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Original Article

ARP-HPLC STABILITY INDICATING METHOD DEVELOPMENT AND VALIDATION FOR THE SIMULTANEOUS DETERMINATION OF NETUPITANT AND PALONOSETRON IN PHARMACEUTICAL DOSAGE FORM

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ABSTRACT

Objective: A simple, precise, sensitive, rapid, accurate, and specific method was developed and validated for the simultaneous determination of Netupitant and Palonosetron in Pharmaceutical dosage form.

Methods: The separation was done on the ODS C_{18} column of dimensions (150 mm x 4.6, 5 µm) with the mobile phase 0.1N potassium dihydrogen orthophosphate pH 3.3 and acetonitrile in a 50:50 ratio, at a flow rate of 1.0 ml/min and injection volume of 10 µl. The optimum wavelength selected was 274 nm and the temperature of the column was maintained at 30 °C.

Results: The retention time was 2.199 min and 2.893 min and they were linear in the concentration range of 75-450 µg/ml and 0.125-0.75 µg/ml for Netupitant and Palonosetron, respectively. The repeatability and intermediate precision were found to be within acceptable limits. Regression equation of Netupitant and Palonosetron is Y=6329x+42914 and Y= 258884x+3103.9, respectively. LOD was found to be 0.33µg/ml, 0.99µg/ml and LOQ was 0.01µg/ml, 0.04µg/ml for Netupitant and Palonosetron. The correlation coefficient (R^2) value was found to be 0.999 and %recovery was obtained as 100.32%and 99.6% for Netupitant and Palonosetron, respectively. Forced degradation studies reveal that the drugs are unstable under acidic conditions.

Conclusion: The flexibility, accuracy, precision of the developed method ensures applicability in routine analysis of pharmaceutical dosage form.

Keywords: Netupitant, Palonosetron, Stability studies, Validation

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INTRODUCTION

Chemotherapy-induced nausea and vomiting (CINV) is a common adverse effect experienced by cancer patients. CINV impacts the quality of life and treatment outcomes. Different treatment regimens are available to treat CINV, including 5HT3 receptor antagonists, NK1 antagonists, and corticosteroids. The combination of Netupitant and Palonosetron is the most effective and safe oral combination for the prevention of CINV [1].

Netupitant (NTP) is in a class of medications called Neurokinin antagonists, which work by blocking neurokinin, a natural substance in the brain that causes nausea and vomiting. Netupitant has a IUPAC name of2-[3,5-bis(trifluoromethyl)phenyl]-*N*,2-dimethyl-*N*-[4-(2-methyl phenyl)-6-(4-methyl piperazin-1-yl)pyridin-3-yl]propenamide. The structure of Netupitant was given in fig. 1 [2].

Palonosetron (PNT) is in a class of medication called $5HT_3$ receptor antagonists, which work by blocking serotonin, a natural substance in the body that causes nausea and vomiting. IUPAC name of Palonosetron (3aS)-2-[(3S)-1-azabicyclo[2.2.2]octan-3-yl]-3a,4,5,6-tetrahydro-3H-benzo[de]isoquinolin-1-one. The structure of the Palonosetron was represented in fig. 2 [3].

The literature survey discloses that the method development and validation of the simultaneous estimation of Netupitant and Palonosetron by using RP-HPLC have limitations like long run time, low resolution, and a few analytical works found on stability-indicating studies [4-8]. The new study was developed for the simultaneous estimation of Netupitant and Palonosetron by using reverse-phase HPLC for the precise, rapid, accurate analysis.

Nowadays, RP-HPLC is the most widely preferred analytical technique that could be applied to separate a wide range of molecules that requires a small sample size.

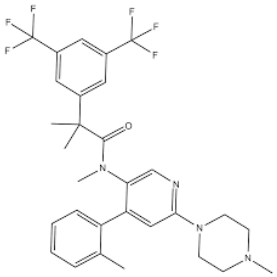

Fig. 1: Structure of netupitant [2]

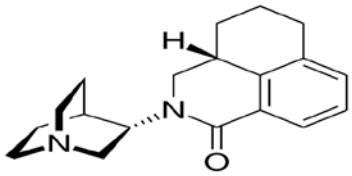

Fig. 2: Structure of palonosetron [3]

MATERIALS AND METHODS

1. The pharmaceutical-grade standards of NTP, PNT are obtained from MSN Laboratories Ltd. Hyderabad.

2. Marketed formulation (Akynzeo, manufactured by Glenmark Pharmaceuticals Ltd. Mumbai)

3. Chemical used in analysis-Acetonitrile (HPLC grade).

Instrumentation

The separation was done on WATERS HPLC 2695 system equipped with quaternary pumps, a Photo Diode Array detector, and Autosampler integrated with Empower 2 Software. The column of ODS C_{18} column of 150 mmx 4.6x 5µm dimensions was used for separation. UV-VIS spectrophotometer PG Instruments T60 with special bandwidth of 2 mm and 10 mm and matched quartz cells integrated with UV win 6 Software was used to measure Netupitant and Palonosetron solutions absorbances. Electronic Balance (Denver), p^H meter (BVK enterprises, India), Ultrasonicator (BVK enterprises, India) were used for the study.

Composition of analytical solutions [9, 10]

Diluent

Acetonitrile and Water taken in the ratio of 50:50 v/v were used as a diluent.

Mobile phase

In the mobile phase, a 50:50 v/v mixture of buffer and acetonitrile was utilized. 1.36g potassium dihydrogen orthophosphate buffer (pH-3.3)–1.36g potassium dihydrogen orthophosphate was accurately weighed and added to 1000 ml of Volumetric flask, followed by 900 ml milli-Q water, degas to sonicate, and finally make up the volume with water, before adding 1 ml Triethylamine and adjusting the pH to 3.3 with a dilute solution of orthophosphoric acid.

Standard solution

Weighed and transferred 300 mg Netupitant and 0.5 mg Palonosetron working Standards into a 100 ml clean dry volumetric flask, added 3/4th volume diluent, sonicated for 5 min, and rounded up to final volume adding diluents (3000 µg/ml of Netupitant and 5 µg/ml of Palonosetron).

Standard working solutions (100% solution)

In a 10 ml volumetric flask, add 1 ml from the above two stock solutions and finally rounded to 10 ml (3000 µg/ml of Netupitant and 0.5 µg/ml of Palonosetron).

Sample solution

5 tablets were weighed and the average weight of each tablet was calculated, then, the weight equivalent to 1 tablet was transferred into a 100 ml volumetric flask, 5 ml of diluents was added and sonicated for 25 min further, the volume was made up with diluent and filtered by HPLC filters (3000µg/ml of Netupitant and5µg/ml of Palonosetron)

Sample working solutions (100% solution)

1 ml of filtered sample stock solution was transferred to a 10 ml volumetric flask and made up of diluent (3000µg/ml of Netupitant and 0.5µg/ml of Palonosetron).

HPLC optimized conditions

Analytes can be separated using a 50:50 v/v mixture of 0.1N potassium dihydrogen orthophosphate (pH-3.3) buffer and acetonitrile flow rate across a column at 30 °C with a detection wavelength of 274 nm. Before injecting the solution, the column was equilibrated with the mobile phase. The injection had a capacity of a 10 µl. The optimized conditions of the discussed study were represented in table 1. The chromatogram for optimized conditions was represented in fig. 3.

Table 1: Optimized conditions of RP-HPLC method

S. No.	Variables	Conditions
1	Mobile phase	50% KH_2PO_4: 50% Acetonitrile
2	Diluent	Water: Acetonitrile (50:50)
3	Column	ODS C_{18} (150 mm×4.6, 5 µm)
4	Wavelength	274 nm
5	Column temperature	30 °C
6	Injection volume	10 ml
7	Flow rate	1 ml/min
8	Run time	5 min
9	Retention time	2.199 min (NTP) 2.893 min (PNT)

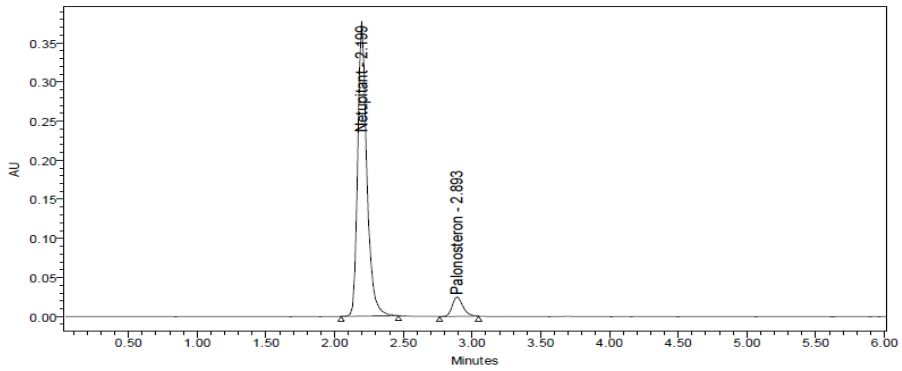

Fig. 3: Chromatogramfor optimized conditions

Method validation [11]

The method validation was performed according to the ICH Q2B (R2) guidelines. The parameters were validated, such as system suitability, linearity, precision, accuracy, the limit of detection, the limit of quantification, robustness, and assay. As per ICH Guidelines, stability studies were performed like acidic degradation, alkali, peroxide, thermal, UV, and aqueous degradation studies for both drugs (NTP and PNT).

System suitability parameters

System suitability is essentially performed before sample analysis to check the specifications of a liquid chromatographic system. That means

knowing that the system is working perfectly before any analysis of the pharmaceutical products on HPLC and other systems. Some of the parameters that were checked using the system suitability testing are resolution, retention time, pressure, column efficiency, repeatability, plate number, tailing factor, and signal-to-noise ratio. The data for the system suitability parameters were represented in table 2.

Table 2: System suitability parameters

Injection	Retention time		Tailing factor		Plate count		Resolution
	NTP[a]	PNT[b]	NTP	PNT	NTP	PNT	
1	2.197	2.891	1.32	1.2	5169	6788	5.2
2	2.197	2.892	1.29	1.23	5252	6778	5.2
3	2.199	2.893	1.27	1.21	5472	6758	5.3
4	2.199	2.896	1.29	1.21	6061	7188	5.1
5	2.2	2.896	1.31	1.19	5401	6462	5.2
6	2.201	2.897	1.3	1.19	5242	6726	5.2

a–Netupitant, b-Palonosetron

Standard solutions of Netupitant (50 ppm) and Palonosetron (25 ppm) were injected six and the above-mentioned parameters were determined. The acceptance limits for the above parameters such as the tailing factor (T≤ 2), theoretical plates (N) should be greater than 2000, resolution (Rs) should be more than 2. The chromatogram was represented in fig. 4.

All the system suitability parameters were passed and they were within the limits.

Specificity

According to ICH, the term specific generally refers to a method that produces a response for a single analyte only. The analyte should have no interference from other extraneous components and be well resolved from them. The standard, placebo, and blank samples were injected into the system separately.

Retention times of Netupitant and Palonosetron were 2.199 min and 2.893 min, respectively. We did not find interfering peaks in blank and placebo at retention times of these drugs in this method. So, this method was said to be specific. The chromatograms were represented in fig. 5, fig. 6, fig. 7.

Linearity

The linearity of a method is a measure of how well a calibration plot of response vs. concentration approximates a straight line. Linearity can be assessed by performing single measurements at several analyte concentrations. The data is then processed using linear least-squares regression. The resulting plot slope, intercept, and correlation coefficient provides the desired information on linearity.

Six linear concentrations of Netupitant (75_450µg/ml) and Palonosetron (0.125-0.75µg/ml) were injected in a duplicate manner. From the data, Linearity equations obtained for NTP and PNT were found to be Y= 6329x+42914 and Y= 258884x+3103.9, respectively. The correlation coefficient obtained was 0.999 for both drugs. The data for the linearity was given in table 3 for Netupitant and Palonosetron. The calibration graphs for both drugs were represented in fig. 8 and fig. 9.

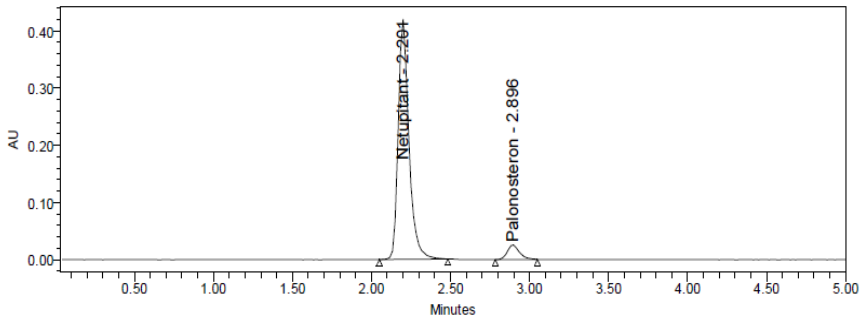

Fig. 4: Chromatogram for system suitability parameters

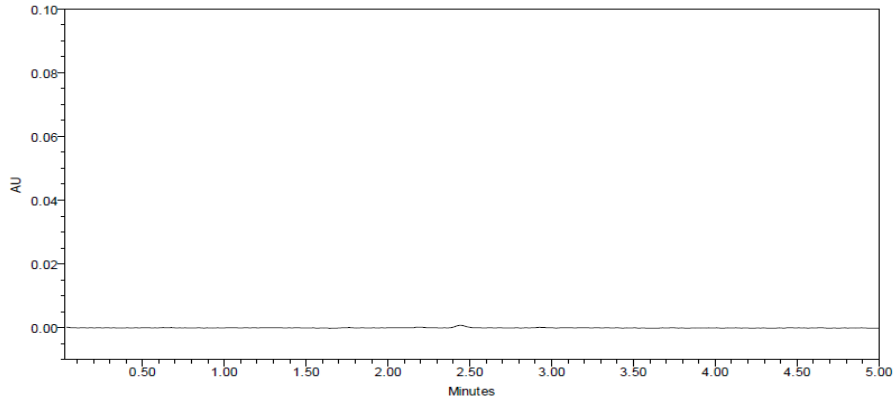

Fig. 5: Chromatogram of blank

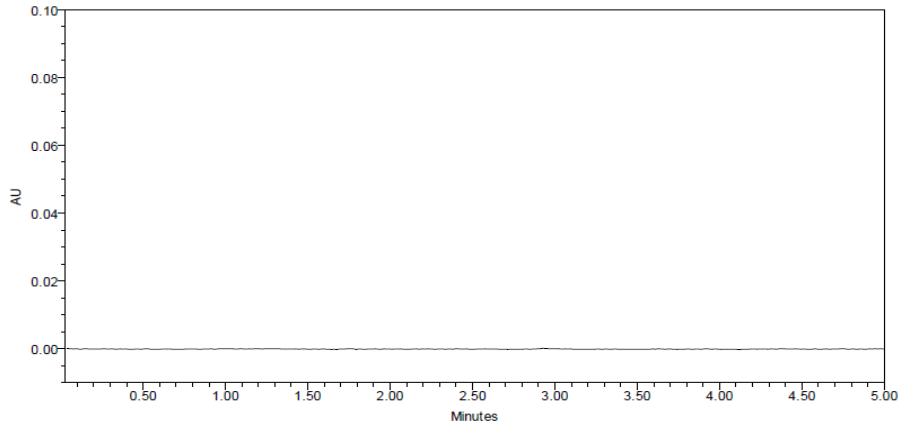

Fig. 6: Chromatogram of placebo

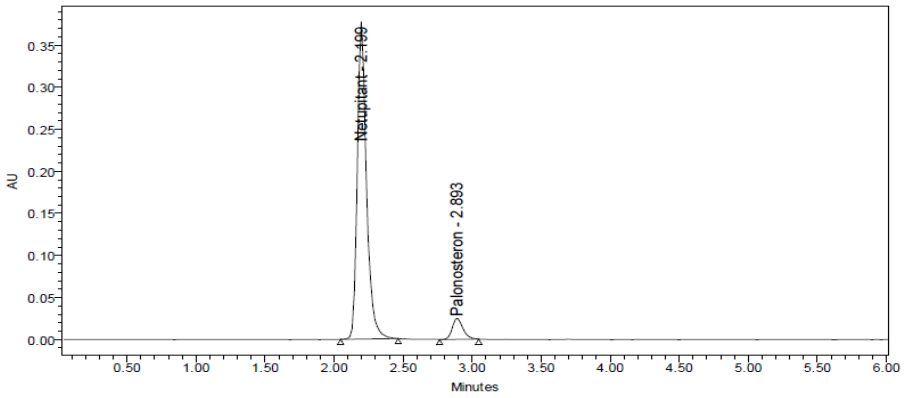

Fig. 7: Typical chromatogram

Table 3: Linearity table for netupitant and palonosetron

Netupitant		Palonosetron	
Conc. (µg/ml)	**Peak area**	**Conc. (µg/ml)**	**Peak area**
75	540274	0.125	35419
150	950694	0.25	67721
225	1502488	0.375	100285
300	1905914	0.5	132317
375	2433799	0.625	165710
450	2892430	0.75	196743

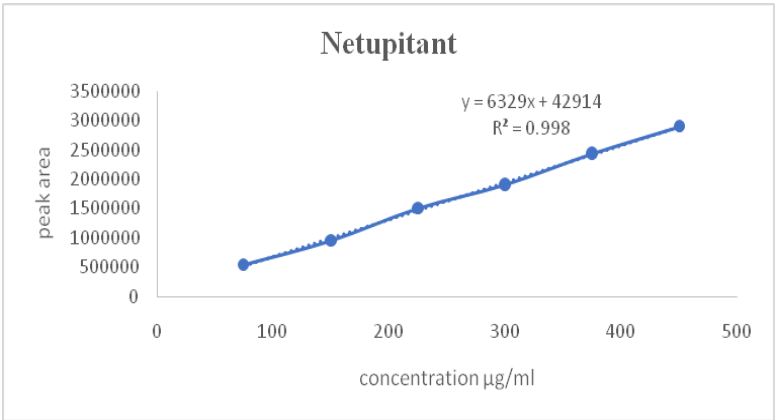

Fig. 8: Calibration graph showing linearity in the range of 75-450µg/ml for Netupitant drug

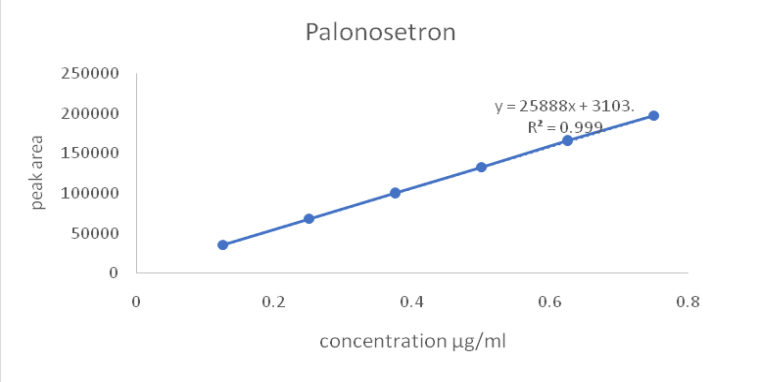

Fig. 9: Calibration graph showing linearity in the range of 0.125-0.75µg/ml for palonosetron drug

Precision

Precision can be defined as "The degree of agreement among individual test results when the procedure is applied repeatedly to multiple samplings of a homogenous sample".

System precision

From a single volumetric flask of working standard solution, six injections were given and the obtained areas were mentioned in the table. Average area, standard deviation, and % RSD were calculated for two drugs. % RSD obtained as 0.4% and 0.5%, respectively for Netupitant and palonosetron. As the limit of Precision was less than "2", the system precision was passed in this method. The data for system precision was reported in table 4 for both drugs.

Repeatability

Multiple sampling from a sample stock solution was prepared and six working sample solutions of the same concentrations were prepared, each injection from each working sample solution was given, and obtained areas were mentioned in the table. Average area, standard deviation, and % RSD were calculated for two drugs and obtained as 0.2% and 0.6%, respectively, for Netupitant and Palonosetron. As the limit of Precision was less than "2", the repeatability was passed in this method table 5. Represents data of repeatability for Netupitant and Palonosetron.

Table 4: This table represents the data for system precision

S. No.	Area of netupitant	Area of palonosetron
1.	1954145	134350
2.	1942166	133115
3.	1948328	133887
4.	1953557	134302
5.	1960732	133362
6.	1964079	132851
Mean	1953772	133645
SD	7989.7	629.1
%RSD	0.4	0.5

SD-standard deviation, %RSD-relative standard deviation

Table 5: Data for the repeatability

S. No.	Area of netupitant	Area of palonosetron
1.	1961234	133397
2.	1953909	131317
3.	1964776	133219
4.	1961325	132805
5.	1961826	133539
6.	1962039	133509
Mean	1960852	132964
SD	3642.3	850.7
%RSD	0.2	0.6

Table 6: Data for intermediate precision

S. No.	Area of netupitant	Area of palonosetron
1.	1896327	130809
2.	1908020	131637
3.	1894903	131822
4.	1913691	131768
5.	1908475	132146
6.	1912622	131399
Mean	1905673	131597
S. D	8115.1	456.7
%RSD	0.4	0.3

Intermediate precision

Multiple sampling from a sample stock solution was done and six working sample solutions of the same concentrations were prepared, each injection from each working sample solution was given on the next day of the sample preparation and obtained areas were mentioned in the above table. Average area, standard deviation, and % RSD were calculated for two drugs and obtained as 0.4% and 0.3%, respectively for Netupitant and palonosetron. As the limit of Precision was less than "2", the intermediate precision was passed in this method. Table 6 represents data for intermediate precision.

Limit of detection (LOD)

The lowest quantity or concentration of a component can be reliably detected with a given analytical method. The acceptance criteria signal to noise ratio (S/N) of LOD within 3:1. LOD was calculated by using the given formula:

$$LOD = 3.3\ \sigma/S$$

Where σ = Standard deviation of Intercepts of calibration curves, S = Mean of slopes of the calibration curves.

Limit of quantification (LOQ)

The limit of quantitation (LOQ) is the lowest concentration of analyte in a sample that can be determined with acceptable precision and accuracy under the stated experimental conditions. The acceptance criteria signal to noise ratio (S/N) of LOQ within 10:1. LOQ was calculated by using the given below formula:

$$LOQ = 10\ \sigma/S$$

Where σ = Standard deviation of Intercepts of calibration curves, S = Mean of slopes of the calibration curves.

Accuracy

Three levels of Accuracy samples were prepared by the standard addition method. Triplicate injections were given for each level of accuracy and mean. % Recovery was obtained as 100.32% and 99.62% for Netupitant and Palonosetron respectively. Table 7 represents the accuracy data for the Netupitant drug in levels of 50%, 100%, 150% in a triplicate manner.

Table 7: Data for lod and loq

Molecule	LOD (ppm)	LOQ (ppm)
Netupitant	0.33	0.99
Palonosetron	0.01	0.04

Robustness

The concept of robustness of an analytical procedure has been defined by the ICH as "a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters". The variable method parameters in the HPLC technique may involve flow rate, column temperature, sample temperature, pH, and mobile phase composition.

Table 7: Accuracy table of netupitant

% Level	Amount spiked (µg/ml)	Amount recovered (µg/ml)	%Recovery	Mean % recovery
50%	150	152.0232	101.35	100.98
	150	151.9569	101.30	
	150	150.4555	100.30	
	300	298.9231	99.64	99.41
100%	300	301.383	100.46	
	300	297.1499	99.05	
150%	450	452.7792	100.62	100.25
	450	450.4292	100.10	
	450	450.1959	100.04	

Table 8: Accuracy table of palonosetron

% Level	Amount spiked (µg/ml)	Amount recovered (µg/ml)	% Recovery	Mean % recovery
50%	0.25	0.249688	99.88	99.71
	0.25	0.247646	99.06	
	0.25	0.249115	99.65	
100%	0.5	0.502165	100.43	99.98
	0.5	0.501515	100.30	
	0.5	0.496166	99.23	
150%	0.75	0.748463	99.80	99.30
	0.75	0.743114	99.08	
	0.75	0.742777	99.04	

Table 8: Robustness data for netupitant and palonosetron

S. No.	Condition	%RSD of netupitant	%RSD of palonosetron
1	Flow rate (-) 0.9 ml/min	0.3	0.9
2	Flow rate (+) 1.1 ml/min	0.4	0.7
3	Mobile phase (-) 45B: 55A	0.7	0.4
4	Mobile phase (+) 55B: 45A	0.2	0.7
5	Temperature (-) 25 ℃	0.3	0.7
6	Temperature (+) 35 ℃	0.4	0.3

Robustness conditions like Flow minus (0.9 ml/min), Flow plus (1.1 ml/min), mobile phase minus (45B: 55A), mobile phase plus (55B: 45A), temperature minus (25 ℃), and temperature plus (35 ℃) were maintained and samples were injected in a duplicate manner. System suitability parameters were not much affected and all the parameters were passed. % RSD was within the limit.

Stability studies [12]

Acid degradation studies

To 1 ml of stock solution Netupitant and Palonosetron, 1 ml of 2N Hydrochloric acid was added and refluxed for 30 min at 60 °C. The resultant solution was diluted to obtain 300µg/ml and 0.5µg/ml solution and 10 µl solutions were injected into the system and the chromatogram was recorded to assess the stability of the sample. The data was reported in table 9.

Alkali degradation studies

To 1 ml of stock solution Netupitant and palonosetron, 1 ml of 2N sodium hydroxide was added and refluxed for 30 min at 60 °C. There sultan solution was diluted to obtain 300µg/ml and 0.5µg/ml solution and 10 µl were injected into the system and the chromatograms were recorded to assess the stability of the sample. The data was reported in table 9.

Dry heat degradation studies

The standard drug solution was placed in the oven at 105 °C for 1h to study dry heat degradation. For the HPLC study, the resultant solution was diluted to 300µg/ml and 0.5µg/ml solution and 10 µl were injected into the system and the chromatograms were recorded to assess the stability of the sample**.** The data was reported in table 9.

Photostability studies

The photochemical stability of the drug was also studied by exposing the 1000µg/ml Netupitant and 50µg/ml Palonosetronsolution to UV Light by keeping the beaker in UV Chamber for 1 d or 200 Watt-hours/m^2 in a photostability chamber. For the HPLC study, the resultant solution was diluted to obtain 300µg/ml and 0.5µg/ml solutions and 10 µl were injected into the system and the chromatograms were recorded to assess the stability of the sample. The data was reported in table 9.

Neutral (aqueous) degradation studies

Stress testing under neutral conditions was studied by refluxing the drug in water for 1 h at a Temperature of 60°. For the HPLC study, the resultant solution was diluted to 300µg/ml and 0.5µg/ml and 10 µl were injected into the system and the chromatograms were recorded to assess the stability of the sample**.** The data was reported in table 9.

Table 9: Degradation data for netupitant

Type of degradation	Netupitant		
	AREA	% Recovered	% Degraded
Acid	1918191	97.98	2.02
Alkali	1826889	93.32	6.68
Peroxide	1834971	93.73	6.27
Thermal	1954569	99.84	0.16
UV	1905678	97.34	2.66
Water	1879424	96.00	4.00

Table 9: Degradation data for palonosetron

Type of degradation	Palonosetron		
	Area	% Recovered	% Degraded
Acid	123400	92.15	7.85
Alkali	124307	92.83	7.17
Peroxide	124769	93.17	6.83
Thermal	132490	98.94	1.06
UV	131818	98.44	1.56
Water	132251	98.76	1.24

CONCLUSION

According to the ICH guidelines, the method was developed validated, which is suitable for the stability-indicating RP-HPLC method development and validation for simultaneous estimation of Netupitant and Palonosetron in the pharmaceutical dosage form. The method developed was found to be accurate, precise, sensitive, rapid, and reliable. The retention time was 2.199 min and 2.893 min and they were linear in the concentration range of 75-450 µg/ml and 0.125-0.75 µg/ml for Netupitant and Palonosetron, respectively. The repeatability and intermediate precision were found to be within acceptable limits. Regression equation of Netupitant and Palonosetron is Y=6329x+42914 and Y= 258884x+3103.9, respectively. LOD was found to be 0.33µg/ml, 0.99µg/ml and LOQ was 0.01µg/ml, 0.04µg/ml for Netupitant and Palonosetron. The correlation coefficient (R^2) value was found to be 0.999 and %recovery was obtained as 100.32% and 99.6% for Netupitant and Palonosetron, respectively. Forced degradation studies reveal that the drugs are unstable under acidic conditions. The method was satisfactory in short retention time and was linear in the concentration range. In addition, all the validated parameters observed were within the limits. Degradation studies disclose that both drugs were stable only under thermal conditions. The data introduced reveals that the method is economical and thus it is applicable for routine analysis in the pharmaceutical dosage form.

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Nil

AUTHORS CONTRIBUTIONS

All the authors have contributed equally.

CONFLICT OF INTERESTS

Declared none

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