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Analytical Quality by Design (A-QbD) Driven RP-HPLC Method for the Development and Validation of Analytical Methods for Ribavirin, Lopinavir, and Ritonavir

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ABSTRACT

Analytical Quality by Design (A-QbD) provides a systematic, science-based, and risk-managed framework for developing robust analytical methods. This study aims to apply A-QbD principles to establish optimized and validated analytical procedures for Ribavirin, Lopinavir, and Ritonavir—drugs widely used in antiviral therapy. Critical Analytical Attributes (CAAs), Critical Method Parameters (CMPs), and Critical Quality Attributes (CQAs) were identified through risk assessment and design of experiments (DoE). Method operable design ranges (MODRs) were established to ensure method robustness and lifecycle sustainability. The results demonstrate that A-QbD-driven method development improves accuracy, precision, linearity, robustness, and overall analytical reliability. The study confirms that A-QbD is essential for modern pharmaceutical analysis, ensuring regulatory compliance and product quality consistency. Successful separation of Ribavirin, Ritonavir and Lopinavir was achieved on Cromosil C18 column (5 µm 250 × 4 mm) with isocratic elution of Methanol:0.1 % Orthophosphoric acid 84:16 (v/v) as a mobile phase. The Ultraviolet detection was monitored at a wavelength of 223 nm at flow rate 0.8 mL/min. The proposed method is found to have linearity in the 40–200 µg/mL for Ribavirin and Lopinavir then 10–50 µg/mL for Ritonavir with correlation coefficients of not less than 0.999 respectively. All method validation criteria were within the range of acceptance. Relative standard deviation (%RSD) was observed to be <2% for inter- and intra-day precision. Besides, the recovery rate was observed close to 100% for both the drugs confirming the accuracy of the method. Minor alterations in the chromatographic conditions have revealed robustness and ruggedness of the developed method.

1. Introduction:

Analytical Quality by Design (A-QbD) is an extension of pharmaceutical QbD principles to analytical methods. It emphasizes method understanding, risk assessment, design space establishment, and continuous improvement. The growing complexity of antiviral drug formulations, especially fixed-dose combinations containing Lopinavir and Ritonavir, necessitates robust analytical methods. Ribavirin—another antiviral agent—also requires precise quantification due to its narrow therapeutic index. Traditional analytical development relies on empirical trial-and-error, often resulting in non-robust methods. A-QbD rectifies this by applying systematic science, risk-based decisions, and statistical optimization. This study

applies A-QbD for method development of Ribavirin, Lopinavir, and Ritonavir, widely used in COVID-19 and other viral infections.

2. Materials and Methods:

2.1 Materials:

Reagents:

Ribavirin, Lopinavir, Ritonavir (pharmaceutical-grade) The HPLC-grade solvents used in this study were obtained from Merck Ltd. in Bangalore, India, including acetonitrile, methanol, Perchloric acid and water. All of the chemicals used were of the highest quality for HPLC.

Instrumentation:

HPLC system with UV detector, C18 column (250 mm × 4.6 mm)

Instrument	**:**	**HPLC**
Column	:	C18, 250 X 4.6 mm, 5 µm, (COSMOSIL is suitable)
Injection volume	:	20 µL
Wavelength	:	UV 223 nm
Column oven temperature	:	25°C
Sample temperature	:	25°C
Needle wash	:	Mixture of Acetonitrile and Water in the ratio of 90:10
Seal wash	:	Mixture of Acetonitrile and Water in the ratio of 10:90

Methods:

Method optimization was carried out using a Box–Behnken statistical design incorporating three critical material attributes (CMPs): mobile phase composition, flow rate, and Wave length, each evaluated at two levels. A total of 17 experimental runs, including five center points, were generated. Flow rate was investigated at 0.7, 0.8, and 0.9 mL/min; wavelength at 222, and 224; and mobile phase concentration at 83%, 84%, and 85%. Retention time (Rt) and peak area were selected as the critical analytical attributes (CAAs). The experimental data were analysed and the model validated using Design-Expert software. The quadratic model demonstrated a strong correlation with the experimental results, enabling effective navigation of the design space. The predicted values showed good agreement with the experimental data, confirming that the optimized conditions fell within the defined design space.

Preparation of Standard Stock Solution:

Weigh and transfer accurately about 100 mg of Ribavirin, 25 mg of Ritonavir and 100 mg of Lopinavir working standard to a 100 mL volumetric flask. Add about 10 mL of methanol, sonicate to dissolve and dilute to volume with methanol.

Preparation of Standard Solution:

Pipette out 3.0 mL of Standard Stock Solution transfer it into 25 mL volumetric flask and dilute to volume with mobile phase and mix well. (Theoretical Concentration: 120 ppm, 30 ppm and 120 ppm of Ribavirin, Ritonavir and Lopinavir)

Preparation of Sample solution:

Weigh and crush 20 tablets to fine powder. Accurately weigh and transfer crushed powder equivalent to 1000mg of Ribavirin, 250mg of Ritonavir and 1000mg of Lopinavir, into 500 mL volumetric flask. Add 300 mL of mobile phase, sonicate for 30 minutes with intermittent shaking. Allow to cool at room temperature and dilute with diluent to volume and mix. Filter the solution through 0.45µm Teflon filter discarding first few mL of filtrate. Pipette out 3 mL of filtrate to 50 mL volumetric flask, dilute with mobile phase to volume and mix.

2.2 A-QbD Workflow:

The analytical development followed a structured A-QbD approach:

2.2.1 Defining Analytical Target Profile (ATP)

The ATP specified:

- Accurate and precise quantification of Ribavirin, Lopinavir, and Ritonavir
- LOQ < 0.5 µg/mL
- RSD precision < 2%
- Resolution > 2.0 for all peaks

2.2.2 Identification of CQAs:

Critical Quality Attributes included:

- Peak purity
- Retention time
- Theoretical plates
- Resolution
- Tailing factor

2.2.3 Risk Assessment:

Using Ishikawa diagrams and FMEA, the following CMPs were identified:

- Mobile phase composition
- Flow rate
- Column temperature
- Detection wavelength

2.3 Experimental Design (DoE)

A **3-factor, 3-level Box–Behnken DoE** was applied.

Factors studied:

Box Behnken Design

Table 1: Parameter to be changed for QBD Trials

	Name	Units	Low	High
A [Numeric]	MeOH conc.	%	83	85
B [Numeric]	Flow rate	ml/min	0.7	0.9
C [Numeric]	Wavelength	nm	222	224

Table 2: QBD trials for Ribavirin

		Factor 1	Factor 2	Factor 3	Response 1	Response 2	Response 3	Response 4	Response 5
Std	Run	A:MeOH conc.	B:Flow rate	C:Wavelength	RT	PA	TP	TF	Resolution
		%	ml/min	nm					
2	1	85	0.7	223	3.73	4381.5	4294	0.82	-
7	2	83	0.8	224	3.018	3521.7	5015	0.84	-
15	3	84	0.8	223	3.024	3915.4	5035	0.89	
6	4	85	0.8	222	3.040	4148.6	6022	0.82	-
14	5	84	0.8	223	3.062	3966.04	5402	0.91	-
11	6	84	0.7	224	3.435	4160.79	5911	0.80	-

12	7	84	0.9	224	2.789	3340.36	5493	0.89	-
10	8	84	0.9	222	2.691	3701.88	5257	0.83	-
8	9	85	0.8	224	3.030	3593.95	5826	0.91	-
1	10	83	0.7	223	3.709	646.985	6117	0.81	-
9	11	84	0.7	222	3.950	5320.73	5428	0.81	-
3	12	83	0.9	223	2.933	3694.04	4894	0.85	-
17	13	84	0.8	223	3.010	3999.76	5901	0.80	-
13	14	84	0.8	223	3.010	3931.39	6115	0.82	-
4	15	85	0.9	223	3.294	3922.74	7878	0.89	-
16	16	84	0.8	223	3.011	3932.19	6345	0.82	-
5	17	83	0.8	222	3.379	4876.38	4183	0.81	-

Table 3: QBD trials for Ritonavir

		Factor 1	Factor 2	Factor 3	Response 1	Response 2	Response 3	Response 4	Response 5
Std	Run	A:MeOH conc.	B:Flow rate	C:Wavelength	RT	PA	TP	TF	Resolution
		%	ml/min	nm					
2	1	85	0.7	223	7.89	2071.00	12198	0.88	16.14
7	2	83	0.8	224	6.577	1703.12	7637	0.84	5.82
15	3	84	0.8	223	6.225	1921.06	7584	0.89	6.74
6	4	85	0.8	222	5.970	2143.24	7110	0.82	1.37
14	5	84	0.8	223	6.374	1929.34	7511	0.86	1.43
11	6	84	0.7	224	7.001	2006.47	8126	0.85	5.68
12	7	84	0.9	224	5.920	1687.52	6729	0.82	1.46
10	8	84	0.9	222	5.524	1859.90	7157	0.89	1.42
8	9	85	0.8	224	5.862	1734.24	7892	0.83	1.37
1	10	83	0.7	223	8.618	155.236	8079	0.83	1.11

9	11	84	0.7	222	9.115	2531.64	10093	0.87	1.11
3	12	83	0.9	223	6.925	1871.74	9477	0.92	1.57
17	13	84	0.8	223	6.140	1898.29	7823	0.87	4.90
13	14	84	0.8	223	6.142	1901.06	7676	0.87	5.34
4	15	85	0.9	223	6.807	1914.95	10424	0.86	1.08
16	16	84	0.8	223	6.148	1937.55	7691	0.88	1.41
5	17	83	0.8	222	8.071	1974.42	8482	0.84	2.39

Table 4: QBD trials for Lopinavir

		Factor 1	Factor 2	Factor 3	Response 1	Response 2	Response 3	Response 4	Response 5
Std	Run	A:MeOH conc.	B:Flow rate	C:Wavelength	RT	PA	TP	TF	Resolution
		%	ml/min	nm					
2	1	85	0.7	223	9.60	1349.921	12001	0.86	5.35
7	2	83	0.8	224	8.525	967.2552	8078	0.85	5.72
15	3	84	0.8	223	7.926	1253.462	7991	0.84	5.30
6	4	85	0.8	222	7.493	1483.727	8253	0.84	1.26
14	5	84	0.8	223	8.159	1324.083	7906	0.82	1.28
11	6	84	0.7	224	8.865	1084.976	8927	0.85	5.42
12	7	84	0.9	224	7.709	1033.891	6752	0.84	1.30
10	8	84	0.9	222	6.984	1339.207	7807	0.84	1.26
8	9	85	0.8	224	7.275	939.3068	8621	0.86	1.24
1	10	83	0.7	223	11.453	81.230	8429	0.84	1.33
9	11	84	0.7	222	11.521	1835.615	12003	0.82	1.26
	12	83	0.9	223	9.022	1226.029	8665	0.86	1.30
3	13	84	0.8	223	7.769	1294.631	8242	0.86	5.25
13	14	84	0.8	223	7.77	1260.551	8255	0.84	5.23
4	15	85	0.9	223	8.234	1126.140	13269	0.87	1.21

16	16	84	0.8	223	7.780	1262.196	8266	0.85	1.27
5	17	83	0.8	222	10.466	1536.414	10305	0.87	1.30

Responses measured:

- Resolution
- Peak symmetry
- Retention time

2.4 Method Validation (ICH Q2 (R1))

Validation parameters included:

- **Linearity:**

For the purpose of evaluating the linearity of Ribavirin, Ritonavir and Lopinavir solution, several concentration solutions ranging from 40–200 µg/mL for Ribavirin and Lopinavir then 10–50 µg/mL for Ritonavir (40, 64, 72, 80, 88, 96, and 120 ppm) were injected into the test tube. Each concentration solution was examined six times in the column under the identical.

Accuracy:

The three separate Ribavirin, Ritonavir and Lopinavir standard and sample solutions were obtained from concentrations ranging from 80 to 120 mg in the concentration range.

Precision:

Analyzing six duplicate concentration solutions from 100 ppm on the same day and three separate days allowed researchers to examine intra-day and inter-day fluctuations in the concentrations

Specificity:

It was necessary to test the specificity and selectivity of the newly devised approach in order to identify the excipients that were interfering with the estimate of the Ribavirin, Ritonavir and Lopinavir. The blank solution, which did not include the Ribavirin, Ritonavir and Lopinavir, was produced and injected. The chromatogram produced from the blank was compared to the chromatograms obtained from the standard and sample, and the difference was examined to determine if excipients interfered with the drug quantification.

Robustness:

In order to determine if any impacts were induced by the changes in parameters such as column temperature, flow rate, and the usage of various percentage ratios of mobile phase, the parameters were varied. The difference in chromatographic conditions was taken into consideration in the current investigation.

The following modifications to the Chromatographic conditions will be evaluated:

- Change in column Temperature (±5°C)
- Change in wavelength (±5 nm)
- Change in Flow rate (± 0.1 ml\min) 10% change.

3. Results:

3.1 DoE Optimization:

DoE indicated:

- Increasing MeOH conc. reduced retention time for Lopinavir and Ritonavir.
- Ribavirin, being polar, required higher aqueous phase.
- pH significantly affected peak shape for Ribavirin.
- Flow rate influenced resolution between Lopinavir and Ritonavir.

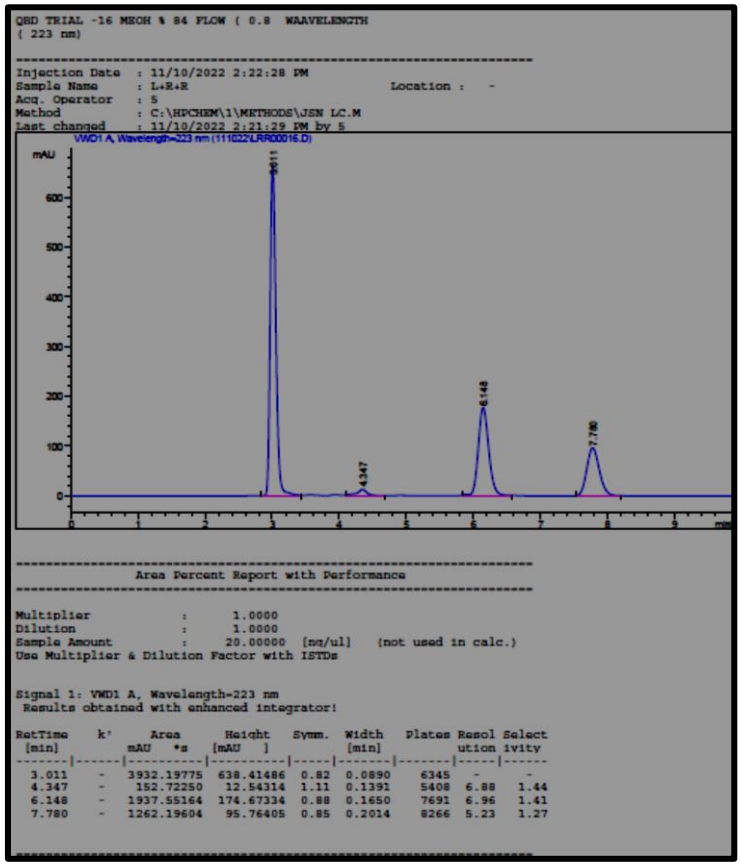
A response-surface model generated a **Method Operable Design Region (MODR)** that satisfied ATP criteria.

3.2 Optimized Chromatographic Conditions:

Instrument	HPLC
Mode	Isocratic
Column	C18, 250 X 4.6 mm, 5 µm, (COSMOSIL is suitable)
Injection volume	20 µL
Flow rate	0.7 mL/Minutes changes to 0.8 mL/Minutes
Run Time	15 Minutes
Wavelength	UV 223 nm
Column oven temperature	25°C
Sample temperature	25°C
Mobile Phase	Mixture of 0.05 % OPA and Methanol in the ratio of 20:80 changes to 16:84
Diluent	Mobile Phase
Needle wash	Mixture of Acetonitrile and Water in the ratio of 90:10
Seal wash	Mixture of Acetonitrile and Water in the ratio of 10:90

Under optimized conditions, all peaks were well resolved (resolution > 2.5)

Figure 1: HPLC chromatogram of Developed AQBD HPLC Method



Throughout the duration of the trial, it was observed that the peak absorbance values for ribavirin, ritonavir, and and lopinavir consistently stayed much below the predetermined limit. This observation was true irrespective of the mobile phase to flow rate ratio, wavelength, or flow rate. Upon modifying the mobile phase ratio, wavelength, and flow rate, it is seen that the USP Tailing factor and the USP Theoretical Plates remain within the permissible ranges of variability as specified in their respective calculations. The experimental results shown that the resolution between peaks may be increased beyond 2.5 by the manipulation of the mobile phase ratio, wavelength, and flow velocity. The task was successfully completed. The task was successfully completed without any difficulties. By manipulating the ratios of the mobile phase, wavelength, and flow velocity, it was feasible to effectively differentiate the primary peaks of lopinavir from the peaks caused by impurities. The impurity peaks and the primary peaks were effectively separated from one other utilizing Quality by Design (QBD) methodology. The impurity peaks observed in the analysis included of ribavirin, ritonavir, and and lopinavir, with several other compounds.

3.3 Method Validation Results:

3.3.1 Linearity:

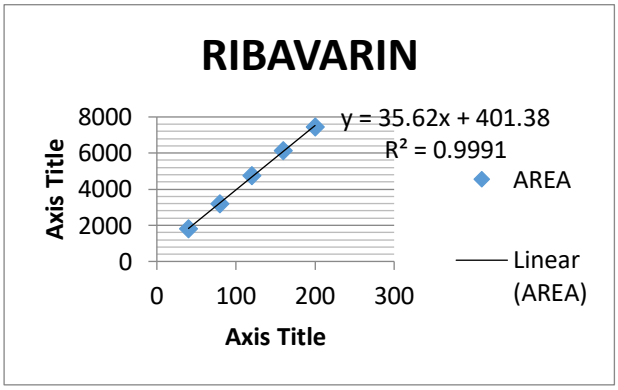


Figure 2: Linearity Graph for Ribavarin

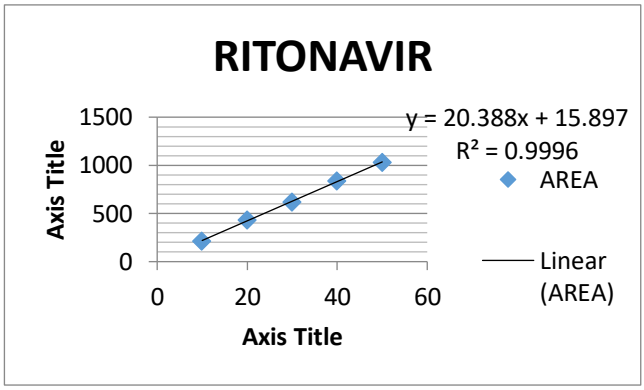


Figure 3: Linearity Graph for Ritonavir

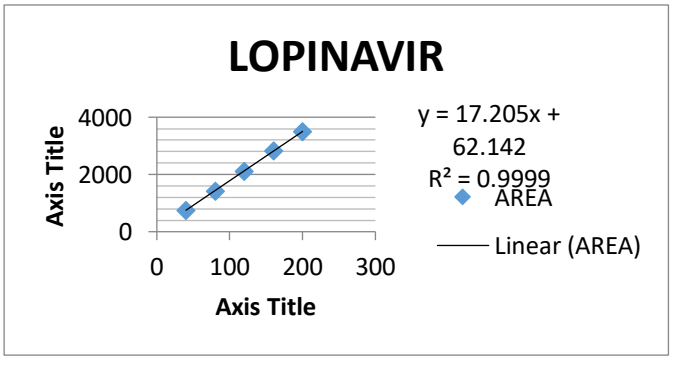


Figure 4: Linearity Graph for Lopinavir

Table 5: Linearity of Ribavirin, Ritonavir and Lopinavir

% Concentration	Lopinavir	Ribavarin	Ritonavir
35%	756.38	1819.40	217.50
60%	1429.55	3190.27	430.84
100%	2122.14	4750.12	618.46
130%	2826.57	6161.12	837.15
160%	3498.77	7457.97	1033.75
Slope	**17.2**	**35.62**	**20.38**
Intercept	**62.14**	**401.3**	**15.89**
Regression	**0.999**	**0.999**	**0.999**

3.3.2 Accuracy (% Recovery)

- Ribavirin: 98.5–101.2%
- Lopinavir: 99.1–101.4%
- Ritonavir: 98.8–102.0%

Table 6: Accuracy studies of developed method

Sample Name	Lopinavir	Ribavarin	Ritonavir
	% Recovery		
Acurracy 80%_Sample 1	97.83	97.06	100.70
Acurracy 80%_Sample 2	97.37	97.61	101.10
Acurracy 80%_Sample 3	98.21	98.24	100.80
Acurracy 100%_Sample 1	100.26	100.03	102.00
Acurracy 100%_Sample 2	99.16	100.40	101.10
Acurracy 100%_Sample 3	99.78	99.01	101.70
Acurracy 120%_Sample 1	102.40	101.75	101.20

3.3.3 Precision:

%RSD of intra-day and inter-day precision was **<2%** for all drugs.

Table 7: Method Precision and Intermediate precision Results

Sample Name	Lopinavir	Ribavarin	Ritonavir
Method Precision % Assay			
Sample 1	99.60	100.69	98.59
Sample 2	101.78	100.63	100.97
Sample 3	100.61	102.65	102.01
Sample 4	99.86	100.43	100.08
Sample 5	102.08	101.84	102.60
Sample 6	100.86	102.25	99.86
Average	**100.80**	**101.41**	**100.68**
RSD	**0.99**	**0.94**	**1.47**
Intermediate Precision % Assay			
Sample 1	100.16	100.03	100.26
Sample 2	100.23	102.21	100.19

Sample 3	100.20	101.04	102.22
Sample 4	99.90	100.29	100.00
Sample 5	100.42	102.51	101.40
Sample 6	100.32	101.29	101.82
Average	**100.21**	**101.23**	**100.98**
RSD	**0.18**	**0.98**	**0.94**
Overall Average	**100.50**	**101.32**	**100.83**
Overall RSD	**0.74**	**0.92**	**1.19**

3.3.4 Robustness:

The percentage recovery of Ribavirin, Ritonavir and Lopinavir for the standard solution was almost identical to 99.0 percent, whereas the percentage recovery of Ribavirin, Ritonavir and Lopinavir for the sample solution was nearly identical to 99.2 percent. According to the findings, the percent RSD was less than 2.0 percent Small changes in flow rate, wavelength, or mobile phase composition did not significantly affect results, confirming robustness.

Table 8: Robustness for Lopinavir, Ribavarin, Ritonavir

Robustness parameter		**% RSD**			**Remark**
		Lopinavir	**Ribavarin**	**Ritonavir**	
Wavelength (nm)	222	0.88	0.48	0.31	Pass
	223	0.36	0.21	0.52	Pass
	224	1.27	0.24	0.84	Pass
Mobile Phase	(83:17)	1.25	1.01	0.91	Pass
	(85:15)	0.36	0.87	0.23	Pass
	(89:21)	1.06	0.43	0.71	Pass
Flow (mL/min)	0.7	0.84	0.72	0.65	Pass
	0.8	0.36	0.65	0.74	Pass
	0.9	0.84	0.32	0.82	Pass

4. Discussion:

A-QbD enabled systematic understanding of the analytical method, improving robustness and lifecycle management. DoE helped identify critical parameters impacting method performance. MODR ensured reliable operation over a defined space.

The study confirmed that:

- Ribavirin required more aqueous content due to hydrophilicity.
- Lopinavir and Ritonavir, being lipophilic, needed higher organic phase.
- A-QbD reduced method variability and improved validation outcomes.

Compared with conventional development, A-QbD demonstrated superior control and predictability.

5. Conclusion:

This research successfully applied the A-QbD framework to develop and validate analytical methods for Ribavirin, Lopinavir, and Ritonavir. The optimized method met all ATP criteria, exhibiting excellent precision, accuracy, linearity, and robustness. The A-QbD approach supports regulatory expectations and strengthens method lifecycle management. Its adoption in antiviral drug analysis ensures reliable quality control and enhances pharmaceutical product integrity.

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