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RP-HPLC METHOD DEVELOPMENT AND VALIDATION FOR THE SIMULTANEOUS ESTIMATION OF FOSNETUPITANT AND PALONOSETRON IN BULK AND COMBINED FORMULATION

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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 Palonosetron and Fosnetupitant in bulk and pharmaceutical dosage form. Palonosetron and Fosnetupitant was separated using Altima C_{18} column (250mm×4.6mm, 5mm particle size), Waters Alliance 2695 HPLC system with 2998 PDA detector and the mobile phase contained a mixture of Methanol: TEA buffer (pH adjusted to 4.5 with orthophosphoric acid) and Acetonitrile (50:25:25 v/v). The flow rate was set to 1ml/min with the responses measured at 225nm. The retention time of Palonosetron and Fosnetupitant was found to be 2.1 min and 3.5 min respectively with resolution of 8.08. Linearity was established for Palonosetron and Fosnetupitant in the range of 5-25µg/ml for FFosnetupitant and 12.5 - 62.5 µg/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 Palonosetron and Fosnetupitant respectively. RP-HPLC method for the simultaneous estimation of Palonosetron and Fosnetupitant in their combine dosage form was established and validated as per the ICH guidelines. Palonosetron and Fosnetupitant are more sensitive towards acidic degradation condition and moderate degradation towards alkaline, thermal and very much resistant towards oxidative, photolytic and water

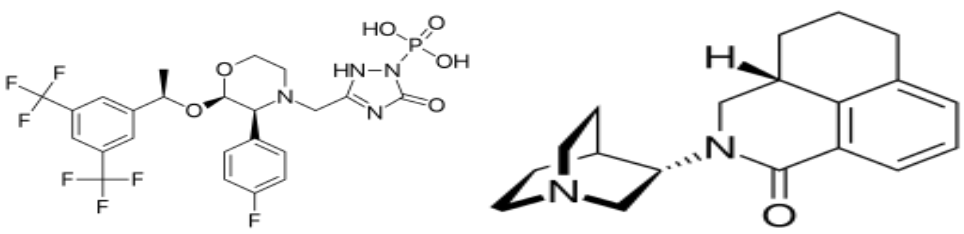
degradation. The developed method was successfully applied for the quantification of Palonosetron and Fosnetupitant in bulk and pharmaceutical dosage form.

KEYWORDS: Palonosetron and Fosnetupitant RP-HPLC-DAD, ICH.

INTRODUCTION

Fosnetupitant: Fosnetupitant is a selective antagonist of human substance P/neurokinin 1 (NK-1) receptors. Upon intravenous administration, Fosnetupitant is converted by phosphatases to its active form. It competitively binds to and blocks the activity of NK-1 receptors in the central nervous system, by inhibiting binding of substance P (SP) to NK-1 receptors. This prevents delayed emesis, which is associated with SP secretion. AKYNZEO is a combination of palonosetron, a serotonin-3 receptor antagonist, and Fosnetupitant (capsules for oral use) or Fosnetupitant (injections for intravenous use). AKYNZEO for injection is indicated in combination with dexamethasone in adults for the prevention of acute and delayed nausea and vomiting associated with initial and repeat courses of highly emetogenic cancer chemotherapy.

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). Palonosetron and Fosnetupitant 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 Palonosetron and Fosnetupitant 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 Palonosetron and Fosnetupitant with forced degradation studies according to ICH guidelines.[16]



(a) **(b)**

Fig 01. Chemical structure of (a) Fosnetupitant and (b) Palonosetron.

Chemical name/Nomenclature/IUPAC Name : [4-[5-[[2-[3,5-bis(trifluoromethyl)phenyl]-2-methylpropanoyl]-methylamino]-4-(2-methylphenyl)pyridin-2-yl]-1-methylpiperazin-1-ium-1-yl]methyl hydrogen phosphate

Molecular Formula : $C_{31}H_{35}F_6N_4O_5P$

Molecular Weight : 688.608 gm/mole.

PHYSICOCHEMICAL PROPERTIES

Description(Physical State) : Solid

Solubility : Water Solubility

pKa(strongest acidic) : -0.11

Metabolism: Fosnetupitant is the prodrug of Fosnetupitant In the human, rat, dog, minipig and marmoset liver microsomal incubations, two major metabolites, an *N-demethylation product (M1) _and an _N-oxidation product (M2),* in addition to hydroxylation products (M3), were identified in all species. CYP3A4 was found to be responsible for the oxidation of Fosnetupitant to the same metabolites observed also in the incubations with human liver microsomes. Metabolism was extensive, with the metabolites generally achieving greater concentrations than parent drug witin 24 hours. M1 and M2 exposure was similar in rat to humans, but higher in dogs, however M3 was lower in both species than in humans.

Pharmacodynamics: The combination drug, Akynzeo, palonosetron prevents nausea and vomiting during the acute phase and Fosnetupitant prevents nausea and vomiting during both the acute and delayed phase after cancer chemotherapy, Neurokinin-1 (NK-1) inhibitor drugs, such as Fosnetupitant, possess unique anxiolytic, antidepressant, and antiemetic properties.

Mechanism of action: The Fosnetupitant in this drug combination is a selective P/neurokinin-1 (NK-1) receptor antagonist Fosnetupitant the active moiety of Fosnetupitant, is a selective neurokinin 1 (NK1) receptor antagonist with antiemetic activity. Fosnetupitant competitively binds to and blocks the activity of the human substance P/NK1 receptors in the central nervous system (CNS), inhibiting NK1-receptor binding of the endogenous tachykinin neuropeptide substance P (SP), which results in the prevention of chemotherapy-induced nausea and vomiting (CINV). Substance P 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 present at high levels 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, Fosnetupitant demonstrated 92.5% NK1 receptor occupancy at 6 hours, with 76% occupancy at 96 hours.

PALONOSETRON

Chemical name/ Nomenclature / 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

Molecular Formula : $C_{19}H_{24}N_2O$

Description(Physical State) : Solid

Solubility : Soluble in Propylene Glycol and Water

Dosage : Tablet

Melting point : 87 to 88 °C

pKa(strongest Basic) : 7.97

Log P : 2.55

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 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

Chemicals and reagents

Fosnetupitant (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 Fosnetupitant 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

Palonosetron and Fosnetupitant was analysed in Kromasil C18 column (250mm×4.6 mm, 5mm 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 225nm. Injection volume was 20µl. The run time was 8 min and the retention time of Palonosetron and Fosnetupitant 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
Mobile phase : Methanol: TEA Buffer pH 4.5: Acetonitrile (50:25:25)
Column : Altima C_{18} column (250mm×4.6 mm, 5mm particle size)
Flow rate : 1mL/min
Wavelength : 225nm
Injection volume : 10 ml
Column oven : Ambient
Run time : 7 Minutes
Column temp : 40ºC
Injection Volume : 10 µl
Run time : 7 minutes

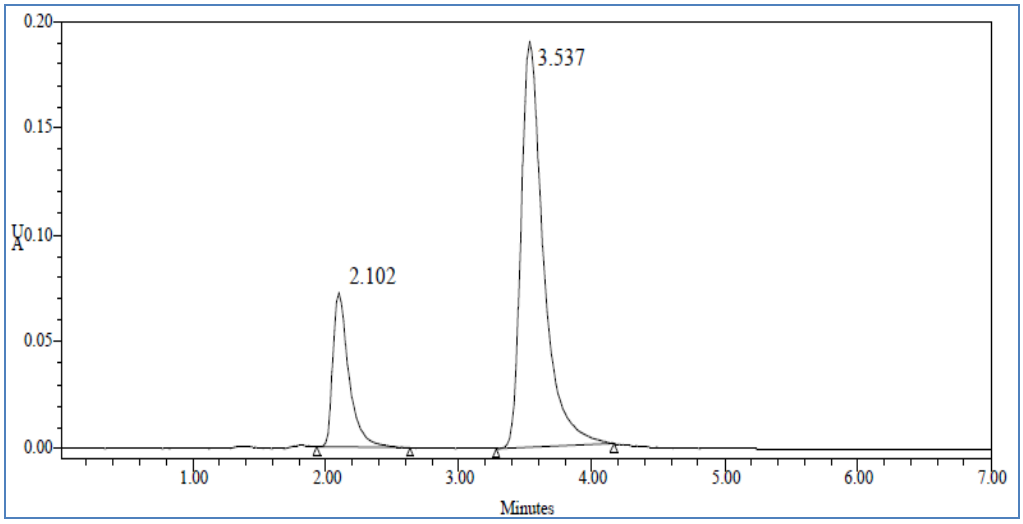


Fig.2: Typical Chromatogram of optimized Chromatogram.

SOLUTIONS AND SAMPLE PREPARATION

Preparation of Triethylamine (TEA) buffer (pH-4.5)

Dissolve 1.5ml of Ttiethyl amine in 250 ml HPLC water and adjust the p^H 4.5. Fliter and sonicate the solution by vaccum filtration and ultrasonication.

Preparation of mobile phase

Accurately measured 400 ml (40%) of Methanol, 200 ml of Triethylamine buffer (20%) and 400 ml of Acetonitrile (40%) were mixed and degassed in digital ultrasonicater for 10 minutes and then filtered through 0.45 µ filter under vacuum filtration.

Diluent Preparation

The Mobile phase was used as the diluent.

Preparation of diluent: Mobile phase was used as diluent.

Preparation of Sample Solution

Take average weight of one Tablet and crush in a mortor by using pestle and weight 10 mg equivalent weight of Palonosetron and Fosnetupitant sample into a 10mL clean dry volumetric flask and add about 7mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent.

Further pipette 0.1ml of the above Palonosetron and 0.375ml of the Fosnetupitant stock solutions into a 10ml volumetric flask and dilute up to the mark with Diluent.

Procedure

Inject the three replicate injections of standard and sample solutions and calculate the assay by using formula:

$$\%ASSAY = \frac{\text{Sample area}}{\text{Standard area}} \times \frac{\text{Weight of standard}}{\text{Dilution of standard}} \times \frac{\text{Dilution of sample}}{\text{Weight of sample}} \times \frac{\text{Purity}}{100} \times \frac{\text{Weight of tablet}}{\text{Label claim}} \times 100$$

PREPARATION OF DRUG SOLUTIONS FOR LINEARITY

Accurately weigh and transfer 10 mg of Palonosetron and 10mg of Fosnetupitant working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution).

Preparation of Level – I (5 ppm of Palonosetron & 12.5ppm of Fosnetupitant)

Pipette out 0.05ml of Palonosetron and 0.125ml of Fosnetupitant stock solutions was take in a 10ml of volumetric flask dilute up to the mark with diluent.

Preparation of Level – II (10 ppm of Palonosetron & 25ppm of Fosnetupitant)

Pipette out 0.1ml of Palonosetron and 0.25ml of Fosnetupitant stock solutions was take in a 10ml of volumetric flask dilute up to the mark with diluent.

Preparation of Level – III (15 ppm of Palonosetron & 37.5ppm of Fosnetupitant)

Pipette out 0.15 ml of Palonosetron and 0.375ml of Fosnetupitant stock solutions was take in a 10ml of volumetric flask dilute up to the mark with diluent.

Preparation of Level – IV (20 ppm of Palonosetron & 50ppm of Fosnetupitant)

Pipette out 0.2 ml of Palonosetron and 0.5ml of Fosnetupitant stock solutions was take in a 10ml of volumetric flask dilute up to the mark with diluent.

Preparation of Level – V (25 ppm of Palonosetron & 62.5ppm of Fosnetupitant)

Pipette out 0.25ml of Palonosetron and 0.625ml of Fosnetupitant stock solutions was take in a 10ml of volumetric flask dilute up to the mark with diluent.

Procedure

Inject each level into the chromatographic system and measure the peak area.

Plot a graph of peak area versus concentration (on X-axis concentration and on Y-axis Peak area) and calculate the correlation coefficient.

PRECISION

Repeatability

Preparation Of Palonosetron and Fosnetupitant Product Solution for Precision

Accurately weigh and transfer 10 mg of Palonosetron and 10mg of Fosnetupitant working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 0.1ml of the above Palonosetron and 0.375ml of the Fosnetupitant stock solutions into a 10ml volumetric flask and dilute up to the mark with Diluent.

The standard solution was injected for five times and measured the area for all five injections in HPLC. The %RSD for the area of five replicate injections was found to be within the specified limits.

INTERMEDIATE PRECISION

To evaluate the intermediate precision (also known as Ruggedness) of the method, Precision was performed on different days by maintaining same conditions.

Procedure

DAY 1

The standard solution was injected for Six times and measured the area for all Six injections in HPLC. The %RSD for the area of Six replicate injections was found to be within the specified limits.

DAY 2

The standard solution was injected for Six times and measured the area for all Six injections in HPLC. The %RSD for the area of Six replicate injections was found to be within the specified limits.

Accuracy

For preparation of 50% Standard stock solution

Accurately weigh and transfer 10 mg of Palonosetron and 10mg of Fosnetupitant working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution).

Further pipette 0.075ml of the above Palonosetron and 0.187ml of the Fosnetupitant stock solutions into a 10ml volumetric flask and dilute up to the mark with Diluent.

For preparation of 100% Standard stock solution

Accurately weigh and transfer 10 mg of Palonosetron and 10mg of Fosnetupitant working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution).

Further pipette 0.15ml of the above Palonosetron and 0.375ml of the Fosnetupitant stock solutions into a 10ml volumetric flask and dilute up to the mark with Diluent.

For preparation of 150% Standard stock solution

Accurately weigh and transfer 10 mg of Palonosetron and 10mg of Fosnetupitant working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution).

Further pipette 0.225ml of Palonosetron and 0.56ml of Fosnetupitant from the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluents.

Inject the Three replicate injections of individual concentrations (50%,100%,150%) were made under the optimized conditions shown Table . Recorded the chromatograms and measured the peak responses. Calculate the Amount found and Amount added for Palonosetron and Fosnetupitant and calculate the individual recovery and mean recovery values were Shown Table 1& 2.

Table 1: The accuracy results for Palonosetron.

%Concentration (at specification Level)	Area	Amount Added (ppm)	Amount Found (ppm)	% Recovery	Mean Recovery
50%	287774	7.5	7.56	100.8	99.6%
100%	551495	15	14.8	98.6	
150%	825175	22.5	22.4	99.5	

Table 2: The accuracy results for Fosnetupitant.

%Concentration (at specification Level)	Area	Amount Added (ppm)	Amount Found (ppm)	% Recovery	Mean Recovery
50%	1104782	18.75	18.73	100%	100%
100%	2105321	37.5	37.4	99.9%	
150%	3211306	56.25	56.21	100%	

ROBUSTNESS

The analysis was performed in different conditions to find the variability of test results. The following conditions are checked for variation of results were shown Tables 3 & 4.

For preparation of Standard solution

Accurately weigh and transfer 10 mg of Palonosetron and 10mg of Fosnetupitant working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 0.15ml of the above Palonosetron and 0.375ml of the Fosnetupitant stock solutions into a 10ml volumetric flask and dilute up to the mark with Diluent.

Effect of Variation of flow conditions

The sample was analyzed at 0.9 ml/min and 1.1 ml/min instead of 1ml/min, remaining conditions are same. 10µl of the above sample was injected and chromatograms were recorded.

Effect of Variation of mobile phase organic composition

The sample was analyzed by variation of mobile phase i.e. Methanol: TEA Buffer: Acetonitrile was taken in the ratio and 40: 40:20, 60:10:30 instead (50:25:25), remaining conditions are same. 10µl of the above sample was injected and chromatograms were recorded.

Table 3: Results of system suitability for Palonosetron.

S.No	Name	Rt	Area	Height	USP plate count	USP Tailing
1	Palonosetron	2.117	608452	71498	5643	1.9
2	Palonosetron	2.118	606820	126412	5432	1.6
3	Palonosetron	2.116	608452	126471	5123	1.6
4	Palonosetron	2.109	595267	129859	5207	1.7
5	Palonosetron	2.102	596608	124691	5481	1.6
Mean			603119.8			
Std. Dev			6607.31			
% RSD			1.09			

Table 4: Results of system suitability for Palonosetron.

Sno	Name	Rt	Area	Height	USP plate count	USP Tailing	USP Resolution
1	Fosnetupitant	3.547	2234724	188631	5043	1.2	2.07
2	Fosnetupitant	3.539	2240080	2614821	5432	1.4	2.05
3	Fosnetupitant	3.547	2234724	2321451	5987	1.5	2.0
4	Fosnetupitant	3.565	2204466	2324710	5845	1.6	2.01
5	Fosnetupitant	3.537	2209574	2531247	5371	1.6	2.01
Mean			2224714				
Std. Dev			16399.05				
% RSD			0.73				

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 Palonosetron and Fosnetupitant were obtained with a mobile phase containing a mixture of Methanol: TEA Buffer pH 4.5: Acetonitrile (50:25:25) was delivered at a flow rate of 1ml/min to get better reproducibility and repeatability. Quantification was achieved with PDA detection at 225nm based on peak area. The retention time of Palonosetron and Fosnetupitant was found to be 2.138min and 3.718min respectively with resolution of 8.08. Linearity was established for Palonosetron and Fosnetupitant in the range of 5-25 g/ml for Fosnetupitant and 12.5-62.5 µg/ml for Palonosetron with correlation coefficients (r2=0.999) and the percentage recoveries were between 99.85% to 100.04% and 99.73% to 100.03% for Palonosetron and Fosnetupitant respectively, which indicate accuracy of the proposed method. The % RSD values of accuracy for Palonosetron and Fosnetupitant were found to be < 2%. The % RSD values of method precision are 0.5% and 0.35% for Palonosetron and Fosnetupitant respectively and % RSD values of system precision are 1.3% and 1.1% for Palonosetron and Fosnetupitant respectively. The % RSD values of reproducibility are 0.04% and 0.02% for Palonosetron

and Fosnetupitant respectively, reveal that the proposed method is precise. LOD values for Palonosetron and Fosnetupitant were found to be 0.06µg/ml and 0.01µg/ml respectively and LOQ values for Palonosetron and Fosnetupitant 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 Palonosetron and Fosnetupitant.

Method validation

The developed method for the simultaneous estimation of Palonosetron and Fosnetupitant 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).

SYSTEM SUITABILITY

Accurately weigh and transfer 10 mg of Palonosetron and 10mg of Fosnetupitant working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution) Further pipette 0.1ml of the above Palonosetron and 0.375ml of the Fosnetupitant stock solutions into a 10ml volumetric flask and dilute up to the mark with Diluent.

Specificity

The effect of excipients and other additives usually present in the combined capsule dosage form of Palonosetron and Fosnetupitant 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).

Blank

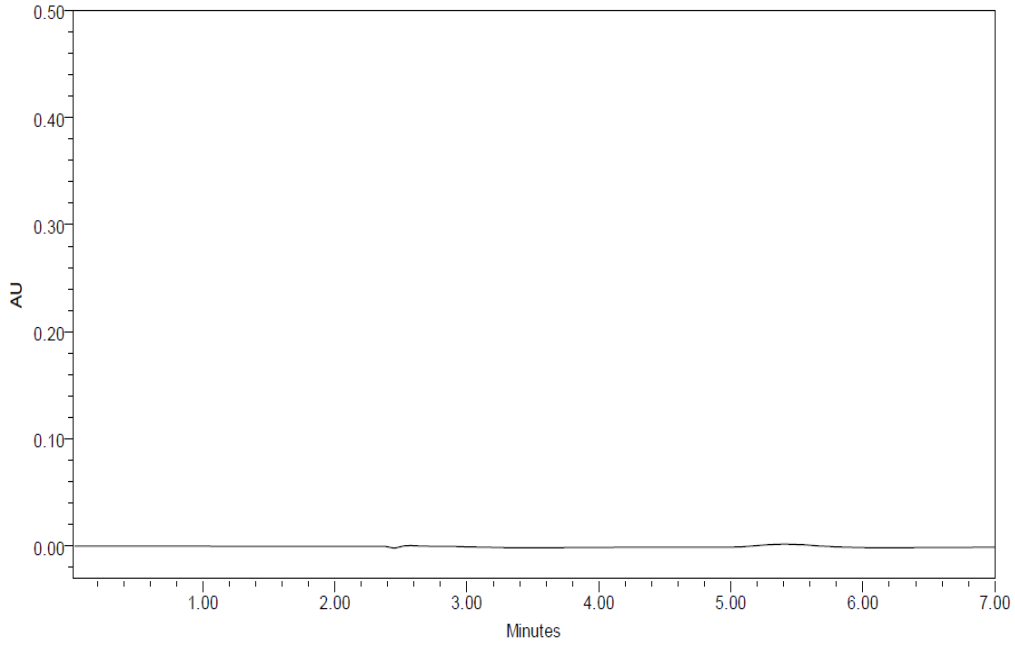


Fig. 3: Chromatogram showing blank (mobile phase preparation).

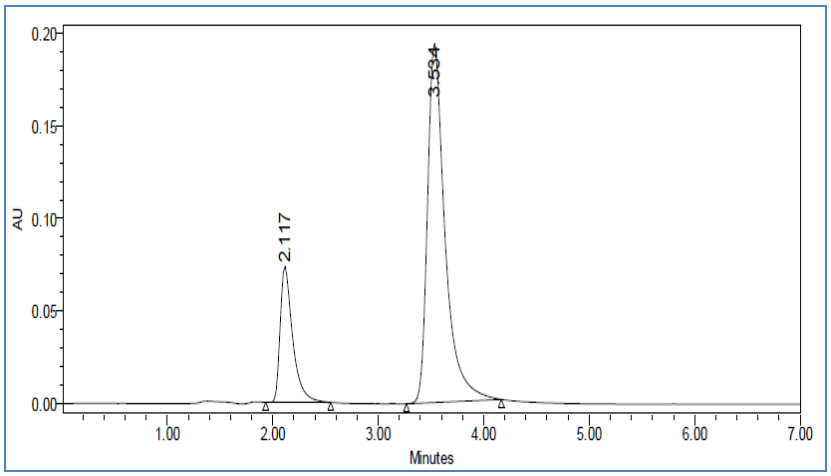


Fig. 4: Chromatogram standard solution.

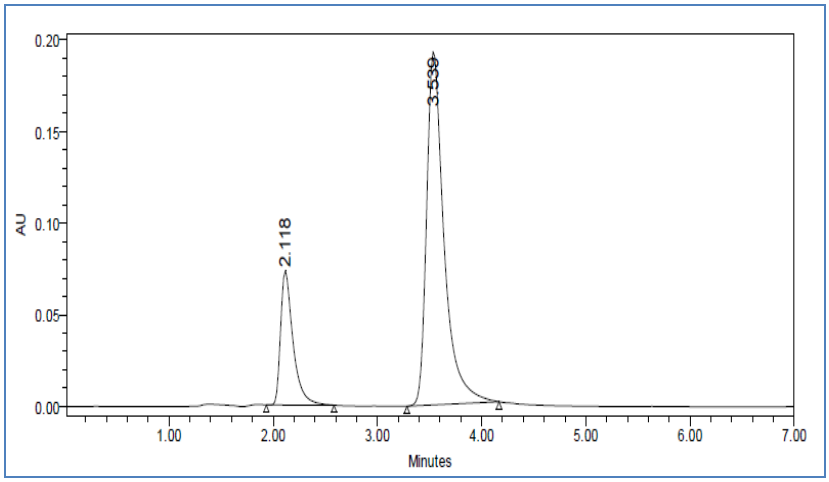


Fig 5: Chromatogram showing sample solution.

Linearity and range for Palonosetron and Fosnetupitant

Aliquots of 0.25, 0.5, 0.75, 1, 1.25 and 1.5ml of mixed standard working solutions of Palonosetron and Fosnetupitant was pipetted out from the standard stock solution of 3000µg/ml of Fosnetupitant and 5µg/ml of Palonosetron and transferred into a series of 10ml clean dry volumetric flask and make volume up to themark with the same diluent to get the concentration of 5,10,15,20 and 25 µg/ml of Fosnetupitant and 12.5, 25, 37.5, 50 and 62.5 µg/ml of Palonosetron. The calibration standard solutions of Palonosetron and Fosnetupitant were injected using a 20µl Hamilton Rheodyne injector and the chromatograms were recorded at 225nm and a calibration graph was obtained by plotting peak area versus concentration of Palonosetron and Fosnetupitant respectively. The linearity data is presented in (Figure 8 and 9) and Table 3.

Table 5: Linearity data for Palonosetron and Fosnetupitant.

FOSNETUPITANT		PALONOSETRON	
Concentration µg/ml	**Average Peak Area**	**Concentration µg/ml**	**Average Peak Area**
12.5	757881	5	205035
25	757881	10	381239
37.5	1458941	15	561128
50	2132457	20	740162
62.5	2901811	25	909922

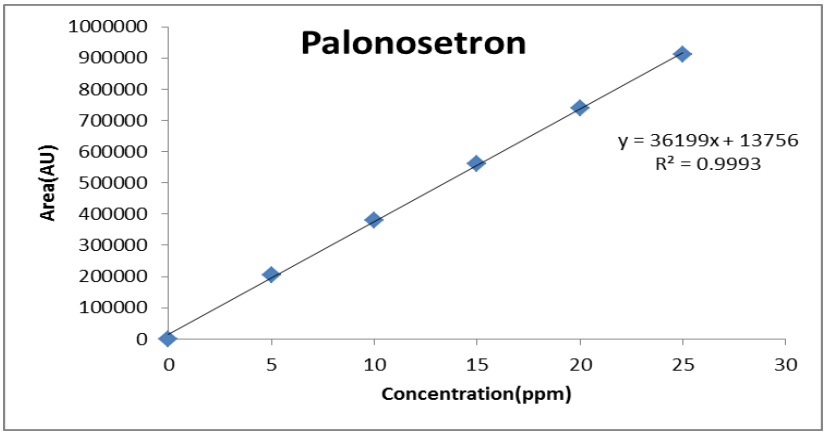


Fig. 6: Calibration graph for Palonosetron.

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) = 36199

Intercept (c) = 13756

Correlation Coefficient (r) = 0.999

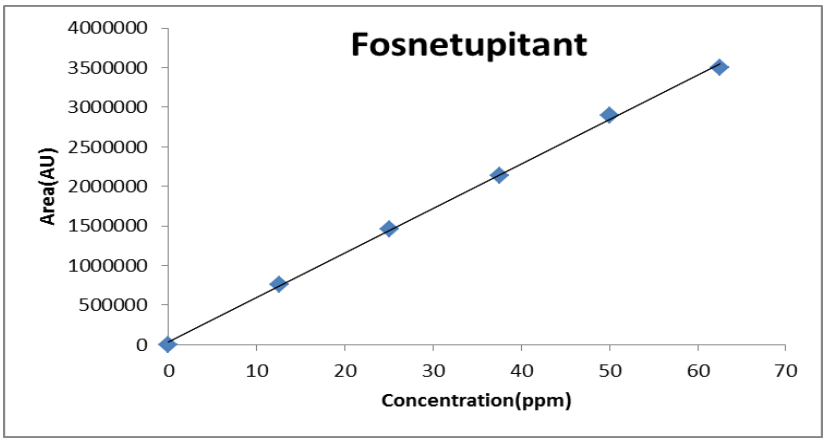


Fig. 7: Calibration Graph For Fosnetupitant.

Accuracy studies for Palonosetron and Fosnetupitant

The accuracy of the method was determined by calculating recovery of Palonosetron and Fosnetupitant by the method of standard addition. Known amount of standard solution of Palonosetron and Fosnetupitant at 50%, 100% and 150% was added to a pre quantified sample solution and injected into the HPLC system. The mean percentage recovery of

Palonosetron and Fosnetupitant at each level was calculated and the results were presented in Table 4.

Table 6: The accuracy results for Palonosetron.

%Concentration (at specification Level)	Area	Amount Added (ppm)	Amount Found (ppm)	% Recovery	Mean Recovery
50%	287774	7.5	7.56	100.8	99.6%
100%	551495	15	14.8	98.6	
150%	825175	22.5	22.4	99.5	

Table 7: The accuracy results for Fosnetupitant.

%Concentration (at specification Level)	Area	Amount Added (ppm)	Amount Found (ppm)	% Recovery	Mean Recovery
50%	1104782	18.75	18.73	100%	100%
100%	2105321	37.5	37.4	99.9%	
150%	3211306	56.25	56.21	100%	

Preparation of pre quantified sample solution for accuracy studies

Capsule powder equivalent to 300mg of Fosnetupitant and 0.5mg of Palonosetron were taken into100ml 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 Palonosetron and Fosnetupitant sample stock solution into a 10ml volumetric flask and diluted up to the mark with diluent to get the concentration of 150µg/ml of Fosnetupitant and 0.25µg/ml of Palonosetron.

Preparation of standard solution of Palonosetron and Fosnetupitant for accuracy studies

Standard stock solutions of Palonosetron and Fosnetupitant were prepared by dissolving 300mg of Fosnetupitant 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 Fosnetupitant and 5µg/ml of Palonosetron.

Preparation of 50% standard solution

From the standard stock solution of 3000µg/ml of Fosnetupitant 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 Fosnetupitant and 0.125µg/ml of Palonosetron.

Preparation of 100% standard solution

From the standard stock solution of 3000µg/ml of Fosnetupitant 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 Fosnetupitant and 0.25µg/ml of Palonosetron.

Limit of Detection

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value.

$$\mathbf{LOD = 3.3 \times \sigma / s}$$

Where

σ = Standard deviation of the response

S = Slope of the calibration curve

Palonosetron: =3.3 × 3188.4/36199 =0.2µg/ml

Fosnetupitant: =3.3 × 39656.07/56304 =2.3µg/ml

Limit of Quantitation

The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined.

$$\mathbf{LOQ = 10 \times \sigma / S}$$

Where

σ = Standard deviation of the response

S = Slope of the calibration curve

Palonosetron: =10×3188.481242/36199 = 0.8µg/ml

Fosnetupitant: =10 × 39656.07/56304 = 7.04µg/ml

Robustness

The robustness was performed for the flow rate variations from 0.9 ml/min to 1.1ml/min and mobile phase ratio variation from more organic phase to less organic phase ratio for Palonosetron and Fosnetupitant. The method is robust only in less flow condition and the method is robust even by change in the Mobile phase ±5%. The standard and samples of Palonosetron and Fosnetupitant were injected by changing the conditions of chromatography.

There was no significant change in the parameters like resolution, tailing factor, asymmetric factor, and plate count.

Table 8: Results for Robustness (Palonosetron).

Parameter used for sample analysis	Peak Area	Retention Time	Theoretical plates	Tailing factor
Actual Flow rate of 1.0 mL/min	607323	2.102	5586	1.7
Less Flow rate of 0.9 mL/min	674735	2.330	5231	1.7
More Flow rate of 1.1 mL/min	1408920	1.950	5234	1.7
Less organic phase	606093	2.290	5643	1.4
More organic phase	603559	1.998	5298	1.5

Table 9: Robustness study of Fosnetupitant.

Parameter used for sample analysis	Peak Area	Retention Time	Theoretical plates	Tailing factor
Actual Flow rate of 1.0 mL/min	558777	3.537	5371	1.6
Less Flow rate of 0.9 mL/min	2505636	3.885	5324	1.7
More Flow rate of 1.1 mL/min	1408920	3.263	5098	1.7
Less organic phase	2239255	4.435	5239	1.2
More organic phase	2300346	3.009	5647	1.0

SUMMARY

The analytical method was developed by studying different parameters. First of all, maximum absorbance was found to be at 225 nm and the peak purity was excellent. Injection volume was selected to be 10µl which gave a good peak area. The column used for study was Altima C_{18} because it was giving good peak. Ambient temperature was found to be suitable for the nature of drug solution. The flow rate was fixed at 1.0ml/min because of good peak area and satisfactory retention time. Mobile phase is Methanol: TEA Buffer pH 4.5: Acetonitrile (50:25:25) was fixed due to good symmetrical peak. So this mobile phase was used for the proposed study. Run time was selected to be 7 min because analyze gave peak around 2.102, 3.537 ±0.02min respectively and also to reduce the total run time. The percent recovery was found to be 98.0-102 was linear and precise over the same range. Both system and method precision was found to be accurate and well within range. The analytical method was found linearity over the range 5-25mg/ml of palonosetron and 12.5-62.5mg/ml of Fosnetupitant of the target concentration. The analytical passed both robustness and ruggedness tests. On both cases, relative standard deviation was well satisfactory.

CONCLUSION

In the present investigation, a simple, sensitive, precise and accurate RP-HPLC method was

developed for the quantitative estimation of palonosetron and Fosnetupitant in bulk drug and pharmaceutical dosage forms. This method was simple, since diluted samples are directly used without any preliminary chemical derivatisation or purification steps. palonosetron and Fosnetupitant was freely soluble in ethanol, methanol and sparingly soluble in water. Methanol: TEA Buffer pH 4.5: Acetonitrile (50:25:25) was chosen as the mobile phase. The solvent system used in this method was economical. The %RSD values were within 2 and the method was found to be precise. The results expressed in Tables for RP-HPLC method was promising. The RP-HPLC method is more sensitive, accurate and precise compared to the Spectrophotometric methods. This method can be used for the routine determination of palonosetron and Fosnetupitant in bulk drug and in Pharmaceutical dosage forms.

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