ANALYTICAL QUALITY BY DESIGN (QBD) APPROACH FOR DEVELOPMENT AND VALIDATION OF HPLC METHOD FOR LOPINAVIR: APPLICATION TO HYDROLYTIC, THERMAL, OXIDATIVE AND PHOTOLYTIC DEGRADATION KINETICS

Humnabadkar Shubham C[1], Patil Pallavi M[2*]

Article History: Received: 23.03.2023 **Revised:** 05.05.2023 **Accepted:** 19.06.2023

Abstract

The goal of the current study is through the use of quality-by-design software a simple, robust, specific, and accurate stability-indicating liquid chromatography (LC) methodology (reverse-phase high-performance LC) was created for the estimation of Lopinavir. Water's HPLC System with a photodiode array detector at 250 nm was used for the chromatographic separation. The method was created using methanol-potassium dihydrogen orthophosphate (pH 3.8; 10 mm, tetra butyl ammonium hydroxide; 10 mm (40:60, v/v)) on a Lichrospher RP C18 (250×4.6, 5 mm) column with a flow rate of 1.55 ml/min at 55°C. According to the International Conference on Harmonization (ICH) criteria. Results were chosen as independent variables (critical analytical attributes) for the method optimization. (20 runs, 3 levels, and 3 factors) were carried out with Design Expert Software. LOD, LOQ, Specificity, and Robustness were carried out. The lopinavir HPLC method that is provided is accurate and simple. The developed method shows good accuracy, precision, linearity, specificity and system compatibility.

Keywords: Column; Flow Rate; Organic Phase; Qbd; Temperature,

[1]Research Scholar, Department of Quality Assurance Technology, P. E. Society's Modern College of Pharmacy Yamuna Nagar, Nigdi, Pune-411044 Maharashtra, India, 411044.
Email: humnabadkarsc@gmail.com
[2*]Associate Professor, Department of Pharmaceutical Chemistry, P. E. Society's Modern College of Pharmacy Yamuna Nagar, Nigdi, Pune-411044 Maharashtra, India, 411044.
Email: [2*]pallavipatil_2007@yahoo.com

***Corresponding Author:**
Patil Pallavi M[2*]
[2*]Associate Professor, Department of Pharmaceutical Chemistry, P. E. Society's Modern College of Pharmacy Yamuna Nagar, Nigdi, Pune-411044 Maharashtra, India, 411044.
Email: [2*]pallavipatil_2007@yahoo.com

DOI: 10.31838/ecb/2023.12.Si6.548

1. INTRODUCTION

As part of Antiretroviral Therapy (ART), the HIV Protease Inhibitor (PIs) Lopinavir is given in combination with a low dose of Ritonavir (RTV) under the trade name Kaletra®. The U.S. Food and Drug Administration authorized the combination[1].

Food and Drug Administration a couple of decades ago Since 2006, Lopinavir has been continuously advised as one of the PIs in second-line regimens by World Health Organization (WHO) guidelines. According to the most recent WHO recommendations (from 2019), LPV/r is still suggested as the best PI therapy for second-line ART regimens, alternative first-line ART regimens in children, and unique conditions in neonates. Most recently, as a result of the worldwide SARS-CoV-2 infection outbreak, Lopinavir is being examined as a viable candidate in various clinical trials[2].

By the end of 2018, 1.7 million youngsters were among the estimated 37.9 million HIV-positive individuals worldwide. In addition, 0.16 million children were among the 1.7 million people who contracted HIV for the first time in 2018 alone. But as of the end of 2018, just US$ 19 billion, or US$ 1 billion less than in 2017, was available for the fight against HIV/AIDS in low- and middle-income nations. The comparatively low financing, combined with the recent stasis in the rate of new HIV infections, poses a barrier to efficient and timely diagnosis and monitoring for people living in resource-limited situations.

To determine Lopinavir in biological materials, several immunological approaches and high-performance liquid chromatography (HPLC) procedures have been developed to date [3–13]. However, Lopinavir's structural resemblance to endogenous molecules makes it more difficult to analyze in human serum. Because it is polar and almost insoluble in the majority of organic solvents, Lopinavir's solvent extraction is challenging [8]. Ion pairing agents [9], column thermostatic detection [10], and fluorometric detection are all used in the previously described analytical procedures. for Lopinavir evaluation. Furthermore, the claimed analytical procedures did not fulfil the current International Conference on Harmonization Q1A (R2) (ICH, 2003) [14]and regulatory criteria.

The stability of drugs and degradation products can be determined using stability-indicating techniques (SIMs), which are becoming a common analytical tool. Under the impact of numerous environmental variables, a pharmaceutical molecule's chemical stability is susceptible to change. Drug molecules' stability may be negatively impacted by degradation processes such as hydrolysis, oxidation, racemization, or reduction. In this case, stress testing offers better insight into the potential degradation of products created during medication product manufacturing or storage[15]. This makes it easier to identify degradation pathways and identifies the process of degradation. It is very beneficial in determining the degradation product's structure and of the degradation product and determining the inherent stability of pharmacological molecules. Stress testing of pharmaceutical products is also advised to create and show the specificity of a stability-indicating method. As a result, the data from stress testing determines the chemical behavior of the drug molecule, which further aids in tormulation and packaging creation[15]. To understand how a drug substance's and drug product's quality changes over time under the effect of different environmental conditions, stability testing data are required, according to FDA and ICH guidance[16].

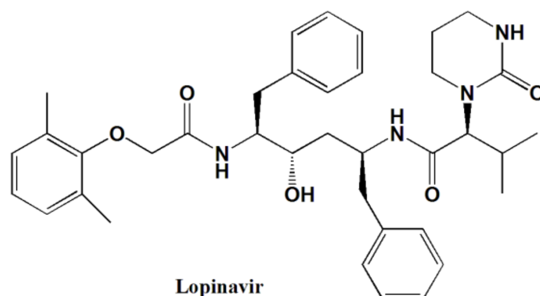

Figure 1. Chemical Structure of Lopinavir

All regulatory organizations for pharmaceutical products place a high value on quality. Quality refers to the satisfaction of the client with the process, product, and services. Many of these quality-related operations reflect the necessity for businesses to succeed in the global marketplace. The customer expects excellence in terms of quality, dependability, low cost, and timely performance[17]. In contrast to the conventional method, the QbD paradigm starts with pre-defined goals to produce a product that meets pre-established requirements. The QbD approach's key elements are product

profile, product quality, design and development, risk management, and manufacturing process control[18].

A thorough literature review revealed no particular, robust, or stability-indicating HPLC technique development in a QbD setting for Lopinavir. As a result, a systematic approach is preferable for the creation of an optimal stability-indicating Chromatographic method based on the QbD approach, which aids in the estimation of Lopinavir and ensures medication quality.

For the first time, we present an accurate, specific, and reproducible HPLC approach for determining Lopinavir that is relevant in the presence of degradation products and stress degradation regimes. The QbD methodology was used to try to design a new specialized HPLC method for Lopinavir.

2. EXPERIMENTATION

2.1 Materials and Methods

2.1.1. Chemicals, reagents and solutions

All the reagents as HPLC grade Water and Methanol (HPLC grade), were purchased from Merck Chemicals, India**.** Reference standards Lopinavir and Ritonavir were procured by Cipla Private LTD Mumbai as gift samples. Where the Marketed Formulation is taken from the Pharma Store. Hydrochloric acid, sodium hydroxide, potassium dihydrogen orthophosphate, o-phosphoric acid, tetra butyl ammonium hydroxide, and 30% hydrogen peroxide were all analytical-grade chemicals that were bought from S D Fine Chem. Ltd. (Mumbai, India). In 1 L of HPLC grade water, dissolve 1360 mg (10 mm) of potassium dihydrogen orthophosphate and 3330 mg (10 mM) of TBAH.

2.1.2. HPLC instrumentation and chromatographic procedure

The mobile phase comprised of methanol-potassium dihydrogen orthophosphate (With pH 3.8; 10 mM, tetra butyl ammonium hydroxide;10 mM (40:60, v/v) at a flow rate of 1.5 ml/min to achieve chromatographic separation of Lopinavir using a Lichrospher RP C18 (250mm 4.6 mm, 5 mm) sonicated for 15 minutes before being filtered through a 0.22 m nylon membrane filter. The ideal wavelength for quantification was 254 nm, and the injection volume was 20 µl. The internal diameter of the photostability chamber model CS-90 (GMP) is 50x40x85 cm.

Expert Design Software (Version 13.5 Trial) - RSM study type response surface optimization.

2.1.3. Preparation of standard solutions

A standard Lopinavir stock solution in methanol was created with a 100mg ml^{-1} concentration. Working standard solutions were made freshly before using a 10 mg ml^{-1} dilution of the mobile phase to achieve appropriate concentration levels.

2.1.4. Construction of the calibration curve

The mobile phase was used to dilute the working standard stock solution to create calibration samples with concentrations between 0.5 and 100 mg ml^{-1}. For each calibration sample, 20 µl injections were produced in triplicate and chromatographed using the predetermined HPLC conditions. The calibration curve was obtained by plotting peak regions against the corresponding concentration.

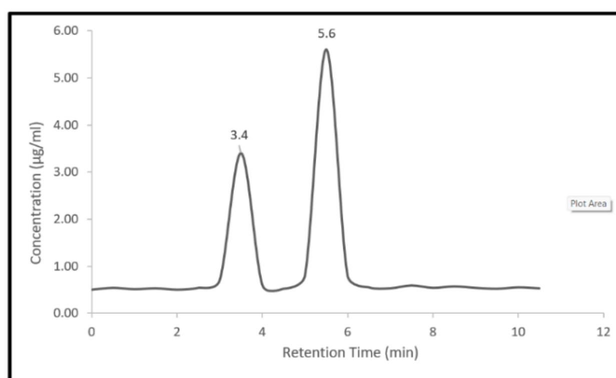

Figure 2. Typical chromatogram of (RT 5.6 min)

2.2. Forced degradation of Lopinavir

Forced degradation studies (FDS) were used to ensure the specificity and applicability of the proposed analytical approach. The current stress testing involves an analysis of the impact of oxidizing and reducing agents and other environmental factors on the chemical behavior of Lopinavir.

2.2.1. Hydrolytic degradation

Hydrolytic degradation is a frequent chemical process that occurs over a wide pH range.

Lopinavir was acidically hydrolysed in 2 N and 5 N HCl. The alkaline hydrolysis was carried out in 2 N NaOH and 5 N NaOH. Water was used for neutral hydrolysis. These samples were then exposed to the conditions listed in Table 1.

2.2.2.Oxidizing degradation

Under normal storage conditions, drug substances typically autoxidize. A chain reaction started by a free radical is known as autoxidation. In stress studies, hydrogen peroxide (H_2O_2) is a frequently utilized oxidant that initiates autoxidation. By adding one mL of the standard stock solution to each of the two 10 ml volumetric flasks, the oxidizing degradation process was carried out. With 3% and 10% H_2O_2 separately, the volume was brought up to the required level. These were put through the conditions listed in Table 1.

2.2.3. Thermal degradation

As temperature rises, chemical reactions typically proceed more quickly. As a result, medicinal products are typically prone to degradation at higher temperatures. Both dry heat and moist heat are used to carry out thermal degradation. Standard stock solution (1 ml) was transferred to each of two 10 mL volumetric flasks for thermal degradation testing, and the volume was filled with methanol. Both of these flasks were treated to the conditions listed in Table 1.

2.2.4. Photolytic degradation

Photolytic degradation products may be created when drug compounds are exposed to light. Photodegradation was performed in this study by transferring a standard stock solution (1 ml) to a 10 mL volumetric flask. The volume was diluted properly with methanol and treated to the conditions listed in Table 1.

Table 1. Hydrolytic, oxidative, thermal and photolytic stress testing conditions for drugs

Stress condition	**solvents**	**Temperature ^{0}C**	**Time (days)**	**Sampling time (days)**
Hydrolytic	H_2O	60	25	1,3,5,9,11,17,21
Neutral	2 N HCL	60	30	1,3,5,9,11,17,21
Acidic	5 N HCL	60	30	1,3,5,9,11,17,21
Basic	2N NaOH 5N NaOH	60	30	1,3,5,9,11,17,21
Oxidizing	3% H_2O_2	60	30	1,3,5,9,11,17,21
thermal	10% H_2O_2	Room temp.	10	1,3,9,21
Moist heat	Methanol	Room temp.	10	1,3,9,21
Dry heat	Methanol	60	15	1,3,5,9,11,17,21
Photolytic	Methanol	60	15	1,3,5,9,11,17,21
Direct sunlight	Methanol	-	25	1,3,5,9,11,17,21

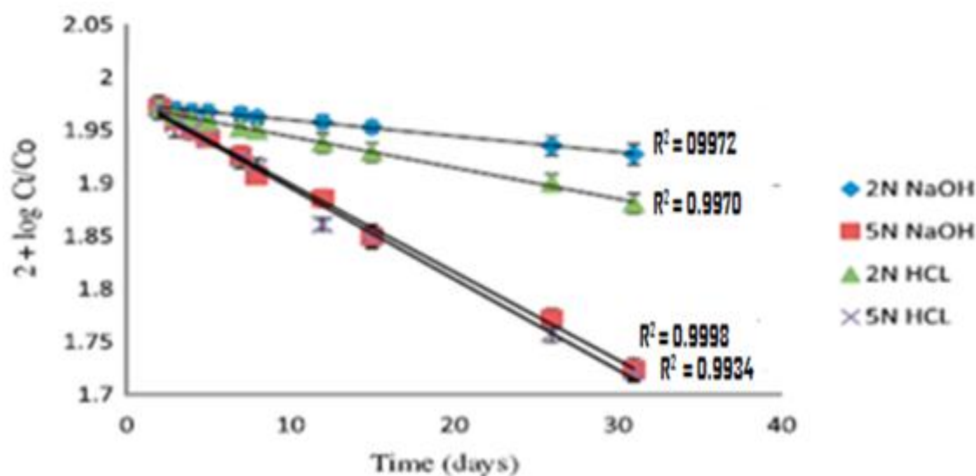

Figure 3. First-order plots for the degradation of Lopinavir under acidic and basic stress conditions (each point represents the mean ± SD, n=3)

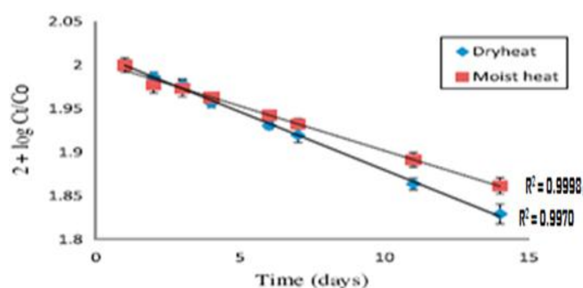

Figure 4. First-order plots for the degradation of Lopinavir under thermal stress conditions (each point represents the mean ± SD, n=3)

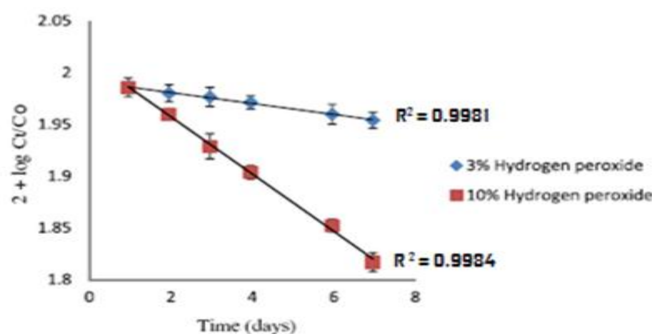

Figure 5. First-order plots for the degradation of Lopinavir under oxidative stress conditions (each point represents the mean ± SD, n=3)

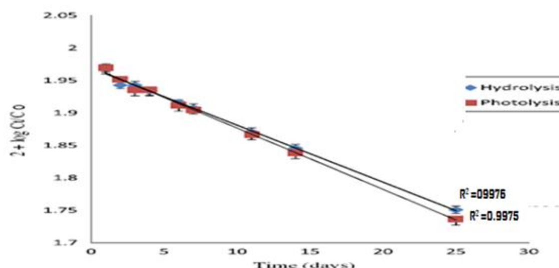

Figure 6. First-order plots for the degradation of Lopinavir under hydrolysis and photolytic stress conditions (each point represents the mean ± SD, n=3)

2.3 Sample collection

The volume was adjusted using the appropriate solvent before collecting samples. Table 1 shows where the sample (200 µl) was taken. Acid and base-induced degrading samples were neutralized with 200 µl of suitable strength NaOH and HCl. All samples were kept in the refrigerator at 2 to 8 °C. Samples were injected three times into HPLC on the day of analysis after being diluted with the mobile phase to a maximum of 10 mL, filtered using a 0.22 µm membrane syringe filter, and purified.

3. RESULTS AND DISCUSSION

3.1. Method development and optimization

The objective of the current QbD work was to analyze the effectiveness of the method and the impact of various variables on method responsiveness. The ideal wavelength of 250 nm was selected to reduce baseline noise at the absorption maximum of lopinavir (208 nm). Methanol was selected as an anionic phase due to the solubility of Lopinavir. First, a mobile phase with a varied content of methanol (80–20% v/v) and water was evaluated on reversed-phase analytical columns (C18 and C8). With a flow rate of 1.5 ml min-1, water was then changed with buffer (10 mm potassium dihydrogen orthophosphate) at various pH levels between 2.5 and 6.0 as shown in Fig 2. Lopinavir failed to obtain a capacity factor (k) under the conditions applied. The retention volume was identical to the void volume because lopinavir was eluted along with the mobile phase. Based on this Lopinavir elution behavior, we used the ion pair method, using TBAH as an ion pair agent at a concentration of 10 mm. With the help of

o-phosphoric acid, the buffer's pH (10 mm potassium dihydrogen orthophosphate) was brought down to 3.2, which is more than two units below the pKa (6.2) necessary to completely ionize lopinavir. Considering the above circumstances, the mobile phase was chosen, consisting of methanol: buffer at a ratio of 30:70 eluted Lopinavir through the C18 stationary phase (Lichrospher, RP C18, 250 mm 4.6 mm, 5 l) with a k of 1.44. 20 experiments were carried out utilizing with the buffer's pH being adjusted between 2.6 and 3.2, the amount of methanol being 20% to 30% v/v, and the concentration of TBAH was adjusted between 5 and 10 mm (3 factors, 2 levels, 20 runs). This makes it easier to analyze logically how the concentration of TBAH, the pH of the buffer, and the concentration of the organic phase affect the capacity factor of lopinavir. Based on earlier univariate investigations and chromatographic intuition, the factors and ranges were chosen for consideration. Design Expert 13 Trial software was used to do a statistical analysis of the collected data.

The effects of each element on the capacity factor are shown in (Figure 3). By linear regression, both the organic phase and TBAH were significant; by quadratic regression, only the organic phase was significant and the TBAH effect was less significant. By both linear and quadratic regression, pH’s influence was insignificant.). These are excellent resources for analyzing how the various components combine to affect the capacity factor. When TBAH concentrations rise, Lopinavir's capacity factor rises as well. The capacity factor increases as TBAH concentration increases and falls when the organic phase concentration increases. The influence of pH on the capacity factor of lopinavir was examined in the pH range of 2.6-3.2, and it has no effect. Within the limits of the experiments, the created model can be utilized to predict the Lopinavir capacity factor.

The residuals are seen often as linear which indicates that the errors are regularly distributed. This demonstrates how well the model matches the data. These plots are typically necessary to verify the fitted model's normality assumption and make sure it accurately approximates the optimization procedure. There is no clear pattern in the difference between the residual and predicted response. The plot indicated that there was a nearly equal scatter above and below the X-axis, indicating that the suggested model is suitable.
A mixture of 10 mM potassium dihydrogen orthophosphate (pH 2.8) comprising 10 mm TBAH and methanol (25:75, v/v), with a flow rate of 1.0 mL min 1, were the optimal chromatographic conditions determined by the design. For Lopinavir, these chromatographic conditions led to acceptable retention (k= 2.06) as well as symmetric peak shape with a retention time of 7.05 min (Figure. 4). Lopinavir retention time was not affected by the excipients in the blank and cream formulation. The formulation yielded a recovery percentage of 100.5±1.8 (n= 6).

3.2. Solution stability

By measuring the Lopinavir standard (60 µg ml min^{-1}) at 0, 3, 6, 9, 12, and 24 h, the stability of Lopinavir in the mobile phase was investigated. There was no discernible change in the standard's peak area. As shown in Table 2.

Table 2. Stability of drug in the mobile phase

Time	Peak area (60 µg/ml of drugs)
0	15479
3	15533
6	15348
9	15533
12	15537
24	15527

Mean +/- SD. RSD (%), n = 6

3.3. Method validation

The ICH guidelines (ICH guideline Q2 (R1), 2005) validated the proposed approach. Establishing the technique's fitness for its intended purpose is the strategy for method validation. Fundamental factors such as system compatibility, linearity, limits of detection and quantification, accuracy, intra-day and inter-day precision, specificity, and robustness were used to conduct the method validation.

3.3.1 System suitability

For six replicate injections of the drug at a concentration of 60 µg ml min $^{-1}$, the retention time (Rt), capacity factor (k), the number of theoretical plates (N), and tailing factor (T) were determined.

Table 3. System suitability data

Property	Mean ± SD, n=6	RSD (%)	Required limits
Retention time (Rt)	8.41 ± 0.03	0.23	RSD≤ 2
Capacity factor (k)	2.02 ± 0.003	0.21	-
Theoretical plates (N)	31466 ±179	0.34	N ≥ 2000
Tailing factor (T)	1.26 ± 0.01	1.46	T ≤ 2

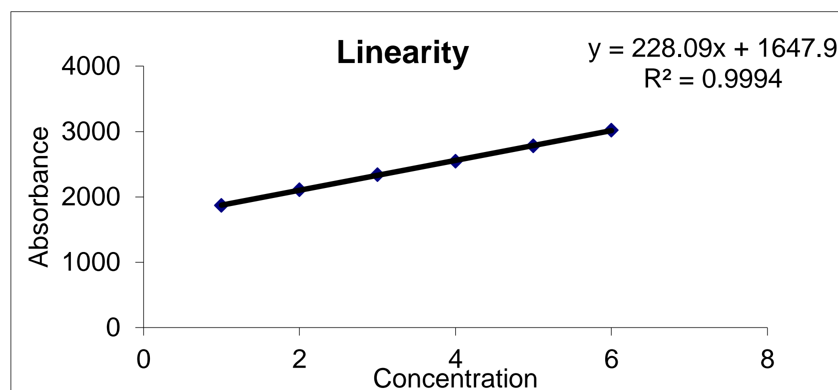

Figure 7. Standard Calibration Curve for Lopinavir

3.3.2 Linearity

The proposed method's linearity was demonstrated throughout a concentration range of 1-10 µg ml min^{-1}, with a coefficient of determination of $r^2 = 0.9994$. The regression equation was determined to be Y= 228.09x + 1647.9, where Y is the absorbance and X is the Lopinavir concentration (g ml^{-1}).

3.3.3 Limits of Detection and Quantification

The limit of detection (LOD) was determined as the lowest concentration of Lopinavir with a signal-to-noise ratio of 3:1, and the limit of quantification (LOQ) as a signal-to-noise ratio of 10:1. Because of the variation in detector response, concentrations ranging from 0.01 to 2 µg ml $^{-1}$ were prepared and evaluated. The obtained LOD and LOQ were 0.78 and 2.37 µg ml $^{-1}$, respectively.

Table 4. Result of Recovery data

Sr.no.	Level (%)	Amt Taken (µg/ml)	Amt added (µg/ml)	Abs Mean±SD	Amt recovered Mean±SD	% Amt recovered Mean±SD
1	80	60	48	29399±0.01	48.26±0.30	100.53±0.15
2	100	60	60	29183±0.00	60.55±0.14	100.91±0.11
3	120	60	72	31885±0.00	72.39±0.14	100.54±0.03

3.3.4. Analysis of Marketed Formulation.

The average weighed amount of tablet powder containing 200 mg of Lopinavir was transferred to a 100 ml volumetric flask and dissolved using a sonicator in a solution of Methanol.

Then, Whatman filter paper no. 41 was used to filter the solution.

On the basis of the label claim, a filtrate solution was further diluted with Methanol.

The final concentration of the stock solution was 1000µg/ml, then (40-90µg/ml) further diluted with Methanol.

Table 5. Result of Label Claim.

Sample	Labeled Claim	% Labelled Claim ± SD	%RSD
Lopinavir	Kaletra 200mg	100.18±0.06	0.18

3.3.5. Accuracy

The method's accuracy was tested in triplicate at 3 distinct concentration levels (80%, 100%, and 120%), and the percentage recovery was calculated. The degraded sample mixture was fortified with known amounts of the drug at 80%, 100%, and 120%. The standard's peak area was calculated as the difference in peak area between fortified and unfortified samples. Three replicate samples were generated for each concentration level, and the % recovery at each level (n = 3) was calculated (Table 4). The recovery standard for Lopinavir is 100% at all three levels, showing the correctness of the recommended method.

3.3.6. Intra-day and inter-day precision

Four different concentrations of Lopinavir (60,70 and 80 μg ml^{-1}) were injected to assess the analysis of within- and between-day variance. Six sets of the four concentrations, each with six replicates, were examined on the same day to evaluate intra-day precision. Data from six replicates that were examined on six distinct days allowed for the calculation of the intraday variation. According to Table 6, the method's accuracy was indicated by the intra-day and inter-day coefficient of variation and the percentage error is less than 2%.

Table 6. Results of intra-day and inter-day precision

Conc	Intraday			Interday		
	Mean ± SD	Amt Found	% Amt Found	Mean ± SD	Amt Found	% Amt Found
60	15341.6±0.00	60.04	100.06	15335 ±0.00	60.01	100.01
70	17398±0.01	69.05	98.65	17553 ±0.01	69.73	99.62
80	20087.3±0.01	80.84	101.05	19793 ±0.01	79.55	99.44

3.3.7. Specificity

The method's specificity is demonstrated by isolating the target analyte even when other sample matrix components are present. The complete separation of Lopinavir in the presence of degradants, illustrating the specificity of the proposed HPLC method. With six replicates, an average Rt standard deviation of 7.05 ±0.04 min was reported for Lopinavir. The obtained peaks were sharp and had a clear baseline separation.

3.3.8. Robustness

Small adjustments were purposefully made, such as varying the C18 columns from different manufacturers, the Flow Rate, temperature, and the percentage of organic solvent in the mobile phase, to test the robustness of the present process. Two analytical columns, Lichrospher C18 column (Germany) and Atlantis C 18 column (USA) were used to test the method's robustness. To estimate the impact, each of the three elements under consideration (temperature, flow rate, and Organic Solvent %) was altered one at a time. Six replicate injections of the same standard solution (60 µg ml^{-1}) were carried out with minor chromatographic parameters (factors). The mobile phase's organic concentration was adjusted by 25 ±1% (v/v), the flow rate by 1.5±0.1 mL min^{-1}, and the temperature by 55 ±5. The observed results are shown in Table 7, which shows that even minor changes to these parameters had no impact on the outcomes. The results from the two columns showed that there is no significant difference between the two results.

Table 7. Results for the analysis of robustness

Factors	level	Retention Mean ± SD (n=3)	Time (min) Mean ± SD (n=3)	Peak area Mean ± SD (n=3)
A Flow rate (ml/min)				
1.4	-1	5.60 ± 0.04	1.65 ± 0.03	15397.16 ±,1.22
1.5	0	5.61 ± 0.04	1.66 ± 0.04	15398.36 ±,1.19
1.6	+1	5.62 ± 0.04	1.67 ± 0.07	15396.87 ±,1.23
mean		5.61 ± 0.04	1.69 ± 0.02	15397.67 ±,1.21
B Organic Solvent (v/v)				
24	-1	5.62 ± 0.04	1.32 ± 0.03	15397.67 ±,1.21
25	0	5.61 ± 0.04	1.31 ± 0.01	15396.16 ±,1.22
26	+1	5.62 ± 0.04	1.31 ± 0.01	15397.50 ±,1.20
mean		5.61 ± 0.04	1.31 ± 0.02	15397.67±,1.21
C Temperature (°c)				
50	-1	5.60 ± 0.04	1.27 ± 0.03	15397.16 ±,1.24

55	0	5.61 ± 0.04	1.24 ± 0.02	15397.86 ±,1.22
60	+1	5.60 ± 0.04	1.26 ± 0.01	15398.16 ±,1.19
Mean		5.60 ± 0.04	1.25 ± 0.02	15397.67 ±,1.21

3.4. Stability-indicating property

When an analytical method can separate all process-related impurities and degradation products, it indicates stability. Lopinavir model chromatograms under acidic and oxidative stress conditions. Lopinavir exhibits the same degradant peaks under acidic and basic stress conditions, with 2.8, 4.1, and 7.9-minute retention times. Two degradant peaks were seen in stress samples subjected to dry heat, moist heat, water hydrolysis, and photolysis at 2.8 and 4.1 minutes. Lopinavir displayed two degradant peaks under oxidative degradation at 2.8 and 6.0 min; the peak seen at 3.1 min is blank. This indicates that the drug is hydrolytic (acid, base, and water), oxidative, thermal, and photolytically degradable. The degradant peaks did not interfere with the Lopinavir peak in any of the reported cases, indicating that the approach enabled specific identification of Lopinavir in the presence of its degradation products. Scheme 1 describes the suggested Lopinavir degradation under stress conditions. 2,4-dichloro-10,11-dihydro-5H-dibenzo[a,d] In hydrolytic/thermal/photolytic stress conditions, cyclohexane-5-of (I) and imidazole (III) may be the primary breakdown products; 2,4-dichloro-10,11-dihydro-5H-dibenzo [a,d] In oxidative stress conditions, the degradation products could be cyclohexane-5-one (II) and imidazole (III).

2 + log C^t/C^o Time (days)

3.5.1 Quality Target Product Profile

It is a prospective overview of a drug product's quality characteristics that also take into consideration factors affecting method performance.

3.5 Quality by Design Approach Application

The main qualities of a drug product that have an impact on how effectively a method works are dose form, colour, and impurity percentage.

Table 8. Critical method parameters

Critical method parameters	Condition
HPLC instrument	Control
Column	Control
Detector	Control
API standard	Control
Glasswares	Control
The pH of the mobile phase	Control
Organic Solvent	Variable X1
Flow rate of Mobile phase	Variable X2
Temperature of column	Variable X3

Table 9. Experimental Factors and levels used in experimental design

Factor	Level (-1)	Level (0)	Level (+1)
Temperature	50 °c	55 °c	60 °c
Organic Solvent(ml)	24	25	26
Flow(ml/min)	1.4ml/min	1.5ml/min	1.6ml/min

3.5.2 Risk assessment

The parameters that affect the quality target profile are the critical method variables. It is a systematic process for evaluating, managing, communicating, and reviewing quality risk throughout the product lifecycle. It is usually carried out and done throughout the life cycle. Table 8 lists the risk factors for the lopinavir technique based on theoretical and practical information.

3.5.3 Design of experiment

The critical analytical parameters were chosen based on the risk assessment of organic concentration, flow rate, and column temperature. The response chosen was drug retention time, which frequently

coelutes and leads to method failure. 2^3 factorial designs were chosen as preferred in the response surface method. Table 9 shows the technique chosen for responses and their levels. A central composite design was chosen, and 20 chromatogram runs were carried out by the DoE design. Table 10 summarises the method responses for each run.

Table 10. Coded values for factorial level and response in central composite design for 20 analytical trials.

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	38.76	9	4.31	44.59	< 0.0001	significant
A-temperature	0.8173	1	0.8173	8.46	0.0156	
B-organic Solvent	32.95	1	32.95	341.14	< 0.0001	
C-flow	0.6751	1	0.6751	6.99	0.0246	
AB	0.1013	1	0.1013	1.05	0.0300	
AC	0.1013	1	0.1013	1.05	0.1300	
BC	1.05	1	1.05	10.88	0.0080	
A^2	0.6602	1	0.6602	6.83	0.0258	
B^2	2.62	1	2.62	27.10	0.0004	
C^2	0.1175	1	0.1175	1.22	0.2959	
Residual	0.9659	10	0.1966			Non-significant
Lack of Fit	0.9659	5	0.1932			
Pure Error	0.0000	5	0.0000			
Cor Total	39.73	19				

3.5.4 Statistical Analysis of Method Response

ANOVA for the method response retention time

The anticipated response surface quadratic model for retention time's ANOVA of regression parameters was generated using DoE software and is shown in Table 11. The Model F-value of 44.59 implies the model is significant. There is only a 0.01% chance that an F-value this large could occur due to noise. P-values <0.0500 indicate model terms are significant. A, B, C, AB, BC, A^2 and B^2 are significant model terms in this case. Values >0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model. This model can be used to navigate the design space.

Based on the results, the response surface model developed in this study for predicting retention time was deemed reasonable. The full regression model and its coded factors are shown in the equation below.

Table no 11. ANOVA for Quadratic model Response: retention time

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	38.76	9	4.31	44.59	< 0.0001	significant
A-temperature	0.8173	1	0.8173	8.46	0.0156	
B-organic Solvent	32.95	1	32.95	341.14	< 0.0001	
C-flow	0.6751	1	0.6751	6.99	0.0246	
AB	0.1013	1	0.1013	1.05	0.0300	
AC	0.1013	1	0.1013	1.05	0.1300	
BC	1.05	1	1.05	10.88	0.0080	
A^2	0.6602	1	0.6602	6.83	0.0258	
B^2	2.62	1	2.62	27.10	0.0004	
C^2	0.1175	1	0.1175	1.22	0.2959	
Residual	0.9659	10	0.1966			Non-significant
Lack of Fit	0.9659	5	0.1932			

Pure Error	0.0000	5	0.0000			
Cor Total	39.73	19				

R^2=5.61-0.2426A-1.55B-0.2223C-0.1125A*B-0.1125A*C+0.3625B*C-0.2140A^2-4262B^2-0.0903E^{-003}*C^2

Polynomial equations can be utilized for predicting the behavior of retention time and process variables using the equation in terms of actual factor

Actual Equation

R^2=-175.65960+1.79284*temperature+15.55512*organicsolvent-53.38539* flow-0.022500*temperature *organic solvent -0.225000*temperature*flow+3.62500*organic solvent*flow-0.008561*temprature^2-0.426169 *organicsolvent^2-9.02931*flow^2

Table 12**. ANOVA Summary**

Std. Dev.	Mean	R^2	Adjusted R^2	Predicted R^2	C.V. %	Adeq Precision
0.3108	5.11	**0.9907**	0.9878	0.8148	6.08	23.8666

3.5.5 3D and contour graph for retention time

The Design-Expert program presented the 3D surface responses and contour plots of the quadratic model, which gives the interactive relationship of two parameters on the response by maintaining the third element constant. According to the contour and 3D surface response in Fig. 8, the greatest retention time was only achieved below 8 minutes by maintaining an organic Solvent of 23.31 ml and flow rate of 1.55 ml/min while maintaining a constant column temperature of 55°C.The drug's retention duration was eluted in more than 6 minutes which is not advised. As illustrated in Fig. 9, the retention time was investigated using a constant flow rate and the interaction of organic solvent and column temperature. It demonstrates that retention time increases to > 6 minutes at low organic solvent and temperature levels. The retention time was 2.6 minutes at high levels of variables

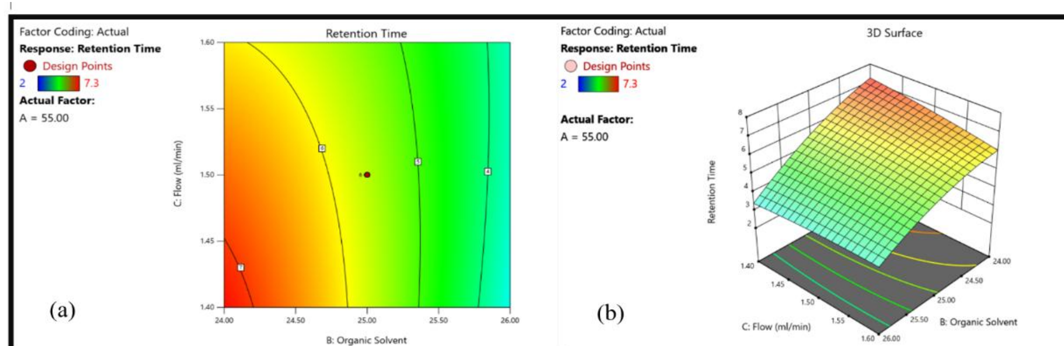

Fig 8. Contour plots (a) and 3D Response Surface (b) for retention time as a function of Organic Solvent and Flow ml/min (Constant Temperature - 55 ºC)

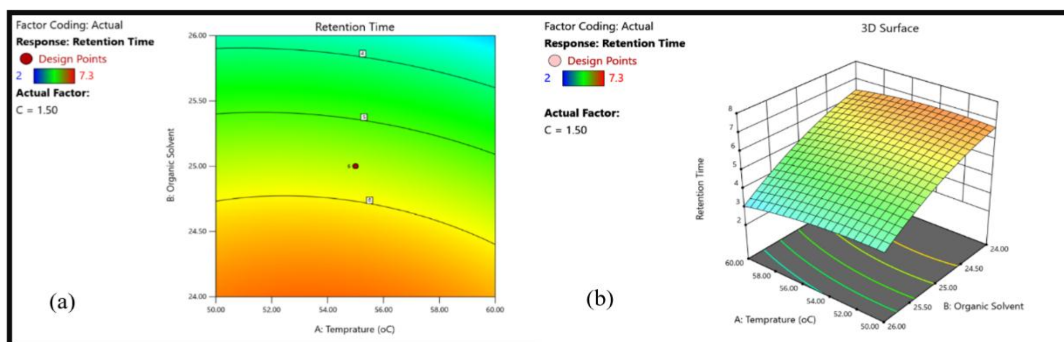

Fig 9.Contour plots (a) and 3D Response Surface(b) for retention time as a function of Temperature and Organic Solvent (Flow -1.50ml/min)

The interaction between the flow rate and the column temperature was investigated Where the organic solvent was maintained at 25. These two factors have the smallest impact on the drug's retention time. The retention period of lopinavir is least affected by raising these two parameters from low to high levels. As a result, these two-term interactions in this model were not significant.

3.5.6 Design Space

A suitable method performance, such as retention time of the technique within the space, can be provided by the design space.

By keeping one variable constant and properly understanding how the two elements interact to affect method performance, technique failure has been identified. The design space for the method that can yield appropriate system suitability parameter values as shown in Table 13 was determined by carefully observing the contour and 3D diagram.

3.5.7 Method optimization through DoE software

The accuracy of the model was tested using the Design-Expert software's numerical optimization method. The desired objectives were set as in range for method response, retention time (2-7.5). 30 possibilities were proposed, but the software chose only one based on desirability 1.0. The experiment was run under the ideal criteria specified by Design Expert.

The Design-Expert recommended chromatographic conditions where the Organic solvent is 25 ml, 1.50 ml/min flow rate, and 55°C for the column temperature. The model projected method responses of 5.61 min for retention time, shown in Fig. 10.

The HPLC system was used under the same experimental conditions. Lopinavir chromatogram technique results were nearly closer to the predicted values. Table 14 provides the verification data for model-predicted values and observed values.

The correlation between predicted and observed values was determined to be 1. As a result, the model successfully predicts the method response within a 95% confidence interval of low and high. The proposed optimized approach was validated using ICH parameters.

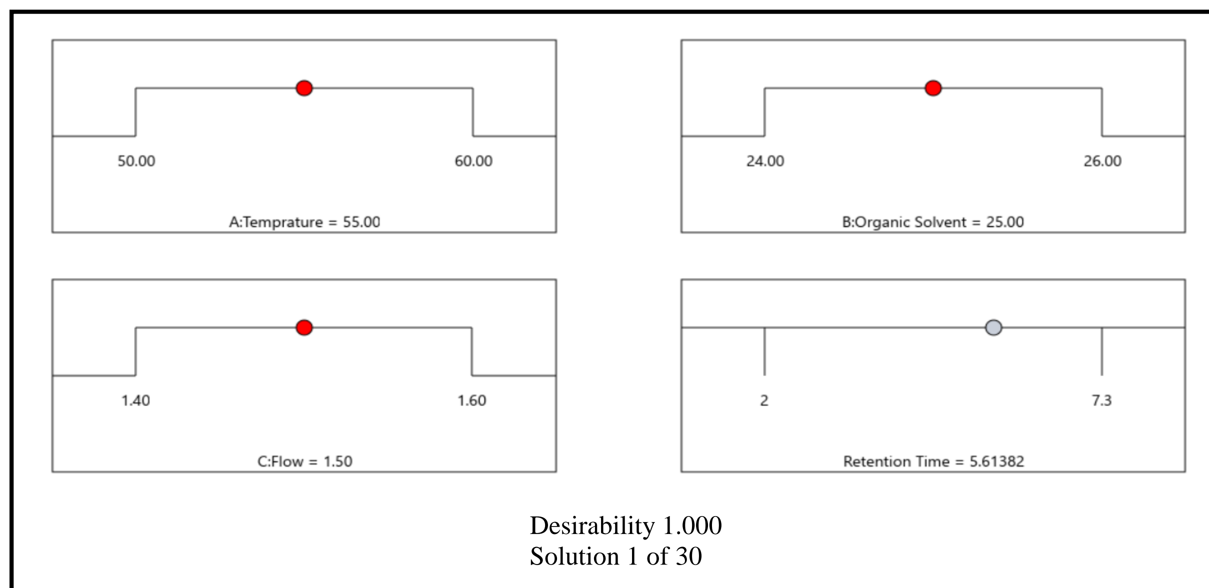

Fig. 10: Optimization and prediction of method responses by model

Table 13: Method operable design region

Constant factor	Variable range for method response		
Y1	**Factor A (Temperature)**	**Factor B (Organic Solvent)**	**Factor C (Flow)**
Temperature	Constant	50-60	1.4-1.6
Organic Solvent	24-26	Constant	1.4-1.6
Flow	24-26	50-60	Constant

Table 14: Verification of experiment at optimum conditions

Analysis	**Predicted**	**95% CI low**	**95% CI high**	**Observed**
Retention time	5.613	5.33	5.90	5.6

4. DISCUSSION

For determining the concentration of lopinavir in bulk drugs by QbD approach, a unique, selective, robust stability-indicating RP-HPLC method was developed the mobile phase comprised of methanol-potassium dihydrogen orthophosphate (With pH 3.8; 10 mM, tetra butyl ammonium hydroxide;10 mM (40:60, v/v) at a flow rate of 1.55 ml/min to achieve chromatographic separation of Lopinavir at 250 nm. The drug was put under a variety of stresses, including thermal energy, peroxide, and hydrolysis (in acid, alkali, and neutral solutions). The chromatogram exhibits a greater number of critical impurity peaks under peroxide stress conditions, which has an impact on the method's selectivity. The design experiment was used to find a solution. Utilising three components as input variables for the response surface method's central composite design are the Column Temperature, Organic solvent, and flow rate. The retention time was the output variable. The statistical tools were used to determine the significance of each factor's impact on the method responses. The retention time was calculated from the polynomial equation. Design-Expert recommended the following chromatographic parameters: Organic Solvent is 25 ml, rate of flow is 1.55 ml/min, and the temperature of the column at 55ºC. Lopinavir's retention time was estimated by the model to be 5.61 min. By carrying out the previously mentioned chromatographic conditions, the method was verified. The retention time was 4 minutes under this condition. The proposed framework can predict the response variables with a 95% confidence interval. According to ICH Q2 (R1) guidelines, the optimized procedure was validated.

5. CONCLUSIONS

With Organic Concentration, flow rate, and column temperature as the method variables, a quadratic model of a central composite design with a high degree of correlation and predicting ability was developed for predicting the retention time of Lopinavir. RSM was used to analyse how all the factors interacted. The organic Concentration showed an impact on retention time. By using the model's recommended chromatographic condition, the model's accuracy was evaluated. The predicted value of retention time by the design was nearly matched with the actual value. The method has been optimized and validated in accordance with ICH guidelines.

6. REFERENCES

1. Yoshiko Usami, Tsuyoshi Oki, 2003. A Simple HPLC Method for Simultaneous Determination of Lopinavir, Ritonavir and Efavirenz, Chem. Pharm. Bull. 51(6) 715-718, DOI-10.1248/cpb.51.715
2. Chaolong Qin, Wanshan Feng, Development and validation of a cost-effective and sensitive bioanalytical HPLC-UV method for determination of lopinavir in rat and human plasma, 2020, DOI-10.1002/bmc.4934
3. A. Sunitha, S. kathirvel and G. ramachandrika, 2011. A Validated RP HPLC method for simultaneous estimation of Lopinavir and ritonavir in combined dosage form, International Journal of Pharmacy and Pharmaceutical Sciences, Vol 3, Issue 1.
4. Shivanand N. Hiremath, and Charushila H. Bhirud, 2015. Development and validation of a stability-indicating HPLC method for the simultaneous analysis of Lopinavir and ritonavir in fixed-dose combination tablets, Journal of Taibah University Medical Sciences, 1-7, DOI-10.1016/j.jtumed.2014.11.006
5. Rishikesan Rathnasamy, Ranjith Pakkath Karuvalam, Etal, 2018. RP-HPLC Method Development and Method Validation of Lopinavir and Ritonavir in Pharmaceutical Dosage Form, American Journal of Clinical Microbiology and Antimicrobials, Article 1002 Volume 1, Issue 1.
6. S. Mohan Varma, R. Vijaya Lakshmi and MD. Dhanaraju, 2012. Development and Validation of an RP-HPLC method for determination of Lopinavir in bulk and pharmaceutical dosage form, IJRPC, Volume 2, Issue 2, 413-417
7. N. Sunitha, Subash C Marihal, 2015. Method Development and Validation of Stability Indicating RP-HPLC Method for Simultaneous Estimation of Ritonavir and Lopinavir in Pure and pharmaceutical dosage form, International Journal of Advanced Research, Volume 3, Issue 5, 649-657
8. R.B. Mardia, B.N. Suhagia, T.Y. Pasha, etal ,2014. RP-HPLC Method for Simultaneous

Estimation of Lopinavir and Ritonavir in Combined Tablet Dosage Form and Spiked Human Plasma, IJPSR, Vol. 5, Issue 8, 3443-3454. DOI- 10.13040/IJPSR.0975-8232.5(8).3443-54

9. Sunkara Namratha, Vijayalakshmi A, 2018. Method Development and Validation of Lopinavir in Tablet Dosage Form Using Reversed-Phase High-Performance Liquid Chromatography, Asian J Pharm Clin Res, Vol 11, Special Issue 4, 1-4, DOI-10.22159/ajpcr.2018.v11s4.31715
10. A.V. Sulebhavikar, U. D. Pawar, 2008. HPTLC Method for Simultaneous Determination of Lopinavir and Ritonavir in Capsule Dosage Form, E-Journal of Chemistry, Vol. 5, No.4, pp. 706-712, 1, DOI-0.1155/2008/539849
11. A.Rajitha, V.Divya Vani, 2022. Development and Validation of a New Stability Indicating Rp Hplc Method for Simultaneous Estimation of Ritonavir and Lopinavir, International Journal of Pharmaceutical Research and Applications Volume 7, Issue 3, pp: 486-492
12. Vegesna Swetha, S. V. U. M. Prasad, 2017. Stability Indicating Method Development and Validation for Simultaneous Estimation of Lopinavir and Ritonavir by Using RP HPLC, European Journal of Pharmaceutical and Medical Research, 4(11), 516-520.
13. Khagga Bhavyasri, V. Murali Balaram, et al, 2015. Development and Validation of Forced Degradation Studies of Lopinavir Using RP-HPLC and Characterization of Degradants by LC-MS/MS, Int. J. Chem. Sci.: 13(1), 551-562
14. International conference on harmonization,2003. Stability testing of new drug substances and products Q1A (R2) (Accessed 28.12.2016)
15. Blessy M, Patel RD, Prajapati PN, Agarwal YK , 2013. Development of forced degradation and stability indicating studies of drugs-a review. *Pharm.Anal.*4:159-165.
16. International Conference on harmonization, 2009.Pharmaceutical development Q8 (R2). (Accessed 28.12.2016)
17. Sangshetti J N, Deshpande M, Zaheer Z, Shinde D. B, Arote R, 2014. Quality by design approach: Regulatory need. *Arab.J.Chem*. 211:11–18
18. Pramod K, Tahir MB, Charoo N A, Ansari SH, Ali. J, 2016. Pharmaceutical product development: A quality by design approach. *Int.J. Pharm.Investig*.6:129-138.