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Research Article

SIMPLE AND RAPID RP-HPLC METHOD FOR SIMULTANEOUS DETERMINATION OF PALONOSETRON AND NETUPITANT IN SPIKED HUMAN PLASMA

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ABSTRACT

Using a high performance liquid chromatography (HPLC), a meticulous, liable and hasty method was developed for the determination of Netupitant (NTPT) and Palonosetron (PLST) simultaneously. Prior to RP-HPLC, the method employs proteins precipitation as sample preparation with acetonitrile only. Waters C18 column was used to chromatograph the analytes at ambient temperature with isocratic elution of ammonium acetate (pH 6.0)-methanol (35:65, v/v) with flow rate of 1ml/min and UV detection at 229 nm. The chromatographic run time was 10 min for the mixture with retention time of 4.91 min for PLST and 3.16 min for NTPT. Over the range of 250-3500ng/ml, the calibration curves were linear. The method was validated in terms of accuracy, precision, absolute recovery, freeze-thaw stability, bench-top stability and re-injection reproducibility. The within- and between-day accuracy and precision were found to be within acceptable limits <15%. The analytes were stable after three freeze-thaw cycles (deviation <15%). The planned method was definite for the simultaneous determination of NTPT and PLST in human plasma.

Keywords: Human plasma, RP-HPLC, Bio analytical, Palonosetron, Netupitant.

1. INTRODUCTION

The 5-HT_3 receptor blocker, Palonosetron is utilized in the inhibition and treatment of chemotherapy-induced vomiting and nausea [1]. It is used for the control of delayed chemotherapy-induced vomiting and nausea (CINV) and it is found to be more effective than granisetron. The mode of drug function of Palonosetron includes the blocking action of serotonin in certain parts of the nervous system and brain, that help to stop or condense nausea and vomiting. The oral combination palonosetron/netupitantis approved for both severe and overdue CINV [2].

The selective NK_1 receptor antagonist [3], Netupitant shows potential antiemetic activity [4]. It prevents nausea and vomiting caused after surgery and also in case of cancer chemotherapy. It inhibits the NK1-receptor binding of the endogenous tachykinin neuropeptide material P (SP)*via* competitive binding and blocks the activity of the human substance P/NK1 receptors in the central nervous system (CNS), that lead to the inhibition of nausea and vomiting (CINV) induced by chemotherapy.

The fixed dose combination of Palonosetron and Netupitant drugs was recommended for the inhibition of severe and tardy nausea and vomiting induced by chemotheraphy [5]. Palonosetron has been reported for RP-HPLC analysis [6-10]. Only a few methods have been reported for simultaneous analysis of palonosetron and Netupitantin combined dosage forms and stability indication studies using RP-HPLC [11-13]. But a very few bio analytical methods have been reported for Palonosetron and Netupitant. Very recently, we have reported an effective RP-HPLC method for the simultaneous determination of venetoclax and obinutuzumab in bulk and pharmaceutical dosage form [14]. In continuation of our efforts, here in we wish to report the development and validation for simultaneous estimation of palonosetron and Netupitant in human plasma by bio analytical method.

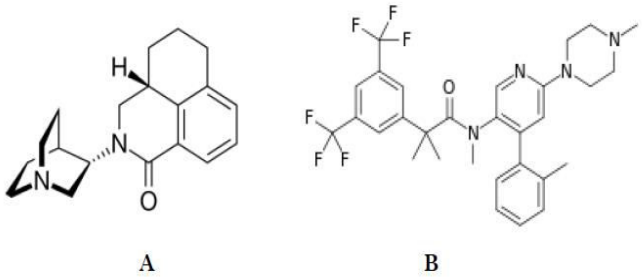

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Fig. 1: Structure of palonosetron and Netupitant

2. MATERIAL AND METHODS

2.1. Chemicals and reagents

HPLC grade acetonitrile and methanol were purchased from Aldrich. Analytical grade CH_3COOH, ammonium acetate and sodium hydroxide of analytical grade, were also purchased from aldrich. PLST and NTPT were kindly supplied by Icon Pharmaceuticals Pvt. Ltd, Vijayawada, and certified to contain 99.80% and 99.79%, respectively, and used without further purification. Human plasma without drugs was obtained from blood bank. The pH 6.0 ammonium acetate buffer was obtained by dissolution of 0.8 g of ammonium acetate in one liter of distilled water and using 0.1 N NaOH and CH_3COOH the pH was adjusted to 6.0.

2.2. Calibration standards (CS) and quality control (QC) samples in human plasma

Stock solutions were prepared by dissolving each of PLST and NTPT in methanol to obtain concentration of 1 mg/ml for each. The solution was prepared by dissolving 100 mg of each drug in sufficient amount of methanol and the volume was made upto 100 ml with the same solvent.

Working standard solutions were prepared by transferring different volumes of stock PLST and NTPT to 10 ml volumetric flask and the volume was completed with methanol. Volumes of 20 µl of working standard solutions were added to 960 µl of drug-free human plasma to obtain drug concentration levels of 150-3000ng/ml.

Separate quality control (QC) samples were prepared and pooled at three diverse concentration levels (150, 1200, 3000ng/ml for both HCT PLST and NTPT) as low, medium and high, respectively. A calibration curve was constructed from a blank sample and non-zero samples covering the total range of 150-3000ng/ml for PLST and NTPT.

2.3. Preparation of plasma sample

Initially, the stored plasma samples were endorsed to thaw at room temperature. The plasma samples were centrifuged at 5000 rpm for 12 min, 1.0 ml of aliquot was pipetted into a 15 ml polypropylene tube and 2 ml of acetonitrile was added to it. After standing for 10 minutes at room temperature, the mixture was centrifuged at 5000 rpm for 30 minutes. Next, the supernatant was injected into HPLC system by transferred into vial watchfully.

2.4. Liquid chromatography/UV spectrophotometry

Chromatographic separation was performed on a PEAK chromatographic system equipped with LC-P7000 pump, UV detector UV7000 and the output signal was monitored and integrated by PEAK Chromatographic Software version 1.06. Waters RP- C18 column was used as stationary phase. Systronics double beam UV-Visible spectrophotometer was used to carry out spectral analysis and the data was recorded by Hitachi software. Denver electronic analytical balance (SI-234), Systronics digital pH meter were also used.

The mobile phase was composed of CH_3OH and ammonium acetate (pH 6.0) in the ratio of 65:35(v/v). 1.0ml/min was the flow rate. 20µl volume was injected. A UV-Vis detector at λ 229nm was used to perform the detection.

2.5. Method validation

The method was validated for selectivity, matrix effect, precision, accuracy, linearity, sensitivity, recovery and stability.

Six individual human blank plasma samples were analyzed to investigate the selectivity of the method. Every blank sample was tested for interference by the present method and was compared with spiked sample whose concentration of the analyte was at the LOQ.

Intra-day precision and accuracy were evaluated through analysis of validation control samples at three different concentrations in six replicates in the same day. Inter- and intra-day precision was expressed as relative standard deviation (RSD). The accuracy was expressed as the relative error (RE) for the determination of the studied drugs in each human plasma sample. The evaluation of precision was based on the criteria.

Calculating the NTPT and PLST concentration in spiked QC samples in six biological matrices individually from diverse drug-free plasma (A) and in mobile phase (B) at dissimilar levels of concentration investigated the matrix effect. Matrix effect was defined as the concentration or peak ratio (A/B × 100). During the preparation of QCs or blank samples at the same concentration level, each individual's biological matrix was used only once.

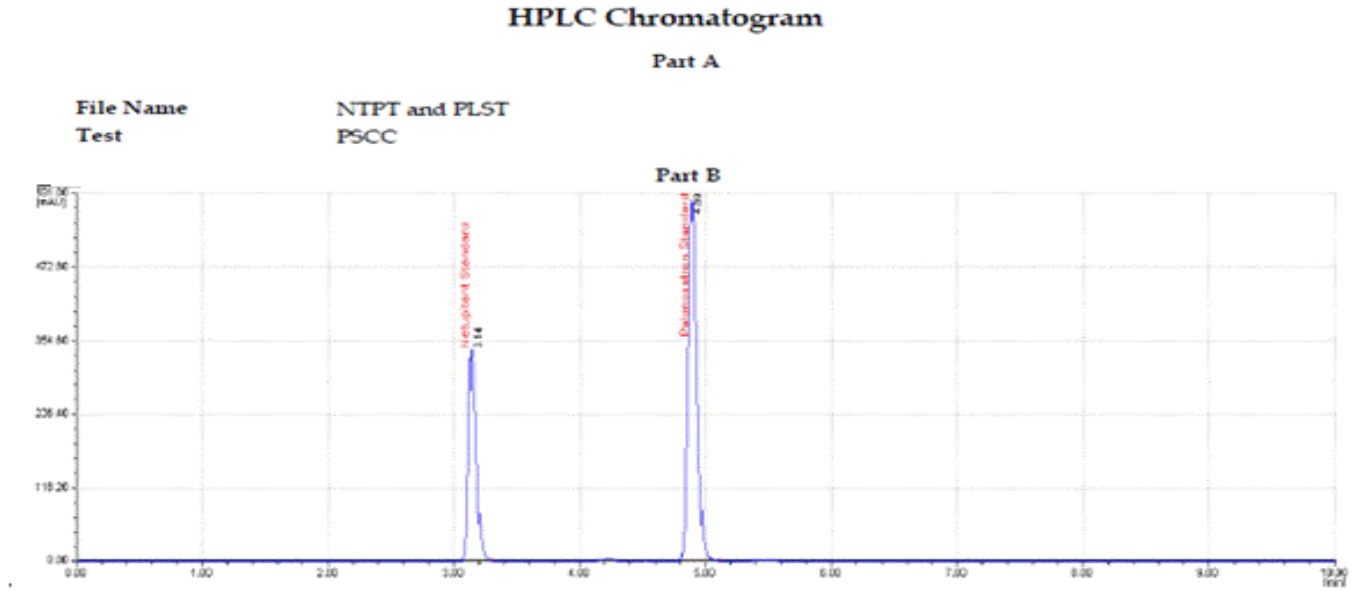
The stabilities of NTPT and PLST in biological matrix and working solutions at different storage conditions were evaluated and the results were expressed as percentage recoveries.

3. RESULTS AND DISCUSSION

To obtain the best chromatographic condition, different columns and mobile phases with different pH and organic modifier were tested to provide sufficient selectivity and sensitivity in short separation time.

The best chromatographic condition took placed on Waters C18 column with mobile phase consisting of ammonium acetate solution (pH 6) and methanol in the ratio of 35:65 (v/v) at flow rate 1 ml/min and UV detection at 229nm (Fig. 2).

The influence of both; organic modifier concentration and pH were carefully studied increasing organic modifier concentration not only improve peak shape and decrease the run time but also decrease the method specificity due to the interference of the PLST peak with endogenous biological substance. Decreasing of organic modifier concentration more than 20%, resulted in high specificity with regard to the separation of the studied drug from endogenous biological substances and more retained the drug on the column that led to excessive tailing of eluting peaks and long run time. Optimized separation was achieved at an organic modifier concentration of 60%.



S NO	Name	RT (min)	Area	Theo Plat	Tail Factor	Resolution
1	Netupitant Standard	3.142	325147.1	3958	0.98	---
2	Palonosetron Standard	4.891	469280.5	5631	1.32	12.25
	Sum		794427.6			

Fig. 2: HPLC chromatogram of PLST and NTPT spiked in human plasma

Variation of pH plays an important role in the separation process, at pH (3.5), PLST become more retained on the column. pH (6.0) was chosen as the optimum value both for resolution of drugs from endogenous biological substances and better peak shape and reasonable run time. The UV absorbance characters show high absorbance at the chosen wave length (229nm) that increases the sensitivity of the method.

3.1. Methods validation

3.1.1. Selectivity

No endogenous source of interference was observed at the retention times of the analytes. Typical chromatogram obtained from a plasma sample spiked with PLST and NTPT is presented in Fig. 3.

3.1.2. Linearity

Linearity was established by analyzing seven concentrations of PLST and NTPT ranging between 150-3000ng/ml by plotting the peak area against the corresponding concentration. Linearity of the calibration graphs was validated by the high value of the correlation coefficient (>0.999) and the intercept value; (Fig. 3). The calibration range for the proposed method was established through considerations of the practical range required and the concentrations of PLST and NTPT present in the pharmaceutical product to give accurate, precise and linear results.

Table 1: Results of plasma spiked calibration curve for PLST and NTPT

Test	Sample ID	Netupitant		Palonosetron	
		Concentration Prepared (ng/ml)	Area Obtained	Concentration Prepared (ng/ml)	Area Obtained
PSCC	PSCC01	150	48148	150	82541
	PSCC02	300	96398	300	132804
	PSCC03	600	169858	600	230746
	PSCC04	1200	325146	1200	469280
	PSCC05	1800	499589	1800	699058
	PSCC06	2400	675818	2400	955049
	PSCC07	3000	828544	3000	1195821
N		7		7	
Slope		276.6		396.5	
Intercept		6638		7978	
r^2		0.999		0.999	

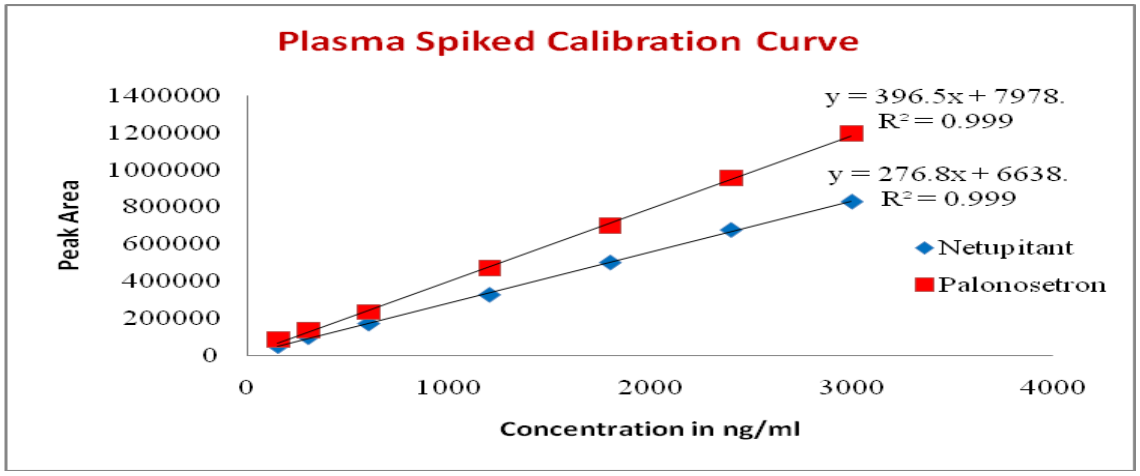


Fig. 3: Plasma Spiked Calibration Curve for PLST and NTPT in the developed method

3.1.3. Recovery

The method was repeated for different concentrations of pure samples (quality control samples) at the low, medium and high concentration levels, each repeated six times. The concentrations were calculated each from its corresponding regression equation. The recovery percentages and the mean recoveries were then calculated and the % recovery was found to be more than 90% for both the drugs PLST and NTPT in the developed method.

3.1.4. Precision and Accuracy

Six replicate QC samples at the low, medium and high concentration levels were used to analyze the precision and accuracy of the method. By calculating relative error (RE), the accuracy of the method was determined and by calculating RSD the precision was calculated. Table 2 and 3 summarizes the precision and accuracy on each of two drugs in human plasma in intraday and intraday precision respectively. The %RSD was found to be 0.318, 0.714 and 0.215 for NTPT and 1.412, 0.569 and 0.389 for PLST in intraday precision in HQC, MQC and LQC respectively (Table 2). The % RSD was found to be 0.255, 0.923 and 0.734 for NTPT and 1.573, 0.657 and 0.368 for PLST in intraday precision in HQC, MQC and LQC respectively (Table 3).

3.1.5. Robustness

The intended HPLC method showed robustness when the authors tried to persuade slight deliberate changes in the organic strength (±1.5%) and the pH (±0.1 unit) of the mobile phase where the retention time of the peaks was not significantly affected (±0.05 minutes).

Table 2: Precision results for PLST and NTPT

S NO	HQC		MQC		LQC	
	NTPT	PLST	NTPT	PLST	NTPT	PLST
1	821685	1190254	326141	467579	48022	82529
2	826184	1193658	324504	466577	48192	82628
3	827643	1199578	322648	462512	48148	82962
4	826423	1175823	320469	463918	48318	82229
5	826548	1165821	325857	460252	48245	82359
6	829756	1157469	326248	463619	48111	82041
CV	2652.75	16723.87	2353.541	2666.16	104.129	321.378
Mean	826381	1180439	324317.5	464081	48146.2	82394.2
%RSD	**0.318**	**1.412**	**0.714**	**0.569**	**0.215**	**0.389**

Table 3: Interday Precision results for PLST and NTPT

S NO	HQC		MQC		LQC	
	NTPT	PLST	NTPT	PLST	NTPT	PLST
1	820348	1152411	325812	462029	48358	82112
2	821354	1142358	321022	466605	48579	82859
3	822363	1168918	326631	463689	48931	82312
4	824709	1125249	328123	469316	48248	82929
5	823321	1139019	321316	460967	48009	82581
6	826511	1174712	326224	463959	48858	82684
CV	2251.77	18786.5	2959.11	3058.78	348.793	321.64
Mean	812907	1140439	323849	463443	48498.3	82476.8
%RSD	**0.255**	**1.573**	**0.923**	**0.657**	**0.734**	**0.368**

Table 4: Results of freeze–thaw stability of NTPT

S NO	HQC		MQC		LQC	
	Peak Area	% Recovery	Peak Area	% Recovery	Peak Area	% Recovery
1	1136243	94.918	461421	97.8238	82131	98.9504
2	1195854	99.902	466011	98.930	83221	99.3221
3	1192523	99.6925	469198	98.9877	84446	99.846
4	1170233	98.462	460112	97.9245	81912	99.4503
5	1163621	97.2976	468568	98.9515	81208	98.9602
6	1169564	98.7109	469212	99.8906	82113	99.5096
CV	1.86021		0.86962		0.2598	
Mean	97.9432		99.2406		99.6120	
%RSD	**1.839**		**0.869**		**0.263**	

Table 5: Results of freeze-thaw stability results of PLST

S NO	HQC		MQC		LQC	
	Peak Area	% Recovery	Peak Area	% Recovery	Peak Area	% Recovery
1	812148	98.0198	318198	97.8761	48023	99.7189
2	816343	98.5285	316298	97.2871	47687	99.0598
3	833198	100.549	320116	98.4453	47867	99.4677
4	816912	98.5989	315698	97.1076	47134	97.898
5	837134	101.026	326243	100.23	47125	97.8998
6	825128	99.5789	316234	97.243	47598	98.9056
CV	1.21356		1.22498		0.76897	
Mean	99.3897		98.0489		98.8214	
%RSD	**1.213**		**1.236**		**0.765**	

3.1.6. Ruggedness

The proposed HPLC method showed ruggedness when we tried to transfer analyst to another lab with another analyst in another city and running the analysis where the same results and retention times were obtained (±0.01 min) proving no significant lab to lab and analyst to analyst and time to time variations and hence ruggedness of the method.

3.1.7. Stability

The stability of the examined drugs in human plasma was measured by analyzing six replicate QC samples at the low, medium and high concentration levels at ambient temperature over 24 hours. The deliberated concentrations of the drugs in these QC samples at room temperature for 24 hours were compared with that obtained with the corresponding QC sample freshly prepared and advanced instantly. The outcomes in table specify that the examined drugs were stable for at least 24 hours in human plasma when stored at ambient temperature. On other side, QC samples experiencing three freeze-thaw cycles were analyzed collectively. The results indicate the stability of the examined drug in human plasma over three freeze-thaw cycles (Table 4 and 5). Also the studied drug showed the stability in human plasma when stored at -20°C for one month as long term stability when compared with the freshly prepared sample.

4. CONCLUSION

Meeting all needs for the validation of an analytical methodology an easy, quick, precise and dependable method was developed for the analysis of PLST and NTPT in human plasma. The method involves the use of proteins precipitation with acetonitrile as the only sample preparation prior to RP-HPLC. This method is cost-effective and suitable for analysis of a large number of samples.

5. ACKNOWLEDGMENT

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