EUROPEAN JOURNAL OF BIOMEDICAL AND PHARMACEUTICAL SCIENCES
http://www.ejbps.com

ISSN 2349-8870
Volume: 7
Issue: 8
419-427
Year: 2020

DEVELOPMENT AND VALIDATION OF STABILITY INDICATING HPLC METHOD FOR SIMULTANEOUS DETERMINATION OF FOSNETUPITANT AND PALONOSETRON IN PHARMACEUTICAL DOSAGE FORM

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Article Received on 30/05/2020 **Article Revised on 20/06/2020** **Article Accepted on 10/07/2020**

ABSTRACT
The present work concerns with the development of stability indicating the RP-HPLC method for simultaneous determination of Fosnetupitant (FTP) and Palonosetron (PNS). In the developed RP-HPLC method separation was achieved using Synchronies C-18 (250mm x 4.6mm, 5µm) column as a stationary phase and Methanol: Acetonitrile: 1% sodium perchlorate 75:20:05 (v/v/v) as a mobile phase at pH 4. The retention times of FTP and PNS were found to be 5.3 min and 9.3 min, respectively with run time 16 min. Calibration curves were drawn relating the integrated area under peak to the corresponding concentrations of FTP and PNS in the range of 94-329 µg/ml and 01-035 µg/ml, respectively. The developed method has been validated and met the requirements delineated by ICH guidelines with respect to linearity, accuracy, precision, specificity and robustness. Both the drugs were subjected to acid, base hydrolysis, peroxide, UV light and thermal degradation conditions. Degradation peak was well resolved from the main peak of drug. The validated method was successfully applied for determination of the studied drugs in formulation and moreover its results were statistically compared with those obtained by the official method and no significant difference was found between them.

KEYWORDS: Fosnetupitant, Palonosetron, RP-HPLC, ICH guidelines, Method Validation.

1. INTRODUCTION
Fosnetupitant is the pro-drug form of netupitant. It is approved as a fixed antiemetic combination of along with PNS.[1-3] In combination FTP is used for prevention of acute and delayed nausea and vomiting associated with initial and repeat courses of cancer chemotherapy.[4, 5] Palonosetron is a serotonin-3 (5-HT3) antagonist used in the prevention and treatment of chemotherapy-induced nausea and vomiting (CINV). Both drug are available as combination drug as injection formulation for intravenous administration and also capsule for oral admistration.[6-10] Most common adverse reactions with usage of this combination include headache, asthenia, dyspepsia, fatigue, constipation and erythema.

There is no method available for the simultaneous quantification of FTP and PNS. Few analytical methods were available for the simultaneous estimation of Netupitant and PNS using HPLC[11-14], HPTLC[15] and LCMS.[16,17] Few methods were available for the estimation of single drug Palonosetron using HPLC[18-24], HPTLC[25], LCMS[26-28] and UV spectrophotometer technique.[29] As both the drugs doesn't have analytical methods for determination and estimation, the present work is aimed to develop a RP-HPLC method for stability indicating analysis of FTP and PNS in standard and pharmaceutical dosage form.

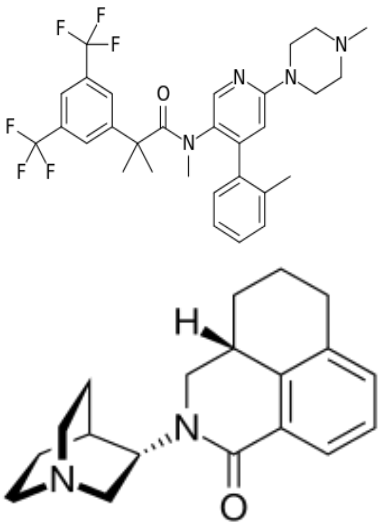


Figure 1: Chemical structure of FTP and Palonosetron.

2. MATERIALS AND METHODS

2.1 Instrumentation

Chromatographic separation was performed on a PEAK chromatographic system equipped with LC-P7000 isocratic pump; Rheodyne injector with 20µl fixed volume loop, variable wavelength programmable UV detector UV7000 and the output signal was monitored and integrated by PEAK Chromatographic Software version 1.06. Teccomp UV-2301 double beam UV-Visible spectrophotometer was used to carry out spectral analysis and the data was recorded by Hitachi software. Sonicator (1.5L) Ultrasonicator was used to sonicating the mobile phase and samples. Standard and sample drugs were weighed by using Denver electronic analytical balance (SI-234) and pH of the mobile phase was adjusted by using Systronics digital pH meter.

2.2 Chemicals and Solvents

The pharmaceutical formulation was procured from local market. Methanol, Acetonitrile and water used were HPLC grade and were purchased from Merck Specialties Private Limited, Mumbai, India. Perchloric acid and reaming buffer solutions used were AR Grade and purchased from Merck Specialties Private Limited, Mumbai, India.

2.3 Preparation of sample solution

10 formulation capsules of PNS and FTP (AKYNZEO®; PNS - 0.25g and FTP - 235mcg) were powder. The capsule powder equivalent to 10mg of FTP was weighed accurately and was dissolved in 5ml diluents and was keep it for solubility for 24H. Then it was filtered and makes up to 10ml with same diluents to get 1000 µg/ml FTP stock solution. From this by proper dilution a concentration of 235 µg/ml of FTP was prepared. As per the label claim of the two drugs a PNS concentration of 0.25 µg/ml was obtained. The resultant solution was used for the simultaneous estimation of PNS and FTP in combined dosage forms.

2.4 Method development and optimization

Initially various mobile phases were tried to obtain the best separation and resolution between PNS and FTP. Different ratios of methanol, water and acetonitrile in different pH ranges and with different pH buffers were studied. The overlay UV absorption wavelength was selected for detector wavelength for simultaneous detection of PNS and FTP and separation was carried on C18 column with different manufactures.

2.5 Method Validation

The method developed for the simultaneous estimation of PNS and FTP was validated by the determination of the following parameters: specificity, linearity, range, recovery, accuracy, precision, limit of detection (LOQ) and limit of quantization (LOQ), and stability studies according to the currently accepted ICH validation guidance.

A different concentration ranges of PNS and FTP were analyzed in the developed method for the determination of linearity range in the developed method. A concentration in the linearity range was analyzed six times in the same day for intraday precision and six times in three different days for inter day precision and six times by change in analyst for ruggedness study. The % RSD of peak area response observed in intraday, interday precision and ruggedness was calculated and the % RSD of less than 2 was considered as acceptance criteria. The robustness of the developed method determined by change in mobile phase ratio (±5 %), mobile phase pH (± 0.1 factor) and detector wavelength (±5nm) in the developed method. Standard concentration of PNS and FTP was analyzed in each changed condition, the change in each condition was calculated by comparing the observed results with linearity results. A % change of less than 2 was found to be the robustness of the method. Signal by noise ratio of the PNS and FTP in the developed method was used for the determination of limit of detection of the method. The drug stability was studied by conducting different stress degradation studies like Acidic (0.1N HCl), Base (0.1N NaOH), Peroxide (3% hydrogen peroxide), Thermal (80^{o}C), UV Light (320 nm) and fluorescent Light. The % degradation and the number of degradation compounds observed were determined in each condition.

3. RESULTS AND DISCUSSION

The goal of the present investigation was to develop a simple, easy accurate, precise, reliable and least time consuming HPLC method for the simultaneous analysis of PNS and FTP in bulk drug and in combined pharmaceutical formulation. The newly developed method has been validated as per guidelines of the International Conference on the Harmonization of Technical requirements for the Registration of pharmaceutical for Human use [ICH 2005].

The optimization of the method was achieved on Methanol: Acetonitrile: 1% sodium perchlorate 75:20:05 (v/v/v) at a pH of 4.9 (using 1% perchloric acid). The mobile phase pumped at a flow rate of 1.0ml/min. The iso-absorption wavelength of 263nm was found to be suitable for the UV detection of PNS and FTP. The overlay iso-absorption wavelength was shown in figure 2. best separation of PNS and FTP was achieved on Synchronies C-18 (250mm x 4.6mm, 5µm) column.

In the optimized chromatographic conditions, well resolved peaks were observed for FTP and PNS at a retention time of 5.3 min and 9.3 min respectively. The number of theoretical plates were found to be 3566, 12779 where as tail factor was found to be 0.81, 1.21 respectively for FTP and PNS respectively. The optimized chromatogram was shown in figure 3.

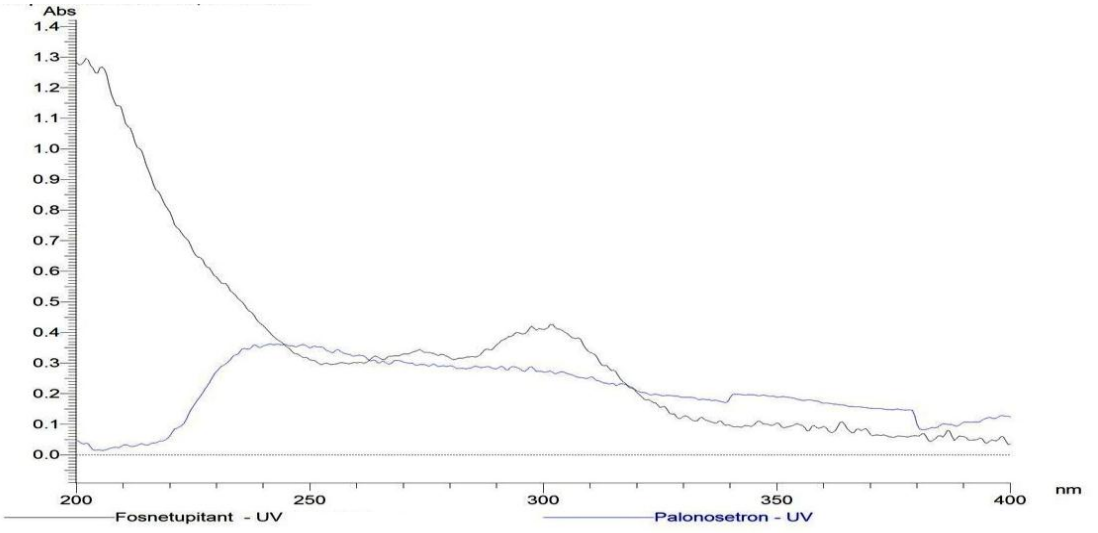
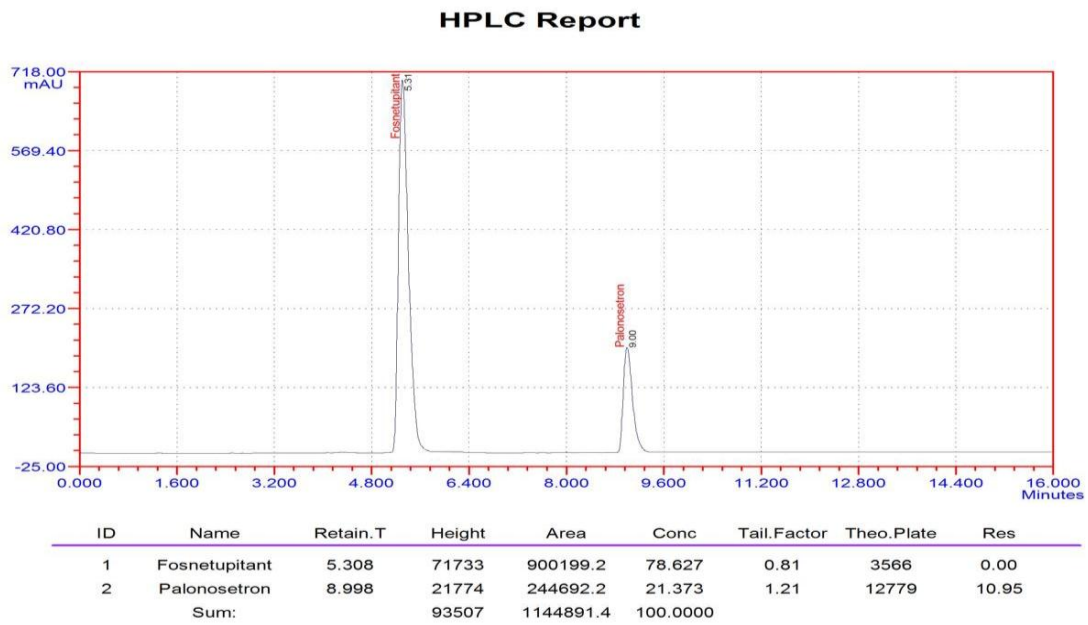


Figure 2: Overlay UV absorption wavelength of PNS and FTP.



ID	Name	Retain.T	Height	Area	Conc	Tail.Factor	Theo.Plate	Res
1	Fosnetupitant	5.308	71733	900199.2	78.627	0.81	3566	0.00
2	Palonosetron	8.998	21774	244692.2	21.373	1.21	12779	10.95
	Sum:		93507	1144891.4	100.0000			

Figure 3: Optimized chromatogram of PNS and FTP in the developed method.

The linearity of an analytical method is its ability to elicit test results that are directly proportional to the concentration of an analyte in the sample within a given range. The linearity of the method was observed in the concentration range of 94-329 µg/ml for FTP and 0.1-0.35 µg/ml for PNS. The regression equation was found to be y = 3372.x + 12102 [R^2 = 0.999] for FTP and y = 1E+06x – 8004 [R^2 = 0.999] for PNS in the developed method. The linearity curve of FTP and PNS was shown in Figures 4 and 5 respectively. Table 1 shows the linearity results of PNS and FTP in the developed method.

Table 1: Linearity results of PNS and FTP.

S No	FTP		PNS	
	Concentration in µg/ml	Peak area	Concentration in µg/ml	Peak area
1	94	445823	0.1	92558
2	141	596390	0.15	139746
3	188	751488	0.2	196479
4	235	900199	0.25	244692
5	282	1071203	0.3	292244
6	329	1240737	0.35	343223

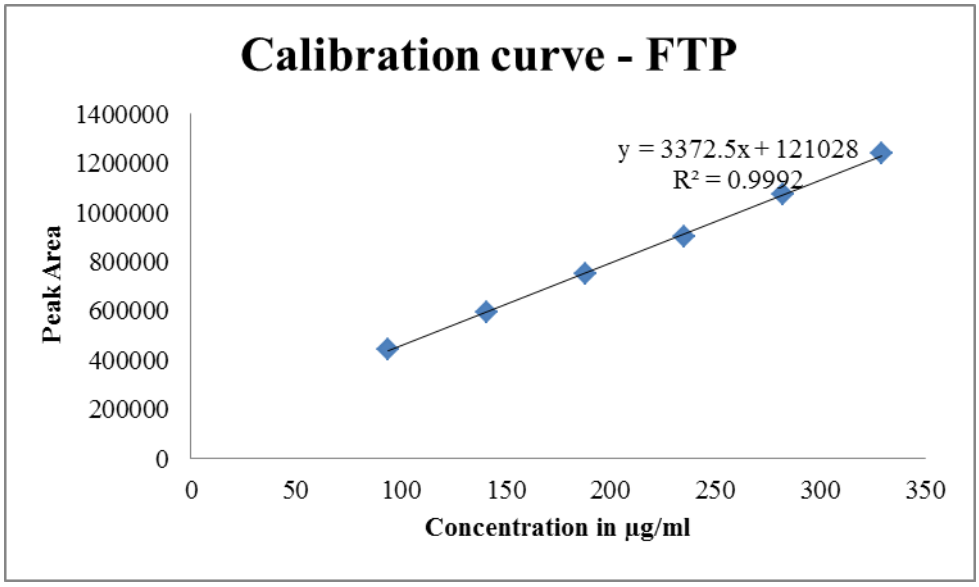


Figure 4: Linearity Graph for FTP.

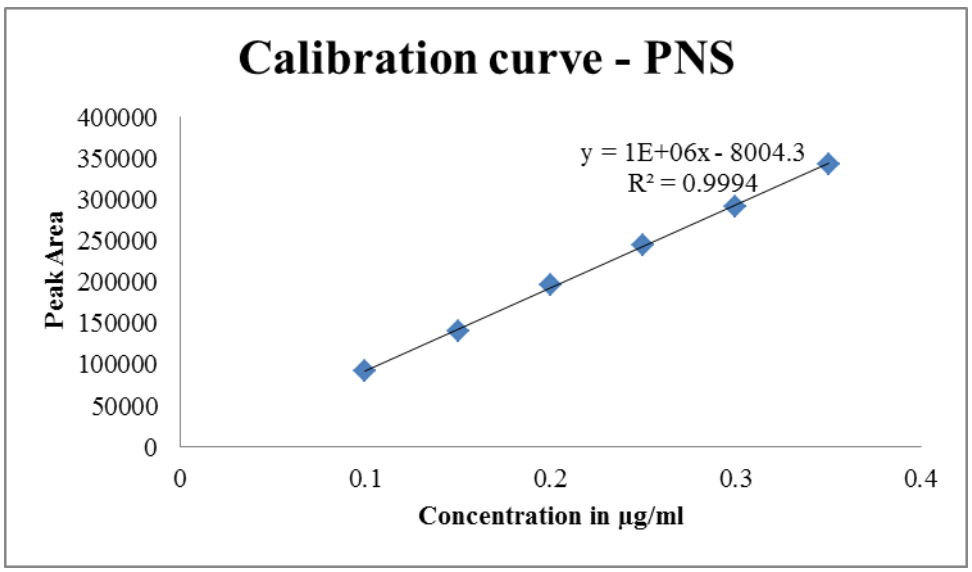


Figure 5: Linearity Graph for PNS.

The % RSD of precision study at a concentration of 235 µg/ml of FTP, 0.25 µg/ml of PNS was found to be 0.82 and 0.90 for FTP, 0.99 and 1.45 for PNS respectively in intra-day and inter-day precision respectively. The results obtained from intermediate precision also indicated a good method precision. All the data were within the acceptance criteria and results were given in table 2.

Table 2: Precision results of PNS and FTP.

S No	Intraday Precision		Interday Precision	
	FTP at 235µg/ml	PNS at 0.25µg/ml	FTP at 235µg/ml	PNS at 0.25µg/ml
1	883902	235973	860219	238253
2	887123	232741	879879	237980
3	871838	234995	872578	230977
4	888342	230013	881343	238474
5	873789	235090	876639	237049
6	886549	235886	879032	231788
RSD	**0.82**	**0.99**	**0.90**	**1.45**

The % RSD in ruggedness study was also found to be 0.71 and 1.63 FTP and PNS respectively. The % RSD was found to be within the acceptance limit of less than 2. Robustness is a measure of the performance of a method when small deliberate changes are made to the conditions of the method and results are summarized in Table 3. The % change in each changed condition was found to be less than 2 and hence the method was found to be robust.

Table 3: Robustness results of PNS and FTP.

Condition	FTP at 235µg/ml		PNS at 0.25µg/ml	
	Area	% Change	Area	% Change
Standard	900199	---	244692	---
MP 1	909949	1.08	243750	0.38
MP 2	909330	1.01	249555	1.99
pH 1	896596	0.40	246466	0.72
pH 2	898910	0.14	244028	0.27
WL 1	908156	0.88	242084	1.06
WL 2	903878	0.41	244934	0.10

MP1: Methanol: Acetonitrile: 1% sodium perchlorate 70:25:05 (v/v/v), MP 2: Methanol: Acetonitrile: 1% sodium perchlorate 80:15:05 (v/v/v); Ph 1: 4.8, Ph 2: 5.0; WL 1: 268nm, WL 2: 258nm

Recovery

Spiked recovery at spiked levels of 50%, 100% and 150% was studied for the determination of accuracy of the method developed for PNS and FTP. The % recovery was calculated and was found to be within the acceptance limit of 98-102% for both PNS and FTP. The % RSD in each spiked levels was found to be less than 2 for each spiked level for PNS and FTP confirms that the method was found to be accurate. Recovery results were given in table 4 and 5 respectively for PNS and FTP.

Table 4: Recovery results of FTP.

S. No	Level	Target	Spiked	Total	Amount found	% Recovery	RSD of Recovery
1	50%	94	47	141	140.567	99.69	0.28
2		94	47	141	139.937	99.25	
3		94	47	141	139.848	99.18	
4	100%	94	94	188	185.721	98.79	0.23
5		94	94	188	186.261	99.07	
6		94	94	188	185.416	98.62	
7	150%	95	140	235	233.120	99.20	0.70
8		95	140	235	233.584	99.40	
9		95	140	235	236.182	100.50	

Table 5: Recovery results of PNS.

S. No	Level	Target	Spiked	Total	Amount found	% Recovery	RSD of Recovery
1	50%	0.1	0.05	0.15	0.150	100.374	0.88
2		0.1	0.05	0.15	0.149	99.2551	
3		0.1	0.05	0.15	0.148	98.6518	
4	100%	0.1	0.1	0.2	0.196	98.1138	0.75
5		0.1	0.1	0.2	0.198	98.8569	
6		0.1	0.1	0.2	0.199	99.6061	
7	150%	0.1	0.15	0.25	0.246	98.3289	0.82
8		0.1	0.15	0.25	0.250	99.8725	
9		0.1	0.15	0.25	0.249	99.5615	

Very sensitive detection limits of 0.6µg/ml and 0.01µg/ml for FTP and PNS respectively. The quantification limit was found to be 2.0µg/ml and 0.04µg/ml for FTP and PNS respectively. This confirms that the method was found to be very sensitive. The solution stability of FTP and PNS was studied up to 24 H in different time intervals and results confirm that both the drugs were found to be stable up to 24 H in the developed method.

Forced degradation studies were studied for FTP and PNS in different stress conditions. The % drug degradation was calculated and the % degradation was found to be less than 10% for FTP and PNS in all the stress conditions. In all the degradation studies, well resolved degradation peaks was observed and there is no disturbances in the separation of FTP and PNS and clear base line was observed. The % degradation was found to be very high in acidic conditions. In peroxide and

thermal conditions, the % degradation of FTP and PNS was found to be very less. The results of forced degradation study was oven in table 6 and formed degradation chromatograms was given in figure 6-11.

Table 6: Forced degradation rersults of PNS and FTP.

S. No	Condition	Number of additional peaks observed	FTP		PNS	
			Area	% Degradation	Area	% Degradation
1	Acidic	3	822950	8.58	222476	9.08
2	Base	3	827153	8.11	222790	8.95
3	Peroxide	2	852649	5.28	228040	6.80
4	Thermal	3	851008	5.46	227230	7.14
5	UV Light	1	859852	4.48	223809	8.53
6	Light	1	875553	2.74	227186	7.15

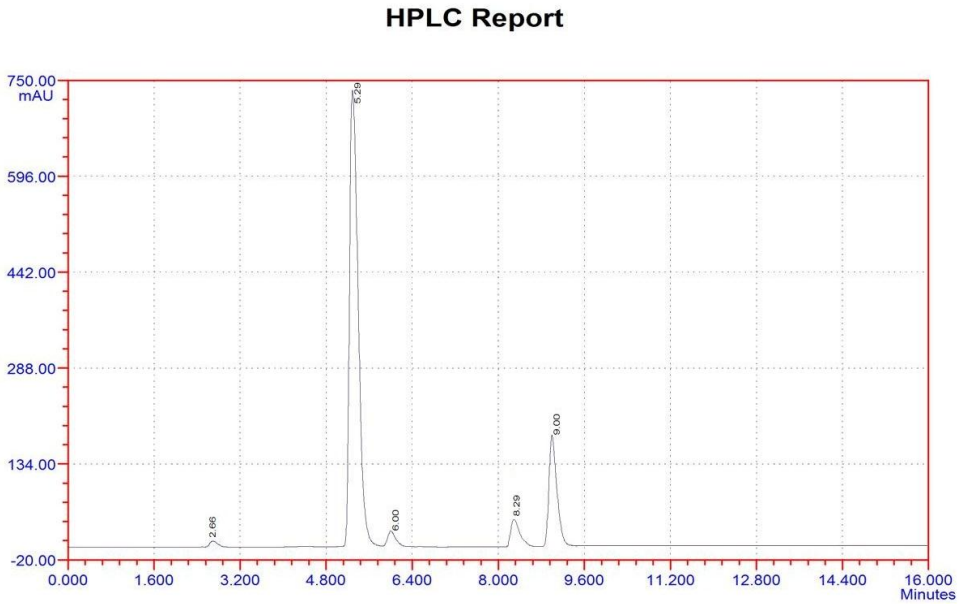


Figure 6: Acidic degradation chromatogram of PNS and FTP.

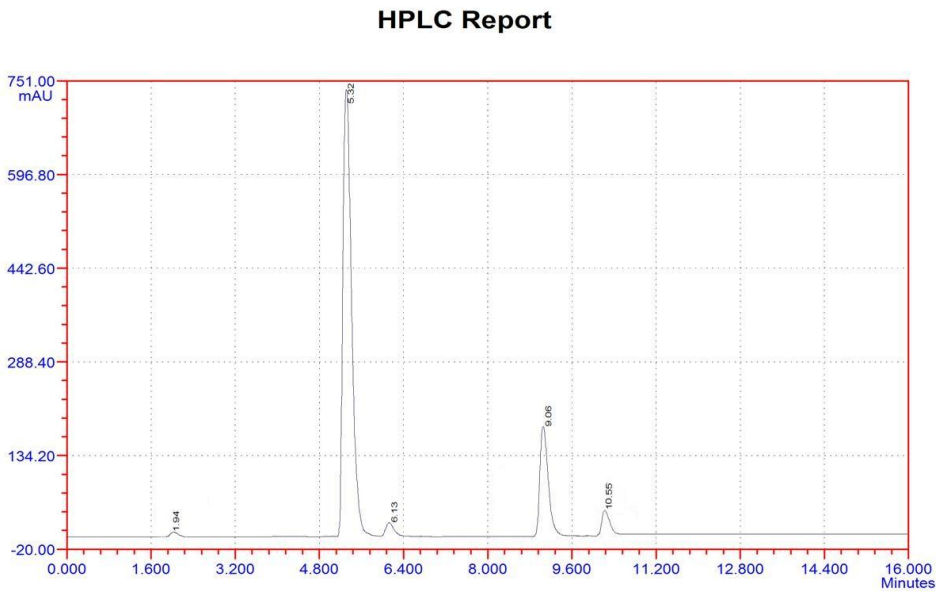


Figure 7: Base degradation chromatogram of PNS and FTP.

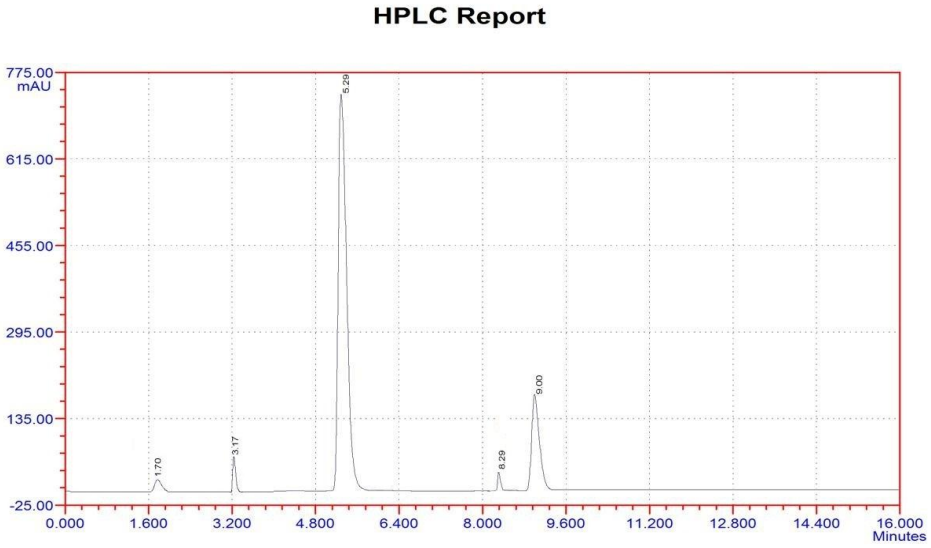


Figure 8: Peroxide degradation chromatogram of PNS and FTP.

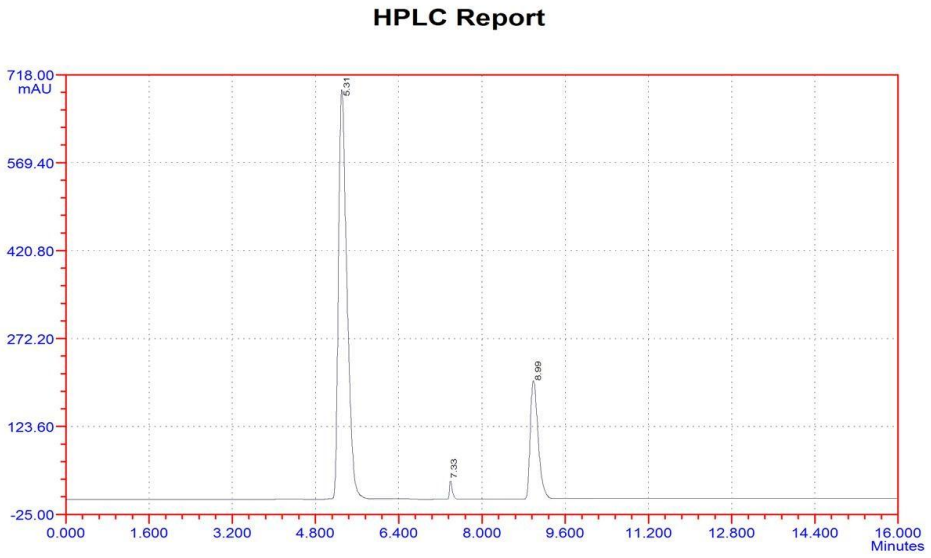


Figure 9: Thermal degradation chromatogram of PNS and FTP.

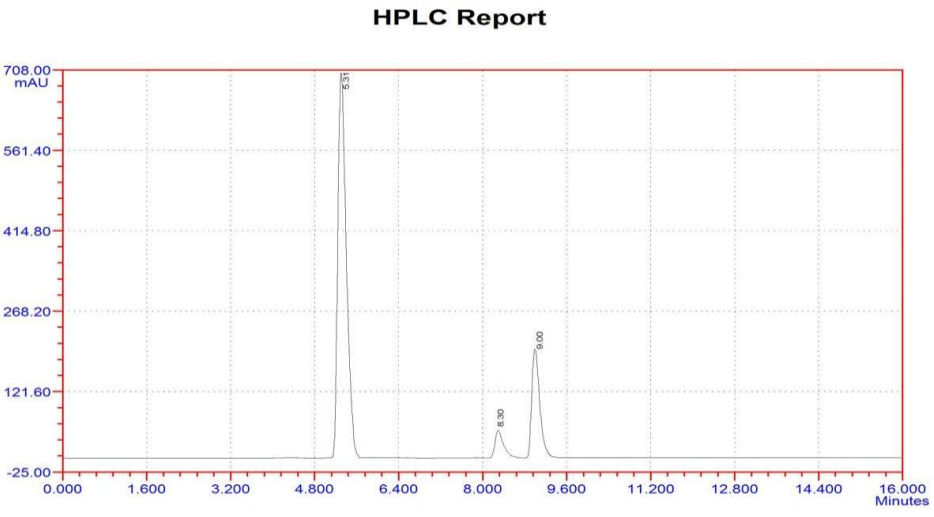


Figure 10: UV Light degradation chromatogram of PNS and FTP.

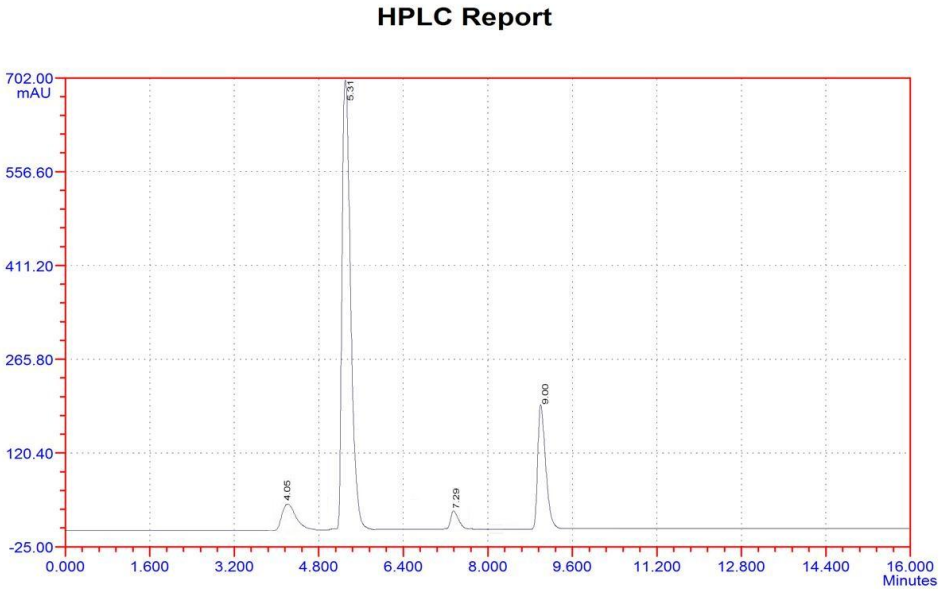


Figure 11: Light degradation chromatogram of PNS and FTP

The formulation solution prepared from AKYNZEO® was analyzed in the developed method. The % assay was calculated and was found to be 99.44% for FTP and 98.40% for PNS. The formulation chromatogram shows clear base line and no other excepients in the formulation was detected. This confirms that the method was specific and can be successfully applied for the routine analysis of PNS and FTP in pharmaceutical formulations. Formulation results was given in table.

Table 7: Formulation rersults of PNS and FTP.

S. No	Drug	Brand Name	Label Claim	Amount Prepared	Amount Found	% Assay
1	Fosnetupitant	AKYNZEO®	235mg	235µg/ml	233.693 µg/ml	99.44
2	Palonosetron		0.25mg	0.25µg/ml	0.246 µg/ml	98.40

4. CONCLUSION

It is a notable that the validation procedure is a basic piece of the logical technique improvement. Along these lines, the developed method was validated as per ICH rules Q2 (R1). In view of the outcomes, it very well may be reasoned that there is no other co-eluting peak with the main peaks and that the method is specific for estimation of PNS and FTP. The proposed method has a linear response in the stated range and is found to be precise, reproducible, accurate and robust. The stability study indicated that the standard stock solution was stable up to 24 hours and less degradation was observed during stress study. Hence the developed stability indicating HPLC method was found to be suitable for the routine analysis of Palonosetron and Fosnetupitant in their combined dosage form.

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