A NOVEL AND ROBUST ANALYTICAL TECHNIQUE FOR DETERMINING COVID-19 MEDICATIONS USED IN EMERGENCIES

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Abstract :

To treat mild to moderate COVID-19, an investigational drug called nirmatrelvir in combination with ritonavir is being researched for which the potential hazards with this are still unknown. Nirmatrelvir has been approved for immediate use by the US Food and drug intake in conjunction with the drug ritonavir for the treatment of mild to medium COVID-19 in grown ups and individuals of more than 12 years who test positive for the virus and are at a high risk to develop severe COVID-19.To quantify the drugs simultaneously in tablet dosage forms, a novel, sensitive and reproducible reverse phase liquid chromatography method has been developed. Thechromatographic separation was performed using Phenomenex (250×4.6mm,5µ particle size) column. The separation and elution were carried out at an ambient temperature using a mobile phase consisting of 0.1% trifluoro acetic acid &acetonitrile in the ratio of 50:50%v/v. The maximum absorbance by UV spectrophotometer shown at wavelength 258.3nm& 271.4nm for nirmatrelvir and ritonavir. Also, 266nm was selected as detector wavelength by a photodiode array detector for the HPLC chromatrographic method.Beer lambert's law obeyed in the linear range of 37.5-225µg/mL(R^2=0.9998) for nirmatrelvir and 25-150 µg/mL(R^2=0.9994) for ritonavir. The method shows method and system precision with % RSD less than 1%.The percentage mean recovery was found to be 99.9-100.2%& 100.0-100.2%. The LOD 1.5 µg/mL &1 µg/mL values indicates the method sensitivity. The proposed stability indicating method was validated for precision,accuracy, specificity, selectivity, robustness and stability studies according to ICH guidelines.

Keywords: RP-HPLC, Nirmatrelvir, Ritonavir, Trifluoro acetic acid, Stability studies.
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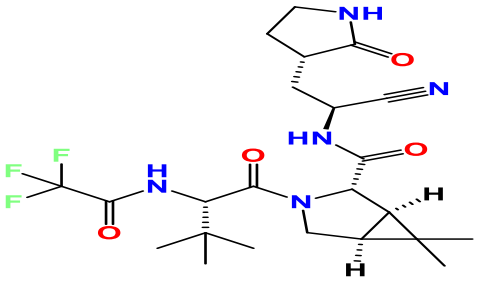
INTRODUCTION

Chemically, Nirmatrelvir[1,2] $C_{23}H_{32}F_3N_5O_4$, (1*R*,2*S*,5*S*)-*N*-[(1*S*)-1-cyano-2-[(3*S*)-2-oxopyrrolidin-3-yl]ethyl]-3-[(2*S*)-3,3-dimethyl-2-[(2,2,2-trifluoroacetyl)amino]butanoyl]-6,6-dimethyl-3-azabicyclo[3.1.0]hexane-2-carboxamide. It is soluble in 1-butanol, methyl isobutyl ketone. The median T_{max} of nirmatrelvir, when given with ritonavir, is 3 hours. Ritonavir[3,4] is known as 10-hydroxy-2-methyl-5-(1-methylethyl)-1- [2-(1-methylethyl)-4thiazolyl]-3,6-dioxo-8,11-bis(phenylmethyl)-2,4,7,12 tetraazatridecan-13-oic acid, 5-thiazolylmethyl ester. Almost insoluble in water, it is easily soluble in methanol, ethanol, and isopropanol.

An oral protease inhibitor called nirmatrelvir is effective against MPRO, a viral protease that cleaves the two viral polyproteins and is crucial for viral replication. All coronaviruses known to cause human infection have been resistant to it's antiviral activity. As Paxlovid, ritonavir, a potent cytochrome P450 (CYP) 3A4 inhibitor and pharmacokinetic booster used to augment HIV protease inhibitors, is packed with nirmatrelvir. To raise nirmatrelvir concentrations to the desired therapeutic range, ritonavir must be coadministered.The drug ritonavir alone has no effect on SARS-CoV-2. The Food and Drug Administration (FDA) authorised the use of ritonavir-boosted nirmatrelvir for the management of COVID-19 on December 22, 2021.

Starting as soon as feasible after a COVID-19 diagnosis and within five days of the onset of symptoms, the suggested regimen is two tablets of nirmatrelvir (150 mg) and one tablet of ritonavir (100 mg) taken simultaneously every 12 hours for five days.The tablets should not be chewed, broken, or crushed; rather, they should be swallowed whole, with or without food. Due to ritonavir's[5,6] ability to stop CYP3A4 from utilising the drug to break it down, nirmatrelvir is largely excreted intact in urine and faeces. When coupled with ritonavir, nirmatrelvir has a mean half-life of seven hours. Nirmatrelvir[7,8] should be administered at a lower dose to people who have mild renal impairment, although there have been no clinical studies on this modified regimen. It is not advised for people to take this combination if they have significant renal or hepatic impairment. Nirmatrelvir and ritonavir should not be used by patients who are taking drugs that are significantly metabolised by CYP3A or drugs that are strong CYP3A inducers.

Using phosphate buffers and methanol as the mobile phase, there are a few HPLC methods for estimating ritonavir both alone[9] and in combination[10-12] with other medications such lopinavir, paritaprevir, and atazanavir. Recently, two techniques for estimating nirmatrelvir and ritonavir simultaneously in human plasma[13,14,15] for pharmacokinetic research employing formic acid as a buffer in conjunction with methanol were published. According to a literature review, there is no HPLC method available for simultaneously estimating nirmatrelvir and ritonavir in bulk and its dose form. Our current study focuses on creating a quick, affordable, and sensitive HPLC approach for the concurrent measurement of ritonavir andnirmatrelvir as well as validating the method in accordance with ICH recommendations[16].



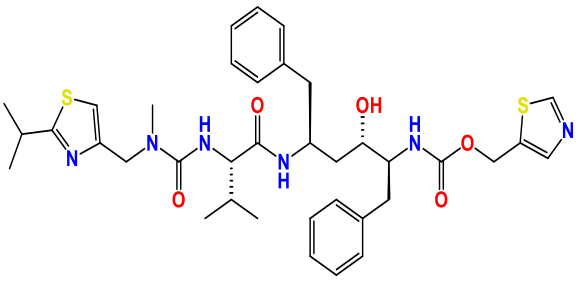


Figure 1 Structure of Nirmatrelvir

Figure 2 Structure of Ritonavir

MATERIALS AND METHODS

Solvents and buffer solutions:

The gratis samples of Nirmatrelvir and Ritonavir were provided by Icon labs (R&Dsamples) while the tablet formulation (Paxlovid with composition of Nirmatrelvir-150mg and Ritonavir-100mg) was brought from local pharmacy. Methanol, acetonitrile, water (HPLC grade of merck) is of HPLC grade. All the chemicals for buffers and diluents used in the trials and optimized method is of analytical grade.

Instruments : A waters RP-HPLC instrument equipped with software (empower 2695, 2.0 version)with PDA detector and auto vial injector , long columns of symmetry and phenomenex C18 (250X4.6mm, i.d. 5µm particle size) in an isocratic mode is employed. The UV/Vis spectrophotometer used was of shimadzu and UV 1700 model. All the glassware employed is of Borosil make and ultra sonicator of UCA701 , Unichrome. Sartorious weighing balance and Eutech pH meter is used in developing the analytical method.

Optimisation of the method:

Nirmatrelvir and Ritonavir were separated chromatographically and quantitatively for analysis using the Alliance model and e 2695-Empower manufacturer from Waters. A quaternary solvent pump, an auto-sampler, and a PDA detector make up the system. The software 2.0 version was used to monitor and analyse the chromatographic signals. On a Phenomenex C18 (250x4.6 mm, 5µ) column, the analytes were separated. By combining 0.1% trifluoroacetic acid and acetonitrile in a 50:50 v/v ratio, the isocratic mobile phase system was created. Eluents were observed at 266 nm wavelength while the flow rate of the mobile phase was changed to 1 ml/min. The mobile phase was used to create the diluted solutions for the sample and the standard. 10 µL was the set injection volume.

Solution Preparation

The standard stock solutions of Nirmatrelvir and Ritonavir were prepared by dissolving individually 15mg of Nirmatrelvir, 10 mg of Ritonavir of the drug in a 10 ml clean dry volumetric flasks which contain an aliquot of acetonitrile.Add the mobile phase as diluent(50:50v/v ACN and TFA) and sonicate to dissolve it completely and make volume up to the mark with the same solvent. Then Pippete in a 10 ml volumetric flask, add 1 ml of the aforementioned stock solutions and diluent to the desired concentration. (Ritonavir 100 ppm, Nirmatrelvir 150 ppm). Prior to being injected into the chromatographic apparatus, each solution was filtered.

Validation:

System suitability: System suitability study was performed to indicate that the system is functioning perfectly before the analysis and this is essential to check the specifications of LC system. The parameters that can be verified using this study are resolution, retention time, theoretical plate number and tailing factor. The test was performed by injecting the standard concentration of nirmatrelvir and ritonavir at various volumes of injection from 10 to 50 µL

Linearity: The linear relationship was observed in the range of 37.5-225 µg/mL and 25-150 µg/mL for nirmatrelvir and ritonavir respectively, the drugs were determined simultaneously by calibration curve method.

Precision: Precision was defined in terms of repeatability and reproducibility for the given measurement. This study was performed by injecting standard concentration of 150µg/mL of nirmatrelvir and 100 µg/mL of ritonavir six replicates (n=6).

Accuracy: Accuracy was performed to verify the recovery studies of active pharmaceutical ingredient. The studies were performed by standard addition method at three different

levels of concentration (80 %, 100 % and 120 %) which covers the range of linearity.

Specificity: Specificity shows the identity of the analyte in the pharmaceutical formulation in which the excipient components does not show interference. It is achieved by analyzing the chromatograms of blank, placebo, active pharmaceutical ingredient and the formulation.

LOD and LOQ: The LOD and LOQ are helpful to express the least concentration of the analyte that can be quantified by the optimized method. These values were estimated by the standard deviation of the responses (σ) and the mean of the slopes (m) of standard calibration curve. The values were calculated by the formulae LOD = 3.3 σ/m and LOQ = 10 σ /m .

RESULTS AND DISCUSSION

Method Development

The separation of all the analytes present in the bulk mixture and the formulation with acceptable resolution is the major goals for the analytical procedure. The chromatographic settings were chosen and tuned based on the physicochemical characteristics and chemical structure.Various trials were conducted in which initially on symmetry column(250×4.6mm,5µm) with acetonitrile and phosphate buffer (80:20v/v) as mobile phase but system suitability is not there with plate count.in the next trial two trials , mobile phase ratio changed to 70:30v/v , but baseline is not achieved and the interference is observed .Now the mobile phase changed to triflouro acetic acid with acetonitrile on phenomenex column (250×4.6mm, 5µm) and found that the response of peaks is very low.The successful separation with the satisfactory peak parameters was achieved using an isocratic mobile phase system, with an injection volume of 10µL containing 0.1% trifluoro acetic acid & acetonitrile in the ratio of 50:50%v/v on Phenomenex (250×4.6mm,5µm) column at a flow rate of 1 mL/min. To demonstrate the suitability and repeatability of the chromatographic system used to determine nirmatrelvir and ritonavir in tablet formulation, parameters including plate count, tailing factor, resolution, and reproducibility (% RSD retention time and area for repetitive injections) are calculated and compared to the guidelines established for the method. Nirmatrelvir peak was observed at 2.873 min with peak area 2152885, tailing factor 1.04, and ritonavir peak was observed at 4.527 min with peak area 1437372, tailing factor 0.99. The resolution was attained at 8.73.

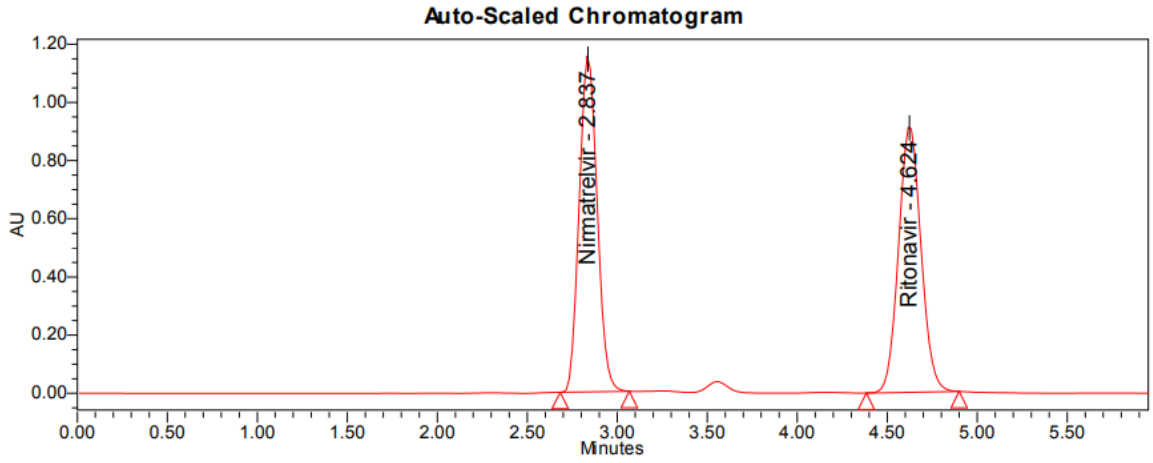


Figure-3: Optimised chromatogram

System suitability

Parameters such as plate count, tailing factor, resolution and reproducibility are determined

and compared against the acceptance limits as per USP. This trial was optimized. The finalized conditions were optimized and preparation of solutions was done by increasing the injection volumes from 10µL to 50µL for method validation

Table 1: System suitability parameters of the optimized chromatogram

S.no	Parameter	Nirmatrelvir	Ritonavir
1	Retention time	2.876	**4.522**
2	Plate count	3651	**8850**
3	Tailing factor	1.07	**0.96**
4	Resolution	----	**8.77**
5	%RSD	0.39	0.44

Specificity

Ritonavir and Nirmatrelvir had retention times of 2.873 minutes and 4.527 minutes, respectively. At the retention durations of these medications using this approach, we did not detect any interfering peaks in the blank or placebo samples. Thus, it was claimed that this procedure was specific.The peak purity plot compares each peak purity plots spectrum in an integrated peak to the spectrum at the peak apex. Peak purity is done on the instrument by comparison of different rates of the spectrum. This is ratio, which measured the absorbance attwo wavelengths, then divides the absorbance at one wave length by the absorbance at another. If the peak is pure, the ratio is a constant value.

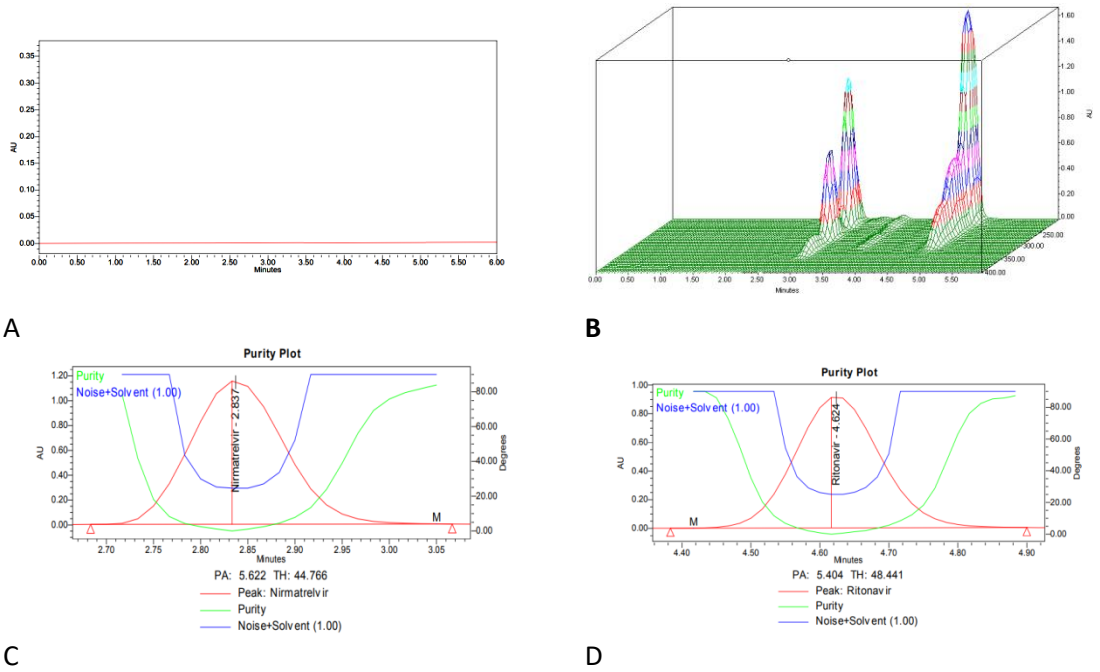


Figure 4: (A).Chromatogram of Blank, (B).3D chromatogram,

(C). Peak purity plot of nirmatrelvir,(D). ritonavir

Linearity

The plot of peak area vs. concentration was linear at a range of 37.5- 225 µg/mL (37.5, 75, 112.5, 150, 187.5 and 225 µg/mL) for nirmatrelvir and 25- 150 µg/mL (25, 50, 75, 100, 125, 150 µg/mL) for ritonavir. The linear regression analysis data are summarized as below.

Table -2 : Results of linearity for Nirmatrelvir & Ritonavir(n=3)

S.NO	Nirmatrelvir		Ritonavir	
	Conc.(µg/ml)	Peak area	Conc.(µg/ml)	Peak area
1	37.50	558753	25.00	359516
2	75.00	1071560	50.00	720128
3	112.50	1662175	75.00	1110473
4	150.00	2166503	100.00	1437233
5	187.50	2687600	125.00	1749370
6	225.00	3198881	150.00	2081243
Regression equation	y = 14237.41x +19073.14		y =13915.06x + 21793.79	
Slope	14237.41		13915.06	
Intercept	19073.14		21793.79	
R^2	0.9998		0.9994	

Precision

The precision of the instrument & method was checked by repeatedly injecting (n=6) solutions of 150ppm of Nirmatrelvir, 100ppm of Ritonavir. The value of RSD found to be 0.39% and 0.44% which is less than 1 indicates the reproducibility of the method.

Accuracy

The results for the recovery studies by the developed method are shown as below. The higher values of the 98 to 102% recovery confirms the accuracy of developed method.

Table- 3 : Accuracy (Recovery) results of Nirmatrelvir and Ritonavir (n=3)

% Concentration (at specification Level)	% Recovery of Nirmatrelvir	% Recovery of Ritonavir
50%	100.0	100.1
100%	100.2	100.0
150%	99.9	100.2

Robustness

Variable method parameters like temperature, pH, ionic strength, or the percentage of an organic solvent are used to assess a method's robustness. The acceptance criteria also include determining whether there is any impact on the method's results and the applicability of the system.

Table-4 : Robustness results of Nirmatrelvir and Ritonavir by RP-HPLC

PARAMETER	CONDITION	NIRMATRELVIR			RITONAVIR		
		R_t (min)	Tailing factor	Plate count (N)	R_t (min)	Tailing factor	Plate count(N)
Flow rate(ml/min)	0.8	3.025	1.06	3728	4.698	1.05	8948
	1.0	2.873	1.04	3646	4.527	0.99	8854
	1.2	2.771	1.00	3602	4.459	0.92	8813
Organic Phase	Less org (45:55)v/v	3.137	1.11	3759	4.762	1.10	8971
	(50:50)v/v	2.876	1.03	3651	4.522	0.96	8850
	(55:45)v/v	2.544	0.97	3574	4.280	0.90	8792

LOD and LOQ

LOD and LOQ values for Nirmatrelvir and Ritonavir are given as below.

Table 5: LOD & LOQ for nirmatrelvir and ritonavir

Sl.No.	Name of drug	LOD(µg/ml)	LOQ(µg/ml)
1	Nirmatrelvir	1.5	4.5
2	Ritonavir	1	3

Assay:

Nirmatrelvir and ritonavir samples were precisely weighed and transferred into a 10 mL clean, dry volumetric flask for the preparation of the test solution. Diluent was added, the mixture was sonicated for three minutes to dissolve it, and then centrifuged for 30 minutes to completely dissolve it and make the volume up to the required level with the same solvent. The 0.45 injection filter is then used to filter it. Pipette 1 ml of the aforementioned stock solutions into a 10 ml volumetric flask, and then add diluent to the mark. (Ritonavir 100ppm, Nirmatrelvir 150ppm). The blank solution, also known as the placebo, was a mobile phase made by mixing 0.1% TFA and ACN in a 50:50 v/v ratio without the addition of any medicinal ingredients. The peak area of the standard chromatogram and the sample were used to estimate the amount of medication present in each unit formulation. It is discovered that 99.9% to 100% of the medication is present in the formulation using the optimised approach. (n=3)

Degradation studies:

The same optimised HPLC settings were used for forced degradation tests on nirmatrelvir and ritonavir. Degradation was found to be less than 30% for both medications, which shows that the proportion of degradation is likewise within US-FDA criteria. The principal peak is clearly distinguished from the degradant, placebo, and diluent peaks, and its strength is relatively low.

Table6 : Forced Degradation results for Nirmatrelvir and Ritonavir (n=3)

	Nirmatrelvir		Ritonavir	
% Degradation results	**Area**	**% Degradation**	**Area**	**% Degradation**

Control	2157823	0	1428118	0
Acid	1843017	14.5	1260512	11.8
Alkali	1861075	13.7	1252849	12.3
Peroxide	1803507	16.4	1222925	14.4
Reduction	1904461	11.7	1280976	10.3
Hydrolysis	2120156	1.7	1418154	0.7

Biological and pharmaceutical samples are evaluated using the HPLC technology, which is significant for both quantitative and qualitative analysis. Due to the lack of a published method for the simultaneous estimation of nirmatrelvir and ritonavir, a straightforward, inexpensive, and reliable method was created using acetonitrile and triflouroacetic acid, for which all of the system suitability parameters were in good agreement with USP guidelines.The sample recoveries were in line with the claims made on their respective labels, and formulation excipients did not interfere with the estimation, this method can be employed in laboratories for regular drug examination. As the system validation characteristics of the optimised HPLC method used for the estimation of Nirmatrelvir & Ritonavir in pure and formulation have demonstrated specific, precise, accurate, and robust results, it is inferred that the method will be most beneficial and recoverable. Also, the current work can be the stability indicating method by RP-HPLCwhich is easy to use, accurate, exact, and specific, and it did not interfere with the placebo or degradation products.

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CONFLICTS OF INTEREST: No conflicts

REFERENCES

1. https://go.drugbank.com/drugs/DB16691
2. https://pubchem.ncbi.nlm.nih.gov/compound/Nirmatrelvir
3. https://pubchem.ncbi.nlm.nih.gov/compound/Ritonavir
4. https://go.drugbank.com/drugs/DB00503
5. Toussi SS, Neutel JM, Navarro J, et al. Pharmacokinetics of Oral Nirmatrelvir/Ritonavir, a Protease Inhibitor for Treatment of COVID-19, in Subjects With Renal Impairment. Clinical Pharmacology and Therapeutics. 2022 Oct;112(4):892-900. DOI: 10.1002/cpt.2688. PMID: 35712797; PMCID: PMC9349773.
6. Marzolini C, Kuritzkes DR, Marra F, Boyle A, Gibbons S, Flexner C, Pozniak A, Boffito M, Waters L, Burger D, Back DJ, Khoo S. Recommendations for the Management of Drug-Drug Interactions Between the COVID-19 Antiviral Nirmatrelvir/Ritonavir (Paxlovid) and Comedications. Clin Pharmacol Ther. 2022 Dec;112(6):1191-1200. doi: 10.1002/cpt.2646. Epub 2022 Jun 7. PMID: 35567754; PMCID: PMC9348462.
7. Reis S, Metzendorf MI, Kuehn R, Popp M, Gagyor I, Kranke P, Meybohm P, Skoetz N, Weibel S. Nirmatrelvir combined with ritonavir for preventing and treating COVID-19. Cochrane Database Syst Rev. 2022 Sep 20;9(9):CD015395. doi: 10.1002/14651858.CD015395.
8. AusPA R Final 24Paxlovidnirmatrelvir/ritonavirPfizer Australia Pty LtdPM20210488012 January 2022

9. Chiranjeevi and Channabasavaraj, Development and validation of RP-HPLC method for quantitative estimation of ritonavir in bulk and pharmaceutical dosage forms ;International Journal of Pharmaceutical Science and Research , 2011; vol. 2(3): 596-600
10. Rathnasamy R, Karuvalam RP, Pakkath R, Kamalakannan P, Sivasubramanian A. RP-HPLC Method Development and Method Validation of Lopinavir and Ritonavir in Pharmaceutical Dosage Form. Am J Clin Microbiol Antimicrob. 2018; 1(1): 1002.
11. M.P.Gandhvi, A.Bhandri, B.N suhagia, U.H.Desai, Developed and validation of RP-HPLC method for simultaneous estimation of Atazanavir and Ritonavir in their combined tablet dosage forms: Research journal of Pharmacy and Technology, Volume 6, Issue 2, Year 2013.
12. Gnana Deepika killi, Ramakrishna maddenapudi, Satish Kumar dinakaran, Avasarala barani. A novel validated UPLC method for quantitation of lopinavir and Ritonavir in bulk drug and pharmaceutical formulations with its impurities. Brazilian journal of pharmaceutical sciences. volume 50,June 2014.
13. Liu C, Zhu M, Cao L, Boucetta H, Song M, Hang T, Lu Y. Simultaneous determination of nirmatrelvir and ritonavir in human plasma using LC-MS/MS and its pharmacokinetic application in healthy Chinese volunteers. Biomed Chromatogr. 2022 Nov;36(11):e5456. doi: 10.1002/bmc.5456.
14. Guyon J, Novion M, Fulda V, Ducint D, Molimard M, Couzi L, Kaminski H, Salvo F, Bouchet S. A UPLC-MS/MS Method for Plasma Biological Monitoring of Nirmatrelvir and Ritonavir in the Context of SARS-CoV-2 Infection and Application to a Case. J Am Soc Mass Spectrom. 2022 Oct 5;33(10):1975-1981. doi: 10.1021/jasms.2c00204
15. Martens-Lobenhoffer J, Böger CR, Kielstein J, Bode-Böger SM. Simultaneous quantification of nirmatrelvir and ritonavir by LC-MS/MS in patients treated for COVID-19. J Chromatogr B Analyt Technol Biomed Life Sci. 2022 Dec 1;1212:123510. doi: 10.1016/j.jchromb.2022.123510
16. International conference on harmonization of technical requirements for registration of pharmaceutical human use column validation of analytical procedures Q2 (R1), 1994, 2007