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Original Article

LC-MS/MS Method for Simultaneous Estimation of Lopinavir and Ritonavir in Bulk and Dosage Form

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ABSTRACT

Aim: This study introduces a novel simultaneous analytical quantification of Lopinavir (LPV) and Ritonavir (RTV) in drug products by using LC-MS/MS. **Materials and Methods:** The present study were carried out on Acquity™ LC system from Waters paired with a triple quadrupole mass spectrometer and employing positive ion mode for optimal mass spectra. The developed method exhibited molecular and product ions for both analytes for improved resolution and signal intensity, choosing an 80:20 v/v mobile phase consists of ACN and 0.1% HCOOH in water. Strong performance was shown by the Acquity BEH C18 column, which ensured distinct peak morphologies and effective elution with retention periods of 1.89 min for LPV and 1.85 min for RTV. **Results:** The approach showed remarkable linearity (r^2=0.998 for LPV and r^2=0.999 for RTV) over the range of 10-150 ng/mL for both medications, confirmed through recovery studies with percentage recoveries of 102.46-110.66% for LPV and 104.50-111.12% for RTV. Selectivity assessments revealed no interference, attesting to the method's suitability for concurrent quantification. Appropriate %RSD values for peak area and retention time were found by precision evaluations to be between 1.92% and 1.33%. Robustness tests revealed stability with %RSD values for both compounds when the Limit of Quantification (LOQ) and Linit of Detection (LOD) were set at 0.010 g/mL and 0.0003 g/mL, respectively varying between 1.98% and 1.67% for peak area and 1.47% and 0.87% for retention duration. When this approach was used to examine tablet samples that were on the market, the percentage recovery values for LPV is 100.8% and 101.99% for RTV and, underscoring its efficacy for routine quality control testing and establishing it as a precise, sensitive, and robust tool for pharmaceutical analysis. **Conclusion:** The suggested method's results were determined to be acceptable and validated in compliance with regulatory requirements. This approach accurately calculated two pharmaceutical analytes simultaneously.

Keywords: UPLC-MS/MS, Method development, Simultaneous estimation, Validation.

INTRODUCTION

Lopinavir (LPV) is a protease inhibitor characterized by its chemical structure as (2S)-N-[(2S,4S,5S)-5-[[2-(2,6-dimethylphenoxy) acetyl]amino]-4-hydroxy-1,6-diphenylhexan-2-yl]-3-methyl-2-(2-methylpropyl)butanamide.[1,2] LPV stops viral particles from maturing and from becoming infectious virions.[3] Antiretrovirals like Ritonavir (RTV) is used to treat and prevent HIV/AIDS.[4,5] In terms of chemistry, it is 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] butanoyl]amino]-1,6-diphenylhexan-2-yl]carbamate.[6] Figure 1 RTV functions inhibiting the activity of the HIV protease, which stops viral particles from maturing and from becoming infectious virions.[3,7] The fixed-dose combination of LPV and RTV in adults is frequently used as an second-line therapy for HIV/AIDS., effectively inhibiting viral replication and aiding in the management of the infection.[8,9] Kaletra is a brand name for a medication containing both LPV, RTV.[10,11] The typical adult dose for LPV/RTV in the treatment of HIV/AIDS is often 400 mg/100 mg (two tablets) taken orally twice daily with food.[12] According to the literature review, Several analytical techniques were reported for LPV and RTV by LC-MS/MS and HPLC.[12-15] In a seminal study by Laura Else *et al*, the individual quantification of LPV and RTV was investigated by LC-MS/MS, and highlighting the suitability of this technique for pharmacokinetic investigations.[16] Phechkrajang *et al*.,[17] Nils von Hentig *et al*.,[18] has reported the quantification of oral formulations by using liquid chromatography. Jagadeeswaran *et al*.[19] and Sankar, D. G *et al*.,[20] have published the HPLC procedures for determining LPV and RTV simultaneously in pharmaceutical products.

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Shivanand N. Hiremath *et al.* has reported, for the purpose of quantifying these two antiviral medications, the stability-indicating RP-HPLC method was created.[21] A few researchers have developed and validated the bioanalytical technique for LPV and RTV in human plasma.[22-25] LC-MS/MS has emerged as a robust analytical technique for the quantification of pharmaceuticals, providing high sensitivity, specificity, and rapid analysis. The objectives of this research include optimizing LC-MS/MS conditions for simultaneous estimation, validating the method according to international guidelines (ICH), and using the technique that was developed to measure LPV and RTV in dosage forms. Although existing methods exhibit extended chromatographic run times exceeding 10 min and values beyond the Limit of Quantification (LOQ) of 1 µg/mL.

The present technique specifies a LC-MS/MS strategy for the simultaneous quantification of LPV and RTV in tablet and bulk dose forms, with a 3.0 min total run time. LOQ for both analytes was set at 10 ng/mL in order to assess the method's sensitivity. Successful application of the method was demonstrated on commercially available tablet dosage forms of LPV and RTV.

MATERIALS AND METHODS

Chemicals and reagents

LPV and RTV API medications were purchased from Dr Reddy Lab, Hyderabad, India as a gift samples. Methanol, Acetonitrile and water of LC grade were obtained from Merck, Mumbai. Formic acid of analytical grade was procured from SD Fine Ltd., India. Kaletra tablets (Label claim: LPV-200 mg, RTV-50 mg) manufactured by Cipla Pharma Ltd, India were purchased from Apollo pharmacy store.

Instrumentation

An Acquity™ LC system from Waters paired with a triple quadrupole mass spectrometer was employed for the study. Using a mass spectrometer running in +ve ion mode, the separated components were quantified. The following parameters were methodically maintained throughout: source temperature (12°C), capillary voltage (3.0 kV), collision energy (25 V), cone gas flow rate (100 L/hr), desolvation gas temperature (400°C), and evaluation.

Standard stock solution Preparation

Transferring 10 mg of each of LPV and RTV into distinct 10 mL volumetric flasks and dissolving them in 7 mL of diluent (CH_3OH: H_2O, 50:50, v/v) resulted in the creation of a standard stock solution. Following extensive agitation, diluent was added to the volume to get each drug's concentration down to 1 mg/mL. To further dilute the stock solution and reach the necessary concentrations for the standard solutions, the mobile phase solvent was utilized.

Sample solution preparation

Twenty tablets each containing LPV and RTV as stated on the label 200 and 50 mg respectively, were weighed, ground into a powder, and mixed with diluent. Further dilutions were carried out the sample solution's ultimate concentration of 10 ng/mL is achieved by employing the mobile phase solvent.

Optimized Chromatography and mass spectrometry conditions

After processing sample was injected into a C18 column (2.1×50 mm, 1.7 µm) in 5 µL aliquots. Mobile phase as ACN:HCOOH, in H_2O (80:20, v/v) at a flow rate of 0.30 mL/min with isocratic elution. The transition from m/z 629.83 ($[M+H]^+$) to 447.38(product ion) was detected using multiple reaction monitoring (MRM) for the identification of ions were observed for LPV, and from m/z 721.35 ($[M+H]^+$) to 426.24 (product ion) were monitored for RTV (refer to Figure 2). Version 4.1 of the Mass Lynx program was used to analyze the data.

Method Validation

According to ICH recommendations, method for quantifying both LPV and RTV simultaneously was validated.[26] Precision, selectivity, accuracy, robustness, limit of detection, limit of quantification, linearity and range, and solution stability were among the criteria that were assessed.

RESULTS

Method Development

In this work, a sensitive, fast, and selective method for LPV and RTV simultaneous quantification utilizing UPLC-MS/MS was developed. While developing the approach, mass spectra for positive and negative ion modes of an electrospray ion source were used to record both analytes. The positive ion mode was chosen because it responded more strongly. A molecular ion peak with the molecular formula. $[C_{37}H_{48}N_4O_5]^+$, was visible in the mass spectra of LPV at m/z 629.83 yielding a product of ions at m/z 447.38 with the disappearance of 3-methyl-2-(2-oxotetrahydropyrimidin-1(2H)-yl) butanal ($C_9H_{15}N_2O_{22}$). For RTV, a parent ion peak in the mass spectrum at m/z 721.35, with the molecular formula $[C_{37}H_{48}N_6O_5S_2]^+$, and the product ion at m/z 426.24 was obtained with the loss of (1,3-oxathiolan-2-yl)methanol ($C_{14}H_{23}N_3O_2S$). For precise LPV and RTV quantification in complicated matrices, this UPLC-MS/MS approach shows promise.

To achieve enhanced resolution and high-intensity signals within a brief run time for all two analytes, optimization of composition of the mobile phase was undertaken. Various combinations of different ratios of acetonitrile were tested in conjunction with acetic acid and formic acid. Ultimately, the optimized mobile phase is set as 80:20 v/v mixture of ACN and 0.1% HCOOH in water, 0.300 mL/min flow rate, providing well-defined peak shapes and expediting elution. This mobile phase configuration

was selected for its suitability. The chromatographic performance of the robust response and peak morphology of the C18 column (2.1×50 mm, 1.7 µm) were observed even at lower analyte concentrations. In order to allow for a 3.0 min total run duration, the retention times for LPV and RTV were found to be 1.89 and 1.85 min, respectively (Figures 3 and 4).

Range and Linearity

The linearity of the procedure was assessed using the injection of seven non-zero concentrations of reference solutions, particularly at 10, 25, 50, 75, 100, 125 and 150 ng/mL for both LPV and RTV. The obtained results affirm the linear nature of the proposed method over the range of 10-150 ng/mL for each of the two medications (Table 1). For LPV and RTV, respectively, the R^2 for the linear connection were found to be 0.998 and 0.999 (Figure 5). The peak area and rising LPV and RTV concentrations are strongly correlated, according to these data.

Accuracy

Recovery studies were conducted to assess accuracy. A predetermined quantity of working standards were added to the blank solution at 50%, 100%, and 150% levels. At every level, three duplicates were injected. The outcomes demonstrated the accuracy of the developed procedure (Table 2). According to Table 2, the percentage recoveries for LPV and RTV were determined to be 102.46- 110.66% and 104.50-111.12%, respectively. The good accuracy of the devised approach is indicated by the percentage RSD in the range of 1.39-4.28 and 1.83-3.61 for LPV and RTV, respectively.

Selectivity

For the purpose of selectivity assessment, the instrument received consecutive injections of both the analytes' blank and distinct standard solutions. The chromatogram obtained from the blank solution revealed an absence of peaks at the retention times corresponding to LPV and RTV. Moreover, there was no discernible interference from one analyte during the retention period of another when different analyte standard solutions were introduced. Consequently, the approach that was created demonstrated selectivity in facilitating the concurrent quantification of the two analytes.

Precision

In evaluating the precision of the developed method, A standard solution containing LPV and RTV was injected six times in duplicate into the UPLC-MS/TS apparatus, with peak area and retention time percentage RSDs computed .The technique demonstrated excellent accuracy, with %RSD values for peak area and retention duration lying between 1.33% and 1.92% and 0%, respectively (Table 3).

LOD and LOQ

LPV and RTV were discovered to exhibit LOD and LOQ with signal-to-noise ratios of 3:1 and 10:1, respectively. The calculated limits for LPV and RTV in two analytes were 0.0003 g/mL and 0.010 g/mL (Figure 3).

Robustness

The suggested method's resilience was assessed through deliberate variations in chromatographic conditions, changes to the composition of the mobile phase and flow rate, among others. Specifically, chromatographic flow rate was adjusted from 0.30 mL/min to 0.32 mL/min, and the organic phase of the mobile phase was increased from 80% to 82%. For each modification, the analysis was performed three times at a concentration of 100 ng/mL for each of the two analytes. After that, percentage RSD values were computed by contrasting the outcomes of the initial and altered processes. The robustness of the approach was demonstrated by the %RSD values, which varied from 1.67%-1.98% for retention time and 0.87%-1.47% for area of the peak.

Table 1: **Linearity data.**

Parameters	Lopinavir	Ritonavir
Range(ng/mL)	10-150	10-150
R^2	0.997	0.997
Slope	127.77	229.68
Y-intercept	158.87	144.73

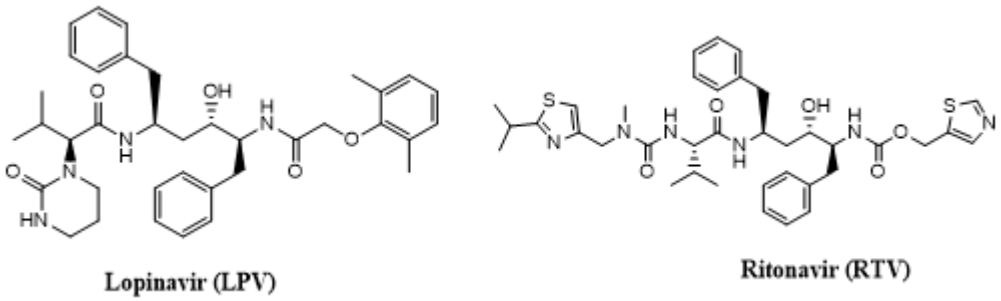

Figure 1: Chemical Structures of LPV and RTV.

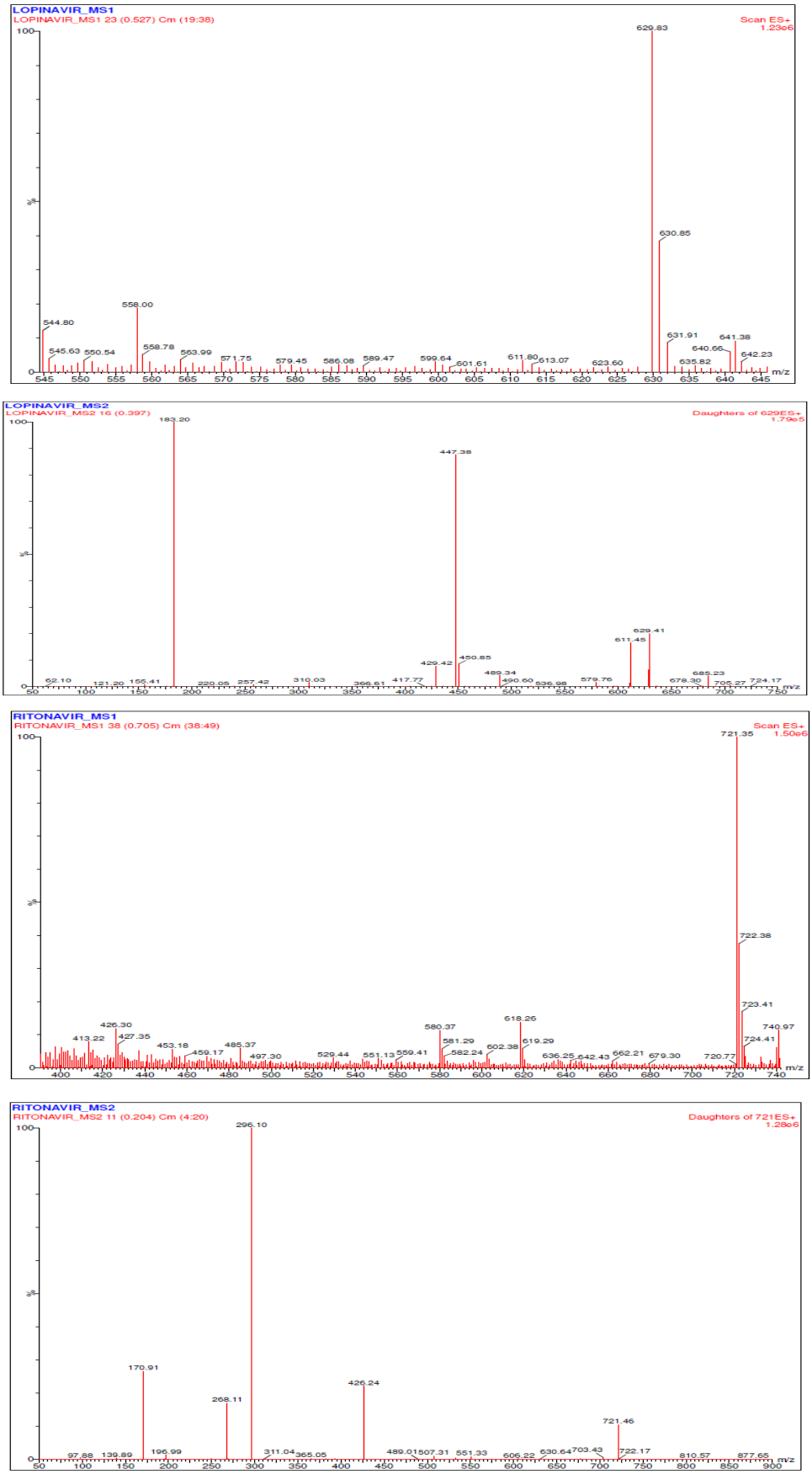

Figure 2: Mass spectra Data of A) Lopinavir (m/z 629.83 ([M+H]$^+$) to 447.38 (product ion) and B) Ritonavir m/z 721.35 ([M+H]$^+$) to 426.24 (product ion).

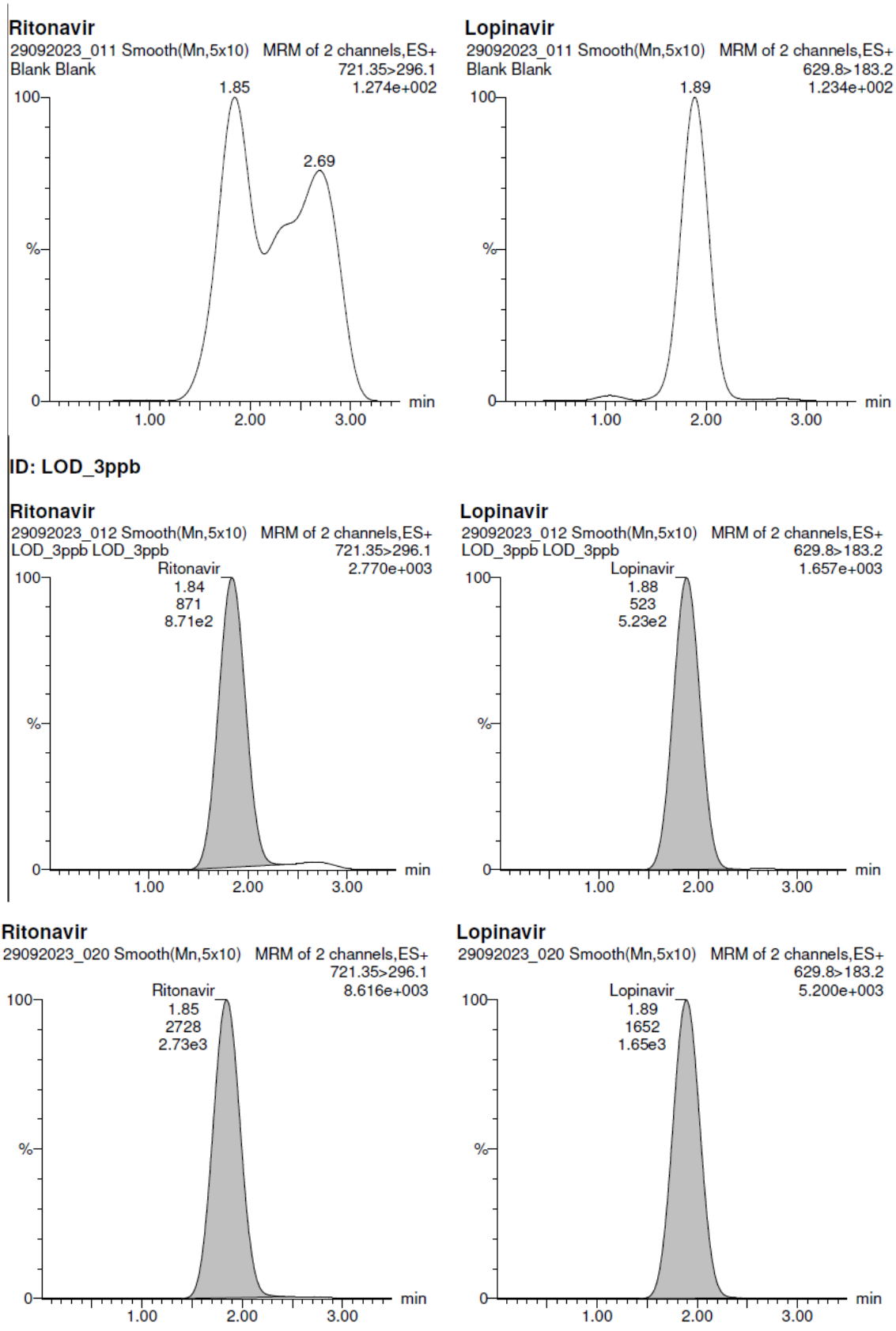

Figure 3: (A) MRM Chromatograms of blank. (B) Analyte peak at LOD at 3ng/mL of RTV and LPV. (C) Analyte peak at LOQ level-10 ng/mL of RTV and LPV.

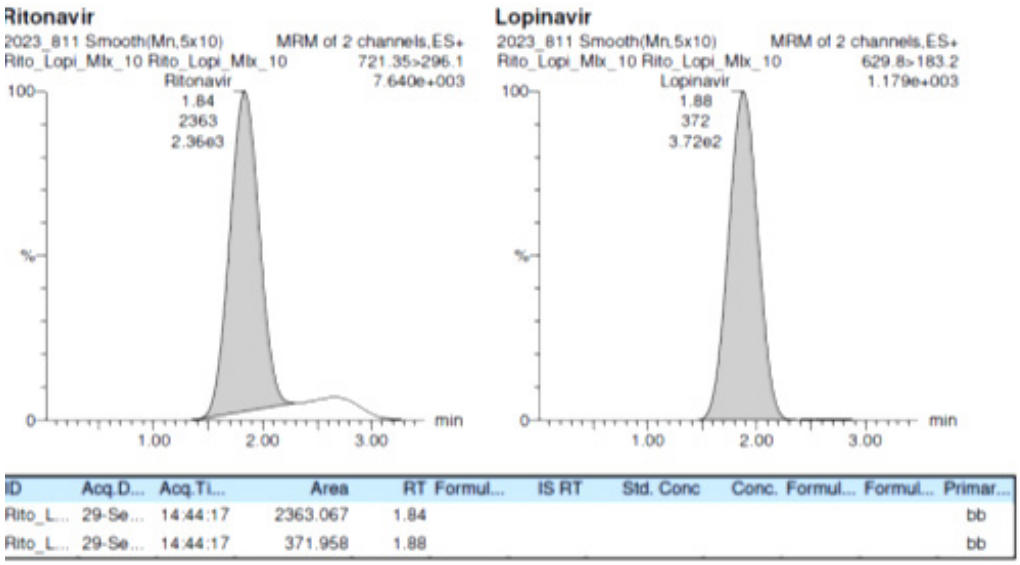

Figure 4: MRM chromatograms of LPV and RTV.

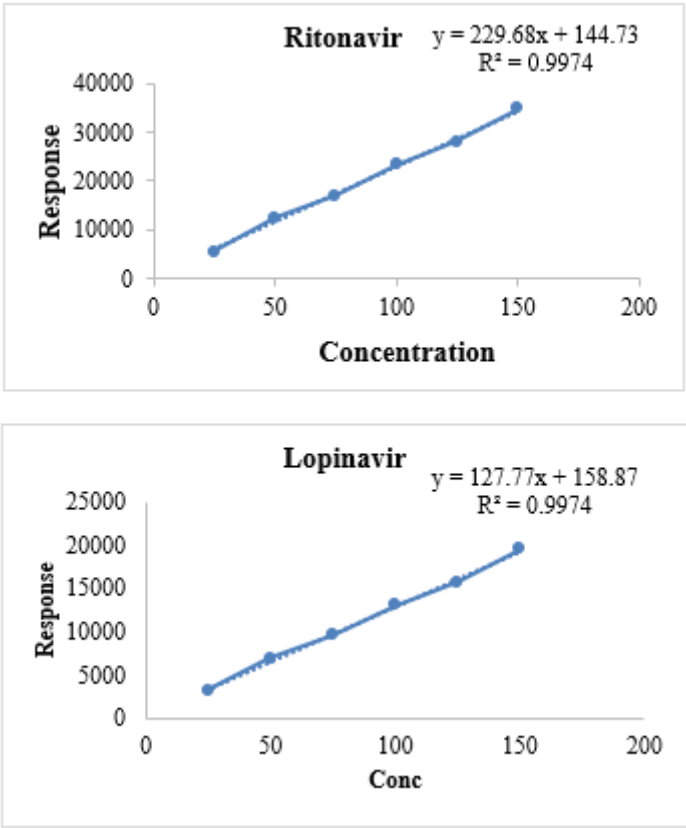

Figure 5: Standard curve of a) LPV and b) RTV.

Table 2: Accuracy results of LPV and RTV at various levels.

Drug	Amount added (ng/mL)	Amount Recovered (ng/mL)	Mean % recovery	%RSD
LPV	50	55.33	110.66	3.37
	100	106.02	106.02	4.28
	150	153.69	102.46	1.39
RTV	50	55.56	111.12	1.83
	100	107.18	107.18	3.61
	150	156.76	104.50	1.93

Table 3: Precision

Drug	Concentration (ng/mL)	Peak Area	%RSD	Retention Time (min)	%RSD
LPV	10	1564	1.92	1.88	0
		1627		1.89	
		1594		1.89	
		1591		1.89	
		1597		1.89	
		1652		1.89	
RTV	10	2756	1.33	1.84	0
		2834		1.84	
		2794		1.85	
		2770		1.85	
		2800		1.85	
		2728		1.85	

Table 4: Analysis of Marketed Drug data.

Drug	Label claim (mg/tablet)	Amount found (mg/tablet)	%Assay	%RSD
LPV	200	198.23	100.8	1.24
RTV	50	49.02	101.99	1.47

Commercial Sample Analysis

The technique which was established was utilized to analyze the tablet dosage form of LPV and RTV available in the market. The % recovery values obtained were 100.8% for LPV and 101.99% for RTV, as detailed in Table 4.

DISCUSSION

The study introduces a new method for simultaneous quantification of Lopinavir (LPV) and Ritonavir (RTV) using UPLC-MS/MS, focusing on sensitivity, speed, and selectivity. Positive ion mode mass spectra were chosen due to their stronger response, revealing distinct molecular and product ions for LPV and RTV. The optimized mobile phase (80:20 v/v ACN and 0.1% HCOOH in water) provided efficient elution with well-defined peak shapes, facilitating retention times of 1.89 min for LPV and 1.85 min for RTV. The method exhibited excellent linearity over the range of 10-150 ng/mL for both drugs, with high correlation coefficients. Recovery studies confirmed accuracy, with percentage recoveries and RSD values indicating precision. Selectivity assessment showed no interference from blank or standard solutions, enabling simultaneous quantification of both analytes. Precision evaluation revealed excellent reproducibility, and robustness testing demonstrated stability under varied chromatographic conditions. Analysis of commercial tablet samples further validated the method's efficacy for pharmaceutical analysis, yielding accurate recovery. The % recovery for commercial samples for LPV and RTV obtained was found to be 100.8% and 101.99%, respectively.

CONCLUSION

A unique UPLC-MS/MS technique was developed and validated for the simultaneous quantification of LPV and RTV in bulk and tablet dosage form, demonstrating precision, accuracy, selectivity, robustness, and linearity for both analytes between 10-100 ng/mL in terms of concentration. The strong correlation observed among the three medications' peak area and concentration suggests the proposed method's suitability for regular testing of these medications' quality control in both bulk and mixed tablet dose forms. The method holds potential for further development to simultaneously detect more than two drugs in pharmaceutical

formulations, and its applications in pharmacokinetic research are being explored.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

LPV: Lopinavir; **RTV:** Ritonavir; **ACN:** Acetonitrile; **UPLC:** Ultra Performance Liquid Chromatography; **MS:** Mass Spectroscopy; **LOD:** Limit of Detection; **LOQ:** Limit of Quantification; **API:** Active Pharmaceutical Ingredient.

SUMMARY

This study introduces a new method for simultaneous quantification of Lopinavir (LPV) and Ritonavir (RTV) in drug products. Using positive ion mode mass spectra and an 80:20 v/v mobile phase of ACN and 0.1% HCOOH in water, the method achieves high resolution and signal intensity. The Acquity BEH C18 column shows strong performance with retention times of 1.89 min for LPV and 1.85 min for RTV. The method demonstrates excellent linearity (r^2=0.999 for RTV, r^2=0.998 for LPV) and accuracy (102.46-110.66% for LPV, 104.50-111.12% for RTV). It is suitable for concurrent quantification with no interference and exhibits low %RSD values for precision (1.33% to 1.92%). Robustness tests confirm stability, and application to tablet samples yields recovery values of 100.8% for LPV and 101.99% for RTV, establishing it as an effective tool for pharmaceutical analysis.

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