Stress Testing of Linaclotide: Development of a Validated Stability-Indicating RP-HPLC Method

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Summary. Linaclotide, a first-in-class guanylate cyclase-C agonist, was recently approved by US Food and Drug Administration (FDA) as a promising pharmacotherapy for the management of constipation-predominant irritable bowel syndrome (IBS). In this communication, we present a novel stability-indicating reverse-phase high-performance liquid chromatography (RP-HPLC) method for the quantitative determination of linaclotide along with its degradation products. During the International Conference on Harmonization (ICH) prescribed stress study, linaclotide was found susceptible to degrade under hydrolytic (acid and base) and oxidative (peroxide) conditions. The separation of the degradants from the analyte was achieved on a Zorbax Eclipse XDB C_8 Column (250 mm × 4.6 mm, 5 µm) using 0.01 N potassium dihydrogen orthophosphate buffer and acetonitrile (80:20 v/v) as mobile phase at a flow rate of 1.00 mL min^{-1} at column temperature of 40 °C. The detection of the column effluents was realized on a photodiode array detector set at 220 nm. Under the above optimal condition, the method was validated with respect to specificity, linearity, range, precision, robustness, and sensitivity in compliance to the regulatory requirements.

Key Words: degradation behavior, linaclotide, RP-HPLC, stability-indicating

Introduction

Oral linaclotide, the cysteine-rich 14-residue peptide, is a novel constipation-predominant irritable bowel syndrome (IBS) therapy [1, 2]. It is evidenced that linaclotide specifically mimics the guanylate cyclase-C receptor for stimulating fluid secretion and transit in the intestine and is effective for chronic gastrointestinal disorders [3–5]. In addition to intestinal secretagogue, linaclotide has been demonstrated to be an efficient antihyperalgesic during stress-induced hypersensitivity (chemical-induced

acute colitis and other stress conditions) in rat models [6]. The exact mechanism of linaclotide in human is obscure; however, a phase-III clinical trial confirmed that it acts by a guanylate cyclase-C dependent inhibition of colonic nociceptors [7, 8].

A population-based study says that chronic constipation and IBS has been profoundly impairing the life quality of general population (~20%) including elderly and women since decades [9]. Since its inception, the increased enthusiasm surrounding linaclotide has been well reflected by the fact that, in the recent years, numerous clinical trials [10, 11], research articles [1, 12, 13], critical reviews [9, 14, 15], and editorials [8] are being published to emphasize it as the best treatment option for the management of constipation-predominant IBS with very low risk of adverse reactions.

A recent literature survey evidenced that no analytical method is available for linaclotide. In this context, a novel stability-indicating method was developed to determine the degradation behavior of linaclotide for the first time.

Experimental

Chemicals and Reagents

Standard linaclotide was provided as a gift sample from Aurobindo Pharma, Hyderabad, Telangana, India. Ultrapure water was obtained from Millipore, Milli-Q plus water purified system (Bedford, MA, USA) and used for making the solutions. High-performance liquid chromatography (HPLC)-grade acetonitrile and potassium dihydrogen orthophosphate were purchased from the local market (Merck, India). All other solvents and chemicals of analytical grade were procured from local market.

Instrumentation

Analysis of the sample was performed on an HPLC system (Waters 2695Alliance, USA) equipped with inbuilt autosampler, quaternary gradient pump, on-line degasser, column oven, photodiode array detector, and Empower software-2 (all from Waters, USA). The chromatographic separation was performed on a Zorbax Eclipse XDB C_8 (250 mm × 4.6 mm, 5 μm) analytical column. A precision water bath furnished with MV controller (Thermostatic Classic Scientific India Ltd, Mumbai, India) was used for hydrolytic study. A humidity chamber

(Labline Sun Scientific Ltd., New Delhi, India) for stability studies and a photostability chamber (95 Th-400G, Thermolab, Mumbai, India) for photolytic study were used. Thermal stability study was carried out in a hot air oven (Kumar Scientific Works, Pune, India). A pH meter (Lab India) was used for pH adjustment of solutions.

Preparation of Analytical Solutions

Stock solution (1 mg mL^{-1}) was prepared by dissolving the appropriate amount of standard linaclotide in diluent [water and acetonitrile (90:10)]. A 5.0 mL aliquote of stock solution was adequately diluted with the diluent (up to 100 mL) to prepare a final working solution of 50 μg mL^{-1}.

Specificity and Stress Degradation Studies

Stock solution of 1 mL each was used to perform all stress degradation studies. Acid and alkaline hydrolysis was executed by heating the drug solutions in 1 mL each of HCl and NaOH (1 N) at 60 °C for 30 min. Stress testing under neutral condition was studied by heating the drug dissolved in water at 60 °C for 6 h. For study in oxidative condition, the drug solution was kept at 60 °C in 1 mL of 20% hydrogen peroxide for 30 min. For photolytic study, the drug solution was exposed to ultraviolet (UV) light (200 W h m^{-2}) for 7 days. Additionally, the drug solution was exposed to dry heat at 105 °C for 6 h in a hot air oven to perform the thermal degradation study. After the stipulated time, the samples were withdrawn and subjected to HPLC analysis soon after suitable dilution (100 μg mL^{-1}) and neutralization.

Chromatographic Separation

A Zorbax Eclipse XDB C$_8$ (250 mm × 4.6 mm, 5 μm) analytical column (40 °C; column temperature) was used with a mobile phase comprised of 0.01 N potassium dihydrogen orthophosphate buffer and acetonitrile (80:20 *v/v*) pumped at a flow rate of 1.00 mL min^{-1} for the HPLC studies on all reaction solutions individually. The detection of the column effluents was realized on a photodiode array detector set at 220 nm. The sample injection volume was 10 μL.

Method Validation

At the optimal condition, the proposed HPLC method has been validated with respect to the following parameters outlined by ICH [ICH Q2 (R1), 2005]:

Linearity and range

Suitable dilutions from the working standard solution were prepared to yield a series of solutions in the concentration range of 25–150% in the diluent (12.5 to 75 µg mL^{-1}). The resultant solutions were chronologically injected in triplicate into the HPLC column. A calibration curve was constructed, thereby plotting the corresponding peak areas against the concentrations to obtain regression equation and correlation coefficient, which is used to indicate the linearity of the method.

Precision

Intra-day precision of the method was determined using quality control samples (50 µg mL^{-1}), each six injections on the same day, and percentage relative standard deviation (%RSD) were calculated. Furthermore, these experiments were repeated on three consecutive days to assess inter-day precision.

Specificity

The specificity of the method was established through study of resolution of the main analyte peak from the nearest degradant peaks.

Robustness

The robustness study was carried out to assess the influence of minor variations in the optimal chromatographic factors. Deliberate variations in the separation parameters, i.e., flow rate (±0.1 mL min^{-1}) and percent acetonitrile in mobile phase (±1%), were experimented. The resultant responses to the variations were statistically compared with the proposed method.

Detection and quantitation limits

The limit of detection (LOD) and limit of quantification (LOQ) represent the concentration of the analyte that would yield a signal-to-noise ratio of 3 and 10, respectively.

Solution stability

The solution stability was studied by keeping the linaclotide solution for HPLC study in a tightly capped flask at room temperature for 24 h. Subsequently, the solution under study was analyzed at 6 h intervals. The results obtained were compared with that of the freshly prepared solution.

Result and Discussion

Development and Optimization of the Stability-Indicating Method

The stability-indicating chromatographic method was optimized to separate linaclotide from its major degradants formed under various stress conditions. Several buffer systems at different pH values were tried in various ratios with MeOH, and MeCN as mobile phase. Separation with good resolutions was studied on different types of columns (C_8 and C_{18}). Decisively after several experimental trials, a Zorbax Eclipse XDB C_8 column was found to contribute best separation than others with 0.01 N potassium dihydrogen orthophosphate buffer and acetonitrile as mobile phase. Among different mobile phases, better selectivity and good peak parameters were observed with 0.01 N potassium dihydrogen orthophosphate buffer and acetonitrile (80:20 *v/v*). Chromatogram of unstressed standard linaclotide obtained at optimal chromatographic condition is presented in *Fig. 1A*. The obtained chromatograms of stressed samples indicate that separation of drug and all degradation products was successful.
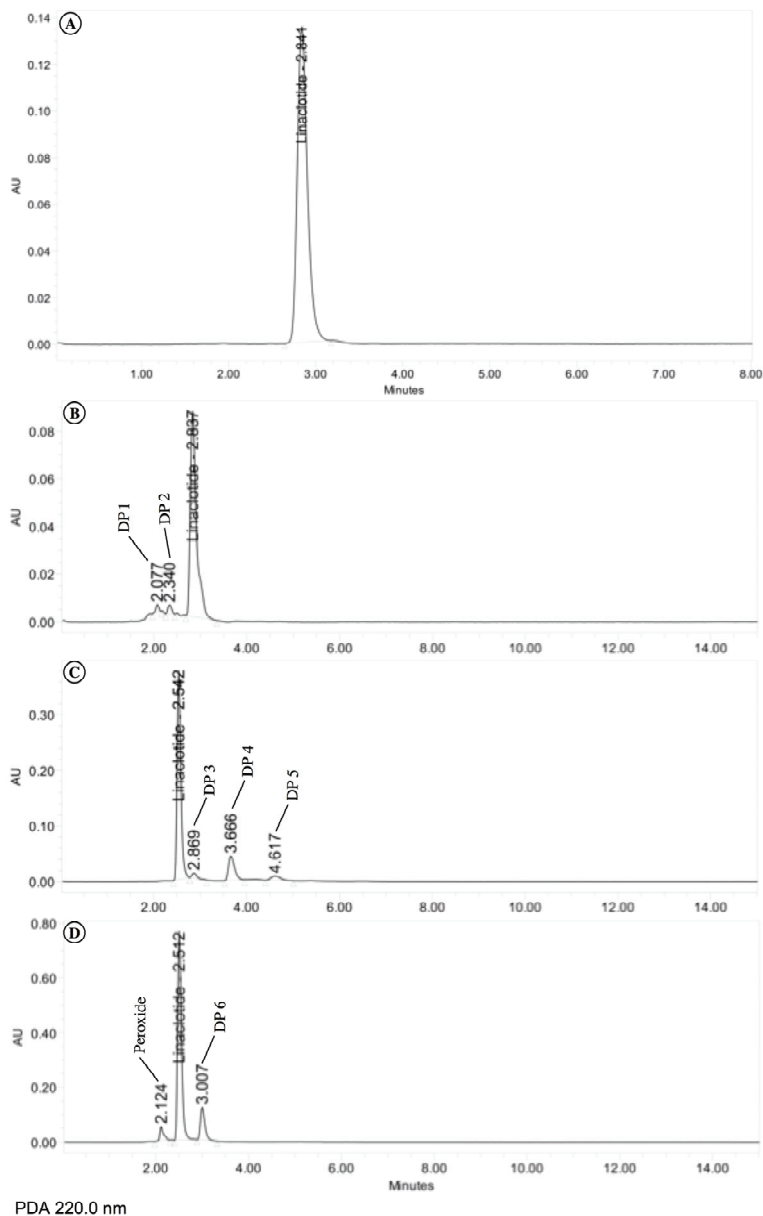
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Fig. 1. (A) Chromatogram of standard linaclotide obtained at optimal chromatographic condition. (B) Chromatogram of linaclotide containing 8.72% of its acid hydrolytic degradants (DP 1 and DP 2). (C) Chromatogram of linaclotide containing 22.43% of its base hydrolytic impurities (DP 3, DP 4, and DP 5). (D) Chromatogram of linaclotide containing 17.82% of its oxidative degradants (DP 6)

Degradation Behavior of Linaclotide

Stress testing of linaclotide under several conditions with the aid of reverse-phase (RP)-HPLC suggested the following degradation behavior:

Acid hydrolysis

Linaclotide is labile to acidic condition forming two degradation products (DP 1 and DP 2) at relative retention time (RRT) of 1.36 and 1.21, respectively. An 8.72% fall in the drug peak area was observed after the stipulated time period (30 min) of acid stress testing *(Fig. 1B)*.

Base hydrolysis

Upon heating the drug solution with 1 N NaOH at 60 °C for 30 min, a sharp fall (22.43%) in the drug peak area was observed. As a result, three major degradation peaks (DP 3, DP 4, and DP 5) at RRT of 0.88, 0.69, and 0.55, respectively were detected *(Fig. 1C)*. Linaclotide was found to be very sensitive to hydrolyze in alkali. During base hydrolysis, the retention time of main peak was observed to deviate from that of unstressed sample. The rate of hydrolysis in alkaline condition was faster as compared to that of acid.

Oxidative

Significant degradation of linaclotide was observed when the drug was subjected to oxidative condition. The degradation was associated with rise in one major degradant peak (DP 6) at RRT 0.83. Upon completion of the stress duration, a substantial fall (17.82%) in the drug peak area was observed *(Fig. 1D)*.

Thermal, light (UV), and neutral (water) hydrolysis

Linaclotide was stable to thermal, light, and neutral hydrolysis stress conditions, and no decomposition was found.

The homogeneity and purity of linaclotide peak in all analyzed stress samples were confirmed by peak purity test (photodiode array [PDA] detector). The data obtained from the degradation study has been enumerated in *Table I*. The obtained chromatograms *(Fig. 1)* endorse that all

the formed degradants were well separated from linaclotide and each other, confirming the specificity and stability-indicating power of the RP-HPLC method.

Table I. Data obtained from the degradation study

Stress conditions	Degradants		Linaclotide				
	RRT	Rs	Rs	T	Purity angle	Purity threshold	%Degraded
Acid hydrolysis							
1.0 N HCl, 60 °C, 30 min	1.36 (DP 1)	–	(LIN-DP2) 2.5	1.81	0.766	0.500	8.72
	1.21 (DP 2)	(DP2-DP1) 1.8					
Alkaline hydrolysis							
1.0 N NaOH, 60 °C, 30 min	0.88 (DP 3)	(DP3-LIN) 1.9	–	1.34	0.166	0.303	22.43
	0.69 (DP 4)	(DP4-DP3) 3.7					
	0.55 (DP 5)	(DP5-DP4) 3.0					
Oxidative degradation							
20% H_2O_2 at room temperature for 30 min	0.83 (DP 6)	(DP6-LIN) 3.0	(LIN-H_2O_2) 2.5	1.36	1.636	0.382	17.82

Rs, resolution; T, USP tailing; LIN, linaclotide

Validation of the Stability-Indicating Method

The summary of method validation data is presented in *Table II*. From the system suitability test results, it has been confirmed that the system was deemed to be suitable as it complies with the limits of peak parameters. Resolution (>1.8) and United States Pharmacopeia (USP) tailing (<1.81) for all the analytes confirm the good selectivity of the method. The peak areas for drug samples were precisely linear in the concentration range between 12.5 and 75 µg mL^{-1} (25 to 150 %). The data were analyzed with least squares linear regression indicating good linearity for linaclotide ($y = 21507x + 1061.9$; $r^2 = 0.999$). The obtained LOD (2.34 µg mL^{-1}) and LOQ (7.09

µg mL^{-1}) results demonstrate that the method is adequately sensitive. The calculated %RSD values for intra-day and inter-day precision were <1.044%, proving that the method was suitably precise. The method was found to be sufficiently robust under the tested conditions *(Table III)*. During the solution stability study, no significant changes were observed, confirming the drug solution is stable for 24 h.

Table II. Method validation summary

Parameters	Linaclotide		Parameters	Linaclotide
System suitability[a]			Sensitivity	
RT (min)	2.840		LOD (µg mL^{-1})	2.34
T	1.27		LOQ (µg mL^{-1})	7.09
N	2887		Precision (%RSD)[b]	
Linearity[a]			Intra-day	0.9
Range	12.5 to 75 µg mL^{-1} (25–150%)		Inter-day	1.044
Slope (mean ± SD; %RSD)	21507.33 ± 7.57; 0.03			
y-Intercept (mean ± SD; %RSD)	1061.9 ± 1.6; 0.15			
r^2	0.999			

RT, retention time; *T*, USP tailing; *N*, number of the theoretical plates; *r*^{2}, correlation coefficient

[a]Average of the three determinations.

[b]Average of six determinations.

Conclusion

A stability-indicating method was developed in the purview of ICH guidelines to separate all the degradation products formed under various stress conditions. The stress study demonstrates that linaclotide is highly labile to oxidative, acid, and alkali hydrolysis (NaOH > H$_2$O$_2$ > HCl). Six degradants were formed under the above stress conditions. The drug remained stable in thermal, light, and neutral hydrolysis stress conditions. The developed method proved to be selective, sensitive, precise, and robust and can be applicable for estimating the possible degradants in bulk and pharmaceutical dosage forms.

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Table III. Robustness test

Condition	Level	Linaclotide ($n = 6$)			
		RT (min): mean ± SD; %RSD	Peak area: mean ± SD; %RSD	*T*: mean ± SD; %RSD	*N*: mean ± SD; %RSD
Optimal	–	2.83 ± 0.004; 0.14	1089688.16 ± 1328.37; 0.12	1.27 ± 0.008; 0.63	2825.83 ± 48.37; 1.71
Flow rate (±0.1 mL min⁻¹)	Decrease	3.21 ± 0.05; 1.67	1239330 ± 5239.5; 0.4	1.52 ± 0.01; 0.66	3163.83 ± 55.12; 1.74
	Increase	2.61 ± 0.005; 0.2	1015469.16 ± 4033.09; 0.39	1.47 ± 0.01; 0.83	2836.16 ± 40.27; 1.42
% Acetonitrile (±1%)	Decrease	3.01 ± 0.001; 0.053	1098303.66 ± 7987.45; 0.72	1.33 ± 0.02; 1.6	2602.83 ± 44.24; 1.7
	Increase	2.72 ± 0.01; 0.38	1114809.66 ± 5206.84; 0.46	1.29 ± 0.01; 1.05	3660.33 ± 55.17; 1.50

RT, retention time; *T*, USP tailing; *N*, USP plate count

Acknowledgment

Conflict of Interest

All the authors have equally contributed to the scientific work and article drafting. All the authors declare no conflict of interest.

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