IJPPR
INTERNATIONAL JOURNAL OF PHARMACY & PHARMACEUTICAL RESEARCH
An official Publication of Human Journals
ISSN 2349-7203
HUMAN

Human Journals
Research Article
October 2020 Vol.:19, Issue:3

RP-HPLC Method Development and Validation for the Simultaneous Determination Netupitat and Palanosteron in Pharmaceutical Dosage Forms

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ISSN 2349-7203
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Submission: 24 September 2020
Accepted: 30 September 2020
Published: 30 October 2020

Keywords: Netupitant, Palonosteron, RP-HPLC

ABSTRACT

A simple, Accurate, precise method was developed for the simultaneous estimation of the Netupitant and Palonosetron in Tablet dosage form. Chromatogram was run through Std Ascentis C18 150 x 4.6 mm, 5µ. Mobile phase containing Buffer 0.1% OPA: Acetonitrile taken in the ratio 60:40 was pumped through column at a flow rate of 1ml/min. Buffer used in this method was orthophosphoric acid. Temperature was maintained at 30°C. Optimized wavelength selected was 260 nm. Retention time of Netupitant and Palonosetron were found to be 2.330min and 3.257min. % RSD of the Netupitant and Palonosetron were and found to be 1.2 and 1.1 respectively. % Recovery was obtained as 99.65% and 100.92% for Netupitant and Palonosetron respectively. LOD, LOQ values obtained from regression equations of Netupitant and Palonosetron were 0.93, 0.002 and 2.93, 0.006 respectively. Regression equation of Netupitant is y = 2997.2x + 7406.4., and y = 143389x + 1519.8 of Palonosteron Retention times were decreased and that run time was decreased, so the method developed was simple and economical that can be adopted in regular Quality control test in Industries.

INTRODUCTION

Netupitant[1]

Description: Netupitant is an antiemetic 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 is a neurokinin 1 receptor antagonist. The combination drug is marketed by Eisai Inc. and Helsinn Therapeutics (U.S.) Inc. under the brand Akynzeo.

Structure:

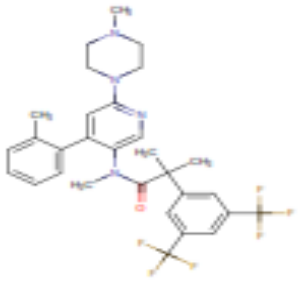

Figure No. 1: Structure of Netupitant

CAS number	290297-26-6
Chemical Formula	$C_{30}H_{32}F_6N_4O$
IUPAC Name	2-[3,5-bis(trifluoromethyl)phenyl]-N,2-dimethyl-N-[4-(2-methylphenyl)-6-(4-methylpiperazin-1-yl)pyridin-3-yl]propanamide
Weight	578.603
Appearance	White solid powder
Solubility	Soluble in DMSO, not soluble in water
pKa (Strongest Basic)	7.6

Mechanism of action: Delayed emesis (vomiting) has been largely associated with the activation of tachykinin family neurokinin 1 (NK1) receptors (broadly distributed in the central and peripheral nervous systems) by substance P. As shown in *in vitro* and *in vivo* studies, netupitant inhibits substance P mediated responses.

Palonosteron[2]

Description: 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.

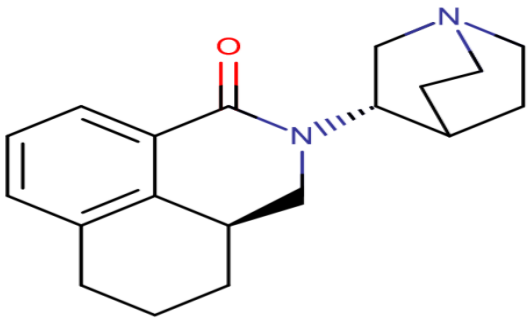

Figure No. 2: Structure of Palonosetron

IUPAC Name:

(5S)-3-[(3S)-1-azabicyclo[2.2.2]octan-3-yl]-3-azatricyclo[7.3.1.0^{5},^{13}]trideca-1(12),9(13),10-trien-2-one

Chemical Formula	$C_{19}H_{24}N_2O$
CAS number	135729-56-5
Physical State	Solid
Appearance	Powder
Storage	Store at room temperature
Melting Point	267-269° C
Weight: Average	296.414
pKa (Strongest Basic)	7.97

Solubility: freely soluble in water and propylene glycol and only slightly soluble in ethanol.

Indication: For the prevention of acute and delayed nausea and vomiting associated with initial and repeat courses of moderately emetogenic cancer chemotherapy, as well as prevention of acute nausea and vomiting associated with highly emetogenic cancer chemotherapy. Also used for the prevention of postoperative nausea and vomiting for up to 24 hours post operation.

Mechanism of action: Palonosetron is a selective serotonin 5-HT_3 receptor antagonist. The antiemetic activity of the drug is brought about through the inhibition of 5-HT_3 receptors present both centrally (medullary chemoreceptor zone) and peripherally (GI tract). This inhibition of 5-HT_3 receptors in turn inhibits the visceral afferent stimulation of the vomiting center, likely indirectly at the level of the area postrema, as well as through direct inhibition of serotonin activity within the area postrema and the chemoreceptor trigger zone. Alternative mechanisms appear to be primarily responsible for delayed nausea and vomiting induced by emetogenic chemotherapy, since similar temporal relationships between serotonin and emesis beyond the first day after a dose have not been established, and 5-HT_3 receptor antagonists generally have not appeared to be effective alone in preventing or ameliorating delayed effects. It has been hypothesized that palonosetron's potency and long plasma half-life may contribute to its observed efficacy in preventing delayed nausea and vomiting caused by moderately emetogenic cancer chemotherapy.

MATERIALS AND METHODS[3-8]

Materials:

- Netupitant and Palonosetron pure drugs (API), Combination Netupitant and Palonosetron (**AKYNZEO**) capsule, Distilled water, Acetonitrile, Phosphate buffer, Methanol, Potassium dehydrogenate orthophosphate buffer, Ortho-phosphoric acid. All the above chemicals and solvents are from Rankem.

Instruments:

- Electronics Balance-Denver
- p^H meter -BVK enterprises, India
- Ultrasonicator-BVK enterprises

- WATERS HPLC 2695 SYSTEM equipped with quaternary pumps, Photo Diode Array detector and Autosampler integrated with Empower 2 Software.
- UV-VIS spectrophotometer PG Instruments T60 with special bandwidth of 2 mm and 10mm and matched quartz cells integrated with UV win 6 Software was used for measuring absorbances of Netupitant and Palonosetron solutions.

Methods:

Diluent: Based up on the solubility of the drugs, diluent was selected, Acetonitrile and Water taken in the ratio of 50:50.

Preparation of Standard stock solutions: Accurately weighed 150 mg of Netupitant, 0.25mg of Palonosteron and transferred to 50ml and 50ml individual volumetric flasks and 3/4 th of diluents was added to these flasks and sonicated for 10 minutes. Flask were made up with diluents and labeled as Standard stock solution. 1ml from each stock solution was pipetted out and taken into a 10ml volumetric flask and made up with diluent. (3000µg/ml Netupitant of and 5µg/ml of Palonosteron).

Preparation of Sample stock solutions: 5 capsules were weighed and the average weight of each capsule was calculated, then the weight equivalent to 1 capsule was transferred into a 100 ml volumetric flask, 50ml 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 and 5µg/ml of Palonosteron).

Preparation of Sample working solutions (100% solution): 1ml of filtered sample stock solution was transferred to 10ml volumetric flask and made up with diluent. (300µg/ml of Netupitant and 0.5µg/ml of Palonosteron).

Preparation of buffer:

0.01N KH_2PO_4Buffer: Accurately weighed 1.36gm of Potassium dihydrogen orthophosphate in a 1000ml of Volumetric flask add about 900ml of milli-Q water added and degtas to sonicate and finally make up the volume with water then added 1ml of Triethylamine then PH adjusted to 4.0 with dil. orthophosphoric acid solution.

System suitability parameters: The system suitability parameters were determined by preparing standard solutions of Netupitant (300ppm) and Palonosteron (0.5ppm) and the

solutions were injected six times and the parameters like peak tailing, resolution and USP plate count were determined. The % RSD for the area of six standard injections results should not be more than 2%.

Specificity: Checking of the interference in the optimized method. We should 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.

Precision:

Preparation of Standard stock solutions: Accurately weighed 150 mg of Netupitant, 0.25mg of Palonosteron and transferred to 50ml and 50ml individual volumetric flasks and 3/4 th of diluents was added to these flasks and sonicated for 10 minutes. Flask were made up with diluents and labeled as Standard stock solution. 1ml from each stock solution was pipetted out and taken into a 10ml volumetric flask and made up with diluent. (3000µg/ml Netupitant of and 5µg/ml of Palonosteron).

Linearity:

Preparation of Standard stock solutions: Accurately weighed 150mg of Netupitant, 0.25mg of Palonosteron and transferred to 50ml and 50ml individual volumetric flasks and 3/4 th of diluents was added to these flasks and sonicated for 10 minutes. Flask were made up with diluents and labeled as Standard stock solution. 1ml from each stock solution was pipetted out and taken into a 10ml volumetric flask and made up with diluent. (3000µg/ml Netupitant of and 5µg/ml of Palonosteron)

25% Standard solution: 0.25ml each from two standard stock solutions was pipetted out and made up to 10ml. (75µg/ml of Netupitant and 0.125µg/ml of Palonosteron)

50% Standard solution: 0.5ml each from two standard stock solutions was pipetted out and made up to 10ml. (150µg/ml of Netupitant and 0.25µg/ml of Palonosteron)

75% Standard solution: 0.75ml each from two standard stock solutions was pipetted out and made up to 10ml. (225µg/ml of Netupitant and 0.375µg/ml of Palonosteron)

100% Standard solution: 1.0ml each from two standard stock solutions was pipetted out and made up to 10ml. (300µg/ml of Netupitant and 0.5µg/ml of Palonosteron)

125% Standard solution: 1.25ml each from two standard stock solutions was pipetted out and made up to 10ml. (375µg/ml of Netupitant and 0.625µg/ml of Palonosteron)

150% Standard solution: 1.5ml each from two standard stock solutions was pipetted out and made up to 10ml. (450µg/ml of Netupitant and 0.75µg/ml of Palonosteron)

Accuracy:

Preparation of Standard stock solutions: Accurately weighed 150 mg of Netupitant, 0.25mg of Palonosteron and transferred to 50ml and 50ml individual volumetric flasks and 3/4 th of diluents was added to these flasks and sonicated for 10 minutes. Flask were made up with diluents and labeled as Standard stock solution. 1ml from each stock solution was pipetted out and taken into a 10ml volumetric flask and made up with diluent. (3000µg/ml Netupitant of and 5µg/ml of Palonosteron)

Preparation of 50% Spiked Solution: 0.5ml of sample stock solution was taken into a 10ml volumetric flask, to that 1.0ml from each standard stock solution was pipetted out and made up to the mark with diluent.

Preparation of 100% Spiked Solution: 1.0ml of sample stock solution was taken into a 10ml volumetric flask, to that 1.0ml from each standard stock solution was pipetted out and made up to the mark with diluent.

Preparation of 150% Spiked Solution: 1.5ml of sample stock solution was taken into a 10ml volumetric flask, to that 1.0ml from each standard stock solution was pipetted out and made up to the mark with diluent.

Acceptance Criteria:

The % Recovery for each level should be between 98.0 to 102.

Robustness: Small deliberate changes in method like Flow rate, mobile phase ratio, and temperature are made but there were no recognized changes in the result and are within range as per ICH Guidelines.

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

LOD sample Preparation: 0.25ml each from two standard stock solutions was pipetted out and transferred to two separate 10ml volumetric flasks and made up with diluents. From the above solutions 0.1ml each of Netupitant, Palonosteron, solutions respectively were transferred to 10ml volumetric flasks and made up with the same diluents.

LOQ sample Preparation: 0.25ml each from two standard stock solutions was pipetted out and transferred to two separate 10ml volumetric flask and made up with diluent. From the above solutions 0.3ml each of Netupitant, Palonosteron, and solutions respectively were transferred to 10ml volumetric flasks and made up with the same diluent.

Degradation studies:

Oxidation:

To 1 ml of stock solution of Netupitant and Palonosetron, 1 ml of 20% hydrogen peroxide (H_2O_2) was added separately. The solutions were kept for 30 min at 60^0C. For HPLC study, the resultant solution was diluted to obtain 300µg/ml & 0.5µg/ml solution and 10 µl were injected into the system and the chromatograms were recorded to assess the stability of sample.

Acid Degradation Studies:

To 1 ml of stock s solution Netupitant and Palonosetron, 1ml of 2N Hydrochloric acid was added and refluxed for 30mins at 60^0C. The resultant solution was diluted to obtain 300µg/ml & 0.5µg/ml solution and 10 µl solutions were injected into the system and the chromatograms were recorded to assess the stability of sample.

Alkali Degradation Studies:

To 1 ml of stock solution 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 & 0.5µg/ml solution and 10 µl were injected into the system and the chromatograms were recorded to assess the stability of sample.

Dry Heat Degradation Studies:

The standard drug solution was placed in oven at 105°C for 1 h to study dry heat degradation. For HPLC study, the resultant solution was diluted to 300µg/ml & 0.5µg/ml solution and10µl

were injected into the system and the chromatograms were recorded to assess the stability of the sample.

Photo Stability studies:

The photochemical stability of the drug was also studied by exposing the 3000µg/ml Netupitant & 5µg/ml Palonosteron solution to UV Light by keeping the beaker in UV Chamber for 1days or 200 Watt hours/m 2 in photostability chamber. For HPLC study, the resultant solution was diluted to obtain 300µg/ml & 0.5µg/ml solutions and 10 µl were injected into the system and the chromatograms were recorded to assess the stability of sample.

Neutral Degradation Studies:

Stress testing under neutral conditions was studied by refluxing the drug in water for 1hrs at a temperature of 60ºC. For HPLC study, the resultant solution was diluted to 300µg/ml & 0.5µg/ml solution and 10 µl were injected into the system and the chromatograms were recorded to assess the stability of the sample.

RESULTS AND DISCUSSION

Method Validation: The validation of the Process carried out was validated as per ICH guidelines and the following parameters were reported as follows:

System suitability: All the system suitability parameters were within the range and satisfactory as per ICH guidelines.

Table No. 1: System suitability parameters for Netupitant and Palonosetron

S. No.	Netupitant			Palonosetron			
Inj	RT(min)	USP Plate Count	Tailing	RT(min)	USP Plate Count	Tailing	Resolution
1	2.318	3545	1.20	3.176	6416	1.07	5.5
2	2.320	3717	1.24	3.187	5541	1.17	5.0
3	2.322	3401	1.21	3.195	5288	1.12	5.2
4	2.325	3494	1.19	3.221	6639	1.10	5.5
5	2.326	3356	1.18	3.222	7400	1.08	5.4
6	2.330	3441	1.19	3.257	5400	1.04	5.2

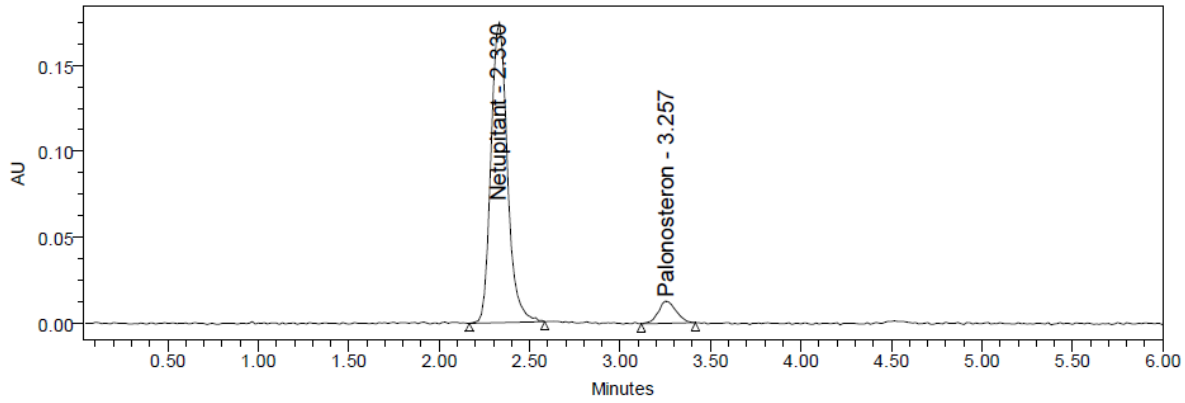

Figure No. 3: System suitability Chromatogram

Accuracy: The accuracy of the method was determined by recovery experiments. Placebo was spiked with known quantities of standard drugs at levels of 50% to 150% of Abel claim. The recovery studies were carried out 3 times and the percentage recovery and standard deviation of the percentage recovery were calculated and presented in table as follows:

Table No. 2: Accuracy table of Netupitant

% Level	Amount Spiked (µg/mL)	Amount recovered (µg/mL)	% Recovery	Mean % Recovery
50%	150	151.0	100.7	99.65%
	150	148.2	98.8	
	150	148.6	99.0	
100%	300	300.0	100.0	
	300	302.7	100.9	
	300	299.4	99.8	
150%	450	446.5	99.2	
	450	447.6	99.5	
	450	444.9	98.9	

Table No. 3: Accuracy table of Palonosetron

% Level	Amount Spiked (µg/mL)	Amount recovered (µg/mL)	% Recovery	Mean % Recovery
50%	0.25	0.249	99.44	100.92%
	0.25	0.251	100.50	
	0.25	0.254	101.59	
100%	0.5	0.509	101.72	
	0.5	0.503	100.56	
	0.5	0.506	101.26	
150%	0.75	0.754	100.57	
	0.75	0.762	101.59	
	0.75	0.758	101.06	

LOD and LOQ

The LOD and LOQ of the developed method were determined by injecting progressively low concentrations of the standard solutions using the developed RP-HPLC method. The LOD is the smallest concentration of the analyse that give the measurable response. The LOD for Netupitant and Palonosetron was found to be 0.97 and 0.002 respectively.

LOQ is the smallest concentration of the analyte, which gives response that can be accurately quantified signal to noise ratio of 10. The LOQ was 2.93and 0.006 for Netupitant and Palonosetron.

Linearity and range

Six linear concentrations of Netupitant (75-450µg/ml) and Palonosteron (0.125-0.75µg/ml) were injected in a duplicate manner. Average areas were mentioned above and linearity equations obtained for Netupitant was y = 2997.2x + 7406.4 and of Palonosteron was y = 143389x + 1519.8. Correlation coefficient obtained was 0.999 for the two drugs.

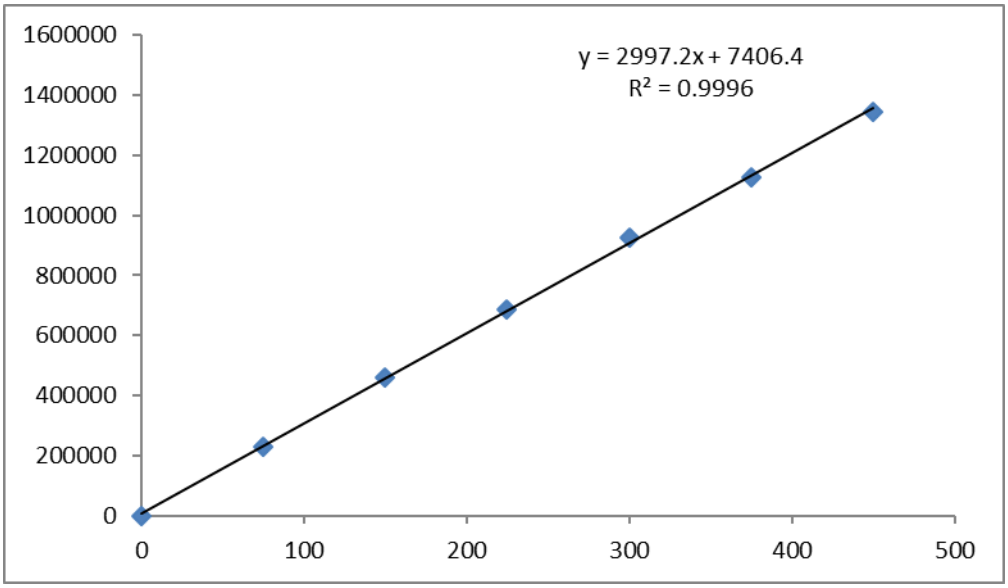

Figure No. 4: Calibration curve of Netupitant

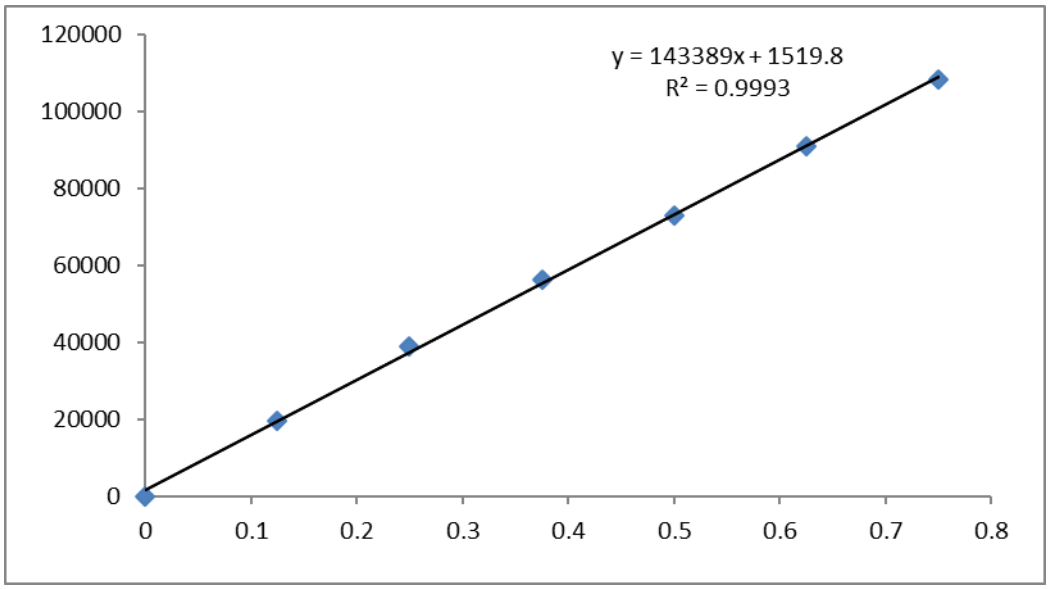

Figure No. 5: Calibration curve of Palonosetron

Table No. 4: Linearity table for Netupitant and Palonosetron

Netupitant		Palonosetron	
Conc (µg/mL)	**Peak area**	**Conc (µg/mL)**	**Peak area**
0	0	0	0
75	230553	0.125	19604
150	458915	0.25	38789
225	685428	0.375	56337
300	925207	0.5	73064
375	1128076	0.625	90928
450	1344264	0.75	108313

System precision:

From a single volumetric flask of working standard solution, six injections were given and the obtained areas were mentioned above. Average area, standard deviation and % RSD were calculated for two drugs. % RSD obtained as 1.2% and 1.1% respectively for Netupitant and Palonosetron. As the limit of Precision was less than "2" the system precision was passed in this method.

Table No. 5: System precision table of Netupitant and Palonosetron

S. No.	Area of Netupitant	Area of Palonosetron
1.	915420	71825
2.	910545	73832
3.	916113	73088
4.	920737	72139
5.	914853	71782
6.	941347	72195
Mean	919836	72477
S.D	11027.7	813.8
% RSD	1.2	1.1

Robustness:

Discussion: Robustness conditions like Flow minus (0.9ml/min), Flow plus (1.1ml/min), mobile phase minus (55B:45A), mobile phase plus (65B:55A), temperature minus (25°C) and temperature plus (35°C) was maintained and samples were injected in duplicate manner. System suitability parameters were not much affected and all the parameters were passed. % RSD was within the limit.

Table No. 6: Robustness data for Netupitant and Palonosetron

S. No.	Condition	% RSD of Netupitant	% RSD of Palonosetron
1	Flow rate (-) 0.9ml/min	0.7	1.1
2	Flow rate (+) 1.1ml/min	1.1	0.6
3	Mobile phase (-) 55B:45A	0.5	0.5
4	Mobile phase (+)65B:35A	0.3	1.8
5	Temperature (-) 25°C	0.9	0.7
6	Temperature (+) 35°C	0.7	1.4

Assay: Arbor Pharmaceuticals Ltd. (AKYNZEO), bearing the label claim Netupitant 300MG and Palonosteron 0.5MG. Assay was performed with the above formulation. Average % Assay for Netupitant and Palonosetron obtained was 99.62% and 100.92% respectively.

Table No. 7: Assay Data of Netupitant

S. No.	Standard Area	Sample area	% Assay
1	915420	929595	100.86
2	910545	919415	99.75
3	916113	918894	99.70
4	920737	908195	98.54
5	914853	913797	99.14
6	941347	919076	99.72
Avg	919836	918162	99.62
Stdev	11027.7	7100.0	0.77
%RSD	1.2	0.8	0.8

Table No. 8: Assay Data of Palonosetron

S. No.	Standard Area	Sample area	% Assay
1	71825	72169	99.38
2	73832	72472	99.79
3	73088	73721	101.51
4	72139	73274	100.90
5	71782	71383	98.29
6	72195	72269	99.51
Avg	72477	72548	99.90
Stdev	813.8	835.2	1.2
%RSD	1.1	1.2	1.2

CONCLUSION

A simple, Accurate, precise method was developed for the simultaneous estimation of the Netupitant and Palonosetron in tablet dosage form. Retention time of Netupitant and Palonosetron were found to be 2.330min and 3.257min. % RSD of the Netupitant and Palonosetron were and found to be 1.2 and 1.1 respectively. % Recovery was obtained as 99.65% and 100.92% for Netupitant and Palonosetron respectively. LOD, LOQ values obtained from regression equations of Netupitant and Palonosetron were 0.93, 0.002 and 2.93, 0.006 respectively. Regression equation of Netupitant is y = 2997.2x + 7406.4., and y = 143389x + 1519.8 of Palonosteron Retention times were decreased and that run time was decreased, so the method developed was simple and economical that can be adopted in regular Quality control test in Industries.

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