RESEARCH ARTICLE

Bioanalytical Method Development and Validation for Determination of Nirmatrelvir in Human Plasma using LC-MS/MS

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ABSTRACT

The term "antivirals" has been associated with COVID-19 during the period when the usage of this specific class of medications has increased. Nirmatrelvir, a protease inhibitor, is used to treat mild to moderate COVID-19 symptoms by stopping SARS-COV-2 reproduction. With deucravacitinib serving as the internal standard, liquid chromatography-tandem mass spectroscopy in human plasma was used to provide a quick, simple, innovative, trustworthy, and sensitive approach. Liquid-liquid extraction was used to separate nirmatrelvir and the internal standard. The extracted sample was then run through a chromatographic system with ACE-C18 column (4.6 × 100 mm, 5 µm); and mobile phase with methanol and 2 mM ammonium formate in a ratio of 80:20, and a flow rate of 1.00 mL/min. The system operates for three minutes in multiple reaction monitoring mode at the ABSCIEX API 4000 mass spectrometer using electron spray ionization. Nirmatrelvir ion transitions are 500.10 to 110.10, while deucravacitinib are 426.30 to 358.20. The validation was conducted using a concentration range of 5.00 to 4000 ng/mL, and the results showed that the selectivity, accuracy, precision, linearity, and selectivity were all within the acceptability limits.

Keywords: Nirmatrelvir, Deucravacitinib, Liquid-liquid extraction, Human plasma, methanol, Ammonium formate.

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INTRODUCTION

Coronavirus is a disease caused by SARS-COV- 2, where the severe illness of this disease causes organ damage, inflammation and problems with the immune system.[1] The symptoms begin to appear from 1 to 40 days, in which most of the patients develop mild to moderate symptoms, while 14% will develop as severe. During this pandemic, the non-availability of proper medicines causes more damage to the health of patients.[2] Then some of the existing medicines were use based on the severity of effects while some are developed.[3] Among these nirmatrelvir in combination with ritonavir, received emergency authorization in December 2021.[4,5] but later on May 25 2023 FDA approved it for treating hospitalized patients.[6] and also for patients who are at high risk of disease progression.[5]

As the first oral medication administered in conjunction with ritonavir for the treatment of COVID-19, nirmatrelvir is an azabicyclohexane. It is a protease inhibitor and an anti-corona viral agent. Nirmatrelvir, specifically targets, binds to and inhibits the action of SARS-CoV-2 M pro after oral dosing. This inhibits the proteolytic cleavage of viral polyproteins, thereby inhibiting the formation of viral proteins, including helicase, single-stranded-RNA-binding protein, RNA-dependent RNA polymerase, 20-O-ribose methyl transferase, endoribonuclease and exoribonuclease. This prevents viral transcription and replication.[7,8]

Being the first oral drug used for COVID 19 new methodologies require estimating the drug concentrations in biological fluids for its pharmacokinetic and pharmacodynamic actions.[9] The determination of plasma concentration is very

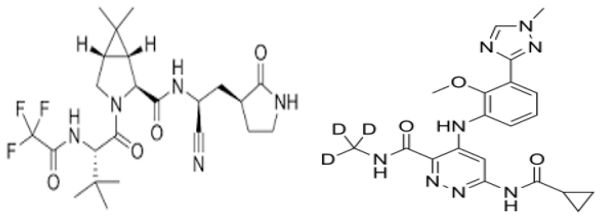

Figure 1: Structure of (A) nirmatrelvir and (B) deucravacitinib

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important for therapeutic drug monitoring to ensure drug safety and use. The simultaneous estimation of nirmatrelvir and ritonavir bio-analytical methods are available, but in this method, nirmatrelvir estimation is developed and validated. As a result, the suggested approach is very repeatable, dependable, sensitive, and selective when using LC-MS/MS.[10] The main benefit of using liquid chromatography-tandem mass spectroscopy (LC-MS/MS) is its exceptional sensitivity and selectivity. On the other hand, the primary flaw of MS/MS detection is the inability to replicate the results in terms of matrix effects. Because of this, the use of internal standards is used in LC-MS/MS to circumvent a few issues, such as those pertaining to ionization efficiency, injection volume, recovery, along fluctuations in the matrix effect. The application of the internal standard is a crucial element in enhancing the choice of an efficient internal standard; this choice has to be predicated on the target analyte's physicochemical properties in order to take matrix effects into account and account for potential variances in ionization efficiency. Deucravacitinib is a free base with readily accessible to the fundamental functional groups for the purposes of extraction and ionization. Its non-polar characteristics make it more susceptible to matrix effects problems. Naturally, lipophilic, deucravacitinib and nirmatrelvir are both pharmaceuticals.[11] Figure 1 represents the structures of Nirmatrelvir and Deucravacitinib.

MATERIALS AND METHODS

Instrumentation

The mass spectrometer used for the investigation was the SCIEX API 4000 mass spectrometer (from AB Sciex LLC, Framingham City, MA, USA), interfaced via Turbo ion spray and equipped with a Shimadzu prominent LC (software version 1.6.3). The optimization of the chromatographic process was conducted with the ACE C18 column (100 * 4.6 mm, 5 µm).

Materials

Dr. Reddy's Laboratories in Telangana, India, provided gift samples of deucravacitinib, and nirmatrelvir, which were purchased from Simson Pharma. Merck (Darmstadt, Germany) provided the HPLC grade methanol, the AR grade ammonium formate, the AR grade tertiary butyl methyl ether, and the HPLC water. Human plasma was acquired from the Om blood bank in Pune, Maharashtra, India, using K2 EDTA as an anticoagulant.

Chromatographic Conditions

A mixture of 2 mM ammonium formate (80:20) and methanol was employed as the mobile phase, with a flow rate of 1.00 mL/min. Quantitation was carried out using a positive ion mode mass spectrometer. Using isocratic elution and a 2 µL injection volume, a highly sensitive and quick multiple reaction monitoring technique was created and verified for the detection of nirmatrelvir in human plasma. The approach for liquid-liquid extraction is used in the method, and deucravacitinib is used as an internal standard. A sample containing 100 µg/mL was prepared in methanol to fine-tune the parameters of mass spectroscopy.

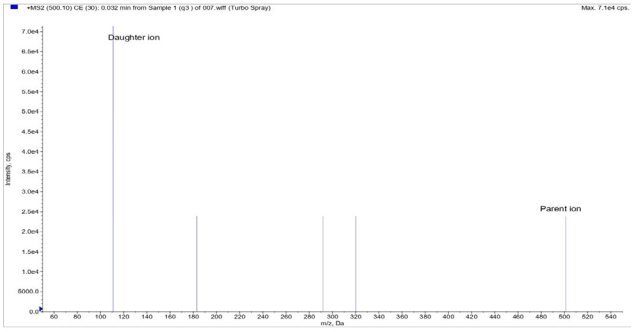

Figure 2: Mass spectra of nirmatrelvir

Preparation of Stock Solutions

The nirmatrelvir as well as deucravacitinib stock solution was made by mixing 1-mg of standard stock solution with 1-mL of methanol to create 1-mg/mL of stock solution. This was then stored in the refrigerator. Additionally, the quality control sample was made using a standard stock solution.

Linear Graph and Quality Control Samples

The analyte was diluted by 0.02 and 0.980 mL of plasma was used to spike the samples. Eight standards, with values ranging from 5 to 4000 ng/mL, were created in order to produce a linear graph. Human plasma samples totaling 9.8 mL and a standard of 0.2 mL were spiked in order to prepare sample solutions and assess accuracy and precision. The respective final concentrations of nirmatrelvir in the QC samples were 5 ng/mL, which is lower limit of quantification (LLoQ), 10 ng /mL, which is LQC. About 448 ng/mL, which is MQC, and 3740 ng/mL, which is HQC

Sample Processing

The selection of liquid-liquid extraction procedures was based on their simplicity, feasibility, and recovery. The selection of the solvent is dependent upon the hydrophobicity of the analytes; non-polar substances, such as hexane, tertiary butyl methyl ether, cyclohexane, etc., were chosen for the extraction of aqueous plasma samples. The most effective solvent chosen is methyl tertiary butyl ether. In the suggested procedure, liquid extraction was chosen as the method of choice for isolating nirmatrelvir and deucravacitinib from human plasma 0.100 mL of K2EDTA plasma is added into RIA (polypropylene) vials, and 0.05 mL of internal standard is added to every sample – aside from the blank and vortexed samples – into each one. Following the addition of 1.000 m: of tertiary butyl methyl ether and vortexing of all samples and to each group of samples, 0.1m: of 2 mM ammonium formate was added. After that, all of the samples were vortexed for 10 minutes at 2500 rpm, and the supernatant was divided into new RIA vials. The samples were evaporated at 40°C while being surrounded by nitrogen gas until they were completely dry. Then they were reconstituted and vortexed using 0.5 mL of mobile phase. Samples were moved to an automatic sampler vial in preparation for analysis. and introduce two microliters of sample for analysis into the chromatographic apparatus.

RESULTS

Mass Spectrometry

Figures 2 and 3 shows the mass characteristics of the compound in positive ionization mode with excellent spectra. The ionization transition values of the analyte and internal standard were 500.10 and 426.30 Q1 mass and daughter mass were 110.10 and 358.20, respectively. Table 1 and 2 shows the parametres of method.

Development of Creative Approach

A number of trials were executed using formate and acetate buffers of assorted pH values. The rationale behind this was that methanol and ammonium formate in an 80:20 ratio were opted as the mobile phase because they eluted peaks with excellent characteristics for nirmatrelvir and internal standard. This methodology yields a peak with retention times of 1.28 minutes for nirmatrelvir and 1.35 minutes for deucravacitinib, and it satisfies all necessary requirements in accordance with USP recommendations.[7]

Chromatography

The measurement of nirmatrelvir in human plasma was conducted by comparing the LLoQ of nirmatrelvir with samples of free plasma and the LLoQ of deucravacitinib. The optimal parameters for estimating nirmatrelvir in human plasma were determined and shown in Table 3.

Method Validation

Specificity

Selectivity was achieved by using blank plasma from six different plasma lots, without spiking with nirmatrelvir. Each and every blank plasma sample was treated and analysed against LLoQ at the retention time of the analytes. The interference of the blank plasma samples was less than 20% of the area of the corresponding LLoQ and 5% of the internal standard retention time. Figure 4 represents the chromatograms of Nirmatrelvir and Deucravacitinib for Specificity.

Linearity

Using a weighting factor of 1/X2, the plasma samples were divided into eight varying concentrations ranging from 5 to 4000 ng/mL. Plotting the area ratios versus the concentration of nirmatrelvir revealed the results for each sample concentration. The least squares regression analysis fit the linearity of the data points. The calibration standards fell

Table 1: Optimizatized data for nirmatrelvir and deucravacitinib using creative method

Name of the sample	*Q1 - Mass*	*Q3 - Mass*	*Dwell*	*DP*	*EP*	*CE*	*CXP*
Nirma trelvir	500.100	110.100	200.00	90	10	36	12
Deucra vacitinib	426.300	358.200	200.00	110	10	32	12

Table 2: Parameters

Parameter	*Value*
CAD (psi)	6.00
CUR (psi)	20.00
GS1 (psi)	40.00
GS2 (psi)	50.00
Ion spray voltage (V)	5500.00
Temperature (^oC)	500.00
Scan type	MRM
Polarity	Positive

Table 3: Optimizatized method developed parameters for nirmatrelvir

S. No	*Parameters*	*Details*
1	Column	Ace C 18, 4.6*100MM, 5 µm
2	Mobile phase	Mobile Phase (80: 20) of proportion A: Methanol B: Buffer (2 mM ammonium formate)
3	Injection volume	2.0 µL
4	Flow rate	1.00 mL/min
5	Column temperature	Ambient
6	Run time	3 minutes
7	Extraction procedure	Liquid liquid extraction
8	Detector	Tandem mass spectrometry (Multiple reaction monitoring)

Table 4 : Linearity table of nirmatrelvir

S.No	*Concentration of nirmatrelvir (ng/mL)*	*Peak: Area*
STD 1	5.02	0.021
STD 2	10.149	0.040
STD 3	30.895	0.119
STD 4	102.983	0.412
STD 5	447.750	1.755
STD 6	1492.500	5.766
STD 7	2985.0	9.980
STD 8	3731.250	12.831
	Slope	0.00201
	Intercept	0.00192
	Correlation coefficient	0.9985

Table 5: Accuracy and precision

0.00192	*LLQC (5. 03 ng/mL)*	*LOQC (14. 537 ng/mL)*	*MQC (1503. 818 ng/mL)*	*HQC (2810. 875 ng/mL)*
Accuracy	98.31	103.9	98.79	92.99
Intraday Precision	101.46	108.09	100.88	101.02
Interday precision	102.82	108.90	103.57	103.21

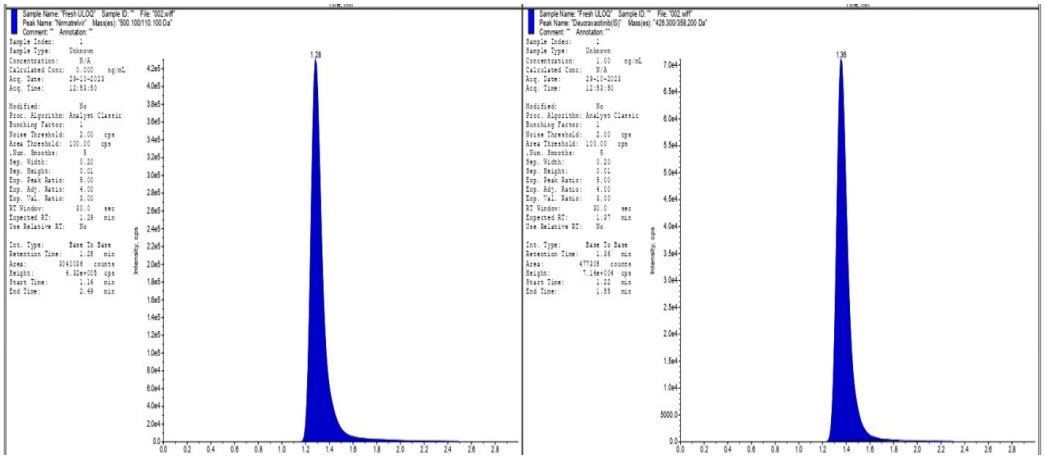

Figure 3: MS peak of nirmatrelvir and deucravacitinib with retention time

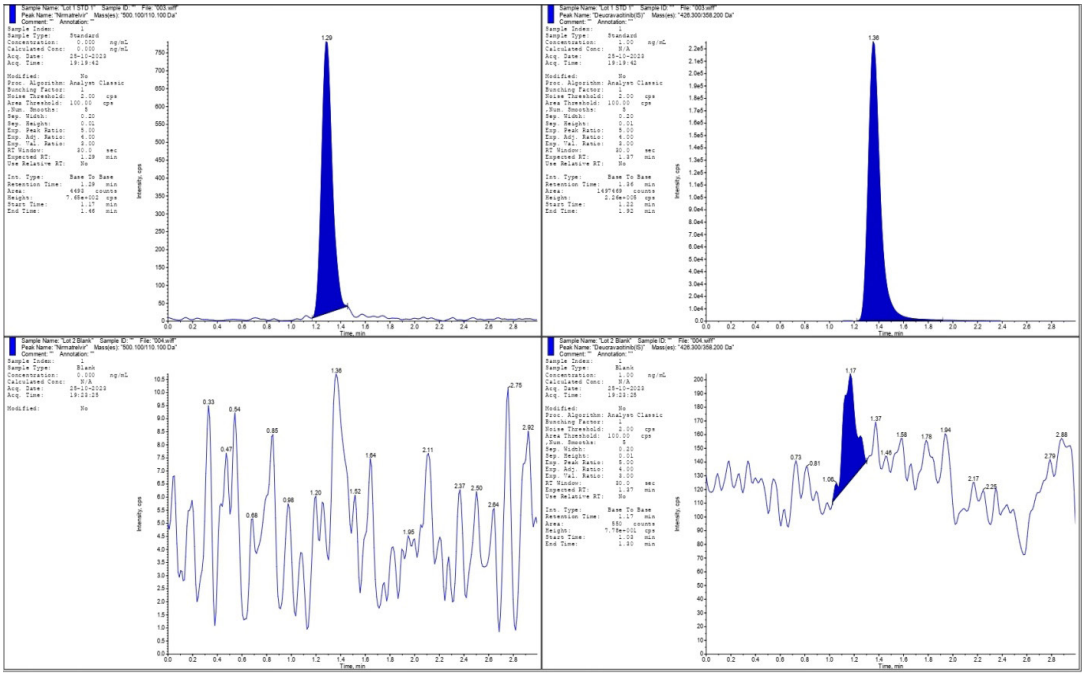

Figure 4: Selectivity chromatogram of nirmatrelvir and deucravacitinib

between 85 and 115% of the range. The average correlation coefficient that was found was 0.9987. As a result, nirmatrelvir may be readily approximated within this concentration, and 5 ng/mL was determined to be the LLoQ for Nirmatrelvir. The Table 4 shows the Linearity Data and Figure 5 shows the Calibration curve.

Accuracy & precision

The accuracy of a measurement can be determined by dividing its mean value by the true value that was collected and indicated as a percentage. The range of values for accuracy is 98.3 to 106.31%.

The sensitivity of nirmatrelvir studied at LLQC is 5.03 ng mL with 98.31% recovery is reasonable. The precision was set for three QC standards of six sets with intraday and interday. These standards were found to be reliable. The Table 5 shows the Accuracy and precision data.

Recovery

The nirmatrelvir extraction recoveries shown in Table 6 at low (LQC), medium (MQC), and high (HQC) were determined using six replicate injections, each of which displayed a percentage recovery of 88.43, 67.79, and 64.56%. The total recovery was determined to be 73.593%. It was determined that the recovery was accurate, appropriate, and repeatable.

Matrix effect

Six different lots of plasma were used, and each plasma LQC and HQC level's extracted and post-extracted blanks were made and examined.

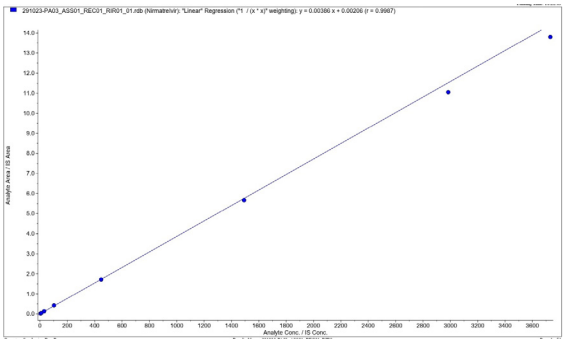

Figure 5: Calibration curve of nirmatrelvir

Table 6: Recovery of nirmatrelvir

S.No	*QC Samples*	*%Recovery*
1	LQC	88.43
2	MQC	67.79
3	HQC	64.56
4	Overall	73.593

Table 7: Matrix factor of nirmatrelvir

Parameter	*Analyte*		*Internal Standard*	
Concentration	LQC	MQC	LQC	MQC
Matrix factor	1.05	0.98	1.05	0.99

Table 8: Stability of nirmatrelvir under varied conditions

Standard (ng/mL)	*Benchtop stability*	*Auto sampler stability*	*Refrigerator stability*
LQC	108.04	108.36	107.86
MQC	102.67	108.04	102.58

There is no indeed significant matrix effect is observed At LQC and HQC levels and the internal standard matrix factor was found to be from 0.98 to 1.14 within the range of LQC and HQC concentration, which is shown in Table 7.

Stability

Two distinct quality control standards, LQC and HQC, were used to determine the stabilities of the analyte following extraction from human plasma. The samples were maintained under autosampler normal circumstances for 24 hours at 10°C, for 12 hours at room temperature on the top of the bench, and for 25 hours in refrigerated storage at 2 to 8°C. The concentration of nirmatrelvir does not significantly decrease following autosampler storage, short-term storage and refrigerator stability. The results validated that nirmatrelvir had reached its optimal stability. The recovery percentage of two different concentrations was established, and shown in Table 8.

CONCLUSION

An innovative method for nirmatrelvir quantitation in human plasma by liquid-liquid extraction technique in human plasma were the concentration range is between 5 to 4000 ng/mL. The range, accuracy, and precision are within the acceptance limits. Recovery for LQC, MQC and HQC have seemed to be appropriate, and the nirmatrelvir is considered to be stable when it is subjected to different stability conditions within the short run time. The drug shows high standards of accuracy, precision, recovery and sensitivity. All the multiple reaction conditions are properly maintained, which is best suitable for method development to be carried out. The methods are less time-consuming, rapid analysis, high throughput, and selective and can be used for routine analysis for the same matrices.

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