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Article

Stability indicating Rp-UPLC method development and validation for the simultaneous estimation of fosnetupitant and palonosetron in bulk and injection dosage form

Poojari Venkatesh[1]*, Umasankar Kulandaivelu[1], GSN Koteswara Rao[1], Guntupalli Chakravarthi[1], Rajasekhar Reddy Alavala[1], Bandlamuri Rajesh[1]

[1]*K L College of Pharmacy, K L Deemed to be University, Vaddeswaram, Guntur District, Andhra Pradesh, India*

A stability indicating UPLC method has been developed and validated for the simultaneous determination of fosnetupitant and palonosetron in bulk and in injection dosage form. This combination is used for the prevention of acute and delayed nausea and vomiting associated with initial and repeated courses of highly emetogenic chemotherapy for cancer. The chromatographic analysis was performed on an HSS, RP C_{18} column (2.1 x 100 mm, 1.8 µm) with an isocratic mobile phase composed of 0.25 M potassium dihydrogen orthophosphate buffer (pH 6.5), pH adjusted with dilute sodium hydroxide:acetonitrile (55:45 v/v), at a flow rate of 0.5 mL/min, and the eluents were monitored at an isosbestic point of 286 nm. The developed method was validated according to the ICH guidelines pertaining to specificity, precision, accuracy, linearity and robustness, and the stability indicating nature of the method was established by forced degradation studies. The retention times of fosnetupitant and palonosetron were observed at 1.390 and 2.404 min, respectively. The developed method proved to be accurate and precise. Linearity was established between 4.70 and 14.10 µg/mL for fosnetupitant and between 0.05 and 0.15 µg/mL for palonosetron. The LOD and LOQ were 0.115 and 0.385 µg/mL, respectively, for fosnetupitant, and 0.005 and 0.016 µg/mL, respectively, for palonosetron. Therefore, the proposed UPLC method was reliable, reproducible, precise and sensitive for the quantification of fosnetupitant and palonosetron.

Keywords: Fosnetupitant. Palonosetron**.** UPLC method. Stability indicating and validation.

INTRODUCTION

Cancer chemotherapy induced nausea and vomiting (CINV) is a common adverse effect of most cancer drug regimens. If this condition is not controlled, it can affect quality of life and contribute to the overall survival of cancer patients (Kuchuk *et al*., 2013; Sun *et al*., 2005); greater importance should therefore be given to antiemetic prophylaxis in the treatment of cancer. This has led to the development of new antiemetics that have substantially changed the current scenario for the prevention of CINV (Basch *et al*., 2017; Basch *et al*., 2016).

With a better understanding of the neuropharmacology of CINV and the development of new agents targeting different receptors involved in the CINV process, multi-agent antiemetic prophylactic combinations are now recommended for the highly emetogenic chemotherapy environment. Unfortunately, due to their apparent complexity, adherence to the antiemetic combinations recommended by the antiemetic guidelines has been very minimal (Aapro *et al*., 2012; Gilmore *et al*., 2014). Several antiemetic drug classes are available on the market in different formulations (*i.e.*, tablets, IV and IM), offering a wide range of options for doctors and patients in various contexts. Alternative drug

***Correspondence:** P. Venkatesh. Research scholar. K L College of Pharmacy. K L Deemed to be University. Vaddeswaram, Guntur District. Andhra Pradesh, INDIA. Phone: +91-9985549421. Email: venkydmm@gmail.com. ORCID: https://orcid.org/0000-0002-4376-6642. Rajasekhar Reddy Alavala – ORCID: https://orcid.org/0000-0002-2610-8111

formulations can help to meet the unaddressed needs of patients and prescribers by promoting greater patient adherence to prescribed drug treatments.

Akynzeo® (Helsinn Therapeutics Inc., USA) for injection is an antiemetic combination containing 235 mg of fosnetupitant (FOS) and 0.25 mg of palonosetron (PAL). It is a freeze-dried powder in a vial and is reconstituted in 50 mL of 5% dextrose injection USP or 0.9% sodium chloride injection USP. Before the start of chemotherapy, a patient is given a vial of reconstituted Akynzeo® as a 30-minute intravenous infusion (Akynzeo® prescribing information, 2020). Figure 1 shows the chemical structures of FOS and PAL.

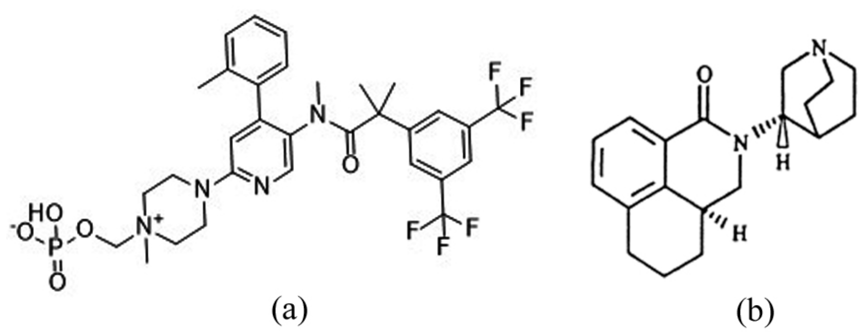

FIGURE 1 - Chemical structures of a) fosnetupitant; b) palonosetron.

Fosnetupitant (FOS), a prodrug of netupitant, is 4-(5-{2-[3,5-bis (trifluoromethyl) phenyl]-N, 2-dimethylpropanamido}-4-(2- methylphenyl) pyridine-2-il)-1-[(hydrogen phosphonooxy) methyl]-1-methylpiperazin-1-ium, with a molecular weight of 688.608, and is an antagonist of the Neutokinin–1 (NK1). Palonosetron (PAL), a 5HT3 antagonist, is (3aS)-2-[(3S)-1-azabicyclo [2.2.2] oct-3-il]-2,3,3a,4,5,6-hexahydro-1H-benz [de] isoquinolin-1-one, with a molecular weight of 332.87. The injection dosage form of FOS and PAL (Akynzeo®) offers some benefits compared to the other antiemetic drugs currently available, including a convenient dosage form, a double target mechanism and a favourable profile of side effects (Abramovitz, Gaertner, 2016) FOS is metabolized to netupitant through CYP3A4, while POS is metabolized through CYP2D6, with small contributions from the CYP1A2 and CYP3A4 systems (Calcagnile *et al.,* 2013)

A literature review reveals that very few analytical methods have been reported for the determination of netupitant and PAL individually and with other combinations. These include HPLC (Inturi, Inturi, Venkatesh, 2011; Zheng Guo-gang, 2010; Murthy *et al*., 2011a; Janaki, Appala, 2012), UV spectrophotometry (Della Grace Thomas Parambi, Ganesan, 2011), micellar electrokinetic chromatography (Tian *et al.,* 2006), chiral HPLC (Radhakrishnanand, Subba Rao, Himabindu, 2009; Murthy *et al*., 2011b; Yu, Song, Hang, 2008), LCMS (Ding *et al.,* 2007; Zhang, Feng, 2008), capillary zone electrophoresis (Wang *et al.,* 2009) and pharmacokinetic studies (Spinelli *et al.,* 2013). However, the existing LC methods were less convenient and time consuming, which is unsuitable for routine individual and simultaneous estimation. The UPLC system reduces the time and the significant costs of analysing samples, with better results. UPLC allows an analyst to work on superior skills with a much wider range of linear speeds, solvent flow rates and system back pressure than traditional HPLC. Considering the growing demand for the aforementioned drugs in the global market, it is necessary to develop a new economic, accurate and rapid UPLC analytical technique for the simultaneous estimation of both drugs in the pharmaceutical formulation and to conduct forced degradation studies in five different conditions, which could be applied to evaluate the quality, efficacy and storage conditions of each molecule.

MATERIAL AND METHODS

Chemicals and reagents

Both drug standards were gifted from Lara Drug Pvt. Limited, Hyderabad, India. Methanol, water and acetonitrile (LC grade), analytical grade sodium hydroxide (NaOH), hydrogen peroxide (H_2O_2) and hydrochloric acid (HCl), and a 0.22 mm membrane filter were purchased from Sigma-Aldrich. Akynzeo® (single-dose vial) containing 235 mg FOS and 0.25 mg PAL was purchased from the native pharmaceutical market. All chemicals were analytical or LC grade.

UPLC instrumental condition

An Acquity UPLC system (Waters, Milford, MA, USA) equipped with a model 2996 PDA detector and Empower software was used to develop the method. The UPLC separation of the two drugs was obtained with an HSS RP-C_{18} analytical column (2.1 mm x 100 mm, 1.8 µm) using 0.25 M buffer of potassium dihydrogen orthophosphate (pH 6.5), pH adjusted with diluted sodium hydroxide:acetonitrile (55:45, v/v) in isocratic mode at a flow rate of 0.5 mL/min and the column at room temperature. The PDA detector was used to monitor the two drugs at 286 nm. The solvents were filtered on a 0.22 mm membrane filter and degassed in an ultrasonic bath before use. The analytical method was optimized using a pure analytical standard (Table I).

TABLE I - Optimized chromatographic conditions for the estimation of FOS and PAL

No.	Parameter	Description/Value
	Stationary phase	HSS, C_{18}, 2.1 x 100 mm, 1.8 µm
	Mobile phase	0.25 M Potassium dihydrogen orthophsophate buffer (pH 6.5) pH adjusted with dilute sodium hydroxide:acetonitrile (55:45, v/v)
	Flow rate	0.5 mL/min
	Detection wavelength (Isosbestic Point)	286 nm
	Detector	Photo diode array
	Injection	Autosampler - Waters, model 717 plus
	Injection volume	3 µL
	Column temperature	30°C
	Run time	3 mins
	Diluent	Mobile phase

Preparation of working standard solution

Standard solutions of 9.4 µg/mL of FOS and 0.1 µg/mL of PAL were prepared using diluent.

Analysis of formulations

A dose equivalent to 100 mg of FOS and 10 mg of PAL was calculated from twenty vials and the contents of the vials were emptied into a clean beaker and mixed well. Measured samples were transferred to clean dry 10 mL standard flasks. Then, 7 mL of diluent was added and sonicated to dissolve completely. Finally, the volume was made up to the mark with the same solvent (primary formulation stock solution). In addition, 9.4 mL of FOS and 0.1 mL of PAL of the respective stock of the respective primary formulation stock solutions were transferred to a standard 10 mL flask and diluted to the mark with the same solvent. Then, 3 µL of both samples were injected into the UPLC system, the peak areas for FOS and PAL measured and the percentage assay of the formulations calculated.

Validation of the chromatographic method

The developed method was validated as per the International Conference on Harmonization (ICH) guideline (ICH Guideline, Q2 (R1), 2005).

System suitability

System suitability parameters were measured to verify the system performance. The precision of the system was determined in six repeated injections of standard preparations. All important characteristics were measured, including the area of the peak, the resolution of the peaks and the theoretical plate number.

Accuracy (recovery)

Accuracy is represented (ICH Guideline, Q2 (R1), 2005) and determined by recovery experiments. In this process, it was tested at three different levels (50%, 100% and 150%) and the chromatogram was analysed.

Specificity

To assess the specificity, a working placebo solution (blank) in the absence of FOS and PAL and a standard solution with a concentration of 9.4 µg/mL FOS and 0.1 µg/mL PAL were introduced into the UPLC system, as well as the formulations, and the chromatograms were analysed.

Precision

The precision (intra-day and inter-day) of the analytical technique was proven using optimized concentrations of FOS and PAL by six replicate injections. The average and % RSD of the peak area and the assay were determined from chromatograms.

Linearity

Linearity was confirmed by preparing and analysing pure analytical standard preparations at five totally different concentrations. The developed method displays ideal linearity over a range of 4.7, 7.05, 9.4, 11.75 and 14.1 µg/mL for FOS and 0.05, 0.075, 0.1, 0.125 and 0.15 µg/mL for PAL.

Limit of detection (LOD) and Limit of quantitation (LOQ)

The LOD and LOQ of FOS and PAL were determined using a signal to noise (S/N) approach, as defined in the ICH guideline (ICH Guideline, Q2 (R1), 2005). An increasingly dilute solution of each drug and impurity was injected into the chromatograph, and the S/N ratio was calculated at each concentration.

Robustness

The robustness, as a measure of method capacity to remain unaffected by small, but deliberate changes in chromatographic conditions, was studied by testing the influence of small changes in flow rate (±5 mL/min), in column temperature (±5°C) and change in the detection wavelength (± 2 nm).

Forced degradation studies

The ICH guideline entitled stability testing of new drug substances and products (ICH Guideline, Q1A (R2), 2003; Reynolds *et al*., 2002) requires that stress testing is performed to describe the inherent stability characteristics of the active substance. The goal of this project was to carry out the stress degradation studies on FOS and PAL using the proposed method.

Acidic and alkaline hydrolysis

From the primary stock solution, 3.0 mL of FOS and PAL were transferred to 2 pairs of 10 mL standard flasks. From the above solution, 1 mL of 0.1 N HCl was added to one pair of 10 mL standard flasks for the acidic condition. For alkaline degradation, 1 mL of 0.1 N NaOH was added to the other set of 10 mL standard flasks. The standard flasks were kept in a water bath at 65 °C for 8 h and 60 °C for 10 h for the acid and alkaline samples, respectively. Both set of solutions were neutralized and made up to 10 mL with diluent, to obtain 9.4 µg/mL of FOS and 0.1 µg/mL of PAL, respectively. The resulting solution was cooled to room temperature, the solution was filtered with a 0.22 mm syringe, and the vials were then introduced to the UPLC system.

Thermally induced degradation

Initially, 3.0 mL of FOS and PAL were transferred to a 10 mL standard flask from the above stock solution, and refluxed at 85 °C for 30 h. Then, the sample was diluted with diluents and made up to 10 mL to obtain 9.4 µg/mL of FOS and 0.1 µg/mL of PAL, respectively. The solution was then cooled to room temperature, and the vials were introduced to the UPLC system, after filtration with a 0.22 mm syringe filter.

Oxidative degradation

Initially, 3.0 mL of FOS and PAL was transferred to a 10 mL standard flask from the above stock solution. Then, 1 mL of 3% (w/v) hydrogen peroxide was added, and the volume was made up to the mark with diluents to obtain 9.4 µg/mL of FOS and 0.1 µg/mL of PAL, respectively. The standard flask was then set aside at room temperature for 5 h, and the resulting solution was introduced to the UPLC system, after filtration with a 0.22 mm syringe filter.

Photodegradation

From above stock solution, 3.0 mL of FOS and PAL were pipetted out to a 10 mL standard flask. The samples were then transferred to a Petri dish and set aside in a photostability chamber 200 Wh/m^2 in UV light and 1.2 million lxh in UV light for 30 h. Finally, the standard flask was made up to 10 mL with diluents to obtain 9.4 µg/mL of FOS and 0.1 µg/mL of PAL, respectively. The final solution was cooled to room temperature, filtered with a 0.22 mm syringe and then introduced to the UPLC system.

RESULTS AND DISCUSSION

Optimization of chromatographic conditions

Numerous trials have been performed based on the physico-chemical properties of the molecules. During the course of the trials, four reliable variables were taken into consideration: the stationary phase, the composition of the mobile phase, the flow rate and the column temperature. The trial was started by keeping one variable as a constant and modifying another variable. The ideal experimental design helps us to optimize chromatographic and robustness parameters. The UPLC resolution of the two drugs was achieved with an HSS analytical column, RP-C_{18}, 2.1 x 100 mm, 1.8 µm and the use of 0.25 M buffer of potassium dihydrogen orthophosphate in mobile phase (pH 6.5), pH adjusted with diluted sodium hydroxide:acetonitrile (55:45, v/v), in isocratic mode at a flow rate of 0.5 mL/min and with the column at room temperature. The detection of the aforementioned drugs were monitored at 286 nm using a PDA detector.

Analysis of formulations

The marketed formulation was analysed, and the assay percentage was calculated. Results were obtained within ICH limits and are summarized in Table II.

Table II - Analysis of formulation

Analytes	Mean Peak area*	% Assay*	%RSD*
FOS	11146828	100.85	0.34
PAL	2062077.83	100.42	1.54

Mean of six replicates. FOS, Fosnetupitant; PAL, Palonosetron; % RSD, percentage relative standard deviation.

Validation of UPLC method

System suitability study

System suitability was attained by checking various parameters and was found to be within the ICH limit. The results are presented in Table III.

TABLE III - System suitability parameters

No.	Parameter*	FOS	PAL
	Theoretical Plate Count	3985	6424
	Average Peak Area	11146828	2062077
	Peak Height	2184745	243682
	RT	1.39	2.40
	Tailing	1.58	1.35
	Resolution	-	11.50
	S/N	3128	482

**Average of 6 replicates.*

Accuracy (recovery)

Accuracy was ensured at three different levels: 50%, 100% and 150%. The results are shown in Table IV. Mean % recoveries at 50%, 100% and 150% were found to be 100.73%, 99.33% and 99.35%, respectively, for FOS and 100.88%, 100.77% and 100.46%, respectively, for PAL.

TABLE IV - Recovery study

Analyte	Accuracy level	Peak area*	Amount added (mg)	Amount found (mg)	% Recovery	Mean % Recovery
FOS	50%	5661523	4740	4774	100.73	99.80
	100%	11165891	9479	9416	99.33	
	150%	16751236	14219	14126	99.35	
PAL	50%	1054830	5.07	5.12	100.88	100.70
	100%	2107492	10.14	10.22	100.77	
	150%	3151438	15.21	15.28	100.46	

*Mean of three determinations at each level; FOS, Fosnetupitant; PAL, Palonosetron;

Precision

The precision of the analytical method was established for both intra- and inter-day using concentrations of 9.4 µg/mL of FOS and 0.1 µg/mL of PAL, with six replicate injections. The results are shown in Table V.

TABLE V - Precision study

Precision	Mean Peak area		% RSD		Mean Assay		% RSD	
	FOS	PAL	FOS	PAL	FOS	PAL	FOS	PAL
Intra-day	11238531.17	2058494.17	0.67	0.28	100.82	99.83	0.67	0.28
Inter-day	11241032.50	2091303.00	0.43	0.29	100.85	101.42	0.43	0.29

Mean of six determinations. % RSD, percentage relative standard deviation. FOS, Fosnetupitant; PAL, Palonosetron;

Specificity

The specificity of the technique established that the chromatogram of the working placebo solution did not show any interference at the retention time of FOS and PAL. Thus, it can be concluded that the main excipients present in the formulations do not interfere with the analytical method for the determination of FOS and PAL. The resulting chromatograms of blank, standard and formulation are shown in Figure 2.

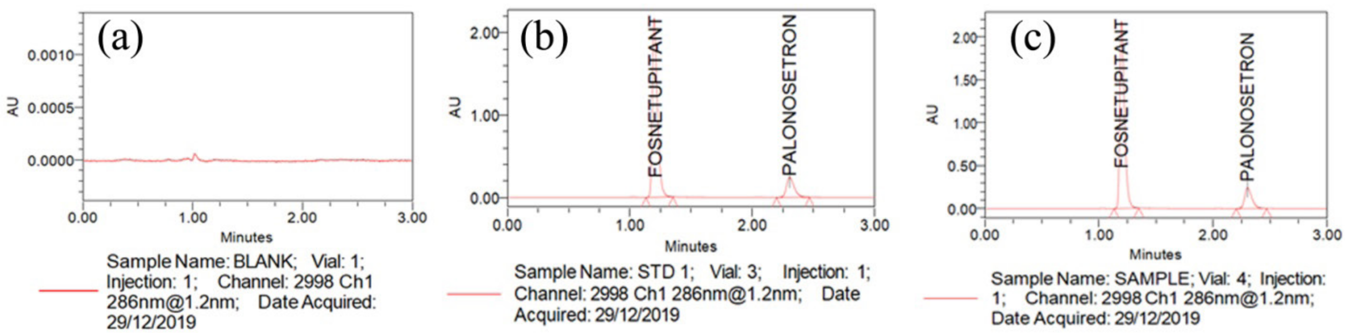

FIGURE 2 - Chromatograms of blank, standard and sample.

Linearity

The projected technique displays ideal linearity over a range of 4.7, 7.05, 9.4, 11.75 and 14.1 µg/mL for FOS and 0.05, 0.075, 0.1, 0.125 and 0.15 µg/mL for PAL, respectively, with excellent coefficient correlation of more than 0.999 for both drugs. A residual plot of both drugs displays that residuals are randomly placed over, below and above the x-axis, signifying that the developed method is a linear model. The results are shown in Table VI.

TABLE VI - Linearity data

Linearity Level	FOS		PAL	
	Concentration	Peak Area	Concentration	Peak Area
50	4.7	6116851	0.05	1035188
75	7.05	9136160	0.075	1601964
100	9.4	12171380	0.1	2139625
125	11.75	15148531	0.125	2659436
150	14.1	18104042	0.15	3207908
Slope	1E+06		2E+07	
Intercept	14069		32341	
R^2	0.9999		0.999	

Limit of detection (LOD) and limit of quantification (LOQ)

The LOD of FOS and PAL was found to be 0.115 µg/mL and 0.005 µg/mL, respectively, and the LOQ was found to be 0.385 µg/mL and 0.016 µg/mL, respectively, indicating that the method was extremely rapid and sensitive. Figure 3 shows the chromatograms of LOD and LOQ.

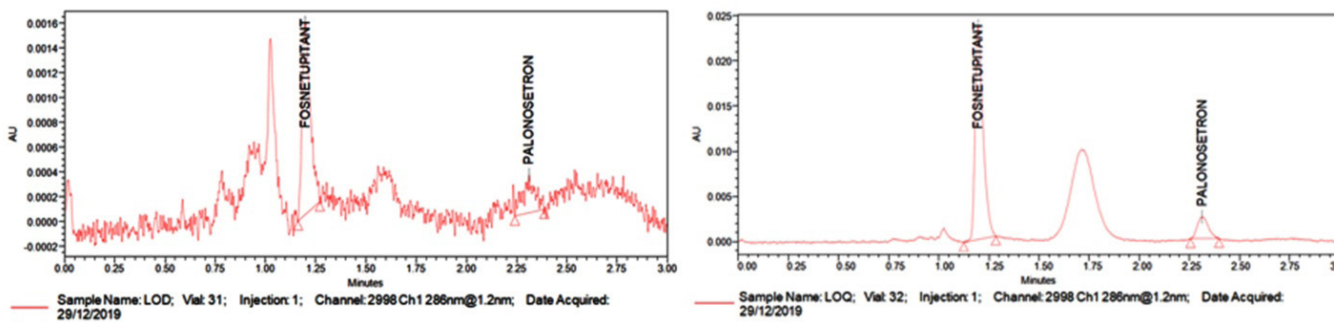

FIGURE 3 - LOD and LOQ Chromatograms.

Robustness study

The robustness study revealed that there was little deviation in the robust chromatograms in comparison with the optimized chromatogram. The results are shown in Table VII.

TABLE VII - Robustness data

Parameter	Condition	FOSNETUPITANT			PALONOSETRON		
		RT	Peak Area	% Assay	RT	Peak Area	% Assay
Flow	0.3 mL/min	2.18	11237314	100.81	3.93	2067984	100.29
	0.5 mL/min	1.39	11146828	100.00	2.40	2062078	100.00
	0.7 mL/min	0.51	11157028	100.09	0.95	2034138	98.65
Temp	25 °C	1.39	11156345	100.09	2.43	2100377	101.86
	30 °C	1.39	11146828	100.00	2.40	2062078	100.00
	35 °C	1.40	11181162	100.31	2.44	2049427	99.39
Wave length	284 nm	1.39	11115692	99.72	2.40	2033621	98.62
	286 nm	1.39	11146828	100.00	2.40	2062078	100.00
	288 nm	1.39	11145007	99.98	2.40	2109865	102.32

Forced degradation study

The degradation studies revealed the specificity of the developed method in the presence of degradation products that were present in the bulk and pharmaceutical dosage form. The studies were performed using the combination of the two drugs, and the purity of the drug peaks was established by purity angles. The formulations were exposed to five different stress conditions.

In acidic and basic conditions, degradation may be due to catalysis of ionisable functional group presents in the drug molecule. Four degradants were detected in the basic condition and three in the acidic condition, but no additional degrading peaks were reported at the retention time of FOS and PAL, respectively. Both drugs were found to degrade more in acidic conditions compared to an alkaline condition.

Two degradants were detected in the oxidative degradation study, and no degradant peaks were reported in the retention time of FOS and PAL, respectively. FOS undergoes more degradation than PAL, with degradation up to 10.06% and 9.30%, respectively. The reason for the high degradation in peroxide may be due to the electron transfer mechanism to form reactive cations and anions. In photolytic stress conditions, degradation may be due to photooxidation by free radical mechanisms whereas, in a thermal condition, it can be explained on the basis of the Arrhenius equation. The results are summarised in Table VIII.

TABLE VIII - Forced degradation study

No.	Condition	FOSNETUPITANT			PALONOSETRON		
		Peak Area	% Assay	% Degradation	Peak Area	% Assay	% Degradation
	Acid	10084887	90.47	9.53	1856340	90.02	9.98
	Base	10137957	90.95	9.05	1888103	91.56	8.44
	Peroxide	10025314	89.94	10.06	1870370	90.70	9.30
	Thermal	10159815	91.15	8.85	1893375	91.82	8.18
	UV	10456783	93.81	6.19	1916893	92.96	7.04

Even if unidentified peaks were observed in the five different stress conditions mentioned above, no degradants were found close to the retention time of FOS and PAL, respectively (Figure 4). Therefore, FOS and PAL are extremely stable in the projected technique, even in stress conditions, up to the specified period of time.

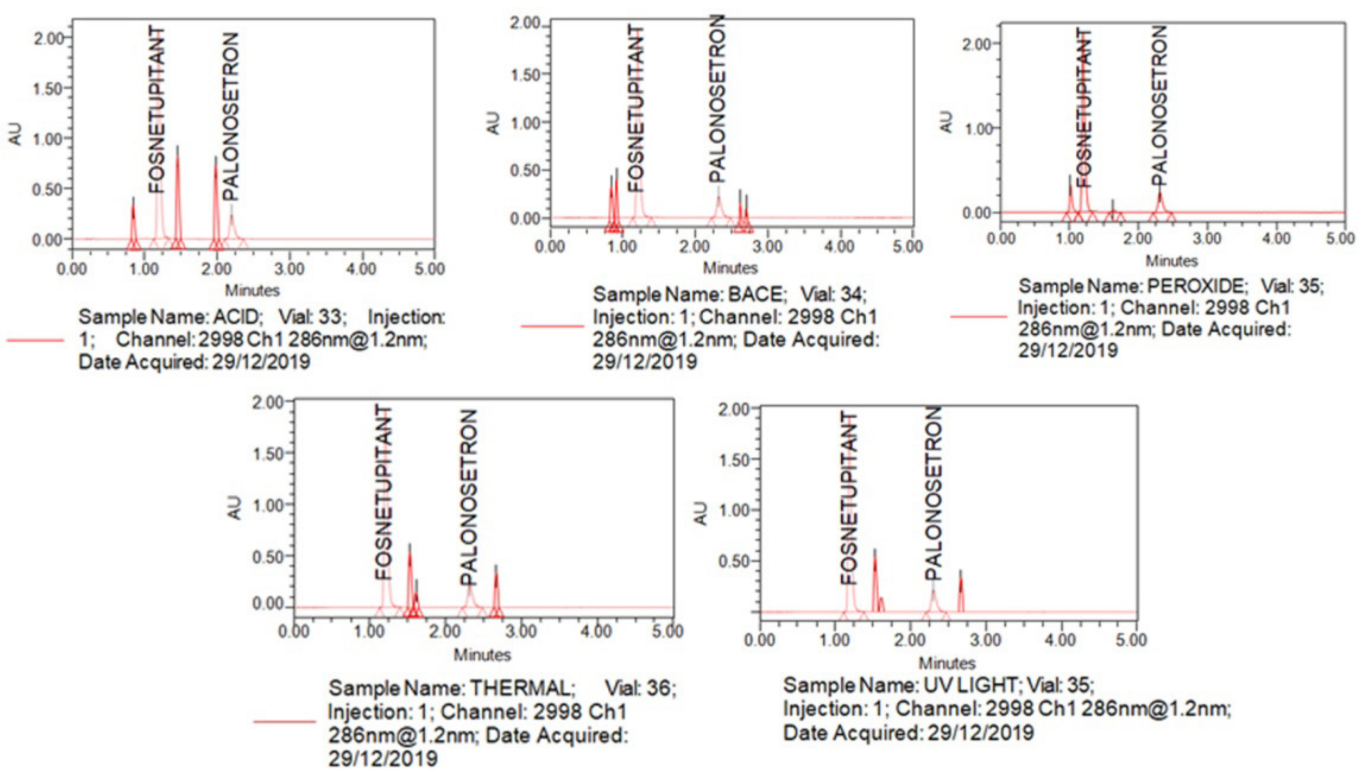

FIGURE 4 - Chromatograms of forced degradation studies.

CONCLUSION

This technique has been demonstrated to be quick, precise, selective, robust and simple, and may be applied to the latest FDA approved pharmaceutical combination of FOS and PAL. This method of analysis could be applied to ensure the safety, efficacy and quality of the drug in a cost effective manner. The established methods were validated as per ICH guidelines, and the stability study revealed that the technique is useful for monitoring drug stability. The method could also be applied for routine analysis in bioanalytical laboratories, by hospital research institutions for the therapeutic drug monitoring of clinical trials, in the quality control division of pharmaceutical companies, in dissolution studies of formulations and in accredited testing laboratories.

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