Development and Validation of A Novel UPLC Method for Simultaneous Estimation of Simnotrelvir and Ritonavir in Bulk and Pharmaceutical Dosage Forms

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Abstract:

A simple, accurate, and precise method was developed to estimate Simnotrelvir and Ritonavir in pharmaceutical dosage form. Chromatography is used on a phenyl column (‘2.1 mm x 100 mm, 1.7 µm’) using a mobile phase consisting of 0.1% formic acid and acetonitrile in 60:40 v/v ratio , with a flow rate of 0.5 mL/min . Retention time of Simnotrelvir and ‘Ritonavir’ were 1.113 minutes & 1.875 min and total run time for the method was 2.50 min. Method exhibited good linearity with correlation coefficients (R^2) of ‘0.999’ for both drugs over the concentration ranges of 93.75–562.50 µg/mL for Simnotrelvir and 25.00–150.00 µg/mL for Ritonavir . The optimized method demonstrated good recovery results for both drugs, confirming its accuracy and precision. The method was validated for specificity, linearity , accuracy , precision , robustness, degradation. Results indicate that the method is suitable for general daily quality control analysis of Simnotrelvir. Ritonavir in pharmaceutical formulations.

Keywords: Simnotrelvir, Ritonavir, Development, UPLC.

1. Introduction:

The pharmaceutical medicine Xiannuoxin, also known as Simnotrelvir [1, 2], is used to treat COVID-19. An inhibitor of ‘SARS-CoV-2’ ‘3CLpro’ and a ‘CYP3A’ inhibitor , simnotrelvir/ritonavir is a combination drug [3]. Phase Ib trial's results are at hand. Its median symptom duration reduction in a phase II/III study was 36 hours, compared to placebo [4, 5].

One of the antiretroviral drugs used to treat HIV/AIDS is ritonavir [6, 7], which is sold with brand name Norvir. Acronym HAART indicates "highly active antiretroviral therapy," which describes this combination of drugs. The current primary use of ritonavir, a protease inhibitor, is to increase the effectiveness of other protease inhibitors. Hepatitis C [8–10] and COVID-19 [11, 12] may both be treated with it or a combination of it and

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other drugs. It is taken by orally. Ritonavir often causes the following adverse effects: numbness in the hands and feet, nausea [13], vomiting, lack of appetite, diarrhea [14, 15], and vomiting. Liver problems, pancreatitis [16, 17], allergic reactions [18], and arrhythmias are among the worst adverse effects. Several additional drugs, such as amiodarone and simvastatin, have the potential to cause serious interactions. Use during pregnancy is thought to be safe at modest levels. Ritonavir is a kind of Medicine called protease inhibitor. On the other hand, it is often used to block the enzyme [19] responsible for the metabolism of other protease inhibitors. The later drugs may be administered at lower dosages because to this inhibition.

1.1. ExperimentalChemicals and Reagents: Merck (India) Ltd. of Worli, Mumbai, India, supplies acetonitrile, formic acid, water, and methanol. The reference standards Simnotrelvir (Purity of the drug 99.99%) and Ritonavir (Purity of the drug 99.98%) were acquired from Glenmark, Mumbai.

1.2. Instrumentation:

1.2.1. UPLC conditions: Acquity UPLC systems with quaternary pumps and PDA detectors were used, together with empower software 2.0.

1.2.2. Chromatographic conditions: Using a phenyl column (100mmx2.1mm, 1.7 µ), chromatographic separation was done at temperature in isocratic mode . The mobile phase was 60:40 v/v combination of 0.1 percent formic acid and acetonitrile , and the flow rate is 0.5 ml/min . The 'PDA' detector was used to monitor the eluents at 266 'nm', and the injection volume was 5µl. The duration was 2.50 minutes.

1.3. Stock Preparation and dealing standards:

1.3.1 Ordinary solution Preparation: After carefully measuring out 375 milligrammes of Simnotrelvir and 100 milligrammes of Ritonavir as working standards, pour 70 millilitres of diluents in 100 millilitre volumetric flask. Submerge the flask in a sonicator for 15 minutes to dissolve the components and fill it up to the mark with diluents. Take 5 millilitres of the aforementioned solution into a 50 millilitre volumetric flask & fill to the top with diluents.

1.3.2. Preparation of sample solution: Weigh 46.4 mg of Simnotrelvir and 14.4 mg of Ritonavir and administer to 10 ml volumetric flask . To this, add 7 ml of diluents sonicate 30 minutes to dissolve the components . Finally, dilute the mixture until it reaches the target concentration. Take 5 millilitres of the aforementioned solution into a 50 millilitre 'volumetric flask', fill to the top with diluents and filter the solution using a '0.22 µ' nylon syringe filter.

1.4. Method Development:

From the literature survey there are no analytical methods was established in combination of Simnotrelvir and Ritonavir. The present study was proposed the estimation of Simnotrelvir and Ritonavir using UPLC. In development process many trials were performed using different buffers like 0.1% Ortho phosphoric acid and 0.1% formic acid, finally 0.1% Formic acid was selected as buffer, pH of 0.1% formic acid was

In development process Formic acid ratio was gradually increased with decreasing of acetonitrile at final stage we get good resolution and exists the system suitable conditions.

1.4.1. Method validation: Tests for system were conducted following ICH Q2 R1 standards to the UPLC analytical technique.

1.4.2. System suitability: To evaluate system performance, parameters such as USP tailing, USP plate count, and percentage of relative variance were assessed.

1.4.3. Specificity: To be highly specific, an assay must be able to identify the analyte with absolute certainty even when additional substances, such as those in the standard and sample solutions, are present. By comparing the chromatograms of unspiked and spiked samples with Simnotrelvir and Ritonavir, it was confirmed.

1.4.4. Accuracy: The degree to which the strategy's test results are near to actual value is defined as its accuracy . Three different concentrations were tested in the recovery experiments. At least three injections were given at each level, and data on drug concentration, percentage of recovery, and associated variability recorded.

1.4.5 .Precision : Precision is of three types ,
1.4.5.1. System Precision: Reference standard solution of Simnotrelvir,Ritonavir injected six times and % RSD was calculated .
1.4.5.2. Method Precision: The sample solution of Simnotrelvir and Ritonavir with concentrations of 375, 100 µg/mL were given and %recovery and %RSD were calculated .
1.4.5.3. Intermediate Precision: The sample solutions Simnotrelvir and Ritonavir with concentrations of 375, 100µg/mL were injected in different days by using different columns. Then, % recovery and % RSD were calculated.

1.4.6. Linearity and range: Analytical methods are considered linear if they provide findings that are, within a certain range, directly proportional to analyte concentration in the sample . In order to determine linearity range, six sets of ordinary solutions were chosen. The regression equation is determined by plotting the calibration curve with peak area vs concentration of the quality solution . The slope, intercept, and correlation coefficient were determined using the least squares approach .

1.4.7. LOD and LOQ: LOQ refers to the minimum quantity of analyte that can be determined with acceptable precision and accuracy, whereas LOD refers to the lowest amount of analyte that can be identified in a sample. The limit of detection and limit of ‘quantification’ for Simnotrelvir, Ritonavir were determined by injecting progressively low concentrations of ordinary solutions using the developed UPLC method. Injecting progressively lower concentrations of ordinary solutions using the new UPLC technology allowed us to estimate the LOD and LOQ for Simnotrelvir and Ritonavir. Using the ICH recommendations as a reference, we determined a LOD of 3.3s/n and a LOQ of 10s/n. The signal-to-noise ratio is denoted as s/n.

1.4.8. Stress degradation: The chromatogram of the forced degradation preparations should not show any interference between the peaks produced from stress degradation. The ICH recommendations Q1A (R2) were followed for conducting the stress degradation investigations. For peak purity of primary peaks to pass , the degradation peaks must be sufficiently spaced from one another, with a resolution of at least 1.0. Various

forms of stress were used in forced degradation investigations, which resulted in a deterioration of around 20%.

1.4.9. Robustness: One indicator of an analytical procedure's dependability under typical conditions is its robustness, which is defined as its capacity to remain unaffected by tiny but intentional modifications in 'method parameters'. Investigation was conducted to determine robustness by infusing a standard solution into UPLC systems and adjusting chromatographic parameters such as flow rate (±10%) and organic content within the 'mobile phase (±10%)'. Through analysis of effects of changed parameters, the separation factor, retention duration, and peak symmetry were computed.

2.1. Results and Discussion:

2.1.1. Method development and Optimization: After optimising the chromatographic conditions for specificity, resolution, retention duration, the isocratic condition that resolved Simnotrelvir and Ritonavir using a phenyl column was a mobile phase consisting of 0.1% formic acid and acetonitrile in a 60:40 ratio. The developed chromatogram shows better baseline noise and tailing of peaks having better proportional to the using mobile phase . At 266 nm wavelength the drugs give more absorbance. Figure 1 shows that Simnotrelvir and Ritonavir were eluted with retention times of 1.119 and 1.870 minutes, respectively, in UPLC, confirming the aforementioned values. See Table 1 for a visual representation of the UPLC chromatographic parameters used in technique.

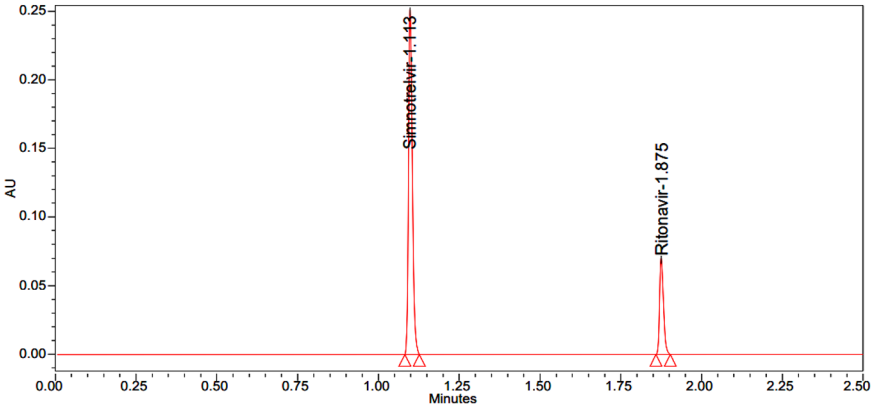

Figure 1: Representative chromatogram of Simnotrelvir and Ritonavir standard

Table 1: UPLC conditions for Simnotrelvir and Ritonavir

Condition	UPLC conditions
Instrument	Waters Acquity
Column	Phenyl (100mmx2.1mm, 1.7µ)
Mobile phase	0.1% Formic acid : acetonitrile (60:40 v/v)

Flow rate	'0.5ml/min'
Injection volume	5µl
Wavelength	266nm
Run time	2.50min
Retention time	Simnotrelvir- 1.119 min Ritonavir- 1.870 min

2.1.2. Method validation: We verified the UPLC technique by standards set forth by ICH guidelines and the proposed industry guidelines for analytical procedures and method validation.

2.1.3. Specificity: By comparing the chromatograms to the blank, we were able to assess the specificity of the assay technique and ensure that it could remove all competing chemicals from the peak findings of Simnotrelvir and Ritonavir (figure 2). The drug contents eluted without any interfering peaks caused by the excipients in the market items, according to the validated procedure.

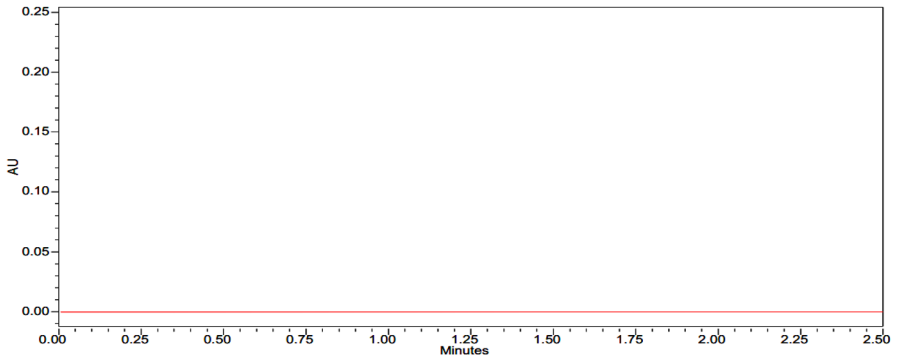

Figure 2: Chromatogram of Blank

2.1.4. System suitability: Standard solution of Simnotrelvir 375µg/ml and Ritonavir 100µg/ml was injected six times record six injections area to calculate % RSD was less than 2.0 and USP tailing is less than 2.0 and USP plate count is more than 2000 for six injections for both Simnotrelvir and Ritonavir in UPLC. The values are tabulated in table 2.

Table 2: System suitability results for Simnotrelvir and Ritonavir

Drug name	USP plate count	USP tailing	USP Resolution	% RSD
Simnotrelvir	8965	0.86	-	0.22
Ritonavir	6324	1.03	5.67	0.16

2.1.5. Linearity and range: Linearity of method is evaluated by preparing a stock solution concentration of 3750 µg/ml of Simnotrelvir and 1000 µg/ml of Ritonavir. The

solutions were diluted in a certain order to achieve concentrations of 25%, 50%, 75%, 100%, 125%, and 150%. The peak regions are used to draw calibration curves against the concentrations since they were injected into UPLC systems. For those analytes, the correlation coefficient was 0.999. In figure 3, you can see the average standard calibration curve from table 3.

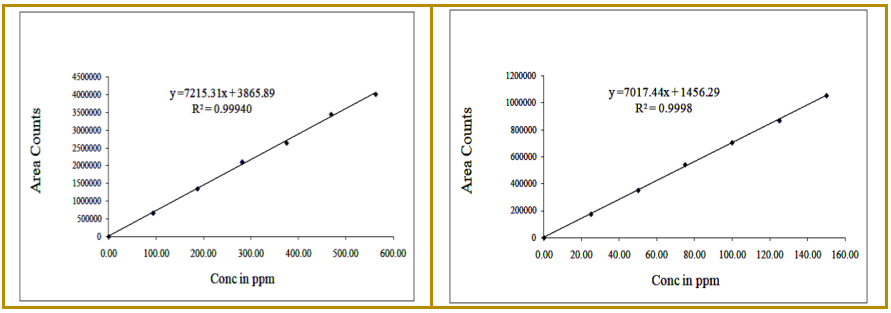

Figure 3: Linearity plot of Simnotrelvir and Ritonavir by UPLC

Table 3: Linearity data of Simnotrelvir and Ritonavir

Linearity	Simnotrelvir		Ritonavir	
	Conc. (µg/ml)	Area	Conc. (µg/ml)	Area
Linearity-1	93.75	661818	25.00	173257
Linearity-2	187.50	1348822	50.00	350216
Linearity-3	281.25	2103374	75.00	541241
Linearity-4	375.00	2645546	100.00	705201
Linearity-5	468.75	3450828	125.00	869568
Linearity-6	562.50	4021815	150.00	1054867
Slope		7215.31		7017.44
Intercept		3865.89		1456.29
Correlation Co-efficient		0.99940		0.99983

2.1.6. LOD and LOQ: The analyte is typically consistently detectable at a minimum concentration level known as the limit of detection and quantification . Estimated using quality-related models. Both Simnotrelvir and Ritonavir had limit-of-detection (LOD) values of 0.675 µg/ml and 0.180 µg/ml, respectively, whereas Ritonavir had LOQ values of 2.250 µg/ml and 0.600 µg/ml.

2.1.7. Accuracy: Table 4 shows three concentration levels 50%, 100%, and 150% were used in the recovery trials that determined the accuracy. APIs containing Simnotrelvir at concentrations of 187.5µg/ml, 375.0µg/ml, and 562.5 µg/ml, and Ritonavir at concentrations of 50.0 µg/ml , 100.0 µg/ml , and 150.0 µg/ml , were produced. Following the protocol, the assay was carried out by injecting the test solution into three

different preparations, one at each spike level. The share recovery figures were determined to fall anywhere between 98% and 102%.

Table 4: Accuracy results of Simnotrelvir and Ritonavir

	UPLC	
% of target concentration	Simnotrelvir (% Recovery)	Ritonavir % Recovery)
50%	99.5	100.4
100%	99.9	100.0
150%	100.0	100.4
Mean % accuracy	99.8	100.3

2.1.8. Precision: Table 5 shows precision was established in two types namely method (intraday) precision and intermediate (inter-day) precision. In order to determine the method's accuracy, six independently produced samples from the same batch were analyzed. The six individual sample solutions were injected into the UPLC system, and resulting peak areas were used to determine the mean assay and percent RSD values . Experiment results showed that the method's % assay precision ranged from 98% to 102% and that the % RSD was below 2.0%.

The instrument, column, and analyst modifications all achieved intermediate precision. Six independently manufactured samples from the same batch were analyzed to assess intermediate precision. In order to determine the mean assay and % RSD values, UPLC was used to inject six different sample solutions and measure the peak areas.

Table 5: Precision results using UPLC

		UPLC			
Analyte	Conc. (µg/ml)	Method precision		Intermediate precision	
		% Assay	% RSD	% Assay	% RSD
Simnotrelvir	375	99.8	0.45	99.9	0.36
Ritonavir	100	100.2	0.34	99.9	0.30

2.1.9. Robustness: The assay's robustness should be between 98% and 102%. The optimized chromatographic settings eliminated minor deviations, such as proportion of 'organic matter' in the mobile phase of around 10% and a flow of about ±10% from table 6.

Table 6. Robustness results using UPLC

Drug name	Flow plus (1.1 ml/min)	Flow minus (0.9 ml/min)	Org plus (44:56)	Org minus (36:64)
	% Assay			
Simnotrelvir	100.2	99.7	99.8	100.2
Ritonavir	99.2	99.7	100.0	99.7

2.1.10. Forced degradation: This strategy has been tested in forced degradation tests, which means it works for deteriorated goods. These studies tell us when the medicine is unstable, so we can take precautions during formulation to make sure it doesn't happen.

2.1.11. Acid degradation: To ten ml volumetric flask , add 46.4 mg of Simnotrelvir .14.4 mg of Ritonavir, then, after 30 minutes in room temperature, add 1 ml of 1N HCl. Finally, add 1 ml of 1N NaOH , and dilute to volume with diluent. Make sure to weigh the samples accurately.

2.1.12. Alkali degradation: Combine 46.4 mg of Simnotrelvir and 14.4 mg of Ritonavir in a 10 ml volumetric flask after accurately weighing samples. Add 1 ml of 1N NaOH and let it sit at room temperature for 30 minutes. Then, add 1 ml of 1N HCl and dilute the volume with diluent.

2.1.13. Peroxide degradation: To a 10 millilitre volumetric flask, add 46.4 milligrammes of Simnotrelvir and 14.4 milligrammes of Ritonavir, then add 1 millilitre of 10% H2O2. Stir and let sit at room temperature for 30 minutes & can be diluted.

2.1.14. Reduction degradation: Measure out 46.4 milligrammes of Simnotrelvir and 14.4 milligrammes of Ritonavir, and then add 1 millilitre of a 10% sodium bi sulphite solution. Stir and let sit at room temperature for 30 minutes & can be diluted.

2.1.15. Thermal degradation: The exposed standards were examined after being subjected to 105°C for 6 hours; the standards for Ritonavir (150 mg) and Simnotrelvir (400 mg) were also tested. In a 100 ml volumetric flask, dissolve 375 mg of Simnotrelvir exposed standard and 100 mg of Ritonavir exposed standard to volume using diluents. Add 5 ml to 50 ml of water and mix well.

2.1.16. Photolytic degradation: The exposed standards were examined after 6 hours of exposure to a photo stability chamber containing 400 mg of Simnotrelvir and 150 mg of Ritonavir. In a 100 ml volumetric flask , dissolve 375 mg of Simnotrelvir exposed standard and 100 mg of Ritonavir exposed standard to volume using diluents. Add 5 ml to 50 ml of water and mix well. Photostability chambers conditions are white fluorescent light exposure of minimum 1.2 million lux hours and integrated near UV energy of at least '200 watt hours/square meter'.

2.1.17. Hydrolysis degradation: From table 7 and 8 Weigh the Simnotrelvir and Ritonavir samples accurately and transfer 46.4 mg and 14.4 mg, respectively, in a 10 ml volumetric flask . Add 1 ml of HPLC water and set in RT for 30 minutes & can be diluted.

Table 7: Forced degradation results of Simnotrelvir

Degradation condition	% Assay	% deg
Control	100	0
Acid deg	88.7	11.3
Alkali deg	88.0	12.0
Peroxide deg	85.1	14.9
Reduction	99.6	0.4
Thermal	99.1	0.9
Photolytic	97.6	2.4
Hydrolysis	96.9	3.1

Table 8: Forced degradation results of Ritonavir

Degradation condition	% Assay	% deg
Control	100	0
Acid deg	98.1	1.9
Alkali deg	89.4	10.6
Peroxide deg	85.1	14.9
Reduction	97.5	2.5
Thermal	95.6	4.4
Photolytic	99.1	0.9
Hydrolysis	99.5	0.5

3. Conclusion:

The developed method for UPLC exists for all variation parameters as per ICH guidelines. They have good resolution between Simnotrelvir and Ritonavir and short run time. Simnotrelvir and Ritonavir were successfully determined using an isocratic approach, which is both accurate and dependable. Based on the measured height area, curve forecast medication concentration of Simnotrelvir within 93.75-562.50 µg/ml and Ritonavir within the range of 25.00-150.00 µg/ml. The approach was verified and used to identify Simnotrelvir and Ritonavir in a typical commercial formulation in a reliable, sensitive, robust, and specific.

4. Authors Contribution:

All authors contributed equally.

5. Acknowledgement:

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