RESEARCH ARTICLE

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Development and Validation of Stability Indicating RP-HPLC Method for Determination of Daridorexant Drug Using AQbD Approach

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Abstract

The current aim of the research is to design and validate the RP-HPLC method systematically for the bulk analysis of Daridorexant which is simple to use, fast, precise, and economical. This employs analytical quality by design techniques to optimize chromatographic parameters for the routine analysis of Daridorexant. The critical process parameters were optimized using a custom design. A risk assessment matrix was developed to identify the risk associated with the development of the method and the simultaneous linkage of critical analytical attributes and critical method attributes. The RP-HPLC column was programmed in the isocratic elution mode with methanol and KH_2PO_4 buffer as mobile phase at the flow rate of 1mL per minute with variable ratio for the process development of the RP-HPLC technique. An Intersil C18 column was employed for the chromatographic separation process. The optimized technique from JMP 17 PRO software yielded a desirability function of 73.70%, using a mobile phase ratio of 70:30 (methanol and 0.1N KH_2PO_4) and a flow rate of 1 ml/min. The results confirmed the LOD and LOQ were found to be 0.232 mcg/mL and 0.773 mcg/mL. It concluded that Daridorexant had a retention time (Rt) of 3.549 minutes. In compliance with ICH Q2 (R1) specifications, this method was developed, approved, and validated. The models were statistically significant with a p-value < 0.0003 for the selected responses. The validation parameters evaluated were within acceptable limits. Stress testing revealed that the drug's stability deteriorated significantly when subjected to heat and acid stress. **Major Findings:** A robust and sensitive RP-HPLC method was developed and validated for the stability analysis of Daridorexant using AQbD principles. The method, optimized with an Intersil C18 column and validated per ICH Q2 (R1) guidelines, demonstrated high precision, sensitivity (LOD: 0.232 mcg/mL, LOQ: 0.773 mcg/mL), and stability-indicating capability. It can be applied to pharmaceutical formulations, bioanalytical studies, and routine quality control.

Keywords: Custom Design, ICH, Orexin Receptor, Retention Time, Stability, Validation

1. Introduction

Quality by Design (QbD) framework is based on a comprehensive scientific and technical understanding of key processes and product characteristics. It uses the scientific knowledge gained during development to establish controls and assessments and ultimately applies this knowledge to the product's lifetime to promote continual improvement[1]. Analytical Quality by Design is a novel approach for optimizing HPLC equipment parameters like flow rate, plate count, retention time, concentration of solvent, buffer, column type, column length, injection volume, temperature and peak area. Analytical techniques must be developed and optimized to ensure the precision and reliability of analytical outcomes[2,3].

Daridorexant (Figure 1) is a selective dual orexin receptor antagonist used to treat insomnia. The wake-

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promoting neuropeptides known as orexins, together with their endogenous ligands, are bound to the orexin receptors OX1R and OX2R by Daridorexant. The drug has the molecular formula of $C_{23}H_{23}ClN_6O_2$ and suppresses excessive wakefulness[4]. The literature review highlights a lack of analytical methods for Daridorexant, both alone and in combination, using Reverse Phase HPLC. Additionally, there is no method development incorporating the Analytical Quality by Design (AQbD) approach. This gap presents an opportunity for future research to optimize and refine these techniques.

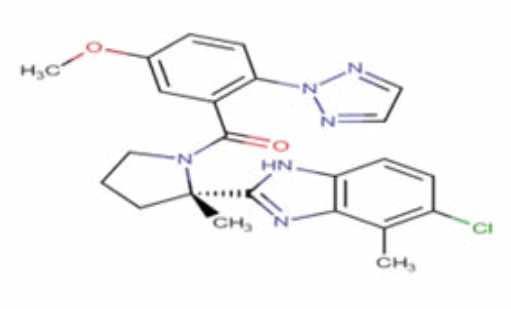


Figure 1. Chemical structure of Daridorexant. (Source: https://go.drugbank.com/drugs/DB15031)

2. Materials and Methods

2.1 Materials

Daridorexant was bought from Sunlight Sciences located in Hyderabad, India. Through Sd Fine-Chem Limited in India, HPLC solvents such as methanol, acetonitrile and orthophosphoric acid were obtained from Merck Specialties Ltd.

2.2 Methods

The Daridorexant method was developed by High-Pressure Liquid Chromatography (HPLC) with a UV detector, which was supplied by Waters Corporation, Massachusetts, U.S.A. Chromatographic separation was executed using an INTERSIL C18 column with a flow rate of 0.7 millilitre/minute at 254 nanometers. The mobile phase was constituted using KH_2PO_4 and methanol (50:50%) at room temperature. RP-HPLC method was optimized employing a custom design of experiment with JMP 17 software[5].

2.3 Risk Assessment

An Ishikawa diagram helps determine the underlying cause of the problem by highlighting the possible causes and their sub-causes that influence the product's Critical Analytical Attributes (CAAs). Risk Estimation Matrix (REM) was used to detect CMPs, that are high-risk and have a strong impact on CAAs. The highest-risk factors were identified by developing the REM and categorized them as high, medium and low[6].

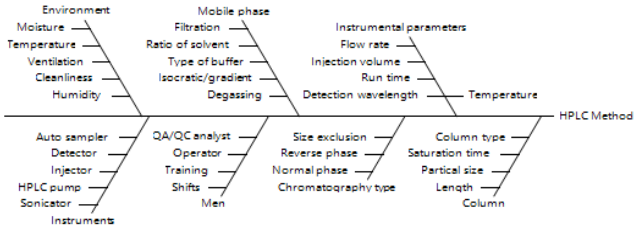


Figure 2. Risk assessment using Ishikawa diagram. (Source: JMP 17 PRO SOFTWARE)

Analytical Risk identification is a systematic method for identifying, monitoring, communicating, and analyzing threats to data integrity throughout the product lifecycle. A thorough effort to identify all pertinent variables that may affect the quality of the data can be ensured by using tools such as Ishikawa diagrams. These variables include standards, reagents, sampling, sample processing, and equipment operating conditions.

2.4 Method Development

2.4.1 Mobile Phase Preparation

A combination of 500 ml of methanol (50%) and 500 ml of KH_2PO_4 (50%) buffer was degassed using an ultrasonic water bath for a period of ten minutes before filtering under vacuum.

2.4.2 Standard Preparation

Weighed exactly 10 milligrams of Daridorexant and transferred it to a dried and clean 25 ml volumetric flask, along with 5 ml of diluent. The flask was then sonicated for 30 minutes, and the volume was adjusted using a diluent to achieve the desired concentration. This solution was further diluted to get different concentrations.

2.4.3 Determining the Absorption Maxima (λmax)

After dissolving the sample in methanol, the solution was analyzed in the UV range from 200 to 400 nm, revealing an absorption maximum of 254 nm when dissolved in methanol.

2.4.4 Method Development

Various trials were developed using Monopotassium phosphate and methanol as mobile phases using various chromatographic columns varying flow rate and run time.

2.4.5 Design of Experiment

The Design of the Experiment (DoE) helps systematically to examine and identify changes in output responses through intentional alteration of process input variables (factors) in one or more trials. DoE is essentially a technique for methodically determining connections between variables. To create trustworthy methods in Quality Control (QC) situations, this framework is essential[7].

2.4.6 Custom Design

Custom Design methodology was employed to conduct experimental studies. During optimization, Critical Process Parameters (CPPs) were monitored along with the acetonitrile ratio in the mobile phase, and ammonium acetate as the aqueous phase, as well as column temperature, flow rate and pH. These factors were chosen based on their identified and predictable influence on chromatographic behavior. It was essential to assess whether minor adjustments could affect optimization quality. The limitations obtained from method development trials were used to guide the symmetric selection of the factor variations' ranges concerning their nominal values.

2.5 Method Validation

2.5.1 Standard Stock Preparation

10 milligrams of Daridorexant were weighed and transferred to the 100 ml volumetric flask. With the addition of 5 millilitre of diluent, the mixture was exposed to 30 minutes of sonication. The solution was subsequently adjusted up to the mark by adding diluent. By diluting the stock solution with diluent, several concentrations of Daridorexant were obtained[8].

2.5.2 Blank

A mobile phase was injected to verify if any peaks would appear on the chromatogram[9].

2.5.3 Suitability of the System

To determine the effectiveness and application of the RP-HPLC system suitability was checked.

2.5.4 Accuracy

Accuracy was evaluated by adding additional quantities of the analytical solution to evaluate the percentage recovery. Solutions of 50 µg/ml, 100 µg/ml, and 150 µg/ml concentrations were prepared at 50%, 100%, and 150% were injected into the HPLC instrument[10].

2.5.5 Precision

The concentration of 100µg/ml is ready; prepare six vials and inject all individually[11].

2.5.6 Linearity and Concentration Ranges

Each vial comprised one injection of various concentrations that ranged from (50-150µg/ml) and was prepared with concentrations of 50% to 150%[12].

2.5.7 Specificity

Specificity is the capacity to distinguish between closely related compounds or compare them against a known reference sample or impurity. It is essential to inject a blank to verify if any peaks appear on the chromatogram[13,14].

2.5.8 Limits of Detection and Quantification

From the stock solution, 100 µg/ml concentrated solution was prepared. Then 0.2 ml is pipetted out for a Limit of Detection (LoD) and 0.6 ml for a Limit of Quantification (LOQ). To find the ideal signal-to-noise ratio, these quantities are diluted with diluent and injected into the 10-millilitre volumetric flask. Signal-to-noise ratios of 3:1 for LOD and 10:1 for LOQ were typically used[15,16].

2.5.9 Robustness

Studies on robustness were conducted by varying the system's technique parameters, including the mobile phase (±5%), pH (±0.1), and flow rate (±0.1%)[17-19].

2.6 Forced Degradation Studies[20-22]

2.6.1 Solution Preparation

Pipette 5 millilitre of stock and dilute up to 50 millilitre to prepare stock solution B.

Acid: 1 millilitre of stock B was pipetted into a 10-millilitre volumetric flask. Inject 5 millilitre of 0.1N HCl, sonicate for 30 minutes, thoroughly mix with diluent to make up the volume and inject into the HPLC.

Base: 1 millilitre of stock B was pipetted into a 10-millilitre volumetric flask. After adding 5 millilitres of 0.1N NaOH, thoroughly mix it with the diluent to adjust the volume, then inject it for 30 minutes, and inject it into an HPLC.

Hydrolysis: Pipette 1 ml from stock B into a 10-milliliter volumetric flask, add 5 ml of water, with diluent make the volume, sonicate for 30 minutes, and then inject into an HPLC.

Peroxide: Pipette 1 ml of stock B into a 10-millilitre volumetric flask, add 5 ml of 1% H_2O_2 mix, dilute up to mark and sonication for 30 minutes, and then inject into HPLC.

Heat: Pipette 1 millilitre from stock B into a 10-millilitre volumetric flask, reconstituted with a 30-minute-heated diluent up to mark, and then injected into an HPLC.

Sunlight: Pipette 1 ml of the diluting agent from stock B into a 10-millilitre volumetric flask and let it remain in the sun for 6 days before injecting it into an HPLC.

3. Results and Discussion

3.1 Risk Assessment

Once the variables have been discovered (as indicated in (Figure 2), they can be managed efficiently.

To assess risks associated with parameters affecting critical attributes such as resolution, accuracy, precision, tailing, etc., risk matrices were employed and the risks were ranked based on severity such as 1 for low risk, 3 for medium risk and 9 for high risk. Initially, basic matrices were developed based on scientific knowledge and methodological experience during the initial stages of development (see Table 1)[6].

3.2 Method Development Trials

A 50:50 (v/v) mixture consisting of potassium dihydrogen phosphate buffer and methanol was used as the mobile phase. The column flow rate is set at 0.7 millilitre/minute. The method was developed to analyze Daridorexant, exhibiting a retention time of 3.549 minutes, a tailing factor of 1.11, and a plate count of 5412 of the chromatograms (Figure 3).

With an injection volume of 10 µl and a total duration of 20 minutes, this technique detects at 254 nm. Various columns, mobile phase ratios, and flow rates are used to maintain sample and column temperatures at 25°C (Table 2).

Table 1. Risk Estimation Matrix (REM)

CPA CAA and CMA	Retention time	Peak area	Theoretical plate	Column oven temperature	Flow rate	Column type	Injection volume
Accuracy	1	9	1	9	3	3	9
Linearity and range	3	3	1	9	3	3	9
Precision	9	9	3	1	1	3	1
Robustness	9	9	3	9	9	3	9
Limit of the Detection (LoD)	3	1	3	1	3	3	1
Limit of Quantification (LoQ)	9	9	1	1	3	3	1
Specificity	3	9	3	1	3	1	3

Table 2. Components and prerequisites for developing an experimental method

Test	Mobile phase	Column	Flow rate (ml/min)	Injection volume (µl)	Retention time	Plate count	Tailing	Inference
1	KH_2PO_4: Methanol (50:50v/v)	AGILANT, C18, 150X4.6mm, 5µm	0.7	10	3.583	3757	1.64	Less theoretical plates and an improperly shaped peak
2	KH_2PO_4: Methanol (70:30v/v)	COSMICSIL, C18, 150X4.6mm, 5µm	0.6	10	6.203	4870	1.61	The plate counts outside the boundaries and has a poor peak shape.
3	KH_2PO_4: Methanol (50:50v/v)	INTERSIL, C18, 150X4.6mm, 5µm	0.7	5	4.557	7086	1.10	A Gaussian peak but higher retention time
4	KH_2PO_4: Methanol (50:50v/v)	INTERSIL, C18, 150X4.6mm, 5µm	0.8	5	3.549	5412	1.11	There is a nice peak shape, and all outcomes are within range.
5	KH_2PO_4: Methanol (50:50v/v)	AVANTOR, C18,250X4.6mm, 5µm	0.9	20	3.186	5179	1.17	Theoretical plates are beyond the Range
6	KH_2PO_4: Methanol (50:50v/v)	SYMMETRY, C18,150X4.6mm,5 µm	0.7	10	4.542	5611	1.13	Higher retention time

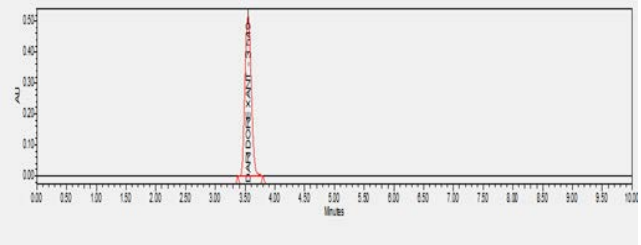


Figure 3. Chromatogram of Daridorexant. (SOURCE: EMPOWER 3 SOFTWARE)

3.3 Custom Design and Optimization of Method

The Custom Design involved 12 runs for the 3 factors and 3 responses (Table 3). Flow rate, solvent composition, and run-time were selected as factors and retention time, theoretical plate count and tailing factor were selected as a response[23].

3.4 Prediction Profiler of The Optimized Method and Statistical Optimization Selected HPLC Method Responses

Numerical optimization shows a continuous constant linkage with different parameters and various responses, as shown in the prediction profiler (Figure 4). The data indicates an overall desirability score of 73.70%, representing a significant chance of succeeding in the intended goals in all three responses. To accomplish these objectives, Figure 3 highlights the importance of the bars that reflect theoretical plate count, buffer and flow rate. The results of 12 trial runs were used to evaluate the model's effectiveness. The results of the statistical analysis utilizing zero-intercept multiple regression models showed that, at the designated significance level, the predicted R-squared values of 0.90 for retention time, 0.90 for theoretical plate count, and 0.91 for tailing factor were statistically significant.

3.5 Contour Profiler

In Figure 5 the white region on the contour profiler shows the optimization and expresses how the method's components affect the results and define the optimum operational design space. To confirm Critical Method Attributes (CMAs) taken in the design have a substantial impact on the Critical Analytical Attributes (CAAs), statistical parameters were examined. This study validated their significance in predicting and optimizing the objectives of the experiment.

Table 3. The method runs by custom design

Sl. No	Flow rate(A)	Solvent Composition(B)	Run time(C)	Retention Time	Theoretical Plate	Tailing Factor
1	0.5	50	5	5.023	6291	1.1
2	1	50	20	3.759	7749	1.7
3	0.5	50	5	4.762	6597	1.13
4	1	50	20	3.157	8572	1.25
5	0.5	70	20	4.612	5612	1.52
6	1	70	5	3.687	7948	1.32
7	0.75	60	12.5	4.372	6839	1.37
8	0.5	70	20	4.781	5697	1.6
9	0.5	50	20	4.472	6371	1.35
10	1	70	5	3.842	7364	1.34
11	0.75	60	12.5	4.478	7285	1.36
12	0.5	70	5	5.326	6439	1.52

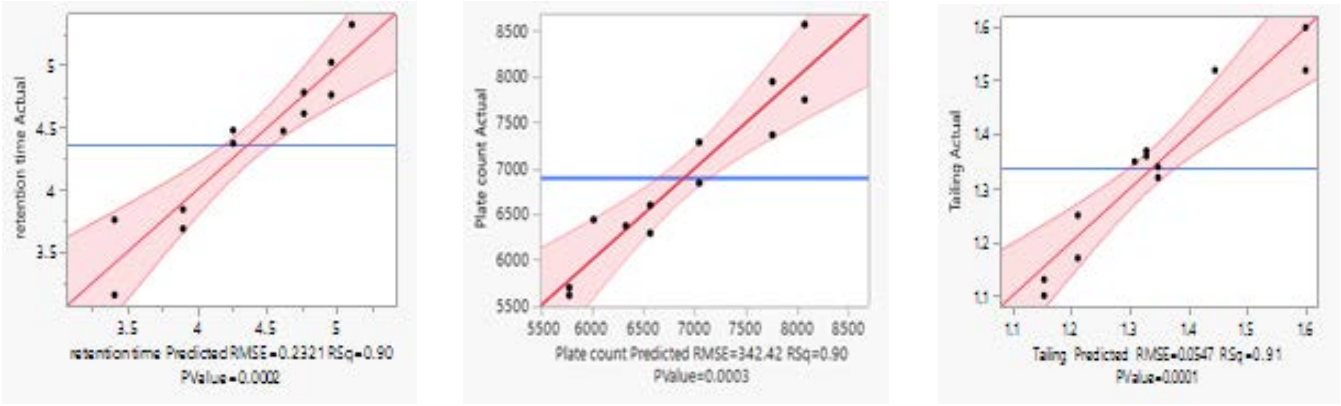


Figure 4. Actual vs Predicted plot for selected responses (retention time, Plate count, tailing).
(SOURCE: JMP 17 PRO SOFTWARE)

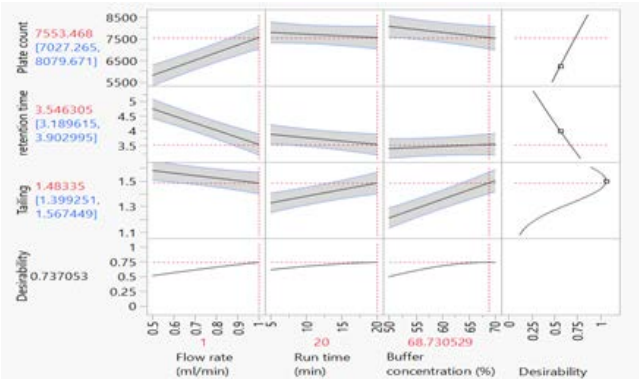


Figure 5. Prediction profiler plot after optimization.
(SOURCE: JMP 17 PRO SOFTWARE)

3.6 Validation of Analytical Technique

Specificity: The specificity of the proposed approach was evaluated by checking the influence of blanks and other substances on the Daridorexant retention period. Consequently, no further peak was seen in the blank, indicating a high level of specificity for the recommended method.

System suitability: According to Table No. 04, the system suitability parameters pass for tailing, which is less than 2.0, acceptable peak area, Rt, plate count, which is more than 2500, and % RSD, which is less than (2).

Accuracy: Three injections at 50%, 100%, and 150% concentrations were used to measure accuracy. As seen in Table No. 05, the developed approach passes this criterion

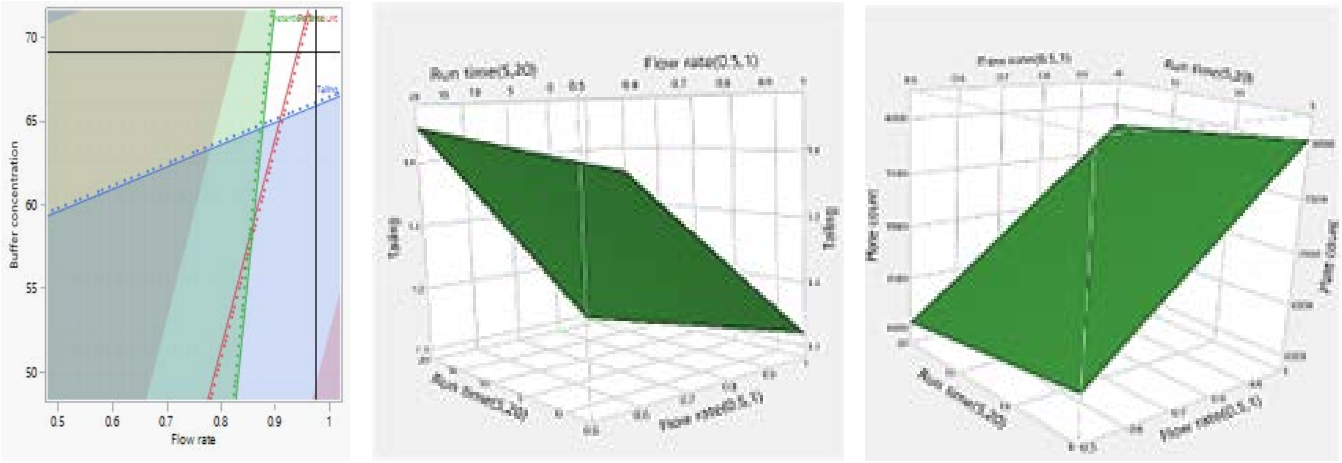


Figure 6. Profiler depicting the effect of method attributes on the responses. (SOURCE: JMP 17 PRO SOFTWARE)

Table 4. Studies on system suitability

Sl. number	Test- sample	Peak	Retention time (min)	Area (µV*sec)	USP - Plate Count	USP- Tailing
1	STD-2	Daridorexant	4.302	1797257	4641	1.27
2	STD-2	Daridorexant	4.445	1844011	4464	1.28
3	STD-2	Daridorexant	4.479	1840937	4672	1.27
4	STD-2	Daridorexant	4.487	1830824	4553	1.27
5	STD-2	Daridorexant	4.526	1838813	4484	1.27
Mean				2576396.1		
%RSD				0.1		

since accuracy at three distinct concentrations was within the range of 97–103 %.

Precision: The accuracy of a system and method was determined by examining the data concordance between the series of measurements. Six injections at 100% concentration were utilized. The precision percentage RSD 0.86% suggested that the approach yielded a high degree of accuracy.

Linearity and range: A solution containing Daridorexant (50 to 150 µg/ml) concentration was injected at a wavelength of 254 nm. Based on the obtained graph, the R2 value was 0.9999. According to this, the Daridorexant exhibits linearity within the 50% to 150% range, as seen in Figure 7.

Limits for Detection and Quantitation: LOD and LOQ were calculated by S/N ratio and found to be 3.2 and 10.6. LOD allows for the detection of sample concentrations as low and found to be 0.232 µg/ml, while the Lowest Quantity of sample that can be quantified (LOQ) is 0.773 µg/ml.

Table 5. Accuracy studies

Spiked level	Weight of sample	The area occupied by the sample	µg/ml added	µg/ml found	% Recovery
50%	67.50	912773	24.750	24.60	100
50%	67.50	913018	24.750	24.58	100
50%	67.50	912039	24.750	24.76	100
100%	135.00	1828451	49.500	49.63	101
100%	135.00	1811593	49.500	49.63	100
100%	135.00	1818509	49.500	49.76	100
150%	202.50	2719992	74.250	74.55	100
150%	202.50	2702244	74.250	74.80	99
150%	202.50	2703496	74.250	74.62	99

Table 6. Robustness assessment

SI.no	Sample	Peak	Retention time (minute)	Area (µV*sec)	Plate Count (USP)	Tailing (USP)
1	FLOW-A	Daridorexant	3.398	13505707	4642	1.22
2	FLOW-B	Daridorexant	5.515	2252770	5277	1.29
3	pH-1	Daridorexant	4.305	1803746	4691	1.26
4	pH-2	Daridorexant	4.375	1837848	4792	1.26
5	COMP-1	Daridorexant	5.515	2252770	5277	1.29
6	COMP-2	Daridorexant	3.724	1598603	4735	1.24

Table 7. Forced degradation study report

Stress Conditions	Area of sample	Assay-%
Acid	1618564	88.16
Base	1677679	91.38
Peroxide	1721329	93.76
Heat	1592564	86.75
Sunlight	1735428	94.53
Hydrolysis	1817277	98.99

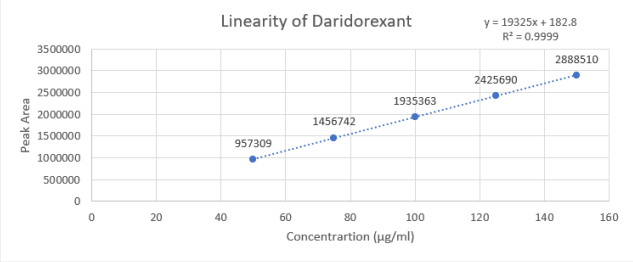


Figure 7. Linearity graph of Daridorexant. (SOURCE: MICROSOFT EXCEL)

Robustness: The results show that the novel approach is robust and does not vary significantly even with mild changes in pH, flow rate, and mobile phase composition. All the results complement the criteria for system appropriateness as indicated in Table 6.

3.7 Studies on Forced Degradation

A series of stress tests were carried out to see if deteriorated products would affect the research. These circumstances included exposure to acid, base, peroxide, heat, and sunshine. According to the degradation data, Daridorexant showed some deterioration in response to the following stressors: heat (13.25%), direct sunlight (5.47%), base (8.62%), peroxide (6.24%), acid (11.84%), and hydrolysis (1.01%). But in every instance, deterioration stayed below the 20% threshold (Table 7). In addition, it proved that the principal peak remained unaffected by the deteriorated products. The findings are summarized as follows: **Hydrolysis stress < Photo stress < Peroxide stress < Base stress <Heat stress < Acid stress**[24].

4. Conclusion

A conventional RP-HPLC technique for Daridorexant estimation was developed by employing analytical quality by design methodology. The injection volume, type, temperature, and flow rate of the columns were chosen as the CMPs. Theoretical plates, peak area and retention time have been identified to be the three critical quality parameters. A custom design method was used to precisely optimize the CMPs. A mobile phase of 30% methanol and 70% 0.1N KH_2PO_4 was injected at a flow rate of 1ml/min to obtain the optimal chromatographic conditions. It was discovered that the theoretical plates and asymmetry were within acceptable range, and the retention time was 3.549 minutes. Based on ICH Q2 (R1) recommendations, the developed technique was validated the drug was found to be acid-sensitive.

5. Acknowledgement

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6. Abbreviations

AQbD: Analytical Quality by Design
HPLC: High-Performance Liquid Chromatography
UPLC: Ultra-Performance Liquid Chromatography

HPTLC: High-Performance Thin Layer Chromatography
LOD: Limit of Detection
LOQ: Limit of Quantification
UV: Ultraviolet
QC: Quality Control
%RSD: Percent Relative Standard Deviation
Rt: Retention time
OX1R: Orexin Receptor 1
OX2R: Orexin Receptor 2

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