2 and mixed.

Figure 1: Chromatogram for mixture of all three standards of Trial 4 at 300 nm

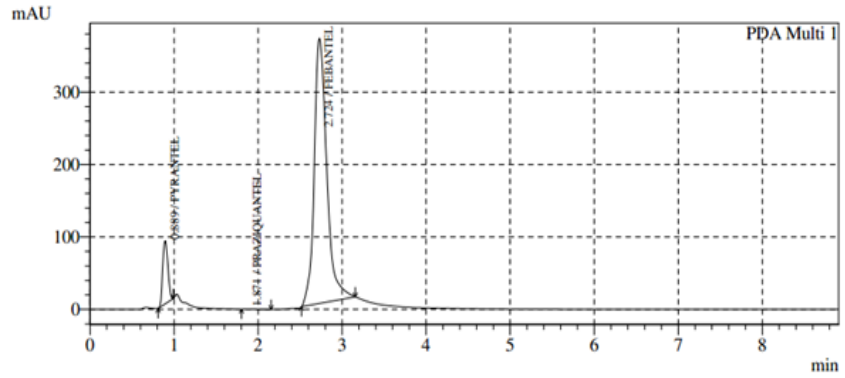


Figure 2: Chromatogram for mixture of standards of Trial 4 at 210 nm

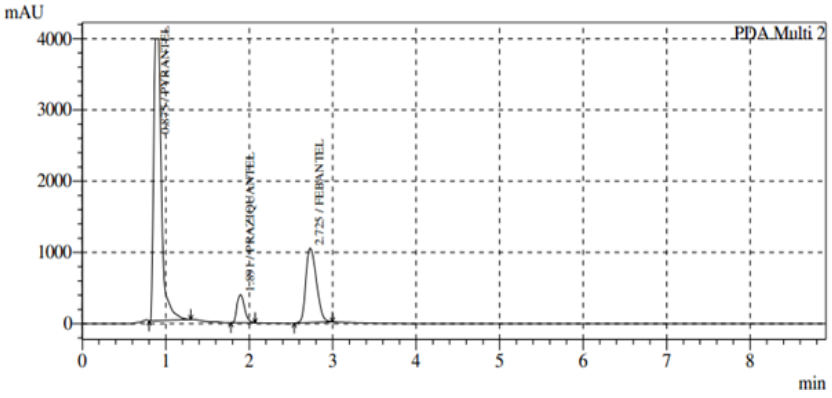


Table 1: Chromatographic conditions & System Suitable Parameters (Isocratic trial)

Chromatographic conditions	Column	Phenomenax Hypersil C18 (ODS) (4.6 x 100 mm) 3 µm				
	MP	60:30:10 (Acetonitrile , buffer and methanol)				
	F rate	1.5 ml/min				
	C Temp	25°C				
	Inj vol	10 µl				
Wavelength	210nm & 300 nm					
Description	Trial 1	Trial 2	Trial 3	Trial 4		
Name of Components	Praziquantel	Pyrantel	Febantel	Praziquantel	Pyrantel	Febantel
Area	2479	1371334	35696511	2463406	25899196	9189041
RT	1.89	0.87	2.71	1.89	0.87	2.72
Tailing Factor	1.34	1.59	1.63	1.33	2.34	1.36
Theoretical plates	1754	672	2557	1701	489	1971

Table 2: Chromatographic conditions & System Suitable Parameters (Isocratic trial)

Chromatographic conditions	MP	60:30:10 (Acetonitrile , buffer and methanol)				
	F rate	1.0 ml/min				
	C Temp	25°C				
	Inj vol	10 µl				
Wavelength	210nm & 300nm					
Description	Trial 5	Trial 6	Trial 7	Trial 8		
Name of Components	Praziquantel	Pyrantel	Febantel	Praziquantel	Pyrantel	Febantel
Area	2967	503669	8269729	559997	5362454	1607340
RT	2.79	1.33	4.027	2.78	1.33	4.03
Tailing Factor	1.05	1.07	1.29	1.24	1.07	1.25
Theoretical plates	3172	787	2503	2346	675	2560

MP: Mobile Phase, F Rate: Flow rate , RT: Retention Time, C Temp: Column Temp , Inj vol: Injection volume

Figure 3 : Chromatogram for mixture of all three standards of trial 8 at 300 nm

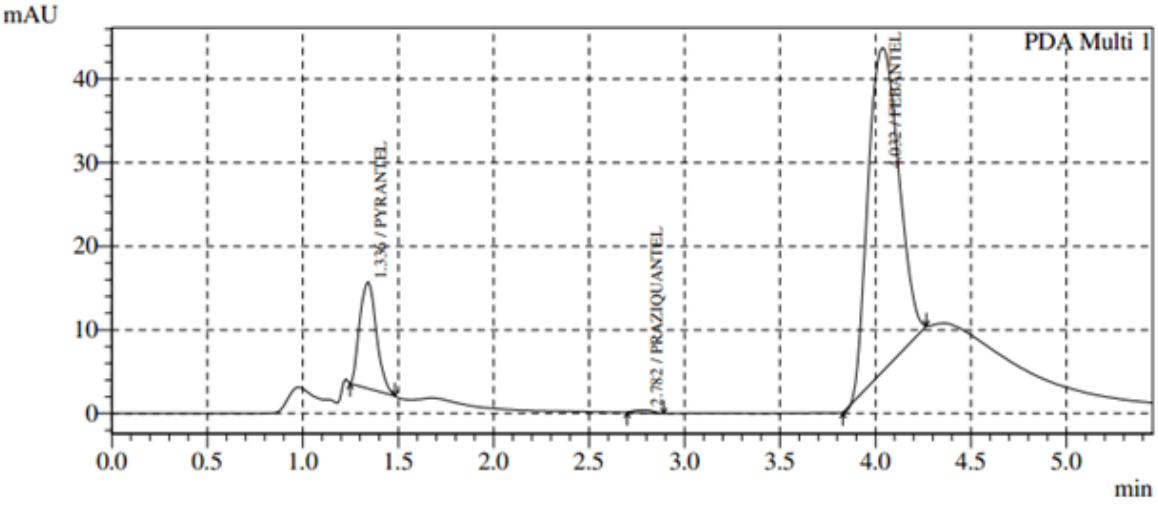
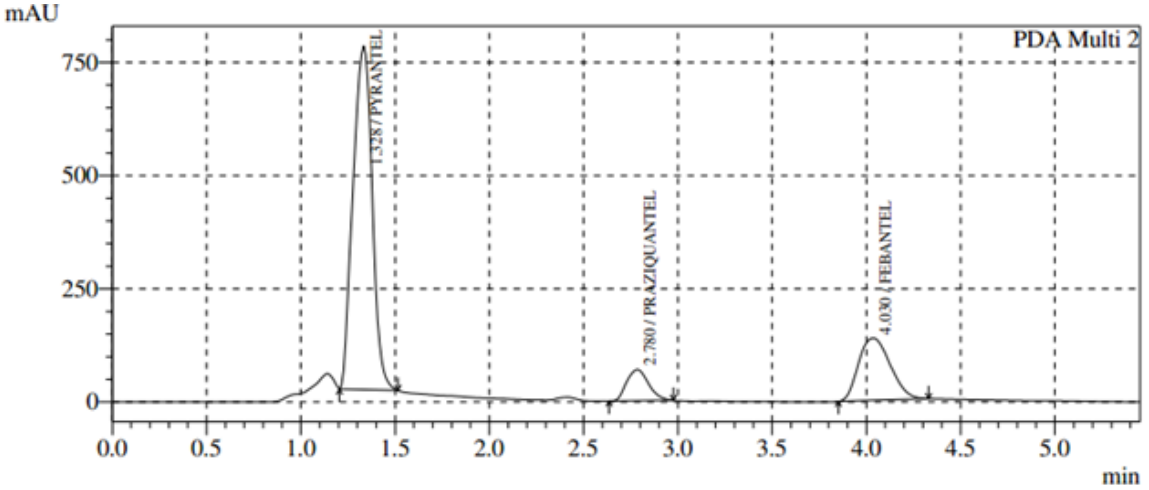


Figure 4: Chromatogram for mixture of all three standards of Trial 8 at 210 nm



Trial 9 with same chromatographic conditions as table 2 with column temperature 45°C and mobile phase ACN and buffer in ratio of 40: 60 was taken

Figure 5: Chromatogram for mixture of all three standards of trial 9 at 300 nm

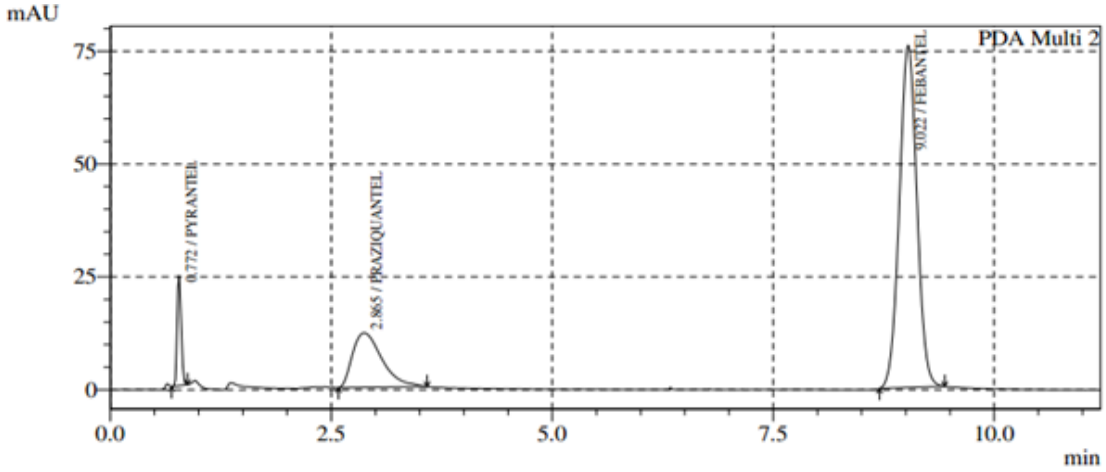


Figure 6: Chromatogram for mixture of all three standards of trial 9 at 210 nm

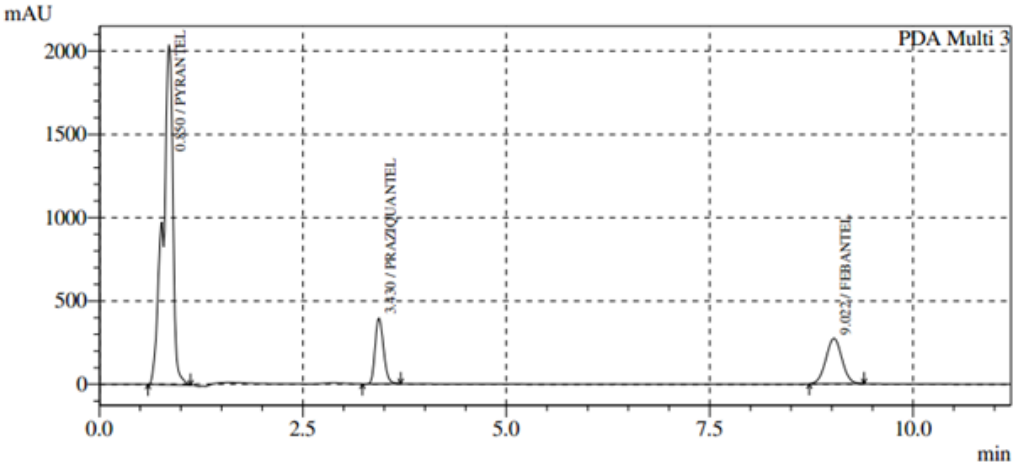


Table 3: Chromatographic conditions: Time –Gradient Programming

Coloumn	Phenomenax Hypersil C18 (ODS) (4.6 x 100 mm) 3 µm.						
Description	Trial 10	Trial 11	Trial 12	Trial 13	Trial 14	Trial 15	Trial 16
Flow rate (ml/min)	1.5	1.0	1.0	1.0	1.0	1.0	1.0
Temperature	45°C	45°C	45°C	45°C	45°C	45°C	45°C
Injection volume	10 µl	10 µl	10 µl	10 µl	10 µl	10 µl	10 µl
Time-Gradient Programming	Table 4	Table 5	Table 6	Table 7	Table 8		

Table 4: Time gradient programming for trial 10

Minutes	ACN	Buffer
0	20	80
2	20	80
2.5	50	50
15	50	50

Table 5: Time gradient programming for trial 11

Minutes	ACN	Buffer
0	30	70
2	50	50
8	50	50
15	50	50

Table 6: Time gradient programming for trial 12

Minutes	ACN	Buffer
0	30	70
2	50	50
8	50	50
9	30	70

Table 7: Time gradient programming for trial 13

Minutes	ACN	Buffer
0	30	70
2	50	50
8	50	50
9	30	70
10	70	70

Table 8: Time gradient programming for trial 14 at 300 and 210 nm

Time (min)	ACN	Buffer	Wavelength
0.00	30	70	300 nm
2.00	50	50	300 nm
2.10	-	-	210 nm
8.00	50	60	210 nm
9.00	30	70	210 nm
10.00	30	70	210 nm
15.00	30	70	210 nm

Figure 7: Chromatogram for mixture of all three standards of trial 10 at 300 nm

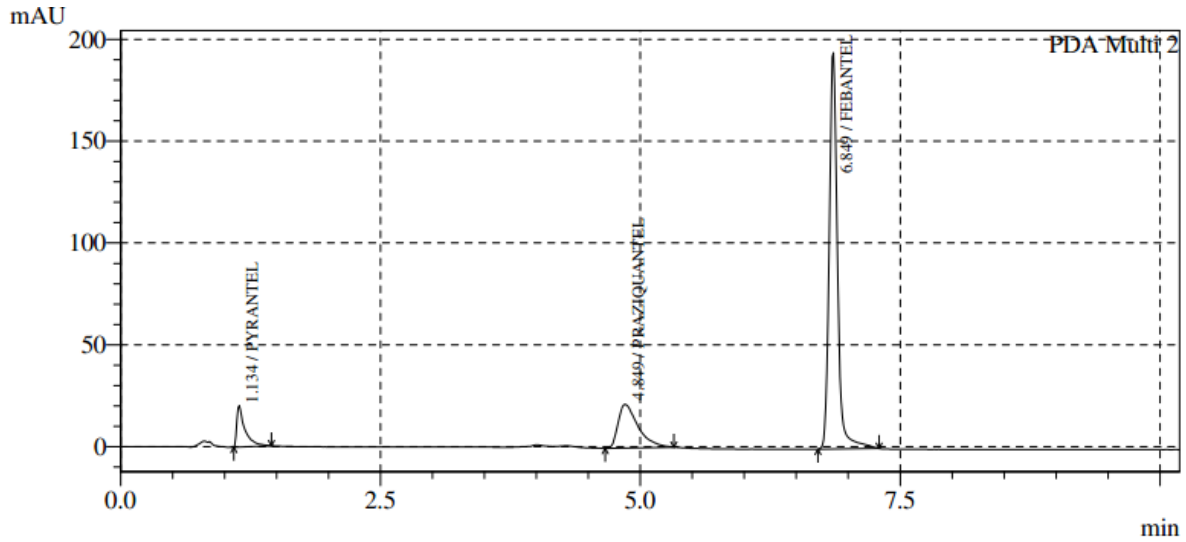


Figure 8: Chromatogram for mixture of all three standards of trial 10 at 210 nm

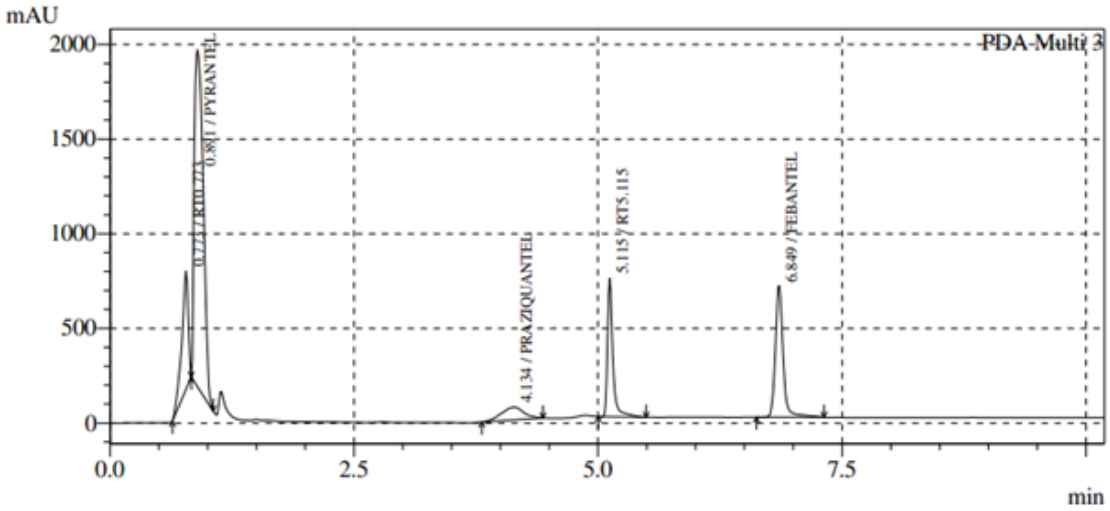


Figure 9: Chromatogram for mixture of all three standards of trial 11 at 300 nm

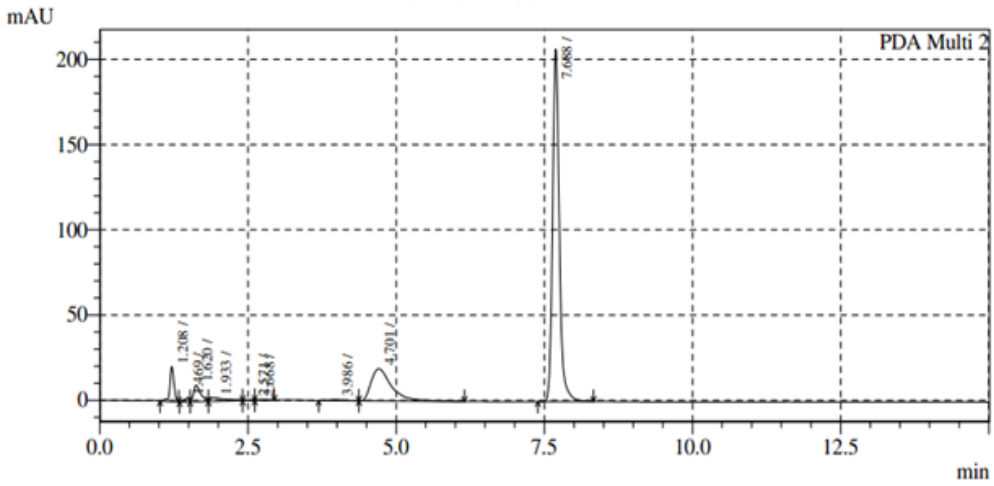


Figure 10: Chromatogram for mixture of all three standards of trial 11 at 210 nm

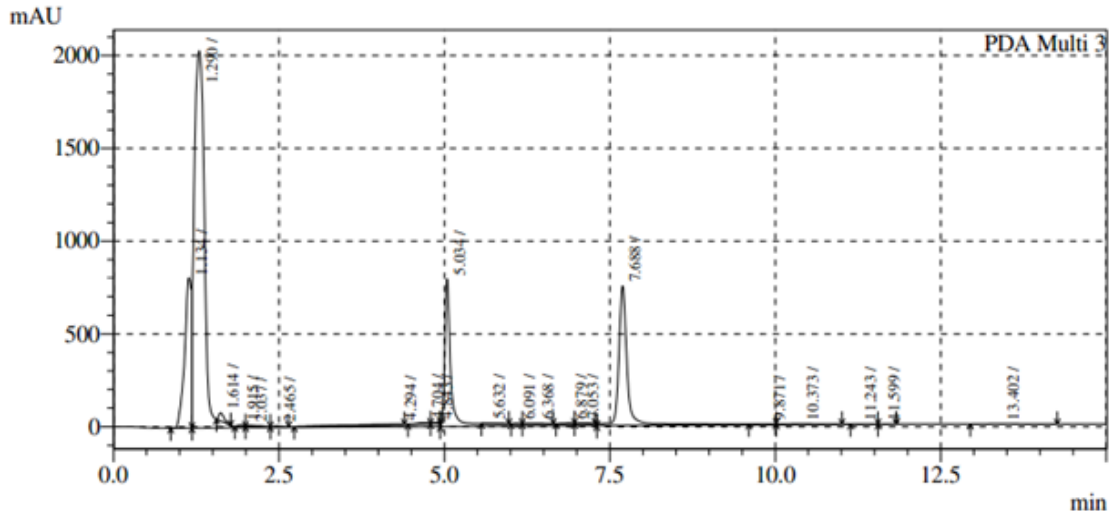


Figure 11: Chromatogram for mixture of all three standards of trial 12 at 300 nm

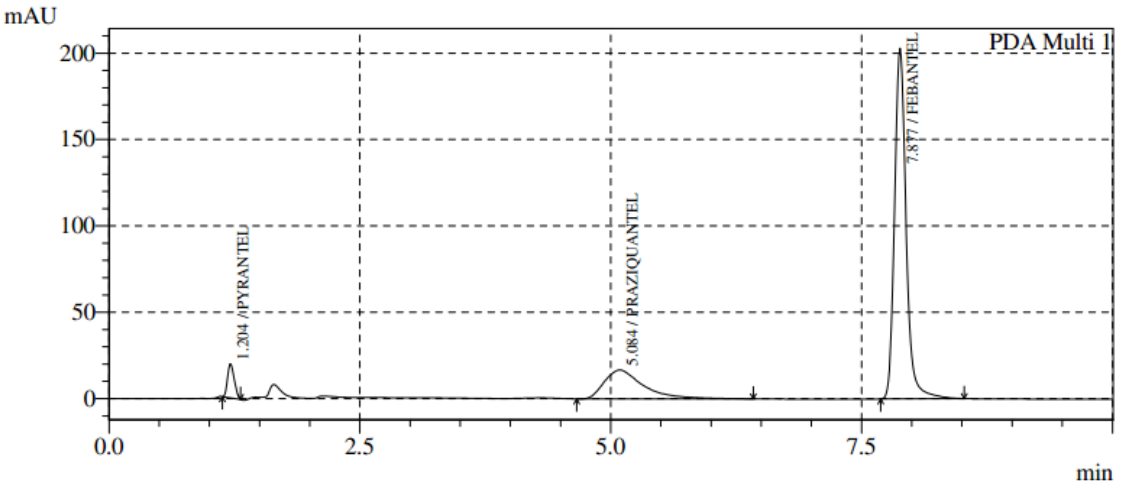


Figure 12: Chromatogram for mixture of all three standards of trial 12 at 210 nm

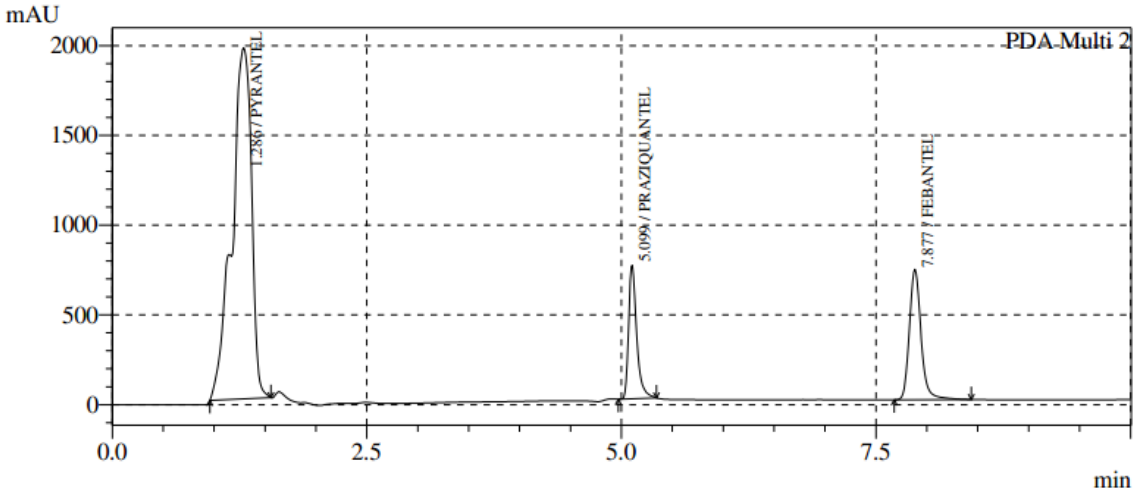


Figure 13: Chromatogram for mixture of all three standards of trial 13 at 300 nm

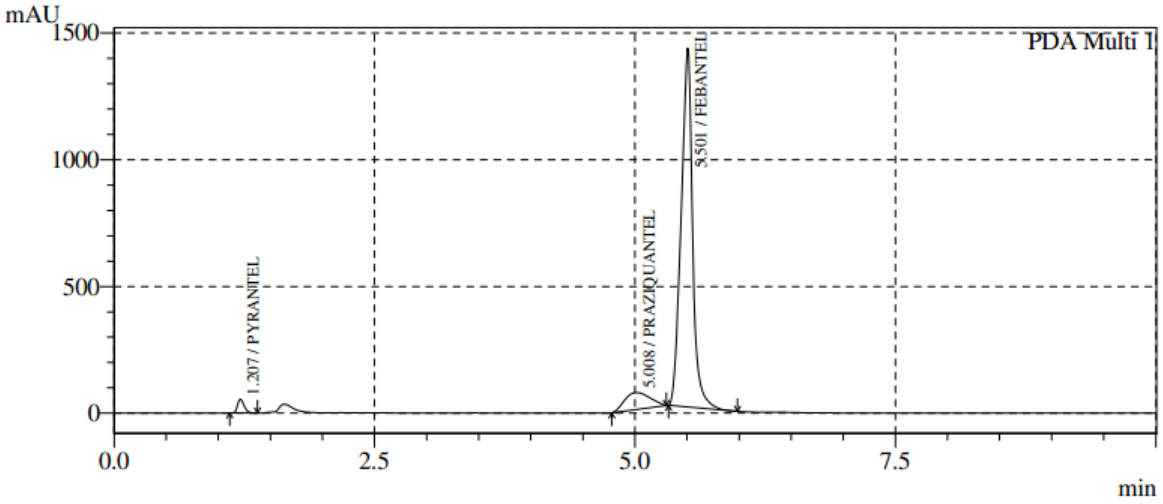


Figure 14: Chromatogram for mixture of all three standards of trial 13 at 210 nm

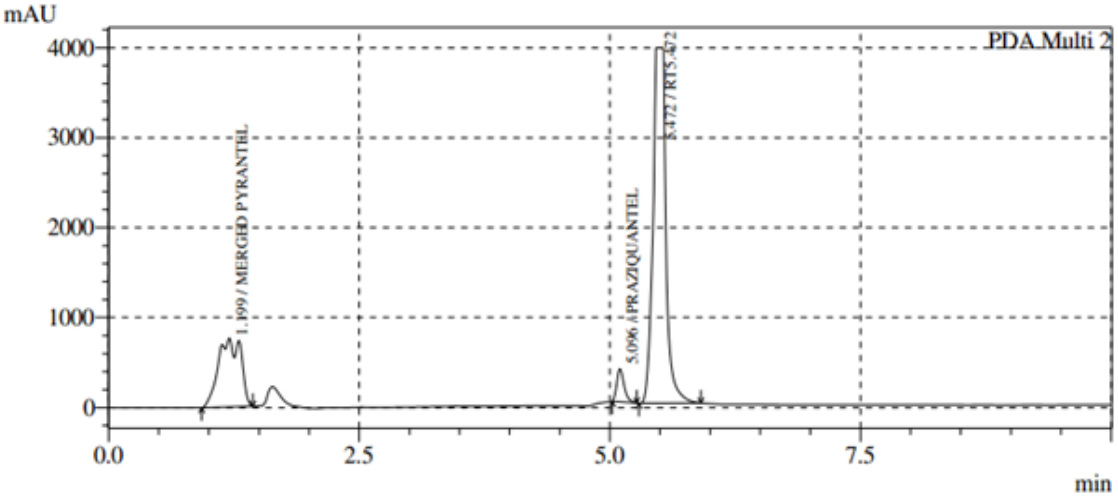


Figure 15: Chromatogram for Standard preparation (mixture of all three standards) at 300 and 210 nm

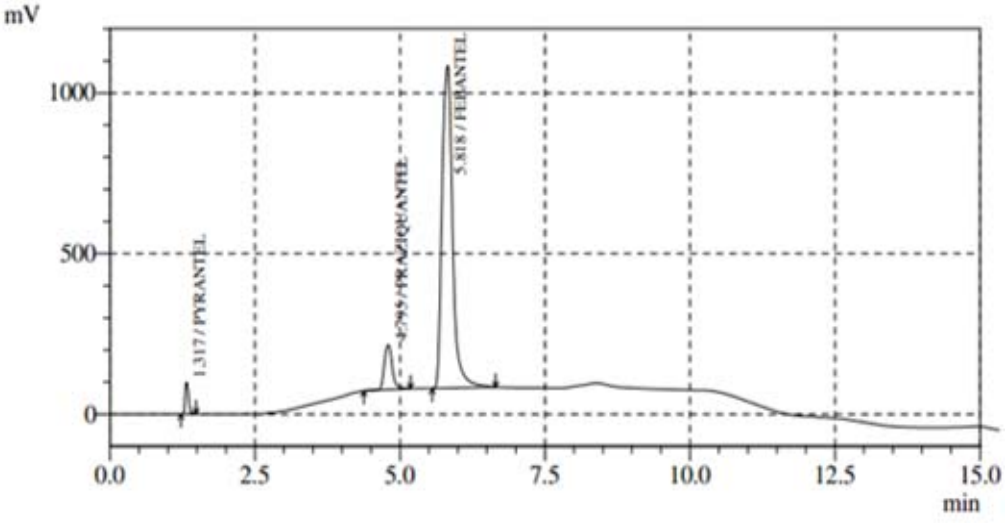


Table 9: System suitability parameters of standard preparation

Name	RT	Area	Theoretical plates	Tailing
Pyrantel	1.32	400365	1987	1.55
Praziquantel	4.79	1335470	6078	1.24
Febantel	5.82	12754959	5743	1.26

Figure 16: Chromatogram for test solution

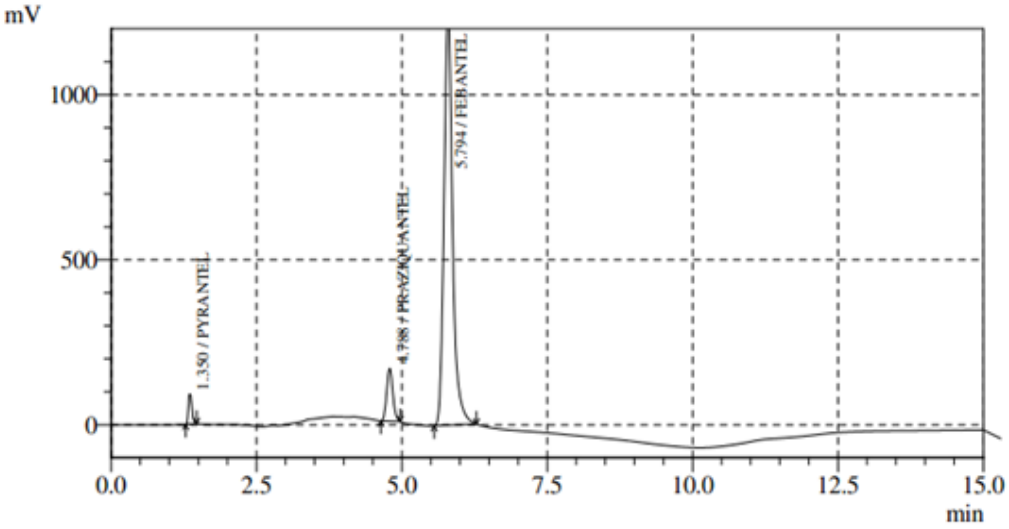
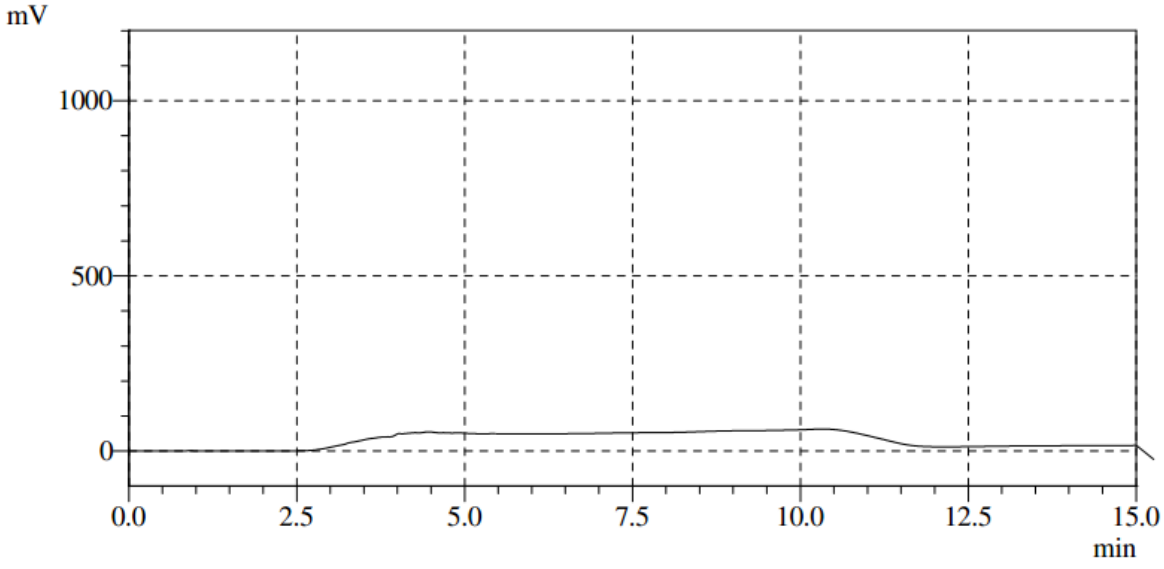


Table 10: System suitability parameters for test solution

Name	RT	Area	Theoretical plates	Tailing
Pyrantel	1.350	388198	2000	1.57
Praziquantel	4.788	1326217	6218	1.18
Febantel	5.794	12899677	5800	1.192

Figure 17: Chromatogram for placebo solution



RESULTS AND DISCUSSION

The method development was started with solubility determination. Pyrantel Pamoate, Praziquantel and Febantel are practically insoluble in water. In reverse phase chromatography acetonitrile (uv cut-off of 190) and methanol (UV cut-off 205) (MeOH) are commonly used solvents. These solvents are miscible with water. Sodium Lauryl Sulphate (SLS) is used as an anionic surfactant to increase the solubility of the molecules in combination of sodium phosphate buffer pH 6.8 and acetonitrile. This diluent is further diluted with mixture of acetonitrile, glacial acetic acid and triethylamine. This mixture works as organic modifier that can alter selectivity and improve peak shape. All analytes have chromophor and are UV active, so UV detector was selected for method development. Organic solvent in mobile phase can decrease the retention time. Similarly retention time can be increased by adding more water or buffer. In the studied method, development is started with the mixture of acetonitrile and water as mobile phase, but satisfactory separation was not obtained. Hence buffer was used in combination with acetonitrile.

In reversed phase HPLC, the retention of analyte is related to their hydrophobicity [6]. The more hydrophobic the analyte, the longer it is retained. When an analyte is ionized, it becomes less hydrophobic and, hence its retention decreases. Acids lose a proton and become ionized when pH increases and bases gain a proton and become ionized when pH decreases. Therefore, when separating mixtures containing acids and/or bases by reversed phase HPLC, it is necessary to control the pH of the mobile phase using an appropriate buffer in order to achieve reproducible results. In the studied method sodium phosphate buffer pH 6.8 was first tried to retain this compound without ion pair reagent, but could not be retained.So ion paring reagent 1-Heptane Sulphonic acid sodium salt was used in the buffer for mobile phase preparation. Triethylamine was used as organic modifiers and orthophosphoric acid was used to adjust the pH of mobile phase to 7. The components were tried to separate using different columns and found that column with smaller size and low particle size provided better efficiency for separation and lower retention time. The separation was better obtained with Phenominax Hypersil C18 (ODS)(4.6 X100mm) 3µm column.

Initially isocratic method was used to understand the behavior of the individual molecules and also in combination at wave length 210 nm and 300 nm. For details refer table 1, 2 and figure 1,2,3,4,5, 6. In the fourth trial mixture of all three components were injected. All three peaks were separated and eluted but Pyrantel peak is merged with one unknown peak at 210 nm. The results are tabulated in Table 1(Figure 1 and Figure 2). To overcome this problem of merging, the flow rate was decreased from 1.5ml/min to 1 ml/min in next trial. When flow rate was decreased the retention time was increased. This can be observed in the fifth, sixth and seventh trials. As a result of decreasing the flow rate of mobile phase the Pyrantel peak which was merged with the unknown peak is slightly separated without proper resolution. So in the ninth trial the ratio of mobile phase acetonitrile and buffer in the ratio was done 40:60.The column temperature was increased to 45°C. The RT of Pyrantel, Praziquantel and Febantel are 0.772, 2.865 and 9.022 min respectively, improper separation of Pyrantel peak at 300 nm. Febantel and Praziquantel peaks were too far with the problem of merging.(Figure 5 and Figure 6)

Elevated temperature was used to reduce analysis time and to improve peak shape. The temperature has a direct influence or the chromatographic result since it influences the RT and shape of the peak. The constant temperature can increase the robustness in HPLC, reproducible RT. Only at constant temperature RT are constant and results are comparable. At temperature above 50°C, an increased noise level was observed. Thus change in temperature can be used to control sample retention.

Thus with isocratic methods satisfactory results were not observed due to improper shape of peaks and improper separation of components. A stronger mobile phase gives shorter run times and weaker gives the reverse of the same. Early bands require a weaker mobile phase, and latter are best separated with stronger mobile phase. Based on these observations, Time-

gradient programming was studied further for simultaneous estimation of the three components and column oven temperature was increased to overcome the problem of merging. In the **tenth trial** different ratio of acetonitrile and buffer was used at flow rate of 1.5 ml/min. The temperature of the column was 45°C. Higher tailing of Pyrantel peak was observed at these chromatographic conditions at 300 nm (Table 3 & 4 and Figure 7 and Figure 8). In the **eleventh and twelfth trials** flow rate of mobile phase was again changed to 1 ml/min. Merging Peak of Pyrantel peak at 300 nm was observed which may be saturation issue. (Table 3, 5, 6 and Figure 9, Figure 10, Figure 11 and Figure 12).In the **thirteenth trial** Praziquantel and Febantal peak was not well separated. (Table 3 & 7 and Figure 13 and Figure 14). Based on these observations, in the next trials the ratio of mobile phase was varied at different time intervals and duel wavelength was used. In the **fourteenth trial,** mixture of all three components were injected using Phenomenax Hypersil C18 (ODS) (4.6 x 100 mm) 3 µm column. Detail of the time- gradient program of mixture of Acetonitrile and buffer was described on table 8. Two wavelengths were selected to get proper separation .These wavelengths used were 210nm & 300 nm. The wavelength in the detector was kept 300 nm initially and at 2.10 min the wavelength was changed to 210 nm. The temperature of the column was 45°C. All the components were well separated. The tailing factor was less than 2 as per compendia requirements. Peak shapes were satisfactory and RT was less than 10 minutes. In the **fifteenth trial,** test preparation was injected with the same chromatographic conditions as **fourteenth trial.** All the components were well separated**.** All the parameters are satisfactory (Table 10 and Figure 16). In the **sixteenth trial,** placebo preparation was injected in the same chromatographic conditions as **fourteenth trial**. No peak was obtained in the gradient (Figure 17).

CONCLUSION

From the above studies, it can be concluded that mobile phase, flow rate , column temperature and gradient programming affects simultaneous estimation of Pyrantel Pamoate, Praziquantel and Febantel by High Performance Liquid Chromatography. The three components can be well separated by gradient programming at wavelength 210 nm and 300 nm at an elevated temperature of 45°C using C-18 column. The run time is 15 min and retention time of Pyrental, Febental, Praziquental is 1.3, 4.7 and 5.8 min respectively that allows simultaneous estimation of the three components in a short period of time. The tailing factors were less than 2 for all the components and comply as per official compendia requirements. This method is also validated and detailed discussions shall be presented in the next article. Hence this method can be used for simultaneous separation, identification and quantitative estimation in quality control.

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