RP-HPLC METHOD DEVELOPMENT AND VALIDATION FOR THE STABILITY INDICATING FOR THE DETERMINATION OF NETUPITANT AND PALONOSETRON IN PURE FORM AND MARKETED PHARMACEUTICAL DOSAGE FORM

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ABSTRACT : A New Analytical Method Development and Validation for Netupitant and Palonosetron in bulk and Combine Dosage Form by RP-HPLC, New method was established for simultaneous estimation of Netupitant and Palonosetron by RP-HPLC method. The chromatographic conditions were successfully developed for the separation of Netupitant and Palonosetron by using Symmetry C18 5µm (4.6 x 150mm), flow rate was 1.0 ml/min, mobile phase ratio was Phosphate buffer (0.02M) pH-3.8: Methanol: Acetonitrile (60:20:20%v/v), detection wavelength was 260nm. The retention times of Netupitant and Palonosetron were found to be 2.324mins and 4.314mins respectively. The % purity of Netupitant and Palonosetron was found to be 99.865% and 99.658% respectively. The analytical method was validated according to ICH guidelines (ICH, Q2 (R1)). The linearity study n Netupitant and Palonosetron was found in concentration range of 0µg-36µg and 0µg-39µg and correlation coefficient (r2) was found to be 0.9995 and 0.9998, % recovery was found to be 100.280, %RSD for repeatability was 0.174 and 0.709, % RSD for intermediate precision was 0.093 and 0.937 respectively. The precision study was precise, robust, and repeatable. LOD value was 1.377 and 1.079, and LOQ value was 4.174 and 3.272 respectively. Hence the suggested RP-HPLC method can be used for routine analysis of Netupitant and Palonosetron in API and Pharmaceutical dosage form.

Keywords: Netupitant and Palonosetron, Method Development, Validation, Accuracy.

I.INTRODUCTION

Netupitant is a selective neurokinin 1 (NK1) receptor antagonist with potential antiemetic activity. Netupitant[1] competitively binds to and blocks the activity of the human substance P/NK1 receptors in the central nervous system (CNS), thereby inhibiting NK1-receptor binding of the endogenous tachykinin neuropeptide substance P (SP), which may result in the prevention of chemotherapy-induced nausea and vomiting (CINV). SP is found in neurons of vagal afferent fibers innervating the brain-stem nucleus tractus solitarii and the area postrema, which contains the chemoreceptor trigger zone (CTZ), and may be elevated in response to chemotherapy. The NK-receptor is a G-protein receptor coupled to the inositol phosphate signal-transduction pathway and is found in both the nucleus tractus solitarii and the area postrema. Netupitant is an antiemitic drug approved by the FDA in October 2014 for use in combination with palonosetron for the prevention of acute and delayed vomiting and nausea associated with cancer chemotherapy including highly emetogenic chemotherapy. Netupitant[2] is a neurokinin 1 receptor antagonist. The combination drug is marketed by Eisai Inc. and Helsinn Therapeutics (U.S.) Inc. under the brand Akynzeo. The IUPAC Name of Netupitant[3] is 2-[3, 5-bis (trifluoro methyl) phenyl]-N, 2-dimethyl-N-[4-(2-methyl phenyl)-6-(4-methyl piperazin-1-yl) pyridin-3-yl] propanamide. The Chemical Structure of Netupitant is following

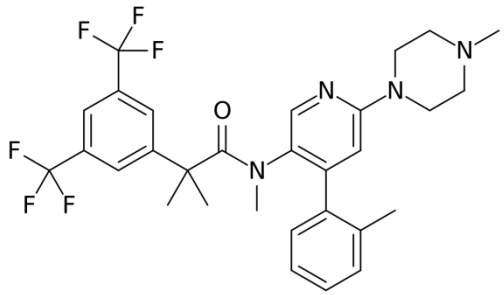

Fig.1. Chemical Structure of Netupitant

Palonosetron is a carbazole derivative and a selective serotonin receptor antagonist with antiemetic activity. Palonosetron[4] competitively blocks the action of serotonin at 5-hydroxytryptamine type 3 (5-HT3) receptors located on vagal afferents in the chemoreceptor trigger zone (CTZ), resulting in suppression of chemotherapy-induced nausea and vomiting. The CTZ is located in the area postrema on the dorsal surface of the medulla oblongata at the caudal end of the fourth ventricle and outside the blood-brain barrier (BBB). Palonosetron (INN, trade name Aloxi) is an antagonist of 5-HT3 receptors that is indicated for the prevention and treatment of chemotherapy-induced nausea and vomiting (CINV). It is the most effective of the 5-HT3 antagonists in controlling delayed CINV nausea and vomiting that appear more than 24 hours after the first dose of a course of chemotherapy and is the only drug of its class approved for this use by the U.S. Food and Drug Administration. As of 2008, it is the most recent 5-HT3 antagonist to enter clinical use. The IUPAC Name of Palonosetron[5] is (3aS)-2-[(3S)-1-azabicyclo [2.2.2] octan-3-yl]-3a, 4, 5, 6-tetrahydro-3H-benzo [de]isoquinolin-1-one. The Chemical Structure of Palonosetron[6] is as follows

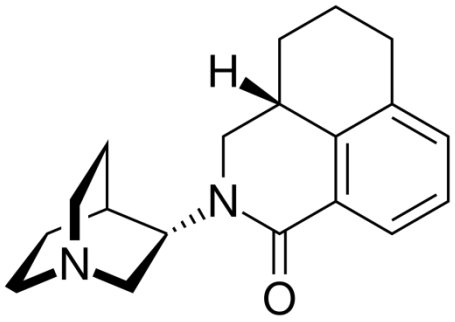

Fig.2. Chemical Structure of Palonosetron

II. MATERIALS AND METHODS

Reagents and Chemicals: All chemicals and reagents used were HPLC grade. Netupitant and Palonosetron standard was obtained from Local Market. Marketed formulation of Netupitant and Palonosetron was obtained commercially. HPLC grade Acetonitrile was procured from Merck Ltd. All other chemical reagents were of analytical grade.

Instrumentation and Chromatographic Conditions: A Waters HPLC system was utilized, consisting of the following components: Binary pump LC – 20 AD, vacuum degasser unit DGU – 20 A5 and a UV/VIS variable detector SPD – 20 A. Separation was carried out on a Symmetry C18 5µm (4.6 x 150mm) under reversed phase chromatographic conditions. The mobile phase consisted of Phosphate buffer (0.02M) pH-3.8: Methanol: Acetonitrile in the ratio of 60:20:20%v/v. The mobile phase was filtered through 0.45 µm membrane filter and degassed by using sonicator for about 15 min before use. The sample solutions were also filtered using 0.45 µm membrane filters. The mobile phase was delivered isocratically at a flow rate 1 mL/min. The column was maintained at ambient temperature. The injection volume was a 20 µL and the total run time was 7 minutes. The detection was carried out at 260 nm.

Wavelength Detection (Or) Selection of Wavelength: The detection wavelength was selected by dissolving the drug in mobile phase to get a concentration of 10µg/ml for individual and mixed standards. The resulting solution was scanned in U.V range from 200-400nm.

Preparation of Phosphate buffer: (pH: 3.8): Weighed 0.136086 grams of KH_2PO_4 was taken into a 1000ml beaker, dissolved and diluted to 1000ml with HPLC water, adjusted the pH to 3.8 with ortho phosphoric acid.

Preparation of Mobile Phase: A mixture of pH 3.8 Phosphate buffer 600 mL (60%), 200 mL of MEOH

(20%) and 200 mL of Acetonitrile are taken and degassed in ultrasonic water bath for 15 minutes. Then this solution is filtered through 0.45 µ filter under vacuum filtration[7].

Diluent Preparation: Mobile phase is used as Diluent**.**

Preparation of the individual Netupitant standard preparation: 10mg of Netupitant working standard was accurately weighed and transferred into a 10ml clean dry volumetric flask and about 2ml of Diluent is added. Then it is sonicated to dissolve it completely and made volume up to the mark with the diluent[8]. (Stock solution). Further 10.0 ml from the above stock solution is pipette into a 100 ml volumetric flask and was diluted up to the mark with diluent.

Preparation of the individual Palonosetron standard preparation: 10mg of Palonosetron working standard was accurately weighed and transferred into a 10ml clean dry volumetric flask and about 2ml of Diluent is added. Then it is sonicated to dissolve it completely and made volume up to the mark with the diluent. (Stock solution). Further 10.0ml from the above stock solution[9] is pipette into a 100 ml volumetric flask and was diluted up to the mark with diluent.

Preparation of Sample Solution: (Tablet) Accurately 10 tablets are weighed and crushed in mortar and pestle and weight equivalent to 10 mg of Netupitant and Palonosetron (marketed formulation) sample into a 10mL clean dry volumetric flask and about 7mL of Diluents is added and sonicated[10] to dissolve it completely and made volume up to the mark with the same solvent. (Stock solution) Further 3 ml of above stock solution was pipetted into a10ml volumetric flask and diluted up to the mark with diluent.

Procedure: 20µL of the standard, sample are injected into the chromatographic system and the areas for Netupitant and Palonosetron peaks are measured and the %Assay[11] is calculated by using the formulae.

RESULTS AND DISCUSSION

Selection of Wavelength: The detection wavelength[12] was selected by dissolving the drug in mobile phase to get a concentration of 10µg/ml for individual and mixed standards. The resulting solution was scanned in U.V range from 200-400nm. The overlay spectrum of Netupitant and Palonosetron was obtained and the isobestic point of Netupitant and Palonosetron showed absorbance's maxima[13] at 260 nm.

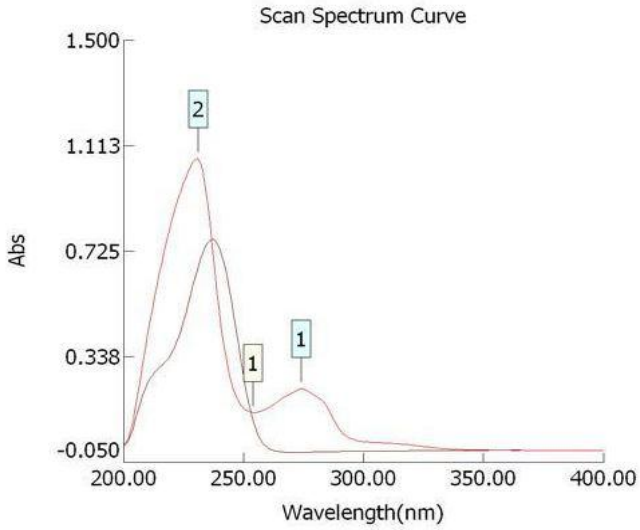

Fig.3. Overlay spectrum for Netupitant and Palonosetron

Method Development

Optimized Chromatographic Method:

Column : Symmetry C18 5µm (4.6 x 150mm)
Mobile phase ratio: Phosphate buffer (0.02M) pH-3.8: Methanol: Acetonitrile (60:20:20%v/v)
Detection wavelength : 260nm
Flow rate : 1ml/min
Injection volume : 20µl
Column temperature : Ambient
Auto sampler temperature: Ambient

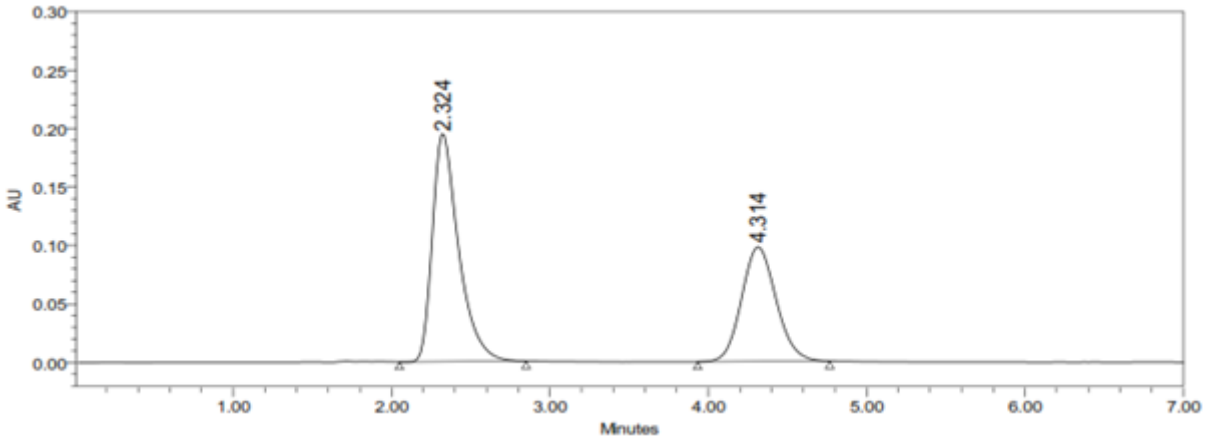

Fig.4. Chromatogram of Optimized Method

Method Validation

System Suitability: System Suitability was the checking of a system to ensure system performance before or during the analysis of unknowns. Parameters[14,15] such as tailing factor, resolution, plate count and reproducibility are determined and compared against the specification suitable for the method.

Table-1: Data of System Suitability Test for Netupitant

S.No.	Injection No.	RT	Area	USP Plate Count	USP Tailing	Resolution
1	Injection 1	2.327	946257	5245	1.3	8.6
2	Injection 2	2.328	946325	5326	1.2	8.7
3	Injection 3	2.319	946859	5124	1.3	8.9
4	Injection 4	2.320	945875	5296	1.3	8.6
5	Injection 5	2.323	946396	5248	1.2	8.9
6	Injection 6	2.328	946548	5295	1.3	8.7
Mean			**946376.7**			
S.D			**325.8936**			
%RSD			**0.034436**			

Table-2: Data of System Suitability Test for Palonosetron

S.No.	Injection No.	RT	Area	USP Plate Count	USP Tailing
1	Injection 1	4.331	112543	3854	1.4
2	Injection 2	4.341	111652	3965	1.5
3	Injection 3	4.299	112854	3874	1.3
4	Injection 4	4.313	111485	3698	1.5
5	Injection 5	4.325	113526	3785	1.4
6	Injection 6	4.341	112985	3965	1.6
Mean			**112507.5**		
S.D			**795.4945**		
%RSD			**0.707059**		

Linearity: Linearity is the ability of the method to elicit test results that are directly proportional to analyte concentration within a given range. Linearity[16] is generally reported as the variance of the slope of the regression line[17].

Table-3: Chromatographic Data for Linearity Study of Netupitant

Concentration µg/ml	Average Peak Area
0	0
12	523864
18	764875
24	999874
30	1235658
36	1488542

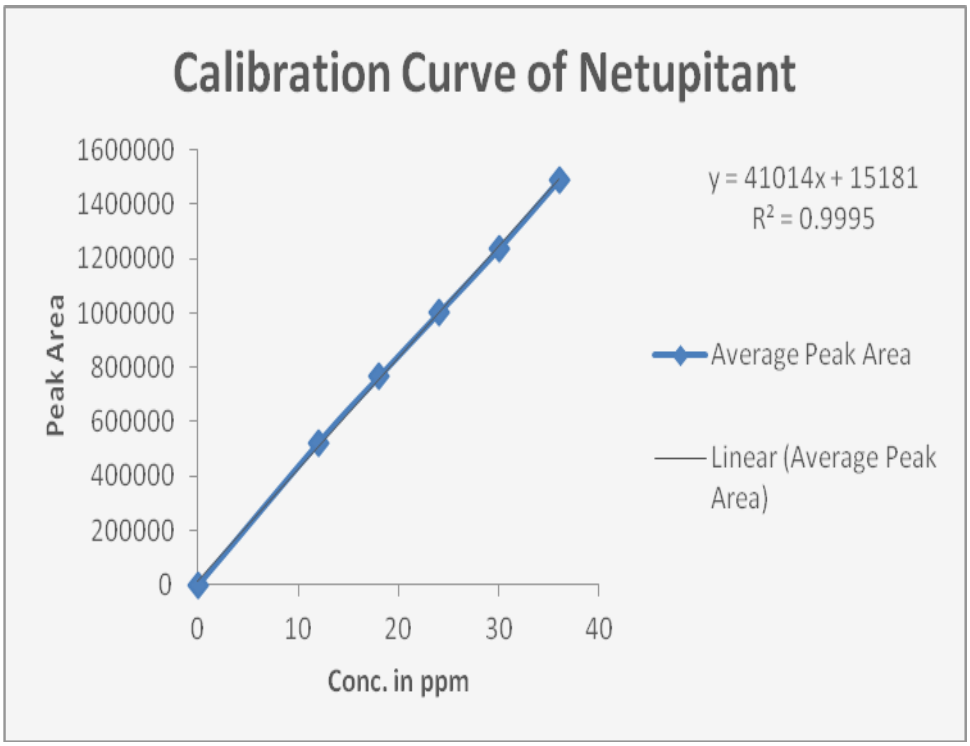

Fig.5.Calibration Curve of Netupitant

Linearity Plot:

The plot of Concentration (x) versus the Average Peak Area (y) data of Netupitant is a straight line.

Y = mx + c
Slope (m) = 41014
Intercept (c) = 15181
Correlation Coefficient (r) = 0.99

Validation Criteria: The response linearity is verified if the Correlation Coefficient is 0.99 or greater.
Conclusion: Correlation Coefficient[18] (r) is 0.99, and the intercept is 15181. These values meet the validation criteria.

Table-4: Chromatographic Data for Linearity Study of Palonosetron

Concentration µg/ml	Average Peak Area
0	0
13	65698
19.5	98254
26	128587
32.5	160648
39	191874

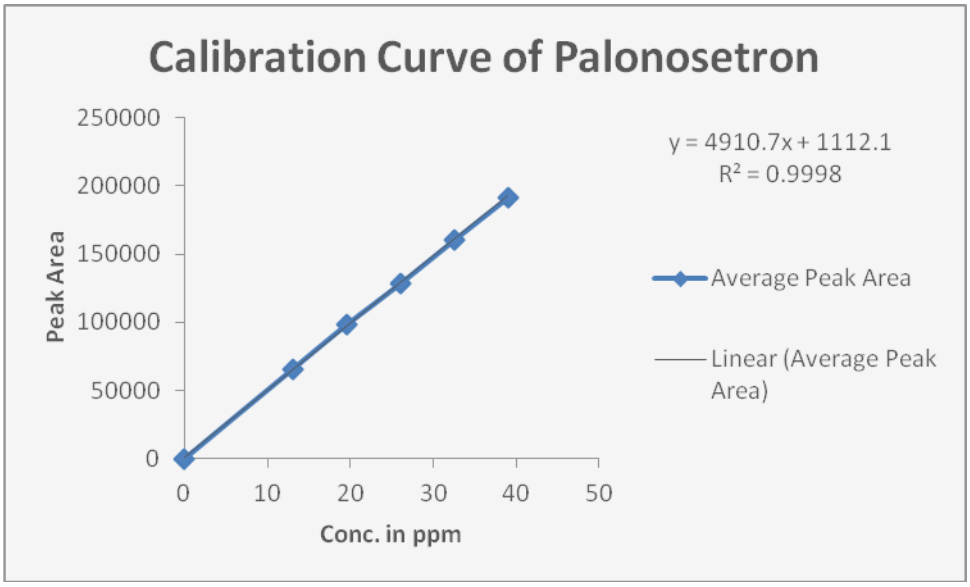

Fig.6. Calibration Curve of Palonosetron

Linearity Plot:

The plot of Concentration (x) versus the Average Peak Area (y) data of Palonosetron is a straight line.

Y = mx + c
Slope (m) = 4910.7
Intercept (c) = 1112.1
Correlation Coefficient (r) = 0.99

Validation Criteria: The response linearity is verified if the Correlation Coefficient is 0.99 or greater.

Conclusion: Correlation Coefficient (r) is 0.99, and the intercept is 1112.1. These values meet the validation criteria.

Accuracy:

The accuracy[19,20] of the method was also studied, at three final concentration levels, i.e., 12, 24, and 36µg/ml for Netupitant, and 13, 26, and 39µg/ml for Palonosetron. In this method, a known amount of the active was added to a determined amount of placebo and was subsequently calculated for Netupitant and Palonosetron, recovered in relation to the added amount of the drugs.

Table-5: Accuracy results of Netupitant

%Concentration (at specification Level)	Area	Amount added (mg)	Amount found (mg)	% Recovery	Mean Recovery

50%	508367	12	12.024	100.200%	100.150%
100%	999100.3	24	23.989	99.954%	
150%	1496200.3	36	36.110	100.305%	

Table-6: Accuracy results of Palonosetron

%Concentration (at specification Level)	Area	Amount Added(mg)	Amount Found(mg)	% Recovery	Mean Recovery
50%	65093.67	13	13.029	100.223%	100.280%
100%	129339.3	26	26.111	100.426%	
150%	178242.7	39	36.070	100.194%	

The results obtained for recovery[21] at 50%, 100%, 150% are within the limits. Hence method is accurate.

Precision:

The precision[22,23] of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions.

Repeatability: Obtained five (5) replicates of 100% accuracy solution as per experimental conditions. Recorded the peak areas and calculated % RSD[24].

Table-7: Results of repeatability for Netupitant:

S. No.	Peak Name	Retention time	Area (µV*sec)	Height (µV)	USP Plate Count	USP Tailing	Resolution
1	Netupitant	2.321	946253	155465	5326	1.36	8.25
2	Netupitant	2.317	947845	154578	5246	1.37	8.26
3	Netupitant	2.323	945867	155845	5478	1.35	8.34
4	Netupitant	2.322	948572	155698	5425	1.38	8.37
5	Netupitant	2.324	949857	154857	5326	1.36	8.39
Mean			**947678.8**				
Std. Dev			**1649.66**				
%RSD			**0.174074**				

Table-8: Results of Repeatability for Palonosetron:

S. No.	Peak Name	Retention time	Area (µV*sec)	Height (µV)	USP Plate Count	USP Tailing
1	Palonosetron	4.304	111563	13254	3869	1.42
2	Palonosetron	4.300	111254	13425	3852	1.43
3	Palonosetron	4.308	111672	13254	3896	1.45
4	Palonosetron	4.310	112654	13265	3962	1.42
5	Palonosetron	4.314	113123	13154	3874	1.48
Mean			**112053.2**			
Std. Dev			**795.2614**			
%RSD			**0.709718**			

Intermediate Precision/Ruggedness:

Table-9: Results of Intermediate precision for Netupitant

S.No.	Peak Name	RT	Area (µV*sec)	Height (µV)	USP Plate Count	USP Tailing	Resolution
1	Netupitant	2.328	956325	156325	5246	1.35	8.24
2	Netupitant	2.326	958741	157854	5367	1.38	8.26
3	Netupitant	2.327	957542	156986	5265	1.34	8.47
4	Netupitant	2.326	956895	158547	5384	1.39	8.29
5	Netupitant	2.331	957486	156985	5297	1.35	8.34
Mean			**957397.8**				
Std. Dev.			**899.5091**				
% RSD			**0.093954**				

Table-10: Results of Intermediate precision for Palonosetron

S.No.	Peak Name	Rt	Area (µV*sec)	Height (µV)	USP Plate count	USP Tailing
1	Palonosetron	4.335	121231	13458	3896	1.52
2	Palonosetron	4.336	121457	13674	3785	1.54
3	Palonosetron	4.334	123142	13485	3969	1.58
4	Palonosetron	4.337	121325	13958	3859	1.57
5	Palonosetron	4.340	123654	13875	3789	1.59
Mean			**122161.8**			
Std. Dev.			**1145.733**			
% RSD			**0.937882**			

Method Robustness: Influence of small changes in chromatographic conditions such as change in flow rate (± 0.1ml/min), Temperature (±2^{0}C), Wavelength of detection (±5nm) & acetonitrile content in mobile phase (±2%) studied to determine the robustness[25] of the method are also in favour of (Table-11, % RSD < 2%) the developed RP-HPLC method for the analysis of Netupitant (API).

Table-11: Result of Method Robustness Test

Change in parameter	% RSD
Flow (1.1 ml/min)	0.96
Flow (0.9 ml/min)	0.84
Temperature (27^{0}C)	0.81
Temperature (23^{0}C)	0.94

Wavelength of Detection (265 nm)	0.56
Wavelength of detection (255 nm)	0.17

Influence of small changes in chromatographic conditions[26] such as change in flow rate (± 0.1ml/min), Temperature[27] (±2^{0}C), Wavelength of detection (±5nm) & acetonitrile content in mobile phase[28] (±2%) studied to determine the robustness of the method are also in favour of (Table-12, % RSD < 2%) the developed RP-HPLC method for the analysis of Palonosetron (API).

Table-12: Result of method robustness test

Change in parameter	% RSD
Flow (1.1 ml/min)	0.58
Flow (0.9 ml/min)	0.64
Temperature (27^{0}C)	0.72
Temperature (23^{0}C)	0.91
Wavelength of Detection (265 nm)	0.86
Wavelength of detection (255 nm)	0.78

Limit of detection (LOD) & Limit of quantification (LOQ):

The detection limit[29] (LOD) and quantitation limit (LOQ) may be expressed as:

$$L.O.D. = 3.3\ (SD/S).$$

$$L.O.Q. = 10\ (SD/S)$$

Where, SD = Standard deviation of the response

S = Slope of the calibration curve

Result & Discussion: The Minimum concentration level at which the analyte can be reliable detected (LOD) & quantified (LOQ) were found to be 1.377µg/ml & 4.174µg/ml respectively for Netupitant.

The LOD was found to be 1.079µg/ml and LOQ[30] was found to be 3.272µg/ml for Palonosetron which represents that sensitivity of the method is high.

Estimation of Netupitant & Palonosetron in TABLET Dosage Form

Twenty tablets were taken and the I.P. method was followed to determine the average weight. Finally the weighed tablets are powdered and triturated well by using mortar and pestle. A quantity of powder which is equivalent to the 100mg of drugs were transferred to a clean and dry 100ml of volumetric flask and add 70 ml of mobile phase and the resulted solution was sonicated for 15 minutes by using ultra sonicator, Then the final volume was make up to the mark with the mobile phase. The final solution was filtered through a selected membrane filter (0.45 µm) and in order to sonicated to degas the mobile phase (Solvent system). From this above stock solution (1 ml) was transferred to five different 10 ml volumetric flasks and volume was made up to 10 ml with same solvent system (Mobile phase).

The prepared solutions were injected in five replicates into the HPLC system and the observations were recorded. A duplicate injection (Blank Solution) of the standard solution also injected into the HPLC system and the chromatograms and peak areas were recorded and calculated. The obtained data are shown in Table 13.

Assay % =

$$\frac{AT}{AS} \times \frac{WS}{DS} \times \frac{DT}{WT} \times \frac{P}{100} \times \text{Average weight} = \text{mg/tab}$$

Where:

AT = Test Preparation Peak Area

AS = Standard preparation Peak Area

WS = Working standard weight taken in mg
WT = Sample weight taken in mg
DS = Standard solution dilution
DT = Sample solution dilution
P = Working standard percentage purity

The assay[31,32] was performed as explained in the previous chapter (Above). The results which are obtained are following:

Table-13: Assay of Netupitant & Palonosetron Tablets

Brand Name of Tablets	Labelled Amount of Drug (mg) Netupitant & Palonosetron	Mean (±SD) amount (mg) found by the proposed method (n=6)	Mean (± SD) Assay (n = 6)
Akynzeo Capsule (Catalent Pharma Solutions)	300/0.5	299.687 (±0.09) /0.493 (±0.08)	99.865 (±0.245) /99.658 (±0.354)

Result and Discussion: The assay of Akynzeo Tablets containing Netupitant and Palonosetron was found to be 99.865% and Palonosetron was found to be 99.658%.

Forced Degradation Studies

The results of the forced degradation studies[33,34] indicated the specificity of the developed method that has been developed. Netupitant and Palonosetron were stable only in acidic and thermal stress conditions. The results of stability studies are given in the following Tables-14 & 15.

Table-14: Results of Force Degradation Studies of Netupitant API

Stress Condition	Time (hours)	Assay of active substance	Assay of degraded products	Mass Balance (%)
Acid Hydrolysis (0.1N HCl)	24Hrs.	87.316	12.684	100.00
Basic Hydrolysis (0.IN NaOH)	24Hrs.	78.155	21.845	100.00
Thermal Degradation (60 ^{0}C)	24Hrs.	86.215	13.785	100.00
UV (254nm)	24Hrs.	76.346	23.654	100.00
3% Hydrogen Peroxide	24Hrs.	75.104	24.896	100.00

Table-15: Results of Force Degradation Studies of Palonosetron API

Stress Condition	Time (hours)	Assay of active substance	Assay of degraded products	Mass Balance (%)
Acid Hydrolysis (0.1N HCl)	24Hrs.	85.155	14.845	100.00
Basic Hydrolysis (0.IN NaOH)	24Hrs.	77.514	22.486	100.00
Thermal Degradation (60 ^{0}C)	24Hrs.	84.522	15.478	100.00
UV (254nm)	24Hrs.	74.251	25.749	100.00

3% Hydrogen Peroxide	24Hrs.	73.015	26.985	100.00

III. SUMMARY AND CONCLUSION

To develop a precise, linear, specific & suitable stability indicating RP-HPLC method for simultaneous analysis of Netupitant and Palonosetron different chromatographic conditions were applied & the results observed are presented in previous chapters. Isocratic elution is simple, requires only one pump & flat baseline separation for easy and reproducible results. So, it was preferred for the current study over gradient elution. In case of RP-HPLC various columns are available, but here Symmetry C18 5µm (4.6 x 150mm) column was preferred because using this column peak shape, resolution and absorbance were good. Detection wavelength was selected after scanning the standard solution of drug over 200 to 400nm. From the U.V spectrum of Netupitant and Palonosetron it is evident that most of the HPLC work can be accomplished in the wavelength range of 240-300 nm conveniently. Further, a flow rate of 1 ml/min & an injection volume of 20 µl were found to be the best analysis.

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