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Quality by Design Based RP-HPLC Method Development and Validation for the Simultaneous Estimation of Atazanavir and Ritonavir in Pure and Pharmaceutical Dosage Form

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ABSTRACT

The method Development and validation for Simultaneous Estimation of Atazanavir and Ritonavir in pure and pharmaceutical dosage form is done by using RP-HPLC (Jasco-Series 2000) based on Quality by Design technique. Chromatographic separations were carried out on Analytical column: C18 column Waters X Bridge (4.6× 250mm id. particle size 5µm), UV detection: 247nm. Injection volume: 20 µL, Flow rate: 1.00 mL min -1, Temperature: Ambient, Run time: 10 min. 2 Level Factorial Design gave 4 runs at different pH and Mobile phase proportion. A mixture of acetonitrile, potassium dihydrogen phosphate (pH-3, KH2PO4) and methanol (90:10) was used as the mobile phase. The pH of the buffer solution was adjusted by phosphoric acid (H3PO4) and triethylamine (TEA). The wavelength of 247nm was used as detection at which both drugs gave good response.

Keywords: *Simultaneous Estimation, Atazanavir, Ritonavir, Factorial Design, Quality by Design*

1. INTRODUCTION

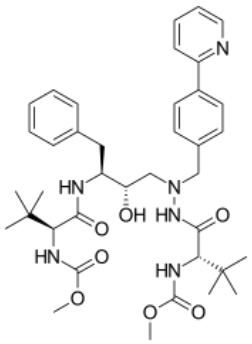
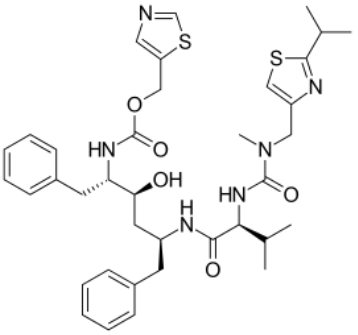
Aim- Quality by Design Based RP-HPLC Method Development and Validation for the Simultaneous Estimation of Atazanavir and Ritonavir in Pure and Pharmaceutical Dosage Form.

Objective-

- Application of Quality by Design (ICH-8) technique for estimation of Atazanavir and Ritonavir by RP-HPLC technique.
- The organic phase of mobile phase and pH of the buffer are minimized with high theoretical plates.
- The Retention time and peak asymmetry of analysis is minimized.
- The developed method is simple, precise, validated and optimized method for estimation of Atazanavir and Ritonavir in bulk and pharmaceutical dosage form
- Statistical analysis of the recovery data obtained from different techniques for Atazanavir and Ritonavir.

Material: Preliminary Analysis of Drug

- The colour and texture of Atazanavir and Ritonavir were matched to known drug bank features. Atazanavir is slightly soluble in water and sparingly soluble in aqueous buffers and soluble in organic solvents ethanol, dimethyl formamide (DMF), dimethyl sulfoxide (DMSO) whereas Ritonavir is little soluble in water, The solutions were subjected to UV examination by scanning them at 200-400 nm.

Name	Atazanavir	Ritonavir
Structure		
CAS NO	98904-31-3	155213-67-5
Molecular Formula	$C_{38}H_{52}N_6O_7$	$C_{37}H_{48}N_6O_5S_2$
Molecular Weight	704.869 g·mol−1	720.95 g·mol−1
IUPAC Name	methyl N-[(1S)-1-{[(2S,3S)-3-hydroxy-4-[(2S)-2-[(methoxycarbonyl)amino]-3,3-dimethyl-N'-{[4-(pyridin-2-yl)phenyl]methyl}butanehydrazido]-1-phenylbutan-2-yl]carbamoyl}-2,2-dimethylpropyl]carbamate	1,3-thiazol-5-ylmethyl N-[(2S,3S,5S)-3-hydroxy-5-[(2S)-3-methyl-2-{[methyl({[2-(propan-2-yl)-1,3-thiazol-4-yl]methyl})carbamoyl]amino}butanamido]-1,6-diphenylhexan-2-yl]carbamate
Category	Anti HIV	Anti HIV
Water Solubility	**4-5 mg/mL**	**0.1 mg/ml**
Storage	68°F to 77°F (20°C to 25°C).	stored below 25°C
Uses	Antiretroviral medication used to treat HIV/AIDS	Antiretroviral medication used to treat HIV/AIDS

Experimental Materials-

Table 1.2: Active Pharmaceutical Ingredient

Sr. No.	Name	Description
1.	Atazanavir & Ritonavir	White Crystalline Solid
2.	Atazor-R 300mg/100mg Kit	300 mg Atazanavir 100 mg Ritonavir

Table 1.3: List of Chemicals

Sr. No	Name of Chemicals	Grade	Manufacturer
1	Methanol	HPLC Grade	Merck Lie Sciences Pvt. Ltd, Mumbai.
2	Acetonitrile	HPLC Grade	Merck Lie Sciences Pvt. Ltd, Mumbai.
3	Ethanol	HPLC Grade	Merck Lie Sciences Pvt. Ltd, Mumbai.
4	Water	HPLC Grade	Merck Lie Sciences Pvt. Ltd, Mumbai.

5	Potassium dihydrogen phosphate	LR Grade	Research Fine Chem. Indu.
6	Sodium hydroxide	LR Grade	Research Fine Chem. Indu.
7	Triethylamine	LR Grade	Research Fine Chem. Indu.

Instrumentation and Chromatographic Conditions

Table 1.4: Instrumentation and Chromatographic Conditions

Sr. No.	Name of Equipment/ Instruments	Model/ Specification	Manufacturer
1	HPLC	Series2000	Jasco
	1. Pump	PU2080	
	2. Sample Injection port	Rheodyne Injector	
	3. UV/Visible Detector	UV 2075 plus	
	4. Software	Borwin	
2	Double beam UV-Visible Spectrophotometer	UV-1800 240V	SHIMADZU
3	pH meter	EQ-636	Equip-Tronics
4	Balance	BL-220H	Shimadzu
5	Sonicator	1.5L 50H	Rolex

Software Experimental Design

The desirability function and data analysis calculations were performed by using Design Expert (Version 8.0.6 Stat-Ease Inc., Minneapolis, MN 55413, USA) trial version statistical software.

Preparation of Phosphate Buffer Solution

6.8 gm of Ammonium Format was dissolved in sufficient water (HPLC grade) with aid of sonicator. Then add triethylamine or orthophosphoric acid was used to adjust the pH to 3.

Preparation of stock solutions

- Stock solution was prepared by dissolving 10 mg of Atazanavir in Methanol in 10 ml volumetric flask to get concentration of 1000 µg/ml. From the resulting solution 0.1 ml was diluted to 10 ml with methanol to obtain concentration of 10 µg/ml of Atazanavir.
- Similarly, stock solution of Ritonavir was prepared by dissolving 10 mg in 10 ml of Methanol with 10 min Sonication and further dilutions prepared by using Methanol.

Chromatographic procedure

- Chromatographic separations were carried out on Analytical column: C18 column Waters X Bridge (4.6× 250mm id. particle size 5µm), UV detection: 247nm. Injection volume: 20 µL, Flow rate: 1.00 mL min -1, Temperature: Ambient, Run time: 10 min
- A mixture of acetonitrile, potassium dihydrogen phosphate (pH-3, KH2PO4) and methanol (90:10) was used as the mobile phase. The pH of the buffer solution was adjusted by phosphoric acid (H3PO4) and triethylamine (TEA). The wavelength of 247nm was used as detection at which both drugs gave good response.

Experimental Design and Response Surface Methodology

- **Design of Experiment**

2 Level Factorial Design is a good design choice when Central Composite and Box-Behnken do not fit your needs. The

factorial experiments, where all combination of the levels of the factors are run, are usually referred to as full factorial experiments. Full factorial two level experiments are also referred to as designs where denotes the number of factors being investigated in the experiment.

Dependent factors were selected as mobile phase (potassium dihydrogen phosphate: Acetonitrile/ Water: Methanol/ Water: Acetonitrile), pH of aqueous phase (3.00 to 5.00 mmol/L) and flow rate were constant at 1 mL/min.

Independent factors were selected as retention time, peak area, theoretical plates and peak asymmetry.

The C18 column is used for proposed method.

2 Level Factorial Design gave 4 runs (Table.1.5) at different pH and Mobile phase proportion. Same procedure followed for each mobile phase. Total runs for three mobile phases were 12. Optimization means finding an alternative with the most cost effective or highest achievable performance under the given constraints, by maximizing desired factors and minimizing undesired ones. In comparison, maximization means trying to attain the highest or maximum result or outcome without regard to cost or expense.

Table 1.5: Trials gives by Software for 2 Level Factorial Design for all Mobile Phase

Sr. No	Mobile Phase Composition (Organic Phase, v/v)	pH of Buffer (mmol/L)
Atazanavir		
1	70.00	3.00
2	70.00	5.00
3	90.00	3.00
4	90.00	5.00
Ritonavir		
1	70.00	3.00
2	70.00	5.00
3	90.00	3.00
4	90.00	5.00

Selection of detection wavelength

- From the standard stock solution further dilutions were done using water and scanned over the range of 200-400 nm and the spectra were overlain. It was observed that Atazanavir & Ritonavir showed considerable absorbance at 247nm.

Preliminary Analysis of Drug

- Atazanavir (PPL) and Ritonavir (HCT) were analyzed and compared with reported in drug bank. Atazanavir is white crystalline solid and slightly soluble in water and soluble in methanol likewise Ritonavir is slightly soluble in water. UV analysis was carried out by scanning the solutions at 200-400 nm.

2. RESULTS AND DISCUSSION

Trials given by Design Expert software

Table 2.1

Sr. No	Mobile Phase Composition (Organic Phase, v/v)	pH of Buffer (mmol/L)
Atazanavir		
1	70.00	3.00
2	70.00	5.00
3	90.00	3.00
4	90.00	5.00
Ritonavir		
1	70.00	3.00
2	70.00	5.00
3	90.00	3.00
4	90.00	5.00

Optimization Result

- **Screening design for suitable chromatographic condition**

Determination of chromatographic condition is based on peak parameters of both drugs.

After taking runs on HPLC, we got following results of different mobile phase with different pH and different flow rate. To have better understanding the peak properties used remarks like Extremely Satisfactory, Satisfactory, More Satisfactory, partially Satisfactory and Dissatisfactory.

Results of various trials, having organic phase composition 70 % v/v are shown in following tables.

Table 2.2: Runs performed at mobile phase (70:30 v/v) with aqueous phase pH 3.

Sr. no.	Composition	Observation	Remarks
1	• Potassium dihydrogen phosphate: Acetonitrile	Good peak properties, less retention time with more theoretical plates and less asymmetric factor	Extremely Satisfactory
2	• Water: Methanol	Only one peak appeared (Atazanavir) another peak is very small (Ritonavir)	Dissatisfactory
3	• Water: Acetonitrile	Lower theoretical plates and less peak height	Satisfactory

Table 2.3: Runs performed at mobile phase (70:30 v/v) with aqueous phase pH 5.00.

Sr. no.	Composition	Observation	Remarks
1	• Potassium dihydrogen phosphate: Acetonitrile	Less peak asymmetry but less theoretical plates	Satisfied

2	• Water: Methanol	Resolution of Peaks is not good	Very Dissatisfactory
3	• Water: Acetonitrile	Greater peak Asymmetry and lower theoretical plates	Partially satisfactory

❖ **Results of various trials, having organic phase composition 90.00 % v/v are shown in following tables.**

Table 2.4: Runs performed at mobile phase (90:10 v/v) with aqueous phase pH 3.

Sr. no.	Composition	Observation	Remarks
1	• Potassium dihydrogen phosphate: Acetonitrile	Less peak asymmetry with more theoretical plates and good retention time	Partly Satisfactory
2	• Water: Methanol	The peak of Ritonavir not appeared	Dissatisfactory
3	• Water: Acetonitrile	Good Peak Properties but Resolution is not Good	Partly Satisfactory

Table 2.5: Runs performed at mobile phase (90:10 v/v) with aqueous phase pH 5.00.

Sr. no.	Composition	Observation	Remarks
1	• Potassium dihydrogen phosphate: Acetonitrile	Less theoretical plates	Satisfied
2	• Water: Methanol	Broad Peak Appeared and noise exist	Very Dissatisfactory
3	• Water: Acetonitrile	Broad Peak Appeared	Partially satisfactory

Optimized trials suggested by software based on desirability value

This methodology is initially based on constructing a desirability function for each individual response. The scale of individual desirability function ranges between i= 0, for completely undesirable response and i =1, for fully desired response. Selection of trial was based on maximum desirability value. Therefore, first trial which was having desirability one (i=1) selected for method optimization. [5-13]

Table 2.6: Trials performed on C18 column at mobile phase (70:30 v/v) with aqueous phase pH 3 are extremely Satisfactory.

Design expert has optimized the following chromatographic conditions with respect to desirability value.

Table 2.6

Sr. No	Mobile Phase Composition (Organic Phase, v/v)	pH of Buffer (mmol/L)	Retention Time	Asymmetry	Theoretical Plates
Atazanavir					
1	70.00	3.00	2.389	1.139	10778

2	70.00	5.00	2.335	1.788	9088
3	90.00	3.00	1.122	1.124	4787
4	90.00	5.00	1.021	1.873	3789
Ritonavir					
1	70.00	3.00	3.996	1.39	9842
2	70.00	5.00	3.982	1.899	9111
3	90.00	3.00	3.227	1.491	6121
4	90.00	5.00	3.298	1.909	5987

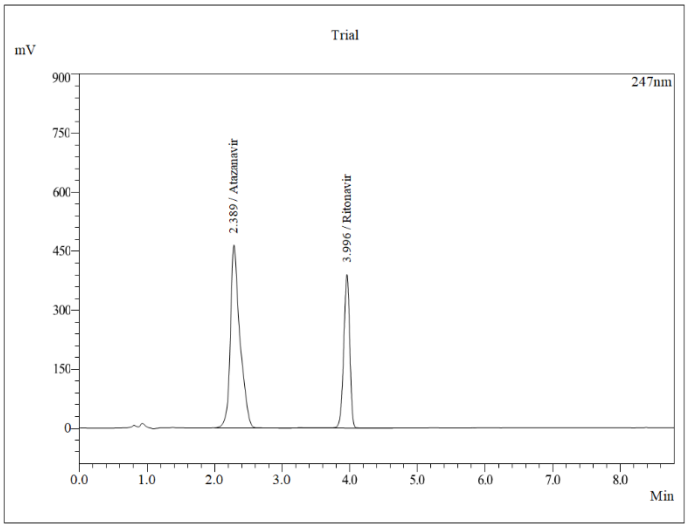


Figure.1

Trial no. 01				
70.00	3.00	3.996	1.39	9842
70.00	3.00	3.996	1.39	9842

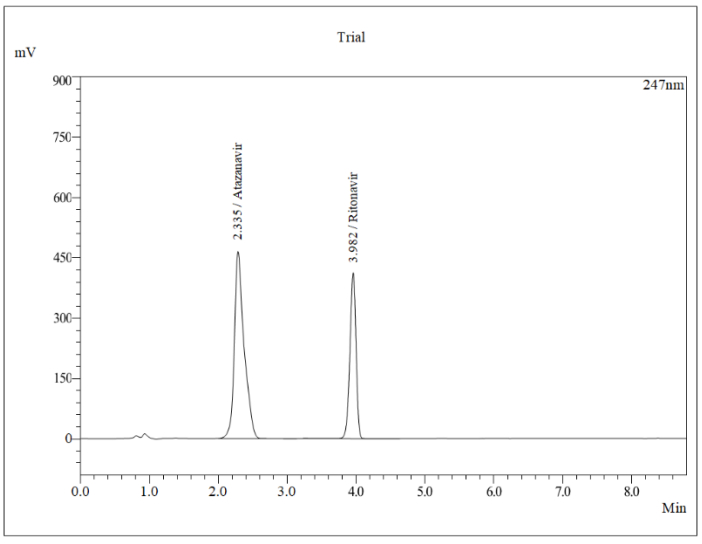


Figure.2

Trial no. 02				
70.00	5.00	2.335	1.788	9088
70.00	5.00	3.982	1.899	9111

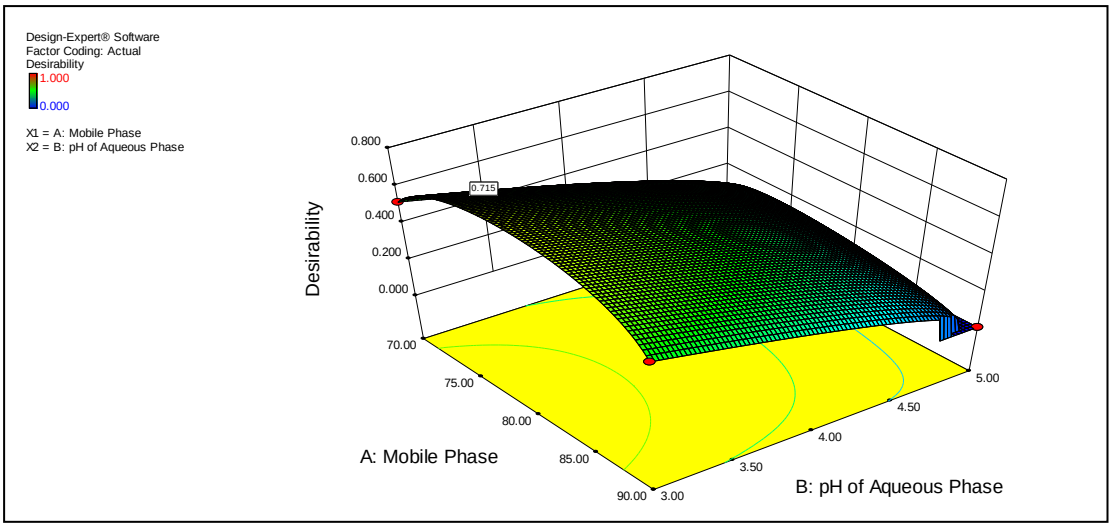


Figure 3: Three-dimensional plot for Desirability Function

Optimized chromatographic conditions

Mobile phase: Potassium dihydrogen phosphate : Acetonitrile (20.76: 79.24 v/v), pH of buffer: 3.00, Analytical column: C_{18} column Waters XBridge (4.6× 250mm id. particle size 5µm), UV detection: 247nm, Injection volume: 10 µL, Flow rate: 1.00 mL min $^{-1}$, Temperature: Ambient, Run time: 10 min

Table2.7: Optimized trials suggested by software based on desirability value

Sr. no.	Amount of CAN	pH of buffer	Flow rate	Retention time	Tailing factor	Theoretical plates	Desirability
Atazanavir							
1	79.24	3.00	1.00	1.767	1.130	7996.87	**0.715**
Ritonavir							
1	79.24	3.00	1.00	3.756	1.438,	8111.47	**0.715**

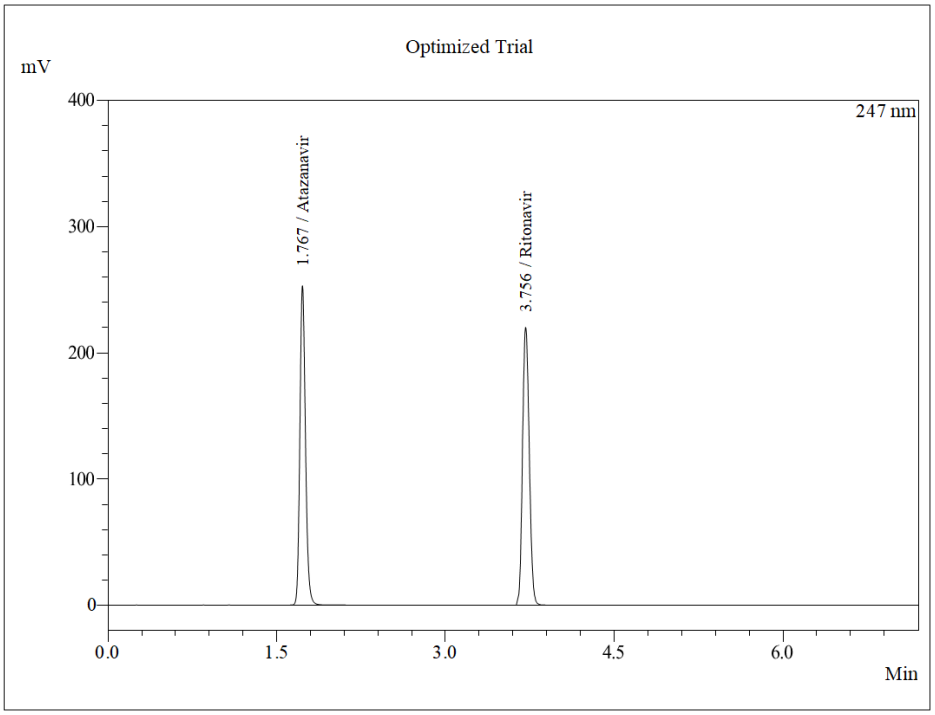


Figure. 4. Optimized Trial

Effect of dependent variables on Retention Time

- **Effect of dependent variables on retention time for Atazanavir**

After applying experimental design, suggested Factorial Model was found to be significant with model F value of 350.94, *p* value less than 0.005 and R^2 value of 0. 0.9986. There is only a 3.77% chance that a "Model F-Value" this large could occur due to noise. Values of % C.V. and adjusted R^2 were 2.78 and 0.9957 respectively.

The model for response

ATVR Retention Time = + 6.77750 - 0.062200 * Mobile Phase - 0.027000 * pH of Aqueous Phase

Fig. shows a graphical representation of pH of buffer (B) and amount of Acetonitrile (A), while flow rate (C) is maintained constant at its optimum of 1.0 mL min^{-1}. An decrease in pH of buffer does not show good on response (X) while increase in amount of acetonitrile showed decreases the retention time.

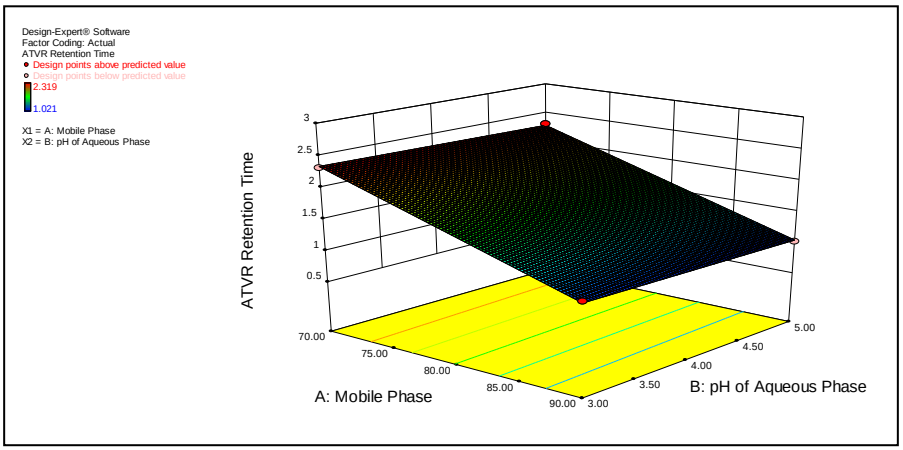


Figure 5: Three-Dimentional plot for Retention Time as a function of pH of buffer and Conc. of Mobile Phase. Constant factor (flow rate- 1mL min^{-1})

Fit summary: Factorial Model was suggested by the software.

ANOVA: ANOVA of developed 2FI model for Retention Time

Values of "Prob > F" (p- value) less than 0.0500 indicate model terms are significant. In this case A is significant model terms.

Table2.8: Significance of *p* value on model terms of Retention Time

Model terms	***p* value**	**Effect of factor**	**Remarks**
Model	0.0377	350.94	Significant
A	*0.0240*	*700.56*	Significant
B	*1.32*	*0.4559*	Insignificant
Overall model	**0.0377**	**-**	**Significant**

❖ **Effect of dependent variables on retention time for Ritonavir**

After applying experimental design, suggested Factorial Model was found to be significant with model F value of 7736.76, *p* value less than 0.005 and R^2 value of 0. 0.9999. There is only a 0.80% chance that a "Model F-Value" this large could occur due to noise. Values of % C.V. and adjusted R^2 were 0.21 and 0.9998 respectively.

The model for response

RTVR Retention Time = + 7.56850 - 0.049600 * Mobile Phase + 0.039500 * pH of Aqueous Phase

Fig. shows a graphical representation of pH of buffer (B) and amount of Acetonitrile (A), while flow rate (C) is maintained constant at its optimum of 1.0 mL min^{-1}.

The change in pH of buffer does not show drastic change on Retention time while increase in amount of acetonitrile showed decreases the retention time.

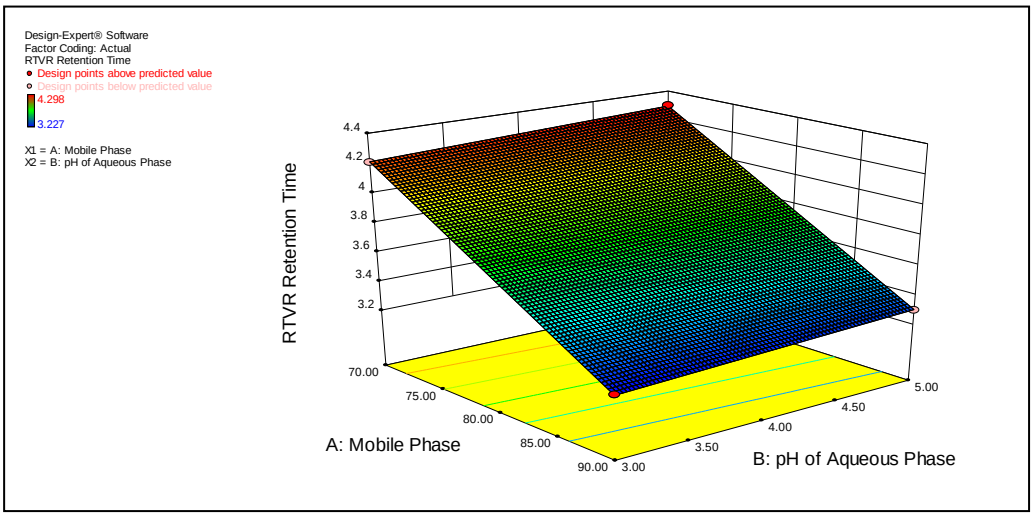


Figure 6: Three-dimensional plot for Retention Time as a function of pH of buffer and Conc. of Organic Phase. Constant factor (flow rate- 1mL min^{-1})

Fit summary: Factorial Model was suggested by the software.

ANOVA: ANOVA of developed 2FI model for Retention Time

Values of "Prob > F" (p- value) less than 0.0500 indicate model terms are significant. In this case A is significant model terms.

Table 2.9: Significance of *p* value on model terms of Retention Time

Model terms	***p* value**	**Effect of factor**	**Remarks**
Model	0.0080	7736.76	Significant
A	0.0051	15376.00	Significant
B	0.0642	97.52	Insignificant
Overall model	0.0080	7736.76	**Significant**

Effect of dependent variables on Asymmetric Factor

- **Effect of dependent variables on Asymmetric Factor for Atazanavir**

After applying experimental design, suggested Factorial Model was found to be significant with model F value of 97.97, *p* value less than 0.005 and R^2 value of 0.9949. There is only a 7.13% chance that a "Model F-Value" this large could occur due to noise. Values of % C.V. and adjusted R^2 were 3.38 and 0.9848 respectively.

The model for response

ATVR Asymmetric Factor = - 0.057000 + 1.75000E-003 * Mobile Phase +0.34950 * pH of Aqueous Phase

Fig. shows a graphical representation of pH of buffer (B) and amount of Acetonitrile (A), while flow rate (C) is maintained constant at its optimum of 1.0 mL min^{-1}. An decrease in pH of buffer show decreases in asymmetric factor while increase in amount of acetonitrile does not have any significant effect.

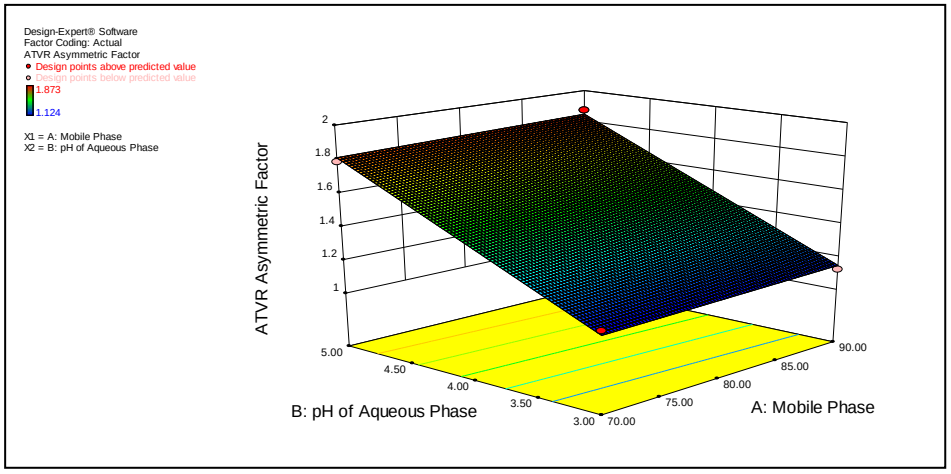


Figure 7: Three-Dimentional plot for Asymmetric Factor as a function of pH of buffer and Conc. of Acetonitrile. Constant factor (flow rate- 1mL min^{-1})

Fit summary: Factorial Model was suggested by the software.

ANOVA: ANOVA of developed 2FI model for Asymmetric Factor

Values of "Prob > F" (p- value) less than 0.0500 indicate model terms are significant. In this case A is significant model terms.

Table 2.10: Significance of *p* value on model terms of Asymmetric Factor

Model terms	***p* value**	**Effect of factor**	**Remarks**
Model	0.0713	97.97	Significant
A	0.6112	*0.49*	Insignificant
B	0.0455	*195.44*	Significant

Overall model	0.0713		**Significant**

❖ **Effect of dependent variables on Asymmetric Factor for Ritonavir**

After applying experimental design, suggested Factorial Model was found to be significant with model F value of 52. 63, *p* value less than 0.005 and R^2 value of 0.9906. There is only a 9.70% chance that a "Model F-Value" this large could occur due to noise. Values of % C.V. and adjusted R^2 were 2.72 and 0.9718 respectively.

The model for response

RTVR Asymmetric Factor = + 0.52325 +2.77500E-003 * Mobile Phase + 0.23175 * pH of Aqueous Phase

Fig. shows a graphical representation of pH of buffer (B) and amount of Acetonitrile (A), while flow rate (C) is maintained constant at its optimum of 1.0 mL min^{-1}. An decrease in pH of buffer show decreases in asymmetric factor while increase in amount of acetonitrile does not have any significant effect.

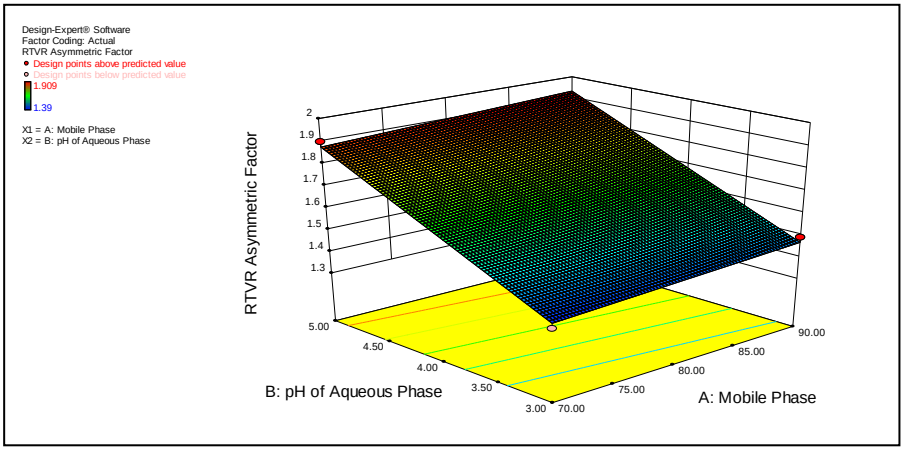


Figure 8: Three-dimensional plot for Asymmetric Factor as a function of pH of buffer and Conc. of Acetonitrile. Constant factor (flow rate- 1mL min^{-1})

Fit summary: Factorial Model was suggested by the software.

ANOVA: ANOVA of developed 2FI model for Asymmetric Factor

Values of "Prob > F" (p- value) less than 0.0500 indicate model terms are significant. In this case A is significant model terms.

Table 2.11: Significance of *p* value on model terms of Asymmetric Factor

Model terms	***p* value**	**Effect of factor**	**Remarks**
Model	0.0970	52.63	Significant
A	0.4372	1.49	Insignificant
B	0.0623	103.77	Significant
Overall model	0.0970	52.63	**Significant**

Effect of dependent variables on Theoretical Plates

❖ **Effect of dependent variables on Theoretical Plates for Atazanavir**

After applying experimental design, suggested Factorial Model was found to be significant with model F value of 140. 63, *p* value less than 0.005 and R^2 value of 0.9965. There is only a 5.95% chance that a "Model F-Value" this large could occur due to noise. Values of % C.V. and adjusted R^2 were 4.87 and 0.9894 respectively.

The model for response

ATVR Theoretical Plates = + 32378.50000 - 282.25000 * Mobile Phase - 672.00000 * pH of Aqueous Phase

Fig.9 Shows a graphical representation of pH of buffer (B) and amount of Acetonitrile (A), while flow rate (C) is maintained constant at its optimum of 1.0 mL min^{-1}. the decrease in pH of buffer show slightly increases the Theoretical Plates while increase in amount of acetonitrile shown antagonist effect like decreases the theoretical plates.

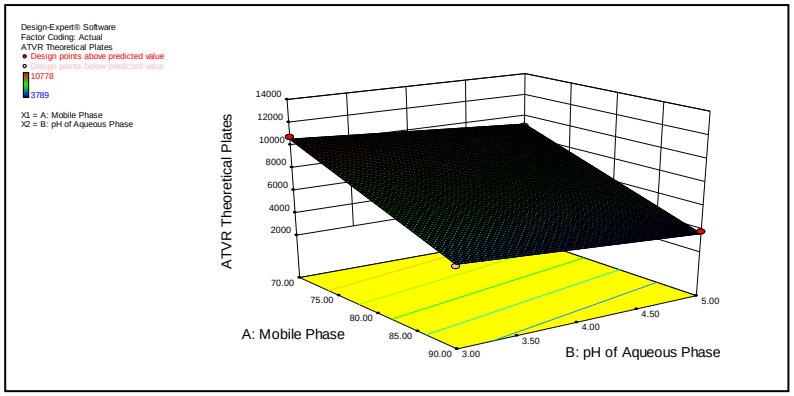


Figure 9: Three-dimensional plot for Rete Theoretical Plates as a function of pH of buffer and Conc. of Acetonitrile. Constant factor (flow rate- 1mL min^{-1})

Fit summary: Factorial Model was suggested by the software.

ANOVA: ANOVA of developed 2FI model for Theoretical Plates

Values of "Prob > F" (p- value) less than 0.0500 indicate model terms are significant. In this case A is significant model terms.

Table 2.12: Significance of *p* value on model terms of Theoretical Plates

Model terms	***p* value**	**Effect of factor**	**Remarks**
Model	0.0595	140.63	Significant
A	0.0390	266.18	Significant
B	0.1604	15.09	Insignificant
Overall model	0.0595	140.63	**Significant**

❖ **Effect of dependent variables on Theoretical Plates for Ritonavir**

After applying experimental design, suggested Factorial Model was found to be significant with model F value of 66.78, *p* value less than 0.005 and R^2 value of 0.9926. There is only a 8. 62% chance that a "Model F-Value" this large could occur due to noise. Values of % C.V. and adjusted R^2 were 3.84 and 0.9777 respectively.

The model for response

RTVR Theoretical Plates = + 22320.25000 -171.12500 * Mobile Phase

-216.25000 * pH of Aqueous Phase

Fig. 10 shows a graphical representation of pH of buffer (B) and amount of Acetonitrile (A), while flow rate (C) is maintained constant at its optimum of 1.0 mL min^{-1}. An decrease in pH of buffer show slightly increases the Theoretical Plates while increase in amount of acetonitrile shown antagonist effect like decreases the theoretical plates.

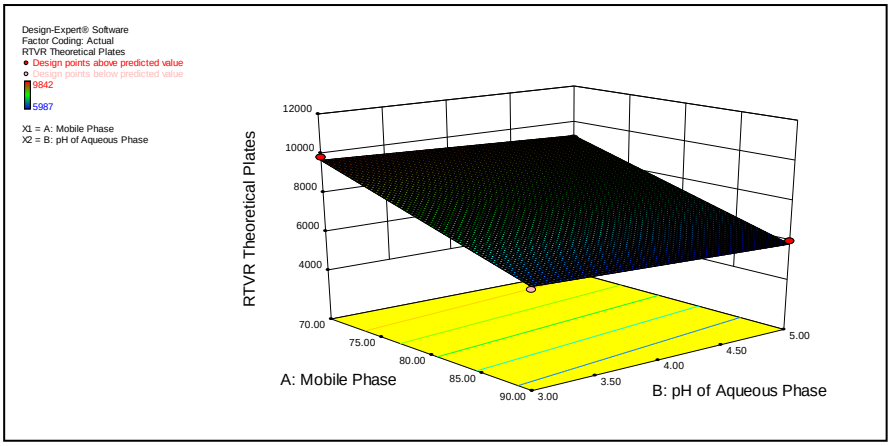


Figure 10: Three-dimensional plot for Theoretical Plates as a function of pH of buffer and Conc. of Acetonitrile. Constant factor (flow rate- 1mL min^{-1})

Fit summary: Factorial Model was suggested by the software.

ANOVA: ANOVA of developed 2FI model for Theoretical Plates

Values of "Prob > F" (p- value) less than 0.0500 indicate model terms are significant. In this case A is significant model terms.

Table 2.13: Significance of *p* value on model terms of Theoretical Plates

Model terms	***p* value**	**Effect of factor**	**Remarks**
Model	0.0862	66.78	Insignificant
A	0.0554	131.46	Significant
B	0.3846	2.10	Insignificant
Overall model	0.0862	66.78	Insignificant

3. ANALYTICAL VALIDATION

The proposed RP-HPLC method was validated as per ICH guidelines.

- **Linearity:** Several aliquots of standard solutions of ATVR and RTVR were taken in different 10 ml volumetric flasks and the volume was made up to the mark with mobile phase such that final concentration of ATVR and RTVR were 10-60µg/ml, respectively. Evaluation was preferred.
- med using the UV-Vis detector at 247nm, peak area recorded for all the peaks. Calibration curve Fig. 2 & 3 was plotted as concentration against peak area.

Table 3.1: Results of Linearity

Linearity			
Sr. No	**Concentration (µg/mL)**	**Peak Area ATVR**	**Peak Area RTVR**
1	10	990521	756412
2	20	1981042	1512824
3	30	2921563	2219236
4	40	3962084	3025648
5	50	4952605	3782060
6	60	5943126	4538472
Slope		99194.96	75784.06
Standard Error		22625.31	22625.31

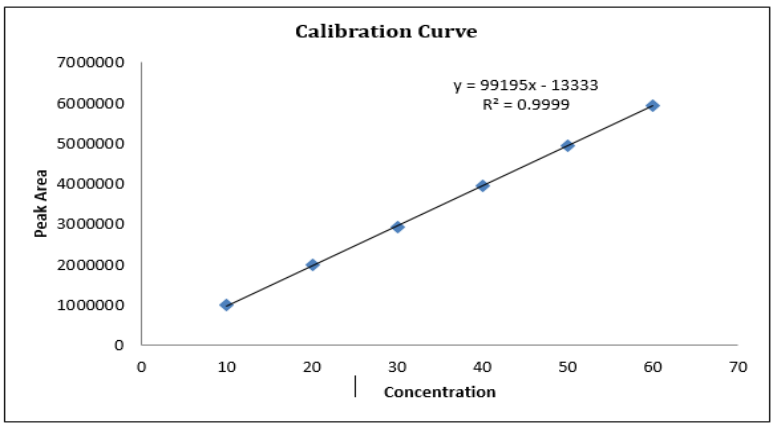


Figure 1 : Calibration curve of Atazanavir

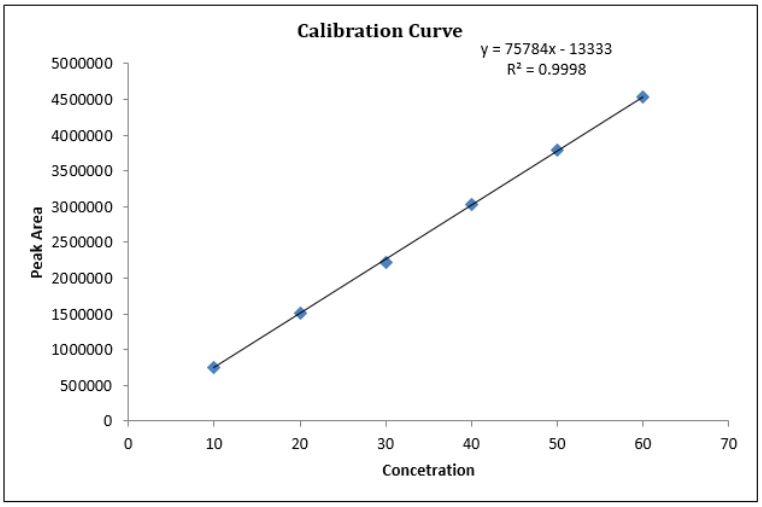


Figure 2: Calibration Curve of Ritonavir

Linearity Trials:

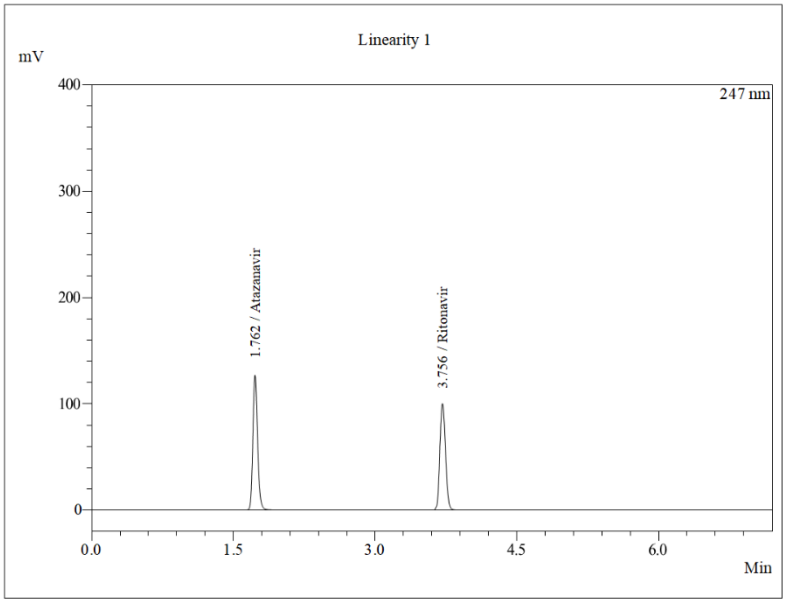


Figure. 3. 10ug/mL

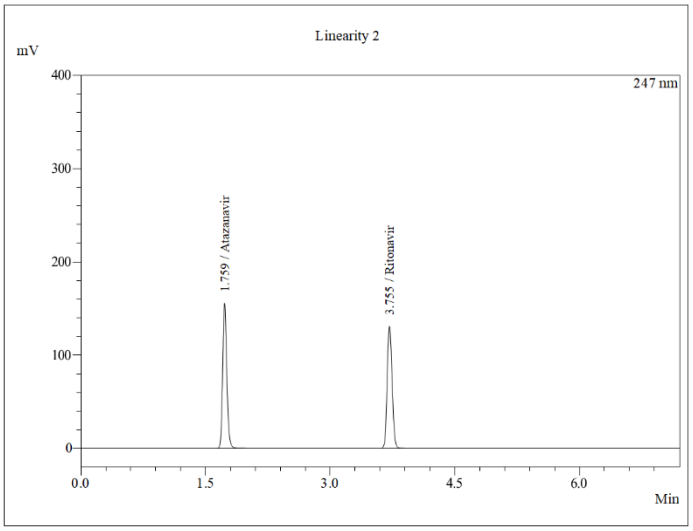


Figure. 4. 20ug/mL

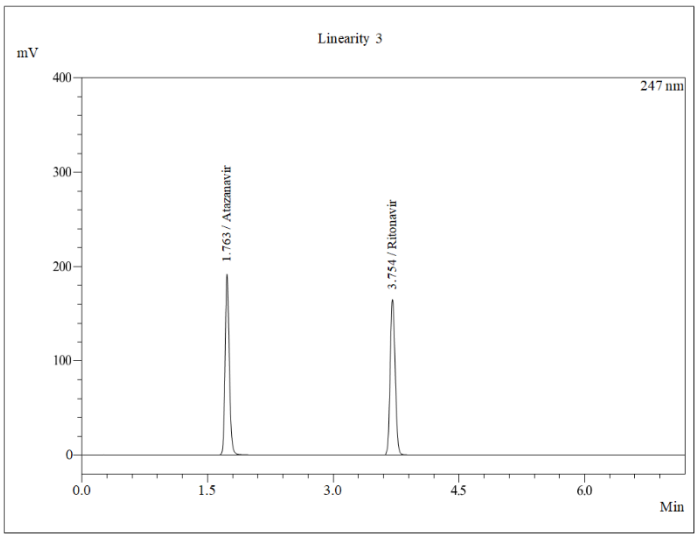


Figure.5. 30ug/mL

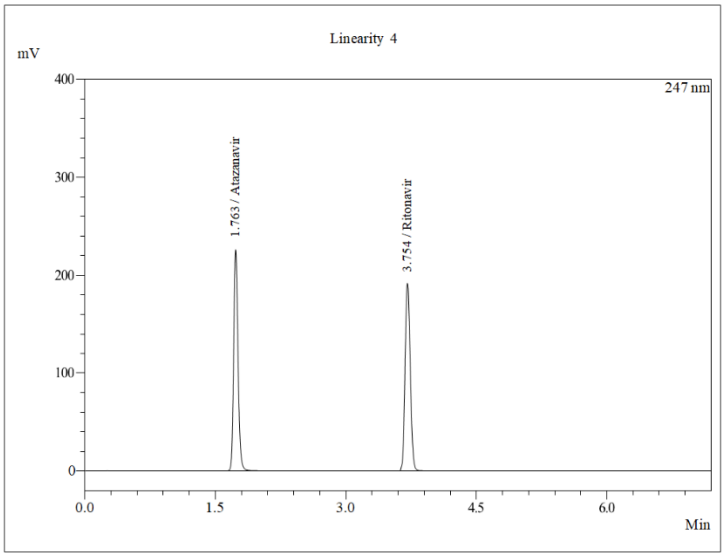


Figure.6. 40ug/mL

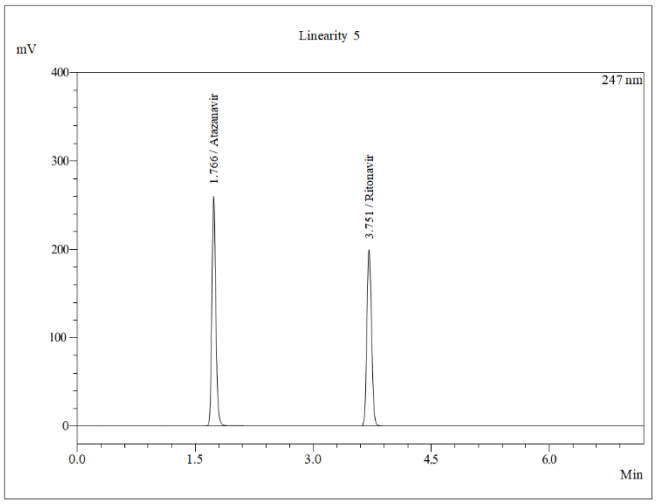


Figure.7. 50ug/mL

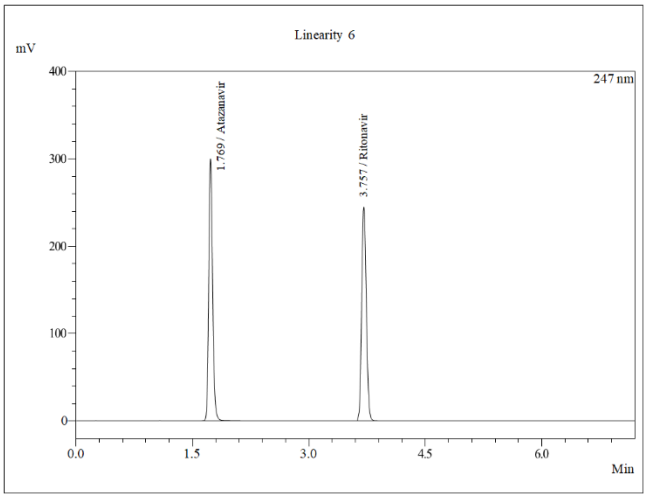


Figure.8. 60ug/mL

- **Specificity:** The specificity of the RP-HPLC method was determined by comparison of the chromatogram of mixed standards and sample solutions. The parameters like retention time, resolution and Area were calculated. Good correlation was found between the results of mixed standards and sample solutions.

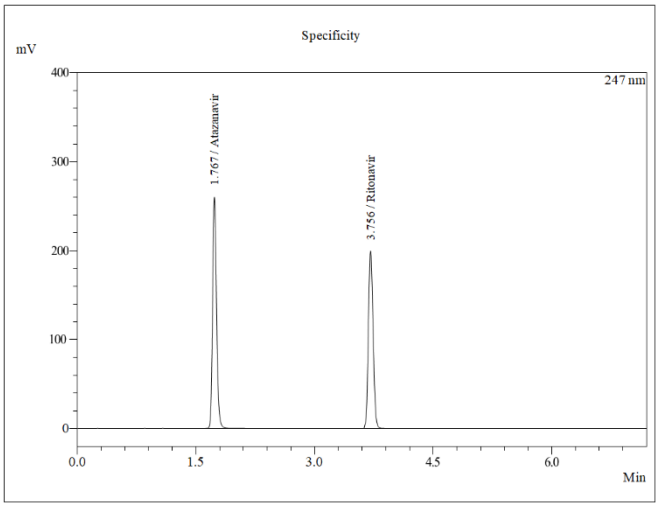


Figure 9: Typical chromatogram of Atazanavir and Ritonavir

Table 3.2: Results of Specificity

Sample	Label Claim (mg)	Amount Found	Recovery	Retention Time (Min)
Tablet	300	299.91	99.97	1.767
	100	99.91	99.91	3.756

❖ **Precision:** To determine the precision of method, six replicates of the sample prepared from the commercial tablets were injected and assay was calculated to measure the repeatability of retention times and peak area of standard and sample. Precision of the method was verified by using tablet stock solution. Intraday and interday precision were determined by repeating assay six times in same day for intraday precision and on different days for interday precision studies. The results of this analysis are as follows.

Table 3.3: Results of Precision

Sr. No.	Concentration (µg/mL)	Intra Day Precession		Inter Day Precession	
		Atazanavir	Ritonavir	Atazanavir	Ritonavir
1	40	3986560.5	3031153	3974923	3081153
2	40	4011585	3024004	4015833.33	3091354
3	40	3838198	3028683	4101246	3152568
4	40	3993565	3009976	3880876	3168038
5	40	4025320	3080895	3961786	3177936
6	40	3998771.25	3028636	3927507	3187817
Average		3975666.6	3033891.2	3977028.6	3143144
SD		62750.91	22140.80	69366.48	41712.85
RSD		1.5784	0.7298	1.744	1.327

❖ **Recovery:**

Accuracy of the method was calculated by recovery studies at three levels (80%, 100% and 120%) by standard addition method. The accuracy was expressed as the percentage of the analyte recovered. Accuracy of proposed method was checked as per ICH guidelines. For PPL, tablet powder equivalent to 5 mg PPL was taken individually into three different 100 ml volumetric flasks and then 8 mg (80%), 10 mg (100%) and 12 mg (120%) of standard PPL were added to each of the volumetric flasks. After that 25 ml of the mobile phase [phosphate buffer solution: ACN (7 v/v)] was added to each of the volumetric flask and sonicated for 5 min. The solutions were then filtered and 1 ml of the filtrate from each was taken in 10 ml volumetric flasks individually and diluted upto the mark with mobile phase. The solutions were injected in triplicates into the chromatographic system and the peak area was evaluated to give percent recovery and standard deviation. Similar procedure was repeated for Ritonavir.

Table 3.4: Results of Recovery for ATVR & RTVR

Recovery				
Sr. No	Amount of Sample	Amount of Drug Added	Amount of Drug Recovered	Recovery %
	(µg/ml)	(µg/ml)	(µg/ml)	
1	40	20	19.989	99.95
2	40	40	39.99	99.98
3	40	60	60.01	100.02

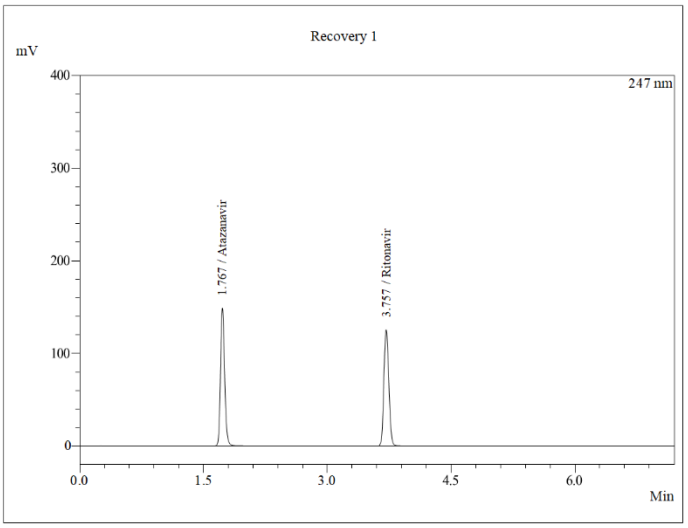


Figure.10

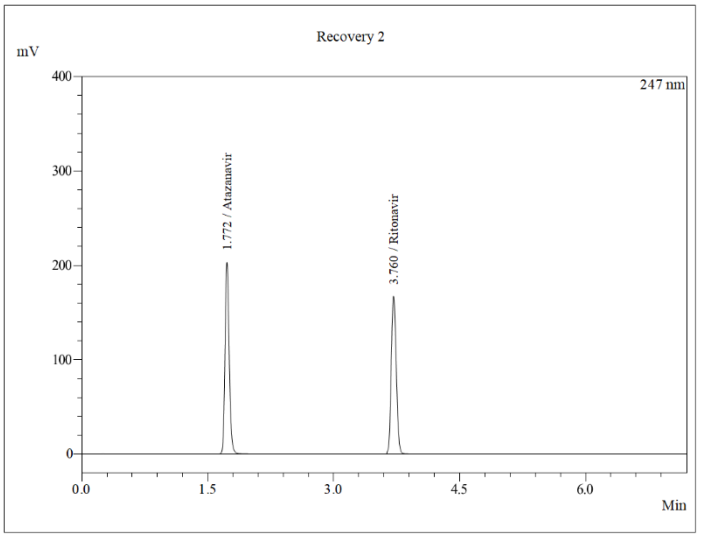


Figure.11

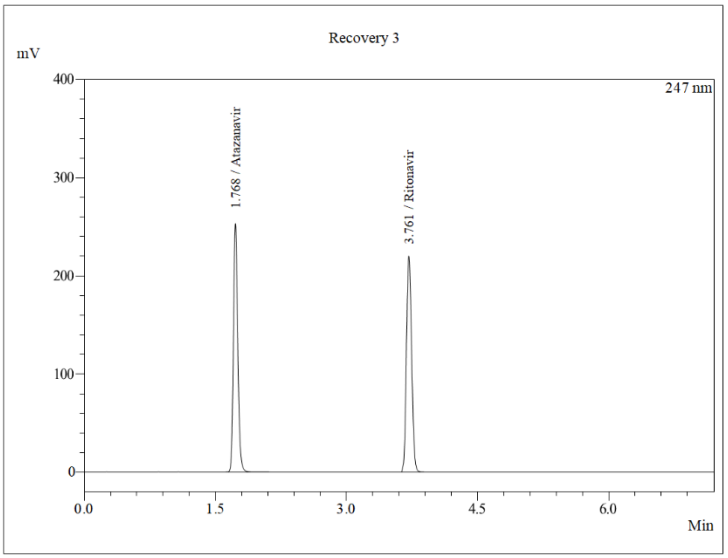


Figure. 12

Robustness: The robustness of the proposed method was verified by varying the solvent ratio in the mobile phase, flow rate and wavelength range. Sample solutions were injected as 10 µl injection into the chromatographic system. The parameters studied were peak area and found their standard deviation & % RSD.

Table 3.5: Robustness for the Atazanavir

Robustness					
Sr. No	**Parameter**		**Response**	**Parameter**	**Response**
Acetonitrile: Buffer			**Retention Time (min)**	**Detection Wavelength**	**Peak Area**
(V/V)				(nm)	
1	78.24	21.76	1.67	245	3903957
2	79.24	20.76	1.767	247	3962272
3	80.24	19.76	1.866	249	3995536
Average			1.768	Average	3953922
Standard Deviation			0.080	Standard Deviation	37850.36
RSD%			4.527	**RSD%**	0.957
Flow Rate			**Retention Time (min)**	**pH of Buffer**	**Peak Area**
(mL/min)				(mmol/L)	
1	0.9		1.898	2.8	3990344
2	1		1.767	3	3962445
3	1.1		1.653	3.2	3886824
Average			1.773	Average	3946538
Standard Deviation			0.1001	Standard Deviation	43733.13
RSD%			5.647	**RSD%**	1.1081

Table 3.6: Robustness for the Ritonavir

Robustness					
Sr. No	**Parameter**		**Response**	**Parameter**	**Response**
Acetonitrile: Buffer			**Retention Time (min)**	**Detection Wavelength**	**Peak Area**
(V/V)				(nm)	
1	69	31	3.659	245	2967521
2	70	30	3.756	247	3025836
3	71	29	3.855	249	3059100
Average			3.757	Average	3017486
Standard Deviation			0.080	Standard Deviation	37850.36
RSD%			2.130	**RSD%**	1.254
Flow Rate			**Retention Time (min)**	**pH of Buffer**	**Peak Area**
(mL/min)				(mmol/L)	

1	0.9	3.887	2.8	3053908
2	1	3.756	3	3026009
3	1.1	3.642	3.2	2950388
Average		3.762	Average	3010102
Standard Deviation		0.1001	Standard Deviation	43733.13
RSD%		2.661	**RSD%**	1.4529

- **Limit of detection and Limit of quantification (LOD, LOQ):** The LOD and LOQ of the proposed method were determined by progressively injecting lower concentrations of the standard solutions under the set chromatographic conditions. L.O.D. = 3.3(SD/S) L.O.Q. = 10(SD/S) Where, SD = Standard deviation of the response, S =Slope of the calibration curve.

Table .3.7

LOD & LOQ (ATVR)		
1	LOD (µg/mL)	0.7527
2	LOQ (µg/mL)	2.2809

Table .3.8

LOD & LOQ (RTVR)		
1	LOD (µg/mL)	0.9852
2	LOQ (µg/mL)	2.9855

4. CONCLUSION

Statistically based experimental designs proved to be an important approach in optimizing selectivity-controlling parameters for the simultaneous determination of Atazanavir and Ritonavir in commercial formulation. The significant factors were optimized by applying Optimal design and response surface methodology. The objective of responses are resolution, capacity factor and the analysis time simultaneously optimized by applying [Derringer's Desirability function] a multi-criteria decision making tool. This method has been evaluated for linearity, precision, accuracy and selectivity, and has proved to be convenient and effective for the quality control of Atazanavir and Ritonavir in raw material and its formulations. The previously reported method addresses only separation of both drugs with traditional approach with longer run time of >10.0 min by using C18 column (5 µm particle size) while our proposed method is able to quantify Atazanavir and Ritonavir within a run time of 8 min.

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