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A novel validated RP-HPLC-DAD method for the simultaneous estimation of Netupitant and Palonosetron in bulk and pharmaceutical dosage form with forced degradation studies

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Abstract: A novel approach was used to develop and validate a rapid, accurate, precise, simple, efficient and reproducible isocratic Reversed Phase-High Performance Liquid Chromatographic (RP-HPLC-DAD) method for the simultaneous estimation of Netupitant and Palonosetron in bulk and pharmaceutical dosage form with forced degradation studies. Netupitant and Palonosetron was separated using Kromasil C_{18} column (250mm×4.6mm, 5µm particle size), Waters Alliance e2695 HPLC system with 2998 PDA detector and the mobile phase contained a mixture of 0.01M Ammonium acetate buffer (pH adjusted to 3.5 with orthophosphoric acid) and Acetonitrile (65:35, v/v). The flow rate was set to 1ml/min with the responses measured at 265nm. The retention time of Netupitant and Palonosetron was found to be 2.438min and 3.718min respectively with resolution of 8.08. Linearity was established for Netupitant and Palonosetron in the range of 75-450µg/ml for Netupitant and 0.125-0.75µg/ml for Palonosetron with correlation coefficients (r^2=0.999). The percentage recoveries were between 99.85% to 100.04% and 99.73% to 100.03% for Netupitant and Palonosetron respectively. RP-HPLC method for the simultaneous estimation of Netupitant and Palonosetron in their combine dosage form was established and validated as per the ICH guidelines. Netupitant and Palonosetron are more sensitive towards acidic degradation condition and moderate degradation towards alkaline, thermal and very much resistant towards oxidative, photolytic and water degradation. The developed method was successfully applied for the quantification of Netupitant and Palonosetron in bulk and pharmaceutical dosage form.
Key words: Netupitant and Palonosetron RP-HPLC-DAD, ICH.

Introduction

Netupitant is an antiemetic drug. It is a selective neurokinin 1 (NK_1) receptor antagonists for prevention of acute and delayed nausea and vomiting associated with cancer chemotherapy[1]. Netupitant is chemically known as 2-[3, 5-Bis (trifluoromethyl) phenyl]-N, 2-dimethyl-N-[4-(2-methylphenyl)-6-(4-

methyl-1-piperazinyl)-3-pyridinyl] propanamide were shown in (Figure 1). Palonosetron is a 5-HT_3 receptor antagonist or serotonin antagonists used in the prevention and treatment of chemotherapy-induced nausea and vomiting. It is used for the control of delayed chemotherapy-induced nausea and vomiting[2]. Palonosetron is chemically known as (3aS)-2-[(3S)-1-Azabicyclo [2.2.2] oct-3-yl]-2, 3, 3a, 4, 5, 6-hexahydro-1H-benz [de] isoquinolin-1-one was shown in (Figure 2). Netupitant and Palonosetron is a fixed dose combination drug for prevention of acute and delayed nausea and vomiting associated with cancer chemotherapy. Literature review reveals that very few analytical methods has been reported for the determination of Netupitant and Palonosetron individually and with other combinations which includes high performance liquid chromatography (HPLC)[3-6], UV-Spectrophotometric[7], Micellar Electro kinetic Chromatography[8], Chiral HPLC[9-11], LCMS[12,13], Capillary Zone Electrophoresis[14] and Pharmacokinetics studies[15]. The present study was aimed to develop a novel, simple, economic and validated method for the simultaneous estimation of Netupitant and Palonosetron with forced degradation studies according to ICH guidelines[16].

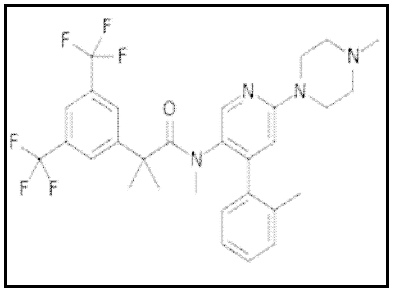


Figure 1: Chemical structure of Netupitant

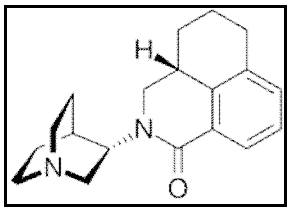


Figure 2: Chemical structure of Palonosetron

Materials and methods

Chemicals and reagents

Netupitant (API) was obtained from A S Bulk Drugs, Hyderabad, India and Palonosetron (API) was obtained from Maps Laboratories Pvt. Ltd., India. HPLC grade of Ammonium Acetate was obtained from Rankem Ltd., India and HPLC grade of Acetonitrile was obtained from Merck Specialities Private Limited, India. HPLC grade of Water and Ortho phosphoric acid was obtained from Rankem Ltd., India. Akynzeo capsule contains Netupitant 300mg and Palonosetron 0.5 mg were kindly supplied by Eisai Inc. and Helsinn Therapeutics (U.S.) Inc.

Instrumentation

The analysis was performed by using a chromatographic system from Waters Alliance e2695 HPLC system with 2998 PDA detector. The HPLC system was equipped with Empower 2 software. Semi-micro analytical balance (India), Ultrasonic bath sonicator (Frontline FS 4, Mumbai, India), Digital pH meter (Systronics model 802) and Whatmann filter paper No. 41 (Whatmann International Ltd., England) were used in the study.

Chromatographic conditions

Netupitant and Palonosetron was analysed in Kromasil C_{18} column (250mm×4.6 mm, 5µm particle size) column for the chromatographic separation. The mobile phase was composed of 0.01M Ammonium

acetate buffer (pH adjusted to 3.5 with orthophosphoric acid) and Acetonitrile (65:35, v/v). Filtered through 0.45µm nylon membrane filter under vacuum filtration and pumped at ambient temperature, at a flow rate of 1 ml/min with UV detection wavelength at 265nm. Injection volume was 20µl. The run time was 8 min and the retention time of Netupitant and Palonosetron was found to be 2.438min and 3.718min respectively with resolution of 8.08.

Chromatographic Parameters:

Equipment	: Waters Alliance e2695 HPLC system with 2998 PDA detector
Column	: Kromasil C_{18} column (250mm×4.6 mm, 5µm particle size)
Flow rate	: 1ml/min
Wavelength	: 265nm
Injection volume	: 20 µl
Column oven	: Ambient
Run time	: 8 Minutes

Solutions and sample preparation

Preparation of Ammonium acetate buffer

A 0.01M Ammonium acetate buffer was prepared by dissolving 0.77gm of Ammonium acetate in 1000ml of HPLC grade water and pH was adjusted to 3.5 with orthophosphoric acid. The buffer was filtered through 0.45µm nylon membrane filter to remove all fine particles and gases.

Preparation of mobile phase

The above prepared Ammonium acetate buffer and Acetonitrile HPLC grade were mixed in the proportion of 65:35, v/v and was filtered through 0.45µm nylon membrane filter and degassed by sonication.

Preparation of diluent

Mobile phase was used as diluent.

Preparation of standard stock solutions of Netupitant and Palonosetron

Standard stock solutions of Netupitant and Palonosetron were prepared by dissolving 300mg of Netupitant and 0.5mg of Palonosetron in 100ml of diluent into a 100ml clean dry volumetric flask and the standard solutions was filtered through 0.45 µm nylon membrane filter and degassed by sonicator to get the concentration of 3000µg/ml of Netupitant and 5µg/ml of Palonosetron.

Preparation of standard solutions of Netupitant and Palonosetron for assay

From the above standard stock solution of 3000µg/ml of Netupitant and 5µg/ml of Palonosetron further pipette 1ml and transferred into a 10ml volumetric flask and dilute up to the mark with diluent to get the concentration of 300µg/ml of Netupitant and 0.5µg/ml of Palonosetron.

Preparation of sample solutions of Netupitant and Palonosetron

Twenty capsules were accurately weighed and capsule powder equivalent to 300mg of Netupitant and 0.5mg of Palonosetron were taken into 100ml clean dry volumetric flask, diluent was added and sonicated to dissolve it completely and volume was made up to the mark with the same diluent and filtered through 0.45 µm nylon membrane filter. Further pipette out 1ml from the above Netupitant and Palonosetron sample stock solution into a 10ml volumetric flask and diluted up to the mark with diluent to get the concentration of 300µg/ml of Netupitant and 0.5µg/ml of Palonosetron. 20µl from standard and sample solution were injected into the chromatographic system and the peak areas were measured for Netupitant and Palonosetron which was shown in (Figure 6 and 7) and the % assay was calculated by comparing the peak area of standard and sample chromatogram by using the formula given below and the assay results was shown in Table 1.

Table 1: Assay of Marketed formulation of Netupitant and Palonosetron

Drug	Akynzeo Label Claim (mg)	Amount Found (mg) (n=6)	% Label Claim ± % RSD (n=6)
Netupitant	300	300.6	100.2± 0.5
Palonosetron	0.5	0.501	100.2± 1.2

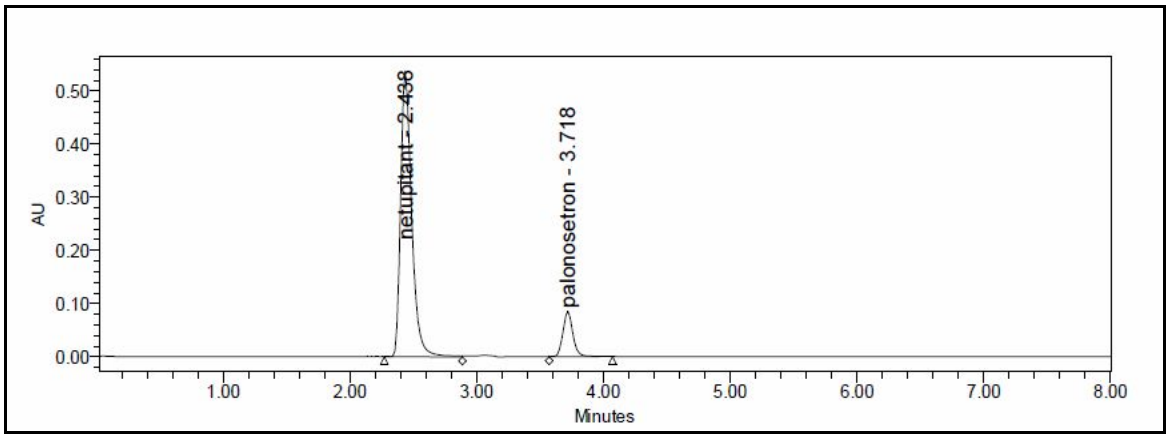


Figure 6: Standard Chromatogram for Netupitant and Palonosetron

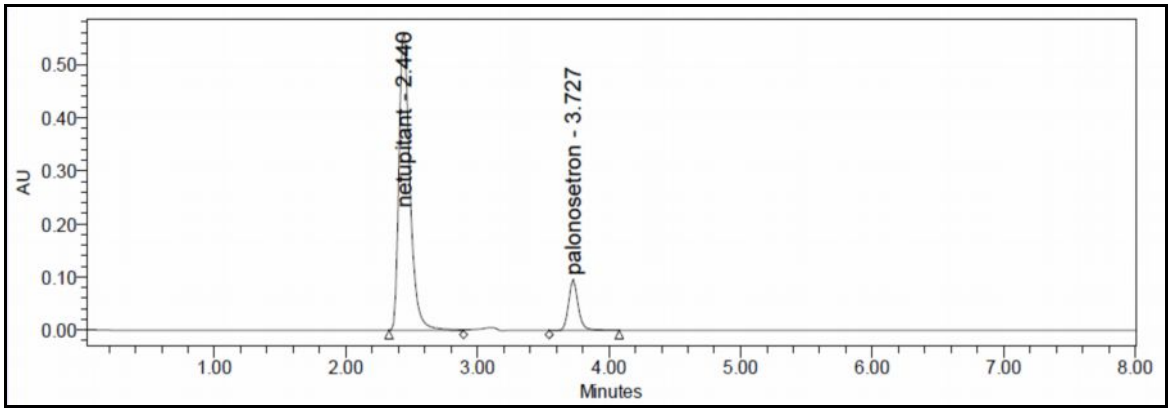


Figure 7: Sample Chromatogram for Netupitant and Palonosetron

$$\text{Assay \%} = \frac{AT}{AS} \times \frac{S}{DS} \times \frac{DT}{WT} \times \frac{P}{100} \times \frac{\text{Avg. Wt}}{\text{Label Claim}} \times 100$$

Where:

AT = Average peak area of sample preparation
AS= Average peak area of standard preparation
WS = Weight of standard taken in mg
WT=Weight of sample taken in mg
P = Percentage purity of working standard
DS= Dilution factor for standard preparation
DT=Dilution factor for sample preparation

Selection of wavelength

In simultaneous estimation of Netupitant and Palonosetron isosbestic wavelength is used. Standard stock solutions of Netupitant and Palonosetron were prepared by dissolving 300mg of Netupitant and 0.5mg of Palonosetron in 100ml of diluent into a 100ml clean dry volumetric flask and the standard solutions was

filtered through 0.45µm nylon membrane filter and degassed by sonicator to get the concentration of 3000µg/ml of Netupitant and 5µg/ml of Palonosetron. From the above standard stock solution of 3000µg/ml of Netupitant and 5µg/ml of Palonosetron further pipette 1ml and transferred into a 10ml volumetric flask and dilute up to the mark with diluent to get the concentration of 300µg/ml of Netupitant and 0.5µg/ml of Palonosetron. The wavelength of maximum absorption (λmax) of 300µg/ml of Netupitant and 0.5µg/ml of Palonosetron were scanned using UV-Visible spectrophotometer within the wavelength region of 200–400 nm against mobile phase as blank. The isosbestic wavelength (λmax) was found to be 265nm for the combination shown in (Figure 3).

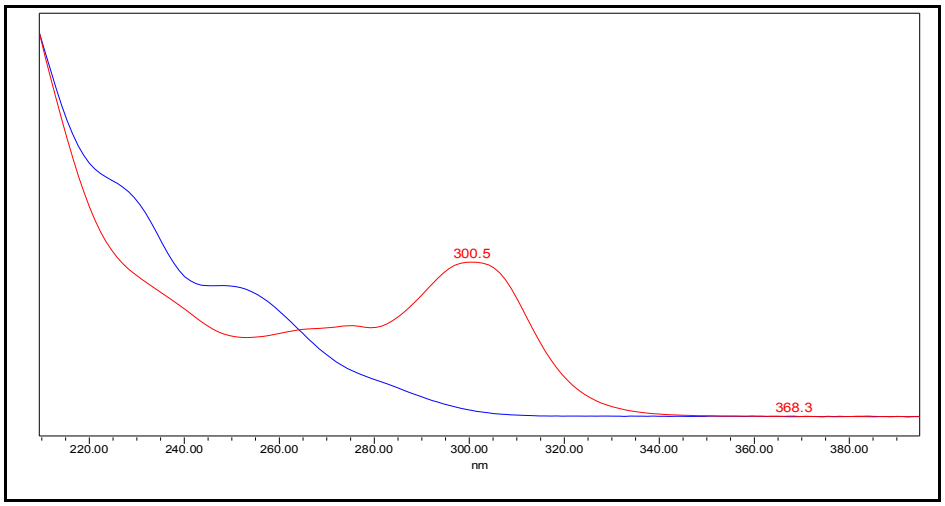


Figure 3: Isosbestic point of Netupitant and Palonosetron at 265nm

Results and discussion

Method Development

To optimize the RP-HPLC parameters, several mobile phase compositions were tried. A satisfactory separation and good peak symmetry for Netupitant and Palonosetron were obtained with a mobile phase containing a mixture of 0.01M Ammonium acetate buffer (pH adjusted to 3.5 with orthophosphoric acid) and Acetonitrile (65:35, v/v) was delivered at a flow rate of 1ml/min to get better reproducibility and repeatability. Quantification was achieved with PDA detection at 265nm based on peak area. The retention time of Netupitant and Palonosetron was found to be 2.438min and 3.718min respectively with resolution of 8.08. Linearity was established for Netupitant and Palonosetron in the range of 75-450µg/ml for Netupitant and 0.125-0.75µg/ml for Palonosetron with correlation coefficients (r^2=0.999) and the percentage recoveries were between 99.85 % to 100.04% and 99.73% to 100.03% for Netupitant and Palonosetron respectively, which indicate accuracy of the proposed method. The % RSD values of accuracy for Netupitant and Palonosetron were found to be < 2 %. The % RSD values of method precision are 0.5% and 0.35% for Netupitant and Palonosetron respectively and % RSD values of system precision are 1.3% and 1.1% for Netupitant and Palonosetron respectively. The % RSD values of reproducibility are 0.04% and 0.02% for Netupitant and Palonosetron respectively, reveal that the proposed method is precise. LOD values for Netupitant and Palonosetron were found to be 0.06µg/ml and 0.01µg/ml respectively and LOQ values for Netupitant and Palonosetron were found to be 0.18µg/ml and 0.03µg/ml respectively. The % RSD values of robustness studies were found to be < 2% reveal that the method is robust enough. These data show that the proposed method is specific and sensitive for the determination of Netupitant and Palonosetron.

Method validation

The developed method for the simultaneous estimation of Netupitant and Palonosetron was validated as per the ICH guidelines for the parameters like system suitability, specificity, linearity, accuracy, precision, ruggedness, robustness, limit of detection (LOD) and limit of quantitation (LOQ) [16].

System suitability test

At first the HPLC system was optimized as per the chromatographic conditions. One blank followed by six replicates of a single calibration standard solution of 300µg/ml of Netupitant and 0.5µg/ml of Palonosetron was injected to check the system suitability. To ascertain the system suitability for the proposed method, the parameters such as retention time, theoretical plates, peak asymmetry and resolution were taken and results were presented in Table 2.

Table 2: System suitability parameters for Netupitant and Palonosetron

Parameter (n=6)	Netupitant	Palonosetron
Retention Time (Mins)	2.438	3.718
Theoretical plates	3871	10816
Tailing factor	1.1	1.1
Resolution		8.08

Specificity

The effect of excipients and other additives usually present in the combined capsule dosage form of Netupitant and Palonosetron in the determination under optimum conditions was investigated. The specificity of the RP-HPLC method was established by injecting the blank and placebo solution into the HPLC system. The representative chromatogram of blank and placebo was shown in (Figure 4 and 5).

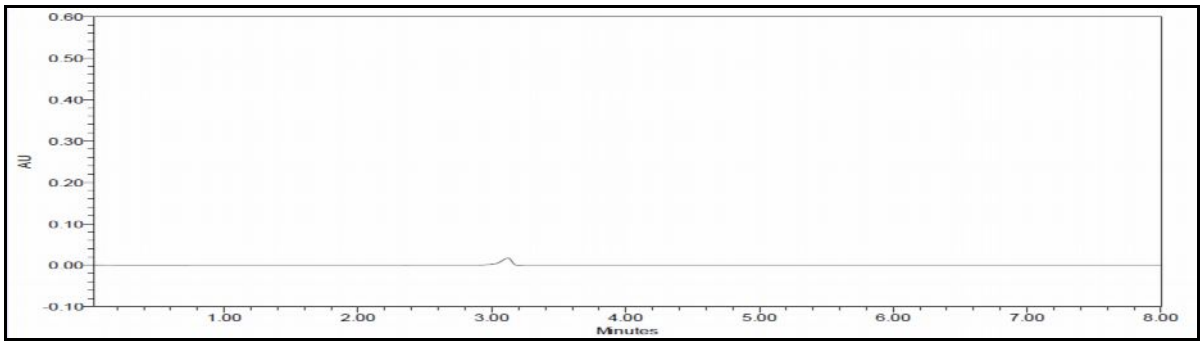


Figure 4: Chromatogram of blank

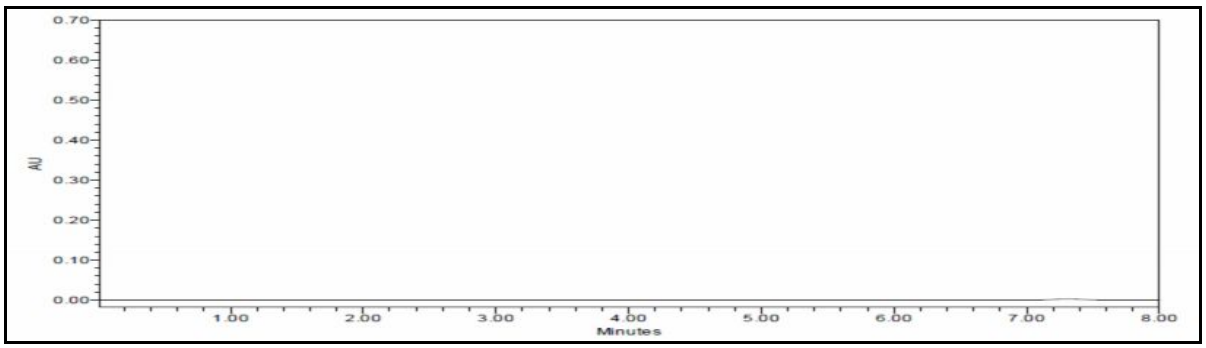


Figure 5: Chromatogram of placebo

Linearity and range for Netupitant and Palonosetron

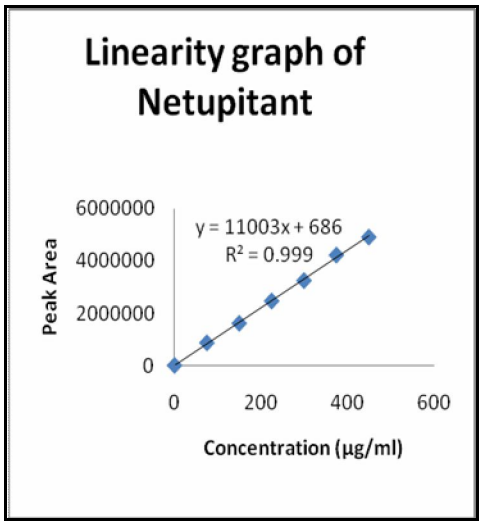
Aliquots of 0.25, 0.5, 0.75, 1, 1.25 and 1.5ml of mixed standard working solutions of Netupitant and Palonosetron was pipetted out from the standard stock solution of 3000µg/ml of Netupitant and 5µg/ml of Palonosetron and transferred into a series of 10ml clean dry volumetric flask and make volume up to the mark with the same diluent to get the concentration of 75, 150, 225, 300, 375 and 450µg/ml of Netupitant and 0.125, 0.25, 0.375, 0.5, 0.625 and 0.75µg/ml of Palonosetron. The calibration standard solutions of

Netupitant and Palonosetron were injected using a 20µl Hamilton Rheodyne injector and the chromatograms were recorded at 265nm and a calibration graph was obtained by plotting peak area versus concentration of Netupitant and Palonosetron respectively. The linearity data is presented in (Figure 8 and 9) and Table 3.

Acceptance Criteria: Correlation coefficient should be not less than 0.999

Table 3: Linearity data for Netupitant and Palonosetron

Linearity of Netupitant		Linearity of Palonosetron	
Concentration (µg/ml)	Peak Area	Concentration (µg/ml)	Peak Area
75	864115	0.125	128061
150	1612752	0.25	245238
225	2466709	0.375	364102
300	3249231	0.5	474414
375	4226134	0.625	612356
450	4915001	0.75	730816



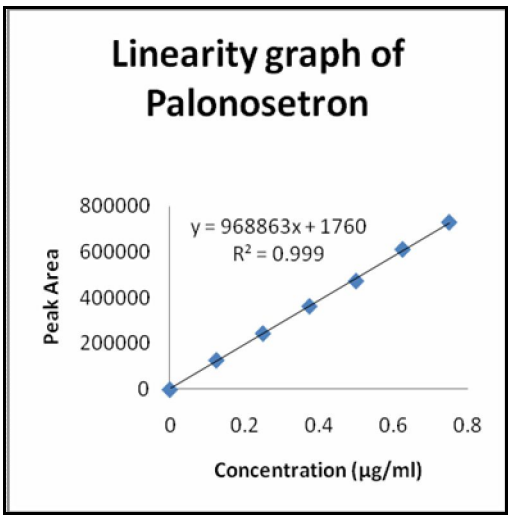


Figure 8: Linearity graph of Netupitant

Figure 9: Linearity graph of Palonosetron

Accuracy studies for Netupitant and Palonosetron

The accuracy of the method was determined by calculating recovery of Netupitant and Palonosetron by the method of standard addition. Known amount of standard solution of Netupitant and Palonosetron at 50%, 100% and 150% was added to a pre quantified sample solution and injected into the HPLC system. The mean percentage recovery of Netupitant and Palonosetron at each level was calculated and the results were presented in Table 4.

Table 4: Recovery studies of Netupitant and Palonosetron

Recovery study data of Netupitant				
Sample name	Amount added (µg/ml)	Amount found (µg/ml)	%Recovery	Statistical Analysis
S_1:50%	75	75.03	100.04	Mean=99.85%(n=3) S.D=0.36 %RSD=0.36
S_2:50%	75	75.06	100.08	
S_3:50%	75	74.58	99.44	
S_4:100%	150	149.97	99.98	Mean=100.04%(n=3) S.D=0.21 %RSD=0.21
S_5:100%	150	149.79	99.86	
S_6:100%	150	150.41	100.27	
S_7:150%	225	224.94	99.97	Mean=99.92%(n=3) S.D=0.12 %RSD=0.12
S_8:150%	225	225.03	100.01	
S_9 :150%	225	224.51	99.78	
Recovery study data of Palonosetron				
Sample name	Amount added (µg/ml)	Amount found (µg/ml)	%Recovery	Statistical Analysis
S_1:50%	0.125	0.1252	100.16	Mean=100.03%(n=3) S.D=0.17 %RSD=0.17
S_2:50%	0.125	0.1251	100.08	
S_3:50%	0.125	0.1248	99.84	
S_4:100%	0.25	0.251	100.40	Mean=99.73%(n=3) S.D=0.61 %RSD=0.61
S_5:100%	0.25	0.249	99.60	
S_6:100%	0.25	0.248	99.20	
S_7:150%	0.375	0.3734	99.57	Mean=99.79%(n=3) S.D=0.32 %RSD=0.33
S_8:150%	0.375	0.3756	100.16	
S_9 :150%	0.375	0.3736	99.63	

Preparation of pre quantified sample solution for accuracy studies

Capsule powder equivalent to 300mg of Netupitant and 0.5mg of Palonosetron were taken into 100ml clean dry volumetric flask and diluent was added and sonicated to dissolve it completely and volume was made up to the mark with the same diluent and was filtered through 0.45 µm nylon membrane filter. Further pipette out 0.5ml from the above Netupitant and Palonosetron sample stock solution into a 10ml volumetric flask and diluted up to the mark with diluent to get the concentration of 150µg/ml of Netupitant and 0.25µg/ml of Palonosetron.

Preparation of standard solution of Netupitant and Palonosetron for accuracy studies

Standard stock solutions of Netupitant and Palonosetron were prepared by dissolving 300mg of Netupitant and 0.5mg of Palonosetron in 100ml of diluent into a 100ml clean dry volumetric flask and the standard solutions was filtered through 0.45 µm nylon membrane filter and degassed by sonicator to get the concentration of 3000µg/ml of Netupitant and 5µg/ml of Palonosetron.

Preparation of 50% standard solution

From the standard stock solution of 3000µg/ml of Netupitant and 5µg/ml of Palonosetron further pipette 0.25ml and transferred into a 10ml volumetric flask and dilute up to the mark with diluent to get the concentration of 75µg/ml of Netupitant and 0.125µg/ml of Palonosetron.

Preparation of 100% standard solution

From the standard stock solution of 3000µg/ml of Netupitant and 5µg/ml of Palonosetron further pipette 0.5ml and transferred into a 10ml volumetric flask and dilute up to the mark with diluent to get the concentration of 150µg/ml of Netupitant and 0.25µg/ml of Palonosetron.

Preparation of 150% standard solution

From the standard stock solution of 3000µg/ml of Netupitant and 5µg/ml of Palonosetron further pipette 0.75ml and transferred into a 10ml volumetric flask and dilute up to the mark with diluent to get the concentration of 225µg/ml of Netupitant and 0.375µg/ml of Palonosetron.

Acceptance Criteria**:** The % Recovery for each level should be between 98.0 to 102.0%.

Precision studies for Netupitant and Palonosetron

Method precision (Repeatability)

Capsule powder equivalent to 300mg of Netupitant and 0.5mg of Palonosetron were taken into 100ml clean dry volumetric flask, diluent was added and sonicated to dissolve it completely and volume was made up to the mark with the same diluent and was filtered through 0.45µm nylon membrane filter. Further pipette out 1ml from the above Netupitant and Palonosetron sample stock solution into a 10ml volumetric flask and diluted up to the mark with diluent to get the concentration of 300µg/ml of Netupitant and 0.5µg/ml of Palonosetron. A homogenous sample of a single batch is analysed six times and was checked whether the method is giving consistent results. The %RSD for the assay of six replicate injections was calculated as mentioned in Table 5.

Table 5: Method precision data for Netupitant and Palonosetron

Netupitant					**Palonosetron**			
S.No.	Concentration (µg/ml)	Retention time (min)	Peak Area	%Assay	Concentration (µg/ml)	Retention time (min)	Peak Area	%Assay
1	300	2.438	3227906	99.66	0.5	3.713	479376	100.18
2	300	2.438	3258393	100.60	0.5	3.713	476760	99.63
3	300	2.439	3227518	99.65	0.5	3.717	481643	100.65
4	300	2.439	3265378	100.82	0.5	3.717	478012	99.89
5	300	2.440	3252181	100.41	0.5	3.718	477938	99.88
6	300	2.442	3239480	100.02	0.5	3.732	479413	100.19
Average		2.439	3245143	100.19	**Average**	3.718	478857	100.07
SD		0.00151	15964.81	0.492913	**SD**	0.00703	1690.934	0.3534
%RSD		0.06	0.49	0.5	**%RSD**	0.19	0.35	0.35

Acceptance Criteria: The % RSD for the assay of six sample injections should not be more than 2%.

System precision

The system precision was carried out to ensure that the analytical system is working properly. The standard preparation concentration of 300µg/ml of Netupitant and 0.5µg/ml of Palonosetron was injected six times into the HPLC system and the %RSD for the area of six replicate injections was calculated as mentioned in Table 6.

Acceptance Criteria: The % RSD for the peak area of six standard injections should not be more than 2%.

Table 6: System precision data for Netupitant and Palonosetron

Netupitant				Palonosetron		
S.No.	Conc. (µg/ml)	Retention time (min)	Peak Area	Conc. (µg/ml)	Retention time (min)	Peak Area
1	300	2.439	3273495	0.5	3.718	467706
2	300	2.440	3280067	0.5	3.732	477735
3	300	2.442	3181747	0.5	3.717	482373
4	300	2.438	3208990	0.5	3.717	476300
5	300	2.439	3183295	0.5	3.713	481557
6	300	2.438	3227906	0.5	3.713	474000
Average		2.439	3225917	**Average**	3.718	476612
SD		0.001506	43020.91	**SD**	0.007033	5387.76
%RSD		0.1	1.3	**%RSD**	0.2	1.1

Intermediate precision/ruggedness

The intermediate precision (also known as Ruggedness) of the method was evaluated by performing precision on different laboratories by different analysts and different days. The sample preparation concentration of 300µg/ml of Netupitant and 0.5µg/ml of Palonosetron was injected six times into the HPLC system and the %RSD for the assay of six replicate injections was calculated as mentioned in Table 7.

Acceptance Criteria: The % RSD for the assay of six sample injections should not be more than 2%.

Limit of Detection (LOD) and Limit of Quantification (LOQ)

Limit of Detection (LOD) and Limit of Quantification (LOQ) were calculated as 3.3×SD/S and 10×SD/S respectively as per ICH guidelines, Where SD is the standard deviation of the response (Y-intercept) and S is the slope of the calibration curve. The LOD is the smallest concentration of the analyte that gives a measurable response (signal to noise ratio of 3). The LOD of Netupitant and Palonosetron was calculated and shown in Table 8. The LOQ is the smallest concentration of the analyte which gives response that can be accurately quantified (signal to noise ratio of 10). The LOQ of Netupitant and Palonosetron was calculated and shown in Table 8.

Table 7: Ruggedness data for Netupitant and Palonosetron

Ruggedness Data for Netupitant								
Laboratory-1 (% Assay)-HPLC-1					Laboratory-2 (% Assay)-HPLC-2			
	Analyst-1		Analyst-2		Analyst-1		Analyst-2	
Conc. (µg/ml)	Day-1	Day-2	Day-1	Day-2	Day-1	Day-2	Day-1	Day-2
300	100.39	100.09	100.11	99.72	99.71	99.66	99.75	100.11
300	100.27	100.28	100.10	100.02	100.29	100.60	100.02	100.03
300	100.28	99.98	99.93	99.96	100.27	100.14	100.41	100.22
300	100.55	99.94	99.82	100.11	99.58	99.69	100.12	100.01
300	100.27	100.13	100.09	100.29	99.79	100.31	100.40	100.09
300	100.54	99.79	99.72	100.08	100.02	100.01	100.03	100.07
Average	100.4	100.03	99.96	100.03	99.94	100.07	100.12	100.09
SD	0.13428	0.16857	0.167939	0.186602	0.296854	0.363239	0.252077	0.073425
%RSD	0.13	0.17	0.17	0.19	0.3	0.4	0.3	0.1
Intermediate precision within-laboratories variations (n=24)								
Laboratory-1 (% Assay)-HPLC-1					Laboratory-2 (% Assay)-HPLC-2			
Average	100.11				**Average**	100.06		
SD	0.199416				**SD**	0.079373		
%RSD	0.20				**%RSD**	0.08		

Reproducibility between laboratories (n=48) (% Assay)								
Average	100.09							
SD	0.0354							
%RSD	0.04							
Ruggedness Data for Palonosetron								
Laboratory-1 (% Assay)-HPLC-1					Laboratory-2 (% Assay)-HPLC-2			
	Analyst-1		Analyst-2		Analyst-1		Analyst-2	
Conc. (µg/ml)	Day-1	Day-2	Day-1	Day-2	Day-1	Day-2	Day-1	Day-2
0.5	100.03	99.80	99.94	99.31	100.03	100.04	100.18	99.68
0.5	100.15	99.96	99.89	100.27	100.13	99.84	99.97	99.59
0.5	99.82	99.86	99.83	100.22	100.02	100.09	100.09	100.02
0.5	100.06	100.03	99.91	99.77	99.64	100.06	99.89	99.47
0.5	100.09	99.93	99.93	99.81	99.92	99.73	100.07	100.13
0.5	99.52	99.72	99.98	99.73	100.04	100.06	100.05	99.56
Average	99.94	99.88	99.92	99.85	99.96	99.97	100.04	99.74
SD	0.237078	0.111872	0.050182	0.355667	0.172074	0.149064	0.100715	0.268716
%RSD	0.2	0.1	0.1	0.4	0.2	0.1	0.1	0.3
Intermediate precision within-laboratories variations (n=24)								
Laboratory-1 (% Assay)-HPLC-1					Laboratory-2 (% Assay)-HPLC-2			
Average	99.90				**Average**	99.93		
SD	0.040311				**SD**	0.129968		
%RSD	0.04				**%RSD**	0.13		
Reproducibility between laboratories (n=48) (% Assay)								
Average	99.92							
SD	0.021213							
%RSD	0.02							

Table 8: Summary of validation parameter for Netupitant and Palonosetron

Parameters	**HPLC method**			
	Netupitant		**Palonosetron**	
Linearity range (µg/ml)	75-450		0.125-0.75	
Slope	11003		968863	
Intercept	686		1760	
Correlation coefficient	0.999		0.999	
LOD (µg/ml)	0.06		0.01	
LOQ (µg/ml)	0.18		0.03	
Method Precision (% RSD, n=6)	0.5		0.35	
System precision (% RSD, n=6)	1.3		1.1	
Ruggedness (% RSD, n=24)	Lab-1	Lab-2	Lab-1	Lab-2
	0.20	0.08	0.04	0.13
Reproducibility (% RSD, n=48)	0.04		0.02	
% Accuracy	99.85-100.04		99.73-100.03	
Robustness (% RSD, n=6)	Less Flow rate	More Flow rate	Less Flow rate	More Flow rate
	0.37	0.11	0.33	0.15
	Less Organic phase	More Organic phase	Less Organic phase	More Organic phase
	0.22	0.3	0.03	0.4

Table 9: Summary of robustness (Change in flow rate) for Netupitant and Palonosetron

Drug	Change in Flow rate (ml/min)	Retention Time (Mins)	Change in flow Rate (0.9ml/min to 1.1ml/min)				
			Average peak area (n=6)	SD	% RSD	USP Plate Count	Asymmetry
Netupitant	0.9	2.726	3641636	13447.05	0.37	4043	1.48
	1	2.438	3225917	43020.9	1.3	3871	1.1
	1.1	2.198	2920162	3162.9	0.11	3562	1.48
Palonosetron	0.9	4.141	513373	1696.35	0.33	12020	1.2
	1	3.718	476612	5387.8	1.1	10816	1.1
	1.1	3.340	422857	615.18	0.15	10861	1.2

Table 10: Summary of robustness (Change in mobile phase) for Netupitant and Palonosetron

Drug	**Change in Mobile Phase**	**Retention Time (Mins)**	**Change in mobile phase (0.01M Ammonium acetate buffer (pH adjusted to 3.5 with orthophosphoric acid) and Acetonitrile) (69:31v/v to 61:39v/v)**				
			Average peak area (n=6)	**SD**	**% RSD**	**USP Plate Count**	**Asymmetry**
Netupitant	10% less Organic (69:31v/v)	2.423	3264268	7113.494	0.22	3986	1.46
	Actual (65:35 v/v)	2.438	3225917	43020.9	1.3	3871	1.1
	10% more Organic (61:39v/v)	2.432	3224224	9654.836	0.3	3879	1.49
Palonosetron	10% less Organic (69:31v/v)	3.623	478099	134.3503	0.03	11948	1.2
	Actual (65:35 v/v)	3.718	476612	5387.8	1.1	10816	1.1
	10% more Organic (61:39v/v)	3.690	473333	1802.5	0.4	11670	1.2

Robustness

As part of the Robustness, deliberate change in the flow rate and mobile phase proportion of ±10% was made to evaluate the impact on the method. The results reveal that the method is robust. The results are summarized in Table 9 and 10.

Stability of solution

The %RSD of the assay of Netupitant and Palonosetron from the solution stability and mobile phase stability experiments was within 2%. The results of the solution and mobile phase stability experiments confirm that the sample solutions and mobile phase used during the assays were stable upto 48hours at room temperature was calculated and shown in Table 11.

Table 11: Summary of solution stability-effect of P^H of mobile phase (0.01M Ammonium acetate buffer and Acetonitrile (65:35, v/v) (P^H adjusted to 3.5 with orthophosphoric acid) for Netupitant and Palonosetron for 48 hours at room temperature.

	Solution stability for Netupitant					
S.No.	Concentration (µg/ml)	Retention time (min)	Peak Area	%Assay	USP Plate Count	Asymmetry
1	300	2.438	3251587	100.39	4235	1.37
2	300	2.438	3247618	100.27	4245	1.37
3	300	2.438	3248094	100.28	4277	1.38
4	300	2.440	3246765	100.24	4276	1.38
5	300	2.440	3247563	100.27	4217	1.38
6	300	2.444	3239481	100.02	4201	1.37
Average		2.440	3246851	100.20	4242	1.4
SD		0.002338	3984.828	0.12303	30.805	0.0055
%RSD		0.1	0.12	0.1	0.7	0.4
Solution stability for Palonosetron						
S.No.	Concentration (µg/ml)	Retention time (min)	Peak Area	%Assay	USP Plate Count	Asymmetry
1	0.5	3.709	478960	100.09	12102	1.13
2	0.5	3.710	478871	100.07	12161	1.15
3	0.5	3.713	478634	100.02	12155	1.15
4	0.5	3.715	479525	100.21	12116	1.15
5	0.5	3.718	479271	100.16	12147	1.16
6	0.5	3.727	479237	100.15	12144	1.14
Average		3.715	479083	100.12	12138	1.15
SD		0.0065929	321.2781	0.0671	23.2959224	0.010328
%RSD		0.18	0.07	0.07	0.19	0.90

Forced degradation studies

Acid Degradation Studies

To 1 ml of stock solution of Netupitant and Palonosetron, 1 ml of 2N Hydrochloric acid was added and refluxed for 30mins at 60^0C. The resultant solution was diluted to obtain 300µg/ml of Netupitant and 0.5µg/ml of Palonosetron solution and 20µl solutions were injected into the HPLC system and the chromatogram were recorded to assess the stability of sample was shown in (Figure 10) and purity plot of acid degradation for Netupitant and Palonosetron was shown in (Figure 11 and 12).

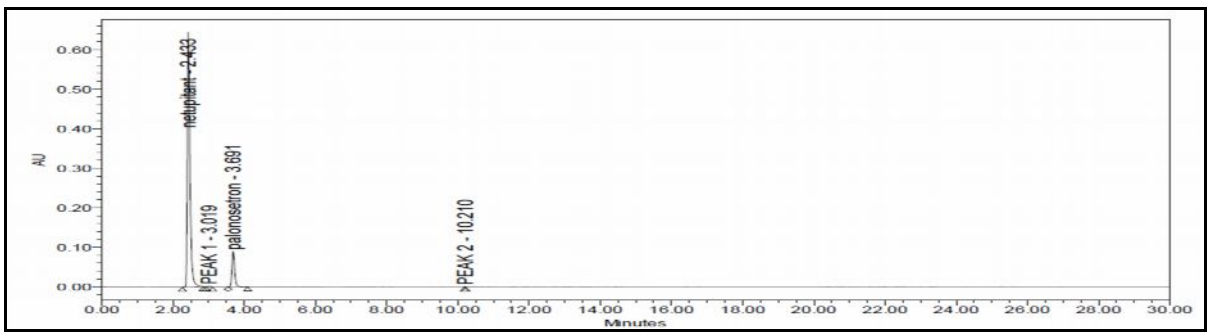


Figure 10: Chromatogram of acid hydrolysis for Netupitant and Palonosetron

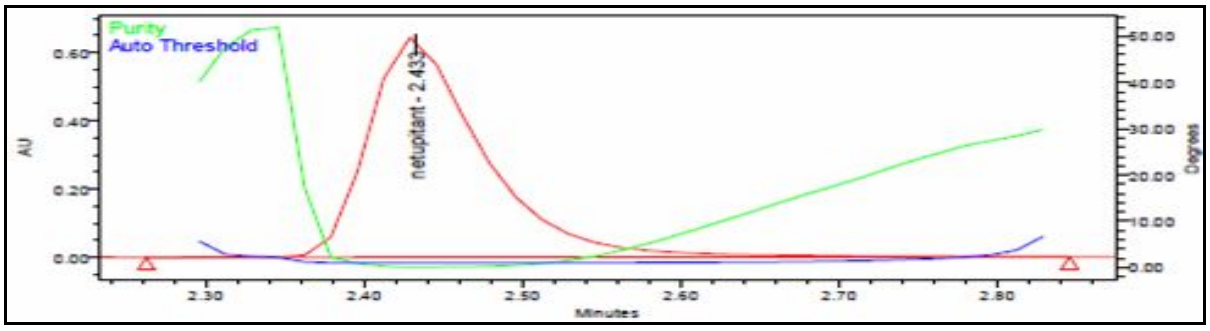


Figure 11: Purity plot of acid hydrolysis for Netupitant

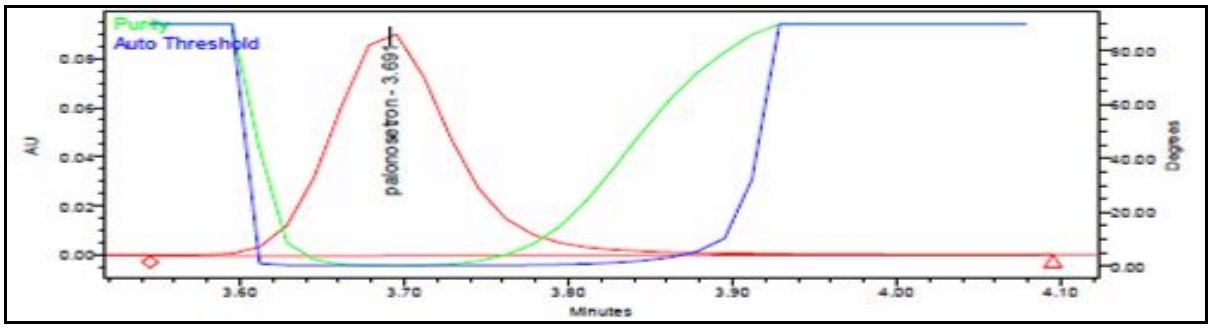


Figure 12: Purity plot of acid hydrolysis for Palonosetron

Alkali Degradation Studies

To 1ml of stock solution of Netupitant and Palonosetron, 1 ml of 2N sodium hydroxide was added and refluxed for 30mins at 60^0C.The resultant solution was diluted to obtain 300µg/ml of Netupitant and 0.5µg/ml of Palonosetron solution and 20µl solutions were injected into the HPLC system and the chromatogram were recorded to assess the stability of sample was shown in (Figure 13) and purity plot of alkali degradation for Netupitant and Palonosetron was shown in (Figure 14 and 15).

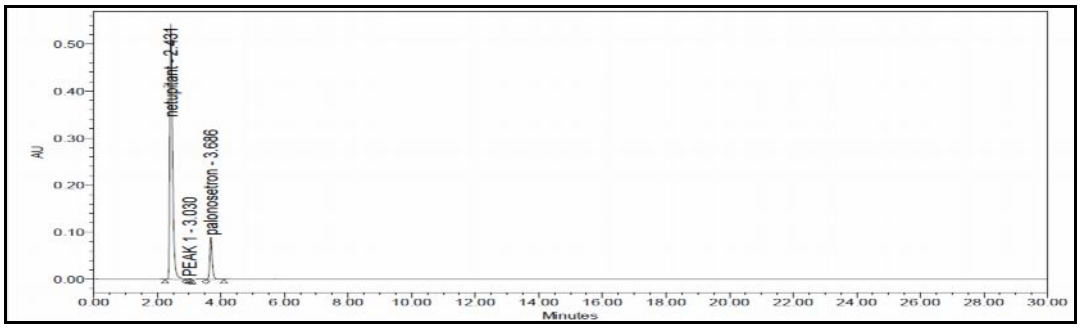


Figure 13: Chromatogram of alkali hydrolysis for Netupitant and Palonosetron

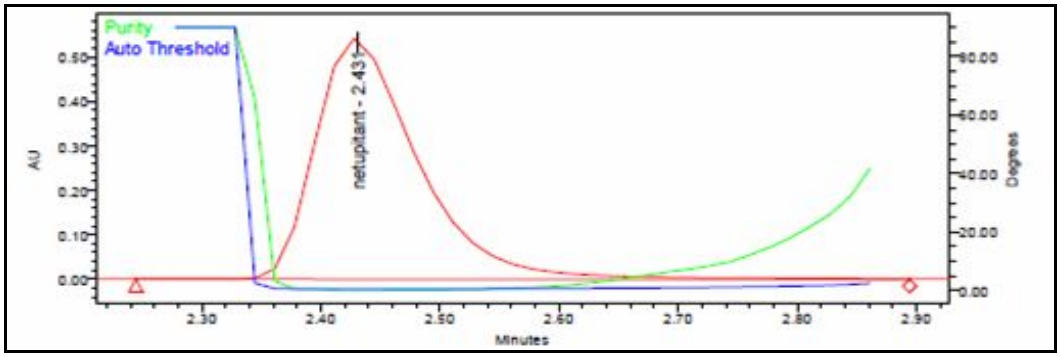


Figure 14: Purity plot of alkali hydrolysis for Netupitant

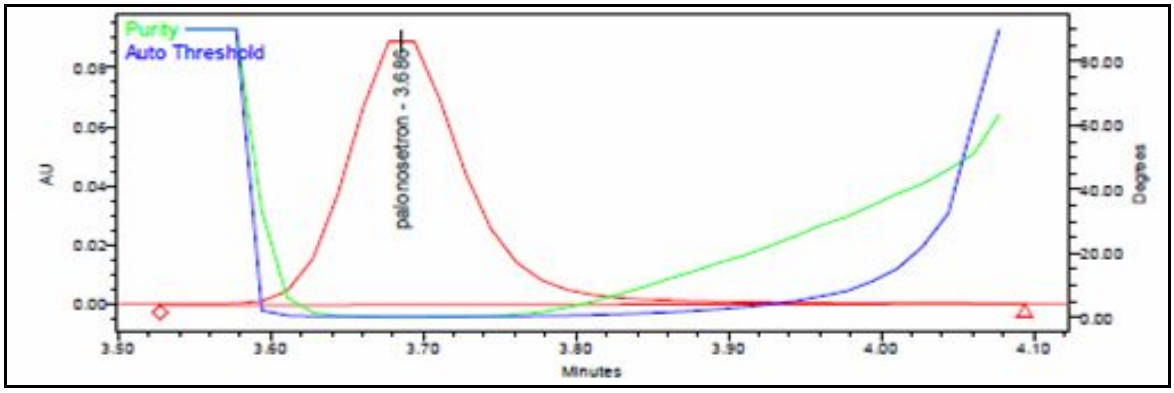


Figure 15: Purity plot of alkali hydrolysis for Palonosetron

Oxidative degradation Studies

To 1ml of stock solution of Netupitant and Palonosetron, 1 ml of 3% Hydrogen peroxide (H_2O_2) was added and the solution was kept for 30mins at 60^0C. For HPLC study, the resultant solution was diluted to obtain 300µg/ml of Netupitant and 0.5µg/ml of Palonosetron solution and 20µl solutions were injected into the HPLC system and the chromatogram were recorded to assess the stability of sample was shown in (Figure 16) and purity plot of oxidative degradation for Netupitant and Palonosetron was shown in (Figure 17 and 18).

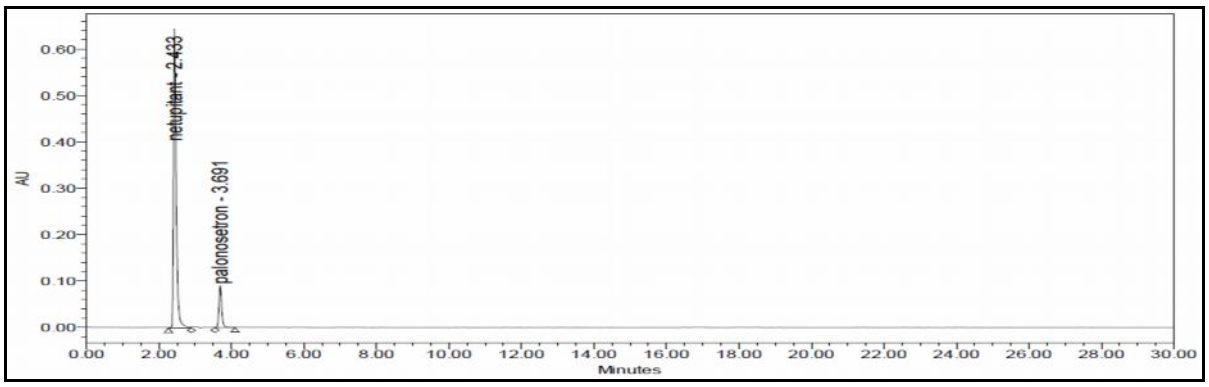


Figure 16: Chromatogram of oxidative degradation for Netupitant and Palonosetron

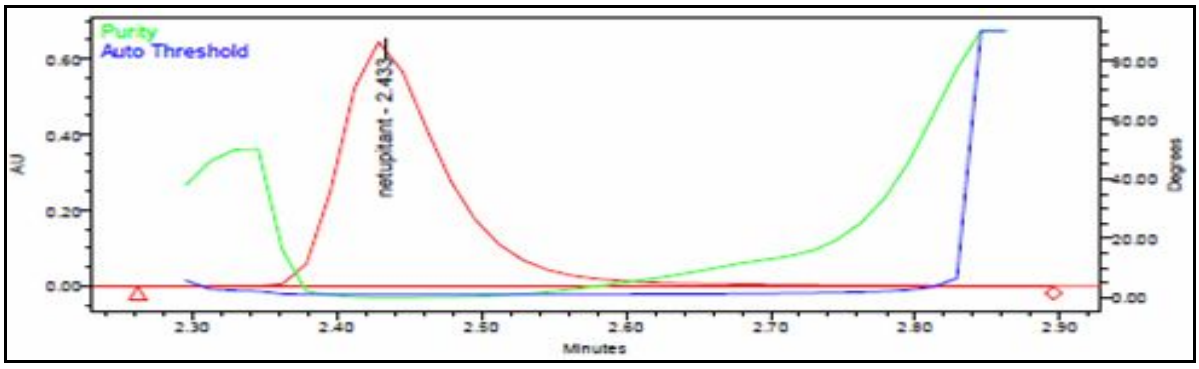


Figure 17: Purity plot of oxidative degradation for Netupitant

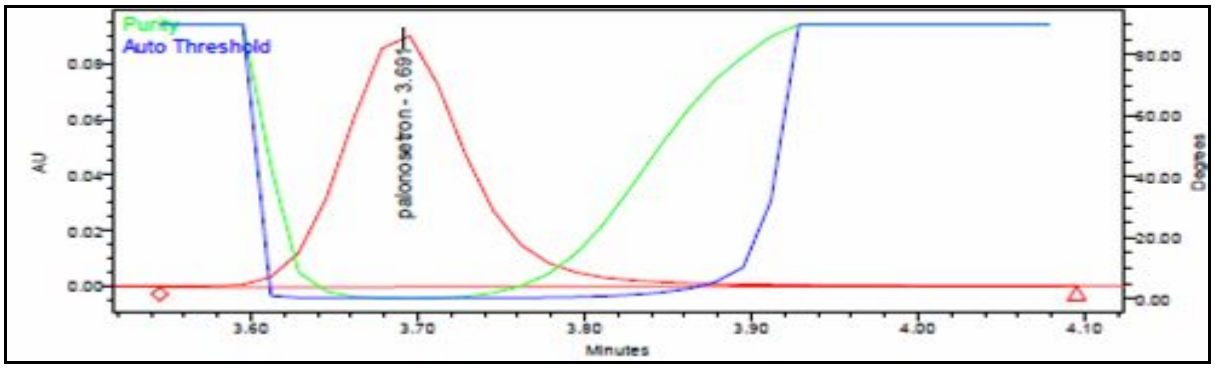


Figure 18: Purity plot of oxidative degradation for Palonosetron

Thermal Degradation Studies

The standard drug solution was placed in oven at 105^0C for 6hrs to study dry heat degradation. For HPLC study, the resultant solution was diluted to 300µg/ml of Netupitant and 0.5µg/ml of Palonosetron solution and 20µl solutions were injected into the HPLC system and the chromatogram were recorded to assess the stability of sample was shown in (Figure 19) and purity plot of thermal degradation for Netupitant and Palonosetron was shown in (Figure 20 and 21).

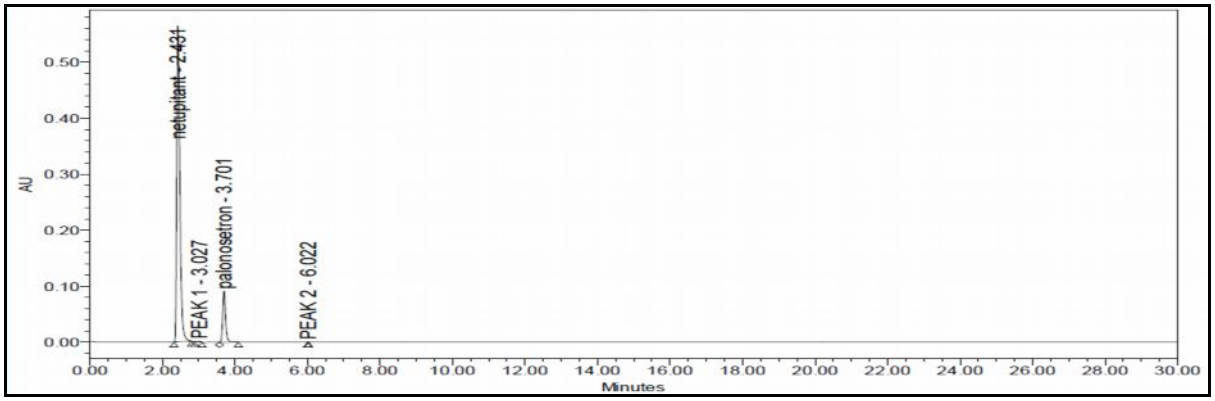


Figure 19: Chromatogram of thermal degradation for Netupitant and Palonosetron

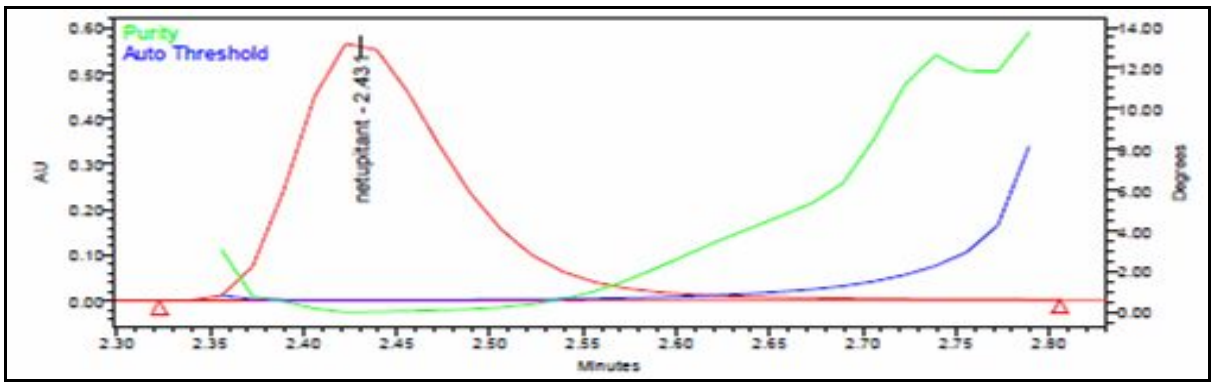


Figure 20: Purity plot of thermal degradation for Netupitant

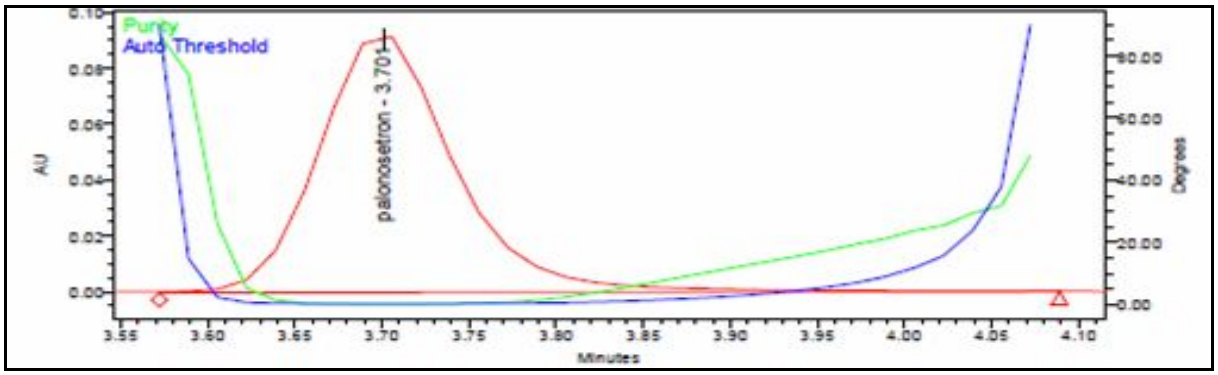


Figure 21: Purity plot of thermal degradation for Palonosetron

Photolytic degradation studies

The photochemical stability of the drug was also studied by exposing the drug solution to UV light by keeping the beaker in UV Chamber for 7days or 200 Watt hours/m^2 in photo stability chamber. For HPLC study, the resultant solution was diluted to obtain 300µg/ml of Netupitant and 0.5µg/ml of Palonosetron solution and 20µl solutions were injected into the HPLC system and the chromatogram were recorded to assess the stability of sample was shown in (Figure 22) and purity plot of photolytic degradation for Netupitant and Palonosetron was shown in (Figure 23 and 24).

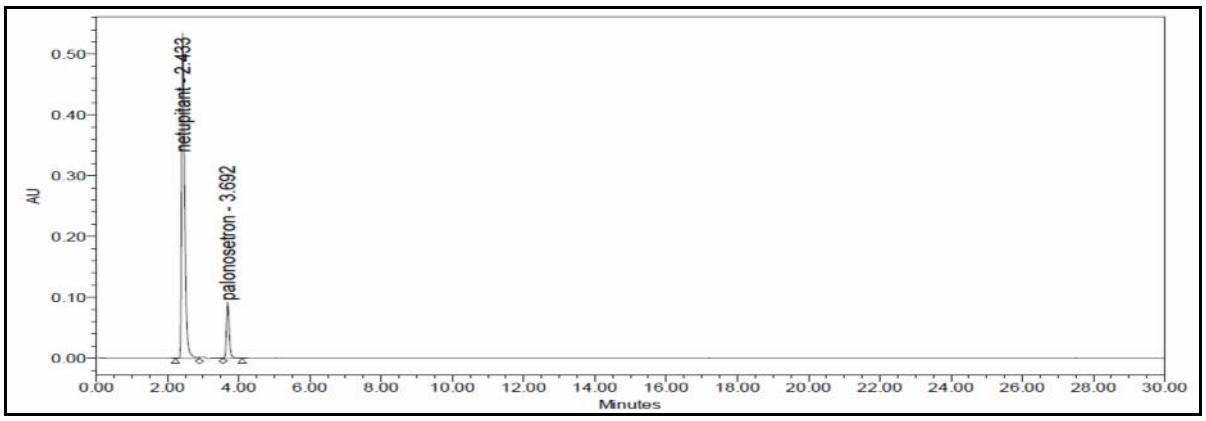


Figure 22: Chromatogram of photolytic degradation for Netupitant and Palonosetron

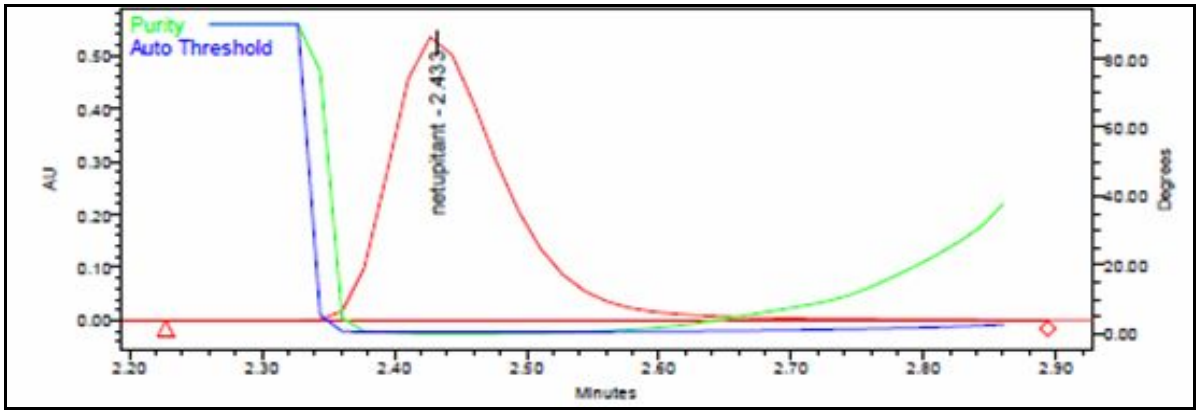


Figure 23: Purity plot of photolytic degradation for Netupitant

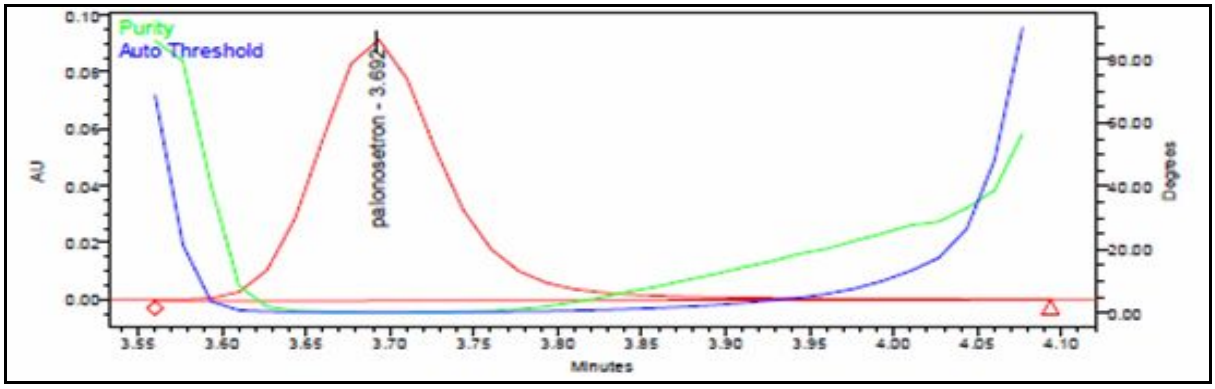


Figure 24: Purity plot of photolytic degradation for Palonosetron

Water Degradation Studies

Stress testing under neutral conditions was studied by refluxing the drug in water for 6hrs at a temperature of 60ºC. For HPLC study, the resultant solution was diluted to 300µg/ml of Netupitant and 0.5µg/ml of Palonosetron solution and 20µl solutions were injected into the HPLC system and the chromatogram were recorded to assess the stability of sample was shown in (Figure 25) and purity plot of water degradation for Netupitant and Palonosetron was shown in (Figure 26 and 27).

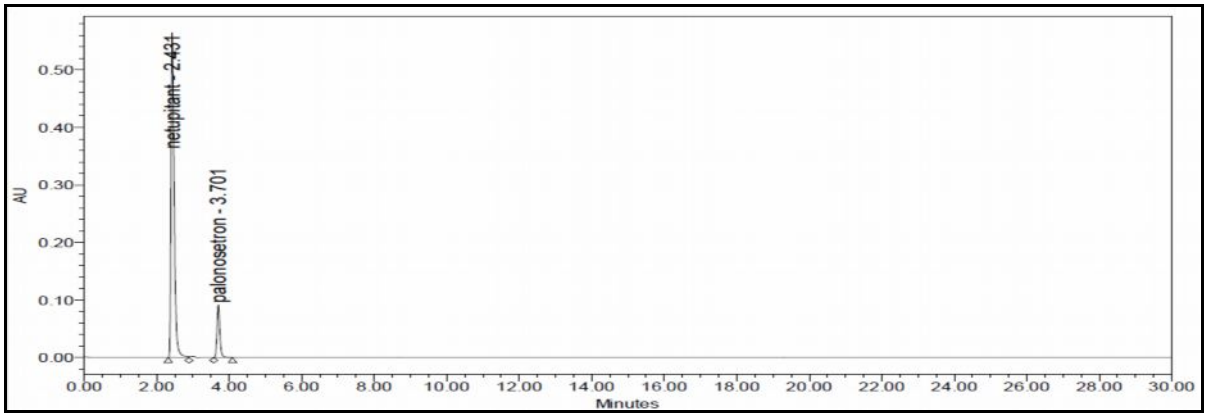


Figure 25: Chromatogram of water degradation for Netupitant and Palonosetron

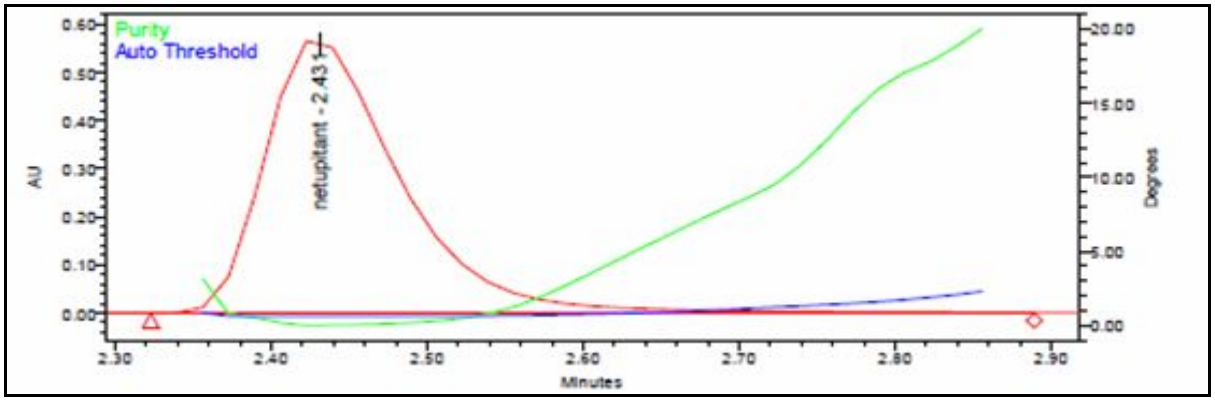


Figure 26: Purity plot of water degradation for Netupitant

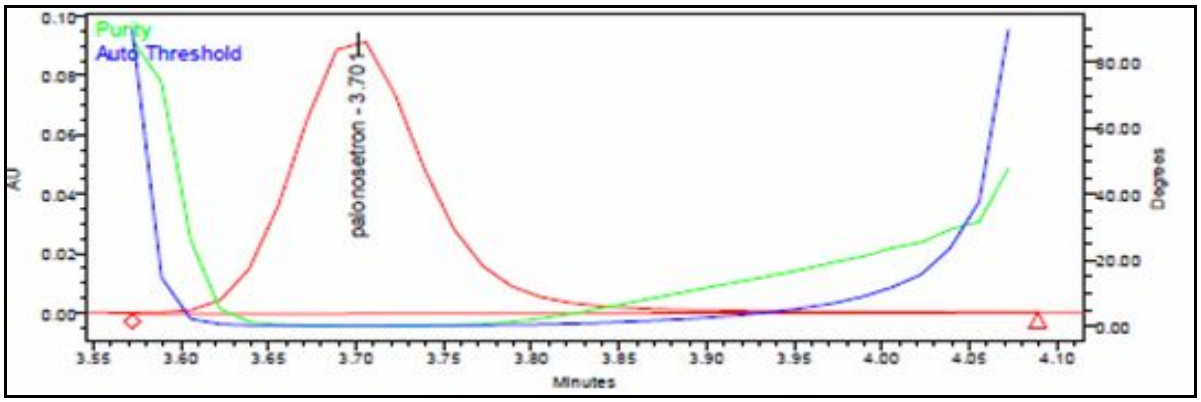


Figure 27: Purity plot of water degradation for Palonosetron

Conclusion

RP-HPLC method for the simultaneous estimation of Netupitant and Palonosetron in their combine dosage form was established and validated as per the ICH guidelines. Linearity was achieved for Netupitant and Palonosetron in the range of 75-450µg/ml for Netupitant and 0.125-0.75µg/ml for Palonosetron with correlation coefficients (r^2=0.999). The percentage recoveries of Netupitant and Palonosetron were achieved in the range of 98-102% which was within the acceptance criteria. The percentage RSD was NMT 2 % which proved the precision of the developed method. The developed method is simple, sensitive, rapid, linear, precise, rugged, accurate, specific, and robust. The forced degradation studies were performed by using HCl, NaOH, H_2O_2, thermal, UV radiation and water. Netupitant and Palonosetron are more sensitive towards acidic degradation condition and moderate degradation towards alkaline, thermal and very much resistant towards oxidative, photolytic and water degradation which was shown in Table 12. No interference from any components of pharmaceutical dosage form or degradation products was observed and the method has been successfully used to perform long term and accelerated stability studies of Netupitant and Palonosetron formulations. Hence it can be used for the routine analysis of Netupitant and Palonosetron in their bulk and combine dosage form.

Table 12: Forced degradation data of Netupitant and Palonosetron in different degradation conditions.

Forced degradation data of Netupitant						
Degradation condition	Retention time	Area	Purity Angle	Purity Threshold	USP Plate Count	Asymmetry
Acid hydrolysis	2.433	3101990	0.526	0.984	5478	1.5
Alkaline hydrolysis	2.431	3197779	0.360	0.547	3858	1.5
Oxidative degradation	2.433	3231281	0.484	0.985	5465	1.5
Thermal degradation	2.431	3149595	0.229	0.619	3919	1.5
Photolytic degradation	2.433	3227453	0.376	0.528	3934	1.5
Water degradation	2.431	3237175	0.285	0.621	3906	1.5
Forced degradation data of Palonosetron						
Degradation condition	Retention time	Area	Purity Angle	Purity Threshold	USP Plate Count	Asymmetry
Acid hydrolysis	3.691	471699	0.290	0.564	12296	1.2
Alkaline hydrolysis	3.686	474060	0.291	0.648	12006	1.2
Oxidative degradation	3.691	476831	0.283	0.571	12116	1.2
Thermal degradation	3.701	473166	0.311	0.498	11733	1.2
Photolytic degradation	3.692	477933	0.300	0.502	11257	1.2

Water degradation	3.701	476173	0.301	0.485	11422	1.2
Degradation condition	Drug Recovered (%)			Drug Decomposed (%)		
	Netupitant	**Palonosetron**		**Netupitant**	**Palonosetron**	
Standard	100	100		100	100	
Acid hydrolysis	95.77	98.57		4.23	1.43	
Alkaline hydrolysis	98.73	99.07		1.27	0.93	
Oxidative degradation	99.77	99.65		0.23	0.35	
Thermal degradation	97.24	98.88		2.76	1.12	
Photolytic degradation	99.65	99.88		0.35	0.12	
Water degradation	99.95	99.51		0.05	0.49	

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