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Short communication

Development and validation of a stability-indicating LC method for determining Palonosetron hydrochloride, its related compounds and degradation products using naphthalethyl stationary phase

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ABSTRACT

A selective and simple reversed phase HPLC method using naphthalethyl stationary phase was developed and validated for the quantitative determination of Palonosetron hydrochloride (PALO), its related compounds and degradation products. Chromatographic separation ($R_s > 2$) was achieved with linear gradient mode of elution at a flow rate of 1 mL/min and with UV detection at 210 nm. The intra and inter-day coefficients of variation were less than 1.0% (RSD). Consistent recoveries were obtained for PALO (99.2–100.5%) and its impurities (90.0–104.8%). All the analytes exhibited excellent linearity with R^2 value greater than 0.998. Limit of detection (LOD) and limit of quantification (LOQ) were determined to be in the range 0.011–0.013 μg/mL and 0.035–0.046 μg/mL respectively. The test solution was found to be stable up to 5 days. Induced degradation methods were applied to study the degradation behavior of the drug. LC–MS was used to analyze the degraded samples and possible structural identifications were assigned based upon known reactivity of the drug and molecular weights. The *m*/*z* values matched with the hydroxylated, keto and N-oxide metabolites of PALO. The stress samples were assayed against a qualified reference standard and the mass balance was found close to 99.9%.

1. Introduction

Palonosetron hydrochloride (PALO), chemically known as (3aS)-2-[(S)-1-azabicyclo [2.2.2] oct-3-yl]-2,3,3aS,4,5,6-hexahydro-1H-benz [de] isoquinolin-1-one hydrochloride (Fig. 1), is a 5-HT3 receptor antagonist and its excellent antiemetic activity in clinical trials is attributed to higher receptor-binding affinity and longer half-life [1–3]. The structure of PALO demonstrates that the compound has two chiral centers. The most active Palonosetron isomer has both chiral centers in the S-configuration (i.e., 3aS, S) [4]. However, (3aS, S) PALO is always contaminated with isomeric and structure related impurities [5,6], which include (3aR, S) diastereomer (Imp-C), unreduced penultimate cyclic amide (Imp-B) and (n-2) stage compound (Imp-A). In addition to this, PALO N-oxide (Imp-D) is a degradation product formed during forced degradation.

Analytical methods for the determination of PALO in biological samples by LC-MS-MS [7–10] were published. A few analytical methods were reported in the literature for enantioseparation of PALO employing micellar electro kinetic chromatography and normal phase chromatography [11–13]. RP-LC methods for quantification of PALO and its related impurities using C18 stationary phase and expensive ion pair reagent were reported [14,15].

In the present work, a naphthalethyl stationary phase (π nap) [16] was used to achieve an optimum resolution between isomeric impurities and PALO. Cosmosil π nap is a reversed phase HPLC column with naphthalene bounded silica packing material. Naphthalethyl group contains two aromatic rings. It has strong π–π and hydrophobic interactions.

In this paper, a simple, precise and accurate HPLC method for the determination of PALO, its related compounds and degradation products in pharmaceuticals was developed. The present work is also intended to study the intrinsic stability of PALO through stress studies as per ICH [17,18].

2. Experimental

2.1. Materials and reagents

Samples of PALO and its related impurities were received from Process Research Department of Integrated Product Development of Dr. Reddy's Laboratories Limited, Hyderabad, India. HPLC grade

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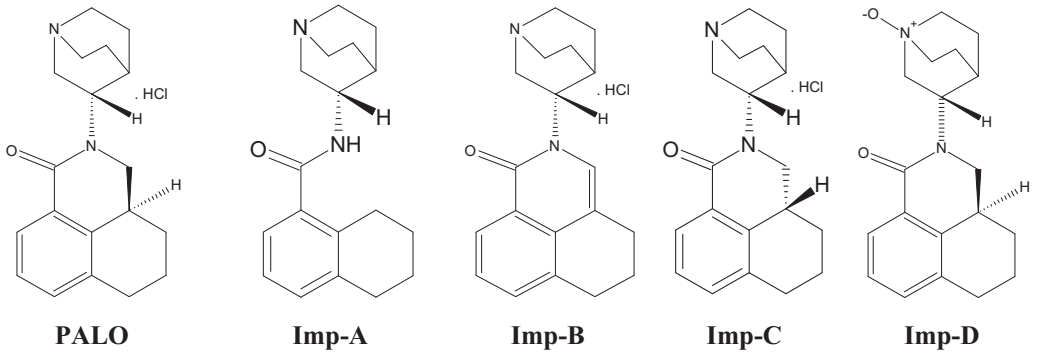


Fig. 1. Chemical structures of Palonosetron hydrochloride and its related substances.

acetonitrile (ACN), dipotassium hydrogen phosphate (K_2HPO_4) and triethyl amine (TEA) were purchased from Merck, Schuchardt OHG, Germany. High pure water was prepared by using Millipore Milli Q plus purification system.

2.2. Equipment

The LC system, used for method development, forced degradation studies and method validation was Waters 2695 binary pump plus auto sampler and a 2996 photo diode array detector. The output signal was monitored and processed using Empower software (Waters) on Pentium computer (Digital Equipment Co). The LC-MS-MS system, used for mass identification of degradation products was an Agilent 1200 series liquid chromatograph coupled with Applied Bio systems 4000 Q Trap triple quadrupole mass spectrometer with Analyst 1.4 software, MDS SCIEX, USA. Stability studies were carried out in a humidity chamber (Thermo lab humidity chamber, India) and photo stability studies were carried out in a photo stability chamber (Sanyo photo stability chamber, Leicestershire, UK). Thermal stability studies were performed in a dry air oven (Thermo lab, Thane, India).

2.3. Chromatographic conditions/LC–MS conditions

The chromatographic separation was achieved on a Cosmosil π Nap, 250 mm × 4.6 mm, 5 μm column. Buffer solution was prepared by adding 2 mL of TEA to 1000 mL of 20 mM aqueous K_2HPO_4 solution and pH adjusted to 2.5 with phosphoric acid. Mobile phase-A was the buffer solution and Mobile phase-B was a mixture of buffer and ACN in the ratio of 50:50 (v/v). Mobile phases were filtered through a nylon membrane (pore size 0.45 μm) and degassed with helium sparge for 5 min. A mixture of water and ACN in the ratio (1:1, v/v) was used as diluent during the preparation of standard and test samples. The flow rate of the mobile phase was kept at 1.0 mL/min with a linear gradient program: time (min)/%B 0.01/40 40/85, with a post equilibration time of 5 min. The HPLC column was maintained at 35 °C and the chromatogram was monitored at a wavelength of 210 nm. The injection volume was 10 μL. Aqueous solution of 0.1% trifluoroacetic acid (TFA) was used as buffer instead of phosphate buffer and TEA in LCMS analysis; remaining conditions were kept constant.

2.4. Preparation of standard and sample solutions

A standard and sample stock solution of 5000 μg/mL concentration was prepared by dissolving an appropriate amount of PALO in diluent. Working solutions of 500 μg/mL and 100 μg/mL were prepared from the above standard and sample stock solutions for related substances and assay determination respectively. A stock solution of impurities at 50 μg/mL was prepared in diluent. Composite solutions of 7.5 μg/mL impurities and PALO were prepared in both low level and test concentration levels for method development purpose. Accelerated and long term stability samples of active pharmaceutical ingredient were prepared similar to the above sample preparation.

Aloxi® injectable solutions (0.05 mg base/mL) were directly used with an injection volume of 100 μL. A placebo solution equivalent to test concentration 0.05 mg base/mL was prepared to check the interference.

2.5. Degradation studies

To study the degradation behavior of PALO, the drug was exposed to 5 N HCl, 2 N NaOH, 3% H_2O_2 solutions and subjected to water hydrolysis at reflux condition. Degradation was also performed under stress conditions like photolytic degradation and thermal degradation. The solutions exposed to HCl or NaOH were neutralized and all the solutions were filtered through a 0.45 μm filter before injection into the column.

2.6. Method validation

Method validation parameters studied were precision, limit of quantitation (LOQ) and limit of detection (LOD), linearity, accuracy and robustness.

The system precision was determined by measuring inter-day (repeatability) and intra-day (intermediate precision) variations. These parameters were investigated by injecting six individual preparations of PALO (500 μg/mL) spiked with impurities (75 μg/mL). The % RSD was calculated from the six measurements. Assay method precision was demonstrated by assaying the test sample preparations (100 μg/mL) in six replicates and the % RSD was calculated.

To establish the minimum concentration at which the analyte was reliably detected (LOD) and quantified (LOQ), the signal-to-noise ratios at 3:1 and 10:1 were determined. Precision and accuracy were studied at LOQ level by injecting six individual and spiked preparations of impurities with the drug. The RSD and % recovery of these responses were calculated.

Linearity was determined by plotting five calibration curves. For the construction of each calibration curve, six calibration standard solutions were prepared and six replicates of each standard solution were injected. Calibration curves were generated by plotting the peak area versus the concentration of the analyte. To establish linearity of the assay method, solutions were prepared from stock solution at five concentration levels 50–150%. The linear regression R^2 values were calculated for PALO. Accuracy of the related substances method was evaluated by spiking known amounts of the impurities to the test sample, analyzed the same and calculated the percent recovered. For recovery experiments, three solutions

Table 1
Maximum resolutions of impurities on various stationary phases.

Stationary phase	R_s[a]	R_s[b]	T_f[c]
C8 (250 × 4.6 mm × 5 μm)/buffer pH 3.0	Less than 1.0	1.10	2.5
C8 (250 × 4.6 mm × 5 μm)/buffer pH 3.0/ion-pair	1.08	1.44	2.4
C18 (250 × 4.6 mm × 5 μm)/buffer pH 3.0	1.20	1.10	2.5
C18 (250 × 4.6 mm × 5 μm)/buffer pH 3.0/ion-pair	1.23	1.39	2.1
C18 (250 × 4.6 mm × 5 μm)/buffer pH 3.0/ion-pair/column temperature 45 °C	1.41	1.80	2.0
C8/C18 (250 × 4.6 mm × 5 μm)/buffer pH 3.0	1.20	1.40	2.0
Phenyl (250 × 4.6 mm × 5 μm)/buffer pH 3.0	1.22	1.03	2.3
CN (250 × 4.6 mm × 5 μm)/buffer pH 3.0	Less than 1.0	Less than 1.0	2.5

[a] Resolution between Imp-B and Imp-C.
[b] Resolution between Imp-C and Palonosetron hydrochloride.
[c] Tailing factor.

were prepared that covered a range 50–150% of the target concentration and injected in triplicate. Accuracy was evaluated at three concentrations 50, 100, and 150 μg/mL for the determination of PALO.

2.7. Robustness and solution stability

Robustness was evaluated by deliberately varying the experimental parameters outlined in the method and determined the subsequent effect on the system suitability parameters that refer to the resolutions between Imp-B and Imp-C (R_s^1) and PALO and Imp-C (R_s^2). The typical parameters investigated include different column, flow rate, mobile phase pH, column temperature and % organic ratio. The same parameters were altered in the assay method to study the system suitability.

Solution stability and mobile phase stability were studied by spiking the test solutions with impurities at ambient and refrigerated conditions. These solutions were repetitively injected up to 5 days and calculated the similarity factor for peak area. Mobile phase stability was assessed by comparative analysis of freshly prepared sample solutions and freshly prepared reference standard solution up to 5 days.

2.8. Analysis of stability samples

Bulk drug stability samples were analyzed as per the test conditions. Long term (12 months) and accelerated (6 months) stability data were generated.

3. Results and discussion

3.1. Optimization of chromatographic conditions

The chromatographic conditions were optimized to develop a stability-indicating method to separate the related compounds and degradation products from the drug. Reversed phase technique was selected based on the high solubility of PALO in water. The wavelength of 210 nm was selected due to maximum absorbance of all the impurities and drug.

During this procedure, three different columns (Zorbax® C8, Betasil® C18, vertisep® C8/C18 mixed stationary phase, Zorbax® phenyl and Zorbax® CN) and different mobile phases with distinct proportions of solvents, with and without ion pair reagent and (TEA), at different pH (3.0–7.0) were evaluated (Table 1). To increase the hydrophobic interactions between solutes and stationary phase, experiments were carried out on the high carbon load and larger surface area column like a Betasil C18 by increasing the ion pair reagent concentration in the mobile phase. A maximum resolution (R_s) of 1.2 was observed with 0.01 M concentration of ion pair reagent, indicating the poor resolution of Imp-B and Imp-C. The results of the above experiments indicated that the hydrophobic interactions per se could not yield satisfactory resolution.

The interactions of Imp-B, Imp-C and PALO with stationary phase were almost same in a variety of chromatographic conditions, it could be because of structural similarities. The active functionalities responsible for hydrophilic interactions like amide group, tertiary nitrogen and the group responsible for hydrophobic interactions like aromatic moiety are common in all the structures. Imp-A was separated well from the other analyte peaks could be due to the less hydrophobic interactions. Presence of electron donor/acceptor moieties within a reversed phase system did not only increase the overall retention times for aromatic solutes, but also lead to an enhanced shape selectivity of the hybrid stationary phase [19]. It was predicted that a stationary phase possesses strong π–π interaction along with hydrophobicity could help to obtain separation between the PALO and its impurities B and C. In view of this, naphthalethyl (π nap) column was chosen for assessment.

K_2HPO_4 (0.02 M) with pH 3.0 and methanol (60:40) (v/v) were chosen for initial trial with a flow rate 1.0 mL/min. When system suitability solution was injected, the resolutions between all the impurities were reasonably good (~1.5) which had shown an indication of separation, but the retention time (~35 min) and the tailing (>2.0) of PALO was very high. To reduce the retention time, ACN was used as an organic modifier and to improve the peak shape, TEA (2 mL) was added to the buffer. To decrease the interaction of PALO with stationary phase (due to hydrophobicity) a gradient program was selected with high quantity of ACN in mobile phase B (ACN: buffer: 50:50, v/v) and buffer as mobile phase A. Different gradient programs were tried to optimize the retention of the drug and resolution between the impurities and arrived to final program, Time (min)/%B 0.01/40 40/85. Buffer pH and TEA played a significant role in enhancing the resolution. Effect of buffer pH (2.0–7.0) and TEA volume (0.5–3.0 mL) was studied. A maximum resolution greater than 2 (Fig. 2a) with good peak shape (T_f < 1.5) was obtained when TEA volume increased to 2.0 mL at buffer pH 2.5. Placebo interference study was performed and no interference was found from the analyte peaks (Fig. 2b).

In the above optimized conditions, PALO and its impurities (A, B and C) were well separated with a resolution greater than 2 and the typical retention times of PALO, Imp-A, Imp-B and Imp-C were about 11, 16, 17 and 17.8 min respectively.

3.2. Results of forced degradation studies (specificity)

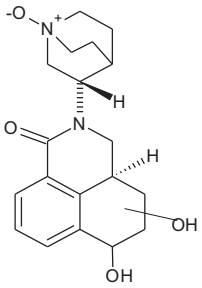
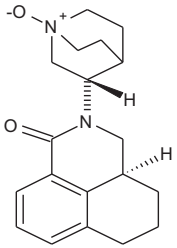
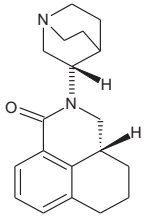
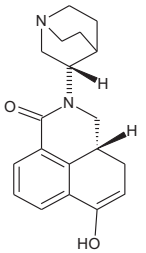
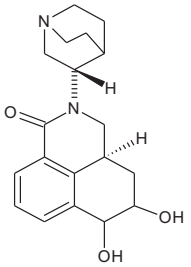
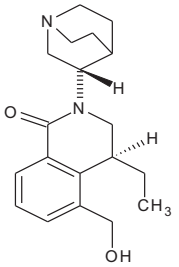
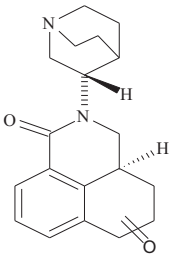
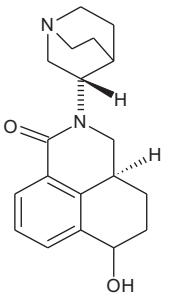
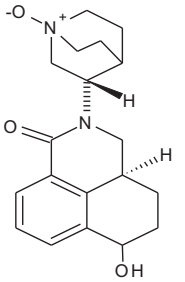
3.2.1. Oxidative conditions

When the drug was exposed to 3% H_2O_2 at 100 °C temperature for 1 h, it was degraded into four major unknown impurities (RRT ~ 0.49, 0.61, 0.72 and 1.12), shown in Fig. 3(a). An LC–MS compatible method was developed, using 0.1% TFA as a buffer to study the mass numbers of unknown degradation products formed (Table 2). The general metabolic reactions are oxidation at the nitrogen in the heterobicyclic ring to produce N-oxide and addi-

Table 2
Summary of forced degradation studies and possible degradation products.

	Peroxide degradation			Base degradation			Acid degradation			Water degradation		
	RRT	*m/z* (mode of ionization + ESI)	M.Wt.	RRT	*m/z* (mode of ionization + ESI)	M.Wt.	RRT	*m/z* (mode of ionization + ESI)	M.Wt.	RRT	*m/z* (mode of ionization + ESI)	M.Wt.
	0.49	345.4–[M+H$^+$]	344.4	0.96	297.4–[M+H$^+$]	296.4	0.25	329.4–[M+H$^+$]	328.4	0.48	311.4–[M+H$^+$]	310.4
	0.61	345.4–[M+H$^+$]	344.4	1.12	313.4–[M+H$^+$]	312.4	0.42	315.4–[M+H$^+$]	314.4	0.49	345.4–[M+H$^+$]	344.4
	0.72	345.4–[M+H$^+$]	344.4				0.48	311.4–[M+H$^+$]	310.4	0.61	345.4–[M+H$^+$]	344.4
	1.12	313.4–[M+H$^+$]	312.4				0.55	329.4–[M+H$^+$]	328.4	1.12	313.4–[M+H$^+$]	312.4
	1.35	313.4–[M+H$^+$]	312.4				1.12	313.4–[M+H$^+$]	312.4			
% Total degradation		5.8			2.7			1.2			4.7	
% Assay		94.0			97.2			98.6			95.0	
Mass balance		99.8			99.9			99.8			99.7	

Possible degradation products for the above mass numbers

 (M.Wt~344.4)	 (M.Wt~312.4)	 (M.Wt~296.4)	 (M.Wt~310.4)	 (M.Wt~328.4)	 (M.Wt~314.4)	 (M.Wt~310.4)	 (M.Wt~312.4)	 (M.Wt~328.4)
Dihydroxy Palonosetron N-oxide –	Imp-D Palonosetron N-oxide Reported as Metabolite M9	Imp-C diastereomer Confirmed by spiking analysis	Keto Palonosetron enol form Metabolite M6 enol form	Dihydroxy Palonosetron Reported as Metabolite M12	Hydroxymethyl compound –	Keto Palonosetron Reported as Metabolite M6	(Hydroxy Palonosetron)[a] Reported as Metabolite M4	Hydroxy Palonosetron N-Oxide Reported Metabolite as M14

[a] Hydroxyl group could also be at 4th or 5th position, which is reported as Metabolite M3.

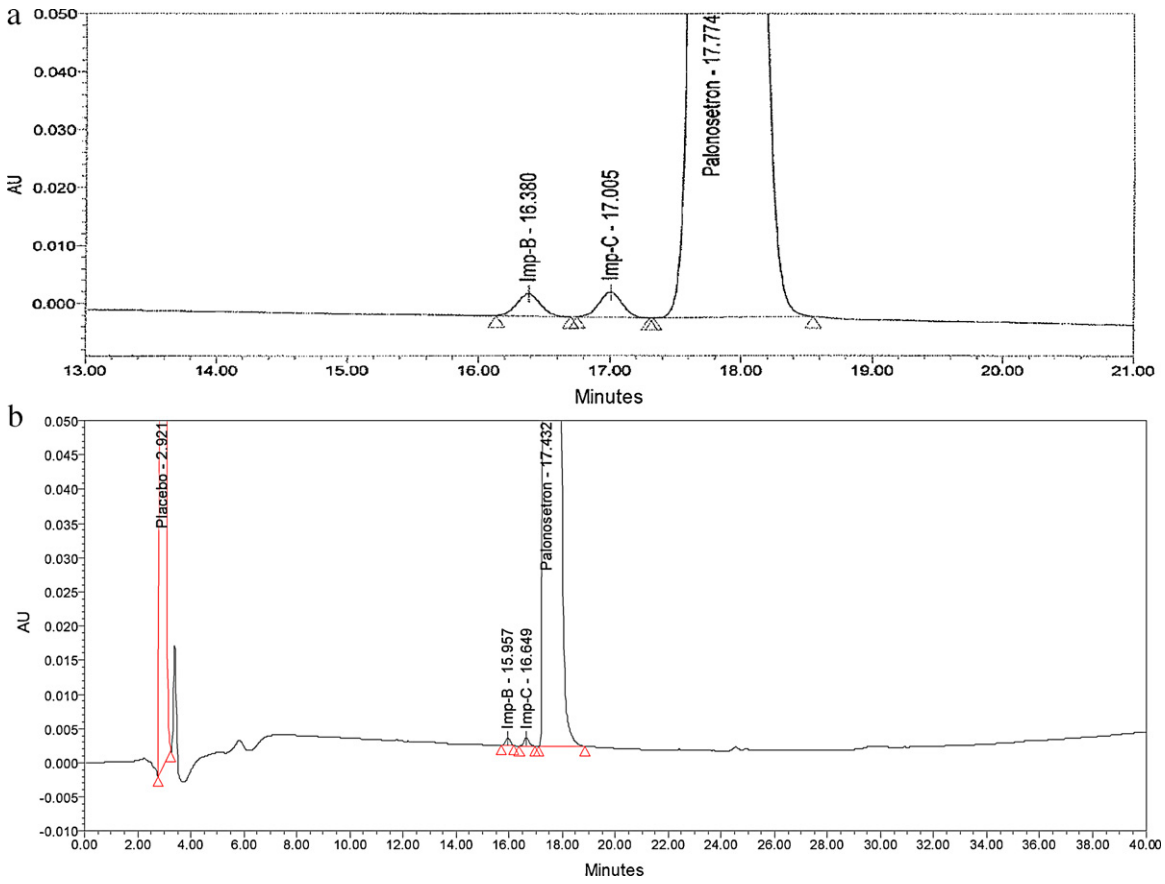


Fig. 2. (a) Base line separation of impurities on naphthalethyl stationary phase. (b) Placebo peak at 2.92 min in direct sample analysis of Palonosetron injection.

tion of hydroxy groups to the molecule. In some cases the hydroxy groups are further oxidised to the corresponding ketone [20]. The data were correlated with the previously reported metabolites [21]. Interestingly, the first three degradation products have the same mass numbers and these products are expected to be the reported hydroxylated metabolites of PALO. The fourth peak at RRT ~ 1.12 (Fig. 3(b)) is N-oxide Palonosetron (Imp-D), which was confirmed by spiking analysis with the reference standard.

In the above optimized chromatographic conditions, Imp-D is closely eluting (R_s < 1.5) with PALO. A change in selectivity with temperature was expected and studied the effect on resolution. An improved resolution (R_s > 2.2) with optimum band spacing was observed, when column oven temperature increased to 35 °C. This change in temperature did not affect the resolution of other impurities.

3.2.2. Degradation in basic conditions

A maximum degradation of 2.7% was achieved when the drug was exposed to 2 N NaOH at 90 °C for 60 h. But the interesting point was, the product slightly converted into its diastreomeric form, which was confirmed by co-injection with Imp-C standard and with spectrum matching. The second major peak obtained was again Imp-D. The *m*/*z* values 297.4 and 313.4 obtained by LC–MS analysis supported this study (Table 2).

3.2.3. Degradation in acidic conditions

The drug was exposed to 5 N HCl at 100 °C temperature for 48 h. The two major peaks are at RRT ~ 0.25 (0.5%) and RRT ~ 1.12 (Imp-D). The mass numbers of major degradation products obtained by LC–MS analysis were 329.4 and 313.4.

3.2.4. Degradation in neutral conditions

Four major degradation products were obtained in neutral water solution under harsh conditions (48 h reflux at 100 °C) with mass numbers 311.4, 313.4, 345.4 and 345.4, indicating the possible formation of Keto, N-oxide and hydroxyl N-oxide derivatives.

3.2.5. Thermal and photo degradation

No degradation was observed when the drug exposed to heat at 100 °C for 10 days and when exposed to light (1.2 million lux h) and an integrated near UV energy (200 W/m h).

The value of purity angle was lower than the threshold angle indicating the peak was pure in all the stress conditions. The mass balance of the stressed samples was close to 99.9%. The assay of PALO is unaffected in presence of all the impurities.

3.3. Method validation results

The intra-day (n = 6) precision showed good results, (mean ± RSD): 0.1634 ± 0.9% (Imp-A), 0.1724 ± 0.9% (Imp-B), 0.1827± 0.9% (Imp-C), 0.1511± 0.5% (Imp-D) and 99.42 ± 0.21%(PALO). The RSD for inter-day precision, performed by assaying the samples in two different days by the same analyst was 0.7% (n = 6) and precision at LOQ was <6.5 (n = 6) for all impurities.

In the accuracy test, three concentrations were evaluated (50%, 100% and 150%) and mean recoveries were 104. 8 ± 0.18 (Imp-A), 90.0 ± 0.52 (Imp-B), 102.3 ± 0.82 (Imp-C), 100.05 ± 0.56 (Imp-D) and 99.86 ± 0.51% (PALO) (mean ± RSD), respectively. The LOD values of Imp-A, B, C, D and PALO were 0.013, 0.011, 0.012, 0.012 and 0.011 μg/mL respectively. The LOQ values of Imp-A, B, C, D and PALO were 0.046, 0.037, 0.039, 0.038 and 0.035 μg/mL respectively.

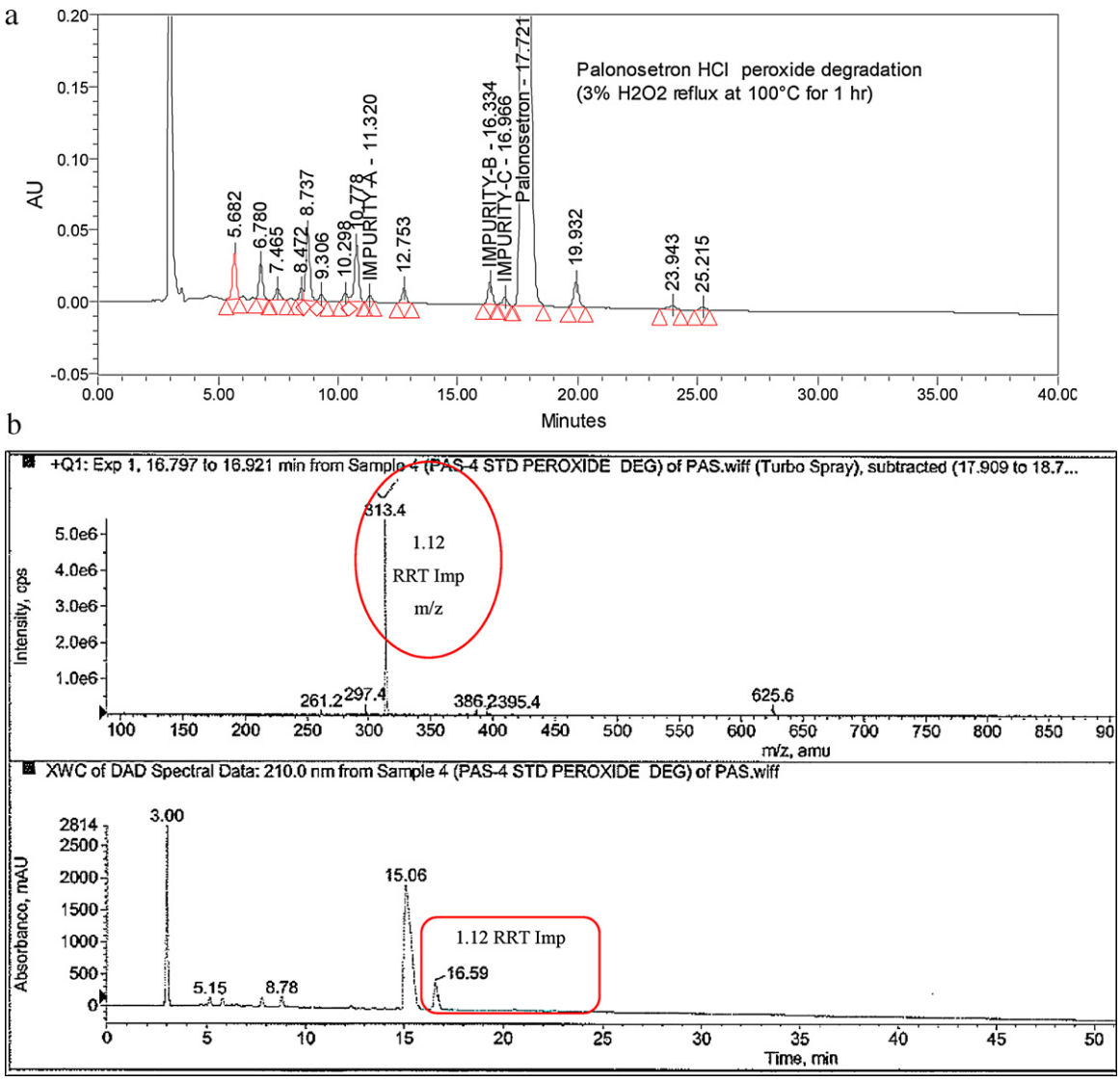


Fig. 3. (a) Peroxide degradation. (b) LC–MS spectrum of 1.12 RRT impurity (N-oxide) in peroxide degradation.

Recoveries of impurities were ranged between 95.2% and 98.6% at LOQ level.

Linearity of related substances method was evaluated at six concentration levels ranging from 0.04 μg/mL to 1.2 μg/mL. The correlation coefficient obtained was greater than 0.999. The best-fit linear equation obtained for Imp-A was $y = 42029x - 0.2$, for Imp-B was $y = 42104x - 0.3$, for Imp-C was $y = 45290x - 0.3$ and for Imp-D was $y = 44899x + 0.0$. Linearity of the assay method was evaluated by determining five concentration levels between 50 and 150 μg/mL and correlation coefficient obtained was 0.999. The R^2 values were greater than 0.998 for all the analytes.

3.4. Results of robustness and solution stability

The results obtained by varying the method parameters (flow rate, pH, and column temperature and % organic ratio) exhibited the similar RRT values (mean ± SD): 0.64 ± 0.01, 0.92 ± 0.004, 0.96 ± 0.003, 1.18 ± 0.03. The theoretical plates (N) value of PALO was (mean ± SD): 24171 ± 2546. The resolution values R_s^1 and R_s^2 were (mean ± SD): 1.98 ± 0.09 and 1.97 ± 0.05, illustrating the robustness of the method. The degree of reproducibility (% RSD < 0.5) and USP tailing factor (<1.1) are within the acceptable range when method parameters were deliberately altered. Robust-

Table 3
Analysis results of Palonosetron hydrochloride initial, stability samples and Aloxi® injection.

Name of the sample ($n = 3$)	Imp-A	Imp-B	Imp-C	Imp-D	SMUI[a]	% Assay (w/w)
PASRS0901– Initial	ND[b]	0.03%	0.04%	ND	0.02%	99.8%
PASRS0901 Acc[c]/6 months	ND	0.03%	0.05%	ND	0.03%	99.7%
PASRS0901 LT[d]/12 months	ND	0.03%	0.05%	ND	0.02%	99.9%
ALOXI#28000065	ND	0.27%	0.30%	ND	0.01%	99.3%
ALOXI#28000063	ND	0.27%	0.30%	ND	0.01%	99.2%

[a] Single maximum unknown impurity.
[b] Not detected.
[c] Accelerated.
[d] Long term.

ness study was performed on two different columns shown the consistent results; the theoretical plates (N) value of PALO was (mean ± SD): 24988 ± 1155. The resolution values R_s^1 and R_s^2 were (mean ± SD): 1.99 ± 0.05 and 1.98 ± 0.02.

Similarity factor was calculated for each impurity and the value was found to lie between 0.98 and 1.02. The % RSD of assay method during solution and mobile phase stability study was less than 1.0 indicating that the solution and mobile phase were stable up to 5 days.

3.5. Sample analysis

Stability samples and Aloxi injection samples were analyzed in the validated method and results were tabulated in Table 3. Stability data showed that drug substance was stable in both long term and accelerated conditions.

4. Conclusion

The selective, stability indicating and simple reversed phase HPLC method using naphthalethyl stationary phase was developed and validated for the quantitative determination of PALO, its related compounds and degradation products. The developed method can be used for synthetic process monitoring and assessing the stability of bulk samples, finished dosage samples of PALO.

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