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ORIGINAL

Enantiopurity Assessment of Chiral Switch of Ondansetron by Direct Chiral HPLC

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Abstract A simple, selective, robust, and rapid enantiospecific HPLC method was developed and optimized for simultaneous determination of ondansetron enantiomers. The chiral separation was performed on Chiralpak AS-3R analytical column (150 mm × 4.6 mm i.d., 3 µm). AD-optimal mixture design methodology was employed to evaluate the influence of solvent mixtures on retention factor of first peak (k_1), resolution between enantiomers ($Rs_{1,2}$) and runtime (tR_2). Solvent mixtures are delivered at 1.5 mL min^{-1} flow rate, and enantiomeric peaks were detected at 222 nm. Experiments were carried out, and results were analyzed by the two-component mix plot graph of the design software. The mobile phase containing methanol/water/diethylamine (85/15/0.1% v/v/v) leads to a best possible combination adequate retention ($k_1 = 1.4$), enantiomeric resolution ($Rs_{1,2} = 2.9$) in shorter runtime (3.5 min). The proposed method was validated according to ICH guidelines and found to be linear, sensitive, selective, precise, and accurate. Furthermore, the pertinence of this developed method was established by analyzing two commercially available tablets: Emeset-8 (racemic mixture) and Zordil-4 (R-ondansetron). Good agreement was found between the assay results and the label claim of the marketed formulations by showing good % recovery and %CV. The study resulted in a better chromatographic system for chiral impurity profiling of ondansetron chiral switch.

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Introduction

Chiral molecules often exhibit different pharmacological and physiological effects on chiral environment (i.e., human body). Therefore, the worldwide drug regulatory agencies have issued guidelines indicating that preferably, only the active enantiomer of a chiral drug should be brought to market [1]. As a result, a number of new chiral entities are developed as enantiomerically pure drug products or obtained as unichiral version by a chiral switch. The enantiopurity assessment of chiral switches aids in avoiding undesirable side effects and assures a better therapeutic index. Recently, the development of direct chiral HPLC method by employing polysaccharide chiral stationary phases (CSP) has gained considerable attention because of their chiral recognition capacity in all common separation modes [2, 3]. Hence, in the present study, chiral separation was carried out using polysaccharide CSP in reversed phase (RP) mode.

Ondansetron (OND), (Fig. 1) chemically designated as (RS)-9-methyl-3-[(2-methylimidazol-1-yl)methyl]-2,3-dihydro-1H-carbazol-4-one, is a 5-hydroxytryptamine type 3 (5-HT_3) receptor antagonist and effective in the prevention and treatment of nausea and vomiting [4, 5]. OND possesses one stereogenic center in the carbazol ring and exists in two enantiomeric forms. R-OND, the eutomer, is a highly selective and more potent 5-HT_3 antagonist which shows approximately eightfold higher activity than S-counterpart (S-OND) and produces comparable therapeutic efficacy to half of the racemic dose [6, 7]. Thus, racemic

OND redeveloped into single R enantiomer (chiral switch) and introduced in Indian market [6]. In addition, R-OND products may contain traces of S-OND, the distomer, residual, or by product from R-OND synthesis. Furthermore, the S-OND attributed to QT prolongation associated with an increased risk of cardiac arrhythmias [7, 8]. Hence, the quantitative determination of OND enantiomers is of great importance from the point of view of chiral quality control.

The determination of OND enantiomers in pharmaceutical formulations and biological matrices was performed by CE [9–11] and LC–MS [12, 13] methods. However, an extensive literature survey revealed that there were few direct chiral HPLC methods reported for determination of OND enantiomers. Zhang et al. [14] proposed a direct HPLC method based on cyclodextrin CSPs. The method suffered from drawbacks of poor enantioresolution (Rs = 1.13) and excess runtime (20 min). Liu and Stewart [15] reported an HPLC method for the separation of OND enantiomers based on Chiralcel OD-RCSP under reversed mode. The method utilized inorganic buffer (sodium perchlorate) for separation, which could affect the life of the column or HPLC system. Kelly et al. [16] proposed a method using Chiralcel OD CSP. The method employed a high-complex mobile-phase mixture which limits its usage for regular analysis. Yin et al. [17] reported a method based on Chiralpak IC CSP. The method suffers from drawbacks of excess enantioresolution (Rs = 6.5) and runtime. Zhang et al. [18] developed a method using two separate CSPs (Ultron ES-OVM and Chiralcel OJ column). This method envisages chiral separation as the only goal, not considering enantiomeric retentivity and analysis time as a major optimization criterion. Furthermore, aforesaid methods were employed a time consuming conventional trial and error approach for the separation of OND enantiomers which could not provide information about possible interactions of the investigated factors and inefficient in determining the true optimal condition for routine analysis. Hence, the present study was aimed to develop an improved direct chiral HPLC method for separation of OND enantiomers by employing chemometric approach. The literature survey addresses the utility chemometric approach in screening and optimization of liquid chromatographic methods for chiral separation [19, 20].

This is the first report describing the application of D-optimal mixture design in the optimization of the mobile-phase composition for analytical separation of OND enantiomers. Furthermore, the application of "Two-Component Mix plot" of the D-optimal mixture design in chiral HPLC analysis may be considered as a novel approach for establishing an optimal chromatographic condition. An additional strength of mixture design is that the design itself suggests the robustness domain. The proposed chiral HPLC method uses a non-buffered reversed-phase mobile-phase system (aqueous/organic solvent) offering the advantage of chiral HPLC/MS transferability and bioanalytical applications.

Experimental

Instrumentation

The chromatographic method development and validation was performed on Shimadzu HPLC (Shimadzu Corporation, Kyoto, Japan). The system consisted of two LC 20 AD solvent delivery modules: an SPD-M 20A PDA detector and a Rheodyne injector (model 7125, USA) valve fitted with a 20 µL loop. The system was controlled through a system controller (SCL-10A) and a personal computer using a Shimadzu chromatographic software (LC Solution, Release 1.11SP1) installed on it. Absorbance spectra were recorded using an UV–visible spectrophotometer (Model UV-1601PC; Japan) using quartz cell of 1.00 cm path length.

Chromatographic Condition

The chromatographic separation was carried out on a Daicel Chiral Pak AS-3R column (150 mm × 4.6 mm i.d., 3 µm) connected with Daicel Chiral Pak AS-3R guard

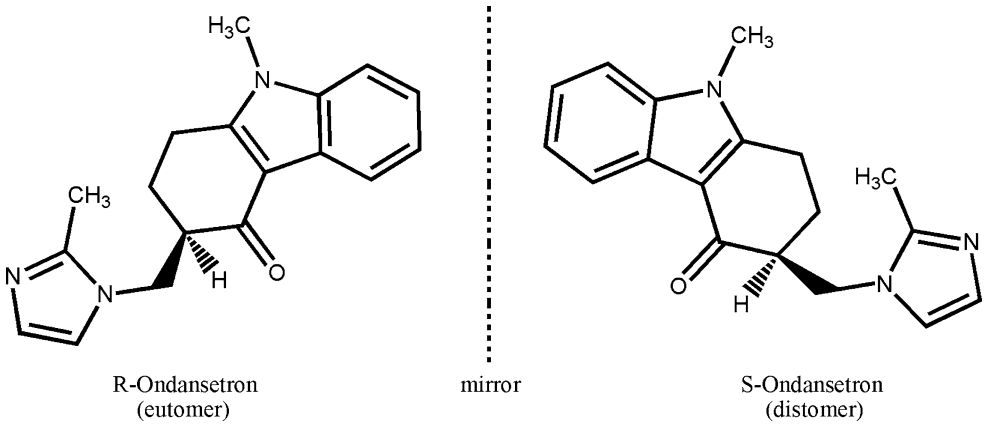

Fig. 1 Chemical structure of ondansetron enantiomers

cadridge. The mobile phase consisted of MeOH/water/diethylamine. Prior to use, the mobile phase was degassed for 15 min in an ultrasonic bath and vacuum filtered through 0.45 µm membrane filter (Gelman Science, India). An injection volume of the sample was 20 µL. The HPLC system was used in an air-conditioned laboratory atmosphere (20 ± 2 °C).

Chemicals and Reagents

The working standard of OND was procured from Yarrow Chemical Ltd, Mumbai, India. Pure enantiomer of R-OND was obtained from Emcure Pharmaceuticals (Pune, India). S-OND was procured from Sigma-Aldrich, India. Methanol (MeOH) was of HPLC grade, and diethyl amine (DEA) of analytical grade was purchased from SD Fine Chemicals, Mumbai, India. High-purity HPLC water was prepared by passing through a Millipore Milli-Q plus system (Millipore, Bangalore, India) was used for the HPLC analysis. The pharmaceuticals: Emeset-8 tablets containing (RS)-OND 8 mg (Cipla Ltd, Mumbai, India) and Zordil-4 tablets containing pure enantiomer of R-OND 4 mg (Emcure Pharmaceticals Ltd, India) were procured from pharmacy retail shop.

Design of Experiments

The D-optimal mixture design was performed using Design expert®, 8.0 version (Stat-Ease, MN, USA). The rest of the calculations were performed using the Microsoft Excel 2010 software (Microsoft, USA).

Stock and Working Standard Solutions

Stock standard solutions of racemic OND and R-OND, at 1000 µg mL^{-1}, were prepared individually using mixture of MeOH and water in 80:20 v/v and stored at 4 °C protected from light. The stock solutions of (RS)-OND further diluted with the mobile phase to give a series of standard mixtures having a final concentration in the range of 4–20 µg mL^{-1}. The solution prepared for the optimization procedure comprised of (RS)-OND, at 8 µg mL^{-1}.

Preparation of the Sample Solution

Twenty tablets of Emeset-8 (RS)-OND and Zordil-4 (R-OND) were weighed and analyzed separately. An amount of powder equivalent to 10 mg was weighed and transferred in a 10 mL volumetric flask, and 5 mL of mobile phase was added. This mixture was subjected to sonication for approximately 15 min to ensure complete solubility of the drugs, and the solution was made up to the mark with mobile phase and further dilutions were made to obtain a concentration of (RS)-OND 8.0 µg mL^{-1} and R-OND 4.0 µg mL^{-1}. The resulted solution was centrifuged at 4000 g for 10 min, and clear supernatant was collected and filtered through a 0.2 µm membrane filter (Gelman Science, India). A 20 µL of the final solution was injected in triplicate and chromatographed.

Assay Method Validation

The analytical performance parameters, such as linearity, precision, accuracy, limit of detection (LOD), limit of quantification (LOQ), selectivity, and robustness, were validated according to ICH Q2 (R1) guidelines [21].

Results and Discussion

The present study attempts to develop a direct chiral HPLC method for the enantiomeric separation of OND using Chiral Pak AS-3R CSP under reversed-phase (RP) mode. The RP mode enantioseparation provides a better solubility for polar analytes, uses nontoxic solvents, and successful HPLC and LC/MS analysis [22]. The chiral selector in Chiral Pak AS-3R is amylose tris[(*S*)-α-methylbenzylcarbomate] coated on 3 µm silica gel. The separation of enantiomers in this CSP may be attributed to hydrogen bonding interactions, dipole–dipole interactions, and pi–pi interactions. The presence of aromatic functionalities could also provide an additional stabilizing effect on the solute-CSP complex by inclusion of the aromatic group into chiral cavity [23–26]. This type of mechanism may operate in OND enantiomer separation.

Initial Screening

Prior to method development, a set of preliminary experiments were performed using different compositions of water, ACN, and MeOH. Under these screening conditions, OND enantiomeric peaks were co-eluted and did not result separation. This might be attributed to poor affinity of the OND enantiomers to the CSP or difficulty in inclusion of the analyte into the chiral cavity.

Effect of Mobile-Phase Additive

Mobile-phase additives play a major role in separating analytes containing basic or acidic functional groups [27, 28]. As OND (Fig. 1) contains the basic functionalities (tertiary amine group in carbazol and imidazole ring), the influence of the basic additives viz, DEA and triethylamine was tested. It was found that the addition of DEA into the mobile phase comprising MeOH and water showed baseline resolution of OND enantiomers. Furthermore, it was

noticed that varying the concentration of DEA (0.1–0.3% v/v) has no significant effect on enantiomeric resolution and peak shape. Thus, mobile phase comprising a mixture of MeOH, water with 0.1% v/v DEA (low level) was used for the enantiomeric separation of OND.

D-Optimal Mixture Design Analysis

A D-optimal mixture design experiments was used to evaluate the effect of changes in mobile-phase compositions on dependent variables and optimization of the response of interest with least number of experiments [29]. In mixture experiments, the factors are the components of a mobile phase, so their levels are not independent. The mixture factors are expressed as the fraction of total amount of their experimental ranges. Based on the preliminary experiments, the proportions of organic modifier MeOH (A: 75–85% v/v) and water (B: 15–25% v/v) were selected for optimization. In mixture design experiments, the sum of the mobile-phase components viz, MeOH and water made equal to 100% v/v. The concentration of DEA was kept constant at 0.1% v/v in the mobile phase. The mobile phase was delivered at 1.5 mL min^{-1} flow rate, and enantiomeric peaks were detected at 222 nm. To judge the quality of the method under different experimental conditions, the following responses of interest were defined: (1) retention factor of the first eluted peak S-OND (k_1); (2) resolution between OND enantiomers ($Rs_{1,2}$); and (3) runtime the method (tR_2).

A total of eight experimental runs obtained from the design were subjected to experiment in order to generate the response variables. Table 1 summarizes the conducted experiments and the responses. All experiments were conducted in randomized order to minimize the effects of uncontrolled variables that may introduce a bias on the measurements. Two replicates were performed for each experiment in order to know the experimental error variance and to test the predictive validity of the model. The effect of organic modifier (MeOH) on the selected responses was then analyzed using "Two-Component Mix" plots (Fig. 2a–c) of the design. Figure 2a–c depicts that changing the fraction of organic modifier MeOH (% v/v) from 75 to 85% resulted rapid decline in retention factor of S-OND (k_1), enantioresolution ($Rs_{1,2}$) and analysis time (tR_2). To obtain a region for normal operation, the following criteria were selected: retention factors k_1 >1.0, resolution between enantiomers $Rs_{1,2} = 2$ and runtime of the method $tR_2 < 4$ min. From Fig. 2a–c, the mobile phase comprising MeOH/water (85/15% v/v) with 0.1% v/v DEA was selected as an ideal condition for regular analysis. The experiments were performed under optimal condition and obtained chromatogram was shown in Fig. 3. The optimized condition gave adequate retention ($k_1 = 1.4$), resolution ($Rs_{1,2} = 2.9$) within a less analysis time (3.5 min).

Validation of the Proposed Method

Linearity

The linearity of the proposed method was assessed at five concentration levels in the range of 4–20 µg mL^{-1} for (RS)-OND (approximately 20–200% of the nominal range of the analyte). The calibration curve was plotted using the linear least squares regression procedure. The obtained mean ($n = 6$) regression equations were $y = 0.484x - 0.132$ and $y = 0.489x - 0.142$ for S-OND and R-OND, respectively. Correlation coefficients were found to be more than 0.998 for both enantiomers (Table 2). To evaluate the linearity performance of the developed method, one way ANOVA ($p > 0.05$) was performed [19]. The computed F values (F_{Calc}) for S-OND (0.48) and R-OND (0.92) were found to be not more than the theoretical F value ($F_{Crit} = 2.62$), validating that there was no significant difference between replicate determinations for each concentration levels.

LOD and LOQ

The LOD was determined based on signal to noise (S/N) ratio using analytical response of three times of the background noise. Calibration curves were plotted at five levels ranging from 0.05 to 1.0% of the nominal analyte concentration. The residual standard deviation of the response (σ) and slope (s) of the calibration curve was used to calculate the LOD as 3.3 σ/s and LOQ as 10 σ/s. The results of LOD and LOQ were depicted in Table 2.

Selectivity

The selectivity of the method was evaluated by assessing the chromatograms of commonly used excipients (starch, lactose monohydrate, methyl cellulose, titanium dioxide and magnesium stearate) with that of the standard drugs. From Fig. 3a it was concluded that there were no excipients peaks co-eluted with the analytes, indicating that the optimized assay method is selective in relation to the excipients used in this study.

Accuracy and Recovery

The accuracy of the method was assessed by analyzing quality control (QC) standards prepared at three levels of 80, 100 and 120% of the expected assay value in the marketed formulation. QC samples (4, 8 and 12 µg mL^{-1}) were prepared as three replicates at each concentration level by

Table 1 Experimental design matrix representing mobile-phase composition and observed responses

Run	Factor levels			Responses		
	Type	MeOH (A)	Water (B)	k_1	$Rs_{1,2}$	tR_2
1	Center	80	20	1.948	3.655	5.355
2	Axial CB	82.5	17.5	1.443	3.338	3.881
3	Vertex	85	15	1.405	2.968	3.581
4	Center	80	20	1.988	3.642	5.322
5	Vertex	75	25	2.879	3.599	7.722
6	Axial CB	77.5	22.5	1.955	3.648	5.403
7	Vertex	85	15	1.402	2.952	3.579
8	Vertex	75	25	2.868	3.586	7.732

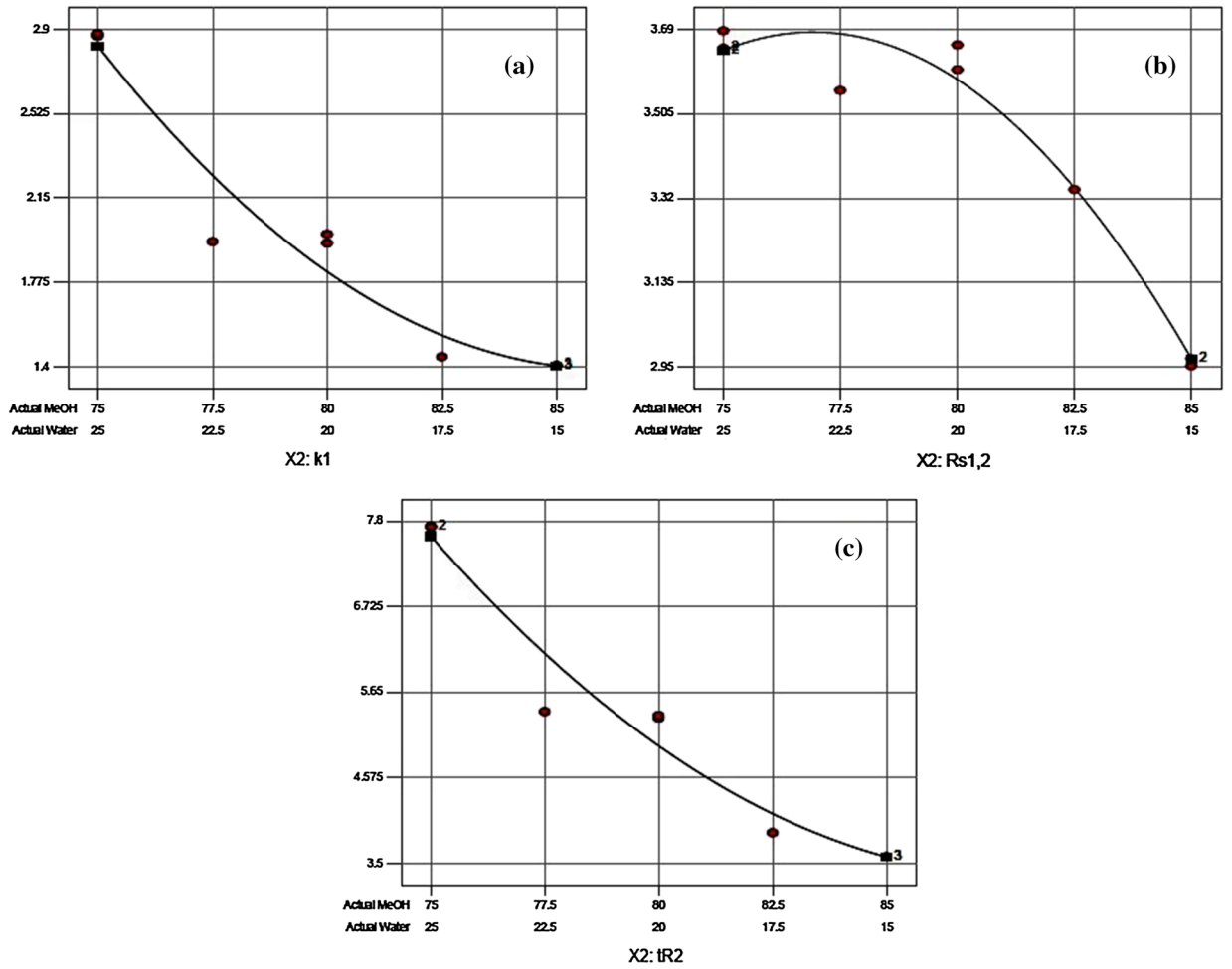

Fig. 2 Two-component mix plot showing the effect of solvent mixture on **a** retention factor of S-OND (k_1), **b** resolution between OND enantiomers ($Rs_{1,2}$), and **c** analysis time (tR_2)

spiking the standard drugs with the placebo excipients, which were left overnight to allow matrix–analyte interactions to occur, and then analyzed as described in "Preparation of the sample solution". The % recovery of the enantiomers at each level ($n = 3$) and mean % recovery ($n = 9$) were determined and data is presented in Table 2, where accuracy (%) was expressed as [(calculated amount/predicted amount) × 100]. The recoveries of both enantiomers at each level were found to lie within the acceptable criteria of the bias ±2% [21]. The mean % recovery ($n = 9$) for each enantiomer was also tested for significance by using Student's t test, the null hypothesis being that the recovery

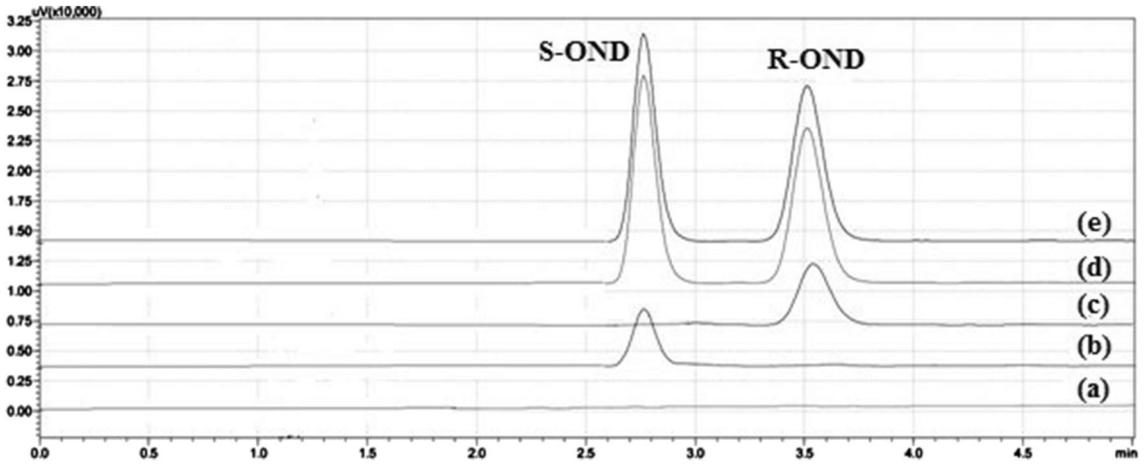

Fig. 3 Representative overlaid chromatograms corresponding to **a** placebo solution, **b** S-OND (enantiopure), **c** Zordil-2 tablets contains R-OND (chiral switch), **d** synthetic mixture of OND enantiomers, and **e** Emeset-8-tablets contains S- and R-OND enantiomers, under optimal condition

is unity or 100%. Since, the calculated t value (t_{Calc}) for S-OND (0.86) and R-OND (0.85) is less than the theoretical t value ($t_{Crit} = 2.776$), at 5% significance level, the null hypothesis was accepted. These results demonstrate that the method is accurate and there was no interference from placebo in this study.

Precision

The precision was established by injecting three concentration levels (4, 8 and 12 µg mL^{-1}) for (RS)-OND each in six replicates, for intra-day precision (repeatability) and on three consecutive days for the intermediate precision [21]. Precision was expressed by the RSD (%) of the analyte peak area. Results for all studied compounds (Table 2) met the proposed requirement %RSD $\leq$3% [30].

Robustness

The robustness of the proposed method was evaluated by using mixture design experiments. The variations in percent mobile-phase components, i.e., %MeOH (85 $\pm$ 2%) and water (15 $\pm$ 2%), flow rate (1.45–1.55 mL min^{-1}) were did not alter the retention factor, enantioresolution and analysis time values more than 2%. So, it could be concluded that the developed method is robust.

Comparison with Reported Methods

The proposed method offers benefit of being rapid, simple and robust liquid chromatographic method for quantitative determination of ondansetron enantiomers. The overall chromatographic runtime of the developed method is shorter when compared to method reported by Zhang et al. [18]. Furthermore, compared to the method proposed by Kelly et al. [16], present method utilizes simple binary mobile-phase system, which suggests its capability for routine chiral quality control analysis of ondansetron. In addition, the present method provides quality enantiomeric resolution against an excess resolution of 7.4 as reported by Zhang et al. Moreover, the use of non-buffered mobile-phase system in the present method may favour a longer life of the column.

Application to Formulation

To assess the applicability of the developed method for intended purpose, an attempt was made to quantitative determination of racemic and enantiopure OND tablet dosage form. Pharmaceutical formulations of Emeset-8 tablets containing R- and S-OND, Zordil-4 containing enantiopure R-OND were analyzed by proposed method. Representative chromatogram for assay was presented in Fig. 3. The results achieved when analyzing Emeset tablets were 3.96 (0.01) mg of S-isomer and 3.97 (0.06) mg of R-isomer, respectively, with the values within parentheses being the %CV of the six replicates. Assay of R-OND was performed on Zordil-4 tablets in which the presence of S-isomer is considered to be a chiral impurity. The results obtained for the assay of R-OND was 3.99 (0.005) mg with the values within parentheses being the %CV of the six replicates. The content of the S-isomer was not more than 0.5%. Good agreement was found between the assay results and the label claim of the marketed formulations.

Table 2 Summary of validation for the determination of S-OND and R-OND

Validation parameters	S-OND		R-OND	
	Concentration	Results	Concentration	Results
Linearity ($n = 6$)	4–20 μg mL^{-1}	$y = 0.484x - 0.132$	4–20 μg mL^{-1}	$y = 0.489x - 0.142$
	$R^2 = 0.998$		$R^2 = 0.998$	
LOD	1.13 ng mL^{-1}		1.49 ng mL^{-1}	
LOQ	4.01 ng mL^{-1}		4.52 ng mL^{-1}	
Specificity	The method is specific with respect to tablet ingredients			
Accuracy	Mean %recovery ± SD (%) ($n = 3$)			
	80% w/w	99.18 ± 0.03	80% w/w	99.24 ± 0.12
	100% w/w/	99.23 ± 0.01	100% w/w	99.25 ± 0.06
	120% w/w	100.18 ± 0.01	120% w/w	100.14 ± 0.05
	Mean %recovery ± SD (%) ($n = 9$)			
	99.53 ± 0.49		99.54±0.45	

Precision ($n = 6$)

	S-OND		R-OND	
	Concentration (μg)	%RSD	Concentration (μg)	%RSD
Intra-day precision				
	4	0.21	4	0.68
	12	0.68	12	0.44
	20	0.45	20	0.50
Interday precision				
	4	0.68	4	0.67
	8	0.44	8	1.45
	10	0.50	10	0.50
Robustness	Variations did not alter response more than 2%, and therefore, it could be concluded that the method conditions are robust			

Conclusion

A simple and rapid direct chiral HPLC method was developed, optimized and validated for the simultaneous estimation of the OND enantiomers in pharmaceutical formulations. The mixture design experiments method provides essential information regarding the effect of solvent variables and their interaction effects on enantioselectivity of OND. The proposed method was validated and found to be linear, sensitive, selective, precise and accurate. The present method offers advantages of being fast (4 min) and efficient non-buffered reversed phase enantiomeric separation of OND. Adequate retention, better resolution and shorter analysis time of the proposed method demonstrate that it can be applied for chiral impurity profiling of OND chiral switch.

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Compliance with Ethical Standards

Conflict of interest The authors declare that there are no conflicts of interest.

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