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SIMULTANEOUS QUANTITATIVE ESTIMATION OF NETUPITANT AND PALONOSETRON HCl BY HPTLC METHOD: DEVELOPMENT AND VALIDATION.

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ABSTRACT
A simple**,** selective, precise High-performance thin-layer chromatographic method for simultaneous estimation of Palonosetron hydrochloride and Netupitant has been developed and validated. The method employed, HPTLC aluminium plates precoated with silica gel as the stationary phase. The solvent system consisted of Dichloromethane: Ethyl acetate: Triethyleamine: Methanol (5: 3: 1.5: 0.5 *v/v*). The R_f value was found to 0.25±0.05 and 0.85±0.05 for Palonosetron HCl and Netupitant. Detection and quantitation were performed by densitrometric scanning at 241 nm for both drugs. The correlation coefficient of Palonosetron HCl and Netupitant was found to be 0.9991 and 0.9999 in a range of (0.01-0.06 µg/ml) & (6-36 µ/ml) respectively. The method was validated for precision, recovery, robustness, LOQ, LOQ and Assay. Statistical analysis proves that the method is repeatable, selective and accurate for the estimation of investigated drug. The proposed developed HPTLC method can be applied for quantitative determination of Palonosetron HCl and Netupitant.

KEYWORDS: HPTLC method, Palonosetron HCl, Netupitant, validation.

INTRODUCTION
D-AKYNZEO® is a marketed formulation which contain Netupitant and Palonosetron (as Hydrochloride) 300mg/ 500 mcg, Hard gelatin capsule available in Germany but not yet in India.

Netupitant(NETU) is a novel antiemetic agent used in cancer chemotherapy with a chemical name 2-[3,5-bis(trifluoromethyl)phenyl]-N,2-dimethyl-N-[4-(2-methylphenyl)-6-(4-methylpiperazin-1-yl)pyridin-3-yl] propanamide. Having Molecular Weight: 578.61 g mol-1 and Molecular Formula is C30H32F6N4O, PKa1 : 2.36, PKa2: 7.65 and Partition coefficient : 5.1. It exist structural formula in (Figure 1).[1]

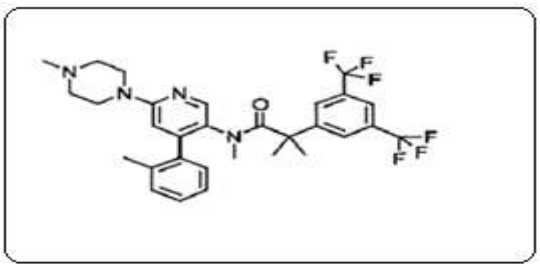

Figure 1: Structural formula of Netupitant.

Palonosetron hydrochloride (PALO) is a antiemetic and antinauseant agent with chemical name (3aS)-2-[(S)-1-Azabicyclo[2.2.2]oct-3-yl]-2,3,3a,4,5,6-hexahydro-1-oxo- 1*H*benz[*de*]isoquinoline hydrochloride. Having Molecular Weight: 332.87, Molecular Formula: C19H24N2O.HCl, PKa: 8.9 and Partition Coefficient 4.3 at pH 7.4. Palonosetron hydrochloride exists as a single isomer and Its structural formula was mentioned in (Figure 2).[2]

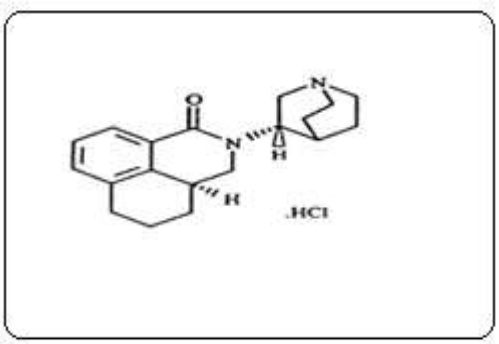

Figure 2: Structural formula of Palonosetron hydrochloride.

Netupitant is a selective high-affinity antagonist of human substance P/neurokinin 1 (NK1) receptors. Palonosetron is a selective serotonin subtype 3 (5-HT3) receptor antagonist with a strong binding affinity for this receptor. Cancer chemotherapy may be associated with a high incidence of nausea and vomiting, particularly when certain agents, such as cisplatin, are used. 5-HT3 receptors are located on the nerve terminals of the vagus in the periphery and centrally in the chemoreceptor trigger zone of the area postrema. It is thought that chemotherapeutic agents produce nausea and vomiting by releasing serotonin from the enterochromaffin cells of the small intestine and that the released serotonin then activates 5-HT3 receptors located on vagal afferents to initiate the vomiting reflex. The development of acute emesis is known to depend on 5-Hydroxytryptamine serotonin (5-HT) and the 5-HT3 receptor has been demonstrated to selectively participate in the emetic response. Delayed emesis 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 and Palonosetron HCL can contribute to the inhibition of substance P mediated response.[3]

Literature survey suggests that methods have been reported for estimation of Palonosetron HCl by RP-HPLC[4], Stability indicating HPTLC[5-6], some spectroscopic methods[7], liquid chromatography-tandam mass spectroscopy for estimation of Palonosetron in human plasma[8]. But for the Netupitant no analytical methods were reported.

This present research work aimed to introduce a simultaneous quantitative estimation of Netupitant and Palonosetron HCl by HPTLC method development and validation. (as per ICH guidelines)[9]

MATERIALS AND METHODS

Instrumentation and Software

The samples were spotted in the form of bands of 6 mm width with a Camag microlitre syringe on precoated silica gel aluminium plates 60 RP-18 F254 (10 × 10 cm with 250 mm thickness, E. Merck), using a Camag Linomat 5 applicator. The plates were prewashed with methanol and activated at 60 0C for 5 min prior to chromatography. The slit dimension was kept at 6.00 × 0.45 mm (micro) and 20 mm/s scanning speed was employed. The mobile phase consisted of dichloromethane: ethyl acetate: Triethylamine: Methanol (5: 3: 1.5: 0.5 *v*/*v*)., and 10 ml of mobile phase was used. Linear ascending development was carried out in a 10 × 10 cm twin trough glass chamber saturated with the mobile phase. The optimized chamber saturation time for the mobile phase was 30 min at room temperature (25ºC±2). The length of the chromatogram run was approximately 9 cm. Subsequent to development; the TLC plates were dried in a current of air. Densitometric scanning was performed on a Camag TLC scanner 3 and was operated by WINCats software.

Reagent and Chemicals

Palonosetron hydrochloride (pure API) was purchased from Swapnroop pharmaceutical & Ltd. Aurangabad and Netupitant (pure API) was provided as a gift sample from Apicore drugs pvt.Ltd. Baroda. and synthetic mixture as specified in German marketed formulation Akynzeo (300mg NETU and 0.50 mg PALO) was made in pharmaceutical laboratory at MSU. Methanol - Triethyleamine (Rankem), Dichloromethane (Fischer) , Ethyl acetate (Rankem) all analytical grade solvents were used for the experiment.

Selection of common solvent

After checking the solubility of drugs (NETU & PALO) in different solvents methanol has been selected as Common solvent for developing spectral characteristics.

Selection of detection wavelength

Selection of wavelength for the NETU & PALO done by scanning of solutions between the range of 200-400 nm. It shows that absorbance was maximum at 258 nm for Netupitant and 241 nm for Palonosetron hydrochloride. Detection wavelength selected for estimation of both drugs was 241 nm respectively for HPTLC method.(Figure.3)

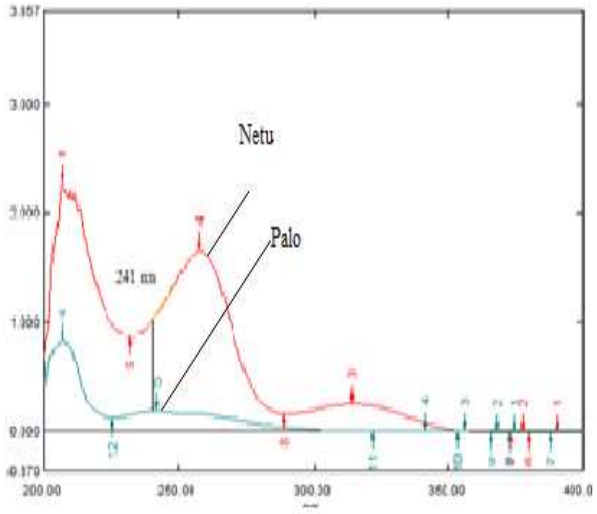

Figure 3: Selection of detection wavelength.

Preparation of standard stock solution

An accurately weighed 25mg of standard NETU and PALO powder, transferred in separate 25 ml volumetric flask and dissolved in methanol. The flasks were shaken and volumes were made up to mark with methanol to give a solution containing 1000 µg/ml of each NETU and PALO.

Preparation of synthetic mixture

Synthetic mixture was prepared by using Active ingredient (Netu 300 mg and PALO 0.50mg) And other

excipients: microcrystalline cellulose, sucrose laurate, povidone, croscarmellose sodium, silicon dioxide, sodium stearylfumarate, magnesium stearate. Glyceryl caprylate, polyglyceryl -3 dioleate, butylated hydroxyanisole.etc. This synthetic mixture was mixed, dissolved in 100 ml methanol, Filtered and used for further analytical process.

Method validation

HPTLC is a widely used analytical technique due to its advantages of low operating cost, high sample throughput, and minimum sample preparation requirement. The major advantage of HPTLC is that several samples can be run simultaneously using a small quantity of mobile phase unlike HPLC, thus reducing the analysis time and cost per analysis.[10-11]

The proposed method was validated according to the International Conference on Harmonization (ICH) guidelines.

Linearity

Linearity was performed by taking from stock solution aliquots in the range of (6,12,18,24,30,36µg/ml) NETU and (0.01,0.02,0.03,0.04,0.05,0.06 µg/ml) PALO then make up to the mark of 10ml volumetric flask. Volume of 20µl was injected five times for each concentration level and calibration curve was constructed by plotting a peak area verses drug concentration.(Figure 4)

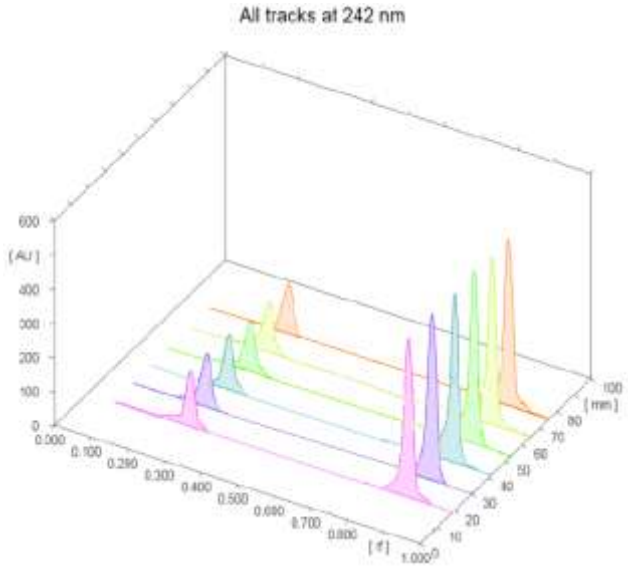

Figure 4: 3D view of different conc. of NETU & PALO.

Precision

Three sample of the mixture were prepared and analyzed as per the test method on same day and different days and calculated the %RSD.

Recovery studies

It was done by recovery study. Sample solution were prepared by spiking at about 80% ,100%,120%.The values of percent recovery and average value of percent recovery for NETU & PALO mixture were calculated.

Limit of detection and Limit of quantification

The parameter LOD and LOQ were determined on the basis of response and slope of the regression.

LOD= 3.3*σ/S

LOQ=10*σ/S

where, σ= standard deviation of intercept, S=slope of calibration curve.5

Robustness

By introducing small changes in the mobile phase composition, mobile phase volume the effects on the results were examined. Robustness of the method was done in triplicate and the %R.S.D. of peak area was calculated.

Applicability of developed method

Synthetic mixture Powder equivalent 300 mg NETU and 0.50 mg of PALO was weighed and transferred to 10 ml volumetric flask. 10 ml methanol was added and sonicated for 10 minutes. After sonication appropriate dilution was made in the range of (6-36 µg/ml) of NETU, (0.01-0.06µg/ml) of PALO and solution was subjected to analysis.

RESULTS AND DISCUSSION

Development of the optimum mobile phase: The TLC procedure was optimized with a view to developing HPTLC method for NETU & PALO mixture. Initially, The mobile phase consisting dichloromethane: Ethyl acetate: Triethyleamine: Methanol (5: 3: 1.5:0.5 v/v/v) gave good resolution, sharp and well defined peak at R_f = 0.85 for NETU and R_f= 0.25 for PALO, Well defined spots were obtained after the chamber was saturated with mobile phase for 30 min at room temperature. The TLC plate was visualized under UV light at 241 nm, without derivatization. The optimized HPTLC parameters was shown in (Table.1).

A photograph of a TLC plate after chromatography of NETU & PALO mixture of standard solution are shown in **(Figure 5).**

Table 1: Optimized HPTLC parameters for NETU & PALO.

Method parameters	Optimized parameters
Stationary phase	Precoated silica gel G 60 F 254 (20X10)
Mobile phase	Dichloromethane: Ethyl acetate: Triethyleamine: Methanol (5: 3: 1.5: 0.5 *v/v*)
Chamber saturation time	30 min
Solvent front	9 cm
Detection wavelength	241 nm
R_f of NETU	0.25
R_f of PALO	0.85

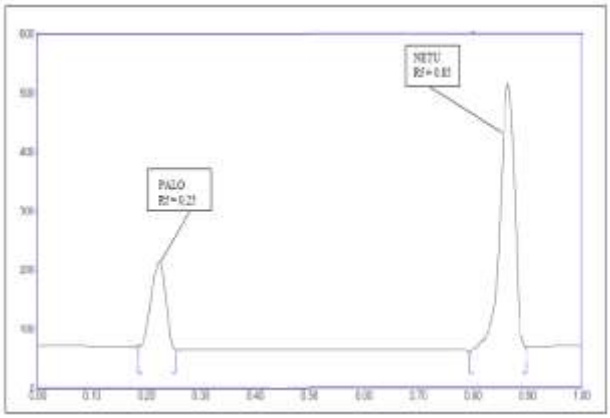

Figure.5: Chromatogram of NETU (R_f = 0.85) & PALO (R_f = 0.25)

Validation of optimized HPTLC method

Linearity

The linear regression data for the calibration curves showed good linear relationship over the concentration range of 6-36µg/ml for NETU and 0.01-0.06µg/ml for PALO. (Table 2) and (Figure.6)

Table.2: Linearity data for NETU & PALO.

Sr No.	Concentration (µg/ml)		Peak area	
	NETU	PALO	NETU	PALO
1	6	0.01	11054.2	4303.9
2	12	0.02	12045.7	4436.8
3	18	0.03	13178.2	4581.9
4	24	0.04	14391.6	4699.2
5	30	0.05	15486.5	4846.2
6	36	0.06	16672.4	4972.8

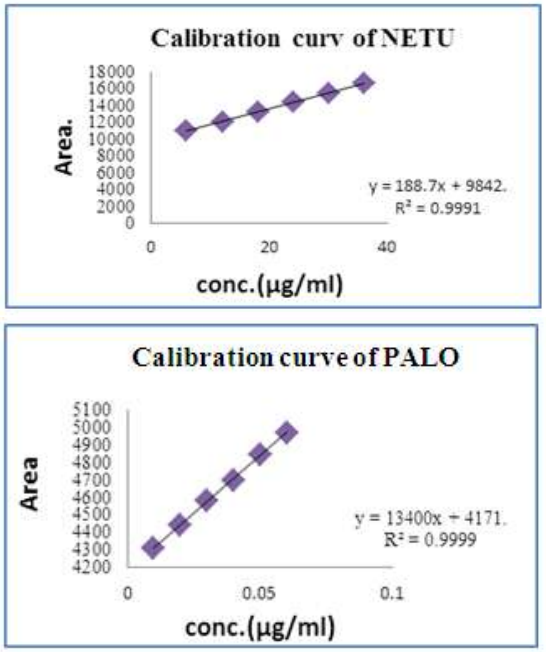

Figure 6: calibration curve for NETU & PALO.

Precision

Standard solutions of NETU & PALO were prepared. Different µL of the standard solutions was applied and chromatograms were recorded. The peak areas of NETU & PALO were calculated for each trial. The experiment was repeated 3 times in a day for Intraday-precision and on 3 different days for inter-day precision. The average % RSD of intra-day and inter-day measurements for determination of NETU & PALO was found to be (0.570 and 0.392) and (0.499 and 0.295) respectively. The low value of SD obtained confirms the precision of the method. (Table 3).

Table 3: Intra-day precision for estimation of NETU & PALO.

Concentration (µg/ml)		Peak area							
		Set 1	Set 2	Set 1	Set 2	Mean	Mean	%RSD	%RSD
Netu	Palo	Netu	Netu	Palo	Palo	Netu	Palo	Netu	Palo
6	0.01	11054.2	10032.2	4303.9	2803.1	10543.2	3553.2	0.532	0.466
12	0.02	12045.7	12079.7	4436.8	3693.8	12062.7	4064.5	0.681	0.331
18	0.03	13178.2	12146.2	4581.9	4511.9	12662.2	4537.6	0.357	0.363
24	0.04	14391.6	15285.6	4699.2	4374.2	14838.2	4729.3	0.582	0.296
30	0.05	15486.5	16510.5	4846.2	5317.2	15998.5	4849.5	0.589	0.519
36	0.06	16672.4	18677.4	4972.8	7623.6	16429.4	5263.7	0.627	0.372
Average %RSD								0.570	0.392

Table 3: Inter-day precision for estimation of NETU & PALO.

Concentration (µg/ml)		Peak area							
		Day 1	Day 2	Day 1	Day 2	Mean	Mean	%RSD	%RSD
Netu	Palo	Netu	Netu	Palo	Palo	Netu	Palo	Netu	Palo
6	0.01	10054.2	10032.2	4303.9	2803.1	10543.2	3453.2	0.482	0.296
12	0.02	12028.7	12183.7	4436.8	4693.8	12082.2	4364.5	0.621	0.331
18	0.03	12934.2	12146.2	4481.8	4721.9	12672.2	4337.6	0.557	0.363
24	0.04	12391.1	13285.4	4592.2	4874.2	12837.2	4479.3	0.482	0.246
30	0.05	13416.7	13510.2	4596.2	4897.2	13948.5	4369.5	0.580	0.219
36	0.06	14672.4	14677.4	4672.8	5823.6	14429.2	5173.7	0.527	0.582
Average %RSD								0.499	0.295

Recovery studies

The proposed method when used for subsequent estimation of NETU & PALO mixture after over spotting with 80, 100 and 120 % of additional drug; afforded good recovery of NETU & PALO. The amounts of drug added, determined and the % recovery are listed in (Table 4).

Table 4: Recovery from Synthetic mixture.

%spiking	C actual (µg/ml)		C added (µg/ml)		C recover (µg/ml)		% recovery	
	Netu	Netu	Netu	Palo	Netu	Palo	Netu	Palo
80	12	0.02	9.6	0.016	9.5587	0.0169	99.12±0.436	99.98±0.383
100	12	0.02	12	0.02	11.596	0.0189	101.31±0.324	100.4±0.417
120	12	0.02	14.4	0.04	15.799	0.0369	101.52±0.381	99.93±0.374

LOD and LOQ

Detection limit and quantification limit was calculated by the method as described above. The LOQ and LOD were found to be (0.159 & 0.146) for NETU and (0.053 & 0.144) for PALO respectively. This indicates that adequate sensitivity of the method.(Table 5).

Table. 5: LOD and LOQ of NETU & PALO.

Parameter	NETU (µg/ml)	PALO (µg/ml)
LOD	0.059	0.053
LOQ	0.146	0.144

Robustness

Robustness of the method was determined by making slight changes in chromatographic conditions. An effect of two different brands of Dichloromethane as it ratio highest amount in mobile phase or different saturation time of the chamber was applied as variable parameters. The retention factor (R_f) remained unaffected by small variation of these parameters. The robustness of the method shows that there were no marked changes in the chromatographic parameters, which demonstrates that the method developed is robust**.(Table.6)**

Table 6: Robustness of HPTLC method.

Factor	Retention factor	
	NETU	PALO
Dichloromethane		
Brand-I	0.85	0.25
Brand-II	0.86	0.24
Mean ±SD	0.855±0.007	0.245±0.005
Saturation time		
25 min	25 min	25 min
30 min	30 min	30 min
Mean ±SD	**Mean ±SD**	**Mean ±SD**

Applicability of developed method

A spot at R_f = 0.85 for NETU and R_f=0.25 for PALO was observed in the chromatogram of the drug samples in synthetic mixture. There was no interference from the excipients commonly present in synthetic mixture. The % drug content and % RSD were calculated. The low % RSD value indicated the suitability of this method for the routine analysis of NETU and PALO mixture.(Table 7)

Table 7: Analysis of Synthetic mixture.

% Assay of Synthetic mixture (±SD) (n=6)		
METHOD	NETU (300mg)	PALO (0.50mg)
HPTLC	101.524± 0.253	99.994± 0.574

Important Parameters for the developed method are listed in **(Table.8)**

Table 8: Results of the developed method in brief including summary of the validation parameters for NETU & PALO.

Sr. No.	Parameters	Netupitant (300mg)	Palonosetron HCl. (0.50 mg)
1	Detection wavelength (nm)	241	241
2	Linearity range(µg/ml)	6-36 µg/ml	0.01-0.06 µg/ml
3	Accuracy	100.65±0.325	100.11±0.371
4	Intraday Precision(%RSD)	0.570	0.392
5	Interday Precision(%RSD)	0.499	0.295
6	Regression Equation- Intercept Slope Correction coefficient	y = 188.7x + 9842. 188.7 9842 0.9991	y = 13400x + 4171. 13400 4171 0.9999
7	LOD(µg/ml)	.059	0.053
8	LOQ(µg/ml)	0.146	0.144
9	Applicability of synthetic mixture.	101.524± 0.253	99.994± 0.574
10	Reproducibility	% RSD less than 2	% RSD less than 2

CONCLUSION

A rapid, simple, accurate and specific HPTLC method for quantitative estimation of Netupitant and Palonosetron has been developed and validated as per ICH guideline. Statistical analysis proves that the method is repeatable and selective for the analysis of Netupitant and Palonosetron HCl in synthetic mixture.

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