World Inventia Publishers

Journal of Pharma Research

http://www.jprinfo.com/

Vol. 6, Suppl 2, 2017 ISSN: 2319-5622 USA CODEN: JPROK3

Research Article Full Proceeding Paper

STABILITY INDICATING METHOD DEVELOPMENT AND VALIDATION FOR THE SIMULTANEOUS ESTIMATION OF PALONOSETRON AND NETUPITANT BY RP-HPLC IN ITS BULK FORM

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Received on: 05-10-2017; Revised and Accepted on: 08-11-2017

ABSTRACT

A new stability indicating RP-HPLC method has been developed for the simultaneous estimation of anti-emetic drugs, Netupitant and Palonosetron. The fixed dose combination of Netupitant and Palonosetron is used in the prevention of Chemotherapy induced Nausea and Vomiting (CINV). HPLC method separation was achieved using C -18 Inertsil-ODS 3V column (250 X 4.6 mm, 5 µm) with mobile phase comprising of Methanol: water in the ratio 45:55 v/v at a flow rate of 1 ml/min and run time of 7 min. Common wavelength of 236 nm was used for detection. Both drugs were properly resolved with resolution of 4.3 and retention time of 3.049 min and 4.317 min for Palonosetron and Netupitant respectively .As per ICH guidelines, the method was validated in terms of Linearity, System Suitability, Accuracy, Precision, Limit of Detection, Limit of Quantification and Robustness .All parameters were found to be within the acceptance limit. The linear regression analysis data for the caliberation curve showed a good linear relationship with a regression coefficient of 0.999 for both the drugs. The method was found to be linear over the range of 20-80 ppm for Netupitant and Palonosetron. The Limit of Detection and Limit of Quantification was 0.051 µg/ml and 0.156 µg/ml for Netupitant and 0.127 µg/ml and 0.386 µg/ml for Palonosetron. Acid, alkali, oxidative and thermal degradation studies were performed. The results obtained show that no significant degradation products were formed thereby showing the stability of the drugs in various stress conditions. This HPLC procedure is economic, sensitive and less time consuming. It is an important tool for analysis of bulk drugs.

KEYWORDS: *Netupitant, Palonosetron, Stability Indicating RP-HPLC, CINV.*

INTRODUCTION

Chemotherapy Induced Nausea and Vomiting (CINV) is one of the leading factors for the non-compliance of anti-cancer therapy. Nevertheless, it has a huge impact on Quality of Life. According to statistics, about 30 % of cancer victims are suggested chemotherapy.70 % to 80 % of these victims are prone to suffer CINV. The fixed dose combination of Palonosetron and Netupitant was approved in the year 2014 by FDA. Palonosetron is a second generation 5-HT3 receptor antagonist while Netupitant is an NK1 receptor antagonist.

Netupitant is a competitive inhibitor which blocks the activity of human substance P/ NK 1 receptors. It inhibits the NK 1 receptor bin ding with the endogenous Tachykinin neuropeptide Substance P which r esults in prevention of CINV. Chemically Netupitant is 2-[3,5-Bis(trifluor omethyl)phenyl]-N,2-dimethyl-N-[4-(2-methylphenyl)-6-(4-methyl-1-pi perazinyl)-3-pyridinyl]Propanamide. The structure of Netupitant is sho wn in figure 1 [1].

Chemically, Palonosetron is (3aS)-2-[(3S)-1-azabicyclo[2.2.2] octan-3-yl]-3a,4,5,6-tetrahydro-3H-benzo[de]isoquinolin-1-one; hydro chloride. 5-HT3 receptors are chiefly located in the postrema and nucleus tractus solitarius .These areas are primarily associated with the vomiting reflex. Upon damage caused due to exposure to chemotherapy and radiation therapy, Serotonin is released by the cells lining the GI tract. As a result, serotonin binds to the receptors on nerves that are responsible in transmitting impulses to the vomiting center in brain thereby stimulating other nerves linked to the vomiting reflex. 5-HT3 receptor antagonists act by binding to 5-HT3 receptors in the small intestine thereby decreasing the incidence of nausea and vomiting. The structure of Palonosetron is shown in figure 2 [2].

Review of literature depicts that few studies have been reported for the simultaneous estimation of Netupitant and Palonosetron. Also, other methods like HPTLC and LC-MS were performed. The present study employs column friendly and cost effective solvents in contrast with the other reported methods [3-6].

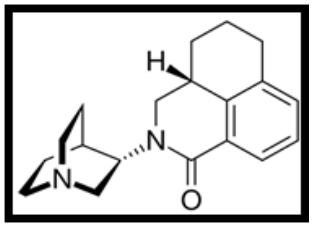

Fig. 1: Structure of Netupitant

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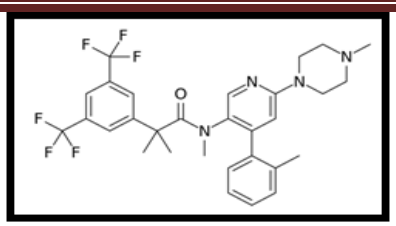

Fig. 2: Strucuture of Palonosetron

MATERIALS AND METHODS

1. Chemicals and Reagents:

- Water, Methanol and Acetonitrile were procured from Standard Reagents Private Limited, Hyderabad.
- All the solvents used were HPLC-Grade.
- Palonosetron and Netupitant working standards were obtained as gift samples from Therdose Pharma Private Limited and Apicore Pharmaceuticals Private Limited respectively.

2. Instrument, Software and chromatographic conditions:

- Reverse-Phase High Performance Liquid Chromatography Seperation module of Waters- Alliance model 2690/95 was used.
- It was equipped with Waters -996 PDA detector.
- Separation was achieved using Thermo Inertsil C 18- ODS 3 V Column with dimensions 250 x 4.6 mm, 5 µ.
- For peak integration, Empower Software version 2.0 was used
- The mobile phase comprised of Water :methanol in the ratio of (45:55 w/v) at a flow rate of 1.0 ml/min and injection volume of 20 µl
- Run time was 7 min with detection wavelength of 236 nm.

3. Preparation of Stock solution:

Accurately 10 mg of Palonosetron and 10 mg of Netupitant were weighed individually and transferred into two 10 ml volumetric flasks and about 6-7 ml of methanol was added separately to each flask. Sonication was performed to dissolve completely and volume was made upto the mark with the same diluent.

4. Preparation of working standard:

From the above stock solutions, 0.4 ml of Palonosetron and 0.4 ml of Netupitant was pipetted out into a 10 ml valumetric flask and the volume was adjusted with diluent.

5. Method Development:

- Trials were performed using different mobile phases and changing their corresponding ratios.
- Various mobile phases like Acetonitrile, Methanol and water were considered. The chromatograms were studied for good peak shapes and resolution.

6. Method Optimization:

The mobile phase was optimized to Methanol: water in the ratio of 45:55 % v/v . The optimized method shows good peaks with resolution 4.3. The optimized chromatographic conditions are reported in table 1 and chromatogram in figure 3.

7. Method Validation:

Validation is an important step in the areas of Quality Control and Quality Assurance. Method Validation was performed in accordance with the Q 2(R 1) guidelines. Parameters evaluated were Linearity, Accuracy, Precision, System Suitability Tests, Limit of Detection, Limit of Quantification and Robustness.

7.1. Linearity:

Linearity demonstrates the linear range of analyte that is reportable. A minimum of five concentrations are to be considered. Linearity evaluation is performed by visually inspecting the plot of concentration of analyte versus the response. The regression line slope shows the sensitivity of regression while the y-intercept provides an estimate of variability of method.

In the present method, seven concentrations from 20 – 80 ppm each of Netupitant and Palonosetron were prepared from the standard solution and injected. The peak area responses were recorded and correlation coefficient value was evaluated and it was found that the value of correlation co-efficient is 0.999 for both the drugs. This indicates good linearity. Results are shown in table 2 and linearity curves in figures 4 and 5.

7.2. System Suitability:

In order to ensure the sensitivity and suitability of the system for the proposed method, System suitability tests are performed. The System Suitability parameters include Tailing factor, USP Plate count, Resolution, etc. For performing this procedure, five replicates of 40 ppm concentration each of Netupitant and Palonosetron were injected and evaluated. All the results obtained show that the method passes the SST. Results are given in table 3.

7.3. Accuracy:

A study of Accuracy was performed in triplicate as per test method with equivalent amount of Palonosetron and Netupitant into each volumetric flask for each spike level to get the concentration of Palonosetron and Netupitant equivalent to 50%, 100%, and 150%.
The average % recovery of Palonosetron and Netupitant were calculated. The results obtained were in the range of 98 % – 102 % which is indicative of the test method being accurate. Results of accuracy are tabulated in table 4.

7.4. Precision:

a) System precision:

- In this, the solution of known concentration is injected to the system at the same conditions (optimized method).
- Five replicates (40 ppm of Netupitant & Palonosetron each) are injected and peak retention times, areas and % RSD are evaluated.RSD can be calculated as follows:

$$\% \text{ RSD} = \frac{\text{Standard deviation}}{\text{Mean}} \times 100$$

b) Method precision:

- Here, six replicates of the solution are injected and % RSD is calculated.
- Method precision is to check the reproducibility of results.

c) Intermediate precision or Ruggedness:

- Precision under defined set of conditions is called as Intermediate Precision.
- Peak area and % RSD for the areas were calculated for the six replicate injections.

The test results obtained for Precision were under the prescribed limits which suggest that method developed is precise. Results are shown in table 5, 6 and 7.

7.5. Robustness:

Robustness is assessed by the effect of small deliberate changes in chromatographic methods. These changes may include change in pH, variation in flow rate, change in mobile phase composition, change in temperature, change in column, etc.

In the proposed method, variation of flow rate (1 ml ± 0.2 ml) showed no significant effect thereby stating that the proposed method is robust and not affected by minor variations.
Results are tabulated in table 8.

7.6. Limit of Detection (LOD) and Limit of Quantification (LOQ):

Limit of Detection and Limit of Quantification indicate the sensitivity of the test method.LOD and LOQ can be calculated by the formulae:

$$\text{LOD} = \frac{3.3\,\sigma}{S}$$

Where,

σ = standard deviation of response
S = slope of caliberation curve

$$LOQ = \frac{10\,\sigma}{S}$$

Where,

σ = standard deviation of response

S = slope of caliberation curve

Results are tabulated in table 9.

8. Forced Degradation Studies:

In forced degradation studies, the sample is subjected to different stress conditions like Acid, alkali, thermal and oxidative degradation. Percent degradation was very low whereby we can conclude that the method is stable in all stress conditions applied. Results are given in table 10 and 11.

1) Acid degradation study :

Acid-induced, forced degradation was performed by adding an aliquot of stock solution (1 mg/ml) of Netupitant and Palonosetron into 10 ml each of methanol and 0.1 M HCl and refluxing the mixture at 60°C for approximately six hours. The solution was then left to reach room temperature, neutralized to pH 7 by the addition of 0.1 M NaOH, and diluted to 100 ml with the mobile phase so as to get a final concentration of 10µg/ml. Chromatogram is shown in figure 6.

2) Alkali degradation study :

Here, forced degradation was performed by adding an aliquot of stock solution (1 mg/ml) of Netupitant and Palonosetron to 10 ml each of methanol and 0.1 M NaOH, and refluxing the mixture at 60°C for approximately six hours. The solution was then cooled to room temperature, neutralized to pH 7 by addition of 0.1 M HCl, and diluted to 100 ml with the mobile phase, so as to get a final concentration of 10 µg/ml. Chromatogram is shown in figure 7.

3) Oxidative degradation study:

To study the effect of oxidizing conditions, an aliquot of stock solution (1 mg/ml) of Netupitant and Palonosetron was added to 10 ml of 30% H_2O_2 solution and the mixture was refluxed at 60°C for approximately six hours. The solution was left to reach room temperature and diluted to 100 ml with the mobile phase, so as to get a final concentration of 10µg/ml. Chromatogram is shown in figure 8.

4) Thermal degradation study :

To study the effect of temperature, approximately 50 mg Netupitant and Palonosetron was stored at 100°C in a hot air oven for 24 hours and then dissolved in 10 ml of methanol and the volume was adjusted to 50 ml with the mobile phase. The above solution was further diluted with the mobile phase, to give a solution of final concentration equivalent to 10 µg/ml of Netupitant and Palonosetron. Chromatogram is shown in figure 9.

Formula for calculating % Degradation is:

$$\% \text{ Degradation} = \frac{Au - At}{Au} \times 100$$

Where: Au=Area of Untreated Solution

At= Area of Treated Solution.

RESULTS AND DISCUSSION

A simple, rapid, sensitive and reproducible RP-HPLC method was developed for simultaneous estimation of Palonosetron and Netupitant in bulk. The optimized mobile phase was Methanol: water in the ratio 45:55 v/v. The wavelength of detection for Palonosetron and Netupitant was 236 nm. The retention time of Palonosetron and Netupitant were found to be 3.049 min and 4.317 min respectively with run time of 7 minutes. The method was evaluated for validation parameters according to ICH guidelines. Linear calibration graphs (Regression equation of Netupitant is y = 97493x – 3818 and Palonoserton is y = 5632.x - 245.0; where y and x are peak area and concentration, respectively) were obtained from concentrations of 20 to 80 ppm. Correlation coefficient was found to be 0.999. LOD and LOQ values from the regression equation were found to be 0.051 µg/ml and 0.156 µg/ml (Netupitant) and 0.127 µg/ml and 0.386 µg/ml (Palonoserton) respectively. The statistical data and recovery data reveal good accuracy and precision of the proposed method .The percentage RSD obtained for precision was below 2. The percentage recoveries of Netupitant and Palonoserton were 100.24 % and 100.20%. From stability studies results obtained, it can be said that % degradation was low and no degradation products were formed.

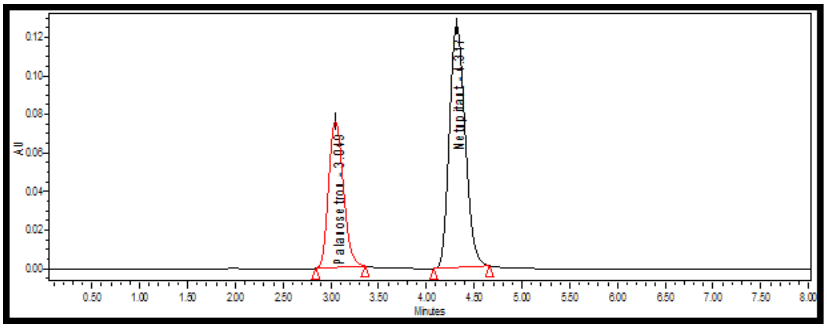

Fig. 3: Chromatogram for Optimized method

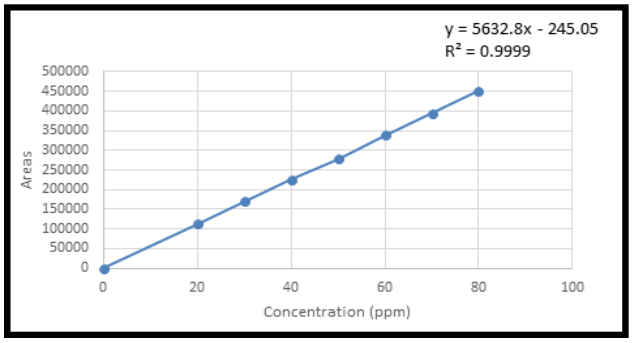

Fig. 4: Linearity Plot (Concentration Vs Response) of Palonosetron

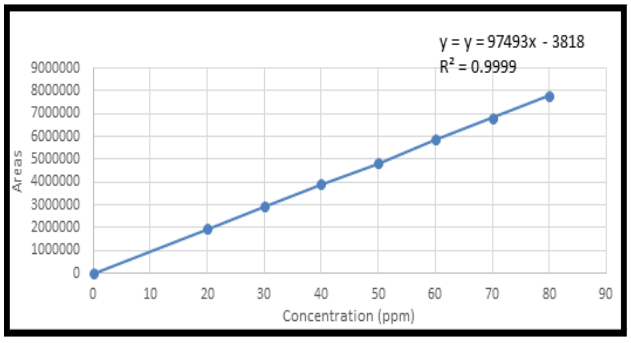

Fig. 5: Linearity Plot (Concentration Vs Response) of Netupitant

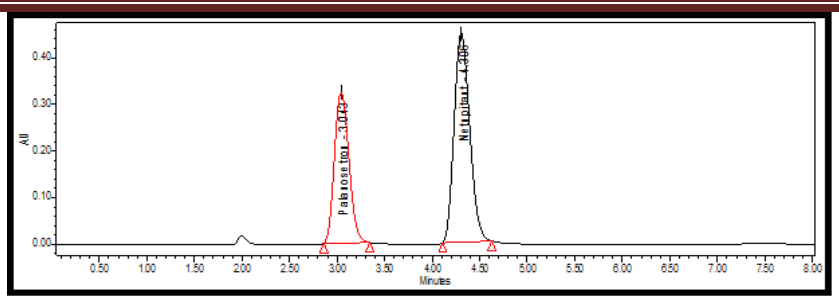

Fig. 6: Chromatogram for Acid Degradation study

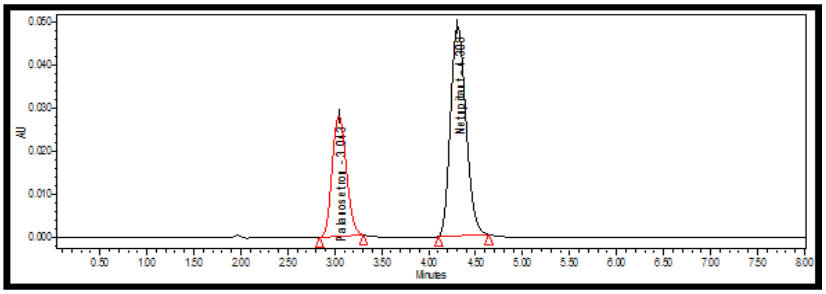

Fig. 7: Chromatogram for Base Degradation study

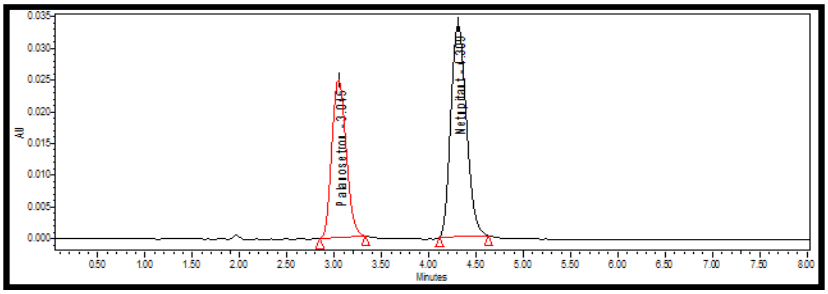

Fig. 8: Chromatogram Oxidative Degradation study

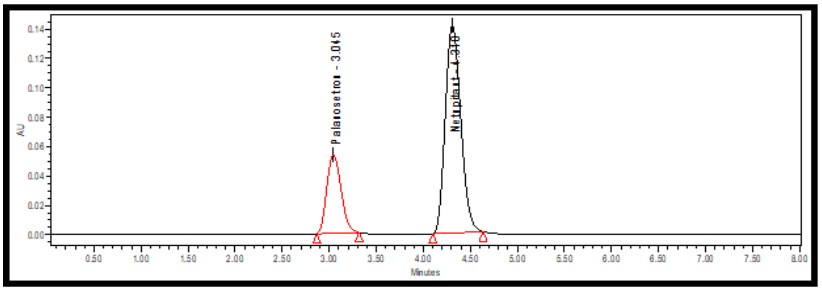

Fig. 9: Chromatogram Thermal Degradation study

Table No. 1: Optimized Chromatographic conditions

Parameters	Method
Stationary phase (column)	Inertsil -ODS 3V C_{18}(250 x 4.6 mm, 5 µ)
Mobile Phase	Methanol : water (45:55 v/v)
Flow rate	1.0 ml/min
Run time (minutes)	7
Column temperature (°C)	Ambient
Detector	Photo Diode Array (PDA)
Volume of injection loop	20 µl
Detection wavelength (nm)	236 nm
Drug Retention time(min)	3.049 min for Palonosetron and 4.317

Table No. 2: Linearity results of Netupitant and Palonosetron

Concentration (ppm)	Average Area (Palonosetron)	Average Area (Netupitant)
0	0	0
20	112744	1951299
30	169116	2926844
40	225488	3902458
50	278368	4823654
60	338232	5853687
70	394604	6829302
80	450976	7804916
Correlation Coefficient	0.999	0.999
Line equation	**y = 5632.x - 245.0**	**y = 97493x - 3818**

Table No. 3: System Suitability results of Netupitant and Palonosetron

S. No.	Drug	Retention time (min)	Mean area	USP Plate count	Tailing	Resolution
1	**Netupitant**	4.317	3904891	8358.8754210	1.064	4.3
2	**Palonosetron**	3.049	225427	10036.825471	1.056	

Table No. 4: Accuracy results of Netupitant and Palonosetron

DRUG	Spiked Level (%)	Amount added (µg/ml)	Amount found (µg/ml)	%RECOVERY(Mean)	%RSD
Netupitant	50	20	20.07	100.41	0.124
	100	40	40.08	100.21	0.023
	150	60	60.07	100.11	0.017
Palonoserton	50	20	20.03	100.17	0.207
	100	40	40.09	100.24	0.047
	150	60	60.12	100.20	0.048

Table No. 5: System precision results

S. No.	Peak Areas of Palonosetron	Peak Areas of Netupitant
1	225601	3903555
2	225550	3905804
3	225458	3902546
4	225688	3906800
5	225248	3903565
6	225452	3904320
Mean	225499	3904431
SD	152.062	1586.200
% RSD	0.067	0.040

Table No. 6: Method precision results

Concentration	Injection	Peak Areas of Palonosetron	Peak Areas of Netupitant
40ppm	1	225646	3905698
	2	225368	3908644
	3	225487	3904801
	4	225598	3902590
	5	225164	3906810
	6	225364	3903654
Statistical Analysis	**Mean**	225437	3905366
	SD	177.040	2186.183
	% RSD	0.078	0.055

Table No. 7: Intermediate precision results

Concentration	Injection	Peak Areas of Palonosetron	Peak Areas of Netupitant
40ppm	1	225480	3901892
	2	225807	3905831
	3	225881	3908465
	4	225980	3903566
	5	226081	3904809
	6	225365	3906500

Statistical Analysis	Mean	225765	3905177
	SD	283.733	2302.648
	% RSD	0.125	0.058

Table No. 8: Robustness results

Parameter(flow rate)	Netupitant (% RSD)	Palonosetron(% RSD)
0.8 ml	0.10	0.13
1.0 ml	0.11	0.10
1.2 ml	0.2	0.11

Table No. 9: LOD and LOQ results

DRUG	LOD(µg/ml)	LOQ(µg/ml)
Netupitant	0.051	0.156
Palonoserton	0.127	0.386

Table No. 10: Forced degradation studies of Palonosetron

Mode of Degradation	Condition	Peak Area	% Degradation as compared with Control
Control sample	**No treatment**	225498	-
Acid	**0.1 M HCl**	220834	2.068
Base	**0.1 M NaOH**	219471	2.67
Oxidative	**30% H_2O_2**	223742	0.78
Thermal	**100°C**	218900	2.93

Table No. 11: Forced degradation studies of Netupitant

Mode of Degradation	Condition	Peak Area	% Degradation as compared with Control
Control sample	**No treatment**	3904856	-
Acid	**0.1 M HCl**	3824672	2.053
Base	**0.1 M NaOH**	3754286	3.856
Oxidative	**30% H_2O_2**	3798564	2.722
Thermal	**100°C**	3784611	3.079

CONCLUSION

A novel stability indicating RP-HPLC method was developed for simultaneous estimation of Netupitant and Palonosetron in bulk. For routine analytical purpose, it is always necessary to establish methods capable of analyzing huge number of samples in a short period with accuracy and precision. The method was validated according to ICH guidelines and from the results obtained we can infer that the method is accurate, simple, precise and robust. Degradation studies were performed and no significant degradants were seen for oxidative, alkali, acid and thermal degradation. Finally, it can be concluded that this method can be employed for routine laboratory analysis.

ACKNOWLEDGEMENTS

The authors are thankful to Therdose Pharma Private Limited, Hyderabad, Apicore Pharmaceuticals Private Limited, Vadodara, Active Pharma Labs, K.P.H.B, Hyderabad for providing samples and chemicals.

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How to cite this article:

P. Sri Haritha et al. STABILITY INDICATING METHOD DEVELOPMENT AND VALIDATION FOR THE SIMULTANEOUS ESTIMATION OF PALONOSETRON AND NETUPITANT BY RP-HPLC IN ITS BULK FORM. J Pharm Res 2017;6(Suppl 2):101-106.

Conflict of interest: The authors have declared that no conflict of interest exists.

Source of support: Nil